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SUPPLEMENT ARTICLE

ISEV2023 Abstract Book

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With a membership of nearly 2000 individuals spanning the globe, the International Society for Extracellular Vesicles (ISEV) stands as the premier professional organization for scientists and researchers engaged in the exploration of extracellular vesicles (EVs). Established in 2012 in Sweden, ISEV subsequently relocated its headquarters to New Jersey, USA. ISEV is dedicated to fostering global consistency and robustness in EV research, as underscored by the MISEV guidelines of 2014 and 2018. The society facilitates this mission through an array of initiatives, including educational offerings, workshops, and summer schools, while also managing two peer-reviewed, gold open access journals—the Journal of Extracellular Vesicles and the Journal of Extracellular Biology. A cornerstone of ISEV's activities is its flagship annual gathering, a focal point that provides a crucial avenue for knowledge exchange. By means of its comprehensive programs and services, ISEV plays an indispensable role in delivering vital training and research prospects for those immersed in the realm of EV research.

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Advancing extracellular vesicle research globally.

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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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The International Society for Extracellular Vesicles is the is the premier international conference of extracellular vesicle research, covering the latest in exosomes, microvesicles and more. With an anticipated 1,000+ attendees, ISEV2023 will feature presentations from the top researchers in the field, as well as providing opportunities for talks from students and early career researchers.

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SUPPLEMENT ARTICLE

Featured Abstract

FA01: Friday Featured Abstract

Location: Ballroom 6BC

08:30 - 10:15

FA01 | EV-DNA is uniquely chromatinized and primes anti-tumor immunity to prevent metastatic progression

Inbal Wortzel[1]; Han Sang Kim[2]; David C. Lyden[3]
[1]Weill Cornell Medicine, New York, USA; [2]Yonsei Cancer Center, USA; [3]Children's Cancer and Blood Foundation Laboratories, Departments of Pediatrics, and Cell and Developmental Biology, Drukier Institute for Children's Health, Meyer Cancer Center, Weill Cornell Medicine, New York, NY, USA, New York, USA

Introduction: Cells actively secrete extracellular vesicles and particles (EVs), transporting selectively packaged functional biomolecules to mediate intercellular communication in normal physiology and pathology. We previously showed that EVs contain DNA (EV-DNA) representing the entire genome and reflecting the mutational status of parental cells. However, the underlying mechanisms of DNA packaging into EVs and their functional consequence in human diseases including malignancy are still elusive.
Methods: Here, via super-resolution imaging of single EVs and biochemical assay, we show that only 30 % of vesicles are DNA positive, and the majority of EV-DNA is presented on the surface of EVs. Histones associated with EVs are remarkably distinct from their cellular counterparts in their variants, size and post-translational modifications, indicating/suggesting unique chromatinization of EV-DNA. To identify factors essential for DNA loading on EVs, we designed a novel strategy of genome-wide CRISPR knockout screening in multiple cancer types. And performed in-vivo experiments to study how EV-DNA affects cancer progression.
Results: The screen uncovered several immune-developmental pathways and genes (e.g., APAF1, NCF1) that play essential roles in EV-DNA packaging. Furthermore, using colorectal cancer as a model system, we found that the amount of EV-DNA is inversely correlated with tumor metastatic potential in animal models. We show that tumor-derived EVs are taken up by Kupffer cells (KCs) in the liver and activates DNA damage response signaling in an EV-DNA-dependent manner, and stimulate the secretion of anti-tumor cytokines from KCs. Finally, analysis of patient-derived tissue EVs showed that the amount of EV-DNA can serve as a predictive biomarker for metastasis.
Summary/Conclusion: Our work suggests that EV-DNA induces immune surveillance in the pre-metastatic organ, and provides molecular insight that tumor-derived EV-DNA activates anti-tumor immunity and prevents metastatic progression.
Funding: This work is supported by the 'Research Assistance for Primary Parents' grant from the Mastercard Diversity-Mentorship Collaborative at Weill Cornell Medicine, and by the 'Worldwide Cancer Research Foundation' (23-0105).
keywords: cancer, metastasis

FA02: Saturday Featured Abstract

Location: Ballroom 6BC

08:45 - 09:30

FA02 | Single EVs sizing and phenotyping via near-wall diffusion analysis using TIRF Microscopy

Yongmin Kwon[1]; Johan Yi[2]; Chungmin Han[3]; Jaesung Park[2]
[1]POSTECH, Pohang-si, Republic of Korea; [2]POSTECH, Pohang, Republic of Korea; [3]POSTECH, Republic of Korea

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Introduction: Total internal reflection fluorescence microscopy (TIRFM) is a powerful technique that enables precise analysis of immobilized EVs with its evanescent field and near-field imaging. Despite recent advances in the single EV analysis via TIRFM, the sizing of EVs in TIRFM is still limited by analysis of immobilized EVs. Analyzing the diffusivity of EVs is a common method for sizing EVs like DLS or NTA. However, Fast diffusivity of EVs and the thin imaging area of TIRFM prevent observation of diffusivity. Here, we present a method enabling high-precision sizing and phenotypic analysis by decreasing the Brownian mobility of EVs with a viscous-enhanced medium in TIRFM.
Methods: EVs were isolated from HEK293T cell line using density gradient ultracentrifugation according to the MISEV2018 guideline. EVs were fluorescently labeled using antibodies, CTB, and NHS ester. The viscosity of the medium was sequentially increased by adding glycerol to stay the particles sufficiently within the imaging area. Optimal medium viscosity was selected experimentally. For the single particle tracking (SPT) analysis in TIRFM, The size of particles is estimated through a modified Stokes-Einstein (SE) equation applying reduced diffusivity near the wall. EV analysis was performed to identify subpopulation and size by visualizing total EV markers and tetraspanin markers simultaneously.
Results: The sizing of beads through the modified SE equation showed similar results to the actual size, but the original SE equation estimated 1.3 times larger. Tracking of beads and virtual tracking simulations shows that the SPT in TIRFM is as reliable as the conventional SPT techniques on both monomodal and multimodal samples. The marker expression ratio and size of EVs were measured. Through cross-validation with multi-fluorescence NTA that is one of the latest single EV analysis technology, we confirmed that SPT of EVs in TIRFM has compatible ability to analysis of subgrouping and precise sizing.
Summary/Conclusion: In this study, we developed the versatile SPT system with the subpopulation analysis capabilities of conventional TIRFM with the sizing and concentration measurement techniques of NTA technology. Our work improves sizing precision with a simple method of increasing the viscosity without the addition of special optical systems and is easy to adapt to existing TIRFM setups.
Funding: This research was supported by the Korea Medical Device Development Fund grant (1711137918).
Keywords: single EV analysis, nanoparticle tracking analysis, total internal reflection fluorescence microscopy, near-wall hindered diffusion

FA03: Sunday Featured Abstract

Location: Ballroom 6BC

08:30 - 10:00

FA03 | Engineering extracellular vesicles for functional ligand presentation over distance to treat tissue injury

Prerak Gupta[1]; Koushik Debnath[1]; Gina Chung[2]; Jae-Won Shin[1]
[1]Department of Pharmacology and Regenerative Medicine, University of Illinois at Chicago College of Medicine, Chicago, IL 60612, USA, Chicago, USA; [2]Department of Pharmacology and Regenerative Medicine, University of Illinois at Chicago College of Medicine, Chicago, IL 60612, USA, USA

Introduction: Extracellular vesicles (EVs) are lipid membrane-bound nanoscale mediators known to present various surface ligands and activate cell membrane receptors over distance. Despite a number of existing strategies, engineering EVs to present full-length surface proteins has not been straightforward. Here, we developed a facile platform to rapidly functionalize surface proteins with correct orientation. We demonstrate the efficacy of this approach by activating the Notch pathway to restore endothelial barrier function upon acute lung injury.
Methods: EVs were obtained from mouse D1 MSCs following EV-TRACK ID: EV150007. We tethered lipid conjugated Fc binding peptides into the membrane of EVs or synthetic liposomes. We used Fc-FITC protein (~50 kDa) to confirm Fc-fusion protein functionalization. We introduced the recombinant Notch ligand Jagged1 (JAG1)-Fc (~150 kDa) to EVs or liposomes, and titrated the number of JAG1 molecules per EV by ELISA. We tested the efficacy of JAG1 functionalized EVs or liposomes by delivering them into mice 4 h after injection of lipopolysaccharide (LPS), followed by evaluating edema (wet/dry ratio) and vascular permeability (Evans blue albumin) after 24 h of treatment.
Results: The results show a dose-dependent membrane tethering of Fc-FITC proteins into EVs (EC50 ~ 1 mM). Strikingly, introducing ~6 JAG1-Fc molecules per EV is sufficient for EVs (1.5 × 108 per 20g) to treat edema and vascular hyperpermeability after LPS injury in mice, while unmodified EVs do not show the therapeutic effect at the same dose. In contrast, JAG1-liposomes were not able to restore edema or vascular permeability even at a higher dose (1.5 × 109 per 20g).
Summary/Conclusion: Our results show that EV-mediated, but not liposome-mediated presentation of JAG1 can treat LPS-induced lung injury. Future studies will test the notion that EVs are biophysically optimized for surface ligand presentation to activate the Notch pathway. This platform is general and can be broadly used to activate membrane receptors over distance.

Funding: This work was supported by National Institutes of Health Grant R01-HL141255 (to J.-W.S.).
Keywords: extracellular vesicles, ligand presentation, notch, vascular permeability

OT01: Nanoflow

Chairs: Joshua Welsh, Marca Wauben

Location: Ballroom 6BC

11:30 - 13:00

OT01.1 | Global inter-laboratory comparison study to standardize EV concentration measurements between 39 flow cytometers

Britta A. Bettin[1]; Edwin van der Pol[2]; Rienk Nieuwland[3]
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Introduction: Extracellular vesicles (EVs) in body fluids are potential disease biomarkers. To measure EV concentrations, most laboratories use flow cytometers (FCMs), but concentrations are incomparable between FCMs. To improve comparability, the METVES II consortium developed reference materials and methods to calibrate FCMs. This developed infrastructure was tested in a global inter-laboratory comparison study including 39 FCMs from 24 different laboratories.
Methods: Concentrations of erythrocyte-derived (CD235a-PE) and platelet-derived (CD61 -APC) EVs were measured in stabilized and pre-labeled human plasma EV test samples (PEVTES). Flow rate was calibrated using solid silica beads, light scattering was calibrated using Rosetta calibration beads, and fluorescence was calibrated using MESF beads. EV concentrations were compared between FCMs within an EV size range of 215 – 1,000 nm and a fluorescence intensity >220 APC MESF, and >50 PE MESF.
Results: Preliminary results from 9 FCMs show that FCM calibration is successful. For the platelet EV concentration, the coefficient of variation decreased from 72% without calibration to 40% after calibration.
Summary/Conclusion: This is the first inter-laboratory comparison study demonstrating that full flow cytometer calibration improves the comparability of EV concentration measurements between FCMs, thereby paving the road to clinically relevant multi-center biomarker studies on EVs.
Funding: This project has received funding from the EMPIR programme co-financed by the Participating States and from the European Union's Horizon 2020 research and innovation program.
Keywords: calibration, extracellular vesicles, flow cytometry, inter-laboratory comparison study, standardization

OT01.2 | Size and fluorescence calibrated imaging flow cytometry: from arbitrary to standard units

Wouter W. Woud[1]; Haley R. Pugsley[2]; Britta A. Bettin[3]; Zoltan Varga[4]; Edwin van der Pol[5]
[1]Erasmus MC Transplant Institute, University Medical Center Rotterdam, Rotterdam, The Netherlands, Rotterdam, Netherlands; [2]Luminex Corporation A DiaSorin Company, USA, Seattle, USA; [3]Department of Clinical Chemistry, Amsterdam UMC location University of Amsterdam, Amsterdam, The Netherlands, Amsterdam, Netherlands; [4]Biological Nanochemistry Research Group, Institute of Materials and Environmental Chemistry, Research Centre for Natural Sciences, Budapest, Hungary, Budapest, Hungary; [5](2) Laboratory of Experimental Clinical Chemistry and Vesicle Observation Center, Amsterdam; The Netherlands; (8) Biomedical Engineering and Physics, Amsterdam UMC, Amsterdam, The Netherlands, Amsterdam, Netherlands

Introduction: Imaging flow cytometry (IFCM) is a technique to detect, size, and phenotype extracellular vesicles (EVs) at high throughput (thousands/minute). However, the generated signals are expressed in arbitrary units, which hinders data interpretation and comparison between instruments. While fluorescence calibration can be readily achieved, calibration of side scatter (SSC) signals presents an ongoing challenge for IFCM. Here, we present an approach to relate the SSC signals to particle size for IFCM, and perform a comparability study between different IFCMs using plasma EV test samples (PEVTES).
Methods: SSC signals for different sizes of polystyrene (PS) and hollow organosilica beads (HOBs) were acquired with a 405-nm 120-mW laser without a notch filter before detection. Mie theory was applied to relate scatter intensities to particle size. Fluorescent calibration was accomplished with 2-μm APC, and PE MESF beads (custom-order, BD). Size and fluorescence calibration was performed for two IFCMs in two laboratories. APC and PE-labelled plasma EV test samples (PEVTES, METVESII) were

used as EV samples. EV concentrations were compared between instruments within an EV size range of 80–1,000 nm and a fluorescence intensity range of 10-10,000 MESF.
Results: 81-nm PS beads could be readily discerned from background signals based on their SSC intensities. Fitting of the obtained PS bead SSC intensities with Mie theory resulted in a coefficient of determination > 0.995 between theory and data. Following size and fluorescence calibration, APC+ and PE+ EV concentrations were compared for both instruments, yielding CV scores of 21.2% and 3.3% (down from 30.5% and 16.2%), respectively.
Summary/Conclusion: Here we demonstrate – for the first time – scatter calibration of an IFCM. The quality of the scatter-to-diameter relation and scatter sensitivity of the IFCMs are similar to state-of-the-art flow cytometers. This development supports the reliability of EV research with IFCM by providing robust standardization and reproducibility, which are prerequisites for understanding the biological significance of EVs.
Funding: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Keywords: extracellular vesicles, imaging flow cytometry, calibration, reproducibility, single EV analysis

OT01.3 | Development of high-throughput microfluidic nanoparticle sorter toward extracellular vesicle analysis

Yoshiyuki Tsuyama[1]; Kenji Hinode[2]; Yusuke Yoshioka[3]; Sadao Ota[2]
[1]Department of Molecular and Cellular Medicine, Tokyo Medical Univesity Institute of Medical Science, Japan; [2]Research Center for Advanced Science and Technology, The University of Tokyo, Japan; [3]Department of Molecular and Cellular Medicine, Tokyo Medical Univesity Institute of Medical Science, Shinjuku-Ku, Japan

Introduction: Selective sorting of extracellular vesicles (EVs) at single particle resolution based on sizes and composites is essential to correlate the characteristics of EVs and their biological functions. In recent years, microfluidic nanoparticle analyzers have been developed and applied to EVs, but selective sorting of nanoscale objects including EVs with integrated microfluidic systems is still challenging. Here, we present a high-throughput microfluidic nanoparticle sorter which implements hydrodynamic focusing and fluorescent detection of nanoparticles in combination with the world's fastest droplet generation and sorting inside 10 μm-scale channels.
Methods: Microfluidic device with 12 μm wide channels was designed and fabricated to have a hydrodynamic focuser, femtoliter droplet generator, and high-throughput droplet sorter, successively. A home-build system of specialized optics, signal processing, and sorting actuator, was used for operating the device. Polystyrene beads with a 1.3 μm diameter were used to validate and optimize the operation. Polystyrene beads with a 300 nm diameter were used for the demonstration of fluorescence-based nanoparticle sorting.
Results: Sensitive particle detection was realized with < 10% signal intensity variation by focusing beads to the center of the microchannel. After detection, each bead was encapsulated in 10–12 μm diameter droplets generated at a speed of >30,000 droplets/sec. Finally, by adjusting the sorting timing, droplets containing beads were successfully sorted based on the measured fluorescent signals. Our system realized sorting of 300 nm beads at more than 90% yield and throughput of >10^7 particles/hour.
Summary/Conclusion: In this study, we developed a microfluidic nanoparticle sorter and demonstrated high-throughput sorting of 300 nm fluorescent beads. We will be soon sorting EV subsets based on fluorescent labels, and the developed system will contribute to a wide range of research and applications targeting EVs.
Funding: This work was supported by JSPS Grant-in-Aid for JSPS Fellows, Grant number: 21J01468, Japan and JST, CREST Grant Number JPMJCR19H1, Japan.

OT01.4 | Novel fluorescent labelling of internal protein targets in ARMMs as a model extracellular vesicle

Ben Peacock[1]; Rebecca J. lees[1]; Joseph Nabhan[2]; Kristin Luther[2]; Wendy Zhao[3]
[1]NanoFCM, Nottingham, United Kingdom; [2]Vesigen Therapeutics, Cambridge, USA; [3]Vesigen Therapeutics, USA

Introduction: Modern extracelullar vesicle (EV) characterization often utilizes fluorescent labelling of proteins, lipids, nucleic acids, or structural components. Labelling of internal EV proteins often requires transgenic co-expression of fluorescent proteins. Here, we present two protocols which allow targeted fluorescent labelling of lumen-side EV proteins, using ARMMs (ARRDC1-mediated microvesicles) as a model EV, and demonstrate detection efficiency as "percentage of targets labelled" through the use of nano-flow cytometry (nFCM).
Methods: HEK (Human embryonic kidney) 293 suspension cells were transfected with a variety of ARMMs loading constructs and EVs were enriched from supernatant of transfected cells using size exclusion chromatography (SEC). Presence of ARMMs

was confirmed by Western blotting and quantified by nFCM using a NanoAnalyzer (optical technique suitable for objects 40–1000nm diameter). Transgenic protein loading was assessed by nFCM as was subsequent fluorescent labelling. Intraluminal localization of the transgenic proteins was confirmed by anti-GFP, anti-SPOT tag, anti-FLAG tag fluorescent labelling.
Results: Loading of ARMMs with GFP tagged SPOT from transgenic lines was shown to be ~70% within EVs >40nm. Fixation and/or permeabilization was shown to have limited impact on particle size and concentration, with EVs retaining a median size between 60–70nm. Incubation with anti-SPOT nanobodies following permeabilization demonstrated that up to 62% of the GFP labelled EVs could be further labelled in two unique protocols. Comparison between antibody and nanobody labelling of internal proteins demonstrated the superior detection efficiency of using smaller targeting reagents, such as nanobodies. Length of incubation, fixative concentration and starting EV concentration were all shown to impact efficiency of detection of EV payloads.
Summary/Conclusion: Here, we describe a novel approach to test the efficiency of immunodetection of intraluminal payloads in EVs and demonstrate consistency with payloading using a quantifiable internal fluorescent protein. We provide two viable protocols for internal labelling with distinct advantages and a potential model to continue exploration of novel approaches.
Funding: N/A
Keywords: flow cytometry, EV lumen, permeabilization, EV cargo

OT01.5 | Single vesicle flow cytometry measures VLP molecular cargo and resolves structural variants

John P. Nolan[1]; Erika Duggan[1]; Tommy Tong[2]; James Binley[2]
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Introduction: EV engineering requires reliable methods to measure the end product. Single vesicle flow cytometry (vFC) provides rigorous and reproducible measurement of EV number, size and cargo, including of virus like particles (VLPs). Here, we extend vFC by using EV light scatter (LS) to resolve EV structural variants.
Methods: HEK cells transfected with HIV Env, MLV Gag, and/or MLV-Gag-GFP were cultured in DMEM (1% EV-depleted FBS) for 48h, and EVs collected by ultracentrifugation (UC-50Kxg, 60'). EV number, size, cargo and LS were measuring using a commercial assay (vFC Assay, Cellarcus), flow cytometer (CytoFlexS, Beckman), and software (FCS Express, De Novo) in a MIFlowCyt-EV compliant manner. LS was calibrated in units of polystyrene (PS)-equivalent scattering cross-section (PS-ESCS, units of nm2) using available beads and software (Rosetta, Exometry; FCSMPASS, NIH). EVs were negative stained and imaged by TEM (H7650, Hitachi).
Results: HEK cells release EVs with characteristic size, surface cargo, and LS distributions. HEK EVs had LS [PS ESCS, median (sd): 16.2 (2.1)] similar to PLT-derived EVs [18.0 (0.5)], and greater than liposomes [4.0 (0.3)]. HEK cells expressing Env, Gag, and Env+Gag produced 10–50 more EVs, and those EVs exhibited higher LS [22.7 (3.0), 40.1 (3.6), and 49 (3.8) respectively]. HEK cells transfected with Env-GAG-GFP produced EVs with GFP that had a median LS of 40 compared to GFP-negative EVs (12.7), indicating increased LS from Gag-containing EVs. TEM provided visual confirmation of protein structures associated with Env and Gag expression.
Summary/Conclusion: vFC has previously been used to measure VLP number, size, and surface antigen expression, and we extend that work here by showing that EV LS, appropriately calibrated and interpreted, can provide information regarding EV structure. These data can be used to guide the design of engineered EVs including VLPs, and to optimize and monitor fractionation procedures designed to enrich EVs.
Funding: NIH AI93278 (JMB)

OT02: Cancer Biomarkers

Chairs: Yu Fujita, Edgar Gonzalez-Kozlova

Location: Ballroom 6A

11:30 - 13:00

OT02.1 | Paving the way towards improving the diagnosis of breast cancer using circulating extracellular vesicles

Nikki Salmond[1]; Wing Sum (Kelly) Tam[2]; Karan Khanna[2]; Renata Moravcova[2]; Jason Rogalski[2]; Karla Williams[2]
[1]University of British Columbia, Vancouver, Canada; [2]University of British Columbia, Canada

Introduction: Breast cancer (BCa) screening uses mammography and ultrasound imaging, and suspicious lesions are investigated using an invasive needle biopsy. Mammograms and ultrasounds are unable to distinguish between benign and cancerous tumors. We need a non-invasive blood test – a liquid biopsy – which can accurately identify BCa at its earliest stage- Stage I. Circulating tumor fragments (extracellular vesicles - EVs) are a promising non-invasive platform for the development of a novel BCa screening test. We isolated EVs from 86 BCa, Stage I, patients, 19 benign and 20 healthy individuals to identify new protein biomarkers unique to BCa.
Methods: Different EV isolation methods were tested to determine the best platform for use with mass spectrometry: EQUltra, SEC, PEG-SEC, EQUltra-SEC, and precipitation. EVs were characterized by Western blot, protein concentration, electron microscopy and mass spectrometry. EVs were isolated by SEC from 86 Stage I BCa patients, 19 benign and 20 healthy (age-matched controls); from 20 μg of processed EVs, 500 ng was spiked with 200 fm yeast glutathione reductase (internal standard) for unlabeled mass spectrometry.
Results: SEC was used for EV isolation with downstream mass spectrometry analysis due to efficiency, purity, and quantity. Mass spectrometry identified 373 unique proteins. A Volcano plot identified 75 proteins with significantly higher expression in BCa relative to healthy/benign. Principal Component Analysis (PCA) and Multiple Logistic Regression analysis identified 26 proteins whose expression could correctly identify 96.5% of cancer patients and 89.7% of healthy/benign individuals.
Summary/Conclusion: Our study has identified protein biomarkers found in the blood of BCa patients with potential utility to generate 'fingerprints' of healthy, benign, and early Stage I BCa. The detection of BCa cases at Stage I, would allow for rapid, curative, intervention reducing the mortality associated with a BCa diagnosis.
Funding: Michael Smith Foundation for Health Research.
Keywords: breast cancer, stage I, biomarker, diagnosis, circulating extracellular vesicles, liquid biopsy, mass spectrometry.

OT02.2 | Small Extracellular Vesicles-GCC2 for the Early Diagnosis of Lung Adenocarcinoma: A Multicenter Trial

Byeong Hyeon Choi[1]; Hyonggin An[2]; Sukki Cho[3]; Sungsoo Lee[4]; Hyeong Ryul Kim[5]; Jong Ho Cho[6]; Hyunku Shin[7]; Ok Hwa Jeon[1]; Yeonho Choi[8]; Hyun Koo Kim[1]
[1]Korea University Guro Hospital, Seoul, Republic of Korea; [2]Korea University, Republic of Korea; [3]Seoul National University Bundang Hospital, Republic of Korea; [4]Gangnam Severance Hospital, Yonsei University, Republic of Korea; [5]Asan Medical Center, University of Ulsan, Republic of Korea; [6]Samsung Medical Center, Sungkyunkwan University, Republic of Korea; [7]Exopert Corporation, Republic of Korea; [8]Korea University, Seoul, Republic of Korea

Introduction: Early diagnosis of lung cancer is important for patients to receive optimal treatment as early as possible and could improve survival rates. Emerging evidence suggests that tumor-derived small extracellular vesicles (sEV) cargo offer an attractive platform for developing cancer-specific biomarkers. We previously reported the identification of GRIP and coiled-coil domain-containing 2-enriched sEV (sEV-GCC2) as a promising biomarker for lung adenocarcinoma in a pilot study. In this multicenter study, we explored the diagnostic and therapeutic potential of sEV-GCC2 in early-stage lung adenocarcinoma.
Methods: A total of 470 blood plasma samples (150 healthy controls and 320 patients with lung adenocarcinoma) were retrospectively obtained from five institutions. sEVs were isolated by size exclusion chromatography, and sEV-GCC2 was quantified by enzyme-linked immunosorbent assay. The effect of sEV-GCC2 on lung cancer cell proliferation was investigated in vitro and in vivo.
Results: sEV-GCC2 concentration was significantly higher in patients than that in controls, with an area under the curve (AUC) of 0.856 (95% confidence interval [CI], 0.820–0.886). In patients at TisN0-T1miN0 stages vs. controls, the AUC was 0.802 (95% CI, 0.734–0.859). The association between sEV-GCC2 and lung adenocarcinoma remained after adjustment for pathological TNM stage and tumor location in the left lower lobe. Immunohistochemical staining revealed that GCC2 expression was significantly higher in lung adenocarcinoma tissues than in the controls ($p < 0.001$). Furthermore, sEV-GCC2 enhanced cancer cell proliferation and accelerated tumor growth and lymph node metastasis.
Summary/Conclusion: We identified sEV-GCC2 as a potential diagnostic marker for very early-stage lung cancer. sEV-GCC2 represents a promising therapeutic target for lung adenocarcinoma and may be quantified as key molecules in the progression of lung adenocarcinoma.
Funding: This research was supported by Basic Science Research Program through the National Research Foundation of Korea(NRF) funded by the Ministry of Education (2021R1I1A1A01052142) and by the Korea Medical Device Development Fund grant funded by the Korean government (the Ministry of Science and ICT, Ministry of Trade, Industry and Energy, Ministry of Health & Welfare, Ministry of Food and Drug Safety) (Project Number: 1711138151, KMDF_PR_20200901_0094_02).
Keywords: small extracellular vesicle, lung adenocarcinoma, biomarker, early diagnosis, liquid biopsy

OT02.3 | Single exosome profiling discovers alteration of exosome-subpopulations related to colorectal cancer progression

Yanling Cai[1]; Wei Guo[2]; Yuge Ji[2]; Cuiyu Zhang[2]; Di Wu[3]; Jingxin Li[2]
[1]Qilu Hospital, Cheeloo College of Medicine, Shandong University,, Shenzhen, China (People's Republic); [2]Department of Physiology, School of Basic Medical Sciences, Department of Colorectal Surgery, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan, Shandong, 250012, China., China (People's Republic); [3]Vesicode AB, Sweden

Introduction: Tumor metastasis is a hallmark of colorectal cancer (CRC). Exosomes play crucial roles in the progression of CRC through the function in cellular communication.
Methods: In our investigation, plasma samples were collected from healthy control (HC) donors, localized primary CRC and liver-metastatic CRC patients. We performed proximity barcoding assay (PBA) for single-exosome analysis. Based on the single – exosome proteomic data, we utilized a machine learning algorithm, FlowSOM, to generate exosome subpopulation. By in vitro and in vivo experiments, the biological impact of these subpopulations on cancer proliferation, migration, invasion, and metastasis was investigated.What's more, we collected samples from another cohort and replicate our findings. We also collected plasma samples from donors with pre-cancerous and stage I CRC to investigate the potential of our biomarker being used as early screening target.
Results: Among 12 subpopulations, we found 2 distinctly abundant subpopulations: one ITGB3-positive and the other ITGAM-positive. ITGB3-positive subpopulation consists 13.6% of detected exosomes in HC group and increased to 23.0% and 52.1% in primary and liver-metastatic CRC patients, respectively. ITGAM-subpopulation, with tendency of co-expression with ITGAL and ITGB2, decreased from 13.5% in HC to 1.13% in CRC groups. By in vitro and in vivo experiments, we proved the ITGB3-positive subpopulation could promote cancer proliferation, migration, invasion, and metastasis. ITGAM-positive subpopulation, which was proved be generated by macrophage, could suppress the development of metastatic sites in our mice model study.The second cohort could repeat our findings. In the test with pre-cancerous and stage-I CRC samples, we investigated the potential of our biomarker being used as early screening target. The area under receiver operator characteristic curve (AUC-ROC) is 0.855 when distinguishing CRC and HC.
Summary/Conclusion: In a sum, the discovery shows potential as diagnostic, prognostic, and therapeutic biomarkers for management of CRC.

OT02.4 | Metastatic patient plasma-derived EV RNA cargo is highly fragmented except for mitochondrial RNAs and YRNAs

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Introduction: Prostate cancer patients almost inevitably develop resistance to first-line hormonal therapy and to second-line castration regimens, entering a state referred to as castration-resistant prostate cancer (CRPC). Liquid biopsies allow for the interrogation of all components shed by tumor cells into biofluids, including extracellular vesicles (EVs). In the framework of PRIME (PRostate cancer plasma Integrative Multi-modal Evaluation), a funded program to develop multi-modal liquid-biopsy tailored assays for advanced prostate cancer, we profiled the EV-associated transcriptome of 16 healthy donors (HDs) and 58 CRPC patients at multiple time points (n = 3) before and during Enzalutamide treatment.
Methods: Plasma samples were collected across multiple Italian institutions with harmonized procedures and processed using ONCE (ONe Aliquot for Circulating Elements), an in-house developed approach for the concomitant isolation of EVs and cfDNA from a single plasma aliquot. RNA was extracted from EVs with the IZON qEV RNA Extraction kit and treated with RNase-Free DNAse I. Libraries were prepared using the SMARTer smRNA-Seq kit for total RNA and sequenced on NovaSeq 6000 with a SR150 protocol.
Results: We observed that the transcripts' integrity depends on the RNA biotype in healthy and disease conditions. Specifically, in EV-associated RNA (EV-RNA) from the HD and CRPC plasmas, we found intact YRNA transcripts with reads spanning the entirety of the transcripts. Also, mitochondrial transcripts show uniform coverage and longer reads than other RNA biotypes. Conversely, lncRNAs represent the RNA biotype with the shortest read length (mean < 50bp) and less uniform coverage.

Furthermore, we observed that the length and coverage uniformity of protein-coding reads depend on the putative cell of origin. Interestingly, erythrocyte-derived, such as HBB, and whole-blood associated EV-RNA transcripts have high uniformity and longer reads with respect to other tissues, in line with mitochondrial transcript features, while prostate-specific genes are highly fragmented.

Stratifying our CRPC patient cohort (n = 58) by the time of response to Enzalutamide, we identified 490 genes differentially represented in plasma EVs between short- or long-treatment responders (FDR< 0.05; n = 431 and n = 59 upregulated in short and in long responders, respectively). The majority of the highly represented genes in short responders (n = 313) are concordantly more expressed in CRPC tissues with respect to the whole blood, in keep with the representativeness of the plasma-derived EVs signal of prostate cancer cell transcriptome.

Summary/Conclusion: While protein-coding and lncRNA from CRPC EV cargo are highly fragmented, their relative abundance represents an informative source for inquiring about the clinical status of cancer patients.

Funding: AIRC (id 22792), CRUK (id 26822).

OT02.5 | Dynamic changes in the miRNA and protein content of circulating extracellular vesicles associated with ovarian cancer progression

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Introduction: Circulating changes in the content and bioactivity of extracellular vesicles (EVs) has been associated with oncogenic transformation of ovarian cancer (OVCA). Thus, characterisation of EV protein and nucleic acids is an essential step for understanding changes to predict cancer outcome. The objective of this study was to describe changes in the EV-associated proteins and miRNAs that change with the progression of the disease and determine biological processes that are disturbed in OVCA.

Methods: A cohort of 97 patients were included in this study from healthy controls (n = 20), benign (n = 20), high grade epithelial ovarian cancer stage I (n = 20), stage II (n = 20), stage III (n = 20), and stage IV (n = 20). EV were isolated using EXO-NET (INOVIQ LTDA, Australia) and characterised by Nanoparticle tracking analysis, protein abundance (CD63, CD9, Alix, TSG101 and CD81) and morphology using NanoSight, Western blot and electron microscopy, respectively. In addition, a Targeted Multiple Reaction Monitoring proteomic approach was designed to evaluate the top 20 proteins associated with EV (exocarta) in our preparations. EV-associated miRNA and protein profile was determined by small RNA sequencing and Mass Spectrometry SWATH Analysis, respectively. Generalised additive modelling was used to model protein and miRNAs abundance as a function of progression of OVCA, while pathway analysis was performed using Ingenuity.

Results: Using an EV capture technology (EXO-NET) around 20% of the total circulating particles was isolated, and an enrichment of proteins CD63, CD9, Alix, TSG101 and CD81 compared to total plasma was observed. A total of 18 out of the top 20 proteins associated with EVs in the Exocarta were identified in our preparations. Of the total proteins identified (1517) within EV, 33% (599) changed in abundance as function of the progression of OVCA (p< 0.05). Our modelling analysis identified a total of 20 clusters with different trends, in which 116 proteins (including Carboxypeptidase E, Adenosine deaminase, and Sex hormone-binding globulin) increased with the progression of ovarian cancer. We identified 703 miRNAs within EV, and 24% (171) changed with the progression of OVCA. A total of 10 clusters with different trends were identified, in which 7 miRNAs (miR-503-5p miR-181d-5p, miR-548ay-5p, miR-548ad-5p, miR-3157-5p, miR-135a-5p, miR-6815-5p) continuously increased with the progression of OVCA. Bioinformatic analysis showed that the top functions associated with the proteins and miRNAs with EV across progression of OVCA are inflammation, lipid metabolism, transport, and binding of tumour cells.

Summary/Conclusion: The EV proteome and miRNA profile across OVCA demonstrates dramatic changes associated with the progression of the disease. Such information is important to understand the physiology of OVCA and the development of biomarkers to differentiate women with early stages of OVCA and determine the response to chemotherapy.

Funding: NHMRC, MRFF, INOVIQ.

OT03: Heart & Lung

Chairs: Augusto Zani, Carlos Salomon

Location: Room 608/609

11:30 - 13:00

OT03.1 | Magnetic resonance imaging and magnetic particle imaging of induced pluripotent stem cell derived EVs in a mouse model of myocardial infarction

Wenshen Wang[1]; Safiya Aafreen[2]; Olesia Gololobova[3]; Kenneth W. Witwer[4]; Jeff Bulte[5]; Robert Weiss[6]; Guanshu Liu[7]
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Introduction: Stem cell-derived extracellular vesicles (EVs) have drawn increasing interest for treating cardiovascular diseases, which would greatly benefit from non-invasive imaging that can define the quantitative, temporal, and spatial distribution of administered EVs. The goal of this study is to develop an MRI/MPI bimodal imaging approach for tracking and quantifying induced pluripotent stem cell (iPSC)-derived EVs in the injured heart.
Methods: iPSC-EVs were isolated from the conditioned medium of iPSCs using commercial size exclusion columns (70 nm qEV column, iZON) and characterized in accordance with MISEV2018. Superparamagnetic iron oxide (SPIO, 20 nm, Superbranche Inc) nanoparticles were conjugated with histidine tags and then encapsulated into EVs by electroporation, followed by Ni-NTA purification. Right after reperfusion in mouse hearts that had undergone a 35-min ligation of the left coronary artery, 1 × 109 (determined by NTA) magnetically labeled iPSC-EVs were administered intramyocardially to peri-infarct areas. MRI and MPI were conducted on day 1 and day 3.
Results: We first optimized the electroporation protocol method to prepare EVs with a high SPIO loading rate and preserved EV characteristics, including size, protein content, RNA content, and immunomodulatory effects on macrophages. We then optimized the imaging procedures and accomplished an MRI and MPI detectability of approximately 4 × 107 EVs, which provided sufficient sensitivity to detect EVs in the therapeutic dose range in mouse hearts.
Summary/Conclusion: We established an MPI/MRI method for tracking iPSC EVs and demonstrated its utility in detecting intramyocardial injected iPSC-EVs in a mouse model of myocardial infarction (MI). The established imaging method provides a theranostic approach for developing and clinical translation of EV-based therapy for combating various cardiovascular diseases.
Funding: NIH R33HL161756 and S10 OD026740.
Keywords: iPSC EV, myocardial infarction, magnetic resonance imaging and magnetic particle imaging

OT03.2 | Novel mouse models to explore the immunomodulatory role of cardiomyocyte-derived extracellular vesicles following myocardial infarction

Lélia Borowski[1]; Cécile Devue[1]; Thomas Nipoti[2]; Paul Alayrac[3]; Jean-Sébastien Silvestre[1]; Stéphane Camus[1]; Chantal M. Boulanger[1]
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Introduction: Cardiovascular diseases remain the leading cause of death worldwide. Following myocardial infarction (MI), inflammatory cells are mobilized from distant compartments towards the ischemic zone to coordinate tissue remodeling. As mediators shaping this inflammatory response are poorly defined, we aimed to determine the role of extracellular vesicles (EVs). We previously observed that fluorescent-labeled EVs released by cultured cardiomyocytes (CM-EVs) are preferentially taken up by spleen macrophages. In order to decipher the mechanisms whereby CM-EVs shape the inflammatory response and influence post-MI remodeling, we developed two genetic mouse models to specifically characterize CM-EV composition and biological tropism on one hand, and to demonstrate the functionality of CM-EV transfer on the other hand.
Methods: These mouse models express in their CM either the Cre recombinase alone (Myh6-Cre) or in combination with fluorescent reporters (Myh6-Cre/mTmG). Intracardiac and circulating EVs were isolated from MI or sham-operated control

mice by differential ultra-centrifugations. CM-EVs derived from Myh6-Cre/mTmG mice were immunopurified while EVs from Myh6-Cre were characterized by Western blot. To investigate the immunomodulatory role of CM-EVs on macrophages, we used peritoneal macrophages isolated from Rosa26/mTmG mice as a model of tissue-resident macrophage. Macrophage GFP expression was used as a proxy for functional transfer of biological information from CM-EVs to macrophages and quantified by flow cytometry.
Results: We showed in Myh6-Cre/mTmG mice that intracardiac and circulating GFP+ CM-EVs were enriched after MI. These data show that CM-EVs accumulate in the heart and reach the circulation after MI. Then we confirmed intravesicular CRE expression in intracardiac EVs isolated from Myh6-Cre mice. Finally, we show that Rosa26/mTmG macrophages express GFP upon incubation with intracardiac or circulating Cre+ EVs, but not from Cre- EVs, confirming functional transfer.
Summary/Conclusion: With these genetic mouse models, we are now able to specifically and accurately characterize, track and decipher the immunomodulatory role of CM-EVs in the context of MI.
Funding: FRM Equipe - EQU202003010767.
Keywords: myocardial infarction, inflammation, cardiomyocytes, macrophages, extracellular vesicles

OT03.3 | Placental extracellular vesicles provide long-term protection against cardiovascular disease in spontaneously hypertensive rats

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Introduction: Cardiovascular disease (CVD) accounts for an estimated 17.9 million deaths yearly. Epidemiological evidence shows increasing numbers of pregnancies protect women against CVD in later life. A precursor to CVD is endothelial cell activation. During normal pregnancy, the placenta extrudes vast numbers of extracellular vesicles (EVs) into the maternal circulation. These EVs are thought to protect endothelial cells from activation. We hypothesised that placental EVs decrease the risk of CVD following normotensive pregnancy.
Methods: EVs isolated from explants of placentae from normotensive pregnancies or control (bovine serum-derived) EVs were administered, i.v. to groups (n = 17) of 12-week-old female spontaneously hypertensive rats (SHR). Systolic blood pressure (SBP) and cardiovascular function were monitored over 12 months using a tail cuff and ultrasound, respectively. Data were normalised to baseline. Small artery remodelling and vasoreactivity of SHRs were investigated by histology and wire myography.
Results: Three months post-EV exposure, SBP in the normotensive group had increased significantly less than the control group ($p = 0.02$), with the difference persisting for 6 months. The SBP of control animals increased by 42.64 mmHg compared to an increase of only 31.88 mmHg, after one year. Echocardiography showed deteriorating cardiovascular function in the control rats that were reduced in the animals that received placental EVs. Compared with the control group, SHRs exposed to placental EVs had significantly decreased small artery remodelling ($p = 0.004$) and renal interstitial fibrosis ($p = 0.02$). Wire myography revealed a significant increase in vasoconstriction in response to acetylcholine (Ach) in vessels exposed to normotensive placental EVs.
Summary/Conclusion: Our data provide the first evidence that long after administration, normotensive placental EVs can mitigate hypertension and cardiovascular damage that occur naturally in SHRs. This work goes some way to explaining why pregnancy protects mothers from later CVD.
Funding: Health Research Council (HRC) 21/209.

OT03.4 | Administration of extracellular vesicles derived from amniotic fluid stem cells promotes growth and maturation of fetal hypoplastic lungs

Lina Antounians[1]; Rebeca Lopes Figueira[1]; Bharti Kukreja[2]; Cadia Chan[3]; Michael Wilson[2]; Brian Kalish[2]; Augusto Zani[1]
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Introduction: Incomplete lung development, also known as pulmonary hypoplasia (PH), is a recognized cause of neonatal death. In search for a regenerative therapy, we reported that administration of rat amniotic fluid stem cell extracellular vesicles (AFSC-EVs) restored features of impaired lung branching morphogenesis in experimental PH. Herein, we aimed to 1) identify if in

vivo AFSC-EV administration promotes lung maturation; 2) determine AFSC-EV cargo miRNAs that induce lung cell-specific responses.

Methods: AFSC-EVs were isolated from rat AFSC conditioned medium by differential ultracentrifugation (100,000g) and characterized by size (NTA), morphology (TEM), and expression of canonical markers (CD63, TSG101, Flot-1; Western blot, WB). We induced fetal PH by nitrofen gavage to dams at embryonic day (E)9.5. At E18.5, fetuses received an intra-amniotic injection of either saline (control+PBS, n = 8; PH+PBS, n = 8) or AFSC-EVs (PH+AFSC-EV, n = 9). At E21.5, lungs were harvested and assessed for branching morphogenesis (mean linear intercept, radial alveolar count) and expression of maturation markers (Pdpn, Sftpc; qPCR/WB).

Lung nuclei were subjected to single nucleus RNA-seq (snRNA-seq; 10X Seurat/R). Cargo-seq of AFSC-EVs was performed with SeraMir/NextSeq. AFSC-EV cargo miRNA levels were correlated with differentially expressed mRNA in PH+AFSC-EV lungs (MultiMiR).

Results: Lung branching morphogenesis was impaired in PH+PBS vs. control+PBS fetuses and restored to control levels by AFSC-EVs. Similarly, Pdpn, and Sftpc expression was reduced in PH+PBS vs. control+PBS group and restored to control levels in PH+AFSC-EV group. snRNA-seq analysis identified macrophages and inflamed epithelial and mesenchymal cells unique to PH+PBS lungs. A network analysis of AFSC-EV miRNAs and downregulated mRNAs in PH+AFSC-EV lungs revealed 820 miRNA-mRNA targets (32 validated) that were involved in inflammatory responses.

Summary/Conclusion: In vivo AFSC-EV administration in experimental PH restores features of impaired lung development and can regulate key genes relevant to pathological processes. AFSC-EV-based therapy could be a promising avenue to treat fetal PH.

Funding: SickKids Foundation, Canadian Institutes of Health Research.

OT03.5 | Activated T cells secrete extracellular vesicles in the allergic airway that enhance eosinophil viability

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Introduction: Extracellular vesicles (EVs) are small, secreted membrane particles that mediate intercellular communication by delivering cell-derived cargoes. We previously found that immune cells secrete EVs into the airways during allergic lung inflammation in mice. The goal of this study was to determine the contribution of T cells to allergic airway EVs, identify T cell EV protein cargoes, and assess the effects of T cell EVs on eosinophils, important cellular mediators of allergic inflammation.

Methods: To determine the contribution of T cells to airway EVs, allergic airway inflammation was induced in mice with T cell membrane labeling, and bronchoalveolar lavage fluid (BALF) was collected and subjected to single EV flow cytometry. To identify T cell EV protein cargoes, EVs were purified from primary mouse T cell culture media by size exclusion chromatography (SEC), ultrafiltration, and density gradient flotation for mass spectrometry. To assess effects of T cell EVs on eosinophils, bone marrow-derived eosinophils were treated with EVs purified from resting and activated T cell culture by SEC and ultrafiltration, and eosinophil viability and degranulation were assessed.

Results: We found that 1% of EVs (6 million EVs per airway) in BALF of mice with induced allergic airway inflammation were of T cell origin, with 75 EVs detected per airway T cell. We identified a total of 957 T cell EV proteins by mass spectrometry, including cell membrane proteins known to be involved in eosinophil survival, such as CD40L, CD47, and CD22. Finally, we found that EVs secreted by activated T cells, but not resting T cells, enhance eosinophil viability with no effect on degranulation. This effect on viability is EV concentration-dependent and can be abrogated with surface protein shaving.

Summary/Conclusion: These results provide evidence that T cells secrete EVs carrying protein cargoes into the allergic airway and that T cell EVs may play a role in allergic airway pathology by improving eosinophil survival through a mechanism that involves EV surface protein(s). T cell EVs may be important and potentially targetable components of allergic and other immune-mediated reactions.

Funding: NIH DP2-HL152426 (HHP), NIH T32GM008554 (KB).

Keywords: T cells, eosinophils, allergic inflammation, asthma,

OT04: Therapeutics

Chairs: Jonathan Anderson

Location: Room 606/607

11:30 - 13:00

OT04.1 | Preclinical study of intraocular EV therapy from stem cell-derived retina pigment epithelium for retinal degeneration

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Introduction: Optimizing bioactivity from different stages of stem cell differentiation and establishing scalable and good manufacturing practice (GMP) compatible EV production are bottlenecks in developing extracellular vesicle (EV) based ocular therapeutics. To overcome this challenge, we examined the bioactivity of stem cell-derived sEV from different days of cell maturation and tested microfluidic tangential flow filtration (mTFF) device for sEV separation.
Methods: EVs were separated from the cell culture supernatant (CCS) of the human embryogenic stem cell-derived polarized retinal pigment epithelium (hESC-PRPE-sEV) by gradient ultracentrifugation (UC) or ExodiscTM, a commercially available microfluidic tangential flow filtration device (mTFF) device. CCS of hESC-PRPE was collected from 16 days (early maturation) or 70 days (late maturation) of cultures at passages # 7–8. The size, numbers and morphology of sEV were characterized by NTA and TEM. Tetraspanin, and multi-surface epitope heterogeneity of sEV were further assessed by Exoview and MACSPlex Exosome Kit. Photoreceptor (PR)-preserving therapeutic efficacy of the intravitreal (IVT) injection of sEV was assessed in the retinal degeneration model, Royal College of Surgeons (RCS) rats by optical coherence tomography (OCT), histopathology, and electroretinogram (ERG).
Results: The EV yield (EV particles/ml of input) and EV preparation time was improved by 5 times in ExodiscTM compared with gradient UC. Meanwhile, EV characteristics and tetraspanin heterogeneity, and multiplexed phenotype remained similar between sEV separated from ExodiscTM and gradient UC. RCS rats treated with IVT injections of hESC-PRPE-sEV delayed retinal degeneration by preserving PR and their function. This therapeutic efficacy was not significantly different between UC and ExodiscTM-separated EVs. Meanwhile, sEV from early matured hESC-PRPE at 16 days of culture had improved therapeutic efficacy than sEV from late matured hESC-PRPE at 70 days of culture.
Summary/Conclusion: Our results suggest that cell maturation of stem cell-derived PRPE affects their PR-rescuing bioactivity in a retina degeneration model. mTFF device holds the potential to enable large-scale and GMP-compatible EV production. Further studies are needed to optimize EV production, stability, and other variabilities for EV-derived therapeutics.
Funding: NIH/NEI K12EY028873 and 1R56EY034193-01;NIH/NEI P30EY029220; Unrestricted Grant to the Department of Ophthalmology from Research to Prevent Blindness, New York.
Keywords: intraocular EV therapy, stem cell, retina degeneration, microfluidic tangential flow filtration device

OT04.2 | Extracellular vesicles as disease mediators and therapeutic agents in Duchenne Muscular Dystrophy

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Introduction: Duchenne muscular dystrophies (DMD) is a recessive, fatal, X-linked disease caused by mutations in the dystrophin gene. which plays a critical role in the maintenance, integrity and normal functions of muscle cells and asymmetric division of satellite cells.
Extracellular vesicles (EVs) are secreted nanovesicles that contain a diverse cargo which reflects the pathophysiological status of the secreting cells and have emerged as circulating biomarkers and disease mediators in pathological conditions. Here, we studied the ability of EVs secreted from DMD muscles to induce pathological processes in healthy satellite and muscle cells.

Methods: The effects of EVs isolated from DMD patient serum and skeletal muscle cultures were analyzed on the myogenic and fibrogenic differentiation of healthy skeletal muscle cells. Expression of specific non-coding RNAs in DMD EVs was analyzed using RNA sequencing and the ability of placenta mesenchymal stromal cell-derived EVs (PL-EVs) to inhibit pathological processes induced by patient EVs was studied.
Results: DMD serum EVs inhibited the proliferation of satellite cells with a moderate effect on their differentiation. In contrast, EVs isolated from cultured DMD muscle cells inhibited the myogenic differentiation of the cells while increasing their fibrogenic phenotype. The DMD muscle EVs expressed higher levels of miR-21, TALNEC2 and lower H19, miR-145 and miR-29c compared with healthy muscle EVs. Using knock down experiments, we found that miR-21 and TALNEC2 mediated some of the DMD muscle EV effects indicating that DMD EVs can induce pathological processes in healthy muscle cells via the delivery of non-coding RNAs. We recently reported that PL-EVs exert therapeutic effects in a DMD mouse model. We therefore analyzed the ability of PL-EVs to inhibit the effects of DMD EVs on the function of muscle cells. We found that PL-EVs altered the cargo of EVs secreted from DMD muscle cells and their pathological effects on normal muscle functions. Similarly, PL-EVs inhibited the pathological impact of DMD serum EVs.
Summary/Conclusion: EVs secreted from DMD muscle cells and patient serum induce pathological changes in normal satellite and muscle cells by transferring specific non-coding RNAs. These effects are inhibited by therapeutic placenta MSC-derived EVs.
Funding: Ministry of Science, Israel and Little Step Association.
Keywords: duchenne muscular dystrophy, miRNA, lncRNA, fibrosis, placenta MSCs

OT04.3 | Reprogramming extracellular vesicles as a therapeutic approach for inflammatory liver disease

Priyanka Ghosh; Kyo Sasaki; Isabel Aranzazu Pulido-Ruiz; Kayla E. King; Steven A. Weinman; Ann L. Wozniak
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Introduction: Macrophage (MΦ)-derived extracellular vesicles (EVs) play a key role in intercellular communication. In the liver, EV signaling is linked to both the progression and resolution of inflammatory diseases including nonalcoholic steatohepatitis (NASH). We previously showed that caspase-1-mediated cleavage of the trafficking adaptor protein RILP changes EV content generating pro-inflammatory EVs. Preventing RILP cleavage with a dominant negative, non-cleavable analog of RILP (ncRILP) reversed this effect. The aim of this study was to determine if manipulation of the RILP-cleavage state in MΦs could prevent the transmission of an injury signal to hepatocytes.
Methods: Cell crosstalk was assessed in transwell co-cultures with LPS/ATP-treated MΦs in the upper chamber and hepatocytes in the lower chamber. MΦs and monocytes were transfected using lentiviral vectors. Effects on target hepatocytes were assessed by RT-PCR and ELISA. EVs were isolated by differential ultracentrifugation and characterized by western blot. Human peripheral blood monocytes were prepared from patients with inflammatory (NASH) and non-inflammatory (NAFLD) fatty liver disease.
Results: LPS-ATP treatment of MΦs caused injury to hepatocytes in the transwell system. Expression of ncRILP in MΦs had no effect on cytokine production but it blocked the transmission of an injury signal to hepatocytes. Similarly, purified EV preparations from ncRILP-expressing MΦs directly suppressed inflammatory effects in hepatocytes. To assess the disease relevance of this finding we examined the RILP cleavage state in circulating monocytes from patients. Monocytes from NASH patients had increased RILP cleavage compared to those from NAFLD patients. Treatment of NASH monocytes with ncRILP-derived EVs abrogated the inflammatory phenotype.
Summary/Conclusion: Macrophages transfer an inflammatory injury signal to hepatocytes via EVs. Prevention of macrophage RILP cleavage reprograms this EV signal and suppresses injury transmission.
Funding: R01AI147276.

OT04.4 | CD39-rich endothelial extracellular vesicles regulate platelet purinergic signaling in sickle cell disease

Tomasz Brzoska[1]; Elizaveta V. Menchikova[2]; Tomasz W. Kaminski[2]; Omika Katoch[2]; Rikesh K. Dubey[2]; Stevan P. Tofovic[2]; Edwin K. Jackson[2]; Mark T. Gladwin[3]; Prithu Sundd[2]
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Introduction: Acute chest syndrome (ACS) is a type of acute lung injury and the leading cause of mortality among sickle cell disease (SCD) patients. ACS is often preceded by thrombocytopenia and involves massive thrombosis across pulmonary artery branches. Although, released during hemolysis, adenosine diphosphate (ADP) is known to activate platelets by stimulating their P2Y1 and P2Y12 purinergic receptors, antagonists of P2Y12 have not shown any benefit in ACS therapy, justifying the need for

better understanding of purinergic signaling in SCD. CD39 maintains ADP homeostasis by degrading excessive ADP. Though CD39 inhibits ADP-dependent platelet activation, its role in the pathophysiology of SCD is still unidentified.
Methods: To evaluate SCD platelet response to ADP in vivo we used a state-of-the-art intravital lung microscopy and a novel in vivo model of ADP-triggered thrombocytopenia in transgenic humanized SCD mice. Additionally, both mouse and human SCD platelet ADP-dependent aggregation was examined using in vitro turbidimetric aggregation assay. Hemin, a major host-derived damage associated molecular pattern associated with SCD, was incubated with Human Lung Micro-Vascular Endothelial Cells (HMVEC-L). Extracellular vesicles (EVs) were obtained from mouse and human plasma samples, and cell culture medium samples using size exclusion chromatography. Obtained EVs were subjected to nanoparticle tracking analysis. EVs CD39 levels and activity were determined using ELISA, western blot and malachite green phosphate assays, respectively.
Results: Intravital lung microscopy and in vivo thrombocytopenia studies revealed that intravascular administration of ADP triggered acute pulmonary thrombosis in control but not in SCD mice. In vitro aggregation study confirmed our in vivo findings and demonstrated impaired SCD mouse and human platelet response to ADP, which was, further, significantly augmented by a specific CD39 inhibitor. Hemin triggered shedding of CD39-bearing EVs by HMVEC-L. Indeed, we discovered that isolated from mouse and human plasma endothelial cell-derived SCD EVs expressed higher CD39 levels and activity in comparison to control EVs.
Summary/Conclusion: Our findings suggest that CD39-bearing EVs prevent ADP-mediated platelet aggregation and pulmonary thrombosis in SCD. Current study explains why P2Y12 blockers are not effective in SCD therapy.
Funding: NIH-NHLBI 1R01HL128297-01, American Heart Association 18TPA34170588, Vascular Medicine Institute startup funds, American Society for Hematology Scholar Award, American Society for Hematology Research Restart Award, Pittsburgh Heart, Lung and Blood Vascular Medicine Institute Pilot Project Program in Hemostasis and Vascular Biology (P3HVB).
Keywords: sickle cell disease, extracellular vesicles, platelets, purinergic signaling, CD39

OT05: Single EV Analysis

Chairs: Tijana Jovanovic-Talisman, Randy Carney

Location: Ballroom 6BC

15:15 - 16:45

OT05.1 | Unravelling the morphology of EVs with super-resolution microscopy by means of model systems

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Introduction: Single-molecule localization microscopy (SMLM) is gaining interest in the extracellular vesicle (EV) field thanks to high resolution. In SMLM, diffraction-limited images are used to localize single fluorophores with nanometric precision, generating a super-resolution picture. Even if used mostly for surface markers, SMLM allows evaluation of several EV features, such as 3D morphology and vesicle cargo.
However, SMLM techniques offer lower axial (Z) resolution (~50nm vs ~15nm in XY), an issue for precise 3D reconstruction of small EVs. Further optimization is thus required. We used large unilamellar vesicles (LUVs) with typical diameter of 100 nm. These synthetic lipid vesicles with controlled size distribution, are used as model system to mimic in a first approach the EVs, to optimize the EV reconstruction and to improve EV characterization with SMLM.
Methods: The LUVs were produced with different lipids composition (DOPC, cholesterol, DHPE-Cy5). To perform SMLM imaging, the LUVs were captured and immobilized on an optimized surface and the effect of the lipid composition on the imaging was also evaluated. In addition, to determine with higher accuracy and to correct the axial reconstruction, we imaged other reference structures with a precise and known size, such as DNA origami. Finally, we applied this procedure to visualize and assess the morphology and the size of EVs.
Results: This approach allowed the accurate size determination of mono- and polydisperse LUV preparations, data confirmed by other established techniques, including NTA. The lipid composition seemed to affect the LUV interaction with the capture surface, representing a potential crucial point in the imaging of EVs with SMLM. The optimized 3D reconstruction allowed an improved evaluation of the size and the 3D morphology of both the LUVs and the EVs.

Summary/Conclusion: In this work we optimized the 3D resolution of our SMLM microscope in imaging LUVs and EVs, allowing a better size and 3D morphology evaluation. The more precise 3D reconstruction will improve the future characterization of EVs, the cargo assessment and the identification of different EV subpopulations with SMLM.
Funding: Research supported by the company Abbelight and the CNRS through the "Plan France Relance."
Keywords: super-resolution microscopy, single-molecule localization microscopy, single-EV imaging, standardization

OT05.2 | Label-free visualization and optical characterization of extracellular vesicles in-situ and in-vivo at single-vesicle resolution using multimodal multiphoton imaging

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Introduction: Label-free multimodal multiphoton imaging is used to image and optically characterize physical and biomolecular properties of extracellular vesicles (EVs). Real-time imaging visualizes the spatial-temporal distribution of EVs in situ in tissue microenvironments, in biofluids, or even in vivo. Optical signatures identify cancer-associated EVs and with AI analysis, demonstrate correlations to disease aggressiveness.
Methods: The platform technology developed by our group for EV imaging and characterization is based on Simultaneous Label-free Autofluorescence Multiharmonic (SLAM) microscopy. Co-registered images are collected from two- and three-photon excited autofluorescence intensities/lifetimes of NAD(P)H and FAD, which reflect EV and parent cell redox state. Second/third harmonic generation provided structural imaging of collagen and lipid/aqueous interfaces. Coherent anti-Stokes Raman scattering imaged vibrational signatures from lipids and proteins. Label-free imaging and characterization of EVs in cell cultures, animal models, and from tissues, serum, and urine from human breast cancer and healthy subjects was performed. All studies were performed under approved IACUC and IRB protocols.
Results: Optical signatures revealed changes in EV properties based on metabolic states (redox ratios). Increased metabolic activity in human breast tumors was reflected in EV signatures from label-free images of the tissue collected intraoperatively using a portable imaging system. AI analysis of multimodal images was applied for spatial biology visualization of the tissue microenvironment and association of EVs to various cell types and vasculature. Label-free characterization of EVs from urine, serum, and tissue of human breast cancer subjects showed trends indicative of the presence and aggressiveness of the disease.
Summary/Conclusion: Label-free multimodal nonlinear imaging/characterization of EVs represents a platform technology. With single-EV resolution spatiotemporal imaging, new investigations into the dynamics, distribution, and trafficking of EVs is possible to elucidate biological processes and generate new optical biomarkers for clinical diagnosis and treatment monitoring.
Funding: NIH P41EB031772 and R01CA241618.
Keywords: label-free, nonlinear imaging, artificial intelligence, spatial biology, microenvironment, cancer

OT05.4 | Imaging of surface microdomains on individual extracellular vesicles in 3-D

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Introduction: EVs, including exosomes, are too small to be seen by conventional microscopy. Direct stochastic reconstruction microscopy (dSTORM) bypasses the diffraction limit of light. This allows us to not only to visualize individual EVs, but also tetraspanin (CD81, CD63, CD9) complexes on their surface. Previous research found that EVs are enriched in tetraspanins and that tetraspanin enriched microdomains (TEMs) are essential for EV biogenesis and cargo sorting.
Methods: EVs were purified using tangential flow filtration and chromatography. We then stained the EVs using a membrane dye, seeded the EVs on slides or in glass-bottom wells, and incubated with fluorescent-conjugated tetraspanin antibodies. EVs were then placed in an oxygen-scavenging buffer and imaged by dSTORM. Images were reconstructed and analyzed using R.
Results: Through dSTORM, we were able to see tetraspanin clusters on the surface of EVs in 3D. We were able to characterize EV heterogeneity and to compute signal clusters on individual EVs. Finally, we used a variety of methods for staining to determine what works well.
Summary/Conclusion: Single particle EV detection of tetraspanins supports the existence of TEM on EVs under physiological conditions.

Funding: This work was supported by R01-CA228172, P01-CA019014 to DPD, and UNC at Chapel Hill. AC and RM received funding through T32 5T32AI007151. DPD has received material support from ONI and Cytiva Inc.
Keywords: tetraspanin, microscopy, dSTORM

OT05.5 | Multiplexed analysis of single EV (MASEV) reveals unique biomarker composition with diagnostic impact

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Introduction: Introduction: Extracellular vesicles (EV) represent a promising avenue for cancer diagnostics because shed vesicles contain cargo derived from parental cells and are abundant in later-stage disease. An important advance has been the introduction of single EV (sEV) analytical techniques. Obtaining multiplexed data has remained challenging yet essential in defining vesicle subpopulations and identifying rare cancer-specific phenotypes early in the disease. We hypothesized that bioorthogonal chemistry could be used to develop an efficient sEV multiplexing tool (MASEV, multiplexed analysis of single EV).
Methods: Methods: The MASEV technology utilizes a specialty C2-symmetric TCO linker between an antibody (Ab) of interest and a fluorochrome (Fl). Upon the addition of functionalized tetrazine scissors (HK-Tz), the fluorochrome is cleaved from the antibody in minutes, leading to fast "de-staining". EV can then be stained for a subsequent round by re-using the same set of fluorochromes. Cycling multiple rounds of Ab-C2TCO-Fl thus enables highly multiplexed biomarker profiling of single EV.
Results: Results: We interrogated the concurrence of proteins in single EV via a 4-cycle, 12-plex panel, consisting of EV and oncogene biomarkers. We developed a simple flow-cell with >99% EV retention and low background. We find 'ubiquitous' tetraspanin proteins present in a fraction of EV: across four cell lines, 39% of EV had no tetraspanins, 39% had one, 22% had two, and only 5% had all three. Profiling oncogene RAS-positive cell line EV indicates pan-tetraspanin capture via affinity purification would miss 35–45% of KRASmut positive EV and up to 80% with CD63 alone. Dimensional analysis of the full 12-plex panel on >12000 sEV data demonstrated clear separation of EV by cell-line origin, whereas limited biomarker multiplexing with CD9, CD47, and EGFR, such as done by spectrally resolved 3-color imaging, does not allow clear separation.
Summary/Conclusion: Summary: Several single EV analytical methods have previously been described, but virtually all of them are limited to a few "color channels" during a single round of analysis. Current research indicates the MASEV method is a vast improvement in multiplexing and performing analyses on simple flow chambers. MASEV is inexpensive, designed for cancer biomarker analysis, and can be extended to other vesicle types. The next step is to use the validated method to analyze clinical samples in prospective well-controlled trials.
Funding: R21CA236561 and P01CA069246. JS, SF, and HP supported by T32CA079443.
Keywords: exosome, extracellular vesicle, liquid biopsy, diagnostic, bioorthogonal click chemistry, single vesicle analysis

OT06: Cancer Immunology and Immunotherapy

Chairs: Robert Coffey, Sheela Abraham

Location: Ballroom 6A

15:15 - 16:45

OT06.1 | Extravesicular CD147 is a major player in orchestrating adaptive immunity-mediated tumor regression in vivo

Laura Patras[1]; Kayleen Bailey[2]; Miho Hatano[2]; Angela Di Giannatale[2]; Ayuko Hoshino[3]; Daniela Freitas[4]; Henrik Molina[5]; Celso Reis[4]; Jack Bui[6]; Doru Paul[7]; Irina Matei[8]; David C. Lyden[9]
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Introduction: Unveiling mechanistic insights into the immunoregulatory role of tumor-derived extracellular vesicles (TEV) could improve the outcome of immunotherapies. To study TEV-mediated crosstalk between cancer and adaptive immunity, we used a murine fibrosarcoma model of progressing and regressing tumors. We hypothesized that regressor and progressor TEV cargo induces functional changes in adaptive immune cells, regulating antitumor immune responses.
Methods: Small EVs were isolated by sequential ultracentrifugation and characterized by nanoparticle tracking analysis, transmission electron microscopy, western blot, and mass spectrometry. CRISPR/Cas9 was used to generate CD147 knockout (KO) progressor cells. TEVs were injected into the footpad for biodistribution and chronic education experiments (10 μg of EV protein every 3 days). Immunofluorescence and flow cytometry were used to assess intratumor immune cell infiltration. Intratumor and lymph node immune cell populations as well as TEV-induced changes in these cells were assessed by scRNAseq.
Results: Our study shows that chronic cross-education of naïve mice with progressor and regressor TEV prior to tumor inoculation could skew tumor growth in vivo towards a regressor or progressor phenotype, respectively, through a direct effect of EVs on T cells. Proteomic analysis of EVs identified CD147, a pan-cancer EV biomarker and immunoglobulin superfamily member with pleiotropic functions as a mediator of progressor phenotype. CD147 ablation in progressor cells was sufficient to induce complete tumor regression in vivo. Chronic education with regressor or CD147 KO TEV inhibited progressor tumor growth and promoted CD4+ and CD8+ T cell tumor infiltration. Importantly, regressor intra-tumor T cells displayed a higher activation state and antitumor functions, whereas T cells isolated from progressor tumors displayed an exhausted phenotype.
Summary/Conclusion: This study reveals a novel role for extravesicular CD147 in modulating antitumor immune responses, identifying this protein as a promising therapeutic target for cancer immunotherapy.
Funding: STOP Cancer Foundation, Hartwell Foundation, Children's Cancer and Blood Foundation.
Keywords: extracellular vesicles, adaptive immunity, tumor regression

OT06.2 | Role of non-small cell lung cancer (NSCLC) tumor-derived extracellular vesicles in the ripple effect leading to hyperprogressive disease upon immune checkpoint inhibition

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Introduction: Hyperprogressive disease (HPD) is a paradoxical boost in tumor growth reported in different types of cancer treated with immunotherapy. In 2018 we identified the role of macrophages (MΦ) in HPD in NSCLC patients. However, mechanisms and effectors underlying the observed HPD-related Tumor microenvironment (TME) reprogramming and its back-effect on tumor cells are still largely unknown.
Methods: We analyzed HPD in vitro in H460, A549, H1299, and Calu-1 NSCLC cell lines, and in vivo in xenografts and in patient-derived xenografts. Extracellular Vesicles (EVs), isolated by ultracentrifuge from 48h-serum free cell conditioned medium, were characterized by NTA (Nanosight NS300), and TEM; and EVs-associated markers (CD9, CD81) by Western Blot. Effects on bone marrow-derived MΦ were evaluated after 24h EVs exposure
Results: In vitro and in vivo analysis showed a 20% increase in proliferation and xenograft growth after Nivolumab treatment for H460 and A549, whereas H1299 and Calu-1 cells were unaffected. HPD cells-EVs polarized MΦ towards a peculiar pro-inflammatory phenotype sharing both M1 and M2 features and characterized also by the over-expression of the CD63/CD33/PD-L1 "metagene", previously identified as a marker of HPD-related MΦ in NSCLC patients. Preliminary data on the back effect on tumor indicated that, before treatment, CD133+ cancer-initiating cells (CICs) expressing PD-1 (CD133+/PD-1+) frequency was higher in HPD than in non-HPD models in vivo (3.5-fold increase), but not in vitro, indicating a role of the TME in modulating HPD-related CICs. Moreover, CD133+/PD1+ cells increased after treatment both in vitro and in vivo (3- and 1.6-fold increase, respectively) only in HPD models.

Summary/Conclusion: Our data demonstrate the role of tumor cell-derived EVs in the reprogramming of MΦ toward an HPD-related phenotype. Moreover, our data suggest a back effect of the TME that may feed a subset of PD-1 expressing CICs involved in HPD.
Funding: Italian Association for Cancer Research (AIRC).
Keywords: non-small cell lung cancer, immunotherapy, hyperprogressive disease, macrophages, cancer initiating cells

OT06.3 | Pancreatic tumors with impaired secretion of extracellular vesicles are driven by a pro-tumorigenic immune response that is sensitive to immunotherapy

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Introduction: Pancreatic ductal adenocarcinoma is characterized by an immune-cold phenotype, making this type of cancer a poor candidate for immunotherapy. However, a subset of PDAC patients have lymphocytic infiltrate. The biological basis for these apparently opposing presentations is not known. Untangling the mechanisms that govern the recruitment of immune cells into hot PDAC tumors may provide new opportunities for treatment and to convert cold into hot PDAC tumors.
The production and secretion of extracellular vesicles (EVs) plays a central role in pancreatic carcinogenesis. Cancer secreted EVs (cEVs) have also been shown to modulate cells of the immune system, both stimulating or inhibiting its action. Here we questioned if targeting secretion of cEVs by pancreatic cancer cells could alter the anti-tumor immune response.
Methods: In order to impair secretion of cEVs we have targeted the GTPase RAB27A. This protein is a central player in EVs exocytosis and it is well documented that RAB27A knockdown results in impaired exocytosis of EVs.
Results: Our work shows that impaired exocytosis of EVs leads to a change in the immune landscape of PDAC tumors characterized by an increase in MRP8+ myeloid cells and Th17 T cells. This change in the immune infiltrate translates into a pro-tumorigenic phenotype with earlier disease onset and worse overall survival. The mechanism underlying this change relies on decreased exocytosis of TSP1+ EVs, which regulate immune cell infiltration and correlate with increased MRP8+ myeloid cell infiltration. MRP8 acts upon CAFs inducing the release of IL6 and recruitment and differentiation of Th17+ T cells. Taking advantage of this knowledge, we demonstrate that treatment with an anti-inflammatory drug or depletion of CD4+ T cells is sufficient to impair disease progression in PDAC GEMMs, specifically in low Rab27a-expressing tumors.
Summary/Conclusion: In keeping with our results, we identified a subset of PDAC patients characterized by low RAB27A expression that could benefit from anti-inflammatory and immunotherapy. These results may pave the way for the use of immune conversion strategies that may turn PDAC into an immunotherapy amenable cancer.
Funding: The work was supported by PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund and national funds through FCT—Foundation for Science and Technology POCI-01–0145-FEDER-32189. Programa Operacional Regional do Norte and co-financed by European Regional Development Fund under the project "The Porto Comprehensive Cancer Center" with the reference NORTE-01-0145-FEDER-072678 - Consórcio PORTO.CCC – Porto.Comprehensive Cancer Center. NB is supported by (SFRH/BD/130801/2017), IB by FCT (SFRH/BD/144854/2019), and BA by FCT (PD/BD/135546/2018).
Keywords: In vivo modelling of disease

OT06.4 | A novel approach of T cell engineering by targeted exosomes delivering CRISPR/Cas9 system for PD-1 knock-out

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Introduction: Among emerging cancer therapies, immunotherapy using T-cell engineering is one of the most promising. The CRISPR/Cas system is one of the most common gene editing tools, but lacking an efficient and safe intracellular carrier is still the

main challenge for in vivo delivery applications. Exosomes as natural, non-immunogenic nanoparticles can be an appropriate candidate for CRISPR/Cas system delivery.
Methods: In this study, targeted exosomes against LFA-1 on T cells were isolated from HEK293T modified cells by Exo-spin kit. The purified exosomes were characterized by DLS, TEM, and western blotting, and their uptake by T cells was evaluated in vitro using flowcytometry. Two gRNAs against the PD-1 gene were cloned into the pX-459 vector, and targeted exosomes were loaded by these CRISPR/Cas9 plasmids. Then, T cells were subjected to exosomes, and disruption in the PD-1 gene was examined by T7 endonuclease assay and sequencing. To evaluate the cytotoxicity and cytokine release of modified T cells, CFSE staining and ELISA were performed, respectively.
Results: Flowcytometry results indicated that newly produced targeted exosomes could enter T cells more than non-targeted ones. pX-459 plasmids were loaded successfully into targeted exosomes and delivered to T cells functionally. CFSE staining and ELISA showed that knocking out the PD-1 gene in T cells, enhanced their cytotoxicity and cytokine release compared to non-treated T cells.
Summary/Conclusion: In the present study, targeted exosomes were applied to transfer the CRISPR / Cas9 system into T cells and were able to modify target cells genetically. This approach may improve and facilitate immunotherapy with no need to extract cells and perform ex vivo tests. To the best of our knowledge, this is the first study of genetic modification in T cells using targeted exosomes containing the CRISPR / Cas9 gene editing system.
Funding: This study was financially supported by Tarbiat Modares University.
Keywords: CRISPR/Cas 9, extracellular vesicle, gene editing, PD-1 knock-out, targeted exosome, T cell

OT06.5 | Unlocking the tumor-immune microenvironment by integrating bulk EV-RNAs with single-cell RNA-seq in liquid biopsies

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Introduction: Extracellular vesicles (EVs) have brought great momentum to the non-invasive liquid biopsy procedure for the detection, characterization, and monitoring of cancer. While our fundamental understanding of EVs exponentially increases, the mechanisms for cargo loading, inter-cellular communication, organ homeostasis, and association with the disease remain unknown. Here, we explore the fundamental question of whether EV-RNA signatures can be used to accurately report the cellular composition of the tumor microenvironment (TME).
Methods: To test our hypothesis, we compared bulk EV-RNAs from urine and serum bio-fluids (n = 10, 40 samples), tumor tissue biopsies (n = 5, 5 samples), and adjacent normal (n = 5, 5 samples) with the composition of the TME (n = 1, 2 samples) through single-cell RNA-seq. We used standardized RNAseq analysis methods (Bowtie, STAR, Samtools, Cell ranger, Seurat & Monocle) combined with mixed linear effect models for differential expression analysis (R, packages Dream, MEGENA). The approach developed during this study represents a methodological and conceptual advance to overcome the technical effects of integrating two different sequencing technologies in addition to unlocking cellular to EV-RNA comparison. While our previous results comparing EV-RNAs with their cellular origin revealed a non-linear correlation, common RNAs and proteins representative of lineage were identified (PMID: 35663013).
Results: We report an in-depth characterization of (1) EV-RNA signatures enriched in urine and serum bio-fluids, prostate cancer tumor, and adjacent normal tissue; (2) The immune landscape of the TME, including the identification of 2 tumor clusters that are potentially indicative of tumor clonal diversity. Next, we used the mRNAs identified in EVs as anchors to compare EV-transcriptomes to single cell clusters. This allowed us to identify EV-RNA signatures correlated (Rho > 0.7, $P < 0.001$) in specific cell types that are potentially informative of tumor and immune phenotypes. At the same time, stratifying EV-RNAs by gene biotype revealed a diversity of non-coding and coding RNAs potentially associated with the TME.
Summary/Conclusion: This proof-of-concept study provides novel insights for using EV-RNAs as minimally invasive tools to characterize the phenotype of the tumor microenvironment. Despite the limitations from our sample size, we lay the ground for more in depth studies unraveling EV-RNAs as a liquid biopsy alternative to report immune and tumor phenotypes.
Funding: Funding: P20CA264076 (NCI/NIH), R21AG078848 (NIA/NIH), Friedman Brain Institute at Mount Sinai.
Keywords: RNAs, transcriptome, single cell sequencing, extracellular vesicles, immune cells, prostate cancer

OT07: Upscaling: Chemistry, Manufacturing and Control

Chairs: Eva Rohde, Mario Gimona

Location: Room 608/609

15:15 - 16:45

OT07.1 | Advancing the manufacture of mesenchymal stromal cell-derived extracellular vesicles in stirred-tank bioreactors through enhanced bioprocess control

Marta Costa[1]; Beatriz Painho[2]; Margarida Costa[2]; Carolina Sousa[2]; Inês Carrondo[2]; Enrique Oltra[3]; Beatriz Pelacho[3]; Felipe Prosper[3]; Inês Isidro[2]; Paula Alves[4]; Margarida Serra[5]
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Introduction: Clinical translation of mesenchymal stromal cells (MSC)-derived extracellular vesicles (EV) is currently limited by their lack of scalability. Although most methods focus on maximizing EV production by increasing the cell culture surface area, besides integrating cell expansion in stirred-tank bioreactors (STB) with scalable downstream EV processing protocols, cell preconditioning strategies could further contribute to boost EV yields.
Methods: In this work, we have evaluated the effect of tailoring glucose concentration throughout MSC culture in STB on EV secretion yields, isolated by tangential flow filtration followed by size exclusion chromatography. Additionally, since the cell microenvironment can influence the properties of their derived EVs, Process Analytical Tools (PAT) tools were integrated in EV manufacturing workflows to monitor critical process parameters in real-time, therefore contributing to ensure that EV-based products retain their potency while manufactured at a clinically-relevant scale.
Results: Cell culture at low glucose concentration levels can maximize EV yields in a scalable bioprocess supported by cell expansion in STB, showing 1.4-fold increase in EVs secreted per cell relatively to MSC cultured at higher glucose concentration. Nonetheless, the distinct glucose levels observed on the harvest day highlight the need to standardize cell culture towards the implementation of more reproducible bioprocesses. To this purpose, we have shown that Raman Spectroscopy can be used to continuously monitor glucose levels in STB as an alternative to off-line glucose concentration measurements ($R2 = 0.923$).
Summary/Conclusion: MSC culture at low glucose concentration can improve their ability to secrete EVs, shedding light on the importance of standardizing cell culture conditions and, particularly, controlling metabolites concentration through the implementation of Raman Spectroscopy tools to develop more robust platforms for EV production.
Funding: This work was performed under the scope of the CardioPatch project (SOE4/P1/E1063) and iNOVA4Health (UIDB/04462/2020 and UIDP/04462/2020), a program financially supported by FCT/Ministério da Educação e Ciência, through national funds.
Keywords: extracellular vesicles (EVs), mesenchymal stem/stromal cells (MSCs), stirred-tank bioreactors, metabolic preconditioning, Raman spectroscopy

OT07.2 | Clinical scale production of exosomes from mesenchymal stromal cells to effectively treat hematopoietic acute radiation syndrome

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Introduction: Ionizing radiation can lead to hematopoietic acute radiation syndrome (H-ARS). As a potential countermeasure, we developed a bioreactor-based, large-scale production of clinical-grade exosomes derived from lipopolysaccharide (LPS) primed bone marrow (BM)-mesenchymal stromal cells (MSCs) employing good manufacturing practice (GMP) standards.
Methods: Exosomes isolated by ultracentrifugation from unprimed or LPS-primed BM MSCs were grown in a Quantum hollow-fiber bioreactor and compared to gold standard, flask-scale exosomes. The physical identity of exosomes included a time course assessment of particle diameter, yield, protein content and surface marker profile by flow cytometry. Comparison of the RNA

cargo in exosomes was determined by bulk RNA-seq. Capacity of exosomes to generate exosome educated monocytes (EEMos) was determined by qPCR and flow cytometry, and potency was assessed using a lethal H-ARS model with NSG mice.
Results: MSC-exosomes produced by flask and bioreactor are similar in terms of purity by particle size/protein content and identity by flow cytometry. RNA–Seq comparison of the cargo within MSC-exosomes indicate flask-derived exosomes upregulate micro-RNAs including the let-7 family and miR-143. MSC-exosomes generated by bioreactor produces much higher yields (up to 50-fold) from relatively small media volumes. Comparing the ability to educate monocytes, MSC-exosomes generated by either platform increase the gene expression of IL-6, IDO and FGF-2. Significant increases in M2 markers like PD-L1 ($p< 0.0001$) and CD163 ($p< 0.0001$) and significant decreases in M1 markers CD86 ($p< 0.0001$) and CD16 ($p< 0.0001$) are present in monocytes by flow cytometry after education using flask or bioreactor MSC-exosomes. Bioreactor MSC-exosomes are as effective as flask MSC-exosomes as treatment in a lethal H-ARS mouse model, significantly extending survival and improving clinical scores ($p< 0.05$). Induction of hematopoiesis is observed in peripheral blood from recipients of bioreactor MSC-exosomes, with enhanced absolute neutrophil and lymphocyte counts at Day 30–31 post-treatment.
Summary/Conclusion: Overall, we describe a GMP-compliant, clinical scale biomanufacturing platform for the production and characterization of therapeutic MSC-exosomes as an "off the shelf" countermeasure for H-ARS.
Funding: Production Assistance for Cellular Therapies (PACT) program from the NIH/NHLBI at the University of Miami (PACT Contract No. HHSN2682016000012I. (J.A.K, P.H.). Bioinformatics analysis by A.L. was supported through the National Library of Medicine (5T15LM007359). This work was also supported by the Don Anderson GVHD fund and Crystal Carney Fund for Leukemia Research (P.H.), St. Baldrick's-Stand Up to Cancer Pediatric Dream Team Translational Research Grant SU2C-AACR-DT-27-17 and NIH/NHLBI R01 HL153721 (P.H. and C.M.C.).
Keywords: exosomes, GMP, mesenchymal stromal cells, TLR4, acute radiation syndrome

OT07.3 | Bottom-up assembly of functional, fully synthetic extracellular vesicles for treatment of atopic dermatitis

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Introduction: The pro-regenerative and immunomodulatory effects of human mesenchymal stem cell (hMSC) EVs are promising for applications such as therapy of atopic dermatitis (AD), an inflammatory skin disease. However, despite large potential, their conventional enrichment from culture medium hampers translation, as EV biogenesis is difficult to control, leading to high EV heterogeneity and challenges in production and mechanistic understanding. Thus, we apply bottom-up synthetic biology to produce fully synthetic EVs (synEVs) mimicking hMSC EVs as potential therapeutic for atopic dermatitis.
Methods: We have isolated EVs from adipose hMSCs by differential ultracentrifugation and characterized them by NTA, dynamic light scattering, cryo-TEM, MACSPlex, Western blot and miRNA sequencing. Based on this and a previously published lipid composition, we have assembled synEVs. We assessed their functions in human organotypic AD models in vitro. These are very physiologically relevant full-thickness skin models at an air-liquid-interface.
Results: We show we can build synEVs mimicking MSC EV diameter, surface charge, lipid composition and certain protein and miRNA contents, while allowing more efficient production. The tested synEVs show great effects in AD models, restoring the physiological skin architecture after only 6 treatment days. This was achieved by direct effects on skin cells, without immune cell contribution. To clarify the mechanisms, we are now performing transcriptomics and expect to identify key pathways whose expression is changing between the healthy, atopic dermatitis and synEV-treated condition until the conference.
Summary/Conclusion: In this work we have developed synEVs with therapeutic functions based on hASC EV analysis to help overcome challenges of clinical translation. Moreover, synEVs allow probing the function of individual EV components. Regarding atopic dermatitis, we may have identified a potential new treatment strategy, which could complement the currently limited options.
Funding: Federal Ministry of Education and Research of Germany, Grant Agreement no. 13XP5073A, PolyAntiBak, MaxSynBio Consortium, which is jointly funded by the Federal Ministry of Education and Research of Germany and the Max Planck Society, Volkswagen Stiftung (priority call 'Life?'), German Science Foundation SFB 1129, Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy via the Excellence Cluster 3D Matter Made to Order (EXC-2082/1 – 390761711), Gottfried Wilhelm Leibniz Award (funding for J.P.S.), Max Planck School Matter to Life.
Keywords: hASC EVs, mesenchymal stem cells, synthetic EVs, EV engineering, atopic dermatitis, skin disease, synthetic biology, biomimetic vesicles

OT07.4 | Comparative studies for determining an optimal fusion protocol for preparing hybrid extracellular vesicles for theragnostic applications

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Introduction: Increasing evidence shows that extracellular vesicles (EVs) are highly advantageous as a natural nanoparticulate system for drug and gene delivery. A number of methods have been developed to load therapeutic or imaging agents into EVs (Armstrong, J. P. K., et al.2018). Among them, the strategy of using membrane fusion with liposomes has been shown effective and gained popularity. Yet, a systematic comparison of different fusion methods, including freeze-thawing, PEG-mediated fusion, and extrusion, is still lacking.
Methods: Model liposomes (PC:PE: DOTAP = 23:17:10) were prepared using a thin-film hydration and extrusion method. KPC-derived EVs were isolated from the adherent pancreatic tumor cell's conditioned media using commercial SECs (qEV, IZON) (Han, Z., et al. 2019). Liposome-EV fusion (1:1 ratio) was conducted according to previously published protocols, including a) freeze-thaw, (Sato, Y. T., et al. 2016) b) extrusion, (Hu, M., et al. 2021) and c) PEG-mediated fusion (Piffoux, M., et al. 2018). Characterization of EVs was conducted in accordance with MISEV2018. Model drugs were SPIO (core size = 5nm), fluorescent FITC-dextran (150 kDa), and GFP plasmid (pcDNA3-EGFP, Addgene). Fusion efficiency was assessed using the FRET (Thorsteinsson, K., et al. 2020) and R18 assay method (Gnopo, Y. M. D & Putnam, D. 2020). Finally, the biodistribution of different hybrid EVs (iv injection, dose = $1 \times 10^{\wedge}9$ particles/animal -quantified by NTA) in mice (n = 3) was studied.
Results: All methods resulted in efficient liposome-EV fusion, with the PEG method being the most efficient. Increased particle sizes were observed in all methods except the extrusion method, which shows a minimal size change and the most homogenous size distribution. The PEG method provided the highest loading efficiency and the highest gene transduction rate. Finally, all methods displayed similar biodistribution.
Summary/Conclusion: We comprehensively compared the efficiencies of fusion, drug loading, and intracellular delivery of hybrid EVs obtained by different methods. Among them, the hybrid EV particles prepared by PEG-mediated fusion exhibited optimal properties allowing efficient therapeutic delivery.
Funding: R33HL161756.
Keywords: theragnostics, imaging, hybrid vesicles

OT07.5 | Enhancement and characterization of RNA loading into EVs using endogenous localization motifs

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Introduction: One of the challenges of actualizing the use of RNA therapeutics is the lack of versatile delivery vehicles that are also non-immunogenic. Engineered EVs could have lower immunogenicity since they are generated by host cells. Unfortunately, methods to load an RNA of interest (ROI) into EVs are not as well explored and a "good-enough" approach is taken wherein an ROI is overexpressed whilst alternative approaches are not tested. This leaves a hole in the therapeutic EV landscape where better cargo-loading methods remain uninvestigated. Sometimes, EV "zipcodes" or motifs recognized by RNA-binding proteins (RBPs) fused to EV-localized proteins are included. Currently, the best method to load mRNA into EVs is to fuse the archaea RBP, L7Ae, to CD63 and have the cognate C/Dbox motif contained in the 3' UTR or end of the ROI.
Methods: Here, we use the designer EV toolkit (DEVKit), a modular cloning toolkit developed in our lab, to test and combinatorically clone endogenous RNA zipcodes and benchmark them against CD63-L7Ae + the ROI with the 3' C/Dbox. Stable H441 epithelial cell lines, and HEK293T cells for orthogonal validation, were created and EVs were purified by differential ultracentrifugation. In concordance with MISEV2018, structure, size, concentration, contents and contamination were evaluated by transmission electron microscopy, nanoparticle tracking analysis, western blotting, and qRT-PCR.
Results: We consistently found four motifs that promote at least an 8-fold increase in the loading of EGFP mRNA into EVs compared to the no-motif control. Importantly, this is a one-plasmid system. CD63-L7Ae is a two plasmid or two-component system which can be severely hampered when using difficult-to-engineer cells.

Summary/Conclusion: This is an endogenous RNA delivery system that can be customized to deliver any mRNA with broad applicability to a variety of diseases. EV-based RNA platforms can be further enhanced by combining other EV-engineering methods such as modified targeting and lysosomal escape.
Funding: Emory I3 Program.
Keywords: RNA therapeutics, cargo loading, designer EVs

OT08: Kidney

Chairs: Dylan Burger, Cristina Grange

Location: Room 606/607

15:15 - 16:45

OT08.1 | Mapping the spatial transcriptomics response of extracellular vesicle therapies in kidney disease

Laura Perin[1]; Charmi Dedhia[2]; Astgik Petrosyan[2]; Benedetta Bussolati[3]; Sargis Sedrakyan[4]
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Introduction: Transcriptomic studies have improved our understanding of the mechanism of action of different therapeutic approaches aimed at slowing down/reversing chronic kidney disease (CKD). Using Spatial Transcriptomics (ST) Visium 10x Genomics, we describe for the first time the spatial kidney transcriptomic map that characterizes the molecular mechanisms underlying the protective effect of extracellular vesicles in a mouse model of CKD characterized by mutation of the col4a5 gene, Alport Syndrome (AS).
Methods: Extracellular vesicles (2.8 × 1010) derived from amniotic fluid stem cells (hAFSC-EVs) were injected in AS mice at the early CKD stage and sacrificed 2.5m after injections. Frozen kidney sessions from injected mice, age-matched non-injected AS mice, 2.5m old AS (early CKD stage), and WT mice were collected and processed for ST following the Visium protocols. Imaging data were collected, and analysis was performed using Space Ranger software v1.0.0, Seurat v3.2 in Rv3.3l, IPA, Panther, and Loupe Browser 6.0.0.
Results: First, using our ST integration data, we determined that the alteration of metabolic pathways in the early AS stage (2.5m) and alteration of the ECM components are the significant glomerular transcriptomics changes that characterize AS progression. Second, our data clearly show that EVs can restore to normal important pathways for glomerular homeostasis like integrin binding, actin filament binding, laminin-binding, collagen binding, fibronectin binding, vasculature development, AGE-RAGE signaling, PI3K-Akt signaling pathway, extracellular matrix organization. ST also allowed the identification of specific genes (Igfbp2, Nupr1, Serpine1) that are modulated by EVs in the AS glomeruli.
Summary/Conclusion: Our ST analysis reveals that hAFSC-EVs are reno-protective since they can stimulate glomerular cell survival and remodeling. We also identified some specific-EV targets that can be used as therapeutics for slowing down disease progression in AS.
Funding: NIH R01:R01DK121037.
Keywords: spatial transcriptomics, kidney disease

OT08.2 | Vascular injury-derived exosomes stimulate renal infiltration and accelerate lupus nephritis

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Introduction: Lupus nephritis (LN) is a severe manifestation of systemic lupus erythematosus (SLE). Insterstitial tertiary lymphoid structure (TLS) formation is an emerging contributing factor to LN renal dysfunction leading to end-stage renal disease. We have demonstrated that apoptotic exosomes derived from vascular injury (ApoExo) trigger the production of

SLE-associated antibodies and LG3-targeted autoantibodies (anti-LG3) in naïve mice. ApoExo also induced TLS formation in a murine vascular allograft model. We hypothesize that ApoExo induce an autoimmune response that accelerates the development of LN.

Methods: Serum-free media conditioned by apoptotic primary murine endothelial cells was fractionated using sequential ultracentrifugation. Comprehensive characterization established ApoExo protein, enzymatic and RNA profiles and demonstrated that active 20S proteasome and LG3 fragment are hallmarks of ApoExo. Routinely, supernatants are monitored by small particle FACS using proteasome 20S probe LWA and CFSE. Resuspended ApoExo are analysed by Western blot for the presence of LG3 and proteasome and proteasome activity assays are performed. Using an established infusion protocol, 20 weeks old NZB/WF1 mice received infusions of resuspended ApoExo or vehicle every second day for 3 weeks. Every 2 weeks, blood samples were collected to quantify circulating anti-LG3 and anti-dsDNA levels by ELISA and ApoExo levels by hs-FCM. At sacrifice, kidneys were collected to evaluate interstitial damage and leukocyte infiltration by immunohistochemistry.

Results: Mice infused with ApoExo show higher levels of circulating anti-LG3 and significant renal inflammatory infiltration compared to the vehicle group. ApoExo triggered the recruitment of lymphocytes to the renal interstitium into nodules reminiscent of TLS. Finally, decreased survival and worsened renal function were observed in ApoExo infused mice compared to the ones infused with vehicle.

Summary/Conclusion: This research is the first to evaluate the contribution of vascular injury derived extracellular vesicles to LN. A better understanding of the impact of vascular injury derived immune mechanisms will improve the identification, prediction and management of LN.

Funding: Canadian Institute of Health Research, CRSNG and Kidney Foundation of Canada.

OT08.3 | Extracellular vesicles restore glomerular endothelial lipid metabolic homeostasis in Alport syndrome

Hasmik Soloyan[1]; Matthew Thornton[2]; Geremy Clair[3]; Janielle Cuala[4]; Senta Georgia[5]; Paolo Cravedi[6]; Andrea Angeletti[7]; Laura Perin[8]; Sargis Sedrakyan[9]

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Introduction: Glomerular endothelial dysfunction plays a key role in the development of chronic kidney disease (CKD). In Alport syndrome (AS, characterized by mutations in collagen IVα3α4α5) damage to the glomerular endothelial cells (GEC) occurs before onset of heavy proteinuria and is characterized by altered fenestration size and abnormal glycocalyx deposition. Despite this evidence, the role of GEC in Alport progression is poorly understood. In the present study we elucidate the role of fatty acid metabolism in glomerular homeostasis and demonstrate how its dysregulation leads to glomerular endothelial injury and disease progression in an animal model of AS.

Methods: The phasor approach technique in FLIM (fluorescent lifetime imaging microscopy) was applied as a novel tool to evaluate metabolic fingerprint in kidneys from AS and WT mice, and human tissue. GEC isolated by FACS from tdTomato-reporter AS and WT mice at 4 months of age were compared by bulk RNA-seq and lipidomics. In vitro, silencing experiments to study the role of fatty acid synthase (FASN) in GEC metabolic dysfunction were performed. FASN-carrying AFSC-EVs and control nanoparticles were applied both in vitro and in vivo to restore lipid homeostasis in GEC. Data were confirmed using AFSC-EVFASN-/-. Biopsy samples from patients affected by AS were used to confirm our findings by immunohistochemistry.

Results: FLIM studies showed distinct metabolic fingerprint in diseased glomeruli. Gene enrichment studies revealed lipid metabolic alteration in GEC in AS mice. Genes important in the transport (CD36, FATP-1, FATP-2, Fabp3) and synthesis (FASN) of fatty acids were downregulated. Lipidomic analysis revealed major disturbances in lipid composition in GEC in diseased mice, while histology showed glomerular accumulation of lipid droplets. We observed similar findings in human biopsy samples from AS patients by histology. In vitro, AFSC-EVs were able to rescue FASN deficiency and improve GEC homeostasis, unlike AFSC-EV FASN-/-.

Summary/Conclusion: We report for the first time a lipid metabolic dysfunction in Alport GEC, and the ability of AFSC-EVs to rescue this phenotype. Therefore, better understanding of the functional role of GEC in AS could lead to the development of targeted new therapies for the treatment of this and other forms of CKD.

Keywords: amniotic fluid stem cells, extracellular vesicles, lipid metabolism, glomerular endothelial cells, chronic kidney disease, alport syndrome

OT08.4 | Human proximal tubular epithelial cell (PTEC)-derived apical small extracellular vesicles (sEV) mediate a 'wave of tubular death' in hypoxic chronic kidney disease (CKD)

Andrew J. Kassianos[1]; Xiangju Wang[1]; Ray Wilkinson[2]; Monica S. Y. Ng[3]; Helen Healy[3]
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Introduction: Hypoxia is a key trigger of tubular cell death and fibrosis, the hallmarks of chronic kidney disease (CKD). Proximal tubular epithelial cells (PTEC) of the kidney are uniquely sensitive to hypoxia and are thus, central players in CKD. Here, we investigated the role of PTEC-derived small extracellular vesicles (sEV) in mediating the 'wave of tubular death' of CKD.
Methods: Ex vivo patient-derived PTEC (n = 8) were generated with ethics approvals and informed patient consent. PTEC were cultured under normoxic (21% O2) and hypoxic (1% O2) conditions on Transwells to separate sEV secreted from apical versus basolateral surfaces. sEV were isolated using size exclusion chromatography (qEV columns). The purity of sEV preparations was confirmed using size/concentration (50-150nm; qNano), morphology (electron microscopy) and sEV tetraspanins/ESCRT components (mass spectrometry). sEV miRNA cargo was examined (sequencing), with cell death profiling of autologous PTEC co-cultured with sEV performed (flow cytometry, microscopy).
Results: Significantly increased numbers of sEV were secreted from the apical surface of hypoxic PTEC compared with normoxic PTEC. No differences in basolateral sEV numbers were observed between culture conditions. Biological pathway analysis of hypoxic-apical sEV cargo identified distinct miRNA linked with cellular injury pathways. In functional assays, hypoxic-apical sEV selectively induced cellular necrosis and ferroptotic cell death (↓glutathione peroxidase-4, ↑4-hydroxynonenal) in autologous PTEC compared with normoxic-apical sEV. The addition of ferroptosis inhibitor, ferrostatin-1, attenuated PTEC ferroptosis. RNAse-mediated digestion of hypoxic-apical sEV also abrogated PTEC ferroptosis, supporting a role for sEV miRNA in the CKD 'wave of death'.
Summary/Conclusion: Our data establish PTEC-derived apical sEV and their miRNA cargo as a mediator of tubular death in hypoxic CKD. This novel concept of how tubular injury is propagated from the initiating insult into a 'wave of death' has significant impact for CKD therapeutics.

OT08.5 | Investigation of temporal patterns of biomarker expression from different segments of the kidney in healthy subjects

Samantha Upson[1]; Margaret Selesky[2]; Morgan Greig[2]; Hayrettin Yavuz[3]; Abdulrahman Alwagdani[2]; Michael Solga[4]; Michael A. A Harding[3]; Luca Musante[5]; Uta Erdbrügger[6]
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Introduction: Urinary extracellular vesicles (uEVs) may parallel physiologic and pathologic processes taking place in their kidney cell of origin. Combined with their ease of access, this makes uEVs excellent candidate biomarkers. It is known that kidney physiology and expression of some proteins follow a circadian pattern. Therefore, normal daily variation in uEV excretion and content of specific protein cargoes must be understood. The goal of this study is to examine changes over 24 hours in uEV urinary excretion, total uEV protein, creatinine concentration, and presence of uEV markers of glomerular (e.g. CD35) and tubular (e.g. SLC12A3) origin. In addition, we examined TSG101 as an EV marker.
Methods: Each void during a 24-hour period for 14 healthy individuals (114 samples total) was collected. uEVs from individual voids were isolated by differential centrifugation at 20,000g and washed with a low ionic strength buffer to remove uromodulin(EV190076). uEV count and sizing was performed on each final pellet using Nanoparticle Tracking Analysis (ZetaView PMX-120; Particle Metrix). Flow Cytometry using SpectralFlow was performed on uEVs stained with an antibody panel consisting of CD35 and CD9. uEV protein lysates were Western blotted, stained for total protein (Revert 700, Licor), quantified, then immunoblotted for SLC12A3 and TSG101. Imaging and quantification were performed using Image Studio software on the Odyssey CLx; Licor.
Results: Total uEV protein excretion and uEV concentration have a strong positive linear correlation ($r = 0.65, p = 0.032$). Preliminary findings suggest some uEV derived biomarkers, like SLC12A3, vary over the day in a potential temporal pattern with a max signal strength at 6 am and min at 4 pm when normalized to total uEV protein. Normalizing to creatinine or total uEV protein yields similar patterns of expression of SLC12A3. CD35, a podocyte and glomerular marker, has a temporal pattern of expression when normalized to creatinine with a max signal at 12 pm and a min at 4 pm. TSG101 did not show a temporal pattern of expression.

Summary/Conclusion: The concentration of uEVs and total uEV protein excretion varies with the time of day. The tubular sodium/chloride cotransporter SLC12A3 has a critical role in kidney function and has a circadian pattern of expression in mice kidneys. Our results demonstrate a possible similar temporal pattern of a large range of uEVs carrying SLC12A3 in humans. In addition, CD35 (complement receptor 1), a podocyte and glomerular marker, shows a temporal variation in uEV expression. Temporal variation of uEV markers needs to be further analyzed for other tubular and glomerular markers in healthy individuals and disease. Characterizing temporal protein expression patterns of uEVs has the potential to accelerate uEV biomarker discovery for kidney diseases.

OF09: Cancer Pathogenesis

Chairs: Serena Lucotti, Muller Fabbri after Serena Lucotti

Location: Ballroom 6BC

10:35 - 12:05

OF09.1 | The lung pro-thrombotic niche drives cancer-associated thromboembolism and metastasis via small EV ITGB2

Serena Lucotti[1]; Yusuke Ogitani[2]; Candia Kenific[3]; Linda Bojmar[3]; Michele Cioffi[3]; Pernille Lauritzen[3]; Henrik Molina[4]; Soeren Heissel[4]; Harry Lengel[5]; Xiaohong Jing[6]; Haiying Zhang[7]; Irina Matei[8]; Eileen O'Reilly[9]; William Jarnagin[9]; David Jones[5]; James Bussel[10]; David Kelsen[9]; Jacqueline Bromberg[11]; Diane Simeone[6]; David C. Lyden[1]

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Introduction: Thromboembolism (TE) is a common complication in cancer patients, especially for those with pancreatic ductal adenocarcinoma (PDAC) and metastatic cancers, and is the second leading cause of cancer-related deaths. The prevention of TE remains an unmet clinical need due to lack of biomarkers predictive of TE risk and the bleeding risk associated with routine anti-coagulants. Cancer cells and cells in the tumor microenvironment release large numbers of small extracellular vesicles (sEVs, 50–150 nm) with therapeutic and predictive value in systemic diseases. However, the role of sEVs in cancer-associated TE remains to be investigated.

Methods: sEVs were isolated by sequential ultracentrifugation from tissues of mice with melanoma (B16F10), breast cancer (MMTV-PyMT), and PDAC (KPC), or cell lines. The pro-thrombotic effect of exosomes was studied in vivo (platelet count, D-Dimer, IHC), and in vitro (LTA and flow cytometry).

Results: sEVs from (pre-)metastatic lungs of mice with melanoma, breast, and PDAC induce TE in mice and express high levels of integrin beta 2 (ITGB2). Remarkably, sEVs from tumor cell lines, primary tumors, or other metastasis-bearing organs did not induce thrombosis. A specific subtype of interstitial macrophages infiltrating (pre-)metastatic lungs were the main source of ITGB2+ sEVs. Blockade of ITGB2 on lung-derived sEVs, or systemically in mice, prevented sEV-induced platelet aggregation and TE, and reduced metastasis. We also found that ITGB2 exists in its active conformation on the sEV membrane and interacts directly with GPIb on platelets, thus inducing their activation and aggregation. Importantly, the levels of sEV-associated ITGB2 are elevated in the plasma of PDAC patients prior to TE events in comparison to patients with no history of TE and might serve as prognostic biomarker of TE.

Summary/Conclusion: Our results provide the first evidence of the establishment of a pro-thrombotic lung niche in different cancer types. Moreover, we identify sEV-associated ITGB2 as a new target for the prevention and/or treatment of TE, as well as a potential "liquid biopsy" analyte for the early stratification of patients at high risk of TE.

Funding: NIH CA232093; Thompson Family Foundation; Vth District AHEPA Cancer Research Foundation; DoD (W81XWH-20-1-0263 to SL).
Keywords: small EVs, thromboembolism, metastasis, platelets

OF09.2 | HIF-1α shuttled from extracellular vesicles of COPD patients promotes lung cancer initiation through the modulation of cancer stem cells phenotype

Orazio Fortunato[1]; Ilaria Petraroia[2]; Francesca Pontis[2]; Patrizia Ghidotti[2]; Luca Roz[2]; Paola Suatoni[2]; Ugo Pastorino[2]; Gabriella Sozzi[3]; Giulia Bertolini[4]
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Introduction: Cigarette smoking is the main risk factor for the development of chronic obstructive pulmonary disease (COPD) or the onset of lung cancer. Reduced air flow in COPD patients promotes the activation of HIF-1α hypoxic signaling pathway that was associated to a reduction in lungs functionality and increased risk to develop lung cancer. The subset of CD133+ cancer stem cells (CSC) was demonstrated to be responsible for lung cancer initiation. Cells co-expressing CD133 and CXCR4 are defined as the subpopulation of metastasis initiating cells (MIC). Recently, the involvement of extracellular vesicles in the pathogenesis of lung diseases gained attention. The aim of this project is to evaluate the pro-tumorigenic role of EVs from COPD patients in the development of lung cancer, exerted through the modulation of CSC subsets properties
Methods: EVs were isolated by ultracentrifugation from plasma of heavy smoker volunteers with (COPD-EVs) or without (HS-EV) COPD enrolled in a LDCT screening trial. EVs characterization was performed accordingly to the MISEV guidelines (Flow cytometry, NTA, TEM and WB). The protumorigenic role of EVs was evaluated on transformed Epithelial Cells (HBEC) with different oncogenic manipulation (P53 and/or KRAS) using 2D and 3D in vitro assays.
Results: Circulating COPD-EVs mainly derived from immune and endothelial cells as indicated by high levels of CD31, CD146, CD45 and HLA. COPD-EVs increased the subset of CD133+CXCR4+ MICs along with the stimulation of 3D growth and acquisition of mesenchymal phenotype. Notably this effect was observed only in HBEC- shP53-KRASV12high cells but not in non malignant parental HBEC-1, single mutated HBEC-KRASV12 or HBEC-shP53 cells. Mechanistically, HIF-1α transferred from COPD-EVs triggers CXCR4 activation that in turn mediates MICs expansion and acquisition of pro-tumorigenic effects. Hypoxia induced a 4-fold increase of MIC cells as observed after COPD-EVs. HIF-1α inhibitor or CXCR4 silencing prevents acquisition of malignant traits induced by COPD-EVs or hypoxia. Interestingly, we found higher levels of HIF-1α inside EVs isolated from COPD individuals that develop lung cancer compared to cancer-free individuals
Summary/Conclusion: Our findings demonstrated that in a context of transformed but not fully tumorigenic lung epithelial cells, COPD-EVs can promote the expansion of MICs through HIF-1α-CXCR4 axis activation, providing those malignant properties that might sustain lung cancer initiation.
Funding: The study was supported by grants from the Italian Association for Cancer Research [Investigator Grant Nos. 18812 and 23244 to G.S.], Italian Ministry of Health (RF-2018-12367824 to G.S.; GR-2019-12369047 to O.F), Fondazione Regionale per la Ricerca Biomedica (Regione Lombardia) (1731093 to G.B.).

OF09.3 | Tumor diffusion and volume may influence the dissemination of mitochondrial DNA variants in plasma extracellular vesicles

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Introduction: Tumor diffusion measured by magnetic resonance imaging (MRI) reflects cell density, which is important for the cell interactions in the tumor microenvironment (TME). Extracellular vesicles (EVs) have been shown to contain mitochondrial

DNA (mtDNA) and proposed as a transfer mechanism for altered mtDNA in various pathologic conditions. Here, we show that tumor diffusion and MRI-measured tumor volume influence the mtDNA detected in plasma EVs.

Methods: From 61 rectal cancer patients (informed consent obtained), DNA was extracted from whole blood (WB) and DNase- and proteinase-treated plasma EVs isolated by size exclusion chromatography prior to mtDNA enrichment and next generation sequencing. The EVs were characterized with transmission electron microscopy, nanoparticle-tracking analysis, and western blot analysis (expression of CD9, CD63, ALIX, and APOA1; absence of GM130). Total variant number (TVN), heteroplasmy (variant frequency < 0.990), and variant effect prediction of mtDNA were calculated as an EV-to-WB ratio (EV/WB), representing potential additional tumor variants as liquid biopsy. Patients received a radiological extramural vascular invasion score and pathological treatment response assessments. 39 of the patients underwent an MRI diffusion sequence and 52 patients had clinical procedure high-resolution MRI used for estimation of whole tumor volume. Spearman's correlation analysis and Mann-Whitney test were performed.

Results: Patients with high tumor diffusion had plasma EVs containing more mtDNA variants (rho = 0.38, $p = 0.02$), typically in a heteroplasmic state (rho = 0.45, $p < 0.01$) and with high/moderate effects on the protein coding regions (rho = 0.43, $p < 0.01$), relative to WB. Interestingly, tumor volume was inversely correlated with EV/WB-TVN and -heteroplasmy (rho = -0.41 and rho = -0.42, both $p < 0.01$), and patients with low EV/WB-TVN and -heteroplasmy at baseline had more aggressive cancer (tumor extramural vascular invasion and regional lymph node metastases after neoadjuvant therapy, both $p < 0.01$).

Summary/Conclusion: Plasma EVs can act as containers for cells to clear out damaging mtDNA variants, and we propose that high diffusion tumors have high ability to expel such mtDNA. Conversely, in low diffusion tumors, EVs will more likely remain in the TME and be taken up by neighboring cells where damaging mtDNA variants can continue to support tumor growth.

Funding: The Norwegian Cancer Society grant 215613, and the South-Eastern Norway Regional Health Authority grants 2018054 and 2019109.

OF09.4 | Upregulated NHE7 promotes uptake of small extracellular vesicle by macropinocytosis in hepatocellular carcinoma and its blockade inhibits tumorigenesis and metastasis

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Introduction: The successful entry of small extracellular vesicle (sEV) into cells is a prerequisite for the influence of sEVs on the recipient cells. sEV derived from hepatocellular carcinoma (HCC) promote cancer progression and metastasis via multifarious pathways. However, how sEV enters HCC cells remains obscure. This study aims to elucidate the mechanistic basis underlying the internalization of sEV by HCC cells.

Methods: The uptake of sEV by cells was examined by fluorescent microscopy. The properties of cells with sEV uptake were evaluated by in vitro and in vivo functional assays. The clinical significance of sEV uptake regulator was studied by multiplex fluorescent immunohistochemistry of HCC patients tissue microarray.

Results: Compared to normal liver and non-metastatic HCC cells, metastatic HCC cells showed the highest capacity to take up TMR-dextran, an indicator of macropinosome, and PKH67-labeled sEV. The uptake of TMR-dextran and PKH67-sEV by cells was compromised by 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) which is an inhibitor of macropinocytosis. EIPA reduced HCC cell-derived sEV in promoting cell growth, motility and tumor formation in mice. EIPA is a Na+/H+ exchanger (NHE) inhibitor that targets solute carrier family 9 family. Among all family members, NHE7 expression was well correlated with the sEV uptake ability of HCC cells. Knockdown of NHE7 in metastatic HCC cells reduced the ability of cells to internalize TMR-dextran and PKH67-sEV resulted in diminished HCC growth, motility and tumorigenesis. Conversely, HCC with overexpressing NHE7 displayed enhanced ability to internalize sEV and increased cancer properties. NHE7 localized at late endosomes revealed by its colocalization with Rab21. NHE7 induced cytosolic alkalinity and regulated expression of Rab21. The increased alkalinity promoted maturation of macropinosome thus facilitated sEV uptake and cancer properties of cells. NHE7 was frequently upregulated in HCC, associated with poorer survival and well correlated with Rab21 expression. Lastly, inducible-knockout of NHE7 in tumors developed in mice remarkedly suppressed liver tumor formation and distant metastasis.

Summary/Conclusion: This study reveals the sEV uptake by HCC cells via NHE7-driven macropinocytosis and provides therapeutic insights into targeting dysregulated NHE7.

Funding: Research Grants Council General Research Fund (Reference number: 17105322).
Keywords: sEV uptake, hepatocellular carcinoma, macropinocytosis, pH regulator

OF09.5 | The characterization and functional analysis of IRSp53-mediated secretion of the extracellular vesicles from cancer cells

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Introduction: The insulin receptor substrate protein of 53kDa (IRSp53) is the Inverse Bin/Amphiphysin/Rvs (I-BAR) domain-containing proteins that deform the plasma membrane to form protrusions. We have reported that the I-BAR domain-containing protein MIM is involved in extracellular vesicle (EV) formation via the scission of filopodia. However, the role of the IRSp53 in the EV generation and tumor progression had been unclear.
Methods: We prepared the IRSp53-knockout head and neck cancer cells (HNCCs) and separated the EVs secreted from these cells to the culture medium by the stepwise centrifugation. The number and size of these EVs were analyzed by using nanoparticle tracking analysis and mass spectrometry was performed to identify the specific cargo proteins. These EVs were also treated to the cells to examine their effect on cell proliferation.
Results: We found that IRSp53 expression level was correlated to the lifespan of head and neck cancer patients through the cancer genomics database. The in vitro cell culture showed that IRSp53 was an important regulator of filopodia formation in HNCCs. Our in vivo study also found that the tumor cells could secrete IRSp53-containing particles to the surrounding tissues in mice. Furthermore, the HNCCs secreted the large-EVs (l-EVs) dependent on IRSp53. Immunoblotting and mass spectrometry analysis showed that the IRSp53-mediated l-EVs contained integrin-α2 and extracellular matrix protein, laminin-γ2. These l-EV led to the activation of ERK kinase and stimulated the proliferation of recipient cells. The depletion of laminin-γ2 and blockade of integrin ligand binding counteracted the l-EV-mediated cell proliferation, suggesting laminin-γ2 as a mediator for the EV-stimulated proliferation of the cells.
Summary/Conclusion: These comparative functional studies demonstrated the effect of IRSp53-mediated EV on HNCCs proliferation and presumably cancer progression via the secretion of l-EV that promote cancer cell proliferation.
Keywords: I-BAR domain protein, IRSp53, integrin, extracellular matrix, cell proliferation

OF10: Tissue Injury and Repair

Chairs: Rachael Batabyal, Johannes Grillari

Location: Ballroom 6A

10:35 - 12:05

OF10.1 | Promotion of regenerative effects of highly enriched-specific extracellular vesicles from salivary gland epithelial stem cells through modular microfluidic device

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Introduction: Stem cell therapy has been suggested for anti-inflammatory, antifibrotic, and regenerative purposes. For successful therapeutic efficacy and reduced side effect, cell-free therapeutic approaches such as extracellular vesicles (EVs) have emerged containing growth factors, cytokines, miRNAs, and lipids. Despite the increased number of recent studies on cell-free therapy and their promising results, it is still necessary to optimize massive purification protocols and evaluation of the purified EVs to determine the treatment dose, frequency, and delivery routes of the EVs.
Methods: We applied to a modular microfluidic technology that enables us to enrich CD9-specific EVs (CD9+ EVs) within 10 minutes. Modular microfluidic platform can selectively capture the specific EVs by CD9 antibody-coated beads in a horseshoe-shaped orifice micromixer (HOMM) chip. Sequentially, a fish-trap-shaped microfilter unit contained the microbeads into weir structure and released the captured EVs from the microbeads. Then, we harnessed the retroductal delivery route for the EV administration as it is a direct, non-invasive approach to the salivary gland for the first time.
Results: In the wound healing assay using epithelial cells, all the experimental groups treated with the EVs showed faster growth than the control group. Especially, the group treated with CD9+ EVs was superior than those with total EVs. In the mouse duct

ligation model (sialoadenitis), the group treated with PBS as a control showed very severe tissue damage, decreased mucus, and increased fibrosis through H&E, PAS, MTC, and sirius red staining. However, the experimental group treated with CD9+ EVs reduced the degree of tissue damage, reduction of mucus, and fibrosis. The proteomic and miRNA profile of CD9+ EVs revealed the regenerative properties and anti-fibrotic effect for epithelial cells.
Summary/Conclusion: The modular microfluidic device successfully enriched the CD9+ EVs and direct administration of the EVs through the retroductal route can open the gate to potential therapeutic strategy for treating salivary gland dysfunction.
Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No.2021R1A2C3011254), Korea Medical Device Development Fund grant funded by the Korea government (MSIT,MOTIE, MOHW,MFDS) (Project Number: 1711139115, KMDF_PR_20210527_0008), and Korea Environment Industry & Technology Institute (KEITI) through Aquatic Ecosystem Conservation Research Program, funded by Korea Ministry of Environment (MOE) (2020003030007).

OF10.2 | Differential effects of two adipose stem cell-derived extracellular vesicle subgroups on tendon healing in a mouse model of Achilles tendon injury and repair

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Introduction: Tendon injury is one of the most common and challenging orthopedic conditions. We previously found that extracellular vesicles generated by inflammation-primed adipose-derived stem cells (iEVs) have the potential to enhance tendon healing by regulating macrophage and tendon cell functions. However, iEVs are heterogenous. We hypothesized that iEVs from different subpopulations exhibit different potency in regulating tendon healing. To test the hypothesis, this study compared the mechanistic roles of two iEV subgroups in regulating tendon healing in a mouse Achilles tendon injury and repair model.
Methods: With IACUC approval, bulk iEVs (iEVA) were prepared from conditioned medium of IFNγ-primed ASC culture via differential centrifugation. CD9+/CD63+/CD81+ (iEVS+) and CD9−CD63−CD81− (iEVS−) small iEVs were separated from iEVA via size exclusion chromatography and microbead-assisted vesicle sorting, and analyzed by flow cytometry, NTA, TEM, RNA-Seq, and TaqMan PCR. The effects of iEVS+ and iEVS− were compared in isolated tendon cells and macrophages, and a mouse model of Achilles tendon injury and repair at varied time points after injury.
Results: iEVS+ reduced a proinflammatory macrophage M1 phenotype by inhibiting TLR4/NF-κB signaling. In contrast, iEVS− activated NF-κB. iEVS+ but not iEVS− also promoted tendon cell proliferation and Collagen I production. In vivo application of iEVS+ reduced injury site NF-κB activity and mononuclear cell infiltration compared to control repair. iEVS− treatment showed opposite effects. Compared with iEVA, iEVS−, and control repair, iEVS+ markedly enhanced collagen regeneration in the injury center and reduced peritendinous scar formation, thus promoting scarless healing. As such, mice treated with iEVS+ experienced the least incidence of postoperative complications with the fastest recovery rate of weight bearing. The diverse effects of iEVS+ and iEVS− were associated with their differences in cargo contents. miR-147b is an active component of iEVs that targets macrophage TLR4/NF-κB signaling. iEVS+ carried 3.2-fold more and iEVS− possessed only half of miR-147b compared to iEVA. iEVS+ also contained higher levels of mRNAs positively regulating cell proliferation, survival, and collagen production than iEVS−.
Summary/Conclusion: Results demonstrated that iEVs are functionally heterogenous. Not all iEVs are necessarily beneficial to tendon healing. Selective application of iEV subgroup specific for tendon repair can markedly enhance the therapeutic efficiency of iEVs and promote scarless tendon healing after injury and repair.
Funding: NIH R21AR075274.
Keywords: stem cell extracellular vesicle, tendon injury and repair, EV heterogeneity

OF10.3 | Wound Macrophage-derived exosomes enable keratinocyte migration for functional wound closure

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Introduction: Exosomes represent a major component of paracrine regulation in tissue repair. We tested the hypothesis that functional wound closure requires successful crosstalk between keratinocytes and wound macrophages (wmf) in vivo.
Methods: Murine wmf-derived exosomes were genetically labeled with RFP reporter (Exowmf) using tissue nanotransfection. Wound-edge (WE) Exowmf were characterized per MISEV 2018 guidelines and reported in EV-track (EV 220292; EV-metric

score 100%). Protein cargo in Exowmf was detected, validated, and quantified using LC-MS/MS, dSTORM imaging, and flow cytometry. Functional wound closure was evaluated using analytical histology and Transepidermal Water Loss.
Results: The Exowmf was localized at the leading-edge keratinocytes post-injury. LC-MS/MS and dSTORM imaging identified presence of outer mitochondrial membrane (OMM) protein TOMM70 in Exowmf. At d5 post-wounding, 92.65 ± 1.34% Exowmf were TOMM70+ ($p < 0.001$; $n = 6$). Such TOMM70-enriched Exowmf increased keratinocyte migration by 72.05 ± 8.50%. Wound hypoxia significantly degraded TOMM70 in WE keratinocytes. Such hypoxic loss of keratinocyte TOMM70 inhibited cytosolic PTEN-induced kinase 1 (PINK1) translocation to the inner mitochondrial membrane causing PINK1 oligomerization on OMM followed by mitophagy. Hypoxic loss of TOMM70 in keratinocytes was compensated by uptake of TOMM70+ Exowmf Downstream PINK1 oligomerization and mitophagy were thus spared in keratinocytes resulting in improved ATP pool necessary to support the metabolic cost of cell migration To block Exowmf uptake by WE keratinocytes, "eat me not" Exowmf were generated using a LysM promoter-driven tetraspanins (CD9/CD63/CD81) plasmid connected via IRES element with "eat me not"-CD47 sequence with in-frame GFP reporter. Selective interruption of exosomal crosstalk between wmf and WE keratinocytes significantly delayed re-epithelialization and impaired functional wound closure.
Summary/Conclusion: This work lays the foundation for a novel paradigm that addresses the molecular bases of cell-cell crosstalk in the wound microenvironment that has a direct bearing on wound tissue bioenergetics, a critical factor necessary to pay for the metabolic cost of wound closure.
Funding: NIH R56DK129592 to SG.
Keywords: macrophage-derived exosomes, functional wound closure, mitochondria, inflammation

OF10.4 | Small extracellular vesicles loaded BDNF treating cerebral ischemia via intranasal delivery

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Introduction: Mesenchymal stem cells (MSCs) are promising for the therapy of cerebral ischemia in animal studies and clinical trials, but their clinical application still faces many challenges. Small extracellular vesicles (sEVs) may overcome the difficulties of stem cells in clinical translation.
Methods: In the study, we overexpressed BDNF in cultured MSCs and purified sEVs using anion exchange chromatography. sEVs were administrated to the mice with ischemic stroke model via intranasal delivery. The behavioral tests, neural repair, the of the mice, the neurogenesis, angiogenesis, synaptic plasticity and fiber preservation, and the neuronal inflammation were examined.
Results: In an ischemic stroke mouse model, sEVs selectively targeted the peri-infarct region after intranasal delivery, and BDNF loading enhanced the effects of sEVs on functional improvement of behavioral tests, neural repair indicated by infarct volume reduction, the increase of neurogenesis, angiogenesis, synaptic plasticity and fiber preservation, and anti-inflammation as shown by lower inflammatory-cytokine expression and glial response. Intranasal administration of sEVs and BDNF-sEVs resulted in upregulation of neuroprotection-related genes and downregulation of inflammation-related genes, and BNDF-sEVs treatment activated the BDNF/TrkB signaling, in the ischemic cortex. Transcriptomic and proteomic analysis of sEVs and BDNF-sEVs disclosed abundant proteins and miRNAs involved in neuroprotection and anti-inflammation, and BDNF-sEVs showed different characteristics from sEVs.
Summary/Conclusion: In conclusion, intranasal delivery of sEVs-loaded BDNF is an alternative strategy for the therapy of cerebral ischemia.

OF10.5 | Mesenchymal stem cells-derived small extracellular vesicles attenuate diabetic retinal fibrosis by suppressing Hedgehog pathway

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Introduction: Retinal fibrosis resulting from proliferative diabetic retinopathy (PDR) is one of the major causes of decreased vision and even blindness. This study aimed to evaluate the effect of human umbilical cord mesenchymal stem cells-derived small extracellular vesicles (hucMSC-sEVs) on PDR fibrosis in vivo and vitro and to investigate the anti-fibrotic mechanism of hucMSC-sEVs.
Methods: Here human umbilical cord-derived mesenchymal stem cells (hucMSCs) were successfully cultured and identified, and small extracellular vesicles were isolated from the supernatant by ultracentrifugation. HucMSC-sEVs were characterized by

transmission electron microscope, immunoblot and nanoparticle-tracking analyses.A streptozotocin (STZ) induced PDR model was established, followed by intravitreal injection of hucMSC-sEVs.
Results: The intravitreal injection of hucMSC-sEVs effectively attenuated retinal fibrosis and collagen deposition in vivo. Furthermore, treatment of retinal pigment epithelium (RPE) and müller cells with hucMSC-sEVs decreased the expression of fibrogenic genes induced by high glucose environment. Mechanism investigation revealed that hucMSC-sEVs deliver E3 ubiquitin ligase to boost glioma-associated oncogene (GLI1) ubiquitination and degradation, thus suppressing hedgehog signaling in both vivo and vitro. While overexpression of GLI1 decreased the repairing effects of hucMSC-sEVs on renal fibrosis.
Summary/Conclusion: In conclusion, these results reveal a novel fibrosis regulation and demonstrates the potency of hucMSC-sEVs in retinal repair.
Funding: This work was supported by the National Natural Science Foundation of China (Grant no. 82272179, 81971757); Zhenjiang Key Laboratory of High Technology Research on Exosomes Foundation and Transformation Application (Grant no. SS2018003).
Keywords: small extracellular vesicles, mesenchymal stem cells, retinal fibrosis, diabetic retinopathy

OF11: Bacteria

Chairs: Yong Song, Mariola Edelman

Location: Room 608/609

10:35 - 12:05

OF11.1 | Bacterial extracellular membrane vesicles: The missing link between microbiota and the host immunity?

Irma Schabussova
Medical University of Vienna, Vienna, Austria

Introduction: Probiotic bacteria such as E. coli O83 (EcO83) have been shown to reduce the development of allergies. Oral administration of EcO83 reduced allergic sensitisation in children but not allergic lung disease. We showed that intranasal administration of EcO83 reduced allergic airway inflammation in mice in a TLR4-dependent manner. Bacteria produce vesicles that mediate the functions of the microbiota by delivering effector molecules into host cells and modulating host signalling pathways in health and disease. The potential of outer membrane vesicles (OMVs) produced by bacteria for the prevention or treatment of allergy is unclear.
Methods: We isolated OMVs from EcO83 (EcO83-OMVs) by ultracentrifugation and investigated their effect in a model of ovalbumin-induced allergic airway inflammation (AAI). The OMVs were analysed by TEM and NTA. HEK293 cells expressing NOD1, NOD2, TLR2 and TLR4 and bone marrow-derived dendritic cells (BMDC) from wild-type (WT) and TLR4KO-BALB/c mice were stimulated with EcO83-OMVs. Cytokines were measured by ELISA.
Results: Stimulation of HEK293 NOD1, NOD2, TLR2 and TLR4 cells with EcO83-OMVs increased the production of IL -8, indicating the involvement of these receptors in signal transduction by EcO83-OMVs. Stimulation of WT BMDC with EcO83-OMVs increased the production of IL-23, IL-12, TNFα, IL-1β and IL-6, while BMDC from TLR4KO mice showed decreased production of these cytokines. Intranasal administration of EcO83-OMVs reduced allergic airway hyperresponsiveness and lung eosinophil counts compared to sham-treated controls and increased pulmonary neutrophil counts.
Summary/Conclusion: Here, we have shown that i) EcO83-OMVs are recognised by NOD1, NOD2, TLR2 and TLR4, ii) EcO83-OMVs induce cytokine production in a TLR4-dependent manner, and iii) intranasal administration of ECO83-OMVs reduces the development of experimental allergy. Our research suggests that probiotic-derived OMVs could be a novel treatment option for allergic diseases in humans.
Funding: Danube Allergy Research Cluster; FWF, OEAD.
Keywords: microbiota, probiotic, allergy, bacterial extracellular membrane vesicles

OF11.2 | Probiotic-derived EV as therapeutic effectors: biogenesis, host internalization and mechanistic targets

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Introduction: Lactobacillus johnsonii N6.2 is a novel probiotic bacterium that was shown to mitigate the onset of type 1 diabetes (T1D) in in rodent models. Our working hypothesis is extracellular vesicles (EV) released in the gut can mitigate the autoimmune response at distal locations. Using SEM, we observed EV produced by L. johnsonii 90–125 nm in size. Untargeted lipidomic and proteomics revealed that L. johnsonii N6.2 EV are enriched in phospholipids and have a unique protein composition. Using the Sdp differentially enriched protein as biomarker, the host response to L. johnsonii EV was evaluated in blood samples of volunteers that ingested L. johnsonii. The individuals consuming the whole probiotic showed increased amounts of IgA and IgG for EV or the Sdp protein. In vitro, EV can induce a tolerogenic M2 phenotype in macrophages, reduce apoptosis in βlox5 human beta cells and stimulate insulin secretion in human islets. RNAseq analysis showed that EV induced the expression of genes associated with the aryl carbon receptor (AHR) as well as the OAS RNA sensing pathways.
Methods: Treatments of βlox5 cell lines with uptake inhibitors or endosome markers were visualized and quantified by confocal fluorescence microscopy. Enzymatic, chemical, and physical methods were used to disrupt EV. RNAseq and qRT-PCR was utilized to quantify gene expression.
Results: L. johnsonii EV are internalized by the clathrin/dynamin mediated endocytosis pathway. Co-localization experiments with the endosome markers Rab5, Rab7 and Lamp as well as calcein indicated that EV escape the endosome shortly after Rab7 fusion. Using the expression of the OAS host pathway, we found that the host cellular responses to the EV are dependent on the integrity of the external components of the EV as well as on the RNA cargo. It was found that the RNA transcripts found within the EV largely represent the most abundantly transcribed genes in the bacterial cells such as those associated with protein synthesis and glycolysis.
Summary/Conclusion: Ongoing experiments are evaluating host-related physiological conditions that affect vesicle biogenesis in L. johnsonii. The shifts in protein, lipid and RNA composition of the EV cargo are expected to have a strong impact in host cellular transit and, potentially, their mechanistic targets.
Funding: This study is funded by NIH NIDDK R01DK121130.
Keywords: probiotic, OAS, RNA cargo, endosome escape

OF11.3 | Role of fecal bacterial-enriched extracellular vesicles in mediating gut dysbiosis-induced inflammation and pain

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Introduction: Gut dysbiosis is associated with several disorders, including systemic inflammation and pain hypersensitivity, yet underlying mechanisms remain unknown. Extracellular vesicles (EVs) are secreted by all cells, including bacteria, with cargo rich in conserved and unique factors. Here, employing a well-established diet-induced obesity (DIO) gut dysbiosis model, we characterized the role of bacterial-enriched fecal EVs (bEV) in systemic inflammation and allodynia.
Methods: We isolated bEV by density-gradient ultracentrifugation or differential ultracentrifugation combined with immune-selection to remove eukaryotic EVs. bEV isolated from control (bEV-C) and gut dysbiotic (bEV-GD) mice were characterized for size and concentration by NTA and surface expression of LPS and OmpC by immunogold labeling and flow cytometry.
Results: bEV-GD injected locally or intravenously caused higher nocifensive pain behavior compared to bEV-C. Notably, DRG sensory neurons treated with bEV-GD showed increased calcium influx suggesting activation of nociceptors. The characterization of plasma cytokines and peritoneal fluid immune cells showed that bEV-GD induced macrophage-driven inflammation with concomitant downregulation of adaptive immune cells. Live imaging of mice receiving enema with fluorescently-labeled bEV-GD and bEV-C showed their biodistribution to distant organs. Transwell-based gut barrier assays showed that bEV-GD strongly disrupt epithelial barriers. Mechanistic studies showed a higher expression of LPS on the surface of bEV-GD compared to bEV-C which was critical for their pro-inflammatory effects. Further, treatment of bone marrow-derived monocytes with bEV-GD increased the expression of LPS-specific receptor, toll-like receptor 4 (TLR4). Lastly, treatment of THP1 monocytes with bEV-GD in the presence of pharmacologic TLR4 inhibitors compromised their pro-inflammatory effect.
Summary/Conclusion: Altogether, our studies highlight that bEV play a critical role in mediating gut dysbiosis-induced systemic inflammation and pain.

OF11.4 | Plasma extracellular vesicles dampen acute inflammatory responses in neutrophils stimulated with bacterial PAMPS

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Introduction: Neutrophils are key players at inflammatory foci, where they exert defensive functions by producing reactive oxygen species and cytokines, phagocytosing microbes and releasing NETs. However, to avoid tissue damage neutrophil activity has to be controlled. We have previously shown that healthy donors' plasma EVs (pEVs) inhibit macrophage inflammatory response to a PAMP. Herein, we studied whether pEVs were also able to modulate neutrophil activation.
Methods: pEVs were purified from healthy donor plasma by size-exclusion chromatography followed by centrifugation. Western blot analysis of both EV and contaminant markers (ApoA1, ApoB1, IgG) revealed that our pEV preparations were highly pure. Neutrophils were isolated from healthy donors' blood by a standard density gradient separation method. Neutrophil degranulation (CD11b/CD66b surface expression) and oxidative burst (dihydrorhodamine oxidation) were analyzed by flow cytometry (FC) following N-Formyl-Met-Leu-Phe (fMLP) stimulation. Phagocytosis of fluorescent Candida sp. and cell viability (Annexin V and propidium iodide labeling) were also assessed by FC. Cytokine production was evaluated by ELISA.
Results: Results showed that pEV exposure did not affect neutrophil viability but induced a dose-dependent reduction of both oxidative burst and degranulation following fMLP stimulation. Likewise, phagocytosis of Candida sp. was impaired in pEV-treated neutrophils. In contrast, pEVs boosted IL-8 production in response to LPS stimulation, as compared to cells not exposed to pEVs.
Summary/Conclusion: In conclusion, pEVs modulate neutrophils activity at the inflammatory foci, contributing to control acute inflammation by diminishing neutrophils' respiratory burst, degranulation and phagocytosis. Concurrently, pEVs could contribute to tissue repair by promoting the secretion of the angiogenic cytokine IL-8. Together with previous observations from our group, these results suggest that pEVs are homeostatic regulators of acute inflammation.
Funding: Argentinean National Agency for Science and Technology Promotion (ANPCYT) under Grants No. PICT 2015-0658, PICT-2018-02202 and PICT-2019-02506.

OF11.5 | Circulating bacterial extracellular vesicles and their associated virulence factors from autoimmune hepatitis patients induces systemic inflammation in experimental mouse model

Swati Thangariyal[1]; Sukriti Baweja[2]; Ashmit Mittal[1]; Chhagan Bihari[3]; Guresh Kumar[4]; Shiv Kumar Sarin[4]
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Introduction: Autoimmune Hepatitis (AIH) is a chronic inflammation of the liver with an unknown cause. Bacterial membrane vesicles (BEVs) in normal physiological conditions induce virulence and aids in cellular communication with the host. In the study, we investigated plasma BEVs from the AIH patients for virulence factors and their role in inducing inflammation.
Methods: BEVs were isolated and characterized from the plasma of AIH patients [biopsy proven, HAI score >2, ALT(148 ± 58.4); AST(160.7 ± 60.2)] and healthy controls[HC], using Iodixanol-Sucrose density gradient ultracentrifugation. Confirmed by TEM(size ranges 50–200nm), western blot(OMP-A and LPS), and quantified using NTA. Proteomics analysis was performed and further analysed for Virulence factors using VFDB. Adoptive transfer of BEVs with different concentrations (10^6-109 BEV/ml) intravenously in 10 weeks old C57BL/6J female mice (mean weight-22gm ± 2) was done. After 8,12 and 24 hours of treatment, mice were assessed for liver functions test, histology of the liver and spleen, and Immune cells frequency and functions.
Results: The proteomics of AIH plasma BEV revealed >10 fold increase in immunoglobins and complement-associated proteins with increased twitching motility protein as virulence factors and none in HC BEV. In vitro, plasma AIH BEV was able to trigger inflammation by activating TLR2 and its downstream signalling genes more than HC BEV. Upon AIH-BEV adoptive transfer at 12hrs liver enzyme activity [AST($p = 0.0286$) ALT($p = 0.0452$)] significantly increased than healthy-BEVs. Interestingly liver and spleen weight also increased [liver index ($p = 0.0086$), spleen index ($p = 0.0218$)]. The histological assessment showed focal acute inflammation after AIH-BEV intervention but no changes were seen by HC BEV.

Summary/Conclusion: The plasma BEV of AIH carries increased virulence factors that induce inflammation in autoimmune hepatitis patients. AIH BEV adoptive transfer in mouse model also induces AIH-like systemic inflammatory condition.
Funding: The funding for the work was provided by ICMR, India.
Keywords: bacterial extracellular vesicles, gradient ultracentrifugation, virulence factors, BEV, systemic inflammation.

OF11.6 | Macrophages infected with Salmonella enterica serovar typhimurium secrete proteolytically active extracellular vesicles

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Introduction: Proteolysis is an integral process of the immune response to infection. While the research focus is on secreted soluble proteases, recent studies have also identified proteases borne by extracellular vesicles (EVs). However, the role of EVs as protease-bearing particles, especially in the context of bacterial infections, is poorly understood. Here we shed light on the composition and function of proteolytically active EVs secreted from macrophages infected with Salmonella enterica serovar typhimurium (S. typhimurium).
Methods: EV samples were isolated by differential centrifugation and characterized using atomic force microscopy (AFM), nanoparticle tracking analysis (NTA), sucrose gradient pelleting, and protein characterization. EV quantification and standardization was performed using NTA and total protein quantification.
Results: We discovered that EVs derived from S. typhimurium-infected macrophages were enriched in proteolytically active Matrix Metalloproteinase 9 (MMP-9). Interestingly, the signaling pathway responsible for MMP-9 upregulation in response to S. typhimurium exposure is selective to immune cells and regulated by Toll-like Receptor 4 (TLR-4) recognition of bacterial lipopolysaccharide (LPS). Co-immunoprecipitation of MMP-9 with numerous membrane proteins indicates co-localization of MMP-9 to the EV membrane. We further demonstrated that specific inhibition of EV-associated MMP-9 significantly decreased macrophage ability to invade through basement membrane in vitro, suggesting that proteolytic EVs may play a role in macrophage response to bacterial infection.
Summary/Conclusion: Our findings reveal that upon exposure to S. typhimurium, macrophages secrete EVs enriched in membrane-associated proteolytically active MMP-9, crucial for macrophage invasion through the extracellular matrix and basement membrane. Future work includes investigating the role of proteolytic EVs on bacterial infectivity.
Keywords: proteolysis, matrix metalloproteinases, immune response

OF12: Fundamental Biology

Chairs: Clotilde Thery, Tom Driedonks

Location: Room 606/607

10:35 - 12:05

OF12.1 | The ATPase activity of the phosphatidylethanolamine flippase TAT-5 inhibits EV budding from the plasma membrane

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Introduction: Cells release extracellular vesicles (EVs) from their surface, but the mechanisms that govern EV release by plasma membrane budding are poorly understood. We previously showed that the lipid flippase TAT-5 inhibits EV release from the plasma membrane and maintains phosphatidylethanolamine asymmetry in C. elegans (Wehman et al., Curr Biol 2011). EV release is also inhibited by a human homolog of TAT-5, ATP9A (Naik et al., PLoS One 2019). However, how a partial loss of flippase activity regulated EV release was unknown.
Methods: Previous studies on mammalian lipid flippases of the P4-ATPase family have shown that mutations in the DGET motif in the Actuator domain can lead to a 3-fold (D to T) to complete (E to Q) loss in lipid transport (Coleman et al., PNAS 2012). Therefore, we generated similar point mutations in the DGET motif of endogenous TAT-5 using CRISPR/Cas9, specifically

D244T and E246Q. We then quantitatively examined EV release in vivo using degron-based EV reporters (Beer et al., Nat Comm 2019).
Results: We discovered that TAT-5(E246Q) mutants were sterile, similar to tat-5 deletion mutants (Wehman et al., Curr Biol 2011). In contrast, TAT-5(D244T) mutants produced normal numbers of embryos, but they arrested during embryonic development. We also found that EV release was increased >50-fold in TAT-5(D244T) mutant embryos, suggesting that even a partial loss of flippase activity significantly promoted EV release.
Summary/Conclusion: Our data suggest that robust flippase activity and phosphatidylethanolamine flipping are required to inhibit EV release. As phosphatidylethanolamine asymmetry is lost on the intercellular bridge during the final steps of cell division, we predict that P4-ATPase activity could be regulated at this site to control EV release and sculpt this structure for abscission.
Funding: This work was funded in part by DFG WE5719/2-1.

OF12.2 | Cellular cholesterol levels regulate RNA contents of extracellular vesicles via ORP1L

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Introduction: RNA carried by extracellular vesicles (EVs) has emerged as a novel mechanism for cell-to-cell communication and drives many physiological and pathological processes, including cancer. Previously, we identified the conserved endoplasmic reticulum membrane contact site (ER MCS) linker proteins VAP-A and the ceramide transfer protein CERT as significant regulators of the RNA and RNA-binding protein content of a subpopulation of small EVs. Since VAP-A also binds the cholesterol transporter Oxysterol binding protein-related protein 1L (ORP1L) and cholesterol may contribute to EV biogenesis, here we explored the role of cholesterol and ORP1L in the regulation of RNA-containing EVs.
Methods: For cholesterol depletion conditions, DKs-8 cells were cultured for 96h in 10% lipoprotein-depleted serum (LDS) supplemented with 250 μM mevalonate and 10 μM mevastatin for 96h. Small EVs were purified by cushion density gradient from control and cholesterol-depleted colon cancer cells. We used confocal, electron microscopy, and various biochemical techniques to analyze EV biogenesis and cargo content.
Results: We observed a substantial alteration of EVs secreted from colon cancer cells cultured in lipoprotein-depleted growth media and inhibited for cholesterol synthesis. QRT-PCR for candidate microRNAs showed a significant alteration in the RNA content of EVs purified from cholesterol-depleted cells. Knockdown of the VAP-A-binding cholesterol transfer protein ORP1L led to a substantial alteration in the RNA contents of small and large EVs. We propose that cholesterol binding and/or transfer via ORP1L at MCS affects the biogenesis of RNA-containing small and large EVs.
Summary/Conclusion: Altogether, these data suggest cholesterol transfer and/or sensing at ER MCS regulates RNA trafficking into small and large EVs.
Funding: Funding was provided by NIH grants U19CA179514 and PO1CA229123.
Keywords: extracellular vesicles, cholesterol sensing, membrane contact sites, extracellular RNA

OF12.3 | Proteome encoded determinants of protein sorting into extracellular vesicles

Dea Gogishvili[1]; Katharina Waury[2]; Rienk Nieuwland[3]; Madhurima Chatterjee[4]; Charlotte E Teunissen[5]; Sanne Abeln[2]
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Introduction: Extracellular vesicles (EVs) are membranous structures released by cells into the extracellular space and are thought to be involved in cell-to-cell communication. While EVs and their cargo are promising biomarker candidates, protein sorting mechanisms of proteins to EVs remain unclear. In this study, we ask if it is possible to determine EV association based on the protein sequence. Additionally, we ask what the most important determinants are for EV association.
Methods: We answer these questions with explainable AI models, using human proteome data from EV databases to train and validate the model. It is essential to correct the datasets for contaminants introduced by coarse EV isolation workflows and for experimental bias caused by mass spectrometry.
Results: In this study, we show that it is indeed possible to predict EV association from the protein sequence: a simple sequence-based model for predicting EV proteins achieved an area under the curve of 0.77 $\pm$ 0.01, which increased further to 0.84 $\pm$ 0.00

when incorporating curated post-translational modification (PTM) annotations. Feature analysis shows that EV-associated proteins are stable, polar, and structured with low isoelectric point compared to non-EV proteins. PTM annotations emerged as the most important features for correct classification; specifically, palmitoylation is one of the most prevalent EV sorting mechanisms for unique proteins. Palmitoylation and nitrosylation sites are especially prevalent in EV proteins that are determined by very strict isolation protocols, indicating they could potentially serve as quality control criteria for future studies. We demonstrate how our trained model can illustrate for individual proteins which factors contribute to their EV association.

Summary/Conclusion: This computational study offers an effective sequence-based predictor of EV-associated proteins with the extensive characterization of the human EV proteome that can explain for individual proteins which factors contribute to their EV association.

Funding: Research of KW, DG, CT, and SA are supported by the European Commission (Marie Curie International Training Network, grant agreement No 860197 (MIRIADE). CT is supported by JPND (bPRIDE)), Health Holland, the Dutch Research Council (ZonMW), Alzheimer Drug Discovery Foundation, The Selfridges Group Foundation, Alzheimer Netherlands, Alzheimer Association.

Keywords: extracellular vesicle sorting, protein sequence-based determinants, machine learning

OF12.4 | The impact of surface protein expression on the stiffness values of extracellular vesicles

Fredrik Stridfeldt[1]; Hanna Kylhammar[2]; Vipin Agrawal[3]; Vikash Pandey[4]; André Görgens[5]; Samir EL Andaloussi[6]; Dhrubaditya Mitra[7]; Apurba Dev[8]

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Introduction: During the last decade, multiple clinical applications of extracellular vesicles (EVs) have motivated broad interest in method development for their characterization. Until now, their mechanical properties received little interest and therefore remained poorly understood. However, researchers expect that they influence the cell surface-EV interaction. The nanoscale size of EVs and the high heterogeneity in their composition are both factors in the slow progress in this field. Here, we investigate the possibility of using nanoindentation with atomic force microscopy (AFM) for reliable measurement of EV stiffness. We then apply the method to explore the influence of surface protein (tetraspanins) composition on the EV stiffness. Finally, we compare the results to DOPC liposomes of similar size.

Methods: We perform the study with small EVs (sEVs) derived from human embryonic kidney (HEK 293) cell line. We pre-clean the cell culture-derived conditioned media by low-speed centrifugation and 0.22 μm filtration. Afterward, we prepare the sEVs by ultrafiltration and size exclusion chromatography and characterize them by nanoparticle tracking analysis (NTA) and bead-based multiplex flow cytometry. To evaluate the impact of tetraspanins on the stiffness of EVs, we compare wild-type (WT) EVs with EVs derived from CD63 knockout (CD63-KO) and with CD9/CD63/CD81 knockout (Pan-KO) EVs. We image all samples with Quantitative Imaging™ mode using a NanoWizard 3 AFM. In addition to imaging, we measure multiple force-distance curves (FDCs) with three different peak force levels (0.4 nN, 0.6 nN, and 0.8 nN). Finally, we also perform the same procedure for the DOPC liposomes.

Results: We find the mean stiffness to exhibit high heterogeneity within the range of 5–25 mN/m, irrespective of the sample type and the size of the sEVs. Data from individual sEVs are generally consistent over multiple FDCs and force setpoint (0.4-0.8 nN), indicating good stability for such measurements. The stiffness values appear to indicate the presence of sub-populations within each type. These sub-populations are most visible in the CD63-KO sample that shows two distinct clusters, with one being the stiffest among all the sEVs studied. We observe that the liposomes are softer and more homogeneous in their stiffnesses.

Summary/Conclusion: We find the stiffness of the sEVs to be heterogenous with the apparent presence of different subpopulations. The stiffness of sEVs is generally higher than those of DOPC liposomes of a similar size range. The CD63-KO sample exhibits the subset with the highest stiffness value.

Funding: Erling Persson Family Foundation, Swedish Research Council (2016–05051), and VR grant (2018-06228).

Keywords: atomic force microscopy, single EV analysis, force distance curves, stiffness, liposomes

OF12.5 | Charge-modulated dual-mode device for label-free isolation of extracellular vesicles from blood plasma

Hyun-Kyung Woo[1]; Young Kwan Cho[2]; Chang Yeol Lee[2]; Haeun Lee[2]; Cesar Castro[2]; Hakho Lee[3]
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Introduction: Blood contains different bio-nanoparticles that can be exploited for clinical diagnoses. Purifying these particles according to type, however, remains technically challenging. EVs are outnumbered >104-fold by low density lipoproteins (LDLs), yet similar in size and density. These fundamental disadvantages often cause LDL spillover into EV isolates, thus confounding assay results. Here, we report that surface charge can be an effective parameter to differentiate EVs from LDLs. To address this, we refined an all-in-one system which performed i) size-exclusion to remove particles smaller than EVs and LDLs and ii) cation-exchange in an acidic elution to retain LDLs longer than EVs.
Methods: We built an enhanced dual-mode device (DMD) which performed i) size-exclusion to remove particles smaller than EVs and LDLs and ii) cation-exchange in an acidic elution to retain LDLs longer than EVs. The performance of the DMD, in comparison to size-exclusion only, was evaluated by analyzing the yield and purity of the isolated EVs.
Results: We estimated the surface charge densities of EVs (-6.2 mC/m2) and LDLs (-3.6 mC/m2) by measuring zeta potentials at different buffer pH, revealing that EVs are more negatively charged than LDLs. In addition, we found that the charge difference between EVs and LDLs was maximal at a weak acidic condition (pH = 6.4). By applying this, we optimized eDMC operation to enrich EVs directly from plasma, depleting >99.8% of LPPs within 30 min, Minimizing LDL contamination improved analytical signals in EV molecular assays, including single vesicle imaging, bulk protein, and mRNA detection.
Summary/Conclusion: We have demonstrated a fast and non-biased way for EV isolation from blood plasma, promoting the translational value of the dual-mode separation.
Funding: This work was supported by NIH Grants R01CA229777, R21DA049577, R01CA239078, R01CA237500, U01CA233360, R01CA264363; and MGH Scholar Fund.
Keywords: extracellular vesicles, lipoproteins, surface charge modulation, size-exclusion, cancer

OF12.6 | Shedding light on the proteolytic processing capacity of ADAM10 on extracellular vesicles from primary brain cells

Christopher C. Reimann[1]; Tomas Koudelka[2]; Michaela Schweizer[3]; Andreas Tholey[2]; Lesley Cheng[1]; Markus Glatzel[4]; Marina Mikhaylova[5]; Andrew F. Hill[6]
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Introduction: Proteases are a common cargo of extracellular vesicles (EVs), however, the effects of their activity towards surface proteins on EVs and the impact on EV signaling are mostly unexplored. Here, we focus on the metalloprotease ADAM10 in cortex cells as it is a key player of brain function and a master regulator of neuronal proteins and adhesion molecules. Continuous activity of EV-associated ADAM10 (EV-ADAM10) could release bioactive fragments away from the sending cell or alter the specificity of EV-cell interaction, and investigating EV-ADAM10's processing capacity will allow for assessment of its functional significance in brain cell communication.
Methods: Primary cortex cells from E18 rat embryos were cultured for two weeks and small EVs were isolated from the cell supernatant by serial centrifugation and characterized by NTA, western blot, and EM. Activity assays with isolated EVs were performed over 24h in presence or absence of an ADAM10 inhibitor and a fluorogenic substrate. To assess processing of EV proteins, assayed EVs were subjected to N-terminal proteomics by HYdrophobic Tagging-Assisted N-termini Enrichment (HYTANE) followed by bioinformatic analysis.
Results: EV-ADAM10 from cortex cells demonstrated activity towards an exogenous fluorogenic substrate as well as EV endogenous proteins over 24h. N-terminomics identified 16 membrane-associated or secreted proteins with reduced numbers of N-termini following inhibition of EV-ADAM10 over 24h compared to control (>2-fold, $p < 0.05$). The affected proteins include cadherins, chaperones, and membrane traffic proteins with roles in cell adhesion, β-amyloid binding, and nervous system development.
Summary/Conclusion: Our findings provide novel insights into EVs as platform of proteolytic processing and the role of ADAM10 as potential modulator of EV function in the brain.

Funding: This work was supported by a La Trobe University Graduate Research Scholarship and Full Fee Research Scholarship, the German Research Foundation (DFG) and by grants from the NHMRC and ARC.
Keywords: ADAM10, brain cell communication, EV proteolysis, metalloprotease, N-terminal proteomics

OF13: Metastasis and Angiogenesis

Chairs: Janus Rak, Valentina Minciacchi

Location: Ballroom 6BC

14:20 - 15:50

OF13.1 | Brain-seeking extracellular vesicles derived from metastatic breast cancer cells modulate the metabolism of the endothelial cells of the blood-brain barrier

Sara Busatto[1]; Marsha Moses[1]; Golnaz Morad[2]
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Introduction: Breast-to-brain metastasis (BrM) affects ~10-16% of breast cancer patients, predicts a dismal patient prognosis and lacks effective therapeutic and diagnostic options. We demonstrated, for the first time, that EVs derived from brain-seeking breast cancer cells (BrEVs) breach the intact blood-brain barrier (BBB) via transcytosis and cause a significantly increased incidence of BrM. Within this context, we are now studying the interactions between BrEVs and BBB endothelial cells (ECs).
Methods: BrEVs were isolated by ultracentrifugation, characterized following MISEV guidelines and tested in vitro using primary human brain ECs and in vivo using our mouse model of BrM. ECs were treated with BrEVs and tested using transwell assays, impedance measurements and genetic engineering. Our in vivo models of BrM were administered intravenously with BrEVs and mouse brain microvessels were isolated and analyzed for protein expression. The results were analyzed using machine learning approaches.
Results: Our mechanistic studies show that (1) BrEVs target BBB ECs both in vitro and in vivo causing significant variations of multiple proteins involved in intracytoplasmic vesicles' long-recycling loop (2) BrEVs are responsible for specific EC barrier function and morphological changes (3) BrEVs mediate, at least in part, their effect through the delivery of specific micro RNAs. All molecules cannot be disclosed due to a pending patent.
Summary/Conclusion: BrEVs can alter BBB basal metabolism and help to prepare the pre-metastatic niche, a microenvironment that promotes and sustains BrM in an otherwise hostile microenvironment. Our mechanistic studies increase our understanding of the early events that facilitate BrM and have the potential to identify key regulators in BrM formation and contribute to the development of EV-based therapeutic and diagnostic strategies for BrM.
Funding: This work was supported by NIH R21 CA253051-01, the Breast Cancer Research Foundation, NIH T32 5T32HL007917-22 and the Nile Albright Research Foundation.
Keywords: breast cancer, breast-to-brain metastasis, extracellular vesicles

OF13.2 | Angiocrine Extracellular Vesicles Impose a Mesenchymal Phenotype Upon Proneural Glioma Stem Cells

Lata Adnani[1]; Jordan Kassouf[2]; Brian Meehan[3]; Cristiana Spinelli[3]; Nadim Tawil[4]; Ichiro Nakano[5]; Janusz Rak[6]
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Introduction: Glioblastoma (GBM) is an incurable form of primary astrocytic brain tumor driven by the glioma stem cell (GSC) compartment which is closely associated with the vascular niche. GSC phenotypes are heterogeneous and range from proneural

to mesenchymal-like, the latter characterized by greater invasiveness. Here, we studied the impact of cues received by GSCs via extracellular vesicles (EVs) derived from vascular endothelial cells.
Methods: Cell culture studies involved EV-depleted serum, inhibitors, Incucyte, xCelligence, bioassay (MTA, sprouting, migration assays) and FACS analysis. Molecular analysis entailed mass spectrometry, protease activity, and immunostaining assays. In vivo studies employed intracranial injections, and tissue analysis. EV analysis followed MISEV2018 guidelines. EVs were isolated by ultracentrifugation, characterized by Nanoparticle tracking analysis (NTA), and EV markers (western-blot). EV uptake was recorded by ImageStream, confocal microscopy and Cre reporter.
Results: We observed that endothelial cells and their derived EVs drive proneural-to-mesenchymal reprogramming of GSCs. Endothelial EVs carry matrix metalloproteinases (MMPs) which lead to inactivation of NOTCH pathway, stimulation of NFκB signaling, altered motility and responsiveness to chemotherapy, and driving infiltrative growth in vivo and in vitro in proneural GSCs. Proneural GSCs which scatter away from the tumor mass become increasingly susceptible to the angiocrine influences of EVs. Notably, endothelial EVs compete for influence on GSCs with cancer cell own EVs.
Summary/Conclusion: Our work documents that endothelial secretome, including, EVs, alters GSC subtype by instigating an infiltrative growth observed in GBM thereby contributing to therapeutic failure. We postulate that a tailored modulation of endothelial cell vesiculation or EV trafficking may improve outcomes in specific subsets of GBM.
Funding: Canadian Institutes for Health Research (CIHR), Foundation Charles Bruneau (FCB), Canadian Foundation for Innovation (CFI10)- to JR. LA received support from Whitehead-Penny Endowment, Fonds de research du Quebec-Sante, RI-MUHC Desjardins Studentship in Child Health and McGill Faculty of Medicine internal postdoctoral award.
Keywords: extracellular vesicles, endothelial cells, glioblastoma, angiocrine, EMT, invasion, angiogenesis, extracellular vesicle competition

OF13.3 | Plasma extracellular vesicles promote lung cancer pre-metastatic niche formation through endothelial modulation

Francesca Pontis[1]; Ilaria Petraroia[1]; Patrizia Ghidotti[1]; Fabio Maiullari[2]; Mattia Boeri[3]; Ugo Pastorino[1]; Paola Suatoni[1]; Roberto Rizzi[2]; Claudia Bearzi[2]; Gabriella Sozzi[4]; Orazio Fortunato[5]
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Introduction: Lung cancer is the deadliest cancer worldwide, primarily because of metastatic spread. Tumor-derived extracellular vesicles (EVs) play a crucial role in creating pre-metastatic niches (pMN), which facilitates the spread and expansion of circulating cancer cells. So far little is known about the role of plasma EVs in lung cancer pMN formation; we aim to elucidate their role in pMN establishment and identify new prognostic biomarkers for early stages patients.
Methods: Plasma-EVs were obtained by ultracentrifugation from 20 early-stage patients survived at 5 years (ESA-EVs) and 20 patients died within two years (ESD-EVs). Heavy-smokers cancer-free individuals were used as control (HS-EVs). EV's characterization was performed by Nanoparticles tracking analysis, Flow Cytometry (FC) and Western Blot. Uptake were evaluated by FC and Imaging FC. Functional experiments were carried out in vitro (2D and 3D-bioprinted models) and in vivo. EVs-miRNAs were evaluated using Nanostring and digital PCR, their prognostic value was evaluated in training and validation cohorts (40 and 40 patients).
Results: Alongside to common EV-related markers, plasma-EVs markedly express CD41b, CD42a, CD62P and CD31 (enriched in ESD-EVs compared to ESA- and HS-EVs) suggesting platelet and endothelial origin. Among other stromal cells, endothelial cells resulted as most avid incorporators of EVs, followed by macrophages and fibroblast (almost no uptake observed in epithelial cells). Indeed, ESD-EVs strikingly induced endothelial cells "pro-inflammatory" phenotype (VCAM1, CXCR4 and CXCL1 expression and release) compared to ESA- or HS-EVs treatments in 2D and 3D models. Moreover, conditioned medium of ESD-treated endothelial cells significantly stimulated fibroblast's cytokines production (CXCL1 and IL6). 3D-bioprinted co-cultures (fibroblast, endothelial and epithelial cells) corroborated ESD-EV-mediated endothelial activation and consequent fibroblast modulation. Importantly, ESD-EVs directly promoted macrophages immunosuppressive phenotype (IL10, CCL2, VEGF and CD206 upregulation). In-vivo biodistribution confirmed endothelial cells as major EV-incorporators within the lung. Likewise, greater lung endothelial activation and immune cells recruitment were observed in ESD-EV-treated compared to HS- and ESA-EVs-treated mice. Nanostring and dPCR analysis revealed 3 miRNAs (miR-1307, miR-199a, miR-29a) enriched in ESD-EVs compared to ESA-EVs in training and validation cohorts.
Summary/Conclusion: Our findings suggested that plasma EVs drive lung pMN establishment modulating directly endothelial cell and macrophages that in turn conditionate other stromal cells and mediated immune recruitment. Moreover, EV-miR-1307, miR-199a, and miR-29a may represent predictive biomarkers for early-stage lung cancer patients.

OF13.4 | Aged fibroblast-derived extracellular vesicles promote melanoma progression in vitro and in vivo

Laura Hueser[1]; Yash Chhabra[1]; Olesia Gololobova[2]; Vania Wang[3]; Mitchell Fane[1]; Murilo Ramos Rocha[3]; Kenneth W. Witwer[1]; Ashani Weeraratna[1]
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Introduction: Age is an important prognostic factor in cutaneous melanoma, commonly arising in the elderly. Melanoma progression is the concerted outcome of changes occurring in tumor cells and within the tumor microenvironment (TME). Aging causes remodeling of the TME, making it conducive to melanoma progression. Due to the important role of extracellular vesicles (EVs) in tumor progression in different cancer types we investigated how the aged TME can alter EV attributes, cargo, and function.
Methods: Conditioned media of age-stratified dermal fibroblasts (dFs), the predominant cell type in the melanoma TME, was concentrated and EVs were isolated via Izon size separation and characterized according to MISEV2018 guidelines. Next, mass spectrometry was performed to investigate how systemic aging of dFs changes the cargo of their secreted EVs. We then evaluated the influence of these EVs on melanoma progression in vitro and in two mouse models using the EV biogenesis inhibitor PDCC and EV injection.
Results: We found that the tetraspanin CD9 was reduced both in aged dFs and EVs released compared to young dFs. Modulating the CD9 expression in dFs was sufficient to alter its levels in EVs. CD9 is a crucial member in cargo sorting of EVs, and mass spectrometry analysis of EVs released by CD9 knock down vs. control cells revealed a significant increase in ANGPTL2 (angiopoietin-like protein 2), a promoter of angiogenesis. Our in vitro analysis of primary endothelial cells confirmed increased sprouting in HUVEC under CD9 KD conditions. Inhibiting EV biogenesis in aged mice reduced the amount mice with metastasis by 27 % compared to vehicle-treated mice. Moreover, injecting aged dF derived EVs in the primary melanoma in vivo resulted in 17% more mice with metastasis compared to the injection of young EVs.
Summary/Conclusion: Our data shows modulation of EV cargo owing to intrinsic aging that promotes tumor metastasis via angiogenesis and identifies novel therapeutic avenues to alleviate age-associated melanoma progression.
Keywords: cancer, aging, tumor microenvironment

OF13.5 | Dissecting vasectasia - extracellular vesicle-driven non-angiogenic vascular growth process in mesenchymal glioblastoma

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Introduction: Glioblastoma (GBM) is a high grade incurable, highly vascular brain tumour driven by transformed glioma stem cells (GSCs). While prominent, the vascular alterations associated with GBM are poorly understood. GSCs are heterogenous and their mesenchymal subtype (MES-GSCs) often express oncogenic form of epidermal growth factor receptor (EGFR) and its variant III mutant (EGFRvIII), while their proneural counterparts (PN-GSCs) resemble neural stem cells and are largely EGFR-negative. Extracellular vesicles derived from both GSC subtypes have been shown to exhibit different proteomes and biological activities. Here we investigated the ability GSC EVs to stimulate angiogenesis in vitro and in vivo.
Methods: EVs were isolated from serum-free conditioned media of MES- or PN-GSCs, following size exclusion chromatography and ultracentrifugation protocol. EVs were characterized for their content of tetraspanin markers, EGFR protein, mRNA and biological activity in endothelial cell migration and sprouting assays. EVs were also embedded in Matrigel pellets and implanted subcutaneously.
Results: Intracranial xenografts of PN-GSC and MES-GSC exhibited dramatic differences in vascular patters, the latter composed mostly of large, dilated vessels and relative scarcity of angiogenic capillaries. This non-angiogenic process (vasectasia) was driven by EV-mediated transfer of oncogenic EGFR from cancer cells to endothelial cells and abrogated by EGFR gene editing and EGFR kinase inhibitor treatment, each of which also obliterated responses of endothelial cells in vitro to the exposure to EGFR-containing EVs. EV-dependent tumour vascular patterns were recapitulated in Matrigel assays in vivo. Single cell sequencing of MES-GSC tumours revealed a distinct composition and molecular make up of vasectasia-associated endothelial cells in glioma xnografts.

Summary/Conclusion: EGFR carrying EVs released from mesenchymal GSCs drive vasectasia, a distinct form of non-angiogenic vascular growth that may represent a new target in glioblastoma therapy.
Funding: Canadian Institutes for Health Research, Fondation Charles Bruneau.
Keywords: angiogenesis, vascular pathology, glioblastoma, endothelial cells, oncogenes, EGFR, EGFRvIII, oncogene-transfer, mesenchymal glioma stem cells, proneural glioma stem cells, cancer therapy

OF14: Neurogenerative Diseases

Chairs: Christian Neri, Koen Breyne

Location: Ballroom 6A

14:20 - 15:50

OF14.1 | Exophers: novel extracellular vesicles in mammalian neurons are highly responsive to tauopathy

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Introduction: We have discovered that exophers, which previously had been described in C. elegans, exist in mammalian neurons. Exophers are thought to be a mechanism for non-autonomous degradation of unwanted cellular material. Unlike other extracellular vesicles, exophers can be as large as the cell itself. They may remain connected to the cell via a nanotube for many hours and continue to receive cellular material. Proteostatic stress has been shown to increase exopher number in C. elegans yet whether this is also the case in mammalian neurons is not known. It is also unknown if the size of exophers is responsive to the stress level.
Methods: Exophers were analyzed using light and fluorescence microscopy in cell culture systems, including human iPSC-derived neurons, primary mouse neurons from three different lines, and in human and mouse brain sections. Human brains included patients with Alzheimer's disease (AD), other tauopathies, and controls. They were stained, as appropriate, for markers of the neuronal cell body, hyperphosphorylated tau, Aβ42, and nuclei.
Results: In cell culture, the number of exophers increases over time and in response to tauopathy, yet the response differs in different cellular models and in neurons from different mouse models. Interestingly, in human brain, although both Aβ and tau pathologies correlate with increased exopher number, only tau pathology appears to increase exopher size, a finding that is recapitulated in cell-culture models. Initial co-culture experiments suggest that neuronal exophers are taken up by microglia for processing of their content.
Summary/Conclusion: Exophers are a newly discovered type of extracellular vesicles, apparently playing important roles in non-autonomous degradation of unwanted cellular material. In the context of AD and other tauopathies, they may be a double-edged sword – helping clear proteotoxic aggregates, but also facilitating cell-to-cell spread of the pathologic protein aggregates.
Keywords: exopher, tauopathy, alzheimer's disease.

OF14.2 | Tau interactome profiling in human brain-derived extracellular vesicles uncovers key molecules potentially contributing to disease progression in Alzheimer's disease

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Introduction: Extracellular vesicles (EV) play critical roles in transferring pathological proteins related to Alzheimer's disease (AD). Recent studies demonstrate tau-containing EVs are transmissible and induce tau propagation in the brain, however, the underlying mechanism regarding EV-mediated tau pathology is still largely unknown.

Methods: We performed the immuno-affinity purification of tau in EVs derived from 14 AD and 14 normal controls (CTRL) brains and mass spectrometry to characterize EV associated tau interactome. We applied the Monolith to validate the interaction of potential EV-tau interactors with purified recombinant tau. We tested super-resolution microscopy (ONI) to further visualize their colocalizations in a single-EV level. We also designed siRNAs to silence EV-tau interactors in human neuronal SH-SY5Y cells overexpressing human P301L tau and assessed tau containing EVs secreted from the cells by Nanoanalyzer (NanoFCM). EVs from cells after silencing tau interactors are evaluated with live-cell imaging in human iPSC-derived neurons to determine tau uptake and seeding.
Results: A total of 764 proteins were identified as tau interactome in brain-EV isolated from CTRL and AD patients. The proteins are enriched in exocytic vesicle, transmembrane transport and cytoskeleton pathways as determined by bioinformatics. The comparisons of tau interacting proteins demonstrated that 65 proteins (e.g.,CYCS, KRAS, SIR2, SYT1), significantly downregulated in AD brain EVs, are enriched in pathways associated with vesicle-mediated transport in synapse; whereas 5 proteins (e.g., ATP1B3, ANXA5, ANK3), significantly upregulated in AD brain EVs, are involved in transmembrane localization. Correlation analysis showed the top differentially expressed EV-tau interacting proteins including CYCS, SYT1, ATP1B3 and ANXA5 were significantly correlated with Braak tangle stage. SYT1 level showed a significant prediction of AD (AUC = 0.86) as determined by ROC curve. The purified recombinant proteins of CYCS (Kd 0.34 μM), SYT1 (Kd 1.19 μM), ATP1B3 (Kd 0.833μM) and ANXA5 (Kd 1.88μM) showed binding affinities with purified recombinant tau in vitro as determined by Monolith. Furthermore, Nanoimager validated the reduced colocalization of SYT1, and increased colocalization of ATP1B3 or ANXA5 with tau in AD-derived EVs at a single-particle level. Finally, we silenced ANXA5 or ATP1B3 in SH-SY5Y cell lines and found reduction of tau+ EV populations.
Summary/Conclusion: We performed a novel EV-associated tau interactome and found the changes in tau interactors in AD-derived EVs: SYT1 and CYCS were reduced to affect synaptic vesicle transportation; Localization of specific membrane proteins ATP1B3 and ANXA5 preferably interacted with tau. Finally, silencing of ANXA5 or ATP1B3, the enriched tau interactors in AD EV, diminished tau loading in EVs. Targeting of these molecules may suppress tau dissemination, highlighting their therapeutic potential in AD.

OF14.3 | Microbially induced exosomes in the oral cavity cross the BBB and contribute to Alzheimer's disease pathogenesis

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Introduction: Exosomes (exo) can cross the blood brain barrier (BBB) and have recently been linked to neuroinflammation and Alzheimer's disease (AD) pathogenesis. Periodontitis (PD) is a chronic oral degenerative disease that affects 70.1% of Americans 65 years and older and has been linked to age-related disease such as AD. P.gingivalis (Pg) is considered a "keystone" pathogen in PD due to its outsized influence on the local oral microflora. Our recent work has shown that exo derived from Pg-infected dendritic cells (DC) transmit immune senescence to bystander cells and induce inflammatory alveolar bone loss in-vivo. The aim of the current study was to test the hypothesis that microbially induced exo in the oral cavity cross the BBB and contribute to neuroinflammation and pathogenesis of AD.
Methods: Isolation of exo from Pg-infected DCs (PgDCexo) was performed using ultracentrifugation and ultra-filtration methods. Quantification and characterization of PgDCexo were performed using NTA analysis, western blot and miRNA array. DiI labeled PgDCexo or exo from immature non-infected DCs (control) were injected intragingivally in wild type (WT) mice or APPNL-G-F knock in mouse model of AD (n = 6 per group). Detection of exo in the brains was performed using the in-vivo live imaging system (IVIS) and confocal microscopy. Brains were dissected and analyzed for signs of AD pathology and neuroinflammation by WB and qPCR. Maxillae were analyzed for bone loss by micro-CT.
Results: In-vitro analysis revealed a~2-fold increase in secreted exosomes from DCs infected with Pg.These exo were enriched in age-related, anti-apoptosis/anti-autophagy miRNAs, Pg fimbrial adhesin protein mfa1, and pro-inflammatory cytokines IL6/TNFa/IL1b. Orally injected PgDCexo were detected in the brains of APPNL-G-F knock-in and WT mice using IVIS and colocalized with Iba1 positive microglial cells in the hippocampus using immunofluorescence. Hallmarks of AD including, neuroinflammation, hyperphosphorylation of Tau (Ptau), and beta amyloid deposition were increased in brains of APPNL-G-F knock-in injected with PgDCexo compared to control. This was associated with inflammatory alveolar bone loss as evident by micro CT 3D analysis.
Summary/Conclusion: Oral microbial infection induces senescence and exo release from DC. These pathologic exo promote paracrine senescence of normal bystander cells, induce inflammatory alveolar bone loss and can cross BBB, contributing to AD pathogenesis.
Keywords: periodontitis, exosome, immune senescence, BBB, alzheimer's disease

OF14.4 | Brain-derived EVs: a window into Parkinson's biomarker discovery

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Introduction: Parkinson's disease (PD) is a progressive neurodegenerative movement disorder. Currently, PD diagnosis relies on late clinical manifestations that overlap with those of other pathologies. Extracellular vesicles (EVs) have emerged as important participants in PD pathophysiology. Interestingly, brain tissue-derived EVs (bdEVs) participate in cell-to-cell communication in the brain and can leave the tissue of origin to more accessible peripheral biofluids such as blood plasma.
Methods: bdEVs were separated per an established protocol (Huang et al., JEV, 2020) from brain tissue (Johns Hopkins Brain Resource Center) of PD (n = 24), progressive supranuclear palsy (n = 25), and control (n = 24). Basic characterization was conducted per MISEV2018 guidelines. bdEV surface proteins were profiled using multiplexed ELISA. A subset of the samples was profiled by mass spectrometry for quantitative protein analyses.
Results: Interestingly, several markers showed differences between pathological groups and control. Microglial markers TMEM119 and CX3CR1 and neuronal markers CD90, NCAM, and NRCAM were significantly (p-value < 0.05) more abundant in PD and PSP. Quantitative proteomics revealed the presence of 26 significantly differentially abundant proteins in PD vs. control and/or PSP (Log 2-fold-change = 0.32 and p-value < 0.05). Biologically, some of these proteins are implicated in solute carrier transport, e.g., SLC8A2 differed between PD vs. PSP and PD vs. Control. In contrast, SLC1A3 and SLC2A1 differed only between PSP and control. Several EV markers were also statistically different, e.g., clathrin protein implicated in endocytosis pathway was significantly less abundant in PD vs. PSP.
Summary/Conclusion: In this study, we identified several proteins that are differentially abundant between PD and control and that may serve as biomarkers and therapeutic targets. We will test abundant cell-specific EV surface proteins for utility in immunoprecipitation-based separation.
Funding: This work was supported by the Michael J. Fox Foundation for Parkinson's Research [00900821]. The Witwer lab is also supported in part by NIH grants AI144997, MH118164, and DA047807.
Keywords: Parkinson's disease, microglia, astrocytes, neurons, extracellular vesicles, ectosomes, exosomes, biomarkers, proteomics, progressive supranuclear palsy

OF14.5 | Mesenchymal stem cell-derived small extracellular vesicles rescue DA neurons through restoration of mitochondrial functions in Parkinson's Disease mouse model

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Introduction: Parkinson's disease (PD) is a common neurodegenerative disease, which is characterized by the irreversible damage of dopamine neurons related to mitochondrial disorder. Emerging studies have reported that mesenchymal stem cells-derived small extracellular vesicles (MSC-sEV) treatment represents a promising cell-free approach to alleviate neurodegenerative disorders. However, the repairing effects of MSC-sEV in PD remain largely unclear. In the present study, we determined the role of MSC-sEV intervention in MPTP-induced DA neurons and the following mechanism.
Methods: GMP grade umbilical cord-derived MSC-sEV were prepared using anion-exchange chromatography, and their characteristics and safety effects were examined. We induced PD mouse model using MPTP, followed by the intranasal administration (IN) of MSC-sEV to determine their effects. Mitochondrial dysfunction of DA neurons were further examined using RNA-seq, transmission electron microscopy.
Results: MSC-sEV exhibited good safety effects. The intranasal administration of MSC-sEV promoted the repair of dopaminergic neurons in MPTP-induced PD mouse model. After IN for 6h, fluorescence labeled MSC-sEV were observed in substantia nigra (SNc). We further found that Homer3 may play important role in the MSC-sEV treatment via RNA-sequencing. Additionally, we identified that MSC-sEV could restore mitochondrial function of DA neurons by upregulating Homer3. Moreover, Homer3 knockdown reversed MSC-sEV-mediated DA neurons therapeutic effects.
Summary/Conclusion: Our findings indicated that MSC-sEV rescued damaged DA neurons through restoration of mitochondrial functions partially via upregulating Homer3, providing the new insights into the treatment of PD.

OF14.6 | ATP1A3 as a novel marker for isolating neuron-specific extracellular vesicles from human brain and biofluids

Tsuneya Ikezu[1]; Yang You[1]; Zhengrong Zhang[1]; Nadia Sultana[2]; Maria Ericsson[3]; Yuka Martens[4]; Min Sun[5]; Takahisa Kanekiyo[4]; Seiko Ikezu[1]; Scott Shaffer[6]

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Introduction: Background: Neuron-derived extracellular vesicles (NDEVs) provide a reliable source for understanding the state of brain and discovering biomarkers of neurological diseases. Over the past several years, there has been a keen interest in capturing neuron-specific EVs from patient-derived biopsies or biofluids and characterizing their contents as a pathological reflection of the central nervous system (CNS). A reliable and reproducible NDEV markers are needed to isolate and characterize bona fide NDEVs from human samples. Our recent study identified ATPase Na+/K+ Transporting Subunit Alpha 3 (ATP1A3) as one of the abundant neuron-specific EV markers. ATP1A3 is mostly enriched in brains, with some specific expression in heart muscles. Here we conduct systematic neuronal EV analyses to evaluate whether ATP1A3 is specific to NDEVs and a reliable marker for NDEV isolation from biofluids for disease monitoring.
Methods: Methods: Immunoelectron microscopy was used to detect ATP1A3, L1 cell adhesion molecule (L1CAM) and neural cell adhesion molecule 1 (NCAM1) in EVs isolated from iPSC-derived excitatory neurons, brain tissue, plasma and cerebrospinal fluid (CSF). Neuronal EV was enriched from isolated brain EVs using immunoaffinity isolation with either anti-ATP1A3, L1CAM or NCAM1 antibodies, and the enrichment of neuronal population was evaluated by quantitative mass-spectrometry and validated by immunoblotting. The enrichment of neuronal markers were compared among EVs isolated from CSF and plasma samples using ExoView and Nanoimager. We also tested if Alzheimer's disease-related amyloid-b peptide (Ab) are enriched in ATP1A3+ plasma EVs using Nanoimager and compared with the values of plasma AD biomarkers as determined by SIMOA.
Results: Results: ATP1A3 is highly enriched in NDEVs isolated from induced human neurons, brain, cerebrospinal fluid, and plasma samples compared to NCAM1 or L1CAM as determined by quantitative proteomics, biochemistry, ExoView and NanoImager. Aβ+ population in ATP1A3+ EVs from plasma can distinguish Alzheimer's disease from mild cognitive impairment and control cases as determined by Nanoimager whereas the conventional quantification of Aβ in plasma by SIMOA show modest difference among groups.
Summary/Conclusion: Conclusion: Our data demonstrate that ATP1A3 is a promising marker to isolate human NDEV from biofluids for diagnostic research in neurodegenerative diseases.
Funding: Florida Department of Health Ed and Ethel Moore Alzheimer's Disease Research Program-22A04 (YY), Alzheimer's Association AARF-22-918114 (YY), Cure Alzheimer's Fund (TI), NIH RF1 AG054199 (TI), R01AG054672, R01 AG066429 (TI), R01 AG067763 (TI), R01 AG072719 (TI).
Keywords: neuron, brain, biomarker, induced pluripotent stem cells, cerebrospinal fluid, plasma, ExoView, nanoimager, proteomics

OF15: Omics

Chairs: Juan Manuel Falcon, David Greening

Location: Room 608/609

14:20 - 15:50

OF15.1 | Visualization and proteomic mapping of extracellular vesicles during intracellular trafficking

Steven Ting-Yu Chuo[1]; Yen-Ju Chen[2]; Wendy Wan-Ting Wong[2]; Yi-Wen Chang[3]; Ju-Chen Chuang[1]; Hsueh-Fen Juan[3]; Charles Pin-Kuang Lai[4]

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Introduction: Extracellular vesicles (EVs) play an important role in cell-to-cell communication under (patho)physiological conditions. However, the fate of delivered EVs, as well as interacting proteins which mediate EV intracellular transport remained largely unexplored. We developed a multi-functional EV imaging and proximity labeling system, PalmGREX, to enable visualization and interactome analysis of EVs with spatiotemporal accuracy.
Methods: Bioluminescence resonance energy transfer (BRET)-based PalmGRET EV reporter (Charles Lai lab) comprising eGFP-NanoLuc (Antonio Amelio lab) and inner membrane labeling palmitoylation moiety of growth cone associated protein 43 was molecularly fused with APEX2 ascorbate peroxidase (Alice Ting lab) to create PalmGREX. Human embryonic kidney 293T cells (293T) were stably transduced to express PalmGREX followed by isolation of small EVs (sEVs) via differential centrifugation. Labeled sEVs were administered to 293T, and the sEVs were tracked subcellularly at super-resolution, followed by proximity labeling and proteomic analysis of EV-interacting proteins during intracellular trafficking. Trypin was applied to EV-recipient cells to mitigate non-internalized sEVs.
Results: PalmGREX enables live-cell tracking of sEVs under super-resolution microscopy, where PalmGREX-sEV signals significantly overlap with the endoplasmic reticulum (ER) and are in close proximity to the mitochondria at 3-hour post-sEV treatment. In addition, PalmGREX-sEVs were semi-quantitated and confirmed to be internalized by the recipient cells via bioluminescent and BRET-fluorescent signals in a time-dependent manner. Concurrently, PalmGREX enables proximal protein labeling of internalized sEV during subcellular trafficking. Downstream proteomics and functional enrichment analyses based on GeneOntology reveal that PalmGREX-sEV proximity-labeled proteins are enriched with the mitochondrial and endoplasmic reticulum (ER) annotated proteins.
Summary/Conclusion: Using PalmGREX, we identified that internalized sEVs are trafficked to the ER and mitochondrial periphery, which is corroborated by enriched ER and mitochondrial proteins as identified by sEV proximity labeling. Efforts are currently underway in identifying sEV-interacting proteins involved in subcellular trafficking and the fate of internalized sEVs following their uptake.
Funding: National Science and Technology Council (NSTC) grants NSTC 111-2628-B-001-004 (C.P.L.), Academia Sinica Innovative Materials and Analysis Technology Exploration (i-MATE) Program AS-iMATE-107-33 (C.P.L.), Institute of Atomic and Molecular Sciences grants IAMS 30-08 (C.P.L.), and Academia Sinica Career Development Award 109-M04 (C.P.L.).
Keywords: EV uptake, small EV, subcellular trafficking, proximity labeling

OF15.2 | Multiplexed inner and outer protein analysis of metastatic colorectal cancer patient plasma EVs on antibody microarrays

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Introduction: Colorectal cancer (CRC) is a leading cause of cancer death, in large part due to its high metastasis rate, notably to the liver. CRC liver metastases (CRCLM) present with distinct angiogenic behaviors that can inform treatment decisions but require a biopsy to determine. Extracellular vesicles (EVs) offer a biomolecular snapshot of the cell of origin and are abundant in easily sampled biological fluids. Previously, we developed the Extracellular Vesicle Antibody Microarray for Multiplexed Inner and Outer Protein Analysis (EVPio), enabling the combinatorial, high-throughput study of cytosolic and membrane proteins in cell line EVs. Here, we introduce the optimization and application of EVPio for the protein phenotyping of EVs from plasma samples from CRCLM patients with differing clinical presentations.
Methods: To optimize EVPio for use with patient samples, three sample processing parameters (plasma dilution or concentration factor, filtration pore size, and presence of size-exclusion chromatography [SEC] purification) were assessed for their impact on assay signal and robustness using EVPio assays of human pooled plasma (with and without HT29 CRC EV spike-ins). The optimized sample processing pipeline was then used to run EVPio on plasma samples from 6 CRCLM patients (3 each of the replacement and desmoplastic subtypes) and 3 non-CRC controls. EVPio assays targeted 20 proteins, including tetraspanins, integrin subunits, and cancer-associated or mutated proteins. The phenotyping data was analyzed using hierarchical clustering to pinpoint differentiating features.
Results: Sample processing featuring a single, lower-speed centrifugation step followed by filtration without further purification was found to yield higher SNRs than SEC isolation, without substantial increases in assay background or variability. EVPio in plasma sample EVs was validated using HT29 EVs spike-ins. Phenotyping of CRCLM plasma samples using EVPio yielded extensive co-expression patterns and identified distinctive proteomic features associated with disease status.
Summary/Conclusion: EVPio was optimized for use with human plasma samples and used to phenotype EVs from CRCLM patients, providing insight into the link between EV proteomic profile and disease presentation. These protein profiles will be leveraged to develop an EVPio study on a larger-scale CRCLM patient cohort.

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Keywords: colorectal cancer, colorectal cancer liver metastasis, proteomics, antibody microarray, plasma EVs

OF15.3 | Mass spectrometry of extracellular vesicles from ascites identifies the composition of ovarian cancer tumor microenvironment

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Introduction: Ovarian cancer (OC) ranks among the deadliest cancers in women. Lack of symptoms, rapid metastases and common chemoresistance contribute to unfortunate fate of majority of OC patients, especially those having high-grade serous carcinoma of the ovary, fallopian tube and peritoneum (HGSC), the most common and most aggressive type of OC. Many HGSC patients have excess fluid in the peritoneum at the stage of diagnosis called ascites. Ascites is basically a tumor microenvironment (TME) containing various cells, proteins and also extracellular vesicles (EVs). Small size and polydispersity of EVs brings various challenges to their isolation and characterization, including method-dependent enrichment of different EV subtypes as well as contaminants.
Methods: Therefore we isolated EVs from ascites of 11 HGSC patients by two different methods: ultracentrifugation coupled to sucrose cushion and size-exclusion chromatography (SEC) using qEV column, as well as the main protein fraction from SEC, serving as negative control for each patient; and analyzed all samples using tandem mass spectrometry.
Results: We identified core ascitic EV proteins present in all patients that contain typical EV markers and are devoid of method-dependent contaminants. To cover interpatient heterogeneity, we expanded these "core proteins" with proteins found in majority of patients. Next, we compared them with proteins of EVs from related control fluids and found proteins present only in/on EVs from HGSC patients. We believe this list of proteins contain both important players of HGSC progression as well as potential biomarkers. Using single cell RNA sequencing data we mapped the origin of EVs to different types of cells present in malignant ascites. Our results suggest that EVs in ascites do not come predominantly from tumor cells, but rather from variety of non-malignant cell types including cancer-associated fibroblasts and tumor-associated macrophages, which presence in ascites we confirmed by flow cytometry.
Summary/Conclusion: Our results emphasize the recently appreciated role of TME in the progression of HGSC. To conclude, this is the first study combining mass spectrometry and scRNA sequencing in an attempt to link EV composition to the cell types producing it. As such it opens numerous avenues both for better understanding of EV role in tumor promotion/prevention and for the improved HGSC diagnostics.
Funding: This work was supported by the Grant Agency of Masaryk University (grant number MUNI/R/1225/2021) and Ministry of Health of the CR / Ministry of Health Research Programme 2020 – 2026 (grant number NU21-03-00306).
Keywords: ovarian cancer (OC), high-grade serous carcinoma of the ovary, fallopian tube and peritoneum (HGSC), extracellular vesicles (EV), ascites, mass spectrometry, tumor microenvironment, macrophage

OF15.4 | Proteomic analysis of sEVs recovered from cow's milk, human milk and infant formula products: Moving towards next-generation infant nutrition

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Introduction: The first thousand days of life are critical to establishing optimal health in the human infant. Up to 75% of infants receive infant formula (IF) by 6 months of age, despite human milk (HM) imparting significant health advantages related to improved immune function. Most IF has a protein base derived from cow's milk (CM), however the difference to HM is not well-understood. Small extracellular vesicles (sEVs; 50 – 200 nm) are present in milk and have been successfully recovered by our group from IF products (unpublished data). Here, we compared the proteomes of sEVs recovered from HM, CM and IF.

Methods: sEVs were isolated from 200 mL of HM, CM and IF by sequential differential centrifugation coupled with size-exclusion chromatography (Vaswani et al., 2017). sEV enriched fractions were pooled and characterised by western blot (WB; FLOT-1, CD9, CD81, SYN-1, TSG101, ALB, GAPDH, CALNX), NTA and TEM. Proteomic analysis was performed on a SCIEX5600+ TripleTOF mass spectrometer using sequential window acquisition of all theoretical mass spectra (SWATH-MS). Raw data were processed in DIA-NN (False discovery rate = 0.01), and output files were processed in R Studio using homologous peptides for cross-species comparison. Gene ontology was performed using the PANTHER online tool.
Results: Mean particles sizes for HM, CM, and IF were 129.9 nm, 124.3 nm and 101.5 nm, respectively. CD81, TSG101, and FLOT-1 were detected by WB and mass spectrometry in all samples. 216 proteins were common to HM and CM; 126 were enriched and 17 depleted in CM compared to HM. Among the enriched were immunomodulatory proteins lactadherin, xanthine oxidase and butyrophilin, G proteins (24%) and metabolite interconversion enzymes (22%). CM and IF had 208 proteins in common, of which enzymes and binding proteins represented 37% and 41%, respectively.
Summary/Conclusion: The workflow captured species-specific differences, thus demonstrating potential for ongoing characterisation of milk sEVs relating to infant health. Lyophilised IF contains sEVs whose molecular contents are preserved regardless of processing, which could be leveraged to optimise IF products in the future. Further investigation into the functional capacity of IF EVs should focus on changes to the EV protein corona and surfaceome, as this may influence cellular uptake of lyophilised sEVs.
Funding: This project was funded by Reckitt Benckiser/Mead Johnson.
Keywords: extracellular vesicles, milk, infant formula, nutrition, proteomics, mass spectrometry, SWATH

OF15.5 | Optimization of plasma extracellular vesicle enrichment for in-depth lipidomic characterization

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Introduction: Lipid dyshomeostasis is associated with various diseases including lipid storage and neurological disorders. The work from our laboratory (Su et al, JEV 2021), and others, suggests that extracellular vesicles (EVs) contain lipid biomarkers that could aid in the diagnosis and treatment of these diseases. Knowing the lipid content of EVs is a key first step to discovering EV-based lipid biomarkers in blood. Some studies have reported the lipid content of ''EVs'' in blood. However, currently used techniques are known to co-isolate free lipid and lipoproteins. Here we: 1) compare the ability of commercially available EV kits to deplete plasma co-isolates and enrich for EVs, 2) demonstrate the importance of size exclusion resin size to improving the signal to noise ratio 3) are developing a reference EV plasma lipidome for the ISEV community.
Methods: Human plasma EVs were isolated using an assortment of commercially available kits or by density fractionation and size exclusion chromatography and characterised by western blot, transmission electron microscopy, and quantitative mass spectrometry based proteomic and lipidome analysis.
Results: Of the high-throughput EV isolation kits tested, the majority were unable to separate lipoproteins from EVs for the purposes of EV profiling. We then relied on comparing labor intensive methods (density gradient and size exclusion) to identify the combination of techniques to produce the analytes of interest, EV proteins and lipids. Quantitative proteome and lipidome analysis strategies are being applied to highly enriched EVs with the goal of providing the ISEV community with the reference lipidome of EVs in plasma.
Summary/Conclusion: To enhance the confidence of EV lipid identification and reveal the lipidome of EVs in plasma, our study compared and identified techniques to enrich EVs and deplete non-EV associated lipid and protein from human plasma. These findings serve as a reference resource and the foundation for future research investigating plasma EV lipids as disease biomarkers.
Funding: This work was supported by grants from the Bethlehem Griffiths Research Foundation to LJV, the Alzheimer's Australia Dementia Research Foundation John Shutes Project Grant to LJV and The Alzheimer's Association (AARF-18-566256) to LJV (U.S.A).
Keywords: biomarkers, plasma, extracellular vesicles, exosomes, lipids, lipidomics, proteomics, mass spectrometry, density gradient, size exclusion chromatography

OF16: Drug Delivery

Chairs: Mario Gimona, Houjian Cai

Location: Room 606/607

14:20 - 15:50

OF16.1 | IEDDA-mediated surface functionalization of extracellular vesicles with immunomodulatory ligands enhances therapeutic efficacy and decreases off-target toxicity

Migara Kavishka Jayasinghe[1]; Yock Sin Lay[2]; Dawn Xiao Tian Liu[2]; Chang Yu Lee[2]; Dong Van Hoang[1]; Rebecca Carissa Prajogo[2]; Minh T.N Le[1]
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Introduction: Extracellular vesicles serve as excellent drug carriers, enhancing biodistribution and cellular uptake of drugs. Current immunotherapeutic approaches for cancer therapy are limited by a balance between therapeutic efficacy and off-target side-effects and can benefit from conjugation onto EVs. In this study, we developed a novel IEDDA-mediated surface functionalization approach to engineer red blood cell-derived EVs post-isolation with immunomodulatory ligands at high efficiency. We hypothesised that the resulting EVs would mimic ligand multimerization, facilitating efficient receptor crosslinking and formation of immune synapses, thereby enhancing the effect of the EV-associated immunomodulatory ligands as compared to free ligands.
Methods: Extracellular vesicles were isolated from human red blood cells and subsequently purified via differential centrifugation, density centrifugation and size exclusion chromatography. The resulting EVs were surface functionalized with a combination of immunomodulatory ligands using bioorthogonal IEDDA click chemistry. These surface functionalized EVs were assessed on their ability to activate immune cells and suppress tumor progression in vivo as compared to treatment with an equivalent dose of free ligands.
Results: IEDDA-mediated conjugation resulted in efficient EV surface functionalization, capable of achieving over 50,000 copies per EV. Conjugation of immunomodulatory ligands such as agonistic CD137 antibodies and IL-2 on the EV surface significantly enhanced their therapeutic efficacy over free ligands via the formation of immune synapses and improved receptor clustering. EVs conjugated with a complementary combination of immune stimulatory ligands and immune checkpoint inhibitors were able to significantly shift the tumor immune milieu towards an anti-tumorigenic phenotype in a lung metastatic B16 F10 allograft and suppress tumor progression to a greater extent than a equivalent dose of free ligands. Conjugation of ligands onto EVs also limited their biodistribution to the tumor microenvironment, resulting in lower off-target toxicity than the free ligand treatment.
Summary/Conclusion: Conjugation of immunomodulatory ligands onto EVs via IEDDA reactions presents a potent and biocompatible approach that can enhance the efficacy of existing immunotherapeutics while addressing much of the drawbacks faced by current immunotherapeutic approaches for cancer treatment.
Funding: Ministry of Health (grant MOH-000643).
Keywords: extracellular vesicles, cancer, immunotherapy, EV engineering, EV surface functionalization, IEDDA

OF16.2 | Functionalizing the membrane of enveloped protein nanocages

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Introduction: Extracellular vesicles (EV) offer a promising alternative to current methods of drug packaging and delivery, but engineering EV is challenging. To enable more precise engineering of EV, we developed Enveloped Protein Nanocages (EPN): EV generated by designed self-assembling proteins which induce their own release from cells inside cell-derived membrane envelopes. EPN proteins provide a genetically addressable and modular platform to produce EV. Here we describe methods that enable the functionalization of EPN membranes through the incorporation of natural and designed Transmembrane Proteins (TMP). In contrast to conventional EV-based technologies which rely on passive pseudotyping, we leverage covalent conjugation of the TMP with the EPN protein to create Transmembrane Protein-Conjugated Enveloped Protein Nanocages (TMP-C-EPN). These TMP-C-EPN ensure efficient incorporation and display of the TMP on the EPN membrane, and can result in the formation of monocage EPN consisting of a single protein nanocage per membrane envelope. Different TMP have been incorporated to functionalize the EPN, allowing cell-specific targeting and vaccine development. EPN are a promising new class of genetically encoded biomaterials, and generally highlight the utility of designed protein scaffolds that induce EV release.

Methods: EPN were produced and purified from adherent HEK 293T and suspension expi293F cells. Use of the SpyCatcher-SpyTag system allowed for conjugation of the intravesicular EPN nanocage with the intravesicular tail of the designed TMPs, ensuring presentation of a particular TMP on the surface of released EPN. Western blots were used to probe for the presence of designed TMP, to check for membrane integrity, and to evaluate conjugation efficiency of the TMP-cage interaction. CryoEM was used to image particles and evaluate EPN morphology. Flow cytometry analysis was done to evaluate cell-specific EPN targeting efficiency.
Results: EPN cages were successfully conjugated via SpyCatcher-SpyTag covalent interaction to a designed minimal TMP. A small population of TMP-C-EPN were seen as monocage EPN. Design of an EGFR-targeting TMP allowed for targeting of the EPN towards only EGFR-expressing cells. Inclusion of the SARS-CoV-2 spike protein resulted in CoV-2 decorated EPN, and delivery of an mRNA encoding for the EPN nanocage and designed CoV-2 TMP resulted in an immune response in mice.
Summary/Conclusion: EPN are a versatile, modular, and tractable platform to induce release of TMP-decorated-EV from producer cells. Here we functionalized EPN by directing them towards target cells in a mixed cell population, and tested their use as a vaccine platform with an mRNA-based vaccine against SARS-CoV-2 to mice. The EPN technology shows promise for efficiently engineering EV as therapeutic and basic science tools.
Keywords: therapeutics delivery vaccine

OF16.3 | Extracellular vesicle loading of proteolysis targeting chimeras for targeted therapeutic delivery

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Introduction: The development of targeted therapies is essential for the production of safe and effective cancer treatments. An emerging targeted therapy approach is proteolysis targeting chimeras (PROTAC), which triggers proteasomal degradation of target proteins implicated in cancer. However, wide-spread clinical use of PROTAC is limited due to considerable instability and poor cell penetration in vivo. In order to enhance the efficacy of PROTAC, we introduce a novel extracellular vesicle (EV) transfection platform for highly efficient loading of PROTAC for enhanced cellular delivery.
Methods: Microfluidic droplet-based electroporation (μDES) was developed and optimized for highly efficient EV transfection of PROTAC. We compared the drug loading efficiency to conventional transfection methods, including simple incubation, chemical transfection through lipofection, and physical transfection through the Neon electrotransfection system (ThermoFisher). The resulting EVs were characterized by nanoparticle tracking analysis for size, concentration, and zeta potential, and by TEM for morphological determinations. Drug transfection efficiency was measured through absorbance spectrometry and PROTAC-loaded EVs were tested in vitro for histone deacetylase (HDAC) 3 and 8 degradation with western blot analysis and in vivo for biodistribution behavior.
Results: The resulting μDES PROTAC-loaded EVs showed little, or no, changes compared to the native EVs and Neon control group in terms of EV characteristics, while offering high recovery and transfection rates. The in vitro therapeutic function of PROTAC loaded EVs from the μDES platform demonstrated high degradation ability of HDAC 3 and 8 in the breast cancer cell line MDA-MB-231, and thus significantly higher therapeutic function.
Summary/Conclusion: PROTAC delivery by EVs could enhance stability, biocompatibility, transportability, and targeting ability of the drug. This serves as a novel PROTAC drug delivery, formulation, and administrative strategy that fills an important gap in current PROTAC use.
Funding: The project is supported by NIH NIGMS MIRA award 1R35GM133794 and USDA-NIFA award 2017-67021-26600. Additional support is provided from the Spiegel Fellowship award.
Keywords: proteolysis targeting chimeras, transfection, extracellular vesicles, therapeutic development

OF16.4 | Development of an EV-mediated mRNA delivery platform, outperforming an equal mRNA dose delivered by LNPs

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Introduction: A key challenge in the development of mRNA-based therapeutics is efficient and safe delivery. The potential for extracellular vesicles (EVs) to be engineered to carry different cargos and targeting moieties, coupled with their non-immunogenic nature, make them a novel and promising modality for delivery of mRNA-based therapeutics. Through protein and RNA engineering strategies, we have developed a platform to load and deliver mRNA via EVs. We have demonstrated functional delivery of erythropoietin (EPO) mRNA through in-vitro uptake assays and have performed an in-vivo comparison of EPO mRNA loaded EVs with lipid nanoparticles (LNPs), the current gold standard in mRNA delivery.
Methods: EVs were enriched from conditioned media of transfected CAP cells using differential centrifugation, tangential flow filtration and size exclusion chromatography. EVs were quantified by nanoparticle tracking analysis, probed for proteins of interest by western blot and mRNA loading quantified by droplet digital PCR. In-vitro assays were carried out in Huh7 cells and in-vivo studies in C57BL/6 mice. Quantification of EPO in conditioned media and mouse blood plasma was performed by ELISA.
Results: Quantification of mRNA by ddPCR revealed enrichment of EPO mRNA in EVs using engineered active loading strategies. In-vitro uptake assays showed delivery and translation of EPO mRNA loaded into EVs. Finally, following IV injection of EVs loaded with EPO mRNA in mice, quantification of EPO in the blood plasma showed detection of EPO protein expression, which showed ~4-fold greater expression compared with an equivalent LNP dose.
Summary/Conclusion: We have developed a platform for actively load specific mRNA within EVs and successfully demonstrated EV-mediated delivery both in-vitro and in-vivo. Further, our results suggest that in-vivo, at an equivalent dose, EV mediated mRNA delivery appears to be more efficient than LNP mediated delivery.

OF16.5 | CD81 -based combinatorial library for selecting recombinant extracellular vesicles to target EGFR and HER2 for drug delivery in vivo

Madhusudhan Bobbili[1]; Johannes Grillari[2]; Gordana Wozniak-Knopp[3]; Florian Rüker[3]; Nuria Gimeno Magan[4]
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Introduction: Despite the recent successes with lipid nanoparticles (LNPs) for drug delivery, LNPs can display toxicity and can be limiting. Thus ineffective drug delivery problem needs to be addressed urgently. For this reason, extracellular vesicles (EVs) as a drug delivery vehicles has become an intense topic of research. EVs have an innate therapeutic potential with the promise of them functioning as target-directed drug delivery vehicles, able to modulate proliferation, migration, differentiation, and other properties of the recipient cell that are vital for health of the host organism. To enhance the ability of their targeted delivery, we employed an intrinsically overrepresented protein, CD81, to serve for recognition of the desired target antigen.
Methods: Yeast libraries displaying mutant variants of the large extracellular loop (LEL) of CD81 have been selected for binders to EGFR and HER2 as an example target. Their specific interaction with EGFR and HER2 was confirmed in a mammalian display system by multiple rounds of screenings. Derived sequences were introduced in to full-length CD81 tagged either with eGFP or luciferase and stably expressed in EVs producer cell line HEK293 to target EGFR and HER2 tumors. EVs were isolated from producer cell lines by tangential flow filtration (TFF).
Results: TFF isolated EGFR and HER2 targeting EVs were characterized for size and number by nanoparticle tracking analysis (NTA) and for surface marker profile by bead-based flow cytometry. Additionally, the purity of the EV preps were confirmed by immunoblotting. To assess the novel functionality of antigen-binding CD81 LEL variants, internalization of such EVs into EGFR and HER2 overexpressing cells was compared with the wild-type CD81 EV internalization. Additionally, EGFR and HER2 targeting EVs loaded with doxorubicin can induce apoptosis in recipient cells more effectively compared to wild-type CD81 EVs. Currently, we are accessing the doxorubicin loaded EVs targeting EGFR and HER2 tumors in xenograft mouse models.
Summary/Conclusion: To our knowledge, this is the first example of harnessing an EV membrane protein as a mediator of de novo target antigen recognition via in vitro molecular evolution, opening horizons to a broad range of applications in various therapeutic settings. The advantage of the method presented here is that it can rapidly deliver binders to any antigen of choice, which can simply be 'clicked' into the full-length CD81, recombinantly expressed on the EV surface, enabling specific EV-mediated delivery to a large variety of cells and tissues.
Keywords: CD81, extracellular vesicles, targeted drug delivery, EGFR, HER2

OS17: Cancer

Chairs: Simon Powis, Takahiro Ochiya

Location: Ballroom 6BC

11:35 - 13:05

OS17.1 | Therapeutic repurposing of mesenchymal stem cells extracellular vesicles (MSCEV) as lipo-drug co-delivery system in amplifying tumour-targeted therapy efficiency

Sahithi Jyothsna Kuravi[1]; Niusha Ansari-Fard[2]; Helen McGettrick[3]; Mohammad Najlah[4]; Nicholas Pugh[1]; Christopher Parris[1]
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Introduction: In this study, we aimed to harness the therapeutic benefits of MSCEV's further by encapsulating nano-liposomal drug carriers, in a co-delivery system. This EV-nanodrug co-delivery system combines the advantages of MSCEV's whilst increasing the efficacy of anticancer drug paclitaxel (PX) and zinc chelated disulfiram (Zn2+-DS).
Methods: PX/Zn2+-DS loaded nanoliposomes were generated using ethanol-based proliposome methods and high-pressure homogenization. MSCEV's loaded nanoliposomes were generated by electroporation or passive transfer over 48hrs, under serum starvation. EV counts were measured using q-Nano. The successful drug loading was detected using Zn2+ specific dye, Fluozin (FZ3) by flow cytometry and fluorescence microscopy. Time dependent and dose dependent effects of MSCEV lipo-drug co-delivery system on MCF-7 was measured by changes in proliferation rates (microscopy) and viability (tested by using MTT assay) over 72h.
Results: We have implemented a successful method to load nanoliposomes into MSCEVs creating lipo-drug co-delivery system. Preliminary results demonstrate dose dependent increase in FZ3 signal correlating to successful loading efficiencies. Fluorescent microscopy shows localisation of FZ3+ MSCEVs in MCF-7 cells. Treatment also resulted in reduced cell viability and proliferation capacity confirming the action of MSCEV lipo-drug derived effects on breast cancer cell line.
Summary/Conclusion: Successful co-delivery of MSCEV-lipo-drug co-delivery system may have potential in clinical applications in tumor-targeted cancer therapy.
Funding: Anglia Ruskin University Research development fund.

OS17.2 | Macrophage reprogramming and immune suppression through ovarian tumor associated extracellular vesicles

Sunila Pradeep
Ob-Gyn, Medical College of Wisconsin, Milwaukee, USA

Introduction: In ovarian carcinoma (OvCa), macrophages are reprogrammed toward pro-tumorigenic phenotypes, including the release of anti-inflammatory cytokines and expression of the immunosuppressive molecules. The expression of genes involved in the inflammatory response is tightly regulated at the transcriptional and post-translational levels. To understand the mechanism driving pro-tumorigenic phenotype in macrophages, we studied the role of tumor cell-derived extracellular vesicles (EVs) in the cross-talk with macrophages in OvCa. We found that EVs from ovarian cancer cells contain eukaryotic translation initiation factor eIF4E, an mRNA 5' cap-binding protein. eIF4E containing EVs from ovarian cells enhanced translational activity and PD-L1 expression in macrophages. However, a detailed mechanism explaining how eIF4E-packaged EVs induce immunosuppressive phenotype in macrophages is necessary to develop novel immunotherapy strategies for advancing OvCa treatment. Our study aims to understand the eIF4E mediated translational mechanism driving pro-tumorigenic phenotype in tumor-associated macrophages.
Methods: We performed EVs characterization following standard techniques and performed a co-culture assay to evaluate the EV uptake by stromal cells. We performed SUnSET assay and polysome fractionation assay to determine whether eIF4E-EVs affect translation in macrophages. Immunoprecipitation and proximity labeling assay were to study the encapsulation of eIF4E in EVs. We used Rab27aKO ovarian cancer in vivo model to study the effect of eIF4E packaged EVs on the immune suppressive ability of macrophages. We confirmed the expression of PD-L1 or other immunosuppressive markers on macrophages by flow cytometry.
Results: Confocal microscopy revealed that macrophages were more susceptible to EV uptake than other stromal cells. Our study shows that tumor cells employ EVs to deliver eIF4E to the tumor microenvironment, facilitating the release of cytokines such as IL-6 and the expression of PD-L1 on macrophages to support tumor growth. In addition, our data confirms eIF4E-EVs enhance

protein synthesis in macrophages. Altogether, our results shows that EVs mediated transfer of eIF4E to macrophages contributes to anti-tumor immune response via PD-L1 expression.

Summary/Conclusion: In summary, we discovered that EVs enhances the immunosuppressive properties. This effect is regulated by EVs encapsulated eIF4E. This immunosuppressive phenotype of macrophages is characterized by increased de novo synthesis of PD-L1, SIRPα and CD206. Our findings provide insight into how EVs orchestrate a pro-metastatic phenotype by translational reprogramming in tumor associated macrophages.

Funding: This work was supported in part by the OCRFA 657713, the WHRP, MCW American Cancer Society (ACS-IRG 19-138-34), the DoD W81XWH-21-1-0361 and NCI R01CA258433.

Keywords: ovarian cancer, extracellular vesicles, protein translation, immunosuppression

OS17.3 | Label-free multimodal nonlinear imaging of EVs following chemotherapy treatment of patient-derived pancreatic tumor xenografts in mice

Marina Marjanovic[1]; Jaena Park[1]; Janet E. Sorrells[2]; Eric Chaney[3]; Edita Aksamitiene[1]; Amro Abdelrahman[4]; Jennifer Leiting[4]; Jennifer Yonkus[4]; Peter Groves[3]; Jonathan Harrington[5]; Isaac Lynch[4]; Colleen Bushell[3]; Heidi Nelson[4]; Mark Truty[4]; Stephen A. Boppart[1]

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is often diagnosed at late stages when surgical and pharmacological treatments become ineffective. Cancer-associated extracellular vesicles (EVs) can potentially serve as a diagnostic and prognostic biomarker to determine effective personalized treatment regimens and improve patient outcome. In this study, the in vivo chemotherapy treatment response of pancreatic tumor patient-derived xenografts (PDX) in mice was evaluated using simultaneous label-free autofluorescence multi-harmonic (SLAM) microscopy.

Methods: Specimens of human pancreatic tumor tissue were surgically placed subcutaneously in NOD SCID mice. A total of 24 mice were used in this 5-week study, divided between treatment groups (responsive or resistant) that received one chemotherapy regimen and control groups injected only with saline. Chemotherapy regimens included either a combination of 5-fluorouracil, irinotecan, and oxaliplatin, or gemcitabine and nab-paclitaxel. Tumors were imaged both intravitally and ex vivo after the animals were euthanized. Label-free multimodal multiphoton imaging was used to provide simultaneous, co-registered structural and functional images of tumors and EVs in untreated samples. Heterogeneous populations of EVs could be identified by their unique optical signatures.

Results: Elevated EV density was observed in both chemotherapy responsive and resistant tumors during the early response (weeks 1 and 3) and remained elevated (thru week 5) in the resistant tumors. The optical redox ratio of EVs decreased with treatment (weeks 1 and 3), but by week 5, returned to baseline in the resistant tumors, and remained low for the responsive tumors.

Summary/Conclusion: These results suggest the potential for using this label-free optical technology and methodology to detect and characterize EVs by their optical signatures, which can be utilized as possible biomarkers for determining chemotherapy responsive vs. resistant tumors.

Funding: Funding was provided by a Seed Grant from the Mayo Clinic & Illinois Alliance for Technology-Based Healthcare, and grants from the National Institutes of Health (R01CA213149, P41EB031772, S.A.B.).

Keywords: multiphoton microscopy, pancreatic cancer, chemotherapy, drug resistance

OS17.4 | Metabolic signatures of biofluid-derived extracellular vesicles in breast cancer characterized using label-free nonlinear optical microscopy

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Introduction: Label-free nonlinear optical microscopy can image and characterize the autofluorescent metabolic cofactors NAD(P)H and FAD in vivo, ex vivo, and in vitro to make inferences about cellular metabolism. One signature of cancer cells is dysregulated metabolism, which can be quantified by NAD(P)H and FAD autofluorescence. Here, we show a multivariate analysis of the optical metabolic signatures of EVs in breast cancer by examining EVs in ex vivo tissue samples as well as EVs isolated from patient serum and urine to determine the potential for using optical metabolic signatures of EVs as biomarkers of cancer.

Methods: EVs were isolated from fresh urine and blood serum samples from human breast cancer patients (n = 31; 18 with invasive tumors, 7 with DCIS, 6 with benign tumors) or breast reduction surgery patients (n = 5). Large EVs (LEVs) were isolated by differential ultracentrifugation: samples were spun at 800 × g for 10 min and 2000 × g for 30 min and pellets discarded. The supernatant was spun at 12000 × g for 60 min and the LEV pellet was resuspended in sterile PBS with 25 mM trehalose. Isolated LEVs and ex vivo tissue (received post-surgery) were imaged with label-free nonlinear optical microscopy.
Informed consent was obtained from participants; the study was approved by the Institutional Review Boards at Carle Foundation Hospital and the University of Illinois at Urbana-Champaign.
Results: LEVs showed optical metabolic differences by cancer status and EV origin. In urinary LEVs, cancer samples showed significantly higher NAD(P)H fluorescence lifetime, indicative of more NAD(P)H-related protein activity, however this trend was not seen in serum EVs.
Summary/Conclusion: The optical signatures of urinary LEVs and ex vivo tissue EVs show potential as biomarkers.
Funding: JES was supported by UIUC Department of Bioengineering and an NIH/NIBIB training grant (T32EB019944). This work was supported in part by NIH (R01CA213149, P41EB031772).
Keywords: multiphoton microscopy, breast cancer, metabolism, single-EV analysis, biofluids

OS17.5 | Tumour-associated antigenic peptides are present in the HLA class I ligandome of EVs derived from melanoma patient blood samples

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Introduction: Melanoma is the form of skin cancer associated with the highest rate of mortality and the incidence of the disease is increasing more rapidly than any other form of cancer. Extracellular vesicles (EVs) are small membrane vesicles that have been indicated as key players in the communication that exists between cancer cells and the host microenvironment, both locally and distally. The MHC class I immunopeptidome present on EVs released by cancer cells may be the defining feature of EVs that allows them to have a significant impact on the anti-tumour immune response. Thus, in this study we isolated EVs from melanoma patient blood samples to characterise their peptide ligandome.
Methods: Twelve stage III and IV melanoma patient blood samples and twelve control blood samples were run through size exclusion columns to collect EVs, which were then lysed. MHC class I peptides were isolated and eluted using the immunoprecipitation technique before being processed by mass spectrometry analysis.
Results: MHC-class I bound peptides derived from blood EVs were successfully eluted and identified. Peptides present on the HLA-I ligandome of EVs derived from melanoma patient plasma stratified into two different peptidomic profiles with varying functional characterizations. Additionally, ten known T-cell epitopes/immunogenic peptides were identified on the HLA ligandome of EVs isolated from melanoma patient blood samples.
Summary/Conclusion: This study indicates the possibility of a peptidomic stratification between the blood EVs of melanoma patients into two distinct groups. Further, the data achieved supports the possibility of identifying peptides on the HLA-I of EVs that can be recognized by T-cells and mediate a reaction. The discovery of these key antigens would be a major step toward the development of a novel immunotherapy for melanoma, as well as possibly providing a novel diagnostic or prognostic tool for the disease.
Funding: The Melville Trust - For the Care and Cure of Cancer.
Keywords: extracellular vesicles, HLA ligandome, T-cell epitopes, tumour associated antigen (TAA), melanoma

OS18: MSC Therapeutics

Chairs: Benedetta Bussolati, Leila Noori

Location: Ballroom 6A

11:35 - 13:05

OS18.1 | Modulations in miRNA packaging of hypoxia primed wharton's jelly MSCs derived small EVs enhances their immunosuppressive abilities

Sujata Mohanty[1]; Yashvi Sharma[2]; Suchi Gupta[3]
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Introduction: Mesenchymal Stem Cells (MSCs) have been recognised widely for their regenerative & immunomodulatory potential. They can be isolated from various sources (Bone Marrow (BM) & Wharton's Jelly (WJ)). Studies have reported that hypoxia exposure to MSCs enhances their therapeutic function, especially via augmenting the release & content of their EVs. Currently the focus of regenerative medicine has shifted to cell free therapy, and EVs serve as the perfect candidate for that. This study explores the comparative immunomodulatory functionality of tissue specific MSCs derived small EVs (less than 200nm), cultured in Normoxia and Hypoxia. Moreover, it also gives a cue into the mechanism of these small EVs to further their applications in immune-implicated disorders.
Methods: BM & WJ MSCs were isolated after obtaining donor consents (IC-SCR/120/21(R)). MSCs were cultured in serum free media & exposed to 1% hypoxia for 24 hours in cGMP compliance. Small EVs were isolated from conditioned media using ultracentrifugation. They were characterized via NTA (Size < 200nm), TEM (Cup shaped morphology) and Western blotting (Surface marker CD63, Cytoplasmic marker ALIX and Negative marker Calnexin). These EVs were tested for their immunomodulatory effect via MLR and macrophage polarization assay. Microarray based mechanistic analysis was performed for identification of miR 125b-5p & its upregulation was done via mimics transfection for further pathway analysis. Furthermore, excisional wound model was created in Wistar rats (226/IAEC-1/2019) and EVs treatment was given for validation.
Results: It was observed that there was a significant immunosuppressive effect of both BM & WJ-MSCs derived exosomes. This activity was augmented significantly when the exosomes were derived forth hypoxic priming of MSCs. Moreover, there was also a significant difference in the immunosuppressive abilities of BM & WJ as a source, where WJ-MSCs derived exosomes were faring better. Furthermore, it was observed that the wound healing and inflammatory scenario was improved upon exosomes treatment in rats. This was evidenced by the increase in miR125b-5p in hypoxia derived EVs of WJ-MSCs.
Summary/Conclusion: This study hereby concludes that hypoxia priming mediated increase in miRNA 125b-5p can be considered as a minimum manipulation strategy to enhance the regenerative capabilities of MSCs & their EVs to further their implication in immune disorders, and while both the sources respond well to this priming strategy, WJ-MSCs exhibit augmented immunomodulation as compared to BM-MSCs. These results could be accredited mechanistically in terms of their enhanced miRNA content and this gives us a hope for developing future therapeutic strategies taking miRNA as a target molecule.

OS18.2 | Mesenchymal stem cell-derived small extracellular vesicles restore innate immune function in a murine model of polymicrobial sepsis

Rachael Batabyal[1]; Wade O'Brien[2]; Laura R. Cechinel[2]; Claire Hoptay[2]; Robert Freishtat[3]; Ioannis Koutroulis[3]; Deepa Rastogi[3]
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Introduction: Sepsis is involved in up to 20% of deaths worldwide. Multiorgan dysfunction in sepsis is attributed to an imbalance of hyperinflammation and immune paralysis. The lungs are one of the most affected organs in sepsis and immune imbalance can lead to edema and compromised gas exchange. Mesenchymal stem cell-derived small extracellular vesicles (MSC-sEVs) have immunomodulatory properties and have demonstrated positive therapeutic potential in several disease processes. In this study, we examined their effects on the septic lung immune response. We hypothesized that MSC-sEVs would address immune dysfunction in a murine model of polymicrobial sepsis and prevent lung dysfunction.
Methods: We administered cecal contents by intraperitoneal injection to 7-10-week-old, C57BL/6J mice to induce sepsis. Septic and control mice received either MSC-sEVs or EV-depleted media intravenously 6 hours post-sepsis induction (n = 3 per group). After 18 hours, lung tissue was harvested and immune cell profile was quantified using flow cytometry. RNA sequencing (RNAseq) was performed on lung tissue from septic treated and non-treated mice as well as non-septic controls. RNAseq data were analyzed on Partek Flow and differentially expressed genes identified using Gene Specific Analysis (GSA). Pathway enrichment analysis was performed using Ingenuity Pathway Analysis.
Results: When we examined the immune cell profile, CD11+ activated macrophages and neutrophils increased in sepsis (23.53 ± 2.75% vs. 7.72 ± 2.06%) and showed a downward trend after MSC-sEV treatment. However, the CX3CR1+ subpopulations of interstitial macrophages were increased in the MSC-sEV treated sepsis group vs. non-treated septic mice (7.04 ± 2.41% vs. 1.38 +/- 0.38%). RNAseq of the lung tissue revealed downregulation of CX3CL1, the ligand for CX3CR1 (FC -2.38, P< 0.05). CD31hi endothelial cells were increased in the lungs of MSC-sEV treated septic mice compared to non-treated mice (12.75 ± 5.85% vs. 4.43 ± 0.35%).
Summary/Conclusion: Exposure to MSC-sEVs decreased CD11+ activated macrophages but increased the CX3CR1+ subpopulation of interstitial macrophages, while simultaneously decreasing the CX3CL1 ligand. These macrophages are likely important in addressing the continuing infectious insult. CD31hi endothelial cells were increased in the MSC-sEV treated septic mice, which may lead to an improved homeostatic regulation and prevention of excessive vascular reactivity. These data suggest that treatment of sepsis with MSC-sEVs may alter the immune profile in the septic lung, alleviating the immune imbalance and potentially preventing respiratory failure.

OS18.4 | Investigating the therapeutic potential of extracellular vesicles derived from mesenchymal stem cells in corneal wound healing and regeneration

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Introduction: Extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs) have shown great promise in the treatment of various clinical disorders. This study describes isolation, purification, and characterization of MSCs derived EVs and functional validation demonstrating their therapeutic potential in healing corneal wounds. Further, the mechanism of action of these EVs in regeneration of corneal tissue is investigated.
Methods: EVs isolated by ultracentrifugation and purified by Iodixanol density gradient separation were evaluated by nanoparticle tracking analysis and electron microscopy for size estimation, and western blot for protein profile as per MISEV2018-guidelines. The therapeutic potential of EVs was established by in-vitro functional assays using human corneal epithelial (HCE) cells and in vivo studies performed in New Zealand white rabbits. The mechanism was elucidated by studying the EV cargo and correlating its activity in corneal wound healing and regeneration.
Results: The prepared MSC-derived EVs showed narrow size distribution (80-150 nm) and appeared as typical cup-shaped vesicles in transmission electron microscopy. They showed characteristic MSC-derived EV-markers CD9, CD63, CD81, TSG101, Flotillin, and Alix in western blots. The EVs showed remarkable activity in terms of anti-inflammation, anti-fibrosis, anti-angiogenesis, and neurogenesis. The EVs stimulated proliferation and migration of HCE cells in vitro while maintaining their phenotype. In vivo studies showed that EVs prepared in this work have a remarkable effect in accelerating wound healing and tissue regeneration in mechanically and chemically injured rabbit corneas. We identified key growth factors and cytokines in the EVs that are critical for cornea regeneration.
Summary/Conclusion: MSCs-derived EVs show a remarkable capacity to stimulate and support transparent regeneration of functional cornea, as seen in vitro and in vivo. They are promising candidates for the clinical translation toward the treatment of various corneal disorders such as inflammation associated with keratopathy and allergy.
Funding: Not applicable.
Keywords: MSC-derived EVs, ultracentrifugation, wound healing, anti-inflammatory, corneal regeneration

OS18.5 | Protein corona bio-inspired mesenchymal stem cell-derived extracellular vesicles for liver regeneration

Revadee Liam-Or[1]; Farid Faruqu[2]; Adam Walters[1]; Julie Tzu-Wen Wang[1]; Francesco Dazzi[1]; Jennifer Oberlaender[3]; Volker Mailaender[3]; Khuloud Al-Jamal[1]
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Introduction: The regenerative and immunomodulatory properties of extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs) have been demonstrated in both clinical and pre-clinical studies of liver pathology. We have previously described the liver accumulation of intravenously administered EVs, but knowledge of their cellular distribution within the liver and factors affecting this are limited.
Methods: Herein, we derive EVs using two culturing methods allowing for collection of EVs generated under different protein environments. We hypothesise that, under these culture conditions, EVs will form distinct protein corona (PC) leading to modulation of their pharmacokinetic profile. MSC EVs were obtained from cells maintained in the presence or absence of serum (EV1 and EV2, respectively).
Results: In each case, EVs exhibited comparable physicochemical properties and marker expression but different protein composition. When EVs were incubated with fetal bovine serum (FBS), to model the secondary corona formed upon systemic delivery, further PC binding patterns could be resolved by liquid chromatography-mass spectrometry. In healthy mouse models, EV1 and EV2 accumulated in the liver and kidney, respectively. Using flow cytometry, it was determined that both EVs were comparably taken up by Kupffer cells. EV1, however, exhibited higher uptake in hepatocytes, liver sinusoidal endothelial cells, and stellate cells. Quantitative proteomics, gene ontology enrichment analysis, and principal component analysis identified that the composition of the PC in EVs was responsible for the differential organ/cellular distribution.

Summary/Conclusion: These findings identify the potential of modifying cell culture conditions as a simple means of retargeting therapeutically relevant EVs to organs or cells. Such an approach offers a solution to a critical challenge facing intravenously administered.
Funding: King's PGR International Scholarship.
Keywords: extracellular vesicles, mesenchymal stem cells, liver therapy, liver targeting, protein corona, hepatocyte uptake, albumin-based drug delivery

OS19: Aging

Chairs: Ursula Sandau, Pamali Fonseka

Location: Room 608/609

11:35 - 13:05

OS19.1 | Age/senescence-related small extracellular vesicles biomarker discovery using multi-omics analysis

Sandip Kumar Patel[1]; Jacob Rose[1]; Rebecca Beres[2]; Joanna Bons[1]; Roland Bruderer[3]; Lukas Reiter[4]; Erin Baker[2]; Judith Campisi[1]; Birgit Schilling[1]
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Introduction: Cellular senescence is a predominant factor causing aging by triggering profound secretory phenotype (SASP), including sEVs. sEVs have recently emerged as drivers and promising therapeutic targets for multiple age-related conditions. Thus have tremendous potential for diagnostics of age-related diseases, which is largely unexplored.
Methods: sEVs multi-omics analysis identifies multicomponent biomarkers for aging/senescence in plasma (Old vs. young) and senescent lung fibroblasts (induced by three stimuli; IR, Doxo, and MiDAS). sEVs are isolated and enriched by size-exclusion chromatography/ultrafiltration and antibody-based methods. sEVs intactness and quality were confirmed based on median size and CD9 and TSG101 western blot.
Results: We report the first comprehensive high-throughput analytical platforms to screen sEVs-specific proteins, lipids, and miRNA for age/senescence and health biomarkers. Deep profiling of sEVs-specific proteins (~2,300), lipids (~350), and miRNA (331) are generated as a resource for the fast-growing gerontology community. Based on machine learning models, sEVs components independently classify aging/senescent and control samples. sEVs proteomics using DIA-MS resulted in 144 differentially regulated proteins in aged plasma and ~1,300 changing proteins from the senescent fibroblasts. sEVs lipidomics on a timsTOF ion mobility MS platform identified >300 lipid species, 23 were differentially regulated in young and old plasma, and 156 lipids were differentially expressed in senescent fibroblasts. In addition, 88 sEVs miRNAs are unique to old plasma.
Summary/Conclusion: Ten potential aging/senescence biomarkers emerged; proteins (Peroxidasin, Hemopexin, Plasminogen activator inhibitor 1, SPARC, Transforming growth factor beta-1-induced transcript1 protein), lipids (ceramide, sphingolipids), and miRNA (miR-532, miR-654-3p, miR-409-3p). In the future, the biomarkers will be validated in aging and age-related disease cohorts.
Funding: U01 AG060906 (Schilling), U54 AG075932 (Campisi/Schilling), S10 OD028654 (Schilling), P01AG017242 and R01AG051729 (PI: Campisi), Glenn Fellowship (Patel).
Keywords: sEVs, biomarker, cellular senescence, aging, multi-omics

OS19.2 | Extracellular vesicles are critical elements in senescent cell removal by recruiting antigen-presenting cells

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Introduction: Standard-of-care chemoradiation limits tumor growth by induction of cellular senescence. However, immediate removal of senescent cells (SCs) by immune cells is crucial to avoid SC-mediated cancer relapse. SC persistence promotes recurrence by fostering chronic inflammation. The paucity of approved senolytic drugs highlights the need for more efficient strategies for SC removal. Here, we hypothesized that SC-derived extracellular vesicles (senEVs) promote immune-mediated SC removal.

Methods: To characterize senEVs in a model of oral squamous carcinoma, we isolated them by ultracentrifugation and analyzed by nanoparticle tracking analysis, electron microscopy, immunoblotting and mass proteomics. To study the effect of senEVs on immune response, first, we suppressed senEV release by expressing Rab35 dominant-negative mutant in SCs, which uncoupled senEV release from secretion of soluble factors. To investigate the role of senEVs in cancer recurrence, we challenged mice orthotopically with EV-competent or EV-deficient SCs (generated in vitro by chemotherapy treatment) mixed with limiting amounts of tumor cells. As read-out, we evaluated cancer relapse and immune infiltrates using both flow cytometry and single cell RNA sequencing. Importantly, we tracked SCs in tissues using transgenic markers.
Results: SenEVs inhibited cancer recurrence in vivo. When senEVs are inhibited, we observed a significant decrease of antigen-presenting cells (APCs) in senescent tissues, which may contribute to the observed persistence of SCs. The results of single cell RNA sequencing highlighted changes in APCs in the presence of senEVs. Proteomic analysis revealed several proteins enriched in senEVs compared to those from their non-senescence counterparts and common senEV biomarkers were found in two different oral SC models.
Summary/Conclusion: These results suggest that senEVs have critical roles in immune-mediated removal of SCs. Future work will identify the receptor-ligand relation between the identified protein markers on senEVs and APCs, which will highlight novel therapeutic candidates for boosting immune-mediated removal of SCs.
Funding: NIH Training Grant T32CA254888, CRUK/OHSU A29681.
Keywords: senescence, extracellular vesicles, cancer relapse

OS19.3 | Extracellular vesicles from frail individuals contain higher levels of mitochondrial DNA and inflammatory proteins

Nicole Noren Hooten[1]; Anjali M. Byappanahalli[2]; Mya Vannoy[2]; Nicolle A. Mode[1]; Ngozi Ezike[1]; Alan B. Zonderman[1]; Michele K. Evans[1]
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Introduction: Frailty is a clinical syndrome that is characterized by a decline in physiological reserve and increased vulnerability to stressors. Although often studied in the elderly, frailty occurs at mid-life and is linked to increased mortality. Few studies have examined the molecular mechanisms that contribute to the chronic inflammatory phenotype observed in frail individuals. In this study, we examined whether there were differences in molecular cargo in extracellular vesicles (EVs) in frail individuals and whether EVs may act as damage-associated molecular pattern (DAMP) molecules in frailty.
Methods: We separated plasma EVs using size exclusion chromatography from non-frail (n = 90) and frail (n = 87) middle-aged (45-55 years) participants from the Healthy Aging in Neighborhoods of Diversity across the Life Span (HANDLS) study. EV concentration was measured using nanoparticle tracking analysis. We isolated EV DNA and quantified mitochondrial DNA (mtDNA) levels using quantitative real time-PCR. Inflammatory proteins in EVs were quantified using multiplex proximity extension assays. Linear regression was used to analyze relationships between EV characteristics and frailty status, race, sex, and poverty status.
Results: Plasma EVs levels were higher in frail White participants. EV mtDNA levels and the presence of inflammatory proteins in EVs were associated with frailty. Furthermore, EV inflammatory proteins were significantly altered by frailty status, race, sex, and poverty status. EV mtDNA and EV inflammatory proteins levels were significantly correlated.
Summary/Conclusion: In our study we report molecular cargo differences in EVs with frailty, suggesting that EVs may act as DAMP molecules in frailty. These data indicate that circulating factors are altered with frailty at midlife and provide mechanistic clues of pathways that may underlie frailty.
Funding: This work is supported by the NIA-IRP, NIH.
Keywords: mitochondria, inflammation, frail, aging, mtDNA, DAMP, health disparities

OS19.4 | Functionalization of MSC-EVs with senolytic drugs as a potential treatment for atherosclerosis

Ainara González-Moro[1]; Elena Cercas[2]; Arturo González-Camuñas[3]; Miriam Morales Rodríguez De Lope[4]; Jordi Royes[3]; Luis A Campos[3]; Milagros Castellanos[3]; Carlos Félix Sanchez-Ferrer[2]; Concepcion Peiro[5]; Álvaro Somoza[3]; Fernando de la Cuesta[6]
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Introduction: Cellular senescence has been associated with the progression of atherosclerosis. Senolytic drugs are compounds that induce apoptosis of such senescent cells and two of the most used are quercetin (Q) and dasatinib (D). Several studies have demonstrated that mesenchymal stem cell-derived EVs (MSC-EVs) exert beneficial effects. Thus, the objective of our study is to develop a novel therapy using MSC-EVs as a vehicle for Q and D delivery in a context of atherosclerosis.
Methods: EVs were isolated from immortalized adipose tissue MSCs by ultrafiltration and size exclusion chromatography (SEC), and characterized by NTA, western blot and TEM. NTA and light dispersion analyses were used to determine the optimal conditions for Q and D internalisation. Functionalization of the EVs with both senolytics was evaluated by HPLC and MRM mass spectrometry. EV's uptake by HUVECs was observed with CFSE staining. β-galactosidase assay was used for analysing a potential senolytic effect. Besides, aptamers conjugated or not with cholesterol were synthesized in order to compare their incorporation into EVs, with the final goal of specifically delivering the treatment to the vascular tissue.
Results: Optimal concentration of the different agents for EVs functionalization were: DMSO 3%, Q 20 μM and D 4 μM. Efficient functionalization of the senolytics was evidenced. CFSE staining showed that MSC-EVs are internalized by HUVECs and in vitro experiments suggest a potential senolytic effect of EVs-QC. Finally, oligonucleotide's modification with cholesterol displayed a better incorporation into EVs.
Summary/Conclusion: We have been able to encapsulate quercetin and dasatinib in MSC-EVs and the first in vitro experiments showed a senolytic effect of the treatment in senescent human endothelial cells. Cholesterol modified aptamers were efficiently incorporated into MSC-EVs.
Funding: Talent Program,Comunidad Autónoma de Madrid (2019-T1/IND-13794); Ministry of Science (PID2021-126274OB-I00).
Keywords: atherosclerosis, senescence, extracellular vesicles, senolytics

OS20: Viruses

Chairs: Dirk Dittmer, Matias Ostrowski

Location: Room 606/607

11:35 - 13:05

OS20.1 | Phosphatidylserine positive extracellular vesicles enhance proliferation and effector CD8+ T cell responses during acute viral infection

Jan Kranich[1]; Lisa Rausch[1]; Lavinia Flaskamp[2]; Ashretha Ashokkumar[1]; Anne Trefzer[3]; Christine Ried[1]; Veit Buchholz[4]; Reinhard Obst[1]; Tobias Straub[5]; Thomas Brocker[1]
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Introduction: While it has been shown in many in vitro studies that extracellular vesicles (EVs) can either directly or indirectly prime naïve T cells, it is still controversial whether EVs play a relevant role in T cell responses in vivo. To study naturally occurring EVs and their target cells in vivo, we have recently developed a novel method, using Ca2+-independent phosphatidylserine (PS) binding reagents based on MFG-E8 and imaging flow cytometry (Kranich, JEV, 2020, Rausch et al., JEV 2021). We use this approach to analyze and characterize EV-T cell interactions during acute viral infections in mice and to assess if T cell stimulation by EVs occurs during immune responses.
Methods: To visualize EV-T cell interactions, we administer fluorescent MFG-E8 intravenously into mice during an acute LCMV infection to label naturally occurring PS+ EVs in situ. We then analyze and characterize T cells carrying PS+ EVs either directly by imaging flow cytometry or perform downstream analyses after FACS-sorting of EV+ T cells. Downstream analyses include RNAseq and dSTORM superresolution microscopy. Furthermore, we use in vitro generated EVs from activated, antigen pulsed bone marrow derived dendritic cells and compare their interaction with antigen-specific and non-specific T cells in vivo.
Results: We observed a strong increase in the abundance of EV+ cells during viral infections in the spleen. There, PS+ EVs interact especially with activated, but not with naïve CD8+ T cells. EV-binding induced antigen-specific TCR signaling and increased nuclear translocation of the transcription factor NFATc1 in vivo. Single molecule superresolution microscopy showed direct interaction of EVs with the TCR complex on T cells. Furthermore, RNAseq analysis showed that EV-decorated but not

EV-free CD8+ T cells are enriched for gene signatures associated with T-cell receptor signaling, early effector differentiation and proliferation.

Summary/Conclusion: Our results demonstrate that PS+ EVs stimulate activated CD8+ T cells in vivo by directly interacting with the TCR complex and thus provide an antigen-specific adjuvant effect to T cells during acute viral responses. The absence of EVs on naïve T cells argues against direct priming of T cells by EVs in vivo. As EV-binding by T cells only occurs during the effector phase, EVs could act as danger signal, indicating to the cells that virus is still present and that the presence of effector cells is still required.

Funding: This project was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research foundation) – Project-ID 210592381 - SFB 1054 (TP B03; TP B07; TP B15; TP Z02).

Keywords: extracellular vesicles, phosphatiylserine, LCMV, T cells, dSTORM, imaging flow cytometry, MFG-E8, lactadherin

OS20.2 | The RNA landscapes of brain tissue-derived extracellular vesicles in simian immunodeficiency virus (SIV) infection and SIV-related central nervous system pathology

Yiyao Huang[1]; Ahmed Gamal Abdelmagid Abdelgawad[2]; Mona Batish[2]; Andrey Turchinovich[3]; Suzanne Queen[4]; Celina Monteiro Abreu[4]; Lei Zheng[5]; Kenneth W. Witwer[6]

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Introduction: Although HIV replication and disease progression can be effectively controlled by antiretroviral treatment regimens, the eradication of HIV latent infection reservoirs in the central nervous system (CNS) is still of great challenge. Extracellular vesicle (EV) RNAs have emerged as important participants in HIV disease pathogenesis. Brain tissue-derived EVs (bdEVs) act locally in the source tissue and may indicate molecular mechanisms in HIV CNS pathology. Using the simian immunodeficiency virus (SIV) model of HIV, we assessed the potential circRNA-miRNA-mRNA interactions in bdEVs and source tissues to understand molecular changes in SIV infection and neuroinflammation.

Methods: Postmortem occipital cortex tissues were obtained from pigtailed macaques not infected or dual-inoculated with SIV swarm B670 and clone SIV/17E-Fr. SIV-inoculated groups included samples collected at different time points during acute infection, chronic infection without (CP-), or with CNS pathology (CP+). bdEVs were separated as previously described (Huang, et al., JEV, 2020) and characterized per the recommendations of the MISEV. RNAs from bdEVs and source tissue were used for sequencing and qPCR analysis to profile mRNA, miRNA, and circRNA levels.

Results: Dysregulated mRNAs were detected in acute (n = 78) and CP+ (n = 48) bdEV groups (FDR< 0.05). Function enrichment showed these mRNAs were mostly involved in inflammation and immune responses, especially interferon pathways. bdEV miRNA dysregulation was confirmed by qPCR assays to show miR-19a-3p, Let-7a-50, and miR-29a-3p changes in acute, and miRs-146a-5p, 449a-5p in CP+ groups. bdEV mRNA changes mostly reflected changes in source tissues. In addition, target prediction showed that several dysregulated circRNAs in source tissue may potentially regulate the small RNA changes in SIV-infected bdEVs.

Summary/Conclusion: RNA profiling of bdEVs and source tissues reveal regulatory networks in SIV infection and SIV-related CNS pathology.

Funding: US National Institutes of Health, National Institute on Drug Abuse (NIDA, DA040385, and DA047807 to KWW). National Institute of Mental Health Grant No.P30MH075673 to the Johns Hopkins NIMH Center, and by the Johns Hopkins University Center for AIDS Research Grant No. P30AI094189 (pilot grants to YH).

Keywords: brain tissue-derived extracellular vesicles, HIV, HAND, circRNAs, miRNAs, mRNAs

OS20.3 | Development of virus-like vesicles as enveloped virus vaccine-candidate

Suyeon Kim[2]; Chungmin Han[1]; Minsu Kang[2]; Yeongju Seo[2]; Minyeob Lim[2]; Seungik Oh[2]; Sehan Kim[2]; Johan Yi[1]; Yongmin Kwon[3]; Jaesung Park[1]

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Introduction: Various vaccine platforms such as subunit, nucleic acid, and virus-like particle (VLP) vaccines have been developed to prevent the spread of virus infection diseases. VLP vaccines are considered one of the most promising vaccine technologies because of their superior safety and strong immunogenicity. However, the development of VLP vaccines for enveloped

viruses has been unsuccessful because of their high complexity and heterogeneity. In this study, we proposed virus-like vesicles (VLVs) as a new enveloped virus vaccine candidate, which enriched with plasma membrane components of the cells and showed higher homogeneity compared to EVs.

Methods: HEK293 cells stably expressing mCherry at the plasma membrane and GFP at the cytoplasm were prepared to establish the VLV production conditions. The characteristics of VLVs were analyzed using DLS, TEM, TIRF, WB, and dot blot analyses. HEK293 cells stably expressing Spike-GFP were prepared to produce SARS-CoV-2-like EV and VLV. Spike expressions of EVs and VLVs were analyzed using TIRF-based single vesicle analysis. Lastly, SARS-CoV-2-VLV was injected into BALBC mice to characterize in vivo pharmacokinetics distribution and to evaluate the immunogenic activities of the VLVs.

Results: The analysis of membrane-mCherry and cytosolic-GFP contents of VLVs demonstrated that the VLVs were mostly enriched with plasma membrane components. The size and morphology of the VLVs were very similar to those of natural EVs. The SARS-CoV-2-VLVs showed higher spike contents than spike-expressing natural EVs. Single vesicle co-localization analysis showed the VLVs were significantly more homogenous that the EVs. The intranasal injection of Cy5.5-labeled VLVs showed robust retention of VLVs at the injection site. In vivo mouse vaccination of VLVs showed significantly increased spike-specific IgG and IgA productions in serum and bronchoalveolar lavage fluid (BALF).

Summary/Conclusion: In this study, we developed VLVs as an enveloped virus vaccine candidate. SARS-CoV-2 VLVs showed enveloped-virus-like characteristics and robust expression of spike proteins. The intranasal injection of SARS-CoV-2 VLVs successfully induced spike-specific humoral responses both at the systemic circulation and the respiratory mucus, suggesting that the VLVs have potential as future vaccines for enveloped viruses.

Funding: This research was supported by the National Research Foundation of Korea (NRF) (2021R1I1A1A01060075) and Korea Medical Device Development Fund grant (the Ministry of Science and ICT, the Ministry of Trade, Industry and Energy, the Ministry of Health & Welfare, and the Ministry of Food and Drug Safety) (202011B12).

Keywords: enveloped virus-like particle(eVLP), Vaccine, SARS-COV-2, virus-like vesicle(VLV)

OS20.4 | An exosome-based SARS-CoV-2 vaccine

Allaura Cone[1]; Yijun Zhou[2]; Anthony Eason[2]; Ryan McNamara[3]; Gabriel Arias[4]; Kyle Shifflett[5]; Justin Landis[6]; Meredith Chambers[6]; Runjie Yuan[7]; Smaranda Willcox[6]; Jack Griffith[2]; Dirk Dittmer[8]

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Introduction: Subunit vaccines have been essential for the control of the COVID-19 pandemic. They use the SARS-CoV-2 Spike protein and differ only by the particulars of the delivery platform. Here, we demonstrate that extracellular vesicles (EVs) can be engineered to function as a vaccine platform. Overexpressing SARS-CoV-2 Spike results in an underdefined mixture of EVs with low-efficiency incorporation and unclear folding of the immunogen. Therefore, we fused Spike to CD81 to specifically target the immunogen to the exosomal biosynthesis pathway.

Methods: The full-length Spike protein was pasted into a recombinant CD81. After creating a stable cell line, the recombinant EVs were harvested and purified using tangential flow filtration and chromatography. EVs were analyzed using MISEV guidelines, and proper folding was ascertained by Cryo-electron microscopy and super-resolution microscopy. Multiple cohorts of mice were immunized and analyzed for antibody creation against Spike.

Results: The Spike-CD81 fusion protein is efficiently incorporated into EVs as Spike-CD81 intracellular localization was driven by CD81, rather than Spike. Expression of Spike-CD81 did not change biophysical EV properties. Spike could be seen on EVs by Cryo-EM and super-resolution microscopy. The recombinant Spike-CD81 EVs bound ACE-2 and elicited a broad immune response, including high titer antibodies to Spike.

Summary/Conclusion: We developed a novel vaccine platform composed of tetraspanin-fused SARS-CoV-2 Spike on EVs. This design altered the native biogenesis pathway of the viral protein and yielded a well-defined, reproducible, scalable, and homogeneous vaccine preparation with in vivo efficacy.

Funding: This work was supported by R01-CA228172, P01-CA019014 to DPD, and UNC at Chapel Hill. AC and RM received funding through T32 5T32AI007151. DPD, RM, YZ and AC declare competing interests with respect to possible commercialization of some of the information presented. These are managed by UNC.

Keywords: CD81, spike, SARS-CoV-2, vaccine

OS21: Innovative Technologies

Chairs: Colin Hisey, Cherie Blenkiron

Location: Ballroom 6BC

15:20 - 16:50

OS21.1 | Sensitive label-free microfluidic biosensor for the multiplexed detection and characterization of small extracellular vesicles

Moein Talebian Gevari[1]; Siddharth S. Sahu[2]; Dhrubaditya Mitra[3]; Petra Hååg[4]; Kristina Viktorsson[5]; Jan Linnros[6]; Apurba Dev[7]

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Introduction: Rapid, sensitive and inexpensive analysis of extracellular vesicles (EVs) with an easy-to-use method holds the key for the lab-to-clinic transition of various EV-based diagnostic opportunities. The method should also be able to address the issues arising from EV-heterogeneity for a robust and reproducible analysis. Here, we demonstrate the fabrication, characterization, and application of a novel silicon microchip-based biosensor that exploits streaming current for the detection and characterization of small extracellular vesicles (sEVs). The method was further optimized and compared with a fluorescence-based single EV technology for a semi-quantitative assessment of EV count, membrane-protein expression, and relative abundance of various EV-subpopulations.

Methods: sEVs were isolated from cell culture media of the non-small cell lung cancer H1975 cell line cultured in RPMI-media with FBS depleted from exogenous exosomes. The media was concentrated and sEVs were isolated by size-exclusion chromatography. The sEVs were characterized for size and amount by nanoparticle tracking analysis and earlier western blot confirmed expression of the targeted proteins. The CD81 and CD9 membrane proteins of the sEVs were profiled using a novel microchip technology. The microchips, composed of four interconnected microchannels, were designed with an open-top geometry where each of the channels could be functionalized with different antibodies for multiplexed measurement. The expression level of target marker was detected by means of immunocapture of sEVs on the sensor surface and measuring the changes in streaming current.

Results: The electric and fluidic characterization of the biosensors showed a linear dependence on the upstream pressure (0.5-3 bar) of the flow. The limit of detection (LoD) was estimated to be 350 sEVs/μL when targeting CD81 exhibiting a dynamic range of over 3 decades. The microchips were tested and optimized for rapid profiling (30 minutes) of CD81 and CD9 membrane proteins along with the assessment of sEV count in the sample and relative abundance of different sEV population. The results were validated using single-EV fluorescence measurement.

Summary/Conclusion: A novel microchip-based electrical sensors was developed for rapid and sensitive analysis of sEVs. The method allows multiplexed analysis of EV-membrane proteins along with the assessment of sEV count in the sample and relative abundance of different sEV populations.

Funding: The Swedish Research Council (no. 2016–05051 and 2018–06228), the Swedish Cancer Society (no. CAN 2018/597 and CAN2021/1469), the Stockholm Cancer Society (no. 221212 and 221383) and Stockholm County Council (no. FoUI-966345, 909121 and 750032), and The Erling Persson Foundation.

Keywords: microfluidics, biosensor, EV characterization, EV detection, biomarker

OS21.2 | Magnetic resonance imaging and magnetic particle imaging guided biohybrid extracellular vesicles for radiotherapy-induced brain injury

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Introduction: Up to 90% of brain tumor patients treated with radiotherapy will suffer from radiotherapy-induced brain injury (RIBI) and there is no effective treatment or prevention yet. Stem cell-derived extracellular vesicles (EVs) are emerging as a new regenerative approach for RIBI. EVs show superior properties compared to synthetic nanoparticles for drug delivery. In the context of these developments, imaging guidance plays a vital role by visualizing the temporal and spatial distribution of EVs and encapsulated drugs. Here, we aimed to develop a magnetic resonance imaging (MRI)/ magnetic particle imaging (MPI) trackable, liposome-EV biohybrid system for image-guided, effective treatment for RIBI.
Methods: Induced pluripotent stem cell (iPSC)-derived EVs were isolated from conditioned media of iPSC culture using size exclusion chromatography (SEC). Liposomes (PC:PS = 2.5:1) were prepared using the thin-film hydration method and loaded with superparamagnetic paramagnetic iron oxide nanoparticles (SPIONs, 20 nm) as imaging agent and IL-10 as therapeutic agent, and then fused with EVs by PEG-mediated fusion. The RIBI mouse model was induced by X-ray irradiation (80 Gy) under CT guidance. Two days post radiation, 108 EVs (2 μL in PBS) were stereotactically injected into the irradiated brain hemisphere, and MRI and MPI were performed over a period of 4 weeks.
Results: We have prepared biohybrid EVs that share similar characteristics (i.e., size and cell internalization) as native iPSC-EVs whilst loaded with therapeutic and imaging agents. Moreover, biohybrid EVs could be readily detected by T2*-weighted MRI and MPI, and the MRI/MPI signal persisted for up to one month.
Summary/Conclusion: Our study demonstrates the feasibility of developing MRI and MPI-guided EV-based therapy or drug delivery systems to ameliorate RIBI.
Funding: NIH R33 HL161756, R01CA262887 and S10 OD026740.
Keywords: radiotherapy-induced brain injury, magnetic resonance imaging, magnetic particle imaging, iPSC EV, biohybrid AND IL-10

OS21.3 | Negative enrichment of extracellular vesicles (EVs) by using high-resolution spiral microfluidic chip

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Introduction: Purification of extracellular vesicles(EVs) from blood plasma is essential to increase the accuracy of the assay in the use of EVs as a diagnostic biomarker. However, lipoproteins, which are lipid and protein complexes that transport lipid molecules in the blood, are not only 104 times more abundant than EVs but also overlap the size and density properties with those of EVs, making it difficult to remove lipoproteins from EVs.
Methods: The EVs were isolated by size-based ultrafiltration and characterized by using NTA. In this work, we proposed a high-resolution spiral microfluidic chip for the negative enrichment of EVs by selectively eliminating lipoproteins. First, before the plasma sample was injected into the microfluidic chip, the size difference between EVs and lipoproteins was created by immobilizing lipoproteins onto 7 μm beads using an immunoaffinity method. Then this pretreated sample was loaded onto the chip. In the microfluidic channel, nanometer-sized EVs were focused on the inner wall due to high-resolution dean flow fractionation force while the lipoprotein-associated 7 μm beads were moved to the outer wall, resulting in separation of pure EVs from lipoproteins.
Results: Based on our chip, over 97% of lipoproteins conjugated 7 μm beads were removed and over 93% of 100 nm beads, representing the average size of EVs, were recovered with a purity of 95% at 53.2: 531.8 μ/min (sample: sheath) flow rates. Based on these experimental settings, the performance of the spiral chip was explored by using A549 cells derived EVs and commercialized lipoproteins, individually. More than 88% of EVs were recovered, and the remaining LDL(low density lipoprotein) levels using the high-resolution spiral microfluidic chip were decreased to 5% of the initial loading while the HDL(high density lipoprotein) content was further lowered to 1%.
Summary/Conclusion: The proposed technique has the potential to improve the accuracy of EVs analysis because the signals from purified EVs are high while the background signal is low.
Funding: This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (No. 22021R1C1C2007646, No. 2021R1A2C3011254) and the Technology Innovation Program (20008829) funded by the Ministry of Trade, Industry & Energy (MOTIE, Korea).

OS21.4 | Multi-parametric characterization of extracellular vesicles by liquid chromatography coupled with in-line light scattering and fluorescence detection

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Introduction: The complex and heterogeneous nature of EVs poses challenges for their analytical characterization, which requires a multi-parameter analysis. The characterization of the necessary attributes required by the MISEV2018 guidelines can be only achieved by combining multiple different analytical methods, that require different equipment, significant hands-on time and different degrees of sample preparation. Here, we demonstrate how liquid chromatography coupled to multi-angle light scattering (MALS) and fluorescence detection (FLD) can provide multidimensional characterization of EVs with limited hands-on time.
Methods: EVs samples were fractionated on a HPLC system via either size (SEC) or anion exchange (AIEX) chromatography and analyzed by in-line scattering and fluorescence detectors.
EV size and particle amount were characterized by multi-angle light scattering and compared to NTA.
The total protein amount in the sample was characterized by native fluorescence of proteins. The lumen of the EVs was labelled with CalceinAM, the lipid membrane with DiO and CD81 with antiCD81 antibody.
GFP was loaded exogenously and an average per particle loading was measured by combining the fluorescence and light scattering data.
Results: The combination of these in-line detections tools with chromatography enables the analysis of sample purity and of EVs directly in crude mixtures such as conditioned media.
MALS detection provides average size and number of particles down to 107 particles. Fluorescence detections enables the label-free and non-destructive measurement of the total protein amount and, when combined with MALS, the protein to particle ratio.
Moreover, by labelling the EVs with fluorescent dyes, fluorescence detection can report on the composition of particles also in terms of specific EV properties, such as the presence of EV specific surface markers, a lipid membrane or a hollow morphology.
Finally, exogenous cargo loading methods were compared. Electroporation was the most successful loading method, leading to an average of 56 GFP per particle.
Summary/Conclusion: We have described the use of liquid chromatography coupled to in-line multi angle light scattering (MALS) and fluorescence detection for the characterization of EVs. With this combination, many necessary attributes required by the MISEV2018 guidelines have been characterized. The core of the approach relies on the multi-dimensional analysis of the sample on the same platform, similarly to microfluidic platform we have shown previously.
Funding: H2020-EU.1.2.2-FET Proactive programme via the BOW Grant agreement 952183.
Keywords: SEC, AIEX, liquid chromatography, light scattering, MALS, fluorescence detection, MISEV guidelines, exogenous loading

OS21.5 | EVAnalyzer: An Open- Source ImageJ Plugin for automated, quantitative, high content single vesicle imaging and its applications for routine characterisation of extracellular vesicles

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Introduction: Extracellular vesicle (EV) research increasingly demands for quantitative characterisation at the single vesicle level to address heterogeneity and complexity of EV subpopulations. Emerging, commercialised technologies for single EV analysis generally require dedicated instrumentation and proprietary software not readily accessible to non-EV specialised labs, limiting their implementation for standard EV characterisation in the rapidly growing EV field.
Methods: We developed 'EVAnalyzer', an ImageJ plugIn for automated, quantitative single vesicle analysis from imaging data, and established a robust protocol for capture, (immuno-) labelling and fluorescent imaging of EVs using exclusively standard reagents and laboratory equipment. The process has been optimised and validated for a number of routine EV applications (Schürz et al, JEV 2022) and is continuously extended for new functions and applications.
Results: We will present single vesicle characterization applications of EVanalyzer including the quantification of EV subpopulations based on immunostaining, validation of EV labelling reagents or optimisation of genetic EV engineering by determining population fractions and loading densities at the single vesicle level. We will further highlight the use of EVAnalyzer for automated quantification of cell uptake at the single cell - single vesicle level, thereby enabling high content EV cell uptake assays and plate- based screens. Additionally, we will show how the program is used for the quantification of EVs from in vivo applications for quantification of EV biodistribution in biofluids and tissue.
Summary/Conclusion: We propose that EVAnalyzer can be used in virtually every lab for routine as well as advanced in vitro and in vivo applications in EV and nanoparticle research. Due to its open access and open-source character, EVAnalyzer is a versatile tool which is open to community innovation within the EV and nano- and advanced material research fields.
Funding: This work was supported by the following grants: EV-TT BPro (County of Salzburg, WISS2025, P1812596) and EVTT (European Union, EFRE/IWB 20102-F1900731-KZP) and NanoCommons (EC H2020 #731032).
Keywords: cell uptake, EV immunolabelling, EV biodistributino, openinnovation, single particle imaging, single vesicle imaging

OS22: Cargo Delivery

Chairs: Masaharu Somiya, Olivier de Jong

Location: Ballroom 6A

15:20 - 16:50

OS22.1 | EV-mediated intracellular delivery of proteins using a light-induced protein release system

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Introduction: Effective intracellular delivery of therapeutic proteins can provide treatments for a wide array of diseases. Exosomes, nanosized vesicles naturally secreted by various types of cells, are emerging as promising nanocarriers for therapeutic biomolecules. However, efficient delivery of functional proteins across the cell membrane remains a challenge.
Methods: In this study, we introduce a new exosome-based intracellular cargo protein delivery system, named 'mMaple3-mediated protein loading into and release from the exosomes' (MAPLEXs). By fusing a photocleavable protein, mMaple3, between a cargo protein and an exosomal marker, we are able to load the cargo proteins into the exosomes and release the cargo proteins from the exosomal markers via 405-nm light-mediated photocleavage of mMaple3.
Results: Using this novel cargo protein release system, we were able to successfully deliver soluble proteins across the target cell membrane both in vitro and in vivo. First, we showed that transcription factors can be delivered to the target cells via MAPLEX and regulate their target genes. Secondly, we achieved in vivo gene editing in Cre reporter mice. As reported previously, without any targeting moiety, most of intravenously injected MAPLEXs accumulated in the liver. Lastly, we showed that in vivo epigenetic editing can be induced in the brain of AD mouse model via MAPLEX.
Summary/Conclusion: Our results suggest MAPLEXs as an efficient protein delivery system, which can be utilized for delivering diverse therapeutic proteins for various types of diseases.

OS22.3 | Endocytosis of red blood cell extracellular vesicles by macrophages leads to heme release and prevents foam cell formation

Thach Tuan Pham[1]; Anh Le[2]; Cong Dang[2]; Vinh Do[2]; Boya Peng[2]; Hong Boon Ong[3]; Chen Huan[2]; Han Wei Hou[4]; Minh T.N Le[1]

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Introduction: Red blood cell-derived extracellular vesicles (RBCEVs) display excellent characteristics for an EV-based drug delivery platform. Many studies have shown efficient delivery of therapeutic cargo using RBCEVs to treat cancer and other diseases in mouse models, paving a way for the application of RBCEVs in clinics. However, the biological functions of RBCEV's endogenous components are little known. In this study, we investigate the biodistribution of RBCEVs, study how RBCEVs are taken up, and track the fate of haemoglobin(the major RBCEV cargo) and their potential therapeutic effects.
Methods: Red blood cells were induced with calcium ionophore to release RBCEVs. After removal of RBCs and debris, RBCEVs were obtained from supernatant by ultracentrifugation (50 000 xg for 70 min at 4oC) and 60% sucrose cushion (50 000 xg for 16 h at 4oC). The concentrations were measured using a haemoglobin quantification kit. Size and number of RBCEVs were measure by nanoparticle tracking analysis. RBCEVs markers were checked using CD235a, BAND3 and ALIX.
Results: We dissected the biodistribution of RBCEVs at the cellular level and found F4/80+ macrophages took up the majority of RBCEVs in livers, while in the spleen, RBCEVs ended up mainly in CD169+ macrophages at the marginal zone. The uptake was strongly mediated by the phosphatidylserine on the RBCEVs. Upon endocytosis, RBCEVs were trafficked to late endosomal and lysosomal compartments, and the proteins were broken down rapidly within 2 hours. Incubating RBCEVs with human peripheral blood mononuclear cell-derived macrophages led to the differentiation of macrophages into a Mheme-like phenotype. The macrophages showed reduced CD86 expression, increased the expression of CD163, a marker of Mheme and M2. They also exhibited anti-inflammatory effects indicated by reduced secretion of the TNF-alpha cytokine after lipopolysaccharide (LPS) stimulation. In addition, RBCEV-induced macrophages upregulated heme oxigenase 1 (HO-1) and a cholesterol efflux protein, ABCG1. Mechanistically, the heme transporter HRG-1 mediated the release of heme into the cytoplasm, which eventually upregulated HO-1 and induced the Mheme-like phenotype in macrophages. Finally, we found that RBCEV treatment prevented the accumulation of oxidized low density lipoprotein in macrophages in vitro and on the artery-wall-on-a-chip model, suggesting an anti-atherosclerosis effects.
Summary/Conclusion: RBCEVs robustly taken up by macrophages in vivo and in vitro. Incubating RBCEVs with macrophages induced them into Mheme-like phenotype which has anti-inflammatory and anti-atherosclerosis effect.
Funding: This project is supported by the Singapore Ministry of Education (MOE-T2EP30121-0016), Ministry of Health (MOH-000643), and the National University of Singapore (NUHSRO/2019/076/STARTUP/02).

OS22.3 | Exosome-mediated delivery of CRISPR/Cas9 and donor vector for targeted gene insertion into AAVS1 locus in Her2-positive breast cancer cell line

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Introduction: Gene editing tools in particular CRISPR/Cas9 system are promising technology as therapeutic agent for genetical disorders. Here, we developed non-viral delivery platform using targeted exosomes. In this study, we used engineered exosomes which express DARPin against Her2 on exosome surface that targets Her2-positive breast cancer cell lines such as SKBR3. Using reporter gene such as GFP, makes it easier to track the internalization of GFP-encoding vector to target cell and entry of the GFP gene into the SKBR3 genome in AAVS1 locus. pCas9-guide-AAVS1 that targets AAVS1 locus and pAAVS1-Puro-DNR/GFP as donor vector that include AAVS1 left and right hand, were used. Exosomes are specifically investigated as a promising targeted nucleic acid delivery system due to their encapsulation capacity and low immunogenicity. In this study, we used targeted exosomes to deliver pCas9-guide-AAVS1 and pAAVS1-Puro-DNR/GFP vectors and then we evaluated the insertion of GFP gene into AAVS1 locus in SKBR3 genome.
Methods: Engineered HEK293T cells that express the DARPin against Her2 on their exosome surface were cultured. Exosome was extracted from HEK293/DARPin according to Exo-spin exosome extraction kit. Purified exosome characterized by TEM, zeta sizer and exosomal CD markers. CRISPR/Cas9 and donor vector, pCas9-guide-AAVS1 and pAAVS1-Puro-DNR/GFP vector were extracted and were loaded to exosomes using electroporation. Plasmids-loaded exosomes were termed Exo/pCas9- GFP-Puro. Exos/pCas9- GFP-Puro were exposed to SKBR3.

Results: Internalization of PKH26-labeled Exo/GFP-Puro and GFP expression in SKBR3 were validated using flowcytometry and confocal microscopy.
Insertion of GFP gene along with puromycin into AAVS1 locus in SKBR3 cells validated using puromycin selection. GFP expression in resulting cells was measured by flowcytometry and almost all cells expressed GFP.
Summary/Conclusion: Taken together, we demonstrated that targeted exosomes are able to deliver CRISPR/Cas9 and donor vector functionally to target cells. Our results suggest that development of exosome-based delivery of CRISPR/Cas9 could be a viable way towards finding a promising therapeutic tool. The ability of the exosome to transfer CRISPR system vectors into target cells for gene therapy opens up a new approach to improve therapeutics for genetic disorders.
Funding: Tarboiat Modares University.
Keywords: exosome- CRISPR/Cas9- nucleic acid delivery

OS22.4 | Extracellular vesicle-associated ROR receptors promote tumor progression independent of vesicle uptake

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Introduction: Extracellular vesicles (EVs) harbour a plethora of different biomolecules, which they can transport across cells. So far, EV uptake and cargo delivery into target cells have been regarded as the main mechanisms for EV function. To test this hypothesis, we investigated the fate of the transmembrane WNT receptors tyrosine kinase-like orphan receptor 1 and 2 (ROR1, ROR2) delivered via distinct EV subpopulations to breast cancer cells and aimed to unravel their impact on tumor progression.
Methods: Large, medium and small EVs were isolated by differential ultracentrifugation and thoroughly characterized by electron microscopy, nanoparticle tracking analysis and immunoblot based on the MISEV criteria. ROR transfer to target cells was observed using microscopy-based as well as cell fractionation assays and the function of EV-associated ROR on tumor cell aggressiveness was tested in migration and invasion assays.
Results: We observed that the supernatant of ROR-overexpressing cells was sufficient for transferring the receptors to ROR-negative cells. Analysing the secretome of the ROR-overexpressing cells, we detected a high enrichment of ROR1/2 on medium and small EVs, but not on large EVs. Interestingly, the majority of ROR-positive EVs remained attached to the target cell surface even after prolonged stimulation and were easily removed by treatment with trypsin. Nonetheless, ROR-positive EVs significantly increased the migration and invasion of breast cancer cells. Blocking EV uptake had no influence on the pro-tumorigenic function of the EVs. ROR-positive EVs were also detected in the plasma of breast cancer patients and increased levels of ROR-positive EVs showed a trend for association with poorer patient survival.
Summary/Conclusion: The oncogenic WNT receptors ROR1/2 are transferred via EVs to ROR-negative cancer cells. Our results indicate that EV-incorporated RORs support cancer progression independent of EV uptake and that they are promising cancer biomarkers.
Funding: This project was funded by Deutsche Forschungsgemeinschaft (DFG, German Research Foundation - project 424252458) and the Else Kröner-Fresenius-Stiftung (project 2019_A162).
Keywords: breast cancer, WNT signaling, EV uptake, tumor progression, biomarker

OS23: Cardiovascular Disease

Chairs: Ahmed G. Ibrahim, Luisa Weiss

Location: Room 608/609

11:35 - 13:05

OS23.1 | Transcardiac gradient of extracellular vesicle derived miRNAs modulates adipose tissue metabolism

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Introduction: The heart is often considered as a recipient of signals from the periphery to fine tune blood pumping in response to physiological and pathological changes. Cardiac secretory function consisting of proteins and peptides termed as cardiomyokines has founded the concept of heart mediated organ crosstalk. Studies have shown extracellular vesicles (EVs) are major players regulating organ crosstalk between peripheral organs and the heart. Nevertheless, whether cardiac tissue can release EVs with unique composition and content to deliver messages to peripheral organs and the heart itself remains unknown.
Methods: Paired serum samples isolated from coronary sinus (CS) and aorta (AO) blood of dogs under normal and tachypacing induced heart failure were used for extracellular vesicle (EVs) isolation, characterization and miRNA array analysis. In vitro functional assays tested the effect of CS and AO EVs under normal and heart failure conditions on cardiomyocytes and adipocytes. Transplantation of EV from CS and AO samples under normal and heart failure conditions in wild type mice was done to determine changes in whole body metabolism by CLAMs, modulation of adipose tissue metabolism, changes in the heart and immune response.
Results: Results showed heart failure promotes increased secretion of EVs in the CS compared to AO blood suggesting heart produces more EVs under diseased conditions. miRNAs array analysis identified top elevated miRNAs as miRNAs 16, 92 and 137 significantly enriches in CS EVs under heart failure conditions. Measurement of miRNAs 16, 92, 137 is different tissues showed significantly increased expression in brown adipose tissue (BAT) compared to all other tissue assessed. Next, 3TC-LI adipocytes treated with CS EVs from heart failure dogs showed decreased OXPHOS measured by Seahorse, reduced expression of mitochondrial markers and low BODIPY labelling together with increase in whole body oxygen consumption rates, food intake and energy expenditure as measured by CLAMS. Mechanistically, EV derived miRNAs 16, 92, 137 significant transcriptomic changes BAT assessed by bulk RNA-sequencing. Top altered genes identified in BAT were UQCR10, CPT1a and PGC1a after administration of CS EVs from heart failure animals and are binding targets for miRNAs 16, 92 and 137 respectively.
Summary/Conclusion: In conclusion, cardiac tissue communicates to peripheral organs including adipose tissue by releasing EVs derived miRNAs enriched differently in response to heart failure or under normal conditions. Top enriched EV derived miRNAs released by the heart regulate metabolic changes in adipose tissue suggesting heart-adipose crosstalk.

OS23.3 | Extracellular Vesicles from hypertensive mice and humans augment renal afferent arteriole response to Angiotensin II and blood pressure

Morgan Greig[1]; Jin Wei[2]; Ruisheng Liu[2]; Thu Le[3]; Luca Musante[4]; Uta Erdbrügger[5]
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Introduction: The effects of extracellular vesicles (EVs) on blood pressure regulation is unknown. We perfused renal afferent arterioles (AAs) of isolated glomeruli from normotensive C57BL/6 (B6) mice with EVs from hypertensive mice and humans to assess AA vasoconstrictive response to angiotensin II (ANG II) ex vivo. We also injected EVs into normotensive mice to measure their effects on renal blood flow (RBF) and systolic blood pressure (SBP) in vivo.
Methods: Circulating EVs were collected from normotensive mice and mice with ANG II induced hypertension II (1000 ng/kg/min via mini osmotic pump, n = 5) and hypertensive humans (n = 2). Differential centrifugation (DCF) (5000G x 30min, followed by 17.000G for 30 min) was applied to generate an EV pellet (p20) which was subsequently run through size exclusion chromatography (SEC). EV size and concentration was determined by nanoparticle tracking analysis and spectral flow cytometry. Following a 30 min perfusion with EVs (p20 EV pellet (10E10 particles/ml) or fraction 1–3 or 4–6 of SEC (doses 1–9 × 10E7 /ml)), dose-response curves of ANG II (10E-12 to 10E-6 mol/L) were measured. Similar doses of EVs were infused into the penile vein of normotensive B6 mice and RBF and SBP were measured.
Results: EVs derived from ANG II treated mice and hypertensive humans, but not from normotensive control mice and humans, significantly enhanced AA vasoconstrictor response to ANG II. Similar vasoconstrictor responses were observed when AAs were perfused with EV fractions from combined DCF and SEC preps (fraction 1–3 enhanced vasoconstriction vs fraction 1–6 did not) from hypertensive animals. Injection of EVs from hypertensive animals in vivo reduced RBF and increased SBP in normotensive B6 mice compared to EVs from control groups.
Summary/Conclusion: Taken together, these data suggest that EVs from hypertensive animals and humans have vasoconstrictive effects to alter renal hemodynamics and blood pressure. Further work is needed to identify the exact EV cargo with vasoactive property.

OS23.4 | Plasma fibrin-exposing extracellular vesicles to diagnose acute coronary syndrome before myocardial necrosis occurs: LEMONADE trial

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Introduction: Acute coronary syndrome (ACS), which comprises acute myocardial infarction (AMI) and unstable angina pectoris (UAP), is one of the main causes of mortality worldwide, caused by coronary thrombus formation. Cardiac troponin, which is the gold standard to diagnose AMI, has two main disadvantages: it is negative in patients with UAP, but positive in many other diseases. During thrombus formation, activated platelets release extracellular vesicles (EVs). We hypothesized that concentration and composition of circulating platelet EVs allow for earlier diagnosis of ACS than troponin. We aimed to determine the diagnostic value of circulating platelet EVs as novel biomarkers of developing ACS.
Methods: We conducted a prospective, multicenter trial enrolling 105 patients who presented to the emergency department with suspected ACS. Blood was collected at admission to measure the concentration of EVs and cardiac troponin. Flow cytometry (Apogee A60-Micro) was used to determine plasma concentrations of platelet EVs exposing P-selectin (CD61+/CD62p+) and fibrin (CD61+/fibrin+).
Results: Plasma concentrations of platelet EVs exposing fibrin were lower in patients with ACS, compared to patients with other causes of chest pain ($p = 0.013$) and lower in patients with an occlusive thrombus compared to non-occlusive plaque ($p = 0.004$). Among patients with initially negative high-sensitive troponin I, fibrin exposing-EVs were lower in ACS, compared to non-ACS patients ($p = 0.013$). Plasma concentration of fibrin exposing-EVs gradually decreased along with the time from symptom onset ($p = 0.059$). Fibrin exposing-EVs were independent predictors of ACS in multivariate analysis (OR 4.31, 95% CI 1.25 – 14.81), allowing to diagnose ACS with 76% sensitivity and 56% specificity.
Summary/Conclusion: Circulating fibrin-exposing EVs are novel candidate biomarkers of developing ACS in patients, especially useful in patients with initially negative troponin I. Incorporation of EV measurements into the ACS diagnostic algorithm might accelerate the implementation of ACS treatment in the emergency setting.
Funding: "Club 30" Specialized Research Fellowship grant to A.G., Polish Society of Cardiology.
Keywords: thrombosis, platelets, acute coronary syndrome, biomarkers

OS23.5 | Embryonic stem-cell derived small extracellular vesicles improve heart function after myocardial ischemia through pro-angiogenic and anti-fibrotic effects in mice

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Introduction: Heart failure is a leading cause of morbidity and mortality, affecting over 60 million patients worldwide. Although several drugs and mechanical devices can improve cardiac function, they do not stop disease progression or regenerate new

cardiac tissue. During the past two decades, several clinical trials have shown stem cells as a promising way to repair damaged myocardium and small extracellular vesicles (SEV) have been identified as one of the major mediators of stem cell-induced effects. Previous studies showed that EV isolated from different cell sources improve cardiac function through cardiac repair after myocardial infarction (MI) in rodents. The SEV components responsible for the effects observed, as well as a comparison of EV from different cell sources, are still unclear. In this work, we seek to understand which of these sources of SEV is the most suitable to be used for cardiac repair.

Methods: We isolated the SEV from the conditioned media of primary bone marrow mesenchymal stromal cells (BM-MSC), an immortalized line of MSC (hTERT-MSC), human embryonic stem cells (ESC), cardiac progenitor cells (CPC), human cardiomyocytes (CM) and human ventricular cardiac fibroblasts (VCF) by a serial ultracentrifugation protocol. This was followed by a characterization of our isolates by western blot, nanoparticle tracking analysis, and electron transmission microscopy. Then, we evaluated the functionality of the different types of SEV in in vitro assays of cardio-protection, angiogenesis, cardiac fibrosis, cardiomyocyte proliferation, and immunomodulation. Furthermore, we evaluated the functionality in vivo in a rodent model of myocardial ischemia-reperfusion injury (IRI) and characterized the composition of the best SEV candidates.

Results: ESC-SEV showed stronger pro-regenerative effects in vitro compared to the other SEV types evaluated. Furthermore, ESC-SEV dampened the increase in the left ventricular end-diastolic volume (LVEDV) in the post-ischemic heart. These effects correlated with reduced fibrosis and increased angiogenesis detected by histological analysis of the post-ischemic heart sections.

Summary/Conclusion: In summary, our data show that ESC-SEV significantly reduced adverse cardiac remodeling after MI by promoting a reduction in fibrosis and an increase in angiogenesis in the post-ischemic heart, representing a promising treatment to promote cardiac repair post-MI.

Funding: AstraZeneca.

Keywords: cardiac repair, small extracellular vesicles, regeneration

OS24: Check This Stuff Out!

Chairs: Sujata Mohanty, Hanne Winther-Larsen

Location: Room 606/607

15:20 - 16:50

OS24.1 | Extracellular vesicles derived from microalgae: bio-compatibility, bio-distribution and bio-activity

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Introduction: Extracellular vesicles (EVs) are considered one of the most promising bio-nanovehicles for the delivery of bioactive compounds. (nano)Algosomes are microalgae-derived EVs that can be used as nanocarriers for precision medicine. Our previous work has studied their bio-chemical and bio-physical characteristics, based on the MISEV2018 guidelines. Moreover, we have shown that algosomes are not cytotoxic and that they have the ability to bypass the cell membrane of different human and C. elegans model. Here, we defined the endogenous bioactivity of algosomes in vitro and their bio-compatibility and bio-distribution in a mouse model.

Methods: Algosomes were separated from a suspension of Tetraselmis chuii microalgal cells using tangential flow filtration and characterized (EV-Track ID: EV200075 exp. 2). Next, algosomes bio-activities and immuno-compatibility were evaluated using biochemical assays, gene expression, and basophil activation tests. Then, pristine algosomes or algosomes labeled using a specific dye (DiR) were used to perform toxicity and bio-distribution analyses on BALB/C and athymic nude mice, respectively.

Results: Our experiments showed that algosomes have an antioxidant feature and do not cause allergic-like responses. The results of gene expression analysis showed no gene altering after algosome treatment, specifically no DNA damage was detected. The analysis of algosome bio-distribution in mice demonstrated a peculiar organotropism, while liver localization was reduced during time. Conclusion: These studies demonstrate how algosomes represent an efficient natural delivery system of bioactive biomolecules, thus promoting the exploitation of such sustainable EVs in theranostic applications.

Summary/Conclusion: These studies demonstrate how algosomes represent an efficient natural delivery system of bioactive biomolecules, thus promoting the exploitation of such sustainable EVs in theranostic applications.

Funding: VES4US and BOW projects funded by the EU-H2020 programmes under grant agreements N. 801338 and N. 952183.
Keywords: microalgae, nanoalgosomes, pre-clinical studies, extracellular vesicles, anti-oxidant bio-activity, mouse model

OS24.2 | Plant-derived extracellular vesicles as delivery platform for RNA-based vaccine: feasibility study of an oral and intra-nasal SARS-COV-2 vaccine

Margherita AC Pomatto[1]; Chiara Gai[1]; Federica Negro[2]; Lucia Massari[2]; Maria Chiara deregibus[3]; Cristina Grange[4]; Massimo Cedrino[5]; Francesco Giuseppe De Rosa[6]; Giovanni Camussi[7]
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Introduction: Plant-derived extracellular vesicles (EV) may represent a platform for delivery of RNA-based vaccines as an alternative to synthetic nanoparticles as they are not cytotoxic, protect nucleic acids from degradation and environmental stress and are incorporated in target cells. In the present study we evaluated whether EV extracted from orange fruit and engineered with SARS-CoV-2 mRNA could be used as a carrier for development of an oral and intra-nasal administered vaccine.
Methods: EV were isolated by orange (Citrus sinensis) juice using differential ultracentrifugation and engineered with mRNA coding for protein S subunit 1 (S1) of SARS-CoV-2. qRT-PCR/PCR and flow cytometry techniques were used to detect mRNA and evaluate immune activation. Immunization of female BALB/c mice was evaluated using ELISA for antibody response, cytofluorimetric assay for splenocytes activation, ELISPOT for IFN-gamma secretion.
Results: The mRNA was efficiently loaded into EV and protected from degrading stress including RNAse enzymes and gastric juice. After incubation with target cells, engineered EV were incorporated and mRNA was translated into protein.
After PBMC incubation with macrophages treated with loaded EV, T lymphocyte activation was observed as shown by increased proliferation and expression of CD25 and HLA DR.
The immunogenic potential of loaded EV was evaluated in mice immunized via intramuscular, oral and intranasal routes. The intramuscular administration elicited a humoral immune response with production of specific IgM and IgG blocking antibodies and a T cell immune response, as suggested by IFN-gamma production from purified spleen lymphocytes. Oral administration by gavage as well as intranasal administration induced a specific lymphocyte activation and the production not only of specific blocking IgM and IgG but also IgA, that are the first mucosal barrier in the adaptive immune response.
Summary/Conclusion: Plant-derived EV may represent a useful platform for mRNA-based vaccine administered not only parentally but also orally and intranasally, two needle-free routes able to trigger the first barrier for viral infection represented by mucosal IgA immune response.
Funding: The research was supported by EvoBiotech s.r.l.
Keywords: vaccine, exosome, vesicle, delivery, Covid, SARS, engineering, RNA, nucleic acid, oral, intranasal, intramuscular, drug

OS24.3 | Vaccination potential of bacterial extracellular vesicles in fish

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Introduction: Secretion of extracellular vesicles from the bacterial body (BEV) is associated with a range of phenotypes including cell-cell communication, host-pathogen interactions, antimicrobial resistance development and microbial biofilm formation. BEV may also elicit a humoral and antibody mediated immune response after immunization of a host making them an interesting vaccine candidate against an infectious agent. We have investigated the use of BEV as a potential vaccine against several intracellular bacterial pathogens creating problems in aquacultured fish, including Francisella noatunensis, Piscirickettsia salmonis and Yersinia ruckeri.
Methods: BEVs were isolated from the bacterial cultures by either ultra centrifugation or tangential flow, and resuspended in PBS. The BEV were further characterized by electron microcopy, mass-spectrometry and differential light scattering. The zebrafish were immunized with 20 ug BEV-PBS solution before the fish were challenged with the specific pathogen. Tilapia, Atlantic cod and Atlantic salmon were immunized with 10 ug BEV resuspended either in PBS or formulated with a mineral oil adjuvant before challenged with the same bacterial species from where the BEV was derived. The fish immune response from the BEV immunization and bacterial challenge was determined together with the cumulative survival.
Results: Immunization with BEV revealed protection against the diseases in a zebrafish challenge model. However, less protection was detected in the natural hosts; tilapia, Atlantic cod and Atlantic salmon, respectively. While the challenge experiments

performed in the natural host was performed in BEV immunized fish together with an oil adjuvant, the zebrafish was immunized with naked BEV.
Summary/Conclusion: We hypothesis that the immune protective potential of BEV is host dependent and that the oil adjuvant may have a destructive effect on the immune-protective potential for BEV.
Funding: The Research Council of Norway and The University of Oslo.
Keywords: bacterial extracellular vesicles, BEV, aquaculture, immunization, therapeutics, vaccine formulation

OS24.4 | The extracellular vesicle regulator Alix is required for regeneration in planarians

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Introduction: Extracellular vesicles (EVs) can enhance the regenerative ability of mammals, but EVs' roles in organisms with greater regenerative capacity are poorly characterized. We asked whether planarian flatworms, renowned for whole-body regeneration, produce EVs, and whether known biogenesis regulators were required for viability or regeneration.
Methods: EVs were isolated from dissociated, whole planarians using various methods: differential centrifugation, microfiltration, and ultracentrifugation (electron microscopy and NTA), or PEG-based precipitation (EV uptake) followed by size exclusion chromatography (marker characterization). Capillary-based western was performed using antibodies against the planarian homologs of ALG-2 Interacting Protein X (Alix) and Tumor Suppressor Gene 101 (Tsg101). EV uptake in primary cultures of planarian neoblasts (stem cells) was monitored using PKH67-labeled EVs, followed by microscopy and flow cytometry. alix and tsg101 were knocked down using RNA interference (RNAi); phenotypes were characterized by microscopy, flow cytometry, and RNA sequencing.
Results: Planarian EV-like nanoparticles had similar size and morphology as in other organisms, carried orthologs of EV biogenesis regulators Alix and TSG101, and were bound/taken up by neoblasts in a time-dependent manner. tsg101 RNAi caused rapid lethality, whereas alix RNAi delayed regeneration, reduced the number of mitotic neoblasts, and downregulated neoblast-enriched transcripts. Flow cytometry analysis showed a decrease in G0/G1 neoblasts and early progeny, with a concomitant increase of cells with >4N DNA content.
Summary/Conclusion: We conclude that planarians produce EVs, and that known regulators of EV biogenesis are required for viability, regeneration, and neoblast cell cycle kinetics and survival, suggesting that EVs might regulate neoblasts. Future studies will assess purified EVs' biological activity and determine how Alix influences EV function.
Funding: NIH COBRE GM103636.
Keywords: planarians, regeneration

OS24.5 | Quantitative proteomic analysis of EVs during infectious cycle of Eimeria falciformis in the host

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Introduction: Secretion of EV has been identified as a mechanistic system of contact during host-pathogen crosstalk and molecular exchange. Similarly, EVs are formed during host-parasite interaction, parasite motility and development. Eimeria falciformis is regarded as the first and most prevalent coccidian parasite of mice. Although E. falciformis developmental stages in mice and the host immune responses during infection have been described, the secretion of EVs and the array of host signalling protein molecules via EVs are yet unknown during E. falciformis developmental stages in the host.
Methods: Caecal and blood samples were collected form E. falciformis-infected mice at the time of oocyst ingestion (0h), 1st generation merozont (68h), 2nd generation merozont (116h) stages, faecal oocyst shedding (7day) and time of host recovery (10day) post infection. EVs were isolated and processed by discontinuous centrifugation, ultrafiltration, differential ultracentrifugation and purified on discontinuous iodixanol gradient solution. Caecum-derived EVs (cEVs) and serum-derived EVs (sEVs) were characterized by NTA, transmission electron microscope and western blotting. EV protein compositions were characterized and quantified by tandem mass tag-based analysis.
Results: The sub-population of isolated sEVs and cEVs increased significantly as E. falciformis life stages progressed in the host. While sEV were circular to spherical in shape, caecum EVs were of diverse shapes and larger in size. Nonetheless, both

sEV and cEV expressed characteristic EV marker including CD9, CD82, Hsp70 and MHCs. Also, there were 861 and 1,024 quantifiable and differnetially regulated proteins in sEVs and cEVs respectively as infection proceeded in the host. Neutrophil gelatinase-associated, CD4-binding, interleukin-18-binding, monocyte differentiation antigen, interferon inducible GTPase, MCSF, complements, and chemokine 8 were among immune-related sEV proteins. In contrast, cEV immune-related proteins include iNOS, neutrophilic granule, TGFβ-induced, STAT1, NLRP6-associated, caspase 3, leukocyte surface antigen, toll-like receptor 8. There were more cell death-related proteins in cEVs and sEVs contained more ion-exchange and metabolic proteins.
Summary/Conclusion: E. falciformis-infected mice cEVs and sEV proteins associated with immunity, metabolism, ion exchange, and cell death were significantly upregulated, at least, during E. falciformis first and second merozont stages in the host. Also, E. falciformis infection co-opt cellular and humoral responses through EV secretions at local and systemic levels in the host. Some of the identified proteins are potential diagnostic molecules for murine and avian coccidiosis.
Funding: Key Technologies Research and Development Program (Key Technologies R&D Program) 2017YFD050040320.
Keywords: EVs, serum, caecum, eimeria falciformis, tandem mass tag, proteins

Oral with Poster Oral

OWP1: Oral with Poster - Session I

Chairs: Ana Claudia Torrecillas, Annalisa Radeghieri

Location: Room 611/612

15:15 - 16:45

OWP1.01=PS10.01 | Differential expression of bacterial small RNAs in severe bronchiolitis

Kylie I. Krohmaly[1]; Marcos Perez-Losada[2]; Zhaozhong Zhu[3]; Kohei Hasegawa[3]; Andrea L. Hahn[4]; Carlos Camargo[3]; Robert J. Freishtat[4]
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Introduction: Bronchiolitis is a heterogenous condition with respiratory bacterial taxa as risk factors for a spectrum of outcomes. Bacteria produce small RNAs (sRNAs) carried via extracellular vesicles (EVs) that can alter human gene expression, similar to human microRNAs. We aimed to define sRNA reference libraries for four important respiratory bacterial species and then determine their association with bronchiolitis severity.
Methods: We used 574 samples from a multicenter, prospective cohort study of US infants hospitalized with bronchiolitis. Nasal swabs collected at the time of hospitalization were sequenced for sRNAs (MiSeq; Illumina). To define the bacterial EV libraries, we isolated EVs from Haemophilus influenzae (Hi), Moraxella catarrhalis (Mc), M. nonliquefaciens (Mn), and Streptococcus pneumoniae (Sp) cultures via precipitation and size exclusion chromatography. Bacterial cell and EV RNA were extracted for sRNA-sequencing (MiSeq; Illumina).
Results: The median age at hospitalization was 4 months (IQR = 4.1); 41% female; 45% White, 23% Black, and 29% Hispanic. Intubation (n = 18) and/or positive pressure ventilation (n = 15) represented higher bronchiolitis severity (total n = 28). We identified reference sRNA libraries from bacterial cells and EVs: 286 sRNAs from Hi (64%), 272 from Mc (57%), 49 from Mn (100%), and 159 from Sp (87%). Of these, 20 sRNAs were significantly different between bronchiolitis severity groups ($p < 0.05$, fold change>|1.1|). Higher severity-associated sRNAs were linked to endocytic processes, whereas lower severity-associated sRNAs were linked to NFkB pathway activation.
Summary/Conclusion: Bacterial sRNAs differ between higher and lower bronchiolitis severity and may be involved in relevant biological processes during acute illness. These data provide new insights into bacteria-host signaling in bronchiolitis, particularly through interspecies RNA communication.
Keywords: bacteria, bronchiolitis, small RNAs

OWP1.02=PS10.02 | Extracellular vesicle mediated cytokine secretion in chronic airway disease

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Introduction: The cytokine interleukin-33 (IL-33) is a driver of chronic airway diseases such as asthma and chronic obstructive pulmonary disease (COPD). This cytokine lacks a signal peptide and is tightly sequestered in the cell nucleus where classically it was thought to be passively released and bioactive upon necrotic cell death, although the exact release mechanism is still unknown. We recently described a chronic disease-associated spliced isoform of IL-33 expressed in airway epithelial cells that can be found secreted as membrane-bound cargo on extracellular vesicles (EVs). This IL-33 isoform lacks exons 3–4, is unable to localize to the nucleus and instead can be found throughout the cytosol where it can then be tonically secreted into the extracellular environment.
Methods: Using both chemical inhibition and genetic depletion approaches, this secretion phenomenon was inhibited by the nSmase2 inhibition (GW4869 and genetic knockdown) in both in vitro and in vivo models. Subsequent proteomic, biochemical and imaging analyses reveal that in this non-canonical secretion mechanism, IL-33 interacts directly with the chaperone HSP70 to facilitate EV binding and associated cellular secretion.
Results: In an IL-33 dependent airway mouse model, co-administering GW4869 with Alternaria allergen significantly reduced airway inflammation compared to Alternaria treatment alone. This result, coupled with our human biospecimen observations, point to nSmase2 and HSP70 dependent EV biogenesis pathway as a potential mechanism for IL-33 is secretion in chronic airway inflammation.
Summary/Conclusion: This work highlights a recruitment pathway for EV-bound signaling cargo and reveals unique therapeutic targets for chronic airway diseases.
Keywords: IL-33 HSP70 nSmase2 GW4869 inflammation

OWP1.03=PS11.01 | Acidovorax temperans outer membrane vesicles promote lung inflammation and tumorigenesis

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Introduction: Dysbiosis is a common feature of solid tumors, however whether this dysbiosis directly contributes to tumor development is largely unknown. We previously characterized the lung cancer microbiome where the Gram-negative Acidovorax temperans was found to be enriched in tumors of smokers and patients harboring TP53 mutations. We showed that A. temperans exposure accelerated tumor development and burden through infiltration of proinflammatory cells in the lungs. Herein, we investigated the involvement of outer membrane vesicles (OMVs) shed by Acidovorax temperans in driving inflammatory dynamics and tumorigenesis.
Methods: OMVs were extracted using standard serial centrifugations and filtrations followed by a sucrose gradient to get rid of flagella and non-vesicular elements. The purified samples were characterized using NTA, electron microscopy and ImageStream flow cytometer microscope. RNA from the OMVs was isolated using miRNeasy kit (Qiagen) and was prepared for RNAseq using the NEBNext Multiplex Small RNA Library Prep (Illumina).
Results: Our results suggest that A. temperans OMVs are taken up by A549 lung cancer cells and that A. temperans OMVs facilitate a strong pro-inflammatory response also in A549 and THP-1 macrophages. In addition, macrophages exposed to A. temperans OMVs overexpress SIRPα which is associated with tumor cell immune escape and tumor progression. Notably, the levels of CD47, the receptor recognizing SIRPα, were elevated in the cancer cells after the OMVs treatment. We also optimized an in vivo protocol where A. temperans OMVs were introduced into the lungs of mice by intranasal administration. Intranasal administration of A. temperans OMVs lead to an increased secretion of proinflammatory cytokines. We used OMVs RNAseq specifically focusing on short RNAs (sRNA) and identified a unique signature of RNA species including various fragmented tRNAs.
Summary/Conclusion: OMVs shed by Acidovorax temperans promoted inflammatory signaling in lung carcinoma cells and elevated CD47 expression on tumor cells and SIRPα levels on macrophages. We currently investigate the capacity of these OMVs to drive lung tumorigenesis and study the molecular cargo shipped between the pathogen and cells of the host.
Funding: This study is funded by the Israel Science Fund (ISF) grant #: 1178/20.
Keywords: outer-membrane-vesicles, lung cancer, microbiome

OWP1.04=PS03.01 | Presence of small infectious extracellular vesicles from a chronically infected T-cell line

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Introduction: In 2021, 1.5 million people acquired Human Immunodeficiency Virus-1 (HIV), and an estimated 37.7 million people were living with HIV worldwide. Despite extensive efforts, successful vaccines and functional cures have yet to be developed, highlighting the need for further viral characterization. One challenge to our understanding of HIV-1 biology is the heterogeneity of virions and extracellular vesicles (EVs) and particles (EVPs) in biological fluids (i.e., bodily and cell culture). Here, we utilized a latently infected T-cell line as a model to investigate the size range, content diversity, and infectivity of EVPs released in cell culture supernatants and identify smaller than previously known infectious EVPs.

Methods: HIV-1 latently infected J1.1LAV T-cells were cultured for four days, then cell culture conditioned medium was collected. Secreted EVPs were separated by differential ultracentrifugation (DUC) to generate the following fractions (Frac): Frac-A (2000g force, 20 min); Frac-B (10,000g force, 45 min); Frac-C (100,000g force, 90 min); Frac-D (167,000g force, 3-hours); Frac-E (167,000 g force, 18-hours). The fractions were characterized for size, viral markers by WB and dSTORM, and HIV-1 infectivity and blocking.

Results: We observed a heterogeneous particle size distribution for all fractions. Frac-C had a modal size of 132 nm (similar to HIV), whereas Frac-E had a modal size of 15 nm. WB analysis indicated diverse distribution of the EVs and HIV markers amongst the fractions, with Frac-E being positive for all of them. We also observed the colocalization of EVs and HIV markers on the same particles by dSTORM. Functional analysis revealed that small-sized EVPs in Frac-E were infectious and sensitive to neutralizing antibodies.

Summary/Conclusion: Utilizing the DUC approach, we obtained small infectious (sub-100 nm) Frac-E EVPs that share EVs and HIV markers.

Funding: This work was supported by a cooperative agreement (W81XWH-18-2-0400) between the Henry M. Jackson Foundation for the Advancement of Military Medicine Inc., and the US Department of Defense (DoD).

Keywords: HIV, small extracellular vesicles and particles (sEVPs), NTA, direct stochastic optical reconstruction microscopy (dSTORM)

OWP1.05=PS07.01 | Differential protein expression in immune cell-derived EVs isolated at different densities

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Introduction: Small EVs (sEVs) are the most studied EV subpopulation, but less is known about other subpopulations e.g. large EVs (lEVs). Consequently, specific subpopulation markers are missing. Therefore the aim of our study was to compare the differential protein expression between EVs of different sizes and densities.

Methods: HMC-1 (mast cells) and THP-1 (monocytes) were cultured in EV-depleted cell culture media. The lEVs (16,500xg, 20min) and sEVs (118,000xg 2.5h) were isolated by differential ultracentrifugation and purified by a density cushion. Low density EVs (LD; ~1.05-1.12 g/mL) and high density EVs (HD; ~1.12-1.15 g/mL) were collected from both lEV and sEV.

Purity, morphology, and yield of EVs were determined by nanoparticle tracking analysis (NTA), protein measurement and transmission electron microscopy (TEM). The proteome of the different samples was analysed with quantitative mass spectrometry (TMT-LC-MS/MS).
Results: TEM and NTA showed different size and concentration of the different EV subpopulations (lEV LD, lEV HD, sEV LD and sEV HD). In total 3972 and 4735 proteins were quantified in HMC-1 and THP-1, respectively. lEV and sEV were well separated, however, the separation between the LD and HD samples was more pronounced for the THP-1 EVs than for the HMC-1 EVs. Several protein groups were enriched, confirming our previous finding. Among these groups we found that mitochondrial proteins were enriched in lEV LD. Namely, TIM/TOM complex, MICOS, ATP5 proteins. KIF proteins were enriched in the lEV HD samples. Tetraspanins, ESCRT, Syndecans and Syndecan binding protein were enriched in the HMC-1 sEV, additionally these proteins were enriched in the THP-1 sEV LD. ADAM proteins were enriched in the sEV HD.
Summary/Conclusion: This study identified several protein groups differently enriched in lEV and sEV, which validate previous findings. In addition the proteome of EVs isolated at different densities had significant differences.
Funding: Swedish Heart Lung Foundation; Emil and Wera Cornell Foundation; Lars Hierta Memorial Foundation.
Keywords: EV subtypes, small EVs, large EV, size, density, TMT-LC-MS/MS

OWP1.06=PS05.01 | Simultaneous size-based fractionation and surface marker detection of extracellular vesicles in a one-step workflow

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Introduction: Extracellular vesicles (EVs) are increasingly studied as diagnostic and prognostic biomarkers. Typically, the analysis of EV-associated biomarkers requires their separation from biofluids, which warrants larger input volumes, leads to longer turn-over times, and increases technical variability. To overcome these issues we aimed to develop a one-step workflow for simultaneous size-based fractionation and biomarker detection.
Methods: We coupled a multi-angle light scattering detector (MALS) and a fluorescent light detector (FLD) in-line with the asymmetrical flow field-flow fractionation (AF4) equipment. We first optimized the AF4-MALS-FLD parameters using recombinant EVs (rEVs) including spacer (350 μm), membrane (10 kDa regenerated cellulose), running buffer (PBS + 0.02% NaN3), detector flow, and cross flow profile. Next, we evaluated the performance of the AF4-MALS-FLD set-up using diverse biofluids including cell culture medium, urine and blood plasma. Fractions were collected and quality was controlled using complementary characterization methods in compliance with MISEV2018 guidelines. Relevant experimental parameters were submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV210212).
Results: We were able to analyze EV surface markers CD9, CD63 and CD81, and cancer biomarkers HER2, EpCAM and PSMA in pre-purified EV preparations from different breast and prostate cancer cell lines. Analysis of increasing numbers of EVs revealed a linear correlation with the number of particles as measured by NTA (4E+9 – 2E+10 particles). Furthermore, the AF4-MALS-FLD workflow allowed to detect EVs in samples with increasing complexity as cell culture supernatant, urine and blood plasma. Finally, we validated the one-step workflow by confirming the presence of PSMA-positive EVs in urine from prostate cancer patients (n = 25), and by demonstrating the presence of EpCAM- or HER2-positive EVs in blood plasma enabling discrimination of breast cancer patients (n = 15) from healthy donors (n = 15).
Summary/Conclusion: We have successfully optimized and validated the AF4-MALS-FLD workflow, that allows simultaneous size-based fractionation and surface marker characterization in a fast, reproducible and sensitive manner.
Funding: This work was supported by UGent, FWO, CRIG, and KOTK (the Flemish cancer society).

OWP1.09=PS13.01 | Multi-omic landscaping of mesenchymal stem cell-derived extracellular vesicles generated from xeno-free culture condition

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Introduction: Mesenchymal stem cell-derived extracellular vesicle (MSC-EVs) are a bright star in regenerative medicine. A growing body of evidence has demonstrated the effectiveness of MSC-EVs in animal models of renal disease and cardiovascular disease by virtue of their cargo. However, before clinical translation, there is a need to optimize MSC culture condition without any substrate of animal origin for EV harvest. In the present study, we aimed to identify the optimal xeno-free culture medium for the clinical-grade production of MSC-EVs and employed multi-omic analysis for the molecular and functional profiling of these vesicles.
Methods: MSCs were isolated from human umbilical cords and cultured with xeno-free media until passages 6 (P6) to evaluate differentiation potential, cell doubling time, metabolic activity, apoptosis and cellular senescence. EVs were prepared from the culture supernatants by differential centrifugation (2000g, 4°C, 30min; 13500g, 4°C, 30min; 100000g, 4°C, 2h) and were further purified via size exclusion chromatography. The purified EVs were characterized by transmission electron microscopy, nanoparticle tracking analysis, NanoFCM and western blotting analysis of exosomal markers. MSC-EVs from four donors were subjected to multiomics analysis, including transcriptomics, proteomics, metabolomics and lipidomics.
Results: First, we analyzed the influence of different xeno-free media on biological properties of MSCs and found that human platelet lysate (HPL)-supplemented media support the isolation and long-term proliferation of MSCs. Compared to FBS-based media, HPL augmented proliferation and metabolic activity of MSCs while improving apoptosis and senescence, suggesting that HPL is an efficient alternative to FBS for MSC culture. Next, we purified the EVs from FBS or HPL-cultured MSCs and verified them according to the MISEV2018 guideline. Nonsignificant differences in protein markers, size, and morphology were found between these two vesicles. Interestingly, multiomics analysis clarified that differences in the composition of MSC-EVs under FBS and HPL culture conditions were mainly focused on proteins and miRNAs but not mRNAs, lncRNAs, cirRNAs, hydrophilic metabolites and lipids. However, more than 80% of differential miRNAs and 90% of differential proteins were not highly expressed in HPL-produced MSC-EVs, indicating that the therapeutic potential of MSC-EVs may not be affected by HPL. GO enrichment analysis of the highly expressed miRNAs showed they are associated with regeneration and development, immune regulation, and extracellular matrix composition. Moreover, principal component analysis revealed a similar molecular content of MSC-EVs from different donors.
Summary/Conclusion: HPL-based culture condition well-maintained the molecular composition and therapeutic potential of MSC-EVs and could serve as an efficient xeno-free alternative for the production of clinical-grade MSC-EVs.

OWP1.10=PS12.01 | RNA modification levels determine the tumor promoting effect of 5'tRF-GlyGCC in colorectal cancer Te-EVs

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Introduction: We have previously shown that 5'tRF-GlyGCC encapsulated in colon cancer EVs acts in a tumor-promoting manner by promoting the production of inflammatory cytokines via TLR8 in macrophages. However, it is not clear how 5'tRF-GlyGCC regulate TLR8 reactivity in macrophages. Since RNA modifications have been reported to modulate TLR8 reactivity, we focused on RNA modifications on 5'tRF-GlyGCC to determine the mechanism of action of tumor EVs on macrophages.
Methods: Clinical specimens: The colon cancer specimens were obtained from patients undergone primary resection at the Osaka Medical and Pharmaceutical University, Japan. Written informed consent was obtained from each patient, and the study was approved by the ethics review board of the Osaka Medical and Pharmaceutical University.
Isolation of tumor tissue-derived EVs: Tissue-immersed medium was centrifuged at 2,000 g, for 30 min, and the collected supernatants were subjected to the ultracentrifuge method (100,000 g x 2) for recovery of tissue-exudative EVs (Te-EVs). The size and concentration of EVs were determined using qNano.
Isolation of 5'tRF-GlyGCC from Te-EVs: EV-RNA were isolated from Te-EVs by using miRNeasy kit. 5'tRF-GlyGCC were collected from EV-RNA by Dynabeads magnetic beads conjugated with anti-sense oligo targeting 5'tRF-GlyGCC.
RNA modification analysis of EV-RNAs: Quantitative analysis of RNA modification levels was performed by UHPLC-UniSpray-MS/MS using 10 ng of EV-RNA sample.
Results: UHPLC-MS/MS analysis identified tumor characteristic RNA modification landscape in tumor Te-EVs. Among the modifiers reported to contribute to TLR8 reactivity, m6A levels were decreased in tumor Te-EVs compared to normal Te-EVs. The 5'tRF-GlyGCC in tumor Te-EVs also showed a significant decrease in m6A content as well. The reduction of m6A levels in tumor Te-EVs by the recombinant protein ALKBH5, an m6A demethylase, promoted inflammatory cytokine production in macrophages.
Summary/Conclusion: The reduction of m6A modification level on 5'tRF-GlyGCC in tumor Te-EVs, was found to act in a tumor-promoting manner by modulating the reactivity of TLR8 in macrophages. To our knowledge, this is the first report showing that dysregulation of RNA modifications in EV-RNA functions as a tumor-promoting factor in colorectal cancer.

Oral with Poster Oral

OWP2: Oral with Poster - Session II

Chairs: Rienk Nieuwland, My Mahoney

Location: Room 611/612

14:20 - 15:50

OWP2.01=PS02.04 | Differential extracellular vesicle gene expression in Next Generation Sequencing targeted panels as potential platforms for biomarker discovery in Parkinson's disease

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Introduction: Parkinson's disease (PD) is the second most common and fastest growing neurodegenerative disease globally. Neuronal extracellular vesicles are in low abundance in hematopoietic-rich plasma after crossing the blood-brain barrier; however, targeted profiling of brain-derived transcripts can help resolve rare isoforms and enrich their signal.
Methods: To that end, we performed transcriptome (WTS), exome (WES), and brain-specific exosomal RNA profiling on 20 PD and 20 healthy control plasma samples. Following RNA-Seq analysis, DEx analysis was conducted alongside Boruta feature selection to see how each enrichment platform influenced the resolution of gene expression and isoform detection.
Results: We observed tremendous neuronal gene expression, orders of magnitude higher in the brain panel over WES or WTS. Over 278 genes, more than a quarter of the brain panel, was undetectable in WTS but shown in the targeted panel. Additionally, Unique Molecular Indexing (UMI) confirmed more unique RNA transcripts profiled as capture enrichment became more specific. Furthermore, the targeted brain panel revealed splice variant events that were not identified with WTS or WES. DEx analysis identified several novel potential biomarkers between PD and healthy controls in all three platforms. Remarkably, extracellular vesicles from PD patients showed significant dysregulation in gene pathways related to locomotory and walking behavior compared to the healthy controls using the targeted brain panel only.
Summary/Conclusion: High TPM expression and UMI count shown in the brain panel contributed to the retention of rare isoforms not seen in the broader panels. Our most targeted platform contributed to high sensitivity and unique differential expression patterns not seen with the other sequencing libraries. This finding will be evaluated in a larger clinical cohort.
Keywords: parkinson's disease, NGS, differential expression, target enrichment

OWP2.02=PS02.03 | Blood-based neuronal and astrocyte extracellular vesicles biomarkers identify treatment responders for Alzheimer's disease in Down Syndrome

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Introduction: Individuals with Down Syndrome (DS) show AD neuropathology and cognitive impairment at an accelerated rate as they age. There are limited resources for treating AD in DS patients (DSAD). Using biobanked plasma samples from a previously completed AD/DS clinical trial of anti-inflammatory treatment, we aimed to characterize AD neuropathology in blood-based small extracellular vesicles (EVs) over time and as a function of treatment to develop a precision medicine approach for use in DS trials. We hypothesized that changes in amyloid β (Aβ) peptides, total tau (taut), Nf-L, and GFAP in neuronal and astrocyte EVs (NDEVs/ADEVs) might reflect treatment response in DSAD individuals.
Methods: We used archived plasma samples (n = 138; with 106 non-demented and 32 demented subjects) from the previously completed Phase 3 clinical trial, Vitamin E in Aged Persons with Down Syndrome (NCT00056329). Small EVs were isolated from baseline and 36-month plasma of DSAD individuals treated with Vitamin E or placebo. NDEVs and ADEVs were enriched with magnetic immunocapture using neuronal and astrocyte-specific proteins CD171 and GLAST, respectively and fluorescence-activated cell sorting. NDEVs and ADEVs were characterized for size, integrity and homogeneity as per the guidelines of the International Society of Extracellular Vesicles using nanoFCM, electron microscopy, immunoblots, and ELISA. AD marker proteins Aβ40–42, taut, Nf-L and GFAP were quantified by SIMOA assays in native plasma, NDEVs and ADEVs of DSAD individuals in treatment vs. control group.

Results: Blood-based NDEVs and ADEVs demonstrated the expected size, shape, and distribution of small EVs i.e. exosomes. Immunoblots and ELISA showed the presence of small EVs marker proteins. Various AD biomarkers were significantly altered in NDEs and ADEs of the vitamin E-treated DSAD group compared to the control.
Summary/Conclusion: Changes in AD protein markers can serve as a biomarker of treatment response in DSAD individuals.
Funding: INCLUDE-NIH R01AG073979.

OWP2.03=PS14.01 | Extracellular vesicles from iPSC-derived hindbrain organoids in Alzheimer's disease

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Introduction: Alzheimer's disease (AD) is a neurodegenerative disorder and the most common cause of dementia. Gradual deterioration of brain function eventually leads to death. Almost all AD patients eventually suffer from neuropsychiatric symptoms (NPS; e.g., agitation, depression) whose emergence correlates with dysfunctional serotonergic systems. Our aim is to generate hindbrain spheroids containing serotonergic neurons using induced Pluripotent Stem Cells (iPSCs) from healthy volunteers or AD patients with and without NPS. The spheroids can be used to study AD, NPS, and to evaluate patient individual differences in disease progression and response to pharmacologic therapies.
Methods: iPSCs were differentiated into hindbrain spheroids. The presence of serotonergic neurons was confirmed by quantitative RT-PCR, flow cytometry, and detection of serotonin in the extracellular environment. Extracellular vesicles (EVs) were isolated from cell culture supernatants by differential centrifugation followed by size exclusion chromatography. EV presence was confirmed by nanoflow cytometry measuring particle yield and size distribution. EVs were further characterized by single particle interferometric reflectance imaging to detect markers such as CD9 and CD81, as well as a neural cell adhesion molecule (NCAM). EV morphology was validated by transmission electron cryomicroscopy.
Results: Hindbrain spheroids containing serotonergic neurons were successfully generated from iPSCs of healthy volunteers (n = 3) and AD patients (n = 3). EVs were consistently isolated, purified and characterized from all samples.
Summary/Conclusion: It is possible to generate hindbrain serotonin neuron spheroids, and associated EVs, from iPSCs of individuals, laying foundation for a precision medicine approach to study AD, NPS, and to predict response to specific pharmacologic therapies.

OWP2.04=PS02.02 | Selenoprotein P regulates extracellular vesicle secretion from neurodegenerative microglia in an animal model of Alzheimer's disease

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Introduction: Microglia are primary innate immune cells in the central nervous system and display a neurodegenerative phenotype (MGnD) in response to amyloid plaque deposition in human and mouse models of Alzheimer's disease (AD). We previously reported that MGnD hyper-secrete extracellular vesicles (EVs) compared to homeostatic microglia in APPN-L-GF knock-in mouse model of AD. We recently identified selenoprotein P (Sepp1), a secreted heparin-binding glycoprotein, as a potential regulator of EV and IL-1B secretion from microglia under a pro-inflammatory stimulus. We hypothesize that silencing of Sepp1 suppresses EV secretion in plaque associated MGnD microglia in vivo.
Methods: In this study, we employed the small interfering RNA (siRNA) to determine the effects of silencing Sepp1 on EV secretion. We monitored EV secretion from BV-2 cells, a murine microglial cell line, constitutively expressing tdTomato-CD63 EV reporter molecule with or without downregulation of Sepp1 by Nanoimager, which can detect the tdTomato signal at a single molecule level. To track EVs secreted by microglia in vivo, a microglia-specific lentivirus expressing mEmerald-CD9 (mEm-CD9) reporter molecule was co-injected into the hippocampus of aged APPN-L-GF mice with lentivirus expressing Sepp1 or scramble shRNA and mCherry. Mice were euthanized at 2 weeks post injection and immunostained for galectin-3 (Mac2, MGnD marker), RFP (mCherry), GFP (mEm-CD9) and fluorostyrylbenzene (FSB, amyloid plaque). The images of mEm-CD9+ voxels (EV particles) in the proximity of Mac2+/RFP+/GFP+ microglia were captured by Lightning super-resolution confocal microscopy and the number of EV particles were quantified after 3D surface rendering of EV particles using IMARIS software.

Results: Under pro-inflammatory stimulation of BV-2 cells with lipopolysaccharide followed by ATP for rapid EV secretion, we observed a reduction in the loading of tdTomato-CD63+ molecules to EVs secreted in three independent Sepp1-shRNA clones. At seven months of age, we mostly detected mEm-CD9+ microglia as Mac2+ MGnD surrounding amyloid plaques in APPN-L-GF mice. Lentiviral shRNA-induced Sepp1 silencing reduces the EV secretion (mEm-CD9+ voxels) from mEm-CD9+/Mac2+/RFP+ microglia compared to the scramble shRNA-transduced mEm-CD9+/Mac2+/RFP+ microglia in the APPN-L-GF mouse brain.
Summary/Conclusion: These data demonstrate that in vivo silencing of Sepp1 suppresses EV secretion from MGnD microglia. Since activated microglia secrete neurotoxic and pathogenic molecules, our data suggest Sepp1 as a potential target for ameliorating microglia-mediated disease progression in neurodegenerative conditions including AD.
Keywords: microglia, alzheimer's disease, selenoprotein P

OWP2.05=PS02.01 | nSMase2 inhibition reduces tau propagation in Alzheimer's Disease mouse models

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Introduction: Mounting evidence correlates the propagation of hyperphosphorylated tau (pTau) along synaptically connected networks in the brain with progressive cognitive decline in Alzheimer's Disease (AD). Recent findings have highlighted extracellular vesicle (EV)s in enabling transcellular transmission of pathological tau and identified the partial inhibition of EV biogenesis via small-molecule inhibitors of nSMase2 as a potential therapeutic avenue. However, there are no suitable compounds for clinical development so far.
Methods: Through high-throughput screening and subsequent chemistry, our lab identified PDDC, a highly selective and potent nSMase2 inhibitor with excellent brain penetration and oral bioavailability. To characterize the potential therapeutic effect of PDDC in vivo, we administered PDDC-containing chow to both PS19 transgenic mice and to wild-type mice stereotaxically injected with an AAV vector encoding for P301L mutant human tau into their hippocampus (AAV-hTau seeded model). After chronic dosing, we quantified tau levels in the hippocampus of PS19 mice and the contralateral dentate gyrus (DG) of the AAV-hTau mice. Neuronally-derived EVs (NEV)s from plasma were isolated via immunocapture against L1CAM/CD171. Intact NEVs were used to determine particle concentration and diameter using nanoparticle tracking analysis (NTA). Total plasma EVs isolated via Size Exclusion Chromatography were subjected to flow cytometry analysis (FCA) with labeling for p262Tau and b-III-tubulin.
Results: PS19 mice exhibited robust elevation of multiple ceramide species and enhanced brain nSMase2 enzymatic activity, both of which were normalized by PDDC treatment. PS19 mice treated with PDDC had significantly reduced total tau and pTau, reduced gliosis, protected synapses, and increased neuronal counts. Plasma NEVs of treated mice were fewer in number, greater in size, and had lower p181-Tau levels than the untreated group; FCA confirmed the decrease of NEVs carrying p262-Tau at the single EV level. Similarly, the AAV-hTau-seeded mice treated with PDDC had reduced tau staining intensity in the contralateral DG.
Summary/Conclusion: Data in two AD models using PDDC provides strong preclinical support for using nSMase2 inhibition as a therapeutic strategy to slow tau propagation in AD.
Funding: NIH R01 AG063831, R01 AG059799, P30 MH075673, R25GM109441 (Hopkins PREP), Tau Consortium (T-PEP-18-579974C), the Maryland Innovation Initiative award (135726), and the Intramural Research Program of the National Institute on Aging, NIH.
Keywords: alzheimer's disease, eV, nSMase2, tau

OWP2.06=PS08.01 | iPSC-derived sEVs rejuvenate senescent blood-brain barrier to protect against ischemic stroke in aged mice

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Introduction: Blood–brain barrier (BBB) breakdown after ischemic stroke exacerbates brain injury and BBB senescence can cause severe neurological deficits in the aged ischemic stroke population. Recent evidence reveals that inducible pluripotent stem cell-derived small extracellular vesicles (iPSC-sEVs) possess the phenomenal antisenescence capability. However, whether iPSC-sEVs can rejuvenate BBB senescence to improve stroke outcomes in aged mice remains unknown.
Methods: Aged mice were treated with iPSC-sEVs for 2 months, and transient middle cerebral artery occlusion (MCAO) was conducted. BBB senescence, BBB leakage, infarct volume, immune cell infiltration, neuroinflammation, neural death, and sensorimotor functions were detected. Next, D-gal was utilized to induce BBB senescence, and oxygen and glucose deprivation (OGD) was performed. BBB senescence and BBB leakage were further evaluated in vitro. Mechanistically, proteomics analysis of iPSC-sEVs was performed to explore the bioactive factors. eNOS inhibitor, AKT1 and CALM inhibitors were used to verify the mechanism.
Results: In aged mice long-term treatment with iPSC-sEVs alleviated aging-induced BBB senescence. In aged stroke mice, iPSC-sEVs significantly mitigated BBB integrity damage, reduced the following infiltration of peripheral leukocytes, and decreased the release of pro-inflammatory factors from the leukocytes, which ultimately inhibited neuronal death and improved neurofunctional recovery. Mechanism studies showed that iPSC-sEVs could activate the endothelial nitric oxide synthase (eNOS) and up-regulate sirtuin 1 (Sirt1) in senescent endothelial cells. Blocking the activation of eNOS abolished iPSC-sEV- mediated rejuvenation of BBB senescence and the protection of BBB integrity. Proteomics results demonstrated that iPSC- sEVs were enriched with bioactive factors including AKT serine/threonine kinase 1 (AKT1) and calmodulin (CALM) to activate the eNOS–Sirt1 axis. Further investigation showed that AKT1 and CALM inhibitors blocked iPSC-sEV-afforded activation of the eNOS–Sirt1 axis in senescent endothelial cells.
Summary/Conclusion: iPSC-sEVs can protect against ischemic stroke in aged mice by rejuvenating BBB senescence, partially, through delivering AKT1 and CALM to activate the eNOS–Sirt1 axis.
Funding: This study was funded by the National Natural Science Foundation of China under Grant Nos. 82071371, 82201543, and 81871833, National Postdoctoral Program for Innovative Talent under Grant No. BX20220356, China Postdoctoral Science Foundation under Grant No. 2022M723562, Natural Science Foundation of Hunan Province under Grant No. 2022JJ40828, Natural Science Foundation of Changsha city under Grant No. kq2202377, Young Foundation of Xiangya Hospital under Grant No. 2021Q01.
Keywords: induced pluripotent stem cell-derived small extracellular vesicles (iPSC-sEVs), blood–brain barrier (BBB), cell senescence, ischemic stroke, aging

OWP2.07=PS04.02 | Cell-secreted extracellular matrix nanoparticles restore endothelial barrier function

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Introduction: Nanoscale mediators secreted from mesenchymal stromal cells (MSCs) were shown to be efficacious in animal models of tissue injury that disrupts vascular function. However, recent studies highlight the heterogeneity of nanoscale mediators, including extracellular vesicles (EVs) and non-vesicular extracellular particles (NVEPs). Restoration of endothelial barrier function requires the activation of extracellular matrix (ECM) signaling. Thus, we tested the hypothesis that an ECM-containing nanoscale fraction is essential for the restoration of vascular permeability upon tissue injury.
Methods: The crude fraction was isolated by following EV-TRACK ID EV150007 from mouse MSCs. We used immunoaffinity-based approaches along with Triton-X sensitivity assay and nanoparticle tracking analysis to quantify the subpopulations that contain fibronectin (FN). FN+ fractions were pulled down via FN antibody-functionalized magnetic nanoparticles (~8 nm) for characterization by transmission electron microscopy (TEM). The fractionated nanoscale subpopulations were delivered to mice 4 h after treatment with lipopolysaccharide (LPS), and the mice were analyzed for edema and vascular permeability in the lungs.
Results: Our results show that MSC-secreted nanoscale mediators consist of Triton-X sensitive CD63+FN- (40%) and Triton-X resistant CD63-FN+ (33%) subpopulations, while the CD63+FN+ population is less than 5%. TEM analysis confirms that the FN+ fraction is non-vesicular with a single particle size of ~30 nm and is more irregular in shape than EVs. We show that FN+ NVEPs are essential for the restoration of endothelial barrier function after LPS-induced lung injury.
Summary/Conclusion: This study reveals the importance of FN+ NVEPs as a novel nanoscale mediator to restore vascular integrity in response to tissue injury. Future studies will investigate the biogenesis mechanisms of ECM-presenting NVEPs and mechanisms of action by which FN+ NVEPs restore endothelial barrier function.

Funding: This work is supported by NIH Grant No. R01-HL141255 (to J.-W.S.).
Keywords: non-vesicular extracellular particles, extracellular matrix, fibronectin

OWP2.08=PS04.01 | Cardiomyocytes-derived EVs for the treatment of cardiac fibrosis in hypertension and COVID-19-derived cardiac damage

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Introduction: Cardiac fibrosis, found in most cardiovascular diseases, including COVID-19 patients, is the result of an erroneous hyperactivation of fibroblasts. This dysfunction evokes excessive fibroblast cell proliferation and extracellular matrix (ECM) accumulation, decreasing cardiac function and eventually causing death. It is known that cardiomyocytes (CM) communicate and affect the surrounding cells through extracellular vesicles (EVs). Therefore, CM-derived EVs may be a promising cell-free system for fibrosis treatment.
Methods: A defined conjunct of chemicals was used to improve human CM culture and ensure a high collection of EVs. Terminal differentiation, as well as senescence markers emergence, were delayed in comparison to the predetermined culture medium without apparent malignant alteration. EVs were isolated by ultracentrifugation, and their characteristics (morphology, particle number, membrane markers, and internalization) were analyzed in accordance with MISEV2018 guidelines. Finally, their effect on fibrosis was tested.
Results: EV secretion and their characteristics were unaffected in chemically-treated CM. Interestingly, CM-derived EVs were specifically internalized by cardiac fibroblasts compared to other corporal fibroblasts, while no apparent differences were observed in mesenchymal stem cell-derived EVs. Treatment of EVs on TGFβ-activated cardiac fibroblasts showed a decrease of fibroblast activation markers at mRNA and protein levels. Furthermore, ECM secretion was also reduced. Consequently, intracardiac injection of EVs reduced the fibrotic area and induced angiogenesis, which translated to an improved cardiac function in a hypertension mouse model. Because the EV content was comprised of not only anti-fibrotic but also anti-inflammatory microRNAs, their use in COVID-19-like infection is also being studied. To date, the anti-inflammatory effect of EVs on macrophages activated by SARS-CoV2 Spike protein was corroborated by cytokine secretion. Correspondent animal experiments are being processed to elucidate the anti-inflammatory and further anti-fibrotic protection effect in SARS-CoV2 infection-like mouse model.
Summary/Conclusion: Our findings indicate that, due to the anti-fibrotic effects and the specificity of the EV cargo, the use of EVs derived from CM is a promising treatment for several types of cardiac fibrosis. In addition, their study may help to understand the biological meaning of CM-derived EVs in the cardiac microenvironment.
Funding: AMED.
Keywords: EVs, cardiomyocytes-derived EVs, cardiac fibrosis, EV therapy, specific internalization.

OWP2.09=PS04.03 | Extracellular vesicles from ischemic heart disease (IHD) patients demonstrate enhanced procoagulatory activity

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Introduction: Extracellular vesicles (EVs) carry unique repertoires of biologically active cargo that hold promising novel biomarkers for cardiovascular diseases (CVDs). However, it is unclear how the number, location, cellular origin, and size of these EVs within the circulation can influence CVDs such as ischemic heart disease (IHD). The current study compares these novel markers in arterial and venous blood in subjects undergoing routine coronary angiography analysis for IHD. EVs were then characterized from those presenting with and without IHD.
Methods: Arterial and venous blood from IHD and control patients was collected from the Cardiac Catheterization Lab at the Royal Berkshire Hospital after obtaining informed consent for the project. This research was approved by the University of Reading Research Ethics Committee and the Human Research Authority (REC 20/NW/0263). Blood was centrifuged at 1500 x g for 15 minutes at room temperature (RT) to generate platelet-poor plasma followed by 13,000 x g for 2 minutes at RT to isolate

platelet-free plasma (PFP). EV fractions were isolated from 500μL of PFP by size exclusion chromatography. EVs were analyzed by Nanoparticle Tracking Analysis, flow cytometry to characterize number, size and cellular origin and a thrombin generation assay was used to assess procoagulatory activity of the isolated circulating EVs.
Results: Coagulatory activity of EVs isolated from IHD patients was significantly higher in IHD patients compared to controls. There were higher numbers of endothelial-derived EVs in arterial blood compared with venous blood. Linear regression models revealed that plasma triacylglycerol concentration and age independently predicted circulating EV numbers in IHD patients, although numbers of EVs were not significantly different in the two groups.
Summary/Conclusion: Although numbers of EVs in IHD patients were not elevated, EVs in IHD patients had greater procoagulant activity, highlighting a potential important role for EVs in IHD.
Funding: This project was funded by the Joint Academic Board between the Royal Berkshire NHS Foundation Trust and the University of Reading.

OWP2.10=PS03.02 | Reduction in dengue virus serotype 2 (DENV-2) replication induced by small extracellular vesicles (sEVs) produced by EA.hy 926 endothelial cells

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Introduction: DENV infection can cause endothelial cell (EC) hyperpermeability and vascular leakage. Small EV of infected EC (sEVIC) may participate in the viral spread and regulate EC response to DENV. Still, there is no information on sEVIC cargo and its effect on non-infected EC. This work aimed to characterize the sEVIC and evaluate their impact on polarized EC.
Methods: EA.hy 926 were infected with DENV-2, MOI 1. The virus was removed, and serum-free medium was added. Cell viability (Resazurin, LDH) and viral infection (IFI and PCR for DENV E protein) were confirmed. After 48h post-infection, sEV were isolated (ultracentrifugation) and characterized by NTA, DLS, Western blot, LC/MS/MS, and small RNA sequencing. For function assay, sEVIC were pretreated with neutralizing antibody 4G2 or UV exposed after pretreatment. Later, polarized EC were exposed to the different sEV for 24h and then infected. TEER and permeability (Dextran-blue 2KDa) were measured at different times, and IFI for ICAM, E-sele, and actin were made along with qPCR for DENV detection. sEV non-infected cells (sEVNIC) were used as control.
Results: EC infection induced a high concentration of EV (2×109) with low ALIX expression. Compared to EVNIC, EVIC had 129 increased proteins (mainly of immune response -IR-), 206 were downregulated (cellular adhesion (CA) and developmental processes), and a viral NS5 peptide was found along with sncRNA like YRNA and miRs that mainly regulate CA and IR genes. Interestingly, miRs found exclusively in EVIC may have a proviral effect. Functionally, EVIC induced ICAM and E-sele expression and a protective effect in the polarized EC that maintained a stable TEER (45Ω) and reduced viral replication.
Summary/Conclusion: EVIC transported DENV elements and sncRNA that may regulate viral response. Our model suggests that EVIC induced a protection response during infection, reducing viral replication and activating the EC in the first 24h.
Funding: MinCiencias – U Bosque: Grant 130884467149, Contract 431–2020.
Keywords: DENV, sEVs, endothelial cells, viral replication

OWP2.11=PS09.01 | Placental extracellular vesicle-vasorin in preeclampsia associated cardiac dysfunction

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Introduction: Women with preeclampsia (PE) are at increased risk of cardiovascular disease (CVD) during pregnancy and postpartum. The mechanisms of heightened risk for CVD are poorly understood. Consequently, approaches for prevention and treatment are unknown. Preliminary data indicates that the protein vasorin (VASN) was highly down regulated in extracellular

vesicles (EV) isolated from patients with severe PE (sPE-EV), as compared to normotensive-EV (NTP-EV). VASN has been implicated in regulating calcium homeostasis and calcium dynamics in cardiomyocytes. Therefore, we hypothesized that the down regulation of VASN protein in EV in sPE may play a role in PE-associated cardiac dysfunction via altered regulation of intracellular calcium dynamics.

Methods: EV from pregnant women with sPE (n = 15) and NTP (n = 15) were separated from plasma using a precipitation-based kit. We used an unbiased proteomic approach to compare the EV protein cargo profile from women with sPE and NTP using a tandem mass spectrometry approach. In adult murine cardiomyocytes (mCM), calcium dynamics were assessed using fura-2AM imaging in an Ion Optics instrument and VASN and calcium sensor proteins were quantified by western blot.

Results: We verified VASN down regulation in maternal plasma EV, and in placental tissue in sPE as compared to NTP. We also found that the levels of stromal interaction molecule 1 (STIM 1) were decreased in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) and in mCM treated with sPE-EV. Treatment of contractile mCM with sPE-EV that have decreased VASN content inhibited levels of STIM 1 while mCM isolated from pregnant mice injected with AD-sFLT-1 (a model of PE) exhibited altered dynamics of calcium signaling as compared to mCM from untreated pregnant mice (UT). Likewise, treatment of hiPSC-CM results in the decrease of STIM1, phospholamban (PLN) and sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) levels. We also found that in CM isolated from AD-hsFLT-1 injected mice had elevated peak Ca2+ ratio when compared to mCM from UT.

Summary/Conclusion: Our data indicate that levels of VASN, a TGF-beta signaling inhibitor, in EV correlate with the effects of EV on expression levels of proteins with roles in Ca2+ signaling and alterations of Ca2+ dynamics in CM. We speculate that that loss of VASN in sPE-EV contribute to the mechanisms of pathological cardiac remodeling, by decreased inhibition of deleterious TGF-beta signaling.

Funding: This study was supported by seed funds from the Department of Anesthesiology and Perioperative Medicine (D.E.B.) and a REINVENT grant (S.M. and T.J).

Keywords: preeclampsia, cardiac dysfunction, extracellular vesicles, vasorin, calcium signalling

OWP2.12=PS09.02 | Detection of uterine lumen-derived extracellular vesicles in bovine plasma in vivo

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Introduction: Embryo-maternal communication is essential for pregnancy establishment. Extracellular vesicles (EVs) are significant molecules in intercellular communication required during early pregnancy. The EVs can directly or indirectly trigger an endocrine response. However, at this stage of pregnancy, the uterine epithelium has a stable adhesion that could alter the exchange of molecules. Accordingly, the present study aims to investigate whether stained EVs leave the uterine environment.

Methods: The experiment used six Nellore heifers. The heifers received an intrauterine infusion of either PBS+PKH26 (control group) or follicular fluid (FF)-derived EVs, isolated by ultracentrifugation twice at 120000xg and stained with PKH26 (treatment group). To isolate EVs, plasma samples were taken from the jugular vein at pre-determined periods (10 min, 30 min, 1 h, and 3h). EVs were isolated by size exclusion chromatography (Izon qEV35) and evaluated by nanoparticle tracking analysis for particle size and concentration and flow cytometry for positive events for PKH26. Controls were performed to discriminate other nanoparticles in flow cytometry, and samples were labeled with CD9-FITC and CD63-FITC. Data were compared by ANOVA followed by Tukey.

Results: Evs from the treatment group had higher concentration and particle size than those from the control group ($P < 0.05$). Considering only the treatment group, size and concentration increased over time ($P < 0.05$). The PKH26-positive events differed by time and group, although the number of events was relatively small ($P < 0.05$). The detection of EVs in the systemic circulation was relatively rapid, peaking at 30 min, and after a longer period, the detection decreased, suggesting that the effect and migration of EVs are timely. The detection of PKH26 in the control animals could also represent the autofluorescence of particles, one of the limitations of the present experiment; however, the values in the treatment group were always numerically higher when subtracted from the negative samples.

Summary/Conclusion: Although this experiment has some pitfalls, our data suggest that stained EVs leave the uterine environment based on their detection in the bloodstream and elicit an endocrine response in as short as 30 minutes.

Funding: FAPESP #2022/01235-0; #2021/06645-0; #22/01505-8; #2015/21829-9.

Keywords: reproduction, pregnancy, extracellular vesicles

The below poster chairs are confirmed for ISEV2023 as of 8 May 2023

Session ID	Day	Date	Session Name	Chair
PT01	Thursday	5/18/2023	EV Separation/Technology 1	Natalie Turner
PT01	Thursday	5/18/2023	EV Separation/Technology 1	An Hendrix
PT02	Thursday	5/18/2023	Milk EVs	Brett Vahkal and Stefano Tacconi
PT03	Thursday	5/18/2023	EVs and Viral Infection	Zach Troyer
PT03	Thursday	5/18/2023	EVs and Viral Infection	Shilpa Buch
PT04	Thursday	5/18/2023	Kidney and Urinary EVs 1	Christina Grange
PT04	Thursday	5/18/2023	Kidney and Urinary EVs 1	Luca Musante
PT05	Thursday	5/18/2023	Muscle Skeletal System	Hua Shen
PT05	Thursday	5/18/2023	Muscle Skeletal System	Wei Seong Toh
PT06	Thursday	5/18/2023	Cardiovascular EVs	Bo Li
PT06	Thursday	5/18/2023	Cardiovascular EVs	Claire Crew
PT07	Thursday	5/18/2023	EV-Cargo Characterization I	David Greening
PT07	Thursday	5/18/2023	EV-Cargo Characterization I	Gololobova Olesia
PT08	Thursday	5/18/2023	Immunity, Autoimmunity, and Inflammation 1	Marie-Helene Normand
PT08	Thursday	5/18/2023	Immunity, Autoimmunity, and Inflammation 1	Sheela Abraham
PT09	Thursday	5/18/2023	EVs from Microorganism 1	Meta Kuehn
PT09	Thursday	5/18/2023	EVs from Microorganism 1	Irma Schabussova
PT10	Thursday	5/18/2023	EV Separation from Biological Sources	Carlos Salomon
PT11	Thursday	5/18/2023	Resistance to Therapy	Laura Patras
PT11	Thursday	5/18/2023	Resistance to Therapy	Joni White
PT12	Thursday	5/18/2023	Techniques and Method Single EV Analysis	Edwin van der Pol
PT12	Thursday	5/18/2023	Techniques and Method Single EV Analysis	Eduardo Reátegui
PT13	Thursday	5/18/2023	Cancer Biomarkers 1	Serena Lucotti
PT13	Thursday	5/18/2023	Cancer Biomarkers 1	Fabrice Lucien
PT14	Thursday	5/18/2023	Biomarkers from DNA to RNA	Nicole Noren Hooten
PT14	Thursday	5/18/2023	Biomarkers from DNA to RNA	Maija Puhka
PT15	Thursday	5/18/2023	EV Sizing and Counting	Tanina Arab
PT15	Thursday	5/18/2023	EV Sizing and Counting	John Nolan
PT16	Thursday	5/18/2023	Immunity, Autoimmunity & Inflammation #1	Loren Erickson
PT16	Thursday	5/18/2023	Immunity, Autoimmunity & Inflammation #1	Paola Decandia
PT17	Thursday	5/18/2023	Cancer Microenvironment	Laura Hueser
PT17	Thursday	5/18/2023	Cancer Microenvironment	Muller Fabbri
PF01	Friday	5/19/2023	Model Systems, Production and Characterization	Migara Jayasinghe
PF01	Friday	5/19/2023	Model Systems, Production and Characterization	Yong Song Gho
PF02	Friday	5/19/2023	Metabolism and Metabolic Disease	Jamelle Brown
PF03	Friday	5/19/2023	Cancer EV Pathogenesis	Sameh Almousa
PF03	Friday	5/19/2023	Cancer EV Pathogenesis	Janusz Rak
PF04	Friday	5/19/2023	Therapy Adipose and MSC EVs 1	Vera Tscherrig
PF04	Friday	5/19/2023	Therapy Adipose and MSC EVs 1	Yu Fujita
PF05	Friday	5/19/2023	Kidney and Binary EVs 2	Wouter Woud
PF05	Friday	5/19/2023	Kidney and Binary EVs 2	Laura Perin
PF06	Friday	5/19/2023	Outreach and Knowledge Synthesis	Marta Monguió Tortajada
PF06	Friday	5/19/2023	Outreach and Knowledge Synthesis	Roger Alexander
PF07	Friday	5/19/2023	Cancer Biomarkers 2	Nikki Salmond
PF07	Friday	5/19/2023	Cancer Biomarkers 2	Caterina Nardella

(Continues)

Session ID	Day	Date	Session Name	Chair
PF08	Friday	5/19/2023	Eye and Liver EVs	Cristina Zivko
PF08	Friday	5/19/2023	Eye and Liver EVs	Sun Young Lee
PF09	Friday	5/19/2023	EV Biogenesis and Bioengineering	Shannon Stott
PF09	Friday	5/19/2023	EV Biogenesis and Bioengineering	Houjian Cai
PF10	Friday	5/19/2023	EV Separation from Blood	Britta Bettin
PF10	Friday	5/19/2023	EV Separation from Blood	Marija Holcar
PF11	Friday	5/19/2023	Neurodegenerative Disease and EVs	Setty Magana
PF11	Friday	5/19/2023	Neurodegenerative Disease and EVs	Claudia Verderio
PF12	Friday	5/19/2023	EV Labels and Imaging	Allaura Cone
PF12	Friday	5/19/2023	EV Labels and Imaging	Charles Lai
PF13	Friday	5/19/2023	EV Heterogeneity	Michael Harding
PF13	Friday	5/19/2023	EV Heterogeneity	André Görgens
PF14	Friday	5/19/2023	Preeclampsia, Reproduction and Development	Paschalia Pantazi
PF14	Friday	5/19/2023	Preeclampsia, Reproduction and Development	Carlos Salomon
PF15	Friday	5/19/2023	EV Separation Technology 2	Rossella C.
PF15	Friday	5/19/2023	EV Separation Technology 2	Heather Pua
PF16	Friday	5/19/2023	Cancer Microenvironment 2	Sara Veiga
PF17	Friday	5/19/2023	EV MiRNA	Edgar Gonzalez-Kozlova
PF17	Friday	5/19/2023	EV MiRNA	Muller Fabbri
PF18	Friday	5/19/2023	Plant EVs	Lida Halilovic
PF19	Friday	5/19/2023	EVs and the Central Nervous System	Huaqi (Kate) Su
PF19	Friday	5/19/2023	EVs and the Central Nervous System	Gagan Deep
PS01	Saturday	5/20/2023	Therapy Adipose and MSC-Derived EVs	Sujata Mohanty
PS01	Saturday	5/20/2023	Therapy Adipose and MSC-Derived EVs	Yves DeClerck
PS02	Saturday	5/20/2023	Neurodegenerative Diseases and EVs 2	Yiyao Huang
PS02	Saturday	5/20/2023	Neurodegenerative Diseases and EVs 2	Ursula Sandau
PS03	Saturday	5/20/2023	EVs and Viral Infection	Janis Muller
PS03	Saturday	5/20/2023	EVs and Viral Infection	Ken Witwer
PS04	Saturday	5/20/2023	Cardiovascular EVs 2	Robert Myette
PS04	Saturday	5/20/2023	Cardiovascular EVs 2	Dylan Burger
PS05	Saturday	5/20/2023	EV Cargo Characterization II	Martin van Rooijen
PS06	Saturday	5/20/2023	Musculoskelatal System	Daniele D'Arrigo
PS07	Saturday	5/20/2023	EV Heterogeneity	Sarah Catherine Baker
PS07	Saturday	5/20/2023	EV Heterogeneity	Dolores DiVizio
PS08	Saturday	5/20/2023	EVs in Neurological Disorders and Injury	Maheedhar Kodali
PS08	Saturday	5/20/2023	EVs in Neurological Disorders and Injury	Tsuneya Ikezu
PS09	Saturday	5/20/2023	Preeclampsia, Reproduction and Development	Metka Lenassi
PS09	Saturday	5/20/2023	Preeclampsia, Reproduction and Development	Rienk Nieuwland
PS10	Saturday	5/20/2023	Lung EVs	Brian Dobosh
PS10	Saturday	5/20/2023	Lung EVs	Augusto Zani
PS11	Saturday	5/20/2023	EVs from Microorganisms 2	Bin Gong
PS11	Saturday	5/20/2023	EVs from Microorganisms 2	Simon Swift
PS12	Saturday	5/20/2023	EVs in Cancer Progression	Alissa Weaver
PS13	Saturday	5/20/2023	EV Production for Therapeutics	Anthony Yan-Tang Wu
PS13	Saturday	5/20/2023	EV Production for Therapeutics	Sander Kooijmans
PS14	Saturday	5/20/2023	EV Therapy	Tom Driedonks
PS14	Saturday	5/20/2023	EV Therapy	Minh Le
PS14	Saturday	5/20/2023	EV Therapy	Sai Kiang Lim

Poster Presentations

PT01: EV Separation/Technology 1

Location: Hall 4A

16:45 - 18:45

PT01.01 | ExoCAS-2: Rapid and Pure Isolation of Exosomes by Anionic Exchange Using Magnetic Beads

MinJu Bae[1]; HyeonAh Seong[2]; JunSoo Park[2]; SeHyun Shin[3]
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Introduction: Extracellular vesicles (EVs) are considered essential biomarkers in liquid biopsies. Despite intensive efforts aimed at employing EVs in a clinical setting, workable approaches are currently limited owing to the fact that EV-isolation technologies are still in a nascent stage. This study introduces a magnetic bead-based ion exchange platform for isolating EVs called ExoCAS-2 (exosome clustering and scattering). Owing to their negative charge, exosomes can easily adhere to magnetic beads coated with a polycationic polymer. Owing to the features of magnetic beads, exosomes can be easily processed via washing and elution steps and isolated with high purity and yield within 40 min.
Methods: Cationic salt is coated on the surface of magnetic beads with carboxyl group surface residues. Inject the cation coated magnetic bead (ExoCAS) into the plasma solution and incubate in 4°C for 30 minutes. By charge interaction, negative charged EVs are attached to the cationic beads. And then, pH 6 washing buffer and 1M NaCl elution buffer were used for EV isolation.
Results: The present results confirmed the isolation of exosomes through analyses of size distribution, morphology, surface and internal protein markers, and exosomal RNA. Compared with the commercially available methods, the proposed method showed superior performance in terms of key aspects, including operation time, purity, and recovery rate.
Summary/Conclusion: This highlights the potential of this magnetic bead-based ion exchange platform for isolating exosomes present in blood plasma.
Funding: This research was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Korean Government, MSIP (2016R1A5A1010148) and was funded by the Ministry of Trade, Industry & Energy (MOTIE, Korea) under the Industrial Strategic Technology Development Program (20012427).
Keywords: exosome, isolation, cationic polymer, magnetic beads, ion exchange

PT01.02 | Reproducibility and efficiency of an extracellular vesicle capture technology for the detection of ovarian cancer

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Introduction: Despite advances in treatments over the last few decades, epidemiological studies indicate only a marginal impact on the course of ovarian cancer (OVCA) due to the lack of reliable early detection methods. Current Extracellular vesicles (EV) isolation methods are time consuming, and quality control can be challenging to achieve. Here, we evaluate the performance and reproducibility of an EV capture technology (EXO-NETÒ) to characterize a suite of EV biomarkers associated with ovarian cancer and the classification accuracy of multivariate models based on EXO-NET -isolated EV biomarkers.
Methods: Plasma samples were obtained from healthy women (controls, n = 20), benign (n = 20), and high-grade serous ovarian cancer (n = 40). EVs were isolated using EXO-NETÒ (INOVIQ LTD, Australia) and characterised by Nanoparticle Tracking Analysis (NTA), protein abundance (CD63, CD9, Alix, TSG101 and CD81), and morphology using NanoSight, Western blot, and

electron microscopy (EM). Optimisation experiments were performed to determine the optimal ratio of plasma/ EXO-NETÒ. The number of EV-CD9+ve were quantified using an ELISA kit. RT-PCR and targeted proteomic analysis were performed against 7 EV biomarkers (patent under review), and spike-in control miRNAs and proteins were used.
Results: EVs isolated using EXO-NETÒ were positive for CD63, CD9, Alix, TSG101, and CD81, confirming the presence of EV-associated proteins. NTA showed a 20% (equivalent 2.5 × 109) decrease in particles between 50 to 200 nm after EV isolation compared with total plasma. Similar results were observed with EM (i.e., plasma before and after incubation with EXO-NETÒ). Dose-response analysis based on CD9+ve EVs showed an optimal concentration using 200μl of plasma and 30μl of EXO-NETÒ. At optimal ratio, a total of 6.3 ± 2.5 mg protein equivalent to 4.3 ± 1.5 mg of peptides, and 7.3 ± 2.5 ng of small RNA was obtained. Treatment with Proteinase K and RNase A did not significantly ($p>0.05$) change the concentration of peptides and small RNA. The coefficient of variation based on the quantification of CD9+ve EVs was 8.2 ± 5.3 %. The classification accuracy rates (i.e., the number of correct predictions) of a select group of EV biomarkers used for the detection of ovarian cancer were 100%, 95%, 100%, 80%, and 100% using LogitBoost, J48, Random Tree, Decision Table, and Random Forest algorithms, respectively. Interestingly, leave-one-out cross validation showed minimal loss of performance.
Summary/Conclusion: We have optimised a simple, rapid, and scalable capture technology (EXO-NETÒ) to isolate EVs with biomarker potential in the context of ovarian cancer. This will allow for the translation of EV-based research into clinical applications and platforms that are available in pathology laboratories.
Funding: MRF1199984), NHMRC 1195451, and INOVIQ LTDA.

PT01.03 | Aqueous two-phase separation for effective extracellular vesicle isolation with optimal vesicular integrity: EV preps for nanomedicine and liquid biopsies

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Introduction: Development of extracellular vesicle (EV)-based liquid biopsies and EV-based nanomedicines requires isolation of ultra-pure EVs with an intact vesicular structure. Ultracentrifugation (UC) does not satisfy these requirements because of excessive damage to EVs, length of time required to pellet EVs, and difficulties in identifying or locating the pellet. Aqueous two-phase separation (ATPS) may be a more effective EV isolation approach.
Methods: Enrichment and recovery efficiency of multiple EV sources (human and mouse cell culture conditioned media, human plasma, etc.) before and after ATPS were measured using nanoscale flow cytometry (nFC). Canonical EV biomarkers were detected using nFC with pre-conjugated antibodies specific for CD9, CD63, and CD81. EV vesicular structure was analyzed using transmission electron microscopy (TEM). EVs were co-cultured with human recipient cells and internalization rate was measured with confocal microscopy. "omics" studies were performed to determine EV molecular cargo profile.
Results: ATPS has greater EV enrichment capability (21.4 × vs. 10.9 × times fold enrichment) and higher EV recovery efficiency (97.6% vs. 69.3% recovery) than UC. ATPS improves EV-antibody labelling efficiency as determined by flow cytometry analysis, possibly by reducing the protein corona on the surface of EVs. EVs isolated by ATPS are monodispersed and exhibit higher circularity than EVs isolated by UC. Maintenance of EV vesicular integrity via ATPS led to higher internalization rates by recipient cells. Transcriptomics and proteomics analyses revealed a strong overlap, thus validating ATPS for effective EV isolation.
Summary/Conclusion: Our study demonstrates ATPS as a more attractive EV isolation method that produces canonical EVs. ATPS isolated EVs show greater vesicular integrity, reduced protein corona, and relatively homogenous and monodispersed qualities. Lastly, the proteome and transcriptome were consistent between the two methods.

PT01.04 | Separation technology for integrating extracellular vesicle isolation, concentration, and buffer exchange in an equipment free format

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DNAiTECH Ltd, Blenheim, New Zealand

Introduction: We describe a separation technology (S-Tech) for integrating EV isolation, concentration and buffer exchange within a single device, the technology prepares samples for downstream processing and is designed for ease of use in point-of-care diagnostics.

Methods: The device design features include low binding hydrophilic membranes within an injection molded unit with the inlet and outlet portals on the same face, enabling setup on ice for preservation of labile samples. Sample processing is by vacuum, mechanically gentle and hands-free, not requiring fraction collectors or centrifuges. The S-Tech is a variable volume device (0.05-20ml) where the sample volume depends on the sample complexity and selected pore sizes. S-Tech comes in a range of pore sizes (specified in nanometers), S-5000, S-1000, S-450, S-100, S-50, and S-20. Larger pore sized devices enable isolation of cells and sequential fractioning of different sized particles. The S-20 uses membrane technology certified for virus clearance down to 20nm making this unit ideal for the isolation of the smallest extracellular vesicles. When processing serum with the S-20 the optimal sample volume is 150ul.
Results: Using TRPS and NTA we demonstrate very high yield of particles from serum using the S-20 device. The TRPS determined particle size distribution had a mode of 64nm and a mean of 81nm while the minimum resolved particle size was 51nm. The full-size distribution including particles below 50nm could not be resolved because of the resolution limit of the TRPS nanopores.
We used particle/protein ratio as a measure of nanoparticle purification from serum and compared S-20 to ultracentrifugation (100k pellet) and size exclusion chromatography (SEC). The S-20 extract measured 4.93E+09 particles/100ug protein compared to 6.75E+08 particles/100ug by ultracentrifugation and 9.4E+08 particles/100ug by SEC. In addition to EV isolation, nucleic acid extractions were performed on S-Tech system enabling lab-free sample preparation without spin columns and centrifuges.
Summary/Conclusion: This is a versatile sample processing and EV isolation strategy ideal for beyond the lab applications such as point-of-care diagnostics.
Funding: We acknowledge funding from Callaghan Innovation, Agmardt and the Ministry for Primary Industry.
Keywords: EV isolation Point-of-care diagnostics

PT01.05 | ExoMicro: Innovative one-step extraction of exosomal miRNA

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Introduction: Extracellular vesicles (EVs) are receiving great attention as biomarkers in liquid biopsies since they are involved in physiological and pathological processes. It is important to obtain a high-yield of nucleic acids because it is possible to prediagnose through the exosome.
Methods: Here, we report an efficient one-step method for extraction of miRNAs by clustering EVs. When cationic salt is added to samples, anionic EVs tend to form clusters and precipitate within a short time. After centrifugation and adding lysis buffer, miRNA can be extracted with a spin column. Commercial methods were compared to determine the yield of the precipitation method using cationic salt. In addition, we compared with two reference methods to determine the miRNA extraction efficiency. The first one is consisted of two-step method of extracting miRNA from isolated exosome (2 step), which is a general method, where as the second reference method is a commercial product consisting of 1-step extracting miRNA from biofluids.
Results: Cluster precipitation efficiency using cationic salt was identified through cluster image, SEM, and Western blot. The present method for extract exosomal miRNA has an EV isolation efficiency of more than 3 times compared to the commercial EV isolation methods. In addition, it has excellent exosomal miRNA extraction efficiency from various biofluids such as Saliva, Urine, and Plasma, and can be used as a sample of breast cancer patients.
Summary/Conclusion: These results might be contributed by the sequence of precipitation of exosomes, exosome lysis, and nuleic acid extraction. The rapid and efficient method for extracting exosomal nucleic acids will make an innovative contribution to clinical applications.
Funding: This research was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Korean Government, MSIP (2016R1A5A1010148).
Keywords: exosome, exosome isolation, exosomal miRNA, precipitation, Cationic salt

PT01.07 | Multiparametric analysis of small extracellular vesicles purified by a rapid and label-free lab on a chip device

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Introduction: Conventional purification methods of small extracellular vesicles (sEVs) suffer from significant shortcomings including low purity and yield, long processing times, and high costs. Our group has previously developed a label-free insulator-based dielectrophoretic (iDEP) device for rapid and selective entrapment of sEVs based on their unique dielectric properties and size (1). Here we report a comprehensive three-fold characterization of sEVs isolated using the iDEP device from human

biofluids by utilizing conventional flow cytometry (cFCM), advanced imaging flow cytometry (iFCM), and RNA extraction and quantification indicating good yield and purity.
Methods: Serum (n = 6), plasma (n = 2), and urine (n = 3) samples from genitourinary tract cancer cases were obtained from the University of Cincinnati Biorepository (IRB#2015-2364). The device was fabricated and sEVs were isolated from biofluids using the device as reported before (1). For cFCM and iFCM, sEVs were stained with CD63-FITC (clone: H5C6, 353005), CD81-APC (clone: 5A6, 349509), and Isotype-IgG1 FITC (MOPC21, 400109) purchased from BioLegend (San Diego, CA). cFCM was performed using a Cytek Aurora flow cytometer and analysis was performed by FlowJo software. iFCM was performed for extracted sEVs using ImageStreamX Mark II and data was analyzed using IDEAS software. Total RNA was extracted from purified EVs isolated from 1 mL of biofluid using the miRNeasy Micro kit. RNA concentration and integrity were measured by the Agilent 6000 Pico Kit using Bioanalyzer.
Results: Analysis for CD63 and CD81 markers using dot plots from cFCM indicated 30 to 55% sEVs isolated from all biofluids to be positive for CD63 and 22 to 34% sEVs to be positive for CD81. Analysis of the scatter profiles of samples from iFCM revealed high mean expressions ~1 × 10^7 of CD63+ EVs/mL and 2.05 × 10^6 to 1.32 × 10^8 of CD81+ EVs/mL. The percentage of sEVs positive for each surface marker were found to be comparable to positive expressions of sEVs harvested from hTERT-immortalized mesenchymal stem cells and A549 non-cell lung cancer (NSCLC) cell lines purchased from ATCC Inc. iFCM analysis of sEVs isolated using the iDEP device was also found comparable to those isolated using three commonly used techniques (2,3). Total RNA concentration obtained from sEVs was ~100 pg/μL for all samples with acceptable integrity numbers.
Summary/Conclusion: The capability of a label-free iDEP device in isolating sEVs from biofluids was demonstrated by performing multiparametric characterization using cFCM, iFCM, and RNA quantification. The iDEP device hence has potential to be further evolved as a simple yet powerful liquid biopsy platform for rapid isolation of sEVs based on their size and dielectric properties in clinical settings.
(1) Shi et al. Lab Chip 2019. (2) Mastoridis et al. Front Immunol 2018. (3) Gorgens et al. J Extracell Vesicles 2019.
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PT01.08 | Comparative study of isolation methods for cell-derived extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are nanoscale, membrane-bound compartments that carry cargo between neighboring cells and distant organs. The growing importance of EVs as diagnostic indicators and drug delivery vehicles has spurred the development of isolation and measurement techniques with high accuracy, sensitivity, and reproducibility. However, isolation and subsequent characterization of consistent populations of cell-derived EVs is very challenging. The aim of this research was to compare various EV isolation methods for increased purity and yield of EVs.
Methods: EVs were isolated from Jurkat cell culture supernatants using differential centrifugation (DC), size exclusion chromatography (SEC), and tangential flow filtration. Total protein content in EV isolates was measured with a Bradford assay. The morphology and size distribution of these EVs were evaluated through microfluidic resistive pulse sensing.
Results: Preliminary results were found that DC and SEC resulted in higher yields of > 1 × 1010 EV/mL whereas TFF resulted in yields of > 1 × 109 EV/mL. DC had higher total protein contaminants than SEC. The protein content from TFF depended on the pore size of the cartridge (30, 50, 100 kDA). For each method, more than 90% of the EV population had an average size distribution of 50–200 nm and median size of 120 nm. The presence of EV protein markers (CD63, CD81, CD9) were confirmed in each EV isolate.
Summary/Conclusion: SEC appears to perform the best in yield and purity of EV isolates. Nevertheless, further evaluation is needed including the use of orthogonal measurement techniques such as nanoparticle tracking analysis (NTA) and accounting for batch-to-batch variability.
Keywords: isolation, purity

PT01.09 | Orthogonal approaches using traditional and innovative technologies to purify cancer-derived large extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are membrane-covered particles of heterogeneous sizes and cargo. Large oncosomes (LO), which are shed by cancer cells with highly metastatic properties, are atypically large (1-10 um). Size exclusion chromatography (SEC) has been successfully used for the isolation and purification of Small-EVs (S-EVs) but not large EVs (L-EVs). The aim of this study was to obtain quantitative and qualitative information about purification of L-EVs derived from cancer cell cultures comparing SEC to density gradient centrifugation while testing microfluidics as alternative approach.
Methods: Differential ultracentrifugation (UC), SEC (qEVsingle/70nm), density gradient centrifugation, tunable resistive pulse sensing (qNano), flow cytometry, western blot, EVHB-Chip, 3D cell culture (Fiber Cell Systems).
Results: We collected three crude subsets of vesicles (2.8K, 10K, 100K) and purified the vesicles with SEC. EVs were collected from the first EV fractions (EV1-4). To exclude the possibility of apoptotic body contamination, especially in the 2.8K fraction, we induced apoptosis by TRAIL in the DU145 cell line to separate apoptotic bodies from large oncosomes. Particle concentration was determined by qNano and flow cytometry, showed that L- (900 - 5700 nm) and S- (30 - 250 nm) particles were most enriched in the first two EV fractions and L-EV proteins were slightly more abundant in fraction EV2. In line with published data, the bulk of the proteins eluted in the protein fractions (P8-11), confirmed the notion that most of the proteins present in crude low and high-speed UC preparations are non-EV contaminants. While both L- and S-EVs eluted in the same fractions, the recovery efficiency was significantly higher for S-EVs (63.1%) than for L-EVs (22%). Flow cytometry analysis showed similar patterns and relatively low recovery for L-EVs, suggesting that SEC is not appropriate for LO purification. Orthogonal experiments enabled to characterize the captured L- and S- EVs with the state-of-the-art microfluidic device (EVHB-Chip) with the ultimate goal of generating a platform that combines the use of multiple EV analytes and different populations of EVs with different biological significance for biomarker identification.
Summary/Conclusion: SEC can successfully separate EVs from contaminating non-EV proteins, but low particle yield affects its use for L-EVs. It is essential to implement the use of the EVHB-Chip to characterize specific subsets of EVs.
Funding: R01CA218526 NIH/NCI, R01CA234557 NIH/NCI.
Keywords: large oncosomes, EV purification, EV separation

PT01.10 | FastEV™, a novel tool determining optimal isolation method for biomarker discovery

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Introduction: Interest in the EV biomarker discovery is constantly expanding. To this end, tens of different EV isolation methods have been developed generating different EV populations, yields, purities, stability, volumes, molecular detectability and downstream applicability. Usually, it is not known which is the best EV isolation method in each individual case and finding a suitable one can take years. Furthermore, as most of the methods are of low-to-medium throughput or expensive, high impact studies and clinical feasibility remain out of reach. Here we introduce a novel high throughput platform, FastEV™, which enables fast screening of various EV enriching precipitation conditions from blood samples.
Methods: Healthy donor plasma and serum samples (~0.1-1 ml) were subjected to EV enrichment with different proprietary FastEV™ conditions, ultracentrifugation or precipitation using polyethylene glycol (PEG). Then, the isolates or plasma/serum controls were analysed for their protein, RNA and cfDNA pattern, or using EV Array, ApoB ELISA, and small RNA and mRNA sequencing.
Results: FastEV™ workflow in 96-well plates took < 1h. FastEV™ isolates presented variable quantities of proteins, lipoproteins, small RNA, mRNA, cfDNA and EV protein markers depending on the FastEV™ condition used. EVArray showed that reproducibility of isolation between days was up to $R2 = 0.99$ for the 14 EV proteins tested. When comparing the results among the different FastEV™ conditions or conditions vs controls, we observed both similarities and differences in individual parameters and also unique multiparametric profiles.

Summary/Conclusion: This work provides a catalogue of isolate compositions obtained from each FastEV™ condition. With this knowledge, the best isolation condition can be selected if the desired biomarker target is known. Additionally, FastEV™ platform can be used to screen for the optimal condition to separate cases from controls. As the isolates were suitable for many downstream analytics, FastEV™ enables a wide range of high throughput biomarker discovery studies.
Funding: This work has received funding from Business Finland and University of Helsinki.
Keywords: biomarker discovery, high throughput, plasma, serum, extracellular vesicles, exosomes, isolation, RNA, miRNA, protein, cfDNA

PT02: Milk EVs

Location: Hall 4A

16:45 - 18:45

PT02.01 | Proteomic and transcriptomic characterization of mare's milk derived exosomes

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Introduction: EVs are biological nanovesicles that are involved in cell-cell communication via the functionally active cargo (such as miRNA, mRNA, DNA and proteins). Because of their nanosize, exosomes are explored as nanodevices for the development of new therapeutic applications. Here we characterize exosomes derived from mare's milk.
Methods: Exosomes were isolated by size exclusion chromatography, isoelectric precipitation and total exosome isolation kit (Invitrogen) from mare's milk. Total mRNA is isolated from EVs using RNA extraction kit (Thermo Fisher Scientific) according to the manufacturer's instructions. To assess the gene expression, the exosomal kappa casein and lactoferrin mRNAs were detected by quantitative real-time PCR (qRT-PCR). Surface marker proteins of isolated exosomes were analyzed by Western blot.
Results: RT-PCR results showed that all EVs obtained using different methods had the patterns of accumulation of amplification products. However, exosomes obtained by size exclusion chromatography (SEC) method compared to isoelectric precipitation method showed 1.3 and 1.75 fold increase of kappa-casein and lactoferrin relative gene expression respectively. Also, SEC exosomes showed 1.6 fold kappa-casein and 1.4 fold higher lactoferrin relative gene expression compared to EVs obtained using total exosome isolation kit. EVs obtained using all methods contained the exosome surface markers MFG-E8 and CD63, according to WB analysis.
Summary/Conclusion: Mare's milk exosomes showed the mRNA gene expression across methods. EVs' surface marker proteins CD63 and MFG-E8 were present in exosomes isolated by all three methods.
Funding: This research was funded by the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan (Grant No. AP13067844) and Nazarbayev University Collaborative Research Program 2021–2023 (Award no. OPCRP2021006).

PT02.02 | Divergence of gut bacteria through the selection of genetic variants by small extracellular vesicles in milk

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Introduction: We reported that 50–75% of orally administered small extracellular vesicles in bovine milk (sMEVs) escape absorption and alter bacterial communities in the ceca of mice. Here, we expanded that line of investigation and tested the hypothesis that sMEVs select genetic variants in gut bacteria leading to altered metabolite production. Changes in purine metabolism were of particular interest, because of the essential roles of adenine and guanine in energy metabolism and cell signaling.
Methods: Gut content of murine ceca was cultured in media containing a nutritionally relevant concentration of sMEVs (sMEV-supplemented, mEVS) or sMEV-free (mEVF) media under anaerobic conditions for six days. Bacterial DNA and RNA were analyzed by shotgun and RNA-seq analysis, respectively, and genetic variants were identified using the MIDAS pipeline. Metabolites were assessed in bacteria and cell-free media supernatant by ultra-high-performance liquid chromatography-tandem mass-spectrometry.

Results: sMEVs selected approximately 55,000 genetic variants in 23 species of bacteria including a cluster of variants in purine metabolism. Genetic variants were transcribed. We identified a total of 10,508 and 12,500 mRNA-level variants in 2,734 and 3,040 unique transcripts in 7 and 5 bacterial species in mEVS and mEVF cultures, respectively. Six hundred eighty-nine and 226 transcripts matched the genetic variants in mEVS and mEVF cultures, respectively, including 15 out of 65 genes in purine metabolism. sMEV-dependent selection of genetic variants was associated with changes in bacterial metabolism. For example, the concentration of purine metabolites and purine-dependent metabolic pathways (glycolysis, tricarboxylic acid cycle energy metabolism, pentose sugar metabolism) were different in cell-free mEVS and mEVF media.
Summary/Conclusion: sMEVs participate in cross-kingdom communication through selecting genetic variants in genes from purine metabolism in gut bacteria.
Funding: Supported by NIH P20GM104320, NIFA (2016-67001-25301 and 2020-67017-30834), USDA Hatch and W-4002, and the SynGAP Research Fund (all to J. Z.). J.Z. is a consultant for PureTech Health, Inc.
Keywords: divergence, genetic variants, gut bacteria, milk extracellular vesicles

PT02.03 | Human milk extracellular vesicles survive neonatal human digestion

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Introduction: Human milk (HM) contains bioactive components, including extracellular vesicles (EVs). HM EVs are putative regulators of intestinal function and offer a mechanism for the transfer of proteins from mom to infant. It is unknown if milk EVs or their protein cargo survive neonatal digestion, which limits the ability to leverage HM EV proteins as modulators of intestinal function, anti-inflammatories, additives to infant nutrition, or therapeutics. The objective of this study was to develop a high-yield, high-purity EV isolation pipeline from small volume (≤1mL) HM and neonatal intestinal contents (digesta) to address our hypotheses that 1) donor milk processing alters HM EVs and 2) HM EVs protect cargo from digestion.
Methods: All studies were OHSU-IRB approved. Digesta were collected from naso- or orojejunal sampling tubes. EVs were isolated from HM (raw vs pasteurized donor milk) and digesta by bottom-up density-gradient ultracentrifugation following two-step skimming, acid precipitation of caseins, and multi-step filtration. EVs were validated by electron microscopy (EM), nanoparticle tracking analysis (NTA), and western blot. EV effects were tested in human neonatal enteroids.
Results: EVs show typical EV morphology by EM and are enriched in CD81, CD9, and TSG101 by WB. HM EVs are depleted of beta-casein and lactalbumin. There is a 37-fold reduction in particle number in Holder-pasteurized vs raw HM samples ($3.1 \times 10\hat{}11$ vs. $8.4 \times 10\hat{}9$ particles/mL, n = 3). HM EVs survive human digestion and are taken up by neonatal intestinal enteroid cultures.
Summary/Conclusion: Our pipeline yields quite high purity EVs from ≤ 1mL of HM and digesta. Our data suggest that HM processing decreases EV number and that HM EVs protect important bioactive cargo during digestion for absorption by the intestinal epithelium. This pipeline allows for the analysis of a greater pool of low volume clinical samples with the goal of developing novel nutrient-based therapies for intestinal inflammation.
Funding: NIH K01DK129401, USDA NIFA, Collins Medical Trust, Medical Research Foundation, OHSU Exploratory Research Seed Grant.
Keywords: human milk, human digestion, extracellular vesicles, human enteroids

PT02.04 | Establishment and validation of mare's milk-derived exosomes extraction method

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Introduction: Mare's milk has a very good hygienic and sanitary status, differs from the milk of other farm animals in that it has the lowest somatic cell content and a very low total number of microorganisms. The main hypothesis was that exosomes obtained from mare's milk can be a beneficious drug transport system compared to well-studied exosomes from bovine milk. Objective of the project was to establish extraction method of exosomes obtained from a mare's milk, for future use as drug-delivery system.
Methods: To obtain pure exosomes of optimal size, three methods were investigated - isoelectric precipitation, size-exclusion chromatography, total exosome isolation. Each approach differs in price, equipment and materials, duration and complexity of implementation as well as mechanism of action.

Results: Exosomes obtained from all methods have smooth membrane and isolated without surface rupture and damage, which indicates their possible further loading of drugs and use for therapeutic purposes in experiments according to transmission electron microscopy (TEM) data. Exosomes were purified well by total exosome isolation (TEI) and size-exclusion chromatography (SEC) methods from dead cells, cell debris, and fat globules, according to Zetasizer data, whereas immunoprecipitation purification was unsatisfactory. Also, exosomes obtained from all three methods were of optimal size. TEI and SEC showed good clearance of casein, the main protein contaminant in milk.
Summary/Conclusion: In our study, we consider that TEI method is the best choice for both laboratory research and large-scale production. This approach requires less time, is less methodology sensitive, is more consistent with small volumes of biological samples, and does not demand any special equipment in comparison with other methods.
Funding: Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan (Grant No.AP13067844), Nazarbayev University Collaborative Research Program 2021–2023 (Award no.OPCRP2021006).

PT02.05 | Enabling milk-derived extracellular vesicle isolation and analysis via temperature-responsive polymeric reagents

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Introduction: The utility of extracellular vesicles (EV) for biomedical applications have been demonstrated. However, their clinical translation is hindered by technical issues—mostly related to isolation. Common processing methods result in variable yield and purity, are time consuming, require complex procedures, or are difficult to scale up. To address these challenges, temperature-responsive (smart) polymeric reagents were developed to enable rapid and specific EV isolation. The reagents diffuse rapidly at room temperature to promote EV binding, and form aggregate at 40°C to facilitate separation via benchtop centrifugation.
Methods: The polymer-antibody (Ab) conjugates were synthesized by covalently linking azido-polymer(N-isopropylacrylamide) to dibenzocyclooctyne modified anti-mouse IgG Ab using click chemistry. Raw cow milk, processed to remove cream, was used as the specimen. Isolation of EVs from milk is done by adding a transmembrane immunoglobin and mucin domain-4 (TIM-4):mouse Fc construct, which targets phosphatidylserine (PS) expressed by all EV. Then, the polymer-Ab conjugates were added to recognize mouse Fc, bound on PS. The EV were separated by centrifuging the solution at 12000 rpm (12,386 × g) and 40°C for 5 minutes. After the supernatant was removed, the captured EV were characterized and quantified by RT-qPCR.
Results: To evaluate the isolation process, microRNA let-7b, found in high abundance in bovine milk EV, was used. An equivalent volume of unprocessed milk was also analyzed to estimate capture efficiency. Ct values for let7-b were normalized to the miR-cel-39 spike-in control, which was added before RNA extraction of the collected EVs. Compared to the control group, mouse IgG1 kappa isotype, isolation via the TIM-4 construct led to a 4–13 fold increase in let-7b. The EV isolation efficiency was estimated to be 51–77%. The total process duration is 75 min and consists only of 4 steps.
Summary/Conclusion: The smart polymeric reagents have demonstrated EV isolation from raw, unpasteurized bovine milk by targeting the lipid PS, expressed on EV membranes. Compared to the common EV isolation approaches, the new method is rapid and can potentially be scaled to larger processing volumes.

PT02.06 | Massive scale industrial isolation of extracellular vesicles from cow milk and their in vitro and in vivo characterization

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Introduction: Cow milk derived extracellular vesicles have recently gained momentum for pharmaceutical and nutraceutical applications. Their accessibility at scale and intrinsic potential for oral bioavailability make them particularly attractive as drug delivery vehicles. Industrial implementation of milk EV isolation is still challenging since current state of the art methods are largely based on ultracentrifugation or require final chromatographic steps.
Methods: For the development of an industrially scalable process for milk EV isolation we (1) evaluated the suitability of different side streams of cheese manufacturing and dairy processing, (2) iteratively optimised the isolation and quality control processes between the lab and the plant and (3) systematically benchmarked the industrially isolated EVs against corresponding samples obtained by standard lab scale isolation of EVs from raw milk.
Results: An industrial, proprietary process was developed for massive scale isolation of EVs from a waste stream of the dairy industry which showed increased yields of EVs, reduced levels of copurified protein particles as compared to standard lab scale protocols. A systematic comparison of the physicochemical properties (NTA, TEM, 3D cryo-TEM, IR 'fingerprint', IFCM, ζ-potential), molecular composition (protein/particle ratios, proteomics, lipidomics, glycomics, metabolomics) and biological function revealed that industrial milk EVs retain the physico-chemical properties, composition, morphology, as well as cell and tissue uptake activity in vitro and in vivo models.
Summary/Conclusion: We present a scalable process for functional milk EV isolation, that uses the available waste streams from the dairy industry, is directly transferable to the x100,000 L scale and is fully compatible with industrial milk processing.
Funding: EV-TT BPro (County of Salzburg, WISS2025, P1812596), EVTT (European Union, EFRE/IWB 20102-F1900731-KZP), CONSONANT (County of Salzburg, WISS2025, F2200397-KZP).

PT02.07 | Extracellular vesicles from bovine milk mitigate the LPS-induced reduction in gut barrier integrity in C2BBe1 cells

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Introduction: Impaired gut-barrier function is hallmark symptom in Crohn disease and ulcerative colitis. Novel therapeutics which protect intestinal integrity are needed. Extracellular vesicles (EVs) present in bovine milk may provide a means by which dietary bioactives may impact intestinal pathology. The purpose of this research was to determine the effects of bovine milk EVs on barrier function of intestinal epithelium and cell viability.
Methods: C2BBe1 cells were grown for 21 d in transwell inserts. Differentiated enterocytes were incubated with EV-depleted FBS for 72 hr. Cells were incubated with EVs (50-200 ug/mL) isolated from raw bovine milk by differential centrifugation for 24 h before exposure to LPS. A Countess II was used to determine cell viability and transepithelial electrical resistance was determined by a Millicell ERS-2 as an index of barrier integrity.
Results: After 72 hours of incubation with EV-depleted FBS, cell barrier integrity was reduced 29% compared to cells incubated with standard FBS. LPS reduced barrier integrity by 26% in cells incubated with EV-depleted FBS; however, when preincubated with 50–200 ug/mL of EVs, barrier integrity only decreased between 4.1% and 12.2%. Preincubation with EVs prior to LPS exposure resulted in 35% cell viability compared to LPS alone (11%).
Summary/Conclusion: EVs are critically important for enterocyte function. Depletion of EVs from culture medium reduces cell viability and impairs barrier function. Additionally, supplementation of EVs from bovine milk had a protective effect on maintaining cell viability and membrane integrity when exposed to inflammatory stimuli. This indicates that supplementation with specific dietary EVs may ameliorate processes characteristic of inflammatory bowel disease.
Funding: USDA GRANT NO. 2020-67018-33315.
Keywords: nutrition, exosome, intestinal permeability, inflammatory bowel disease

PT02.08 | Antioxidant and cytoprotective properties of quercetin-loaded mare's milk exosomes

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Introduction: Mare's milk is the national drink of the indigenous population of Kazakhstan. Similar to other milk types, mare's milk is a rich source of EVs, which can be loaded with flavonoids such as quercetin that may have some valuable therapeutic properties. In this study, we examine cytoprotective properties of quercetin-loaded exosomes (ExoQuer) on doxorubicin induced cellular toxicity as well as antioxidant and radical scavenging activity.
Methods: EVs obtained 3 different methods: total exosome isolation kit (Invitrogen™), isoelectric precipitation and size exclusion chromatography (Izon™). Radical scavenging activity was measured using the DPPH (2,2-diphenyl-1-picrylhydrazyl) test. ABTS assay kit was used to measure antioxidant activity (Sigma-Aldrich). Cell viability was determined by MTT assay. HDFn cells were seeded in a 96-well plate at a density of 105 cells/well and incubated at 37°C for 24 h. Toxicity was induced by doxorubicin and left for 24 h. The cells were then treated with ExoQuer, free quercetin for 24 h. Subsequently, 0.5 mg/mL MTT reagent dissolved in DMEM was added to each well, and the plate was further incubated at 37°C for 3 h. Each well was measured at 570 nm.
Results: Antioxidant activity of free quercetin and ExoQuer did not differ significantly, 2.4 and 2.2 TEAC respectively. The DPPH radical scavenging activity (ES50) of free quercetin was 5.5 μM and for ExoQuer it was 5.9 μM. Viability of cells treated with ExoQuer was 71% while cells treated with free quercetin was 64%, compared to control.
Summary/Conclusion: Whereas ExoQuer and free quercetin have the same level of antioxidant and radical scavenging activity, ExoQuer has higher cytoprotective activity on the model of doxorubicin-induced cellular toxicity.
Funding: This research was funded by the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan (Grant No. AP13067844) and Nazarbayev University Collaborative Research Program 2021–2023 (Award no. OPCRP2021006).

PT03: EVs and Viral Infection

Location: Hall 4A

16:45 - 18:45

PT03.01 | Syntenin mediated ZIKV transmission through regulating sEVs release and uptake

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Introduction: ZIKV (Zika virus) were emerging from 1947. Since its outbreak, ZIKV has infected countless people with fever, rash, joint pain and Guillain-Barré syndrome, including severe microcephaly in newborns from infected pregnant women.
Small extracellular vesicles (sEVs) are small, nanoscale, natural phospholipid bilayer vesicles secreted by various cells and are widely found in fluids such as blood, urine et al. Large of studies have shown that sEVs are capable of transport ting a range of bioactive molecules that mediate cellular and inter-tissue communication and participate in processes such as viral infection,immune regulation and tumor therapy. It has been found that sEVs derived from virus-infected cells can encapsulate viral components and host factors, and that such sEVs can act on adjacent cells or enter the bodily circulatory system to effect distal target cells, thereby modulating viral infection. For example, sEVs released from host cells infected with hepatitis C virus (HCV) can encapsulate the complete viral genome and can fuse with distal dendritic cells and establish effective infection. We found the sEVs released by ZIKV-infected cells contained viral genomes and proteins and was capable of establishing productive infection in non-receptor expressed cells.
The biogenesis of sEVs involves a complex series of formation mechanisms. Viruses may hijack vesicle sorting pathways to alter sEVs secretion and contents. In this research, we found ZIKV infection affected the expression of syntenin, a cytosolic adapter interacted directly with Alix, and supported the intraluminal budding of endosomal membranes.
Methods: Transmission electron microscopy (TEM), Nanoparticle Tracking Analysis (NTA), Exosome isolation, purification., in Vivo Imaging, Flow Cytometry
Results: 1: ZIKV infection increased the production of sEVs. 2: sEVs derived from ZIKV-infected cells contain viral components. 3: sEVs-ZIKV could establish productive infections in recipient cells 4: ZIKV promoted sEVs production by upregulating syntenin expression. 5: Syntenin regulated sEVs uptake through affecting SDC2 and GPC1.

Summary/Conclusion: We found that ZIKV infection result in packaging of the viral genomic RNA and partial viral proteins into sEVs and upregulates sEVs secretion, and demonstrated that the viral genomic RNA in sEVs could be transferred to and establish productive infection in a new target cell. Importantly, our study reveals that ZIKV-induced syntenin expression enhances sEVs secretion and sEVs-mediated virus transmission. Additionally, our data show that syntenin may be involved in the uptake of the infectious-sEVs to facilitate ZIKV transmission through the vesicles. Taken together, our results suggest a novel mechanism involving syntenin and sEVs-mediated ZIKV intercellular transmission.

PT03.02 | Proteomic profile of circulating plasma extracellular vesicles in SHIV-infected rhesus macaque indicates the development and progression of neuropathogenesis

Partha K. Chandra[1]; Manesh Kumar Panner Selvam[2]; Stephen E. Braun[3]; Ibolya Rutkai[1]; Jorge A. Castorena-Gonzalez[1]; Suresh C. Sikka[2]; David W. Busija[1]
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Introduction: Combined antiretroviral therapy suppresses HIV replication, but 30–60% of human immunodeficiency virus (HIV) infected patients suffer from HIV-1 associated neurocognitive disorders (HAND). Using proteomics, we investigated possible circulating plasma extracellular vesicles (crEVs) protein links to neuropathogenesis in simian-human immunodeficiency virus (SHIV)-infected rhesus macaque (RM).
Methods: crEVs were isolated from plasma of SHIV-infected (SHIV-crEVs) and uninfected (CTL-crEVs) RM (N = 3/group) by QIAGEN exoEasy kit, characterized by the qNano-IZON system. Proteomic analysis was performed by liquid chromatography/mass spectrometry (LC-MS/MS). A significant number of differentially expressed proteins (DEPs) in both groups were subjected to functional annotation and enrichment analysis. Some EV/exosome markers and DEPs were validated by western blotting.
Results: A total of 5,654 proteins were identified in crEVs, with 236 (~ 4%) DEPs, distributed between SHIV-crEVs and CTL-crEVs. Ingenuity Pathway Analysis (IPA) demonstrated that DEPs in SHIV-crEVs were involved in several diseases and functions including neuroinflammation and neurological diseases. Bioinformatic analysis revealed that the DEPs in SHIV-crEVs were involved in abnormal morphology of cerebrum, synapse, early-onset neurological disorder, seizure disorder, and progressive neurological disorder. Functional network analysis displayed the interaction among DEPs involved in cognitive impairment, mental retardation, and dementia, which are the most common features in HAND. IPA analysis also showed that several DEPs in SHIV-crEVs are involved in progressive encephalopathy and leukoencephalopathy. Furthermore, enrichment analysis revealed the DEPs in SHIV-crEVs are associated with Alzheimer, Parkinsonism, and Huntington diseases.
Summary/Conclusion: Our novel findings suggest that plasma crEVs may elucidate the development and progression of HAND, possibly providing novel therapeutic targets.
Funding: NIH: AG075988 (DWB), AG063345 (DWB), HL148836 (DWB), AI110158 (SEB), P51OD011104 (TNPRC).
Keywords: SHIV, rhesus macaque, plasma extracellular vesicles, proteomic analysis, neuropathogenesis

PT03.03 | Molecular profile of circulating small extracellular vesicles in the pathological trajectory of COVID-19

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Introduction: The aim of the present study is to identify the longitudinal changes in the small extracellular vesicle (sEV) proteins and miRNAs profile in COVID-19 patients at the time of diagnosis and post-recovery.

Methods: Plasma samples were obtained from COVID-19 patients at the time of diagnosis and 12 months after infection, and healthy controls. sEVs were isolated using differential centrifugation followed by size exclusion chromatography. sEVs were characterized by size distribution using Nanosight N500, electron microscopy (morphology as spherical vesicles), and the presence of EV enriched markers (i.e. CD9, CD63, and CD81). sEV-associated miRNAs and proteins were characterized by Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH) mass spectrometry and next generation sequencing respectively. Differentially expressed proteins and miRNAs were analyzed by bioinformatics using Ingenuity Pathway Analysis.
Results: The levels of circulating sEVs were higher in COVID-19 patients at the time of diagnosis compared to 12 months post-infection. A specific set of sEV-associated proteins and miRNAs were differentially expressed in COVID-19 patients compared to healthy controls, both in the acute and post-infection phases. A set of 98 proteins and 131 miRNAs were significantly altered in circulating sEVs in the post-infection phase compared to acute phase. Interestingly, a specific profile of sEV associated proteins and miRNAs differentially expressed in COVID-19 patients compared to healthy controls was maintained up to 12 months post-infection. These proteins and miRNAs were associated with glucose metabolism and insulin signalling.
Summary/Conclusion: The findings from this study elucidate the COVID-19 induced unique changes in the expression of proteins and miRNAs in the circulating sEVs. This suggests the biomarker potential of sEVs in predicting the metabolic and glycaemic dysregulation upto 12 months post-recovery in COVID-19 infection.
Funding: NHMRC 1195451.
Keywords: COVID-19, miRNA, proteins

PT03.04 | Exosomal extracellular vesicles interact with delta-9-Tetrahydrocannabinol (THC) and cannabidiol (CBD) to modulate HIV latency in microglial cells and neuroinflammation

Mickensone Andre[1]; Allen Caobi[2]; Adrianna Yndart-Arias[2]; Madhavan Nair[2]; Andrea Raymond[3]
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Introduction: Despite successful virological suppression by antiretroviral (ARV) therapy, people living with HIV/AIDS(PLWHAS) develop neurological, cognitive disorders and can suffer from adverse side effects of ARVs. These side effects include nausea, gastrointestinal, peripheral neuropathy, and toxicity in the central nervous system (CNS). PLWHAs are prescribed (or used recreationally) marijuana to alleviate some of these adverse effects. However, the impact of marijuana on HIV cellular reservoirs is unknown.
Methods: Here we investigate how some of the active components of marijuana (delta-9-tetrahydrocannabinol [delta-9-THC] and cannabidiol [CBD]) affect HIV latency and exosomal extracellular vesicles(xEVs) in microglia, the major cellular reservoir of HIV in the CNS. Using a microglia model of HIV latency, the effect of delta-9-THC and CBD (alone or in combination) on HIV reactivation and xEVs was assessed. The capacity of microglia-derived xEVs to reactivate HIV latency in the context of delta-9-THC and CBD was also assessed.
Results: Our preliminary findings show that cell viability was not affected by THC and CBD at doses 0.1 μM, 1 μM, 5 μM and 10 μM. However, THC and CBD in combination at 10 μM each significantly decreased cell viability. Interestingly, xEVs derived from CBD and delta-9-THC treated microglia modulated HIV latency reactivation. However, direct CBD and THC exposure to the cells no reactivation occured.
Summary/Conclusion: This suggests that THC/CBD modulate microglia xEVs cargo such that these vesicles may modulate the CNS neurotoxicity while direct exposure to THC/CBD promoted HIV latency.

PT03.05 | Exosomes engineered with SARS-CoV-2 spike and nucleocapsid proteins induce strong immune response against multiple variants of concern (VOCs)

Justin B. Nice[1]; Mafalda Cacciottolo[1]; Yujia Li[2]; Michael LeClaire[2]; Ryan Twaddle[2]; Ciana Mora[2]; Stephanie Adachi[2]; Esther Chin[2]; Meredith Young[2]; Jenna Angeles[2]; Kristi Elliott[2]; Minghao Sun[2]
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Introduction: Here we designed an exosome-based, bivalent vaccine with the goal of producing broader immunity against SARS-CoV-2 using Capricor's Stealth X technology. Spike, in its naturally folded structure presented on the surface of exosomes, was designed to protect against newer spike mutations. In addition, nucleocapsid is a more conserved SARS-Cov-2 protein than spike. Both cell lines produced exosomes highly enriched in the desired protein, and when combined in a in vivo model produced a strong immune response and T-cell memory with only nanogram levels of protein presented on the surface of exosome.
Methods: Cells were engineered using lentiviral transduction and expanded for exosome production and the resulting cell-free supernatant was concentrated using a tangential-flow filter (TFF), proteins were then removed using Sephacryl size exclusion chromatography, and the resulting exosome-containing fractions concentrated again using TFF. Exosome size and concentration

was measured using ParticleMetrix ZetaView. Spike or nucleocapsid protein concentration was measured using an ELISA assay. Mice were injected with two rounds of spike-expressing and nucleocapsid-expressing exosomes (day 0 and 21) and immune response was measured by ELISAs for IgG against spike or nucleocapsid at days 14 and 35.
Results: Results showed that IgG production against spike increased up to 1,500-fold while IgG against nucleocapsid increased up to 7-fold. Neutralizing of different VOCs of SARS-CoV-2 was carried out by Retrovirox, Inc. The results showed that mouse plasma could neutralize delta variant at 100%, as well as partial protection against omicron BA.1 and BA.5.2.1 variants.
Summary/Conclusion: This study demonstrates that exosomes can be engineered with viral antigens to be used for rapid vaccine development with implications beyond COVID-19 and to other vaccines that combine the power of Stealth X targeting, multiplexing, low dosage, better safety profile and short turnaround time.
Keywords: COVID-19, SARS-CoV2, engineered-exosomes

PT03.07 | Extracellular vesicles in body fluids expose phosphatidylserine to mimic viral membranes and inhibit infection and transmission

Janis Müller[10]; Rüdiger Groß[1]; Dan Albers[2]; Pascal von Maltitz[2]; Laura Schneider[3]; Hanna Bley[3]; Markus Hoffmann[4]; Mirko Cortese[5]; Miriam Deniz[6]; Jae-Yeon Choi[7]; Jenny Jansen[8]; Stefan Pöhlmann[4]; Dennis R Voelker[7]; Christine Goffinet[8]; Ralf Bartenschlager[5]; Samir EL Andaloussi[9]; Konstantin MJ Sparrer[2]; Eva Herker[3]; Stephan Becker[3]; Frank Kirchhoff[2]; Jan Münch[1]
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Introduction: It is poorly understood why some viral pathogens are rarely transmitted orally or sexually despite their presence in various body fluids. One potential explanation is their inhibition by extracellular vesicles (EVs), which are abundant in semen and saliva and have been shown to interfere with Zika virus (ZIKV) infection. The antiviral mechanism and spectrum, as well as the conservation of this activity in EVs from other sources are unclear.
Methods: Here, we analyzed the antiviral activity of EVs from semen, saliva, urine, breast milk or blood against ZIKV and viruses of different families. EVs were purified by tangential flow filtration combined with bind-elute size exclusion chromatography, analyzed for abundance and size by nanoparticle tracking analysis, and characterized for cargo by western blotting, bead-assisted flow cytometry and lipidomics. Antiviral activities were assessed in vitro by in-cell ELISA and confocal microscopy and ex vivo in human vaginal tissue explants by determining viral RNA release.
Results: We show that EVs from all sources inhibit ZIKV infection with those from semen and saliva being most potent. The inhibitory activity of semen EVs was confirmed on primary cells and human vaginal tissue. Analysis of the lipid composition revealed that the antiviral EVs are rich in surface-exposed phosphatidylserine (PS). PS-exposing EVs and liposomes prevent virion attachment and infection by competing with Zika virions for cellular PS receptors. This activity is not restricted to ZIKV but directed against a wide range of pathogens that use PS receptor interaction for infection and immune invasion. Consequently, EVs inhibited attachment and infection of Zika, Dengue, West Nile, Chikungunya, Ebola, and Vesicular stomatitis viruses, but were inactive against SARS-CoV-2, HIV-1, herpes and hepatitis C viruses.
Summary/Conclusion: Our results identify PS exposure as an EV-based innate defense mechanism that may play a key role in restricting sexual and oral virus transmission.
Funding: German Research Foundation.
Keywords: virus transmission, sexual transmission, innate immunity, phosphatidylethanolamine, zika virus, dengue virus, west nile virus, ebola virus, chikungunya virus, viral apoptotic mimicry

PT03.08 | Purification of virion-free extracellular vesicles from human cytomegalovirus-infected cells, guided by flow nanoanalysis

Paschalia Pantazi[1]; Vladimir Bokun[1]; Blair Strang[2]; Yan Liu[3]; Beth Holder[1]
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Introduction: Investigation of extracellular vesicles (EVs) produced by virus-infected cells is challenging due to the variety of particle types that are released into the cell culture supernatants and their similar physical and biochemical features. In this work, we developed a simple centrifugation-based approach for isolating EVs in human cytomegalovirus (HCMV) -infected cell conditioned medium free from HCMV virions and dense bodies, and validated our results using a state-of-the-art flow nanoanalysis (FNA) method.
Methods: We utilized uninfected or HCMV-infected human foreskin fibroblast (HFF) cells to generate conditioned culture media, which was clarified by low-speed centrifugation and 0.45-μm filtration. Cell-free extracellular particle suspensions were fractionated by centrifugation at 25,000xG for 2.5hr over 16% iodixanol cushions. EVs were then purified from resulting supernatants by ultrafiltration and size exclusion chromatography, and fractions analyzed by nanoparticle tracking analysis (NTA), ELISA for EV markers, electron microscopy (EM), and FNA for sizing and nucleic acid content.
Results: Nucleic acid labelling combined with FNA enabled visual separation of virions and EVs. As evidenced by FNA and EM, a near-complete fractionation of the particles following the centrifugation step was achieved. Due to higher particle density, HCMV virions and dense bodies penetrated through 16% cushions and formed pellets, while less dense EVs were remained in the supernatant, thereby affording a successful separation of viral particles and EVs. EVs purified from the post-centrifugation supernatants were positive for beta-actin and CD63 by ELISA.
Summary/Conclusion: Our method for purification of EVs from HCMV-infected cells is simple, gentle, does not require specialized equipment, and could be adapted to other viruses. These methods will enable future wide-ranging functional and biochemical investigation of EVs produced by infected cells.
Funding: This project was supported by Imperial College President's PhD scholarship awarded to Vladimir Bokun and a Genesis Research Trust grant to Vladimir Bokun, Yan Liu and Beth Holder. Acquisition of nanoFCM instrument was supported by a Wellcome Trust multi-user equipment grant awarded to Dr Beth Holder and Dr Richard Kelwick.
Keywords: human cytomegalovirus, extracellular vesicle isolation, viral infection

PT04: Kidney and Urinary EVs 1

Location: Hall 4A

16:45 - 18:45

PT04.02 | Effects and mechanism of renal targeted delivery of MSC exosomes in the treatment of AKI

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Introduction: AKI occurs in approximately 15% hospitalized patients and over 50% intensive care unit patients. However, effective therapeutic strategies for AKI are urgently needed. The exosomes derived from Mesenchymal stem cells (MSC-exos) have achieved great focus as a cell-free therapy. As foremost metabolic organs, MSC-exos mainly collect in the liver and spleen. More attention should be considered how to induce more exosomes targeted to the injured kidney. In addition, the potential role of MSC-exos in injured kidney and the underlying mechanisms still need further research.
Methods: Transmission electron microscopy, western blotting, and nanoparticle tracking analysis were used to identify the properties of human umbilical cord mesenchymal stem cells (hucMSCs) derived exosomes. Kidney targeting peptide was chosen by IVIS spectrum imaging system. Moreover, Kidney targeting peptide (Peptide- CGA) and CD63 targeting peptide was loaded to the MSC- exos by co-incubated (CGA-exos). Flow cytometry and immunofluorescence staining was utilized to confirm this. IVIS spectrum imaging system was used to assess MSC-exos distribution in vivo.
Results: In vivo imaging showed that Peptide- CGA was efficiently homing to the ischemic kidney and predominantly accumulated in proximal tubules. Further Immunoprecipitation study showed that Peptide- CGA treatment group produced a specific stripe in silver staining compared to other groups. CGA-exos was successfully constructed. MSC-exos could alleviate murine ischemic kidney injury and reduced the renal tubules injury. Furthermore, in vivo imaging showed CGA-exos treatment group could attract more MSC- exos into the injured kidney, and its therapeutic effect was significantly better than that of the MSC-exos treatment group. without causing significant organ side effects.
Summary/Conclusion: Novel Kidney targeting MSC-exos was constructed. It exhibited preferential tendency to injured kidney and localized to proximal tubules in AKI. We demonstrate that MSC-exos ameliorate ischemic AKI. But its further mechanisms need more insight. All in all, this highlights the potential of MSC-exos as a promising therapeutic strategy for AKI.
Keywords: MSC, AKI, exosomes, peptide

PT04.03 | Extracellular vesicles derived from human liver stem cells counteract chronic kidney disease development and cardiac dysfunction in remnant kidney murine model

Elena Ceccotti[1]; massimo cedrino[2]; giulia chiabotto[3]; Cristina Grange[4]; alessandra ghigo[5]; alessandro gambella[6]; maria Felice MF brizzi[7]; Giovanni Camussi[8]; Stefania Bruno[9]
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Introduction: Chronic renal disease (CKD) is a serious clinical hurdle without adequate therapeutic strategies to prevent its progression.
The murine model of 5/6th partial nephrectomy with pole ligation (PNx) is the most suitable approach to mimic the progressive renal failure after loss of the kidney mass in humans. In this model, uremic cardiomyopathy develops 4 weeks post-surgery.
In this study, we investigated the effect of different doses of extracellular vesicles (EVs) derived from human liver stem cells (HLSCs) on CKD development and on cardiac dysfunction.
Methods: EVs were purified by ultracentrifugation and characterized in accordance with ISEV-guidelines. PNx was performed in 10 weeks old SCID mice by ligation of both poles of the left kidney, followed by removal of the right kidney one week after. PNx mice were treated with EVs weekly for 4 weeks, starting 1 month after the nephrectomy, when interstitial fibrosis and glomerular sclerosis had been already established. Mice were sacrificed 8 weeks after the second surgery.
Renal and cardiac function were evaluated using specific biochemical assays and echocardiography. Histological analyses were performed to quantify renal and cardiac interstitial fibrosis and glomerular sclerosis. The expression levels of genes involved in the development of fibrosis and inflammation were evaluated by real time PCR.
Results: PNx mice treated with EVs had an amelioration of renal function and showed a reduction of both interstitial fibrosis and glomerular sclerosis. This trend of improvement was also confirmed by the molecular analyses of specific markers of fibrosis and inflammation. Moreover, EV treatment ameliorated cardiac functionality and significantly reduced interstitial fibrosis, which is a key hallmark of diastolic dysfunction.
Summary/Conclusion: In PNx murine model, EV administration interferes with the development of CKD and cardiomyopathy.
Funding: Unicyte AG (Switzerland).
Keywords: stem cells, chronic kidney disease, interstitial fibrosis, glomerular sclerosis, cardiomyopathy

PT04.04 | Role of Extracellular vesicle in kidney disease through modulation of miR-93

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Introduction: Modulation of miRNAs in cells of the glomerular filtration barrier is associated with renal diseases. We investigated the role of EVs derived from human amniotic fluid stem cells (hAFSC-EVs) in disease-modifying activity in vitro and in vivo by regulation of miR-93. Our data indicate that miR-93, a potent regulator of glomerular damage, is down-regulated in glomeruli of mice with Alport syndrome (AS, characterized by a mutation of the col4a5 gene) and in glomeruli of AS patients.
Methods: hEVs were isolated by UC (from multiple consented donors), characterized by Exoview, ONI, and PBMC immunemodulatory activity (Mixed Lymphocyte Culture). KD hEVs for miR-93 (hEVs mir-93-/-) were generated; RNA-seq and proteomics were performed in normal and KD hEVs. hEV therapeutic effect was evaluated in vitro and in vivo by biodistribution, renal function, and survival.
Results: hEV yield is ~2.8 × 1010EVs/1 × 106cells/24hrs. Our analysis shows that hEVs express CD9, CD63 and CD81, VEGFR1, CD73, CD44, and CD90 and do not initiate activation PBMC or downregulate PBMC activation. Proteomics showed high DE of VPS29, PLEC, IGF2BP3, FLNC, COL5A2, IGF2BP2 in hEVsmir-93+/+ identifying pathways involved in the homeostasis of glomerular structure (extracellular region, extracellular space, ECM structure). RNA seq showed high DE of miR-451a miR-142-3p, miR-142-5p, miR-486-5p, miR-93-5p identifying pathways involved in VEGFA-VEGFR2, vasculature development, and protein binding. In vitro, hEVsmir-93-/- did not prevent damage in human glomerular cells vs. hEVsmir-93+/+. When injected in AS mice, hEVs localized in the kidney corrected proteinuria and prolonged the lifespan compared to hEVsmir-93-/-. No side effects were noted.

Summary/Conclusion: hAFSC-EVs containing miR-93 modulated pathways central to glomerular homeostasis and preserved glomeruli structure with improved kidney function. This suggests the possibility of using hAFSC-EVs as a new therapeutic option for treating AS.
Funding: R01DK121037.
Keywords: kidney disease, miRs

PT04.05 | Therapeutic potential of urine exosomes derived from rats with diabetic kidney disease

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Introduction: Kidney diseases are prevalent in diabetes. Urinary exosomes (uE) from animal models and patients with Diabetic nephropathy (DN) showed increased levels of miRs with reno-protective potential
Methods: In this study (study-1) we performed microarray profiling of miRNA in uE and renal tissues in DN patients and subjects with diabetes without DN (controls). In study 2, diabetes was induced in Wistar rats by Streptozotocin (i.p. 50 mg/kg of body weight). Urinary exosomes were collected at the 6th, 7th, and 8th weeks, and injected back into the rats (uE = 100ug/biweekly, n = 10) via tail vein on weeks 9 and 10. Equal volume of vehicle was injected into controls (vehicle, n = 6). uE from the human and rat was characterized using exosome-specific proteins (CD9, CD63, CD81, and TSG101) by immunoblotting and Size distribution and concentration analyzed by Nanoparticle Tracking Analysis (NTA)
Results: Microarray profiling revealed a set of 15 miRs with high levels in the uE, while lower in renal biopsies, from DN, compared to controls (n = 09/group). Taqman qPCR confirmed the opposite regulation of miR-200c-3p and miR-24-3p between uE and kidneys from DN patients (n = 15). A rise in 28 miRs levels, including miR-200c-3p, and miR-24-3p were also observed in the uE of DN rats collected between 6th-8th weeks, relative to baseline (before diabetes induction). Diabetic rats treated with uE (collected from diabetic rats between the 6th-8th week) had a significant reduction in urine albumin-to-creatinine ratio after 2 weeks of treatment, relative to vehicles. Treated rats had attenuated renal pathology, and reduced levels of fibrotic genes (TGF-beta, Collagen IV), inflammatory marker (IL-6), and miR-200c-3p, and miR-24-3p target genes, relative to vehicle. The bioinformatic analysis confirmed the Renoprotective potential of the miRs, upregulated in DN uEs
Summary/Conclusion: We examined whether urinary loss of such miRs is associated with reduced renal levels in DN patients. We also tested whether injecting uE can leverage kidney disease in rats. Patients with diabetic nephropathy had reduced renal levels, while higher uE abundance, of miRs with reno-protective potential. Reverting the urinary loss of miRs by injecting uE attenuated renal pathology in diabetic rats
Keywords: Diabetes, Diabetic nephropathy, Kidney, miRNA, Exosomes, Microarray

PT05: Muscle Skeletal System

Location: Hall 4A

16:45 - 18:45

PT05.01 | Neurotrophic receptor TrkA-containing extracellular vesicles derived from sympathetic neurons promote spinal cord survival

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Introduction: In the developing peripheral nervous system, sympathetic circuits are tuned by limiting amounts of nerve growth factor (NGF) that is secreted by target organs (e.g. salivary gland or eye). NGF binds its receptor, TrkA, at the distal axon of innervating sympathetic neurons and internalizes onto retrogradely trafficked signaling endosomes. Insufficient NGF uptake leads to cell death. These neurons are innervated by autonomic motor neurons from the spinal cord, the survival of which is also NGF-dependent, despite lacking TrkA or access to soluble NGF. How these motor neurons survive without access to NGF is unknown. We hypothesize that TrkA positive extracellular vesicles (EVs) secreted from sympathetic neurons provide this neurotrophic support.
Methods: Using cultured sympathetic neurons from mouse superior cervical ganglia, we isolated EVs from serum-free, conditioned media by differential centrifugation and characterized them using nanoparticle tracking analysis, immunoblot assays, and

cryo-electron microscopy. To assess neurotrophic capacity, mouse spinal cord neurons were cultured with sympathetic EVs and cell death in ChAT+ motor neurons was assessed by Annexin V staining.
Results: The sizing distribution of isolated EVs show a predominant peak at 45–75nm with a shoulder of larger sizes, while protein analysis shows an enrichment of EV markers (Alix, CD63, CD81) and absence of intracellular markers (Cytochrome C, calreticulin). We identify TrkA as an EV cargo, which can be present in its active, phosphorylated state. Using a compartmentalized culture system, we show that TrkA originating from distal axons can be packaged in EVs secreted from the somatodendritic domain. In addition, sympathetic EVs promote motor neuron survival, suggesting they provide neurotrophic support.
Summary/Conclusion: We have rigorously characterized EVs from sympathetic cultures and shown that retrogradely trafficked TrkA can be a cargo. These EVs are capable of supporting autonomic motor neuron survival, suggesting a novel mechanism in sympathetic circuit tuning.

PT05.02 | Extracellular vesicles derived from plasma and skeletal muscle of SOD1-G93A mice models of Amyotrophic Lateral Sclerosis alter the phenotype and redox balance of recipient motor neuron-like cells

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Introduction: Amyotrophic Lateral Sclerosis (ALS) is a multifactorial neurodegenerative disease in the pathogenesis and progression of which extracellular vesicles (EVs) seem to play an important role. We investigated the effects of circulating and skeletal muscle-derived EVs from SOD1-G93A ALS mice models on motor neuron degeneration.
Methods: EVs from wild-type (WT) and SOD1-G93A mice (ALS model) at early and late stages of ALS development (90 and 120 days, respectively) were isolated from plasma and skeletal muscle (SkM) by ultrafiltration combined to size exclusion chromatography (UF/SEC), and characterized by Transmission Electron Microscopy (TEM), Atomic Force Microscopy (AFM) and EV-marker expression by Western Blot. To assess the contribution of plasma- and SkM-EVs in damage spreading, differentiated NSC-34 motor neurons were treated with SOD1-G93A-EVs for 24h and neurite length, neuron degeneration and oxidative stress were evaluated.
Results: TEM and AFM morphological analysis of plasma- and SkM-EVs revealed the presence of round-shaped EVs, expressing CD63 and CD9 and validating the quality of isolation procedure, with an increased amount and larger size distribution for SOD1-G93A EVs compared to the WT. The treatment of NSC-34 motor neurons with SOD1-G93A plasma- and SkM-EVs from 90 or 120 days induced a significant decrease in the neurite length, with a more prominent effect from EVs isolated at 90 days, vs EVs from WT animals. Furthermore, treated-motor neurons showed an increased oxidative stress, in terms of reduced antioxidant enzymes activity and increased downstream oxidative processes (i.e., protein carbonylation) when treated with SOD1-G93A-derived EVs vs WT-EVs.
Summary/Conclusion: Our results showed that during ALS development plasma- and SkM-EVs have detrimental effects on motor neuron from the onset of the disease, emphasizing the role of the systemic and local EVs crosstalk in ALS pathogenesis. Additional analyses are in progress to propose a mechanism of action of SOD1-G93A-derived EVs in the recipient motor neurons.
Funding: No external funding.
Keywords: amyotrophic lateral sclerosis (ALS), extracellular vesicles (EVs), neurodegeneration, oxidative stress, SOD1-G93A mice, disease progression

PT05.03 | Ectosomes from Schwann cells enhance the regeneration of neuromuscular junction

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Introduction: Neuropathy occurs when the peripheral nervous system (PNS) loses its ability to repair nerves by aging. While neurons are regenerating, Schwann cells work with immune cells to aid neurons. Inflammatory factors accumulate with age, causing chronic inflammation and interfering with nerve regeneration. Although Schwann cells have been suggested as a key player in PNS regeneration, how Schwann cells regulate the PNS in chronic inflammatory conditions is poorly understood. In

the present study, we hypothesize ectosomes of Schwann cells are influenced by inflammation, which affects nerve regeneration. Neuromuscular junction (NMJ) is the synapse between motor neuron and muscle fiber in the PNS. Recently, interest has shifted from the research of neuronal regeneration to the study of the NMJ recovery mechanism in order to overcome peripheral neuropathy. Thus, we investigated the role of Schwann cells in the formation of NMJ and the effect of chronic inflammation on Schwann cells-dependent NMJ function.

Methods: S16 Schwann cell line and mouse primary Schwann cell were treated with 2,4,6-Trinitrobenzenesulfonic acid(TNBS) to induce inflammation and Mice were treated with dextran sulfate sodium (DSS) and cardiotoxin (CTX) to induce both inflammation and neuromuscular junction regeneration. We observed NMJ defects with abnormal ectosome-related gene expression in aged mice. Schwann cell media were collected for ectosome extraction using ultracentrifuge and identified their composition.

Results: We treated mice and S16 Schwann cell line with chemicals and examined the levels of inflammatory cytokines to determine whether chronic inflammation was induced. Intriguingly, we found that in chronic inflammatory conditions, Schwann cells cannot make amount of ectosomes comparing with normal condition. We used Schwann cell ectosomes to rescue neuromuscular junction defects in zebrafish and found there were enhanced synaptic regeneration in zebrafish trunk.

Summary/Conclusion: In summary, we showed Schwann cell-derived ectosomes can rescue the defects of neuromuscular junction and can be negatively affected by chronic inflammation.

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Keywords: schwann cell, ectosome, neuromuscular junction, chronic inflammation

PT05.04 | Exosome-mediated delivery of Cas9 ribonucleoprotein complexes for gene editing in skeletal muscle

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Introduction: Due to the large range of genetic problems relating to muscles, muscle tissue is a crucial target for genetic engineering. However, gene targeting frequently had poor effectiveness and had transient effects. The toxicity and immune response were amplified by the large vector doses required to achieve the desired target effect. Furthermore, there are issues with the delivery strategy because the gene-carry virus tends to target the liver rather than muscle cells in high doses, which can have extremely harmful side effects.

Exosomes, naturally released nanovesicles, are the next generation of delivery tool because of their inherent biocompatibility, transportation capability, circulatory stability and engineerability. Exosome secretion and uptake into by skeletal muscle cells through an autocrine pathway have both been demonstrated. As a result, we created a technique to deliver Cas9 RNP with myoblast-derived exosome employing the ability to target homologous tissues.

Methods: For myoblast-secreted exosome isolation, exosome-depleted FBS was prepared by ultracentrifugation at 100,000g, 4°C for 20 hours. C2C12 myoblast were cultured in DMEM containing exosome-depleted FBS. Then, exosomes were isolated from the culture media using differential centrifugation. The exosomal protein was quantified by BCA assay and the presence of exosome was determined by western blot analysis. Cas9 proteins were and sgRNA were thoroughly combined to create Cas9 RNP complexes. Then, RNP complexes were loaded onto exosome using electroporation. Exosome RNP complexes were added into C2C12 culture medium to investigate the delivery of exosomal RNP. After incubation, the effectiveness of gene targeting was confirmed by western blot. For tissue targeting, exosome RNP complexes were injected into muscle and skeletal muscle tissue were collected for further gene targeting analysis.

Results: We identified the lysate from C2C12-derived exosomes expressed exosome specific markers but not the Golgi apparatus-associated protein. The delivery of exosome RNP resulted in the targeted gene indel frequency in vitro and in vivo. The targeting property of exosome RNP was demonstrated sequencing and target protein quantification in skeletal muscle. In addition, we observed that the deletion caused a failure in myogenic tube fusion.

Summary/Conclusion: In this study, we created an RNP-based CRISPR-Cas9 genome editing delivery tool that allows the generation of genetic disease models. RNP can be successfully electroporated into exosomes secreted from C2C12 and transported to skeletal muscle.

Funding: This work has been done by Molecular & Medical Genomics lab of Ji Eun Lee in Sungkyunkwan University and supported by the National Research Foundation of Korea government's MSIP (2021R1A4A2001389 and 2021R1A2C3004572 to J.E.L., 2021R1A6A3A13039817 and 2022K1A3A1A12080469 to S.W.).

PT05.05 | Macrophage-derived extracellular vesicles affect skeletal muscle homeostasis under lipoglucotoxic stress associated with high-fat diet

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Introduction: Obesity-induced diabetes is associated with systemic inflammation, increased numbers of skeletal muscle (SkM) resident macrophages, and SkM insulin-resistance (SkM-IR). Here, we determined whether lipoglucotoxicity induced by high-fat diets affects the release and function of macrophage-released extracellular vesicles (MEVs) and contributes to SkM-IR.
Methods: To mimic in vivo lipoglucotoxicity, THP-1-derived macrophages were treated with free fatty acids (FFA = palmitate+oleate) with/without 15 mM D-glucose (FFA/G15). Polarization markers, lipid profile, insulin-induced AKT phosphorylation (IIAP) and oxidative stress were quantified in treated-THP-1. Small and large EVs (sEV and lEV), collected by differential centrifugation, were used to treat C2C12 SkM cells. Lipid composition, insulin-sensitivity and RNA sequencing were performed on recipient C2C12.
Results: FFA-polarized THP-1 into a M2-CD163+phenotype. M2-CD163+ expressed pro-/anti-inflammatory cytokines, accumulated triacylglycerols (TAG), FFA, and had altered IIAP vs untreated-THP-1. C2C12 treated with lEV-FFA accumulated TAG, FFA and had reduced insulin-sensitivity vs untreated C2C12, mimicking FFA action on THP-1. lEV-FFA also modulated component from extracellular matrix in C2C12. sEV-FFA triggered TAG accumulation without affecting muscle insulin-sensitivity, reduced lipid oxidation and mitochondrial respiration in recipient C2C12. Thus, in a context of lipotoxicity, MEVs participate in maintaining muscle integrity. In the presence of FFA/G15, THP-1 exhibited a more pro-inflammatory phenotype, altered lipid composition, and increased oxidative stress. In addition, the beneficial action of MEVs was lost as MEVs from FFA/G15-treated THP-1 induced SkM-IR.
Summary/Conclusion: MEVs mirror macrophage phenotypic plasticity during the transition of lipo- to lipoglucotoxicity which is associated with the development of SkM-IR.
Funding: This work is supported by the FRENCH AGENCY OF RESEARCH (# ANR-MEXID-21-CE14-0081).
Keywords: high-fat diet, lipoglucotoxicity, macrophage-derived extracellular vesicles, muscle homeostasis

PT05.06 | Development of therapeutic extracellular vesicle enveloped-AAV vectors for muscle gene therapy

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Introduction: Translating gene therapy for muscular disorders is challenging because systemic delivery of high Adeno-Associated Virus (AAV) doses is required, increasing the risks of treatment-related immune responses and adverse events. To overcome these limitations, we developed muscle-targeting extracellular vesicle enveloped-AAV vectors (myoEV-AAV), aiming to increase transduction potency to skeletal muscle and shield AAV from neutralizing antibodies (NAbs).
Methods: We purified AAV using iodixanol (IDX) density gradient and anion exchange HPLC. We purified EV-AAV using IDX density gradient or size-exclusion chromatography (SEC). We characterized EV size, frequency, purity, and surface markers profile using NTA and western blots. We used Transmission Electron Microscopy (TEM) to determine EV-AAV structure and validate AAV packaging. We used a reporter system to determine EV-AAV transduction efficiency into HEK293 cells, myoblasts and muscle. We performed a neutralizing antibody assay (NAA) to determine EV-AAV resistance to NAbs.
Results: Our data showed better EV-AAV purification using SEC with which we obtained one Log higher EV yield and a 5-fold higher AAV yield. SEC had better resolution in separating eluates. Using TEM, EV-AAVs had an inflated rather than a collapsed structure. EV-AAV was ~2-fold more efficient than AAV in transducing HEK293s but had similar efficiency in transducing myoblasts when we used EVs packaging myotropic AAVs (MYOAAV). Intramuscular injection of EV-MYOAAV into the tibialis anterior of C57BL/6 mice showed higher transduction efficacy than that of MYOAAVs. Using NAA, EV-AAV showed resistance to serum AAV-NAbs. Finally, we successfully targeted EV-AAVs to myoblasts using muscle-specific peptides.
Summary/Conclusion: We have proof-of-concept for designing and producing muscle-specific EV-AAVs that transduce with high efficiency skeletal muscles and shield AAV from NAbs. Next, we will validate our results using systemic delivery of EV-AAVs.

Funding: Not applicable.
Keywords: adeno-associated virus (AAV), extracellular vesicles, muscular disorders, gene therapy, translational research, peptides, skeletal muscles

PT05.07 | Regionally regulated secretion of sonic-hedgehog-containing extracellular vesicles creates a concentration gradient in developing limb buds

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Introduction: Digit formation is essentially determined by a posteriorizing Sonic hedgehog (Shh) protein gradient in the mouse embryo limb buds at 10.5 days postcoitum. The most distal AER secretes FGF8 proteins to form a distal-to-proximal gradient of PI3K signaling. In perpendicular to this, Shh protein is propagated from the posterior organizer toward the anterior region through the mesenchyme, via EVs and/or cytonemes. However, the switching mechanism between these two propagation modalities and its relevance in development has been little understood.
Methods: The polydactylous KIF3B kinesin hypomorph mouse embryos were analyzed by immunohistochemistry, whole mount in situ hybridization, and electronmicroscopy. FGF8b-soaked bead injection to limb buds of wild type embryos, followed by whole embryo culture, was also performed. The primary cultured mesenchyme and KIF3B/talpid3-knockdown fibroblasts were subjected to cell biological and pharmacological analyses. Talpid3 activity and its KIF3B binding capacity were investigated by biochemistry and FLIM/FRET microscopy.
Results: In the limb buds of KIF3B-hypomorph mouse embryos, the distal-to-proximal PI3K signaling gradient was lost by its overall activation. In addition, the posterior-to-anterior gradient of Shh protein was also lost by its global dispersion. Experimental elevation of PI3K signaling by implantation of FGF8b-soaked beads in the inner layer reproduced the disorganization of Shh gradient. We detected that KIF3B essentially terminates PI3K signaling by transporting and stabilizing the PTEN-family protein Talpid3. Interestingly, PI3K signaling generally facilitated Shh-EV release through the general exosome releasing pathway involving nSMase2, and simultaneously suppressed Shh vesicle transport into the cytonemes. Thus, endocytosed Shh protein tends to be secreted as EVs in the outer layer, while retained into cytonemal punctata in the inner layer. This may provide differential Shh diffusion rates between these two layers, constituting a diffusion-and-trapping system for gradient formation.
Summary/Conclusion: Here we show the experimental evidence for a regionally regulated secretion of Shh-EVs creating a concentration gradient in developing mouse limb buds. We will also discuss on possible application of this PI3K-mediated Shh-EV biogenesis by presenting some preliminary data.
Funding: This work was supported by JSPS KAKENHI grant numbers JP23000013, JP16H06372, and JP22K06246 to N.H; by Strategic Research Program for Brain Sciences from AMED (JP20dm0107084) to N.H. and Y.T., and by a GAP-Fund (8th and 12th terms) from Univ Tokyo and an AMED Grant (JP22ym0126805) through the TR Center, The University of Tokyo Hospital, to Y.T.
Keywords: sonic hedgehog, morphogen gradient, regulated EV biogenesis, FGFR, PI3K signaling, polydactyly

PT05.08 | Comparison of human and rat mesenchymal stem-derived extracellular vesicles in motor recovery and body growth following severe spinal cord injury in rat

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Introduction: Traumatic spinal cord injury (SCI) results in neurological deficits and metabolic complications. The high incidence of SCI in young adults presents special challenges related to bone and muscle growth in the young SCI patient population. Several studies report that systemic pro-inflammatory cytokines are associated with growth failure and muscle wasting. Previously, we demonstrated that intravenous (IV) mesenchymal stem/stromal cell (MSC) treatment has a therapeutic effect on recovery in experimental models of SCI, as does IV delivery of small extracellular vesicles (sEVs) derived from cultured rat MSCs. Mechanistically, we demonstrated that IV-delivered rat MSC-sEVs were taken up by M2 macrophages at lesion sites and were associated with an increase in production of anti-inflammatory cytokines. In this study, we evaluated the therapeutic efficacy of human and rat MSC-sEVs on functional motor recovery and body growth following SCI in young adult rats.
Methods: Severe SCI rats were randomized into three different treatment groups (human and rat MSC-sEVs and a PBS group) on day 7-post SCI. We evaluated functional motor recovery using Basso–Beattie–Bresnahan (BBB) score and body growth by measurements of food consumption and body size weekly until day 70 post-SCI. Trafficking of sEVs after IV infusions in vivo or

uptake of sEVs in vitro utilized sEVs labeled with DiR. For evaluation of macrophage phenotype at the lesion and cytokine levels, we analyzed gene expression by RT-qPCR and protein expression levels by western blots, ELISA and immunohistochemistry.
Results: IV delivery of both human and rat MSC-sEVs improved functional motor recovery after SCI and restored normal body growth in young adult SCI rats, indicating a broad therapeutic benefit of MSC-sEVs and a lack of species specificity for these effects. Mechanistic analyses revealed that human MSC-sEVs are selectively taken up by M2 macrophages in vivo and in vitro, consistent with our previous observations of rat MSC-sEV uptake. Furthermore, infusion of human or rat MSC-sEVs resulted in an increase in the proportion of M2 macrophages and a decrease in the production of the pro-inflammatory cytokines TNF-α and IL-6 at the injury site, as well as a reduction in systemic serum levels of TNF-α and IL-6. Growth hormone receptors and IGF-1 levels were increased in the liver.
Summary/Conclusion: Both human and rat MSC-sEVs promote recovery of motor function and body growth after SCI in young adults, a vulnerable SCI population, via cytokine modulation of growth-related hormonal pathways. This represents an important step towards developing a human cell-related product for therapeutic applications in SCI.

PT06: Cardiovascular EVs

Location: Hall 4A

16:45 - 18:45

PT06.01 | Rivaroxaban in combination with low-dose aspirin is associated with a reduction in pro-inflammatory and pro-thrombotic circulating vesicle signatures in patients with cardiovascular disease

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Introduction: Despite secondary prevention with aspirin (ASA), patients with stable cardiovascular disease (CVD) remain at heightened long-term risk of major cardiovascular events (MACE). The Cardiovascular Outcomes for People Using Anticoagulation Strategies (COMPASS) double-blind randomized clinical trial, including nearly 28,000 CVD patients, demonstrated that aspirin combined with low-dose Rivaroxaban (R) significantly decreased the incidence of MACE by 24% and cardiovascular mortality by 18% compared with ASA alone. However the underlying molecular mechanisms remain elusive. Extracellular vesicles (EVs) are crucial messengers regulating a myriad of biological/pathological processes and are highly implicated in CVD. We hypothesised that circulating EV profiles reflect the cardioprotective properties of adding R to ASA.
Methods: A cohort of stable CVD patients (n = 40) who participated in the COMPASS trial (NCT01776424) and were previously randomised to receive ASA, were prospectively recruited following informed written consent according to the declaration of Helsinki and assigned a revised regimen of open label ASA+R. Blood samples were obtained at baseline (ASA only) and 6-months follow-up (ASA+R). Plasma EV concentration, size and origin were analysed by NTA and flow cytometry. EVs were enriched by ultracentrifugation for proteomic analysis.
Results: ASA+R fundamentally altered small (< 200nm) and large (200-1000nm) EV concentration and size, compared to ASA alone. Crucially, levels of platelet-derived, tissue-factor positive and myeloperoxidase-positive EVs significantly decreased at follow-up, potentially contributing to the reduced incidence of thrombotic events associated with adding R to ASA. Comparative proteomic characterisation further revealed a significant decrease in highly pro-inflammatory protein expression at follow-up.
Summary/Conclusion: The observed changes in EV subpopulations, together with the differential protein expression profiles, suggest amelioration of an underlying pro-inflammatory and pro-thrombotic state when low-dose R is added to ASA in patients with CVD, which may be of clinical relevance towards understanding the fundamental mechanism underlying the previously reported superior cardiovascular outcomes associated with this antithrombotic regimen.
Keywords: cardiovascular disease, anti-thrombotic therapy, rivaroxaban, aspirin

PT06.02 | Stem cell exosomes target HIF-1α and Runx2 to attenuate vascular remodelling in pulmonary arterial hypertension

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Introduction: Pulmonary arterial hypertension (PAH) is characterized by extensive pulmonary arterial remodelling. Although mesenchymal stem cell (MSC)-derived exosomes provide protective effects in PAH, MSCs exhibit limited senescence during in vitro expansion compared to the induced pluripotent stem cells (iPSCs).In this study, we determined the efficacy and mechanism of iPSC-derived exosomes (iPSC-Exos) in attenuating PAH in rats with monocrotaline (MCT)-induced pulmonary hypertension.
Methods: Exosomes were isolated from the supernatant of the iPSC culture. The MCT-treated groups of rats were randomized to four groups, that is, (1) saline; (2) iPSC-CM prophylactic treatment initiated after MCT injection; (3) iPSC-Exo prophylactic treatment initiated after MCT injection; and (4) iPSC-Exo therapy initiated 2 weeks after MCT injection. The potential impact of iPSC-Exos on MCT-induced pulmonary arterial remodelling and PAH were evaluated in vitro and in vivo.
Results: Both prophylactic and therapeutic iPSC-Exo treatment effectively prevented the wall thickening and muscularization of pulmonary arterioles, improved the right ventricular systolic pressure, and alleviated the right ventricular hypertrophy in MCT-induced PAH rats. Pulmonary artery smooth muscle cells (PASMCs) derived from MCT-treated rats (MCT-PASMCs) developed more proliferative and pro-migratory phenotypes, which were attenuated by the iPSC-Exo treatment. Moreover, the proliferation and migration of MCT-PASMCs were reduced by iPSC-Exo with suppression of PCNA, cyclin D1, MMP-1, and MMP-10, which are mediated via the HIF-1alpha and PAK1/AKT/Runx2 pathways.
Summary/Conclusion: IPSC-Exo are effective at preventing and reversing pulmonary hypertension by reducing pulmonary vascular remodelling and may provide an iPSC-free therapy for the treatment of PAH.
Funding: 1. The Ministry of Science and Technology, Taiwan (grant numbers: 108-2314-B-075B-009 and 108-2314-B-075B-007-MY2). 2. The Kaohsiung Veterans General Hospital (grant numbers: VGHKS110-063 and VGHKS110-077, and VGHKS110-078).
Keywords: pulmonary arterial hypertension, induced pluripotent stem cells, exosomes, vascular remodelling

PT06.03 | Vascular injury derived exosomes triggers systemic autoimmunity

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Introduction: Apoptotic exosomes (ApoExo) are vascular injury derived extracellular vesicles (EVs) released by apoptotic endothelial cells (EC). Their comprehensive characterization showed distinct size, protein, ARN profile and enzymatic activity from classical apoptotic bodies. We have shown that ApoExo accelerated rejection in association with circulating anti-LG3/perlecan autoantibodies (AutoAb). We hypothesize that ApoExo stimulate specific B cells that exist in the normal immune repertoire to secrete AutoAb and that pro-inflammatory condition prevalent in the context of organ transplantation and autoimmune diseases can amplify this response.
Methods: B cells from spleen or peritoneal cavity (pc) were isolated from C57Bl/6 mice. After in vitro stimulation with Toll-Like-Receptor (TLR) agonists, anti-LG3 levels were assessed in supernatants by ELISA. Serum-free medium conditioned by apoptotic murine EC from C57Bl/6 mice is fractionated based on sequential centrifugations up to 200 000g and ApoExo are isolated and quantified according to MISEV2018 requirements. C57Bl/6 mice were infused with ApoExo or vehicle every second day for 3 weeks. At sacrifice, splenocytes and pc lymphocytes were analyzed as above and by flow cytometry. Circulating AutoAb were quantified by ELISA /microarray and cytokine levels were evaluated by multiplex.
Results: B cells specific to LG3 were found in the pc of C57Bl/6 mice and produced anti-LG3 AutoAb when stimulated with TLR 1/2, 4, 7 and 9 agonists. Interestingly, these cells disappeared from the pc of mice infused with ApoExo. ApoExo infusion also triggered circulating IL-23 –IL17 autoimmune axis, increased splenic Germinal center B cell, increased total circulating IgG and significant production of classical AutoAb such as anti-dsDNA, anti-SSA-B anti-Sm/nRNPand anti-LG3.
Summary/Conclusion: These observations suggest a specific role for vascular injury derived EVs ApoExo in modulating the production of AutoAb of importance in autoimmune diseases and transplant rejection.
Funding: Canadian Institutes of Health Research (CIHR) and Natural Sciences and Engineering Research Council of Canada (NSERC).

PT06.04 | Human saphenous vein smooth muscle cells uptake extracellular vesicles from adeventitial cells

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Introduction: The human saphenous vein (HSV) is the most commonly used conduit in patients undergoing lower extremity arterial bypass surgery (LEABS). We examined the role of extracellular vesicles (EVs) in vein wall cell communication as a potential mechanism for vein graft adaptation to the arterial environment.
Methods: HSV remnants were obtained from patients undergoing LEABS, and the intimal-medial layer containing SMCs was dissected from the adventitia after wiping off the endothelium. Primary HSV SMC lines were created from cells migrating from the tissues were cultured in DMEM with 20% fetal bovine serum (FBS) and then changed to SMC complete medium (CELL APPLICATION). Human aortic SMCs and fibroblasts were also used. EVs were extracted from each cell line using ultracentrifugation (350,000 rpm, 75mins, 4°C), and their diameters measured by a nanoparticle tracking system. SMC proliferation was measured by MTS assay or cell counting. PKH-26 staining for uptake of EVs by SMCs from fibroblasts and SMC cell proliferation after addition of EVs were assessed. RNA sequencing was performed to assess microRNA differential expression from EVs derived from HSV adventitial cells vs HSV SMCs.
Results: HSV adventitial cells co-cultured with HSV SMCs significantly inhibited the proliferation of SMCs (N = 5). EVs diameters from each cell line were approximately 100 - 110nm, indicating small EVs (sEVs). PKH staining demonstrated sEVs derived from the adventitial cells underwent uptake into HSV SMC cell bodies. Aortic SMC proliferation was significantly inhibited by adding sEVs derived from human aortic fibroblasts (N = 3); whereas HSV SMC proliferation was patient dependent; with increase for one patient and decrease for two patients. In total, 2143 microRNAs were detected from EVs derived from HSV adventitial cells or SMCs. A >2-fold difference was detected in 45 microRNAs out of 127 microRNAs with significantly different expression between the two cell types.
Summary/Conclusion: sEVs derived from the adventitial cells influenced on SMC cell proliferation. EVs could be a new target to understand and possibly modulate vein graft adaptation through HSV wall cell communication.
Keywords: human saphenous vein, cell-cell interactions, small extracellular vesicle

PT06.05 | Intravenous injection of surface modified exosomes delivers siRNA selectively to pro-atherogenic endothelial cells

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Introduction: Exosomes (EXOs), one of extracellular vesicles derived from cells, have been focused on as a promising drug delivery system. However, a systemic delivery technique for efficiently delivering EXOs to a specific tissue or cell has not yet been developed. We previously identified a dysfunctional endothelium-specific peptide (DSP) that specifically binds to the surface of dysfunctional vascular endothelial cells (ECs) caused by low oscillatory shear stress (OSS), and1 we developed a method for successfully loading diverse therapeutic materials into EXOs using extracorporeal shock waves (ESW). In this study, we investigated the targeted delivery of short interfering (siRNA) by systemic administration to disturbed flow-induced atherosclerotic dysfunctional ECs using DSP-coated EXO as the delivery vehicles.
Methods: Using click chemistry, DSP was conjugated to the surface of EXOs derived from cow milk (DSP-EXO). ICAM-1-specific siRNA (siICAM-1) was encapsulated into the DSP-conjugated exosomes (DSP-EXO-siICAM-1) by ESW. EXO and DSP-EXO are treated to ECs cultured under pro-atherogenic oscillatory shear stress (OSS, ± 5 dyne/cm^2) and anti-atherogenic laminar shear

stress (LSS, 20 dyne/cm^2), respectively. A mouse model of flow-induced atherosclerosis was produced by partial ligation of the left carotid artery (LCA).
Results: DSP-EXO, unlike EXO, preferentially attached to ECs cultivated under OSS rather than LSS. Following injection into the tail vein, DSP-EXO attached preferentially to the OSS area of the LCA in the mouse model, whereas EXO did not. Furthermore, DSP-EXO-siICAM-1 significantly reduced endothelial ICAM-1 expression in ECs under OSS conditions and in the LCA region in the mouse model.
Summary/Conclusion: The results of this study demonstrate that surface-engineered EXOs can be delivered selectively to specific tissues or cells by systemic delivery, such as intravenous injection. This is anticipated to be a promising strategy for future development of EXO-based therapies.
Keywords: exosome, target delivery, surface engineering, dysfunctional endothelial cell-specific peptide (DSP), atherosclerosis

PT06.06 | The effect of UVA light/8-methoxypsoralen exposure used in ECP treatment on platelets and extracellular vesicles

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Introduction: Extracorporeal Photopheresis (ECP) is a leukapheresis based treatment for Cutaneous T-Cell Lymphoma (CTCL) which takes advantage of the cellular lethal effects of UVA light in combination with a photoactivated drug, 8-methoxypsoralen. 25% of patients treated with ECP do not respond, however the underlying mechanisms are unknown. Platelets, along with leukocytes, are both processed through ECP and subsequently transfused back into the patient, delivering potent immunomodulation. Platelets are a rich source of EVs and are key mediators in thromboinflammatory oncological progression. Previous work completed in our lab has shown the mechanical ECP processes activates platelets, thereby releasing EVs to be exposed to UVA light/8-methoxypsoralen treatment. The effect of exposing platelets and their EVs directly to UVA/8-methoxypsoralen is currently unknown.
Methods: Healthy aged and sex matched donors were recruited, facilitating platelet-rich plasma (PRP) isolation for ECP dose UVA light and/or 8-methoxysporalen in-vitro exposure. Platelet activation was assessed in PRP by 96-well plate aggregometry and activation markers quantified in plasma using immunoassays. EV size and concentration was characterized by Nanoparticle Tracking Analysis and Flow Cytometry.
Results: We found that UVA light and 8-methoxypsoralen treatment of PRP does not induce platelet aggregation or a change in cleavage of soluble p-selectin. Interestingly, secreted platelet factor 4 levels subtly increase upon UVA exposure alone(p-value = 0.029), which was reversed upon dual UVA/8-methoxypsoralen treatment(p-value = 0.094). Strikingly, levels of small and large EV size/ concentration remain unchanged by UVA/8-methoxypsoralen treatment.
Summary/Conclusion: We have found that the combination of UVA light and 8-methoxypsoralen used in ECP to treat CTCL reassuringly does not alter important circulating EVs. Slightly increased levels of anti-angiogenic PF4 denoted may be beneficial, having previously been found inhibit tumor proliferation and migration.
Funding: Kindly supported by an investigator-led award from Mallinckrodt to PM.

PT06.08 | Analysis of the effect of foam cell-derived extracellular vesicles on the environment surrounding coronary artery plaques

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Introduction: Ischemic heart disease (IHD) has been the leading cause of death worldwide for more than several decades, and the research for effective treatments for IHD is an important issue. To prevent the development of IHD, it is necessary to clarify the molecular mechanism of plaque progression and rupture. The purpose of this study was to clarify the molecular mechanism of plaque progression and rupture by analyzing the effects of extracellular vesicles (EVs) derived from foam cells (FCs) on cells surrounding the plaque.

Methods: Smooth muscle cells (SMCs) and THP-1 cells, a human monocyte-derived cell line differentiated to resemble macrophages, were loaded with enzyme-modified non-oxidative low-density lipoprotein for 96 hours for the formation of FCs. Then, the cells were cultured in a serum-free medium for 48 hours, and EVs were isolated from the culture supernatant by ultracentrifugation (>100,000 × g for 70 minutes). Particle size and particle concentration of the obtained EVs were confirmed by nanoparticle tracking analysis. CD9 and CD63 were confirmed by Western blotting. The FC-derived EVs were added to SMCs and endothelial cells (ECs), and we analyzed expression levels of atherosclerosis-related genes by qPCR. We also quantified the cell migration and proliferation by IncuCyte (SARTORIUS). In addition, we measured cholesterol efflux capacity with the cholesterol efflux assay kit (Sigma-Aldrich). SMCs and ECs treated with non-FC-derived EVs or PBS were used as control.
Results: The FC-derived EVs-added ECs decreased the expression of genes related to intercellular adhesion ability compared to the non-FC-derived EVs-added ECs. Compared to the PBS-added SMCs, the FC-derived EVs-added SMCs increased cell migration and cholesterol efflux capacity. Both effects were more significant in the group that was treated with EVs from THP-1-derived FCs.
Summary/Conclusion: FC-derived EVs may demonstrate anti-atherosclerotic effects by affecting SMCs and ECs around plaques with EVs.
Funding: This work was supported by the Project for Promotion of Cancer Research and Therapeutic Evolution (P-PROMOTE) grant number: JP22ama221405 (to Y.Y.) from the Japan Agency for Medical Research and Development (AMED) and Practical Research for Life-Style related Diseases including Cardiovascular Diseases and Diabetes Mellitus grant number: JP20ek0210145 (to T.O.) from AMED.
Keywords: extracellular vesicles, foam cells, smooth muscle cells, endothelial cells, atherosclerosis

PT06.09 | Reduced loading of Annexin A1 in extracellular vesicles derived from atherosclerotic plaques in lower extremity artery disease results in the release of various chemokines and the expression of ICAM-1 in vascular smooth muscle cells

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Introduction: There is limited research on the role of extracellular vesicles (EVs) derived from atherosclerotic plaques, which are composed of various cell types including endothelial cells, smooth muscle cells (SMCs), and macrophages and may contribute to the progression of atherosclerosis. To investigate the roles of EVs from atherosclerotic plaques, we compared the function of EVs derived from plaques removed by femoral endarterectomy (FEA) to EVs derived from healthy arteries in vitro.
Methods: Plaques and healthy samples (iliac arteries) were collected from 12 patients undergoing FEA and from 5 patients with abdominal aortic aneurysm undergoing open surgical repair, respectively. The sampling was approved by the local ethics committee. Single cell RNA sequencing was performed on 3 plaques and 1 healthy sample. 4 samples from each group were incubated in serum-free medium with 10% oxygen for 4 hours. EVs were isolated from the supernatants by ultracentrifugation (210,000 g, 70 min) and characterized by Nanosight and Western blotting (WB). EVs from both groups were added to SMCs, and their RNAs were analyzed by qPCR. The chemokine profiles were analyzed using a Human chemokine array kit, which can profile 31 chemokines. The adhesion ability of THP-1 cells to SMCs treated with EVs was evaluated using a THP-1 cell adhesion assay. Proteome analysis of EVs derived from 5 plaque samples was performed.
Results: Single-cell RNA sequencing showed that the proportion of CD8 T cells and pro-inflammatory macrophages tends to be higher in plaques. Several chemokines that induce immune cell migration were significantly upregulated in SMCs treated with plaque-derived extracellular vesicles (P-EVs) as determined by qPCR and chemokine array. In addition, the mRNA level of ICAM-1, which is involved in immune cell adhesion, was upregulated in the P-EVs group. Functionally, P-EVs increased the ability of SMCs to adhere to THP-1 cells. Proteome analysis revealed that P-EVs carried several types of annexins. The expression level of Annexin A1, which has anti-inflammatory effects, was significantly lower in P-EVs by WB.
Summary/Conclusion: The amount of Annexin A1 in P-EVs was decreased, indicating that the anti-inflammatory effects of arteries may be attenuated via the upregulation of ICAM-1 and several chemokines.

PT07: EV-Cargo Characterization I

Location: Hall 4A

16:45 - 18:45

PT07.01 | Mining the proteome of ovarian cancer extracellular vesicles with complementary thermolysin proteolysis

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Introduction: The proteome of extracellular vesicles (EVs) is comprised of a diverse range of protein ligands/receptors, enzymes, and cellular machinery that is often characterized by the identification of tryptic peptides during tandem mass spectrometry (MS/MS). As a result, proteomic depth (# of proteins and sequence coverage) is limited to peptide products from proteolysis at the C-terminal of lysine or arginine. Thermolysin cleaves at the N-terminal of aliphatic amino acids (FMVAIL) and our in silico analyses predict the parallel detection of these peptides will increase proteomic depth of EVs during exploratory MS/MS. Specifically, we hypothesized that thermolysin would provide complementary sequence coverage to TrypLysC, in return increasing proteomic depth.
Methods: EVs were purified using ultracentrifugation from conditioned media of primary ascites cell lines or ascites fluid from donors with high-grade serous carcinoma. EV number and size distribution were characterized by nanoscale flow cytometry using Apogee A60. EVs were lyophilized and 25μg of protein was digested either with TrypLysC at 37^{0}C for 18hrs or Thermolysin at 75^{0}C for 1hr followed by desalting using C18 stagetips. Offline high-pH reverse phase or strong cation exchange in stagetips was used to decomplexify the peptide pool. 500ng of each peptide fraction was analyzed across 75 or 260min gradients using reverse-phase ultraperformance liquid chromatography coupled to MS/MS on a QE Plus operating in data-dependent acquisition. Peptide/protein identifications were compared between MaxQuant, FragPipe, and PEAKS de novo sequencing software.
Results: Thermolysin proteolysis identified >100 additional proteins compared to TrypLysC alone. PEAKS] provided largest number of unique protein IDs for both TrypLysC and Thermolysin. Notably, Thermolysin provided complementary peptide identification, in return, a >2-fold increase in mean sequence coverage was obtained. Limitations of Thermolysin proteolysis included a need for missed cleavages, an increased number of peptides per protein, and a narrow distribution of peptides based on length, charge, or hydrophobicity. As a result, we are currently investigating the use of high-field asymmetric waveform ion mobility spectrometry to provide additional peptide fractionation and removal of low-charge peptide species during MS/MS.
Summary/Conclusion: Our data indicate that complementary proteolysis of the EV proteome with Thermolysin can be used as a tool to increase the proteomic depth obtained during MS/MS analyses.
Funding: Canadian Institute of Health Research, NSERC, Canadian Cancer Society.
Keywords: ovarian cancer, proteomics, mass spectrometry, sample preperation

PT07.02 | Proteomic analysis of cerebrospinal fluid size-exclusion chromatography fractions

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Introduction: Cerebrospinal fluid (CSF) is located near the central nervous system and is secreted mostly by cells in the choroid plexus. The cargo of extracellular vesicles (EVs) reflects the cells of origin, making CSF EVs a promising way of studying the brain. Here, we isolated EVs from canine CSF and analysed the proteome of three size-exclusion chromatography (SEC) fractions and CSF with (CSF depl.) and without depletion of common plasma proteins.
Methods: EVs were isolated by ultrafiltration combined with SEC from three 6ml pools of CSF collected from euthanised dogs donated for research. SEC fractions were analysed with nanoparticle tracking analysis, transmission electron microscopy

(TEM), and immunoblotting. Proteomic analysis was performed using liquid chromatography with tandem mass spectrometry on fractions 3–5, CSF, and CSF depl.
Results: Characterisation indicated fraction 3 to contain the highest number of EV-like particles as expected. Still, TEM imaging showed populations of smaller particles in fractions 4 and 5. The proteomic analysis revealed that the groups shared 537 proteins and that the highest number of unique proteins (n = 211) was in fraction 3. Gene set enrichment analysis (GSEA) showed greatest enrichment of EV associated proteins in fraction 3. Fraction 4 was also significantly enriched but with a higher p-value. GSEA with a supermere related protein list revealed enrichment only in fraction 3 and no enrichment with an exomere list. Heatmap analysis of proteomic data showed highest intensity of apolipoproteins in fractions 4 and 5, whereas markers for small and large EVs peaked in fraction 3.
Summary/Conclusion: These data indicate that fraction 3 contains the majority of EVs and that the later fractions are more enriched with lipoproteins, although the contents also overlap. Highest number of individual proteins in fraction 3 shows the potential of SEC purification for analysis of lower abundant proteins in CSF EVs.
Funding: The study was funded by Academy of Finland and Finnish Veterinary Foundation.
Keywords: canine, extracellular vesicles, cerebrospinal fluid, proteomics

PT07.03 | Proteomic analysis and biomarker discovery in plant cell suspension culture derived extracellular vesicles

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Introduction: Despite promising therapeutic applications, our knowledge of plant extracellular vesicles (EVs) are severely lagging compared to their mammalian counterparts. The harsh isolation methods that are common in plant EV studies, such as blending or juicing, contaminates the final EV isolates with large quantities and varieties of cellular materials, casting doubt as to whether the results observed are solely due to plant EVs. A lack of standardized isolation methods and biomarkers for the characterization of plant EVs further exacerbates this issue. Further developments in the field of plant EV research depend on the advancement of high-fidelity isolation and characterization methods.
Methods: To address this, we used plant cell suspension cultures and density cushion ultracentrifugation to isolate EVs of N. tabacum, S. rebaudiana and V. vinifera, conducted a proteomic analysis of N. tabacum sEVs, and identified potential plant EV biomarker proteins through an orthologue discovery study of eight plant EV proteomes against well-defined human EV biomarker proteins.
Results: Suspension cultures provided a more abundant, cleaner, and more standardized source of plant EVs. A total of 198 proteins were identified in N. tabacum sEVs, which exhibited similar gene ontology enrichment profiles to human EVs. Using BLAST analysis between human and plant EV proteins, we identified 24 potential plant EV biomarkers, conserved across at least five of the eight plant species studied.
Summary/Conclusion: Here, we demonstrated the potential of plant cell suspension cultures as a promising alternative to plant lysates for the isolation of plant EVs. Our proteomics studies suggest several potential plant EV biomarkers, which show a degree of conservation between different plant species. Finally, the high degree of homology observed between human and plant EV proteins suggests that they may take part in cross-kingdom interactions, with potential therapeutic effects.
Keywords: plant EVs, plant EV characterization, EV biomarkers

PT07.04 | Analytical toolbox for reliable characterization of extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are membrane vesicles secreted by cells and distributed widely in all biofluids as well as conditioned media. Due to their role in intercellular communication, they are receiving attention for therapeutic and diagnostic applications. The first step to better understand EVs and to utilize them as therapeutic and diagnostic tools is to purify them from variety of biofluids. Robust and well-established orthogonal techniques for quantification and characterization of individual EVs are required to utilize them as therapeutic and diagnostic tools.

Methods: We have established capabilities to reliably analyze EVs samples using three different orthogonal techniques; 1) Nanoparticle tracking analysis (NTA), a scattering and fluorescence-based technique to determine the size and concentration of nanoparticles, is the mostly widely used technique in the field. 2) Virus Counter 3100 platform (VC3100), a fluorescence-based technique with similar principles as flow cytometry with critical enhancements to enable the effective detection of smaller particles. 3) Analytical HPLC (PATFix), a powerful technology to analyze the EV samples using three different detectors: Multi angle light scattering, UV and fluorescence. Using these three platforms, EV samples can be analyzed comprehensively, reliably, and reproducibly.
Results: We have evaluated the performance of this analytical toolbox to characterize EV samples. Fundamental studies were first performed to understand the detection range as well as their limitations to understand the results more reliably. We showed that this analytical toolbox could be used to characterize EV samples from the MSC conditioned media to each step through the purification process including clarification, tangential flow filtration, and chromatography.
Summary/Conclusion: This analytical toolbox will allow researchers in the field to understand the EV samples, in particular, the relevant ratio of EVs compared to other non-EV components of the conditioned media. Additionally, this toolbox allowed us to optimize the downstream processing to achieve high yield and high purity EV samples.
Funding: N.A.
Keywords: analytical toolbox, reliable characterization, downstream processing

PT07.05 | Single EV SERS profiling for monitoring molecular progression in Glioblastoma

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Introduction: Technologies that enable non-invasive monitoring and diagnostic accessibility are essential for cancers with low survival rates such as Glioblastoma (GBM). Liquid biopsy offers the potential for rapid, non-invasive continuous assessment of cancer status in GBM with clinically actionable results. EVs, lipid-based heterogeneous structures shed by cells, are a promising platform for liquid biopsy application in GBM reflecting their properties and diversity.
Methods: By combining the working principles of plasmonic nanocavities, and the chemistry of materials, we have developed a sensitive and easy-to-use micro-chip platform to perform surface-enhance Raman spectroscopy (SERS) identification on single EVs derived from glioblastoma stem cells and blood samples in a less than 10 μl amount of fluid. The models used in this study, include glioma U373 parental cell line and its isogenic counterpart expressing oncogenic EGFRvIII receptor (U373vIII cells, as well as another unrelated glioma U87 parental cells) and their corresponding variants obtained by transfection of EGFRvIII oncogene. In addition, glioma stem cells (GSC83) naturally expressing wild-type EGFR and EGFRvIII, and GSC1005 (wild-type) cells and their counterparts in which EGFRvIII was knocked out, were analyzed.
Results: The micro-chip performance was benchmarked by identifying and distinguishing signals from single-EVs with an accuracy of 91.37% in distinguishing EVs populations. This information was then combined to differentiate between tumour EVs and platelet-derived particles in patient blood samples with 87% accuracy.
Summary/Conclusion: single EVs SERS can capture remarkable and clinically significant information about cellular heterogeneity in cancer through the information contained in their populations.
Funding: Canadian Cancer Society (255878 CCSRI), Natural Science and Engineering Research Council (NSERC, G247765), New Frontiers in Research Fund(250326), Canada Foundation for Innovation (CFI, G248924).
Keywords: single-EV profiling, surface-enhanced Raman spectroscopy, glioblastoma

PT07.07 | "Lumin-EV, a multiplexed assay for the analysis of EV surface proteins"

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Introduction: We developed an immunoassay based on Luminex principle, in order to evaluate in a multiplexed format, surface protein composition of intact EVs in unprocessed plasma.
Methods: plasma samples. EVs bound to capture beads are detected with biotinylated antibodies against distinct EV surface proteins followed by streptavidin-PE. Luminex reader separates capture beads by index colors and measures PE levels

associated with each color range. We developed assays that capture tetraspanins, synaptic proteins, or cell-specific markers, which are subsequently detected with one more tetraspanin antibody. Each assay also contains an internal negative control (IgG).
Results: Assay specificity for EVs was shown by abolishing signal by EV depletion via size exclusion or detergent treatment. Capture specificity is indicated by detection of neuronal markers GAP43 and L1CAM in EV preparations from neuronal but not HEK293 cell culture. GAP43-positive neuronal EVs are detected with antibodies for tetraspanin or another neuronal (L1CAM) but not erythrocyte marker (CD235). Our assay for synaptic EV proteins GluR2, Syntaxin1, NRGN, and GAP43, with CD63 and CD9 for normalization and isotype control to exclude non-specific binding generates signals for all analytes, significantly above non-specific background and shows dilution-dependent linearity. Importantly, the results were not affected by multiplexing, as similar values were obtained in a multiplexed and single-plexed formats. When applied to plasma samples from patients with Parkinson's disease, Alzheimer's disease (AD) and age-matched controls (N = 20 each) GluR2, Synataxin1, and GAP43 showed significant reduction in AD.
Summary/Conclusion: The intact EV LuminEV assay has high potential as a user-friendly medium-throughput method for surface protein detection on plasma EV, to be used in fundamental studies and biomarker analysis.
Keywords: intacg = t EV assay, luminex

PT07.08 | Simultaneous analysis of protein and RNA cargo of EV by flow cytometry

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Introduction: The Thermo Fisher PrimeFlowTM RNA assay was originally developed to allow for simultaneous detection of specific RNA and protein targets by flow cytometry. After antibody staining (optional intracellular staining), sequence specific probes are hybridized to miRNA or mRNA sequence of interest. This step is followed by detection of fluorescent branched DNA (bDNA), leading to an amplification of the signal, even at low copy numbers. This assay enables detection of up to four distinct RNA targets at the single-cell level.
Methods: As PrimeFlowTM has been established for cells and extracellular vesicles (EVs) are significantly smaller, permeabilization required for shuttling bDNA and probes into the lumen may cause loss of cargo, surface protein, and/or elimination of smaller vesicles. We are currently optimizing this critical step for EVs alongside evaluation of the conventional PrimeFlowTM protocol.
Results: Our reference is an established PrimeFlowTM kit for a miRNA target that, in literature, has been described as cargo for cells and EVs alike.
Summary/Conclusion: miRNA cargo of EVs has been described as having biomarker potential in context with different types of cancer, or infectious diseases. Using PrimeFlowTM for EVs can enable analysis of liquid biopsies and biomarker discovery of cancer specific miRNA signatures. This technology allows for the use of one application that is widely available to researchers to look at two different types of biomolecules, with the added bonus of conserving precious sample. In conclusion, PrimeFlowTM will allow for tailored solutions to different research questions and help streamline production workflows in the rapidly growing field of EV-based diagnostic therapeutic approaches.
Keywords: flow cytometry, RNA cargo, PrimeFlow

PT07.09 | Transcriptomics of Cerebrospinal Fluid (CSF) Extracellular Vesicle (EV) RNA – A Low Volume Pilot Study

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Introduction: RNA sequencing of EV RNAs isolated from small CSF volumes is challenging and the number of publications on this topic are limited. Traditionally, RNA-seq of CSF has been performed either on total CSF or from EVs enriched from larger volumes, typically milliliters. Due to the small volumes available from human biorepositories, methods that allow complete sequencing of EV RNA are especially needed.
Methods: The Institutional Review Board of the Oregon Health & Science University approved all human participant procedures. CSF from six healthy participants was collected via lumbar puncture under fasting conditions. A volume of 300 μL from each donor was pooled, mixed and aliquoted into three 500 μL samples. Each replicate sample was concentrated using a 30 kD cut-off ultra-filtration column to a final volume of 150 μL. Each concentrated sample underwent size exclusion chromatography (SEC)

using 35 nm resin. The EV enriched fractions were pooled from each replicate at a final volume of 800 μL. RNA was isolated using the Urine miRNA purification kit (Norgen) and eluted with 30 μL of elution solution. RNA QC, library prep, RNA-seq and data analysis were performed at the Icahn School of Medicine using their liquid biopsy analysis pipeline.
Results: Ultra-filtration and EV enrichment by SEC followed by RNA isolation yielded 2–7 ng of RNA and passed input RNA Bioanalyzer QC metrics. Following successful cDNA library construction and sequencing, the data analysis revealed a diversity of coding and non-coding RNAs. The majority of sequences were mapped to known genes (~75%), while the remaining RNA species consisted of lncRNA, rRNA, and pseudogenes. As observed with other studies, miRNA represented a very small percentage of the mapped sequences. Pathway analysis of the top 10% most expressed genes revealed that these EV RNAs are associated with regulation of neuron differentiation, projection development, transmembrane transport and glutamate signaling.
Summary/Conclusion: Here we report that EV RNA from 0.5 mL of CSF is suitable for RNA-seq. Future aims include performing RNA-seq on CSF EVs from larger cohorts of disease and control donors.
Funding: NIH/NIA, R21AG07848.
Keywords: cerebrospinal fluid, RNA, transcriptomics

PT07.11 | The exploration of droplet digital branched rolling circle amplification based ultrasensitive biosensor for gastric cancer cell-derived extracellular vesicles detection

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Introduction: Techniques for the ultrasensitive detection of cancer cell derived extracellular vesicles (EVs) surface protein are limited. Detection of cancer EVs directly from clinical samples is even more challengeable. Here we developed a droplet digital branched rolling circle amplification (ddBRCA) platform for the rapid and sensitive detection of cancer EVs separate by ultracentrifugation.
Methods: The design includes a hairpin structure DNA containing a cancer EVs specific aptamer sequence and BRCA primer sequence. The presence of target EVs could cause the conformational switching of the hairpin DNA, which subsequently triggers the BRCA. The reaction mixture was encapsulated in nanoliter droplets and allowed to incubate at 30 c° for 45 min. The fluorescent signal of each droplet was then measured using a microchip scanner.
Results: The results showed that our platform could detect EVs effectively with a limit of detection down to 12 particles/ml. Furthermore, the platform was tested using serum samples to validate its good prospect for clinical applicability.
Summary/Conclusion: To sum it up, our platform was the first droplet digital RCA method so far, with advancements such as favorable rapidity (~2 h), high sensitivity, and no washing step requirement, the proposed biosensor is expected to become a promising tool to be applied in clinical routines to assist in cancer diagnosis.

PT07.13 | Presenting novel technique for EV research: versatile non-labeled MP-SPR for native state studies

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Introduction: Surface Plasmon Resonance (SPR) has been used already a few decades for label-free detection and quantitation of biochemical kinetics and affinities for many different types of analytes. SPR is a well-established technique to measure biomolecular interactions, such as drug-protein binding kinetics and affinity, in real-time, without labels and with high-purity analytes.
Methods: The new Multi-Parametric Surface Plasmon Resonance (MP-SPR) technology is a technique utilizing full SPR angular spectra in the interaction measurements, allowing for the characterization of several parameters from the SPR curve in real time. Uniquely MP-SPR collects TIR (Total Internal Reflection) angle and SPR peak angle minima, thus monitoring changes in the bulk liquid. PureKineticsTM 1 allows real-time bulk effect subtraction producing high-quality results. MP-SPR extends the applicability of the SPR technique to even more challenging interaction studies of high relevance in life sciences, quality assurance, and in vitro diagnostic applications.
Results: Beyond the traditional use of MP-SPR in biomolecular interaction studies, we present results among new application fields, such as cell-based assays2,3, extracellular vesicle quantification3 and uptake4, and degradation of materials5 with MP-SPR. The versatility of MP-SPR for EV studies has been realized just lately3,4. Since the technique is powerful in detecting particles

below 150 nm in their native state (e.g., without labels), it has enormous potential even for diagnostic use in this segment. We will also offer a new regenerable avidin for surface modification6,7. Regenerable avidin is an ideal reagent for reversible surface immobilization with high precision.
Summary/Conclusion: In conclusion, MP-SPR is a powerful label-free technique for studying biomolecules such as EVs and could be used to study EV and EV corona interactions.
Keywords: multi-parametric surface plasmon resonance, biomolecular interactions, label-free detection, small particle analyses

PT08: Immunity, Autoimmunity, and Inflammation 1

Location: Hall 4A

16:45 - 18:45

PT08.01 | Milk-derived extracellular vesicles attenuate pro-inflammation and inflammasome activation in the microglia

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Introduction: To reduce maternal obesity (MO)-related health problems in children, milk-derived extracellular vesicle (MEVs) supplementation is proposed as a solution. MEVs convey pro-survival effects to neonates, but its association with neuroinflammation is unknown. MO leads to pro-inflammatory responses including NF-κB pathway activation and NLRP3 inflammasome formation. My objective is to investigate the pro-survival properties of MEVs in response to MO-induced pro-inflammatory pathways.
Methods: Immortalized human microglia (HMC3, ATCC #CRL-3304) were activated by 10 or 50 ng/μL interferon-γ (IFN- γ, Millipores-Sigma #01-172), to mimic a MO-induced pro-inflammatory (M1) phenotype. Activation was confirmed by measuring abundance of M1 markers (IL-1β, SAA), anti-inflammatory (IL-10, CD200R1) and general activation markers (CD68, IBA-1). Post M1 transition, HMC3s were supplemented with 200ug MEVs and harvested 0/6/12/24 for downstream analysis (n = 6/treatment). MEVs were isolated via serial centrifugation, ultra filtration, combined with differential ultracentrifugation (Beckman Coulter XL-100). MEV concentration, size, and particle distribution were measured via tunable resistive pulse sensing (TRPS) technology (IZON: The Exoid). MEVs were visualized via transmission electron microscopy (TEM, Thermo Fisher: F200X S/TEM). Western immunoblotting (WB) was used to characterize exosome markers (CD63, CD9, CD81) and negative control (Calnexin). NF-κB and NLRP3 inflammasome markers were quantified via RT-qPCR and WB.
Results: Post IFN-γ treatment, abundance of M1 markers increased, indicating a M1 transition. With MEVs supplementation, NF-κB (TLR4, TRAF6, IκBα, NF-κB p65) and NLRP3 inflammasome (pro-caspase-1, IL-1β, and IL-18) decreased in abundance.
Summary/Conclusion: MEV supplementation may reduce the activation of NF-κB and NLRP3 inflammasome formation and promote pro-survival effects in the microglia. MEVs could potentially combat negative health effects caused by MO.
Funding: This work was funded by Natural Sciences and Engineering Council (NSERC) Discovery Grant (Fund#:03805) and Manitoba Medical Service Foundation New Investigator Research Grant (Fund #:2021-18 awarded to Dr. Wijenayake.
Keywords: milk-derived extracellular vesicle, neuroinflammation, human microglia, NF-κB pathway, NLRP3 inflammasome

PT08.02 | Pooling umbilical cord mesenchymal stromal cell donors as a strategy of improving extracellular vesicle yield and therapeutic benefit in inflammatory arthritis

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Introduction: Mesenchymal stromal cells (MSCs) are immunomodulatory, meaning they can be effective at treating autoimmune disorders, such as rheumatoid arthritis (RA). This is replicated using their extracellular vesicles (EVs), yet we are limited by our ability to yield enough EVs for therapeutic application. Hence, we explored the effect of pooling on MSC EV harvest and therapeutic potential, in comparison to single donors.
Methods: Sucrose cushion ultracentrifugation isolated umbilical cord MSC EVs were characterised by particle concentration and sizing, cryo-EM, nano flow cytometry (NanoFCM) and mass spectrometry. Therapeutic potential was assessed by reduction

of swelling and improvement of histological parameters in an antigen-induced arthritis (AIA) model. Here, the 'best donor' was compared with pooled MSCs, and their derived EVs, against control mice injected with serum free DMEM.
Results: Pooled MSCs yielded significantly more particles than single donors ($p< 0.001$). These were of EV nature due to characteristic morphology, which showed a lipid membrane bilayer, and mode size, 114 ± 3.4nm. Higher particle yield encouraged the increase in EV associated tetraspanins (CD9, CD63, CD81) positive particles, as shown by nano flow cytometry, but there was no real trend in MSC positive particles (CD73, CD90, CD105), perhaps reflecting MSC heterogeneity. All conditions contained proteins common to EV preparations, but some differences were identified. When injected into our AIA model, swelling was significantly reduced for both single donor ($p< 0.05$) and pooled EVs ($p< 0.0001$) 48-hours after peak swelling. Yet, all conditions were significant at 72 hours, MSCs ($p< 0.05$) and EVs ($p< 0.001$ and $p< 0.0001$), in comparison to control. Still, only EVs significantly reduced arthritis index ($p< 0.05$), and associated RA inflammatory phenotype, with pooled MSC EVs appearing superior ($p< 0.001$).
Summary/Conclusion: Pooling MSCs is an effective strategy to increase EV yields for therapeutic application and may be more beneficial when treating inflammatory arthritis.
Funding: EPSRC/MRC DTC in Regenerative Medicine, The James Richardson Studentship, Institute of Orthopaedics Ltd., Oswestry, ISTM and ACORN funding, Keele University.
Keywords: mesenchymal stromal cells, extracellular vesicles, inflammatory arthritis

PT08.03 | Identification of an NKG7+ subset of extracellular vesicles derived from cytolytic granules of Natural Killer cells

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Introduction: Natural killer (NK) cell-based nanotherapies, including NK cell-derived extracellular vesicles (NK-EVs), could be a promising therapeutic tool for targeting solid tumors. Indeed, NK-EVs that contain cytolytic proteins and tumor-targeting molecules have been shown to directly interact with and kill malignant cells.
Methods: We have isolated distinct subpopulations of NK-EVs from cytokine-stimulated NK-92 cells or primary NK cells based on their difference in density using density gradient ultracentrifugation. Moreover, NKG7+ EVs are isolated by affinity-based capture and are imaged in the NK cells by electron microscopy. Mass spectrometry-based and small RNA sequencing analyses are used for the identification of differentially expressed proteins and microRNAs.
Results: Comparative quantitative proteomic analysis indicates the separation of a plasma membrane-derived subset, an intracellular organelle-derived subset, and an as-yet unclassified subset. The intracellularly derived EV subset encompasses the main bulk of cytolytic activity, and we have within this subset identified an NKG7+ population. NKG7 is a tetraspanin that is preferentially expressed in cytolytic granules of NK cells and T cells, and we demonstrate by electron microscopy that NKG7 is expressed in intraluminal vesicles within the granules. NKG7+ EVs isolated by affinity-based capture showed an enhanced capacity to induce apoptosis of cancer cell lines compared to bulk EVs. In support, we find an enrichment of cytolytic proteins such as Granzyme B within the NKG7+ EVs. Further, there is a striking enrichment of certain miRNA forms within the NKG7+ EVs that could play a role in the induction of cancer cell apoptosis.
Summary/Conclusion: In conclusion, NKG7+ EVs may be a specialized subset of EVs involved in the killing of tumor cells and will be further exploited as a potential therapeutic agent.
Funding: Our study was supported by the Research Council of Norway NANO2021-program (grant number: 303256).
Keywords: natural killer cell-derived EVs, tumor killing, NKG7

PT08.04 | Effective isolation and tumor killing activity of EVs derived from immortalized human natural killer (NK) cell line NK3.3

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Introduction: NK3.3 is a human NK cell line derived from the blood of a healthy donor; it has the same strong anti-tumor activity as primary NK cells. We demonstrated that EVs derived from NK3.3 cells kill leukemia, lymphoma, myeloma, breast cancer and glioblastoma cells without harming healthy cells. This makes NK3.3 EVs a potential new treatment for cancer. However, as a normal cell line, NK3.3 eventually undergoes growth arrest. Another challenge to clinical development is large scale production

and processing of EVs. To begin addressing these issues, we immortalized NK3.3 and isolated EVs using polyethylene glycol (PEG)-acetate precipitation to simplify processing and increase yield.
Methods: NK3.3 cells were immortalized by transduction with htert lentivirus (NK3.3-LTV). EVs were isolated from culture supernatants by differential high-speed centrifugation, sterile filtration, followed by precipitation using either a commercial kit (ExoQuick-TC) or homemade PEG-acetate. EVs were characterized by nanoparticle tracking analysis, protein composition and yield. Viability and proliferation of tumor and normal cells after NK EV treatment were evaluated by cell counts, cytotoxicity assays, and flow cytometry.
Results: NK3.3 EVs from all preparations ranged in size from 100–200nm and had identical protein profiles. PEG isolation resulted in a higher protein and particle concentration than ExoQuick isolation. EVs from NK3.3-LTV cells had similar protein and particle concentration as EVs from non-immortalized cells. All NK3.3 EVs decreased tumor growth and viability equally and none affected healthy cells.
Summary/Conclusion: EVs from immortalized NK3.3, isolated by centrifugation, filtration, and precipitation with PEG-acetate, displayed the same strong anti-tumor activity as EVs from non-immortalized cells, with no toxicity to normal cells. This study is the first step towards large-scale production of NK3.3 EVs for clinical use.
Funding: VA and NIH grants to JK.
Keywords: natural killer, cancer, cytotoxicity, tumor

PT08.06 | Human milk-extracellular vesicles promote heat shock protein response following heightened pro-inflammation

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Introduction: Perinatal stress can result in neuroinflammation triggered by microglia activation. Breast milk is proposed as a solution to counter these effects due to the anti-inflammatory properties of milk extracellular vesicles (MEVs). However, associations between MEVs and pro-survival pathways in the brain need investigation, e.g., heat shock response (HSR) for misfolded proteins. I aim to investigate how MEVs may attenuate neuroinflammation in glial cells. I hypothesize that MEV supplementation will enhance basal HSR in stressed microglia.
Methods: Aim 1: Human microglia cell lineage 3 (HMC3; ATCC CRCL-3304) were plated (2×105 cells/well; $n = 8$/treatment) and treated with 10 or 50 ng/mL IFNγ to induce a pro-inflammatory (M1) phenotype. Expression of general (IBA1/CD68), M1 (IL6/MHCII) and anti-inflammatory (M2) (CD206/IL10) markers were analyzed via RT-qPCR to determine degree of inflammation. MEVs were isolated from human donor milk (NorthernStar) using serial centrifugation and filtration as per Wijenayake et al. (2021), with differential ultracentrifugation (Beckman-Coulter XL-100; 100,000 x g, 120 min, 4°C; no brake; SW55TI swing-bucket rotor; 2x). MEVs were characterized via Tunable Resistive Pulse Sensing (Izon: Exoid) (concentration, size, Zeta potential); transmission electron microscopy (FEI Talos F200x S/TEM) with negative staining (morphology); and westerns to detect MEV markers (CD9/CD63/CD81/Hsp70) and negative control (ER marker calnexin). Aim 2: M1 HMC3 cells from Aim 1 were supplemented with 200 μg of MEVs and harvested at 0/6/12/24h.
Results: 50 ng/mL IFNγ had highest microglial activation ($p < 0.05$). IFNγ-treated cells had higher HSF1 activity than controls and nuclear translocation. MEV-supplementation further increased HSF1 expression/translocation and expression of target HSPs (27/40/70/90).
Summary/Conclusion: MEV-supplementation enhances HSR during pro-inflammation and breastmilk provides cellular protection to offspring.
Funding: This work was supported by Natural Sciences and Engineering Council (NSERC) Discovery Grant (Fund#: 03805) and Manitoba Medical Service Foundation New Investigator Research Grant (Fund #:2021-18) awarded to Dr. Wijenayake. Storm, JA holds the University of Winnipeg President's Distinguished Graduate Student Scholarship.
Keywords: neuroinflammation, human microglia, pro-survival, heat shock proteins, human milk extracellular vesicles, microRNA

PT08.07 | Dendritic cells mediated by small extracellular vesicles derived from MSCs attenuated the ILC2 activity via PGE2 in patients with allergic rhinitis

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Introduction: Mesenchymal stromal cells-derived small extracellular vesicles (MSC-sEVs) have recently attracted considerable attention because of their therapeutic potential in various immune diseases. We previously reported that MSC-sEVs could exert immunomodulatory roles in allergic airway inflammation by regulating group 2 innate lymphoid cell (ILC2) and dendritic cell (DC) functions. Therefore, this study aimed to investigate the therapeutic effects of MSC-sEVs on mature DC (mDC)-ILC2 interplay in allergic rhinitis (AR).
Methods: Here, we isolated MSC-sEVs from induced pluripotent stem cells (iPSC)-MSCs using anion-exchange chromatography for the generation of sEV-mDCs. sEV-mDCs were co-cultured with peripheral blood mononuclear cells (PBMCs) from patients with AR or purified ILC2s. The levels of IL-13 and GATA3 in ILC2s were examined by flow cytometry. Bulk RNA-sequence for mDCs and sEV-mDCs was employed to further probe the potential mechanisms, which were then validated in the co-culture systems.
Results: sEV-mDCs showed weaker capacity in priming the levels of IL-13 and GATA3 in ILC2s when compared with mDCs. Furthermore, there was higher PGE2 and IL-10 production from sEV-mDCs, and the blockade of them especially the former one could reverse the inhibitory effects of sEV-mDCs.
Summary/Conclusion: We demonstrated that MSC-sEVs were able to dampen the activating effects of mDCs on ILC2s in patients with AR. Mechanismly, the PGE2-EP2/4 axis played an essential role in the immunomodulatory effects of sEV-mDCs on ILC2s. Herein, we provided new insights into the mechanism underlying the therapeutic effects of MSC-sEVs in allergic airway inflammation.
Funding: National Key R&D Program of China (2022YFA1104900).
Keywords: Small extracellular vesicles, mesenchymal stromal cells, dendritic cells, group 2 innate lymphoid cells, prostaglandin E2, allergic rhinitis

PT08.09 | Extracellular vesicles as markers of blood-brain barrier dysfunction in systemic lupus erythematosus

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Introduction: Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by production of autoantibodies to nuclear antigens and cytokine dysregulation. Disease presentation, particularly, that of neuropsychiatric lupus (NPSLE) is highly heterogenous; NPSLE pathology can initiate before other clinical manifestations of systemic disease are apparent. Over 80% of SLE patients are estimated to have some neurological manifestation of their disease. Imaging studies using dynamic contrast-enhanced MRI (DCE-MRI) revealed blood-brain barrier (BBB) disruption in SLE patients, including those without clinically overt NPSLE. This BBB disruption permits passage of autoantibodies and cytokines into the brain and likely contributes to SLE neurological disease pathogenesis.
Although DCE-MRI is invaluable for obtaining a snapshot of the brain and BBB, it is costly, often contraindicated in patients with lupus nephritis, and cannot be performed at frequent intervals. Thus, developing a safe and accessible method to characterize BBB dysfunction in SLE patients is imperative. Here, we use samples from SLE patients and healthy controls to demonstrate the feasibility of quantifying serum extracellular vesicles (EVs) derived from brain microvascular endothelial cells (BMECs).
Methods: Using nanoparticle tracking analysis downstream of size exclusion chromatography-based EV enrichment, we performed an initial enrichment and characterization of EVs in the sera of SLE patients and healthy controls. The presence of EVs in the enriched product is observed on transmission electron microscopy. This is followed by a novel method of immunoaffinity capture, first described in Mitchell et al. (2021), to specifically select EVs derived from BMECs.
Results: We show that biobanked serum is comparable to freshly processed serum in nanoparticle content, that our methods effectively isolate EVs and select for highly enriched BMEC-derived EVs, and that patients with active lupus disease differ in EV number and content compared to healthy controls.
Summary/Conclusion: We will further explore how BMEC-derived EVs can be indicators of BBB activity and integrity. As we have patients on whom imaging studies were performed, we can examine number and composition of EVs derived from the brain microvasculature to see if these correlate with functional measures of the BBB. Identifying EV biomarkers corresponding to breaches in blood-brain barrier integrity in lupus could yield novel, minimally invasive indicators of disease activity.
Funding: This project uses funding from NIH grants.
Keywords: lupus, SLE, biomarkers, blood-brain barrier, blood brain barrier, BBB, vascular endothelial cells, microvascular endothelial cells, endothelial cells

PT08.10 | A novel reporter mouse model for evaluating B cell-specific extracellular vesicles (EVs) in healthy and autoimmune disease states in vivo

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Introduction: EVs are abundant, membrane-bound particles that contain nucleic acids and proteins and freely circulate thus enabling intercellular communication. However, genetic tools to interrogate cell type-specific EVs in vivo are lacking. Using a new mouse strain that enables GFP labeling, isolation, and high-fidelity probing of cell type-specific EVs in vivo, we made reporter mice that allows us to study EVs derived from B cells that have class-switched to immunoglobulin G (IgG) in response to foreign and self-antigens. These mice were bred onto a lupus-prone strain to test the potential role of B cell-derived EVs to promote inflammatory immune responses in murine lupus.
Methods: Taking advantage of the EV marker CD63, we crossed CD63-emGFP(loxP/stop/loxP) knock-in mice with Cγ1-Cre mice to generate a new reporter strain (BIgG:CD63GFP) that enables the specific GFP labeling of CD63+ EVs derived from class-switched IgG+ B cells. EVs were isolated from the serum or supernatant of B cells stimulated in vitro obtained from mice immunized with foreign antigen or with spontaneous lupus. EVs were characterized according to ISEV-guidelines by nanoparticle tracking analysis, flow cytometry, ELISA, and western blot.
Results: Immunization of BIgG:CD63GFP reporter mice with foreign antigens revealed the presence of GFP+ EVs expressing antigen-specific membrane IgG in both serum and supernatant of cultured B cells. Lupus-prone BIgG:CD63GFP mice demonstrated spontaneous development of GFP+ IgG+ EVs that bind to self-antigens. GFP+ EVs were further detected in neutrophils and localized to the kidneys.
Summary/Conclusion: We describe a new mouse strain useful for testing the function and distribution of antigen-specific IgG+ EVs derived from B cells in vivo. Our data suggest that these EVs are another source of functional antigen-specific antibodies that may contribute to humoral immunity and the pathogenesis of lupus.
Funding: This work was supported by NIH R21 AI153990-01A1.
Keywords: humoral response, autoimmunity, CD63, GFP

PT09: EVs from Microorganisms 1

Location: Hall 4A

16:45 - 18:45

PT09.01 | The interaction of EVs released by Trypanosoma cruzi insect infective forms with host mammalian cells and establishment parasite infection in the vertebrate host

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Introduction: Chagas disease (CD) caused by the protozoan Trypanosoma cruzi is transmitted to humans by metacyclic-trypomastigotes (MT) released by the feces of infected triatomines insects, which infect mammalian cells to proliferate. The cells release trypomastigotes that circulated in blood (TCT) and perpetuates the infection. Our group showed that EVs shedding from cell released TCTs induce pro-inflammatory activity, which mediate host innate and chronic immune responses, important for disease outcome. However, the generation of immune responses induced by EVs released by MT, the initial infective form is poorly understood. The aim of our research was to characterize the structure and composition of EVs and verify their uptake by mammalian cells
Methods: The MT from T. cruzi were obtained from proliferating epimastigote forms cultivated in liver infusion medium containing 10% FBS after differentiation and purification in DEAE-cellulose columns equilibrated in saline buffer containing glucose. EVs were obtained from MT supernatants and purified by ultracentrifugation and size elution chromatography (SEC). The EVs were characterized by NTA and chemiluminescent-ELISA using specific MT surface antigens. EVs were then labeled with PKH-26 to follow uptake by human monocytes (THP-1) cells for 5 to 60 minutes by flow cytometry (FC) and confocal microscopy (CM).
Results: MT EVs range from 186 to 331 nm and contained MT gp82 and gp90, specific stage specific surface glycoproteins. Labeled EVs were uptaken by THP-1 cells over time as shown by FC and CM.

Summary/Conclusion: Conclusion: Our results suggest that T. cruzi EVs isolated from MT could be fusing to the human monocyte, which might affect early parasite infection.
Funding: Financial Support: FAPESP, CAPES, CNPq.

PT09.02 | L. plantarum EVs as a platform for specific anti-allergic immunotherapy – characterization of EVs

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Introduction: Allergy is still a pressing issue and the only causative and disease-modifying treatment for allergies is immunotherapy, which is burdened with several drawbacks e.g. risk of anaphylaxis induction. The beneficial properties of probiotics in fighting allergies have been recognized, however, the use of living organisms is not without its flaws. Derivatives of probiotics, such as extracellular vesicles (EVs), due to their modulatory and adjuvant activity, can be an excellent replacement of whole bacteria, but there is a gap in the knowledge on Gram+ probiotic EVs.
Methods: L. plantarum NCIMB8826 was grown anaerobically in MRS medium (37°C) until it reached the OD of 0.5, 1.0, 1.5 or 2. L. plantarum EVs (LpEVs) were isolated by centrifugation (4 000 x g, 20 min), filtration (0.22 μm filter), concentration by Amicon Stirred Cell (300 kDa filter) and ultracentrifugation (3 h, 150 000 x g). LpEVs were purified with IZON qEV original column and characterized by Zetasizer (size and particle counts), TEM, SDS-PAGE (with MS protein identification) and Bioanalyzer (RNA, DNA and protein content). In vitro analysis of immunostimulatory properties and stability studies are ongoing.
Results: Our data show that the profile of produced LpEVs is OD-dependent. Longer cultures of bacteria result in a heterogenic profile of isolated LpEVs. We are able to isolate up to 2 × 1012 of EVs from a 5 l culture of bacteria. Produced EVs are less than 100 nm in size and they contain diverse proteins and small RNA. Interestingly, we did not detect DNA.
Summary/Conclusion: L. plantarum species were already shown to have an immunomodulatory effect. We have shown that this Gram+ bacteria produces EVs and we characterized them. This is the first step in designing a platform for antigen-specific, intranasal, anti-allergy therapy based on probiotic EVs.
Funding: This research was funded by HORIZON-MSCA-2021-PF (project no. 101066450), Danube Allergy Research Cluster and Foundation for Polish Science (FNP).
Keywords: allergy, probiotics, extracellular vesicles

PT09.03 | Characterization of EVs in the apicomplexan parasite Cystoisospora suis

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Introduction: Cystoisospora suis, a porcine apicomplexan parasite, causes severe economic losses in the livestock industry worldwide. It is characterized by a complex life cycle, with asexual and sexual development in the epithelium of the host gut and an environmental phase as oocyst. All parasite stages vary greatly in their morphology and function during the life cycle and presumably excrete different bioactive molecules as intercellular communication cargo. Due to the complexity of the parasite's development we hypothesize that the cargo transported by parasite EVs varies with the life cycle stage. Hence, this study aimed to characterize EVs of all developmental stages of C. suis.
Methods: C. suis parasite stages were obtained from intestinal porcine epithelia cell cultures (IPEC) during their respective expression and incubated for two hours in a host cell-free environment. All EVs were obtained by several centrifugation steps, and particle numbers and size distributions of stage-specific parasite EVs were analysed by nanoparticle tracking analysis (NTA). Transmission and scanning electron microscopy were used to visualise the respective EVs locations on the developmental stage. Furthermore, correlations between the lipidomic profile of EVs derived from C. suis asexual and sexual stages and their Fourier-transform infrared (FTIR) spectra were compared with profiles of other apicomplexan species.

Results: Asexual, sexual and transmissible stages of C. suis expressed different EVs during the parasite's life cycle. In addition, we could show that the EVs of asexual and sexual stages, which occur in the host animal, are more similar to each other than to the transmissible environmental stage, the oocyst. We could also show a cargo of polysaccharides, which are known to influence the conversion of parasite stages, and the occurrence of fatty acids in EVs, which presumably down-regulate the toxic activity of parasites.
Summary/Conclusion: This study presents the first characterization of C. suis EVs and links them with the respective developmental stages of the parasite and putative functions.

PT09.04 | Extracellular vesicles of potentially pathogenic free-living amoebae: a comparison between Acanthamoeba and Naegleria fowleri

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Introduction: Free-living amoebae (FLA) are protozoa widely distributed in nature, which can be found in a variety of environments such as water, soil and dust, among others. Four genera are recognized as causal agents of infections in humans and animals: Naegleria, Acanthamoeba, Sappinia and Balamuthia.
Acanthamoeba produce granulomatous amoebic encephalitis (GAE), mostly in immunocompromised patients, as well as amoebic keratitis. Naegleria fowleri is the etiologic agent of primary amoebic meningoencephalitis (PAM) in both healthy children and young adults, a fatal infection of rapid progression.
For infections of the central nervous system (CNS) with these amoebae, diagnosis is usually performed post-mortem. In this sense, a rapid and reliable diagnostic panel using extracellular vesicles (EVs) could improve on time intervention.
Methods: EVs of trophozoites of Acanthamoeba T5 and Naegleria fowleri were isolated by ultracentrifugation, and characterization of these vesicles was performed using transmission electron microscopy, atomic force microscopy, nanoparticle tracking analyisis and dynamic light scattering. Protein profiles and protease activity of EVs of both amoebae were compared, and proteomic analyses were also performed. Finally, the effect of EVs over cell viability was also analyzed.
Results: The secretion of EVs by both Acanthamoeba T5 and Naegleria fowleri was confirmed, and protein profiles, protease activity and types of proteases in EVs were compared. Proteomic analyses revealed differences in protein numbers and types of proteins in EVs of Acanthamoeba T5 and Naegleria fowleri. Differences in the effect of EVs over cell viability were also observed.
Summary/Conclusion: EVs secreted by FLA Acanthamoeba T5 and Naegleria fowleri were successfully isolated and these vesicles were compared. The results obtained precede functional analyses that are still ongoing in our laboratory. With these results, the possibility of employing EVs as biomarkers of infections of the CNS could be tested in future experiments.
Funding: This work was funded by the "Vicerrectoría de Investigación" of the "Universidad de Costa Rica". Research projects related to this work: -C-1061: "Caracterización de antígenos de excreción/secreción y antígenos somáticos en amebas de vidalibre mediante empleo de anticuerpos policlonales producidos en roedores" -C-2600: "Secreción de vesículas extracelulares por Naegleria fowleri y evaluación de su potencial rolinmunomodulador en un modelo in vitro."
Keywords: Extracellular vesicles, free-living amoeba, Acanthamoeba, Naegleria fowleri

PT09.05 | Association of bacterial extracellular vesicles among human stool, plasma, and gut microbiota samples studied using a composite purification method

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Introduction: Bacterial extracellular vesicles (BEVs) play a crucial role in neurodegenerative diseases, suggesting they may be involved in the gut-brain axis. However, little research has examined the relationship between gut microbiota, stool-derived BEVs (stBEVs), and plasma-derived BEVs (plBEVs). In this study, we aim to isolate and analyze stBEVs from healthy individuals and explore the connection to their gut microbiota and plBEVs.

Methods: Four models were employed to optimize the purification process for isolating stBEVs: culture medium from gram-positive bacteria, gram-negative bacteria, eukaryotic cells, and human stool specimens. The composite protocol, which included differential centrifugation, filtration, and density gradient centrifugation, yielded high amounts of stBEVs. The isolated stBEVs were characterized using TEM, NTA, BCA assay, WB, and NanoFCM. Functional tests were also performed on gram-positive and gram-negative bacteria using endotoxin, PBMC stimulation, and LTR2 receptor assay to ensure successful isolation of stBEVs. DNA was extracted from stBEVs and plBEVs, and PCR was used to target the v3-v4 and full-length regions of the 16s rRNA gene for sequencing. Electrophoresis confirmed the success of the PCR amplification. PacBio sequencer or NGS platform Illumina MiSeq was used to sequence DNA from stools, stBEVs, and plBEVs.
Results: Our analysis revealed that stBEVs in density gradient fractions F5&6 contain exosomal markers (CD9, syntenin-1), while F8&9 contain bacterial markers (OmpA, LTA). Notably, Flotillin and TGS101 were present not only in eukaryotic cell-derived EVs, but also in bacterial EVs. PCR results indicated that plBEVs were able to amplify the v3-v4 region, but not the full-length region, suggesting that the plBEVs may no longer have an intact 16s rRNA sequence. Metagenomic analysis results showed a correlation between the relative species abundance of stBEVs and gut microbiota at the phylum level.
Summary/Conclusion: Eukaryotic-EVs and Bacteria-EVs from stool were distinctly separated. A comparison of stBEV DNA and plBEV DNA revealed that plBEV DNA had become fragmented. Sequencing results suggest that BEVs may act as a means of transporting information from the microbiota.
Funding: This work was supported in part by funding from National Taiwan University's Advanced Education Deep Cultivation Plan - Core Research Group Plan (grant agreement: ntu-cc-111L890203).

PT09.07 | Identifying size-dependent toxin sorting in bacterial outer membrane vesicles using fluorescence microscopy

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Introduction: Gram negative bacteria produce EVs known as outer membrane vesicles (OMVs) which are involved in cell-cell communication and virulence. OMVs are heterogeneous in their size, toxin content, and cargo. Many traditional assays measure ensemble properties, therefore, heterogeneities can be obscured. We developed a method to identify size dependent toxin/protein sorting in OMVs using fluorescence microscopy. Here we show that the oral bacterium Aggregatibacter actinomycetemcomitans (A.a.) produces OMVs with a bimodal size distribution, and only the larger OMVs contain leukotoxin (LtxA).
Methods: A.a. OMVs were harvested, followed by biotinylation and membrane labeling. A coverslip passivated with streptavidin was used to capture OMVs, and an anti-LtxA antibody was used to detect toxin containing OMVs. Integrated fluorescence intensity of single OMVs was measured and their diameters were calculated. OMVs were sorted into two categories (toxin positive and toxin negative), and the size distribution of each category was determined.
Results: A.a. OMVs showed a bimodal size distribution, where the major population was ~100 nm in diameter, and the minor population was ~350 nm in diameter. Interestingly, LtxA was predominantly present in larger OMVs, and the smaller diameter OMVs did not contain any LtxA. Specifically, no LtxA negative OMVs were larger than 220 nm and no LtxA positive OMV was smaller than 60 nm. These results were verified with western blot. Among the toxin positive OMVs, no correlation between toxin density and size was found.
Summary/Conclusion: To analyze OMV or EV heterogeneity, it is important to study single vesicles. Our method of single OMV analysis demonstrates that LtxA is preferentially sorted to OMVs on the basis of size, where larger OMVs tend to be toxin positive, while smaller OMVs are more likely to be toxin negative. Our method enables detection of size-based toxin sorting, without the need for separation of EV fractions or specialized equipment.
Funding: National Institutes of Health (R21GM134414 (NJW) and R21DE025275 (ACB)), Lehigh University (CORE grant).
Keywords: single-EV analysis, single particle sizing, fluorescence microscopy, outer membrane vesicles, toxins

PT09.08 | Extracellular vesicles derived from T. gondii infected primary cortical neurons alter astrocyte composition

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Introduction: Toxoplasma gondii (T. gondii) is an obligate intracellular parasite that can invade any nucleated cell. However, cyst formation in the central nervous system only occurs in neurons allowing the parasite to remain latent for the lifetime of the host.

Though infected neurons appear intact, it is not yet known how infection disrupts the crucial communication between astrocytes and neurons. Astrocytes are fundamental to neuronal health by providing nutrients and structural support. Extracellular vesicles (EVs) function in intracellular communication and contain proteins, lipids, DNA, miRNA, and other RNAs subtypes.
Methods: EVs were isolated from primary murine cortical neurons infected and uninfected with T. gondii through a series of centrifugations and the Qiagen exoEasy Maxi Kit. EV size and concentration were confirmed with Nanoparticle Tracking Analysis (NTA) and a CD63 ELISA. EVs were stained with PKH67 and added onto primary murine cortical astrocytes to observe uptake. EVs, from infected and uninfected neurons, were added onto primary murine cortical astrocytes for 6 and 24 hours, and bulk RNA Sequencing (RNASeq) was done on the astrocytes to compare gene expression changes.
Results: EVs from neurons infected with T. gondii had a significant decrease in concentration compared to uninfected EVs as seen with both NTA and CD63 ELISA (p-value = < 0.0001 and 0.0104, respectively). Immunocytochemistry proposes colocalization of both uninfected and infected EVs with primary astrocytes. RNASeq suggests the addition of EVs, sourced from either uninfected or infected neurons, alters astrocyte gene expression.
Summary/Conclusion: T. gondii infection of primary cortical neurons decreases the concentration of EVs. Infection does not alter colocalization of EVs and astrocytes. The addition of EVs, in general, can lead to alterations in the receiving cells gene expression. These results help better understand how a parasitic infection in the brain alters EV production and neuronal-astrocyte communication.
Keywords: toxoplasma, parasite, astrocyte, neuron, brain

PT09.10 | Intranasal immunization with extracellular vesicles released by leishmania amazonensis partially protected BALB/c mice after subcutaneous challenge with the parasite

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Introduction: Extracellular vesicles (EVs) released by Leishmania are able to interact with immune system cells and can modulate cytokine production and experimental infection. Intraperitoneal immunization with EVs released by Leishmania amazonensis (LamaEVs) induced partial protection after challenge with the parasite. Herein we evaluated the effects of intranasal immunization with LamaEVs obtained from parasites with different virulence.
Methods: Attenuated parasites were obtained after long term culture passages (LT-P) and virulent parasites were derived after 3 consecutive mice infection (in vivo derived - IVD-P). EVs released by LT-P and IVD-P were obtained after several cycles of centrifugation and ultracentrifugation of culture supernantants. EVs concentrations and sized were determined by nano particle tracking analysis (NTA) and protein quantification were determined by BCA method. BALB/c mice were intranasal immunized with 2 doses in 2 weeks-interval of each EVs (LT-P or IVD-P). Then, after 2 weeks of last immunization, mice were subcutaneously infected with the IVD-P parasites in the paws.
Results: Mice immunized with EVs from IVD-P promastigotes showed similar lesion size to non-immunized controls. However, animals immunized with LT-P EVs showed significantly smaller lesions compared to the group immunized with IVD-P EVs. The parasite load analysis showed a significant reduction in the parasite load in the lesions of animals immunized with LT-P compared with the group immunized with IVD-P EVs. Cytokine dosage showed a modulation of pro and anti-inflammatory cytokines, with change in the immune response profile in immunized animals, as compared to non-immunized mice.
Summary/Conclusion: This work may contribute to a better understanding of the possibility of using the intranasal route in immunization protocols with EVs for cutaneous leishmaniasis.
Funding: CAPES, CNPq and FAPESP (2019/21614-3).
Keywords: leishmania amazonensis, immunization, EVs

PT09.10 | Effects of EVs released from virulent and non-virulent Trypanosoma cruzi in the acute phase to experimental model in Chagas disease

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Introduction: Extracellular vesicles (EVs) released from trypomastigote forms of Trypanosoma cruzi modulate the invasion and host immune response. The parasite can be maintained in tissue culture infected cells but loses mice virulence. We have regenerated virulence by 20 serial passages in mice. Therefore, we compared the effects of EVs released by non-virulent trypomastigotes maintained in vitro (P0), with virulent trypomastigotes maintained in vivo (P20).

Methods: BALB/c mice were pre-treated with 108 total EVs 3 days before infection with 500 either P0 or P20 trypomastigotes forms. Parasitemia was counted at 5, 7, 9, 11, and 13-days post infection (dpi). Mortality was monitored until 45 dpi. For histopathology, the organs (heart, lungs, spleen, rim, liver, bladder, skeletal muscle, and intestine) were collected at 15 dpi to count amastigote nests
Results: Pre-treatment with EVs released by virulent trypomastigote (P20) increased blood parasitemia and anticipated mortality when compared with non-virulent (P0) and control groups (without EVs). Animals pre-treated with P0 EVs did not show significant increase in the quantity of parasites in peripheral blood compared with animals only infected with P0 parasites. Besides that, animals pre-treated with P20 EVs have doubled the number of amastigote nests in heart and bladder, followed by significant difference in intestine and lungs when compared to other groups.
Summary/Conclusion: We show for the first time that T. cruzi virulence is related to EVs released by the parasite. It increases parasitism in heart, followed by bladder, intestine, and lungs.
Funding: FAPESP, CAPES and CNPq.

PT09.11 | EVs immunization effect on the acute experimental model of Chagas disease

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Introduction: Chagas disease or American trypanosomiasis agent is Trypanosoma cruzi. The parasite releases extracellular vesicles (EVs), which are crucial for intracellular communication and the host's immune response. In our previous research, we have shown that EVs shed from trypomastigote forms of the parasite induced pro-inflammatory cytokines (IL-12, TNF-alfa, and NO) in macrophages and increased parasite invasion via TLR2. Our research aims to verify the potential of EVs for the development of new therapies employing an the acute experimental model of Chagas disease.
Methods: BALB/c mice were pre-treated with three doses of EVs released from Y strain T. cruzi trypomastigotes, then infected with 500 virulent trypomastigotes forms. We used three experimental designs with different immunization doses, intervals, and quantity of particles. First, EVs with or without the addition of Alum adjuvant were administered intraperitoneally (i.p.). The doses (5 × 105 and 1 × 107 particles/dose/animal) were administered at intervals of 15 days each, following the infection of the animals. Next, the first and second doses dispersed with or without Alum were administered intradermally (i.d.), while the third dose was administered intraperitoneally. After 8 days of the last dose, the animals were infected. The parameters analyzed were parasitemia and mortality of experimental and control groups
Results: Parasitemia was lower in animals pre-treated i.p. biweekly with 5 × 105 and 107 particles and adjuvant compared with the control group. Nevertheless, the control group showed higher survivability than animals pre-treated. Furthermore, animals pre-treated weekly (i.d. or i.p.) with a higher number of particles (108) and adjuvant delayed the peak of parasites in the blood, while the mortality was the same between the groups
Summary/Conclusion: Our findings shed new light into the complex mechanisms of parasite interaction with the host, involving multiple events to facilitate cell invasion and control host innate immune responses, and ultimately promote long-term parasite survival.
Funding: FAPESP, CAPES, CNPq.

PT09.12 | EVs shedding from infective trypomastigote forms of Trypanosoma cruzi interact with host epithelial and human THP-1 monocyte cells

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Introduction: Infective trypomastigote forms of T. cruzi release EVs that are rich in α-galactosyl epitopes (alpha-Gal), TS/gp85, and mucin-like glycoproteins. Our research showed that EVs release from T. cruzi induce a potent host innate immunity via Toll-like receptor 2 (TLR-2)-dependent pathways. In addition, our research showed that EVs increase host cell invasion. However, the mechanism by which EVs affects invasion is still poorly understood. To understand the effects of EVs, here we studied their interaction with epithelial and human monocytes cells.
Methods: EVs were incubated with epithelial cells (LLC-MK2) for 5 min and 1 hour, at 37oC. The cells were washed, fixed and probed with anti-α-Gal or anti-vinculin antibodies. Alternatively, EVs were incubated with THP-1 containing transfected NF-kB-GFP for 2, 4, 24 and 48 hours, at 37oC and the EVs uptake and translocation of the NF-κB factor from the cytoplasm to the cell nucleus analyzed by Flow Cytometry (FC) and Confocal microscopy (CM).
Results: We detected that T. cruzi EVs were internalized after 1 hour incubation with epithelial cells. After 24 hours we detected maximum fluorescence emission of GFP fluorescence and NF-kB-GFP translocation nuclear translocation.

Summary/Conclusion: The EVs isolated from infective trypomastigote interacted with epithelial cells and human monocytes. The interaction modulates immune cells by activating inflammatory response related to NF-κB transactivation.
Funding: financial support: FAPESP, CAPES, CNPq.

PT09.13 | Compounds GPQF-815 and GPQF-817 regulatory role in the EVs formation in T. cruzi

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Introduction: Trypanosoma cruzi, the etiologic agent of Chagas disease, releases EVs and containing surface molecules represented by glycoproteins anchored to the membrane via GPI (glycosylphosphatidylinositol). Our group previously showed that EVs released by infective T. cruzi forms modulate inflammatory responses in host cells and during in vivo infection. However, the biogenesis and mechanism of shedding of the epimastigotes EVs is poorly understood. Aiming to better understand EV release in this parasite, we studied the effects of GPQF-815 and GPQF-817, which are derivatives of N-acylhydrazones. GPQF-815 and 817 are synthetic compounds that do not yet have well-determined biological activities, but which have already shown anti-prion and antimicrobial actions.
Methods: Epimastigotes forms of the Y strain of T. cruzi were incubated with different concentrations of the compounds (250, 125 and 62.5, 31.25, 15.625 and 7.8125 μg/mL) during 2 h, at 28oC. Released EVs were characterized in size and concentration by NTA. In parallel, we measure the protein content.
Results: The results showed that released EVs concentration and size were not affected in any case. However, the amount of protein released with EVs increased for both compounds relative to the control, which indicated that the number of released particles is not dependent on the protein concentration.
Summary/Conclusion: Compounds GPQF-815 and 817 could have a possible regulatory role in the EVs formation in T. cruzi and consequently be used to control the inflammatory response during parasite infection.
Funding: FAPESP, CNPq and CAPES.

PT10: EV Separation from Biological Sources

Location: Hall 4A

16:45 - 18:45

PT10.01 | Separation and identification of protein complexes in small extracellular vesicles (sEVs) from human urine

Olusanya Olasehinde[1]; Fuquan Yang[1]; Adefarati Oloruntoba[2]; Joshua Seun Olajide[3]; Xiaoxing Huang[1]; Helen Binang[4]
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Introduction: In the past decades, Extracellular vesicles (EVs) have been discovered as heterogeneous, nanosized membrane bilayer which are mostly released by all kinds of cells. The vesicles have been isolated and analyzed in many different biological fluids as further study revealed them as vehicular agents that mediate communication between cells. Imperative advances have been made in the proteomics of EVs, however, there is dearth of knowledge as regards the investigation whether the proteins inherent in EVs function by forming protein complexes (protein machine) or not. Our research group had this insight that protein machine in EVs has not been explored considering the critical roles of proteins in the body.
Methods: sEVs were separated from human urine of health status using the optimized combination of differential ultracentrifugation (DUC) and 0.22 mm membrane filtration. Subsequently, 1D-BN-PAGE was employed for the first time to separate protein complexes in urinary sEVs across seven protein bands in native conditions, and LC-MS/MS was then conducted to identify proteins in the seven protein bands respectively after in-gel digestion.
Results: A total of 1323 proteins were identified from the 7 protein bands of 1D-BN-PAGE. Proteins were detected protein isoforms that are selectively associated with distinct subsets of protein complexes. Amazingly, there was clear enrichment for specific Gene Ontology terms correlated with differential size classes of protein complexes. Bioinformatic analysis of these protein complexes from seven protein bands of 1D-BN-PAGE revealed sEVs in urine were involved in the glycolysis and gluconeogenesis

pathways as such may be used for controlling the high glucose level in humans. In this study, the optimized and combined approach experimentally permitted an efficient strategy to study the protein complexes in human urine sEVs for the first time.
Summary/Conclusion: In general, the technique for the purification of sEVs, separation of protein complexes in sEVs in native conditions, and their downstream identification of potential candidates using LC-MS/MS have emerged as powerful tools to delineate the cellular components, especially the protein complexes, biological processes, molecular functions of sEVs from urine. Our findings demonstrate that 1D-BN-PAGE can be used to annotate protein complexes systemically and to predict potential interactions among isoforms.
Funding: 1. The National Key R&D Program of China (2018YFA0507801 and 2018YFA0507103) 2. Chinese Academy of Sciences (CAS) and The World Academy of Sciences (TWAS).
Keywords: small extracellular vesicles (sEVs), differential ultracentrifugation (DUC), protein complexes, 1D-BN-PAGE, LC-MS/MS

PT10.02 | Separation and identification of protein complexes in small extracellular vesicles (sEVs) from human urine

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Introduction: In the past decades, Extracellular vesicles (EVs) have been discovered as heterogeneous, nanosized membrane bilayer which are mostly released by all kinds of cells. The vesicles have been isolated in many different biological fluids. Imperative advances have been made in the proteomics of EVs. However, there is dearth of knowledge as regards the protein machine in sEVs. Our research group had this insight that protein machine in EVs has been explored considering the critical roles of proteins in the body.
Methods: sEVs were separated from human urine of health status using the optimized combination of differential ultracentrifugation (DUC) and 0.22 mm membrane filtration. Subsequently, 1D-BN-PAGE was employed for the first time to separate protein complexes in urinary sEVs across seven protein bands in native conditions, and LC-MS/MS was then conducted to identify proteins in the seven protein bands respectively after in-gel digestion.
Results: A total of 1323 proteins were identified from the 7 protein bands of 1D-BN-PAGE. Proteins were detected interacting with one another forming complexes, as typified by the string interaction network which was modified by Cytoscape. Additionally, the data revealed protein isoforms that are selectively associated with distinct subsets of protein complexes. Amazingly, there was clear enrichment for specific Gene Ontology terms correlated with differential size classes of protein complexes. Bioinformatic analysis of these protein complexes from seven protein bands of 1D-BN-PAGE revealed sEVs in urine were involved in the glycolysis and gluconeogenesis pathways as such may be used for controlling the high glucose level in humans. In this study, the optimized and combined approach experimentally permitted the development of an efficient strategy to study the protein complexes in urine sEVs.
Summary/Conclusion: In general, the technique for the purification of sEVs, separation of protein complexes in sEVs in native conditions, and their downstream identification of potential candidates using LC-MS/MS have emerged as powerful tools to delineate the cellular components, especially the protein complexes, biological processes, molecular functions of sEVs from urine. Our findings demonstrate that 1D-BN-PAGE, and SEC can be used to annotate protein complexes systemically and to predict potential interactions among isoforms. The data on native protein complex separation have been integrated into DAVID, a multidimensional data-sharing resource.
Funding: This study was supported by: 1. The National Key R&D Program of China (2018YFA0507801 and 2018YFA0507103) 2. Chinese Academy of Sciences (CAS) and The World Academy of Sciences (TWAS).
Keywords: small extracellular vesicles (sEVs), differential ultracentrifugation (DUC), protein complexes, 1D-BN-PAGE, LC-MS/MS

PT10.03 | A rapid and simple isolation assay of urinary extracellular vesicles

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Introduction: Cancer cell-derived extracellular vesicles (EVs) are promising biomarkers for cancer diagnosis and prognosis.However, the lack of rapid and sensitive isolation techniques to obtain EVs fromclinical samples at a sufficiently high yield limits their practicability.

Methods: Herein, we report the fabrication of chimeric nanocomposites of LF-conjugated bis-MPA dendrimer-coated MNPs (LF-bis-MPA-MNPs) for EV isolation based on a combination of electrostatic interaction, physical absorption, and biorecognition. Electrostatic interaction occurs between the positively charged LF and negatively charged surface of the EV, while Van der Waals forces (interaction between LF and other molecules such as lipoproteins on the plasma membrane) provide the driving force for physical adsorption. In addition, the LF receptors on the EV surface can bind with LF via an immunoaffinity mechanism. Therefore, based on the electrostatic interaction, physical absorption, and targeted binding of LF to the EV, we speculate that LF has great potential for EV isolation.
Results: The speed, efficiency, recovery rate, and purity of EV isolation by the LF-bis-MPA-MNPs are superior to those obtained by using established methods. The relative expressions of exosomal microRNAs (miRNAs) from isolated EVs in cancerous cell-derived exosomes are verified as significantly higher than those from noncancerous ones. Finally, the chimeric nanocomposites are used to assess urinary exosomal miRNAs from urine specimens from 20 prostate cancer (PCa), 10 benign prostatic hyperplasia (BPH), patients and 10 healthy controls. Significant up-regulation of miR-21 and miR-346 and down-regulation of miR-23a and miR-122-5p occurs in both groups compared to healthy controls. LF-bis-MPA-MNPs provide a rapid, simple,
Summary/Conclusion: Taken together, the chimeric nanocomposites offer a promising method for EV isolation from human specimens. The developed method is rapid, simple, and highly efficient.We envision that there is great potential for EV-related applications, including stem cell treatment, transplantation, immune-based treatment, and theranostics.
Funding: This study was supported by the Ministry of Science, ICT and Future Planning (MSIP) through theNational Research Foundation ofKorea (NRF) (2020R1A2C2007148 and 2016R1A5A1010148), and also supported by the Yonsei University Research Fund of 2021 (2021-22-0051).
Keywords: clinical applications, extracellular vesicles, magnetic nanoparticle, prostate cancer, urinary exosomes

PT10.04 | Optimizing urinary extracellular vesicles isolation and characterization: a comparison of three protocols

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Introduction: Urinary extracellular vesicles (uEVs) are potentially clinically valuable disease biomarkers, particularly in patients with kidney and urinary tract problems. uEVs encapsulate their cargo, including protein and nucleic acids, and stabilize it from enzymatic degradation. Polymeric Tamm Horsfall protein (THP) was reported to interfere with the number of uEVs isolated and protocol reproducibility.
Methods: We optimized uEVs isolation from healthy volunteers using precipitation-, pH and precipitation-, and size-exclusion chromatography (SEC)-based method, with and without THP removal. uEVs were characterized by nanoparticle tracking analysis (NTA).
Results: Among three commercial kits utilized, Total Exosome Isolation (from urine) Reagent (TEIR) resulted in the highest uEVs concentration, followed by SEC and Urine Exosome Purification Kit, in samples with and without THP removal. The implementation of the THP removal protocol reduced the uEVs isolated with TEIR and Urine Exosome Purification Kit, but not with SEC. The number of small-size uEVs was less in THP-removed isolates than in the samples without THP removal. A homogenous population of small uEVs was best obtained with Urine Exosome Purification Kit, followed by SEC and TEIR. Urine Exosome Purification Kit needed the smallest dilution to reach the requirement of nanoparticle tracking analysis, followed by SEC and TEIR.
Summary/Conclusion: TEIR is beneficial in studies requiring large numbers of uEVs regardless of their size. Urine Exosome Purification Kit is useful in research aiming to study small uEVs. THP removal protocol causes loss of uEVs isolated with TEIR and Urine Exosome Purification Kit, in contrast, it improves uEVs isolation with SEC.
Funding: The project is a part of Dr. Ambarsari's PhD research. The PhD study is funded by the Indonesian Endowment Fund for Education.
Keywords: centrifugation, chromatography, exosomes, methods, nanoparticles, proteins

PT10.05 | A multi-method approach to investigate pathological tau abundance in brain-derived extracellular vesicles isolated from Alzheimer's Disease patients

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Introduction: Extracellular vesicles (EVs) are small membranous structures derived from the lipid bilayer of cells to facilitate the transport of cellular cargo. Recent studies demonstrate that tau oligomers accumulate in brain-derived EVs (BDEVs) in Alzheimer's Disease (AD) patients, and EVs can spread tau pathology in the brain. However, the effective ways to assess and manipulate tau in EVs are not well established. Here, we aim to investigate the levels of pathological tau found in BDEVs and establish the method to isolate tau enriched EV to understand it's differential expression between AD and control (CTRL) patients.
Methods: EVs were isolated from the temporal cortex of human AD and CTRL brains by discontinuous sucrose gradient ultracentrifugation (SG-UC), and characterised by transmission electron microscopy (TEM). We detected tau in EV samples by western blot using antibodies targeting different epitopes on tau protein, and assessed populations of Tau13+ EVs by Nanoanalyzer (NanoFCM). We further developed an immuno-affinity method to purify tau protein from total BDEVs by conjugating Tau13+ or IgG antibody beads. The overall protein content of the immunoprecipitated (IP) samples was assessed by silver staining, and IP tau was confirmed by western blot with the Tau13+ antibody. Finally, the total tau precipitated from the EVs was quantified using ELISA.
Results: Purified EVs showed cup-shaped morphology by TEM. Western blot confirmed the enrichment of tau protein in EVs isolated from AD and CTRL brains. Tau13+ EV populations (5%) were detected by NanoFCM in human BDEVs. Western blots also showed the proper immunoprecipitation of tau by the immuno-affinity method, as no tau signal was detected using the control IgG antibody. Our ELISA assay revealed that from 1.0 g of human brain tissue, 80 μg of EV protein was extracted, of which 5.8 μg was the total tau. Silver staining quantified that 1.2 μg of IP protein contained around 1.8 ng tau protein, suggesting the successful enrichment of tau in IP samples, which were further applied to investigate the tau interactome profile using mass spectrometry (MS).
Summary/Conclusion: We identified and validated tau in human BDEV samples with a multi-method approach. We successfully established the immuno-affinity method to precipitate tau from BDEVs, which reached the sufficient protein level for MS analysis. This study may aid further investigation of the EV tau interactome to understand the role of EVs to mediate tau pathology in neurodegenerative diseases.

PT10.06 | A comparison of enzymatic digestion strategies for the extraction of brain-derived extracellular vesicles

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Introduction: The choice of isolation technique is a critical step for the study of extracellular vesicles (EVs) and can have implications when it comes to the interpretation of results. In particular, the extraction of EVs from tissue samples requires mechanical homogenisation and enzymatic digestion in order to dissociate tissue and release EVs. The process of enzymatic digestion is an important point to consider as it is unclear how different enzymes may affect the integrity and profile of isolated EVs.
Methods: The performance of papain and collagenase IV as digestive enzymes was compared in an EV extraction protocol from whole mouse brains. EVs were subsequently enriched from homogenate samples using a density-based sucrose gradient. Nanoscale flow cytometry, western immunoblot and TEM were employed in order to characterise the phenotype and purity of EVs from both conditions.
Results: Overall, EV samples that were extracted using collagenase had a lower concentration of particles in comparison to the EV samples extracted with papain. However, collagenase treated samples had a higher purity of EVs as indicated by a reduced level of free protein and increase percentage of MemGlow+ events. Moreover, samples extracted using collagenase had a higher proportion of particles expressing typical EV markers (CD81, CD9 and CD63) compared with papain treated samples. Interestingly CD81 was particularly susceptible to degradation by papain digestion.
Summary/Conclusion: In light of these findings, we recommend the use of collagenase in order to increase the purity of EVs from tissue samples.
Funding: Nouvelle-Aquitaine.
Keywords: EV isolation, enzyme, flow cytometry, brain.

PT10.07 | No difference in purity and protein content when extracellular vesicles are isolated from fresh or frozen human melanoma tissue

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Introduction: Solid tissues are emerging as a relevant source of extracellular vesicles (EVs). EVs have been isolated from both fresh and from frozen tissues, even though a thoroughly analysis of the potential effects introduced by the freezing process on EVs has not been performed yet. The aim of this study is therefore to evaluate the effects of tissue freezing process on EV purity and composition.
Methods: Human melanoma metastatic tissue (n = 6) were collected and divided in two parts. The first part was immediately used to isolate EVs by enzymatic treatment followed by ultracentrifugation and density cushion. The second part was frozen in dry ice and stored at -80°C for 2 weeks. After thawing, the EVs were isolated in exactly the same way as from fresh tissue. Tissue-derived EVs from both fresh and frozen tissues were characterized by transmission electron microcopy (TEM) and nanoparticle tracking analysis (NTA), and the ratio particle/protein was calculated to evaluate sample purity. Moreover, quantitative mass spectrometry was used to compare the EV protein composition from the two conditions in depth.
Results: From both fresh and frozen melanoma tissue, TEM showed the presence of round elements with typical EV size (80-500 nm), and no significative difference in particle concentration, size and purity was observed. The proteomic analysis showed strong similarity between the two groups and few proteins were differently expressed. Moreover, no significant difference was observed when typical marker of EVs (CD9, CD63, CD81, ADAM10 and Mitofilin) were analyzed.
Summary/Conclusion: Our results demonstrated that the purity and the protein composition of EVs isolated from frozen tissue was not affected by the freezing process, and they were comparable to those of the EVs obtained from the same fresh sample. This opens the possibility to isolate and analyze EVs from tissues stored in biobanks, further strengthening research studying EVs as potential biomarkers for disease.
Keywords: extracellular vesicle from tissue, human melanoma, fresh tissue, frozen tissue

PT10.08 | Proteome analysis of human salivary extracellular vesicles (EVs) for lung cancer

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Introduction: Human salivary extracellular vesicles (sEVs) have emerged as a potentially rich source of disease-related molecules for biomarker discovery and disease diagnosis. In the context of lung cancer (LC), in-depth analysis of sEVs could potentially yield robust and specific biomarkers that are critically needed to improve diagnostic routines and clinical outcomes. The current clinical assessment of lung cancer relies heavily on a few clinical biomarkers (EGFR, ALK, KRAS) determined through tumor tissue examinations. Quantitative comparison of changes in sEVs proteins of the lung cancer and control group could help to discover protein candidates related to lung cancer.
Methods: In this work, we collected clinical saliva samples from seven normal subjects and seven lung cancer patients. EVs were isolated from the saliva samples through high-speed ultracentrifugation at 110,000 g for 2.5 hours at 4C. The sEVs size and distribution were analyzed through NTA analysis, while the morphology of the EVs was evaluated with TEM.
Results: The nanoparticle tracking analysis (NTA) revealed the mean size of sEVs was 197.9 nm and concentration 2.31e+009+/-9.45e+007, respectively. Transmission electron microscopy (TEM) analysis revealed the size of sEVs was 105 and 145 nm, correspondingly. The label-free quantification analysis revealed the identification of 64 up-regulated (FC>1.5) and 174 down-regulated (FC< 0.66) proteins in normal and lung cancer groups. Moreover, the sEVs proteomic data revealed the identification of 47 unique sEVs proteins by comparing them against Vesiclepedia and Exocarta databases.sEVs biomarkers (CD9, TSG101, and CD63) were verified by western blot (WB). It was observed that the up-regulated proteins were involved in signal transduction, organismal death, and morbidity. In contrast, down-regulated sEVs proteins were involved in tumor cell proliferation, cell death

of tumor cell lines, and apoptosis. Qualitative analysis revealed that the lung cancer sEVs protein cluster pattern was different from the normal.

Summary/Conclusion: In summary,the work demonstrates the great potential of using mass spectrometry (MS) based quantitative proteomics approaches to study and describe the role of the sEVs protein in signaling networks in cancer research. In particular, using LC as a model system, we demonstrate that sEVs-proteomics is a very powerful technology offering great prospects to discover novel protein candidates, molecular mechanisms, and protein-protein interaction networks involved in cancer progression, metastasis, and treatment. It is a step forward in the field of liquid biopsy for the non-invasive study of biomarkers.

Funding: This work is supported by the Key Scientific Project of Shanghai Jiao Tong University (No. TMSK-2020-130, No. YG2017MS80).

Keywords: saliva, extracellular vesicles, proteins, lung cancer

PT10.09 | Salivary small extracellular vesicles role in screening of Parkinson's disease

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Introduction: Parkinson's disease is generally asymptomatic at earlier stages. At an early stage, there is an extensive progression in the neuropathological hallmarks, although, at this stage, diagnosis is not possible with currently available diagnostic methods. Therefore, the pressing need is for susceptibility risk biomarkers that can aid in better diagnosis and therapeutics as well can objectively serve to measure the endpoint of disease progression. The role of small extracellular vesicles (sEV) in the progression of neurodegenerative diseases could be potent in playing a revolutionary role in biomarker discovery.

Methods: The salivary sEVs of the subjects (PD = 70, healthy controls = 26 and prodromal = 08) were isolated by chemical precipitation followed by antibody-based validation through CD63, flotilin, CD9 (universal surface marker) and confirmed neuronal origin by CD171 as well morphologically characterized through cryo-EM. The sEVs quantification via fluorescence-tagged sEVs NTA and antibody-based NTA using CD63. The α-syntotal in sEVs cargo was determined by ELISA. The confirmation of the disease severity staging was done by 99mTc-TRODAT-SPECT.

Results: We observed a significant increase of fluorescence-tagged sEVs in PD ($p< 0.0001$) than the HC via NTA (sensitivity of 94.34%) as well it was in line with the antibody-tagged sEV $p = 0.006$ (sensitivity of 94.12%). A significant increase of α-syntotal concentration in the sEVs of PD when compared to HC (sensitivity of 88.24%). The fluorescence-tagged sEV depicted a positive correlation with the hallmark protein of PD α-syntotal $r = 0.4709$, $p = 0.0486$. The striatal binding ratios in the 99mTc-TRODAT-SPECT shown to have a positive correlation with the fluorescent sEVs concentration $r = 0.3000$, α-syntotal concentration $r = 0.8000$. Based on our analysis, we were speculating that some of the recruited healthy controls could be cases of prodromal Parkinson's disease. We osbserved the increased concentration of sEV (particle/ml) in these prodromal compared to HC but less than PD patients and that further validated by the α-syn protein.

Summary/Conclusion: This study is the first to address that the sEVs can screen the early as well progression of the disease with clinically acceptable sensitivity and can be a potent early detection method for PD.

Funding: The funding provided by Department of Health Research (DHR), Indian Council of Medical Research (ICMR), India and Lulea University of Technology, Sweden.

Keywords: Screening method, neurodegenerative disease, extracellular vesicle

PT10.10 | Exhaled breath condensate and sputum exosomes: as noninvasive diagnostic isolations superseding invasive ones for lung cancer diagnosis

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Introduction: Being the leading cause of worldwide mortalities, the burden of early diagnosis of lung cancer is the utmost requirement of the present research. Due to the invasive nature of Blood and Biopsy samples, noninvasive sample sources such as Exhaled breath condensate (EBC) can be screened for early predictive lung cancer biomarkers.

Methods: Human EBC (HE) and Sputum (HS) samples were correlated with invasive sample sources (human plasma (HP) and tissue biopsy) to check their efficacy as biomarker sources. Samples were collected after permission from the institute's human ethical committee and with the consent of patients. Exosomes were isolated from all the samples and characterized for their size, concentration, and surface markers. Exosomal protein was also quantified to check the downstream reliability of noninvasive exosomes as compared to invasive sources.

Results: Exosomal size analysis through DLS (HE 61.08 ± 24.12 nm, HS 50.32 ± 6.20 nm, HP 85.92 ± 25.45 nm) and NTA (HE 129.2 ± 10.91 nm, HS 114.3 11.09 nm, HP 105.0 3.92 nm) results showed that EBC and Sputum exosomal dimension range was similar as that of plasma exosomes i.e., 30–150 nm. TEM showed characteristic morphology of EBC and sputum exosomes. NTA concentration results provided valuable data that along with plasma samples (HP1.2E+11-8.69E+09 particles/mL), EBC (HE 2.6E+09-2.64E+08 particles/mL) and sputum samples (HS6.44E+09-4E+09 particles/mL) can also provide adequate exosome quantity to proceed for downstream processing. Bradford assay confirms that EBC (31.75 ± 8.09 μg/20μl) and sputum (27.91 ± 2.30 μg/20μl) exosomes have a quantifiable amount of protein.
Summary/Conclusion: Results conclude that EBC and sputum could be novel and efficient sample sources for exosomes to be screened for early lung cancer biomarkers. We can avoid invasive samples such as blood and tissue biopsies and proceed with these noninvasive samples to effectively diagnose lung cancer
Funding: Indian Council of Medical Research, India.
Keywords: exosomes, exhaled breath condensate, plasma, lung cancer

PT10.11 | Extracellular vesicles from bovine follicular fluid separated by different methods have distinct morphological, molecular and functional characteristics

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Introduction: Advances in the use of extracellular vesicles (EVs) for diagnosis and treatment are restricted by the challenge of defining an accurate and reliable protocol for the isolation of these EVs. Therefore, we investigated if different separation methods can influence EVs' morphological, molecular, and functional characteristics.
Methods: Extracellular vesicles were isolated by ultracentrifugation (UC), size exclusion chromatograph (SEC) and precipitation reagent (P) from follicular fluid (FF) and had size and concentration evaluated by nanoparticle tracking analysis as previously described by 1, in addition to transmission electron microscopy and flow cytometry for CD81, CD9 and CD63 analysis. After, EVs pellets were used for total RNA extraction to analyze 383 miRNAs using RT-qPCR (5 biological replicates). Moreover, cumulus-oocytes complex (COCs; 4 biological replicates) retrieved from 3–6 mm follicles from bovine ovaries were in vitro matured (IVM) under different medium conditions: 1) control (base medium (BM); 2) UC (BM supplemented with FF EVs separated by UC); 3) SEC (BM supplemented with FF EVs separated by SEC); and 4) P (BM supplemented with FF EVs separated by P). The COCs were IVM for 22 hours, followed by maturation rate evaluation.
Results: The FF nanoparticles' size and concentration differed among the groups depending on the isolation method used ($p< 0.0001$), being higher in P compared to UC and SEC, respectively 1. A total of 225 miRNAs were detected, being 160 miRNAs expressed in all isolation groups. Of these 106 miRNAs were differently expressed ($p< 0.05$). When we supplemented COCs in IVM with EVs separated by different methods, we observed increased oocyte IVM rates in SEC (78.9% ± 7.1) group when compared to control (59.3% ± 7.5) ($p = 0.02$).
Summary/Conclusion: The isolation method can influence EVs' morphological and molecular characteristics as well as improve oocyte IVM rate. Therefore, it is important to consider the research goal (supplementation, diagnosis, and others) to choose the adequate separation method. 1 - Dos Santos et al. Anim Reprod. 2022: e22150.
Funding: FAPESP 2021/06645-0; 2021/12560-7; 2015/21829-9; 2022/01433-7; 2022/01505-8; CAPES 001.
Keywords: bovine embryos, reproduction, in vitro embryo production

PT10.12 | Evaluation of extracellular vesicle concentration methods following size exclusion chromatography of human stool supernatant

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Introduction: Colorectal cancer (CRC) is the 2nd most fatal cancer in the U.S., but when caught early it is highly curable. Stool-derived extracellular vesicles (EVs) are a novel biomarker source that could augment the sensitivity for detection of CRC precursors. We previously reported that size- exclusion chromatography (SEC) followed by ultrafiltration (UF-100kDa) was the optimal isolation method for human stool supernatant. In this study, we assessed alternative EV concentration methods to determine if we could further maximize EV recovery/purity.
Methods: EVs were isolated from stool supernatant (1ml; n = 4 healthy patients) using SEC followed by ultrafiltration (UF; 4ml/15ml;10kDa, 30kDa, 50kDa, 100kDa) or speed vacuum (SV) after IRB approval. Particle recovery/size was evaluated using nanoscale flow cytometry and microfluidic resistive pulse sensing. Electron microscopy was used to visualize the presence

of contaminants. RNA composition was assessed both pre/post- proteinase K/RNase treatment. Western blot characterized human, bacterial and contaminating proteins. Samples were also submitted for mass spectrometry to compare soluble protein contamination and reveal the host/bacterial proteome.
Results: UF-30kDa and SV had the highest post-SEC recovery (mean ± SEM; %; SV: 68.9 ± 13.3, 10kDa: 62.5 ± 7.4, 30kDa: 78.3 ± 13.1, 50kDa: 51.2 ± 9.3, 100kDa: 40.4 ± 5.0). SV had the highest protein yield (mean ± SEM; ug; SV: 55.3 ± 5.2, 10kDa: 37.2 ± 8.3, 30kDa: 29.3 ± 1.9, 50kDa: 22.5 ± 1.9, 100kDa: 17.2 ± 2.3). There was no difference in RNA yield pre- and post-enzymatic treatment among methods ($P > 0.98$). CD63, TSG101, flagellin, and ompA proteins were present in all concentration methods. Ongoing proteomic analysis will further evaluate the presence of non-vesicular proteins.
Summary/Conclusion: These findings indicate that EV concentration methods primarily impact protein content with limited influence on non-vesicular RNA contamination. In conclusion, this study serves as methodological framework for RNA/protein biomarker discovery.
Funding: This work was supported by the National Cancer Institute under CA214679 to JBK. Additional support was provided by Exact Sciences (Madison WI), under a sponsored research agreement with Mayo Clinic.
Keywords: extracellular vesicle concentration, biomarker, colorectal cancer, gastrointestinal microbiome, proteomics

PT11: Resistance to Therapy

Location: Hall 4A

16:45 - 18:45

PT11.01 | Extracellular vesicles transfer resistance to vincristine in sonic hedgehog medulloblastoma

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Introduction: Medulloblastoma is the most common malignant paediatric brain tumour accounting for 20% of all childhood tumours; approximately one-third of patients present with metastatic disease at diagnosis and the outcome for these patients remains very poor. The high frequency of recurrence and metastatic relapse in medulloblastoma supports the idea of intrinsic drug resistance within cells. This work looks at the transfer of vincristine resistance, primarily focusing on the ATP-binding cassette (ABC) transporters and we hypothesise that extracellular vesicles (EVs) may promote multidrug resistance within tumours by mediating intercellular transfer of ABC transporters.
Methods: Drug-tolerant cell lines were generated by continuous culture in drug-containing culture medium. ABC transporter gene and protein expression was assessed via qPCR and western blot. EVs were isolated from EV-depleted medium using size-exclusion chromatography. Nanoparticle tracking analysis was used to quantify and determine size and flow cytometry was used to identify EV markers. Cytotoxicity analysis was used to assess the role of EVs in the transfer of resistance.
Results: Analysis of drug-tolerant cell lines revealed significant increases in specific drug transporter gene expression across medulloblastoma subgroups. ABCB1 protein expression was significantly higher in drug-tolerant sonic-hedgehog (SHH) lines and inhibition of ABCB1 in a drug-tolerant SHH line abolished acquired resistance to vincristine. Cytotoxicity assays on sensitive cells, after pre-treatment with drug-tolerant EVs, showed a significant increase in vincristine resistance, implicating EVs in the transfer of resistance.
Summary/Conclusion: Data to date supports the hypothesis that multidrug transporter carrying extracellular vesicles may transfer their multidrug resistant phenotype to surrounding cells in medulloblastoma. We are currently testing this using inhibitors and knockout lines.
Funding: Biotechnology and Biological Sciences Research Council, University of Nottingham, The James Tudor Foundation.
Keywords: drug resistance, neuro-oncology

PT11.02 | Conferred radioresistance via treatment trained extracellular vesicles in pediatric H3K27M-driven Diffuse Midline Glioma

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Introduction: H3K27M-driven Diffuse Midline Gliomas (DMG) are a subset of uncurable malignant pediatric gliomas. In all cases, patient tumors evolve to be resistant to radiation therapy, the current standard of care. DMGs are multi-clonal, and each subclone has distinct genetic and transcriptional profiles. Data suggest that activation of oncogenic pathways through sub-clonal communication, specifically extracellular signaling, impacts how tumors respond to therapy and evolve. Secreted extracellular vesicles (EVs) are a mode of intratumor communication. However, EVs in H3K27M-DMG have not been extensively characterized, and their role in multi-clonal tumor evolution and development of radioresistance is unknown.
Methods: We isolated 35–350nm EVs from a panel of primary and relapsed patient-derived H3K27M-DMG cells and individual sub-clones from a primary patient-derived cell line. We utilized Next-Gen sequencing and mass spectrometry to profile miRNA, proteins, and metabolites in H3K27M-DMG EVs. Additionally, we identified H3K27M-DMG EV membrane signatures using surface-enhanced Raman spectroscopy. We are using DMG cells that express a genetically encoded death indicator (GEDI) and a cell cycle indicator (FUCCI) for longitudinal imaging to assess the functional impact of EVs on radiation-induced cell death and cell senescence, two dynamic processes.
Results: EVs isolated from radioresistant H3K27M-DMG conferred radioprotective effects to radio-sensitive DMG cells given 8 Gy ionizing radiation, preventing apoptosis. These EVs also induce enhanced glycolysis in radiosensitive H3K27M-DMG, compared to treatment with autologous EVs.
Summary/Conclusion: Pediatric DMG-derived EVs induce functional changes in radiation-naive DMG cells and confer radioresistant phenotypes.

PT11.03 | Molecular analysis of extracellular vesicles for the prediction and monitoring of paclitaxel pharmacological resistance in breast cancer

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Introduction: Paclitaxel (PX) is a widely used agent for breast cancer (BrCa), but its resistance is a major cause of treatment failure. Making early clinical decisions of continuing with the current therapy or pivoting will improve patient care. Extracellular vesicle (EV) analysis in liquid biopsies provides unique opportunities to frequently monitor treatment responses. Our study aimed to elucidate the ability to predict drug sensitivity by molecular EV analysis.
Methods: We determine the IC50 of 6 BrCa cell lines (HCC1954-WT, HCC1954-RT, BT474, MCF-7, MDA-MB-231, and HCC1937) by methyltransferase (MTT) assays. EVs were isolated from culture supernatant by ultrafiltration followed by size exclusion chromatography. EV concentrations and sizes were analyzed through nanoparticle tracking analysis. We screened gene expressions of multiple candidate markers in cell lines and their EVs by real-time PCR assay. We also measured corresponding protein counterparts in EVs using beads-based flow cytometry (FC). Finally, we selected key protein markers and applied a plasmon-enhanced single EV analysis to compare their levels in EVs.
Results: We measured significant increases in key marker levels in PX-resistant cell lines compared to the more sensitive ones. The EV analysis showed a similar profile. In counterpart protein measurements, the FC confirmed the relatively high levels of key markers in EVs derived from the more resistant cell lines. The single EV analysis with our nanoplasmonic sensors showed an increase in marker-positive EV subpopulations in the more resistant cell lines.
Summary/Conclusion: These results demonstrate a correlation between EV biomarker levels and PX sensitivity, suggesting that evaluating these markers in EVs may serve as an avenue for early detection of PX resistance in BrCa patients.
Funding: A sponsored research agreement between Canon and Massachusetts General Hospital (agreement # 2020A013232).
Keywords: paclitaxel, breast cancer, resistance, biomarker

PT11.05 | The exosomal microRNA miR-503: a key modulator in resistance to chemotherapy in breast cancer cells

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Introduction: Aiming to determine the impact of microRNAs (miRs) secreted by the endothelium on tumor development we identified miR-503, which exhibited downregulated levels in extracellular vesicles (EVs) released from endothelial cells cultured under tumoral conditions (Bovy et al, Oncotarget, 2015). We previously highlighted an EV-dependent transfer of miRNAs from endothelial to tumor cells (Perez-Boza et al., CMLS, 2020). In this work we focus on the impact of miR-503-EV on resistance to chemotherapy.

Methods: EVs were purified by differential ultracentrifugation from endothelial cells. Functional tests were performed by BrdU assay (proliferation), scratch assays (migration) and spheroid assays. Anti-tumoral activity was assessed in vivo in a xenograft mouse model of breast cancer using MDA-MD-231 cells.
Results: To investigate the potential impact of miR-503 in the resistance to chemotherapy, we used epirubicin- or paclitaxel-resistant triple breast cancer cells. Interestingly, the basal level of miR-503 is lower in resistant cells compared to the sensitive ones. To evaluate the impact of miR-503 on cancer cells, we transiently transfected the MDA-MB-231 cells with a microRNA mimic designed to generate the mature miR-503-5p (miR-503) and with an anti-miR targeting miR-503. These results indicate that miR-503 overexpression reduces significantly breast cancer cell proliferation, migration and invasiveness while blocking the miR shows the opposite effects in resistant cells. Similar effects were obtained when cells were treated with miR-503-loaded EVs. In vivo experiments showed that EVs enriched in miR-503 could reduce tumor growth drastically. Finally, clinical data revealed that the deletion of miR-503 decreased the survival of breast cancer patients.
Summary/Conclusion: Taken together, these results suggest that EVs enriched in miR-503 exert anti-tumoral functions in cells resistant to chemotherapy.
Funding: This project has been financed by the Televie (to AB), the Fonds Leon Fredericq, the FNRS and ULiege.
Keywords: cancer, chemotherapy, microRNA

PT11.06 | Harnessing oncolytic exosomes for tumor cell-preferential cytoplasmic delivery of misfolded proteins to induce immunogenic cancer cell death

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Introduction: In order to utilize exosomes to be an ideal drug modality, it is necessary to improve the fusion rate of exosomes with target cells. In this study, we engineered oncolytic exosomes (bRSVF-Exo) loaded with misfolded proteins (MPs) and expressing respiratory syncytial virus F protein, enabling the exosomes to fuse to cancer cells in a nucleolin-dependent manner and directly dumping MPs into cancer cells, inducing immunogenic cell death (ICD). We evaluated the therapeutic efficacy and antitumor immunity of bRSVF-Exo by itself and combined with PD-1 blockade. Taken together, this study suggests an effective therapeutic strategy for using tumor-targeting oncolytic exosomes for cancer immunotherapy.
Methods: Exosomes were isolated by differential centrifugation and ultracentrifugation. Exosome markers and RSVF expression were confirmed using ProteinSimple analysis, along with particle numbers, conformation and size of exosomes. In vivo experiments were conducted with CT26.CL25 inoculated mice intratumorally injected with exosomes three times.
Results: bRSVF-Exo was engineered by isolating exosomes from RSVF-expressing HEK293T cells treated with bafilomycin, an autophagy inhibitor which increased the expression of RSVF and MPs in exosomes. RSVF expressing exosomes spontaneously fused with cancer cells via nucleolin. Therefore, bRSVF-Exo could preferentially fuse with cancer cells and direct dumping MPs, leading to the accumulation of aggresomes inside cancer cells, triggering ER stress and ICD. bRSVF-Exo showed a 70% tumor regression effect in vivo. This antitumor effect was amplified when combined with PD-1 blockade, leading to complete remission in some cases.
Summary/Conclusion: Our results show that bRSVF-Exo is a potential ICD inducer with tumor targetability that elicits anti-tumor immunity. We present a previously unidentified strategy for effectively delivering MPs into cancer cells by tumor-targeting oncolytic exosomes, resulting in potent ICD-mediated immune responses against cancer. Combined therapy with bRSVF-Exo and PD-1 blockade augment a powerful tumor-specific immunity.

PT11.07 | Exosomal cannabidiol: A promising candidate for targeted oral delivery against breast cancer

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Introduction: The current cancer therapies are limited by lack of specificity and the numerous adverse effects. Cannabidiol (CBD) is garnering attention in the scientific field due to its potential as an anti-cancer therapeutic agent. However, its application

is limited due to low oral bioavailability. Here, we show that CBD can be administered orally in folic acid (FA) functionalized tumor targeted exosomal formulation to enhance its efficacy and minimize toxicity.
Methods: Exosomal formulation of CBD (ExoCBD), prepared by incubation of CBD with bovine colostrum exosomes, were characterized for size, charge and hallmark protein markers. Cell culture assays (MTT, colony forming) were performed to analyze the anti-cancer activity of the CBD and ExoCBD against drug sensitive and resistant breast cancer cells. In the ongoing in vivo study, free CBD and ExoCBD formulations, with and without a targeting ligand, FA are being tested for efficacy against orthotopic breast cancer xenograft in a murine model.
Results: Size of exosomes (78 ± 2.4 nm) somewhat increased in ExoCBD (123 ± 1.4 nm) and FA-ExoCBD (122 ± 1.3 nm) formulations. Drug load, as measured by UPLC, was 15% and 25% with Exo and FA-Exo, respectively. ExoCBD showed enhanced antiproliferative activity vs. free CBD against MCF7 and MDA-MB-231; this finding was supported with colony forming assay. CBD and exosomal CBD chemosensitized the drug-resistant MCF7-TR and MDA-MB-231-TR cells to paclitaxel. Analysis of cell lysates by Western blot showed downregulation of CB2 and MDR-1 receptors. We hypothesize that FA-ExoCBD would enhance the anti-cancer activity of CBD by enhancing oral bioavailability and targeting breast tumors in the murine model.
Summary/Conclusion: The ExoCBD showed enhanced antiproliferative activity in breast cancer cells accompanied by downregulation of CB2 and MDR-1 receptors.
Funding: Supported from Duggan Endowment: RCG and University's IPIBS fellowship: DNM.
Keywords: colostrum exosomes, breast cancer, CBD, folic acid functionalization

PT11.08 | Therapeutic application of extracellular vesicles containing EGFR isoform D as a co-drug strategy to target squamous cell cancers with tyrosine kinase inhibitors

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Introduction: Cancers of the head and neck (HNSCC) are primarily driven by the overexpression of EGFR. In the case of these cancers, targeted therapies such as tyrosine kinase inhibitors (TKIs) have limited success. We had previously shown that exceptional responders to TKIs have a genetic aberration that results in the over-expression of an EGFR splice-variant, isoform D (IsoD). We showed that this splice variant is expressed in extracellular vesicles (EV) and that the application of these IsoD-containing EVs sensitizes cancer cells that were previously resistant to TKI treatment.
Methods: We grow donor cells in defined serum-free media and purify EVs using an Amicon Ultra 15, 50kD MWCO filter. Cancer cells grown in EV-free RPMI are co-treated with IsoD EVs and TKIs for 72 hours. Using donor cells, we expressed and knocked down IsoD in order to determine whether IsoD is necessary for sensitizing cancer cells to TKI. The EVs collected from these experiments were used for co-treatment of recipient cancer cells. Xenograft models with HNSCC cancer cells were established in order to determine the efficacy of IsoD EV in vivo, and mice were treated with oral gefitinib and intraperitoneal injections of IsoD EV respectively.
Results: In our study, we demonstrated that the IsoD protein on the EV is necessary and sufficient to increase the sensitivity of cancer cells to TKI treatment. Interestingly, there is a direct correlation between co-treatment sensitivity and the dose of EV added. In vivo data have also demonstrated significant tumor regression following the administration of IsoD EV in combination with TKI.
Summary/Conclusion: We found that the novel expression of EGFR isoform D on EVs resulted in an increase in the killing of cancer cells when this isoD-containing EV was co-treated with TKIs. Our proposal is to use IsoD-containing EVs as a co-drug to treat cancers previously refractory to TKIs.
Funding: NCC Research Fund (NCCRF-YR2019-JAN-PG1).
Keywords: EGFR, tyrosine kinase inhibitor, splice-variant, cancer

PT11.09 | Inhibition of tumor growth using mesenchymal stem cell-derived exosomes loaded with KRAS siRNA and surface-modified with tumor-targeted proapoptotic peptide

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Introduction: Exosomes are extracellular vesicles naturally secreted by cells and serve as carriers of RNAs and proteins for cell-to-cell communications. KRAS is frequently mutated and plays a vital role in many tumors. Receptor expressed in lymphoid tissue (RELT), also known as tumor necrosis factor receptor superfamily 19-like, is upregulated in certain tumors such as lung tumor. To selectively deliver cytotoxic agents to tumor, we herein loaded mesenchymal stem cell-derived exosomes with a siRNA against KRAS and surface-modified them with RELTpep-KLA comprising a RELT-binding peptide (CRQTKN, named RELTpep) and proapoptotic peptide ((KLAKLAK)2, named KLA).
Methods: Exosomes were isolated from cell culture medium using ultracentrifugation and transfected with the KRAS siRNA using ExoFect reagents. Exosomes were then surfaced-modified with the RELTpep-KLA using a phospholipid-based membrane anchor, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol-maleimide. Cytotoxicity assays were performed using CCK reagents. Antitumor therapy was performed using Balb/c nude mice bearing subcutaneous human tumor xenografts.
Results: Compared with control peptide-KLA, mesenchymal stem cell-derived exosomes surface-modified with RELTpep-KLA more efficiently internalized into A549 lung tumor and Panc-1 pancreatic tumor cells highly expressing RELT on their surface and exhibited cytotoxicity to these tumor cells. Exosomes loaded with KRAS siRNA as well as surface-modified with RELTpep-KLA reduced the phosphorylation of Erk and exhibited cytotoxicity in A549 and Panc-1 cells and inhibited tumor growth in Panc-1 tumor-bearing mice more efficiently than those with either control siRNA or control peptide-KLA.
Summary/Conclusion: These results demonstrate that RELT-targeted mesenchymal stem cell-derived exosomes carrying proapoptotic peptide and KRAS siRNA hold a potential for exosome-based targeted therapy against cancer.
Keywords: exosomes, KRAS, proapoptotic peptide, RELT, siRNA

PT11.10 | Characterization of the homing of homotypic stem cell-derived exosomes in pancreatic cancer cells

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Introduction: To date, To date, efforts to systemically deliver in vivo siRNA or gene editing to cancer cells has been hampered by different issues limiting therapeutic efficiency and generating off-target toxicity. We explore an innovative delivery approach that leverages pancreatic stem cell-derived exosomes.
Methods: in the current study, we target pancreatic cancer cells combining exosome delivery with CRISPR-Cas9 payload to specifically knockout mutated KRAS, i.e. 95% of pancreatic cancers. We assess the homing of exosomes derived from cells that are matched to the target pancreatic cell type, sharing similar transmembrane proteins, such as tetraspanins and integrins: 1) AsPC1 cancer-cell-derived exosomes as a control; 2) exosomes derived from homotypic pancreatic stem cells (StemCellerant, Cambridge MA). We target AsPC1 cell line harboring G12D (ATCC: CRL-1682™) and Mia-PACA-2 (ATCC: CRL-1420™) harboring G12C. We optimized exosome isolation and loading with CRISPR-Cas9, with guide RNA we previously set up for targeting G12D and G12C and demonstrated efficacy leading to > 90% cell death related to KRAS knockout. We assess and compare the "homing" of exosomes derived from pancreatic stem cells and from AsPC-1 pancreatic cancer cells in vivo in AsPC1 and Mia-PACA2, in Harlan athymic nude mice models (Charles River), approved by the relevant animal use committees, using tracking exosomes via ExoGlow™- Protein Green (System Biosciences).
Results: We optimized exosome release, isolation and loading parameters. Exosome quality was controlled by nanoparticle tracking analysis (Par¬ticle Tech Labs, Downers Grove IL). We assess and compare the homing of pancreatic stem cells in vivo as well as AsPC-1 cancer cells, in AsPC1 and Mia-PACA2 mice models. Final data will be presented at the meeting.
Summary/Conclusion: Our objective is demonstrate the homing of exosomes from pancreatic stem cells targeting pancreatic cancer cells. The ultimate goal is to use this system as a platform for delivering CRISPR to treat different cancer types.
Funding: So far we raised $200,000.00.
Keywords: exosomes, homotypic, drug delivery, therapeutics, gene editing, oligonucleotides, pancreatic cancer

PT11.11 | Therapeutic potential of ROS amplifier-loaded sEVs to overcome multi drug resistance

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Introduction: The lipid bilayer structure of small extracellular vesicle (sEV) allows the loading of drugs. sEVs exhibit high degree of tropism to parental cells and great biocompatibility, which offers great potential as a drug delivery carrier. Overexpression of P-glycoprotein (P-gp), an adenosine triphosphate (ATP) binding cassette transporter, in cellular membrane after chemotherapy plays as a drug efflux pump to remove the drugs from the cancer cells. Multi-drug resistance (MDR) cancer cells have a higher level of antioxidants, including glutathione (GSH) as well as reactive oxygen species (ROS) than normal cancer cells. Oxidation

therapy through depletion of GSH can induce the lack of ATP supplementation by rapid depolarization of mitochondrial membrane potential (MMP), resulting in apoptosis. Herein, we developed benzoyloxy dibenzyl carbonate (B2C)-encapsulated sEVs (B2EVs), that bypass the efflux pump and increase oxidative stress.
Methods: B2EVs were formulated from B2C and OVCAR-8 cell-derived sEVs. Cell viability after B2EVs treatment was evaluated using MTT assay. B2EVs-induced ROS generation was evaluated using DCFH-DA. The tropism of B2EVs was studied using various ovarian cancer cell lines. OVCAR-8 cells were treated with endocytosis inhibitors (methyl-β-cyclodextrin and chlorpromazine) to confirm the endocytosis mechanism of B2EVs. B2EVs-induced apoptosis was evaluated using annexin V-FITC and PI staning. The disruption of MMP after B2EVs treatment was measured by a JC-1 MMP detection kit. After B2EVs treatment, the levels of ATP and NAD+/NADH were measured. MDR/ABCB1 were stained with a MDR/ABCB1 antibody after B2EVs treatment. Various formulations of B2EVs were intravenously injected into OVCAR-8/MDR xenografted mouse model every three days for 4 times. The body weight and tumor volume were measured. Histological analysis was performed using TUNEL, Cellrox, and H&E staining. The biocompatibility of B2EVs was evaluated by the measurement of CK, ALT, and LDH.
Results: B2EVs were formulated from B2C and OVCAR-8 EVs. B2EVs have spherical shape with membrane integrity of EVs. B2EVs showed higher anticancer activity than free B2C by utilizing the advantages of EVs, including tropism and bypassing P-gp. B2EVs induced the imbalance of redox status with the malfunction of P-gp pump by disruption of mitochondrial membrane potential. In mouse tumor xenograft model, B2EVs accumulated in tumor tissue and suppressed tumor growth.
Summary/Conclusion: We developed B2EVs for MDR cancer therapy. B2EVs induced GSH depletion and led to the disruption of MMP. In the mouse tumor xenograft model, B2EVs significantly suppressed tumor growth with strong biocompatibility. Thus, the therapeutic activity of B2EVs provides a strong translational potential for MDR cancer therapy.
Keywords: oxidative stress, tropism, glutathione, multi-drug resistance

PT11.12 | Delivery of miRNAs that placental extracellular vesicles carry may contribute to the reduction of ovarian cancer cell growth

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Introduction: Ovarian cancer is a leading cause of death in gynaecological cancers, and current treatment has limited efficacy. There is a similarity between the human placenta and tumours, as the placenta is invasive and develops its own vasculature. Unlike tumorus, the development of the placenta is strictly controlled. One control mechanism is placental extracellular vesicles (EVs). Placental EVs carry many functional proteins, regulatory RNAs, DNA, and lipids. Previous studies have shown that placental EVs significantly reduced the growth of ovarian cancer cells. However, the mechanism underlying this inhibitory effect is unclear. In this study, we investigated the levels of miRNAs and their target mRNAs in ovarian cancer cells (SKOV-3 cells) after exposure to placental EVs.
Methods: Placental EVs (n = 8) were collected from the first trimester placentae by differential centrifugation. SKOV-3 cells were cultured with or without placental EVs for 24 hours and total RNA was then collected for miRNA and mRNA sequencing and quantification.
Results: There was a significant increase in the level of miRNA-519a-5p and miRNA-143-3p in SKOV-3 cells after exposure to placental EVs. miRNA sequencing showed high levels of miRNA-519a-5p and miRNA-143-3p in placental EVs. The mRNA levels of Ras-GTPase-activating protein binding protein 1 (G3BP1) and E3 ubiquitin-protein ligase CBL-B (CBLB) which are target genes of these two miRNAs were significantly reduced in SKOV-3 cells after exposure to placental EVs. Both G3BP1 and CBLB are associated with the development of ovarian cancer. Transfection of SKOV-3 cells with mimics of miRNA-519a-5p or miRNA-143-3p significantly reduced the viability of SKOV-3 cells.
Summary/Conclusion: Our data demonstrate that the delivery of miRNAs that are associated with the promotion of cancer cell death by placental EVs could contribute to the reduction of ovarian cancer cell growth. Our findings provide a novel pathway for therapeutic intervention in ovarian cancer.

PT11.13 | Small EV for ovarian cancer therapy through tropism

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Introduction: Exosomes, one of the subtypes of extracellular vesicles, range from 50 to 200 nm in diameter and regulate cell-to-cell communication in biological and pathological processes. Although exosomes derived from tumors have various functions in cancer progression, resistance, and metastasis through cancer exosome-derived tropism, there is no quantitative information on cancer exosome-derived tropism that will be beneficial to guide cancer therapy by inhibiting exosome release or uptake.
Methods: Using ovarian cancer-derived exosome, exosome tropism mechanism and their therapeutic applications were investigated through various in vitro and in vivo experiments.
Results: Multifold exosome uptake from ovarian cancer cells into the "parent" ovarian cancer was observed compared to non-cancer cells through in vitro and in vivo tumor-bearing mice models. Also, their therapeutic application using this advantageous tropism showed that payloads encapsulated exosomes from ovarian cancer significantly deliver the payloads to the parent ovarian cancer tumor.
Summary/Conclusion: Quantification of the release of cancer-derived exosomes and the uptake of the exosomes into their "parent" cancer cells, displayed the target tropism of cancer-derived exosomes. These results will be beneficial for future diagnosis and therapeutic applications.
Keywords: exosome, tropism, drug delivery, cancer therapy

PT12: Techniques and Methods Single EV Analysis

Location: Hall 4A

16:45 - 18:45

PT12.01 | Quantitative serum pharmacokinetics of EVs from diverse sources using high content single vesicle imaging

Tanja Plank[1]; Melanie Schürz[1]; Danmayr Joachim[2]; Cristian-Tudor Matea[3]; Anna Müller[1]; Jana Kiefer[4]; Laurens Kober[1]; Eva Klinglmayr[1]; Vesna Stanojlovic[1]; Petra Reinthaler[5]; Angelika Sales[5]; Martin Wolf[6]; Dirk Strunk[6]; Mario Gimona[7]; Martin Hintersteiner[8]; Nicole Meisner-Kober[3]
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Introduction: Research on the use of EVs from diverse sources as therapeutics or for drug delivery exploded within the last decade, but knowledge on quantitative in vivo pharmacokinetics/-dynamics (PK/PD), bioavailability and biodistribution are still limited. Here we present a rapid, simple, robust, miniaturized and cost-effective method to determine the PK of EVs in serum with absolute quantification at the single vesicle level.
Methods: Different EV labels were characterized for in vivo use based on selectivity, stoichiometry and serum stability and a miniaturized 384well plate assay was developed for labelled EV quantification directly in serum using high content single vesicle imaging (HC-SVI) with quantification by EVAnalyzer (Schuerz et al 2022). EVs from multiple sources (HEK, bovine milk, goat milk, umbilical cord MSC) were covalently labelled and administered i.v. to mice. Blood was collected at various time points and serum was examined by HC-SVI.
Results: The miniaturized assay was validated for different labels and serum from several species (mouse, rat, nonhuman primates and human). NIRF dyes provided the best signal-to-noise, resulting in a limit of detection of 1–5 × 10e7 EVs/mL serum. With sample volumes below 20μl the assay allows multiple bleedings, reducing the animal numbers. A short serum half-life was observed for all EVs tested in mice (T1/2 ~ 3–5min), which we also confirmed in non-human primates for the most relevant sample. Since these short half-lives suggested rapid extravasation rather than renal clearance, we additionally confirmed EV penetration into tissue using quantitative HC-SVI on liver sections.
Summary/Conclusion: The HC-SVI serum assay enables quantification of the circulation half-life of EVs from diverse sources and in different species at the single vesicle level. The assay is performed directly in small volumes of serum without further purification and uses exclusively standard laboratory equipment, fluorescence microscopes and an open source software.
Funding: This work was supported by the following grants: EV-Quant (County of Salzburg, WISS2025, 20102-F2100572-FPR); EV-TT BPro (County of Salzburg, WISS2025, P1812596), EVTT (European Union, EFRE/IWB 20102-F1900731-KZP); CONSONANT (County of Salzburg, WISS2025, F2200397-KZP).

Keywords: pharmacokinetics, EV bioavailability, EV biodistribution, high content single vesicle imaging, extracellular vesicles, absolute serum quantification, open source

PT12.02 | CRISPR/Cas13a-based microRNA detection in tumor-derived extracellular vesicles

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Introduction: Extracellular vesicle (EV) microRNAs (miRNAs) play important roles in cancer initiation and progression via cell-to-cell communication. Thus, quantitative measurements of EV miRNAs is critical for cancer diagnosis and longitudinal monitoring. Traditional PCR-based methods, however, require an RNA extraction process and remained as bulk analysis. Here, we introduce an amplification-free and extraction-free EV miRNA detection method by delivering liposome-encapsulated CRISPR/Cas13a components into the EVs through EV-liposome fusion.
Methods: We isolated EVs from 5 different ovarian cancer (OVCA429, SkOV3, ES-2, CaOV3, and OV90) and 1 benign (TIOSE4) cell lines using size exclusion chromatography. The isolated EVs were characterized by nanoparticle tracking analysis and western blot analysis. Next, we incorporated CRISPR sensing components (Cas13a, CRISPR RNA/crRNA, and fluorescence-quencher probes) in liposomes and delivered them to EVs through EV-liposome fusion to detect miR-21-5p. We also applied the method for EpCAM-positive tumor-derived EVs captured on gold disk arrays for multiplexed analysis.
Results: We showed that miR-21-5p-positive EV counts are in the range of 2∼10% in ovarian cancer EVs (OV90, ES-2, OVCA429, SkOV3, CaOV3,), which is significantly higher than the positive EV counts from the benign cells (< 0.65%, TIOSE4). The result showed an excellent correlation between bulk analysis with the gold standard method, RT-qPCR. We also found that EpCAM-positive EVs showed significantly higher abundances of miR-21-5p in OV90 compared with the TiOSE4.
Summary/Conclusion: The developed EV miRNA sensing system provides the specific miRNA detection method in intact EVs without RNA extraction and the possibility of multiplexed single EV analysis for protein and miRNA markers.
Funding: NIH R21CA217662 and R01GM138778.
Keywords: extracellular vesicle, microRNA, CRISPR, Cas13a, liposome, ovarian cancer

PT12.03 | Urinary EVs as biomarker for renal injury and premature aging evaluated by Super Resolution Microscopy

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Introduction: Extracellular vesicles (uEVs), and in particular urinary EVs are gaining considerable importance as biomarkers in the clinical world, as efficient indicators of the overall state of the kidneys. The development of single vesicle analyses will allow correlation of the type of EV-releasing cell with its cargo. In this study, we focused on the expression of Klotho, an anti-aging hormone released by the kidney with anti-fibrotic properties, to predict the development of multi-organ dysfunction. As a model of fibrotic/aging disease, children with congenital heart disease, known to be predisposed to the development of multi-organ damage and premature aging, including liver and kidney failure (CKD), was studied.
Methods: We isolated uEVs from first morning urine of pediatric patients affected by single ventricle defects (n = 50), and from healthy subjects. We characterized uEVs, combining super resolution microscopy with the quantification of Klotho measured by ELISA. Moreover, the cytofluorimetric analysis, was also performed.
Results: We observed significantly reduced level of urinary Klotho as an indication of aging-associated diseases and progression of fibrosis. We observed the presence of renal progenitor markers using super resolution microscopy (SSEA4, CD133, CD24) in both experimental conditions. However, the expression of renal uEVs markers was decreased in single ventricle patients. Klotho levels and the expression of classical exosomal and renal/stem markers were confirmed by single-molecule super resolution microscopy and correlated with clinical patient's parameters.
Summary/Conclusion: The results presented here suggested that uEV characterization and Klotho expression might predict pre-clinical damage to the renal tissue. Klotho loss could be involved in the systemic alterations observed in these patients. Super resolution microscopy is a reliable method to study uEV biomarkers.
Keywords: super resolution microscopy, uEV biomarkers

PT12.06 | Development and characterization of a liquid biopsy assay for the detection of colocalized biomarkers on extracellular particles

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Introduction: Detection of early-stage cancer, when it is most treatable, remains a significant challenge. Current liquid biopsy approaches can be used for screening or response monitoring but suffer from limitations, including the abundance and stability of the analyte, large sample requirements, and insufficient specificity. Extracellular particles (EPs) derived from cancer cells offer a unique analyte for diagnostic tests given their abundance and stability relative to other liquid biopsy-based analytes (ctDNA, mRNA), and their representation of the cell of origin. We hypothesized that measurement of cancer-associated biomarkers on single EPs may improve upon existing methods for the non-invasive detection of cancer. Here, we describe the development of Mercy Halo™, an immunoassay designed to capture and detect up to three distinct, colocalized biomarkers on tumor-derived EPs from human blood samples.
Methods: EPs isolated from conditioned media or plasma by size-exclusion chromatography were captured in solution with antibody-functionalized beads targeting a cancer-specific biomarker. Captured EPs are incubated with additional cancer-associated biomarker antibodies conjugated to double-stranded DNA probes. After proximity ligation, the abundance of biomarkers on the EPs is read out using qPCR.
Results: In proof-of-concept testing, we isolated EPs from cell lines or human plasma by SEC and demonstrate the assay can detect known membrane-associated surface proteins on detergent-sensitive and -insensitive particles correlated with gene and protein expression. We further confirm the ability of Mercy Halo to detect colocalized, cancer-associated biomarkers on EPs, and systematically demonstrate that detection of multiple, distinct, colocalized targets improves signal/noise relative to a single biomarker. Additional assay characterization was performed to evaluate linearity and reproducibility. Finally, our proof-of-concept assay shows promise for the sensitive and specific detection of stage I and II high-grade serous ovarian cancer (HGSOC) in human plasma, outperforming CA125 in the discrimination of benign and malignant ovarian masses.
Summary/Conclusion: The Mercy Halo platform represents an extensible technology that could be used to measure colocalized biomarkers on EPs as a diagnostic or response monitoring tool.
Keywords: liquid biopsy, cancer, biomarker

PT12.07 | Single particle analysis to investigate and optimize fusion of liposomes with extracellular vesicles for therapeutic application

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Introduction: EVs have proved difficult to translate to the clinic, in part due to low production yields. In contrast, liposomes are inexpensive to produce in great quantities, yet lack the biological complexity responsible for imparting significant function desired for drug delivery. But combining the two systems into engineered EVs (eEVs) may effectively dilute the complex EV membrane with functional or structurally supporting synthetic lipids, decreasing the cost per vesicle while maintaining complexity critical for biological function.
Methods: EVs derived from cell culture and isolated by differential ultracentrifugation were fused with synthetic liposomes to create eEVs. Several fusion pathways were explored and compared, including simple mixing, mechanical extrusion, freeze-thawing, sonication, and microfluidic mixing. Soluble dyes were used as surrogate cargo to investigate potential therapeutic loading. Multiple single particle tools were used to quantify fusion and loading efficiencies, including laser trapping Raman spectroscopy (LTRS), flow cytometry (Beckman Cytoflex), interferometric reflectance/fluorescence imaging (ExoView R100), resistive pulse sensing (Spectradyne nCS1) and super resolution microscopy (ONI Nanoimager S).
Results: We found that each fusion method resulted in differential size profiles, loading efficiencies, and particle-to-particle distributions. In general, extrusion maintained lower size ranges while freeze-thawing appeared to produce the most efficient fusion of EVs and liposomes (based on tetraspanin expression).
Summary/Conclusion: Given the rising importance of analytical tools able to characterize extent of molecular loading for engineered EVs, we believe our multi-pronged characterization scheme will push forward our understanding of eEV analysis at the single particle scale.
Funding: NIH NCI (R01 CA241666), NHLBI (T32 HL007013), and NINDS (F31 NS120590).

PT12.09 | Non-contact microfluidic analysis of the stiffness of single large extracellular vesicles from glioblastoma cells

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Introduction: Most fundamental extracellular vesicle (EV) research has characterized nanoscale EVs and focused on EV biochemical content. There is much less understanding of large microscale EVs and EV mechanical properties. Here we test our new microfluidic technique to distinguish between large EV populations (L-EVs, >1 μm diameter) from glioblastoma cell lines with mutations in isocitrate dehydrogenase 1 (IDH1) that is known to disrupt lipid metabolism and after temozolomide (TMZ) treatment.
Methods: L-EVs were purified via centrifugation from cell culture supernatants of GBM cell lines. L-EVs were characterized per MISEV18 using dynamic light scattering, Western Blot, and optical microscopy (L-EV morphology, size, and concentration). Drug-treated T98G cells were exposed to 90 μM TMZ for 3 days. Lipidomics assessed L-EV lipid content. Non-contact stiffness measurements of single L-EVs were done using microfluidics in which L-EVs were gently stretched by the suspending fluid.
Results: L-EVs from IDH1-mutated Gli36 and MGG cells were stiffer than wild-type (~20% higher median, small overlap of median 95% confidence intervals, classical and bootstrap hypothesis tests $p < 0.05$). Lipidomics analysis indicated a reduction of unsaturated lipids and relatively more saturated lipids in L-EVs from IDH1-mutated cell lines. L-EVs from TMZ-treated T98G cells may be softer than the control, though the statistical evidence was less strong.
Summary/Conclusion: The changes in lipid composition (more saturated lipids) with the IDH1 mutation is consistent with L-EV stiffening; tighter-packing saturated lipids increase biomembrane rigidity. Microfluidics could be a complementary technique with atomic force microscopy to measure EV stiffness, specifically for microscale L-EVs, with the advantages of higher throughputs and a non-contact mode that eliminates substrate adhesion effects.
Funding: NIH (R21CA217662, R01GM138778 to H.I.), Nat. Res. Fdn. of Korea (NRF-2021R1A6A3A14039686 to M.H.J.).
Keywords: large extracellular vesicles, microfluidics, stiffness, glioblastoma, IDH1 mutation

PT12.10 | Multiplexed analysis of cell-specific single extracellular vesicles for pulmonary fibrosis diagnosis

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Introduction: Idiopathic pulmonary fibrosis (IPF) is a lung disorder in which various cell types are intertwined. Extracellular vesicles (EVs) in bronchoalveolar lavage fluid (BALF) could play important roles as cellular surrogates for molecular IPF diagnosis. However, the current bulk EV analysis prevents the accurate evaluation of EV biomarkers derived from various cell types. Here, we developed a single EV sensing platform based on plasmon-enhanced multi-color fluorescence detection to evaluate IPF markers on cell-specific EVs.
Methods: We developed in vitro IPF models using lung epithelial cells (Beas-2B), macrophages (THP-1), and fibroblasts (MRC5) that represent epithelial-mesenchymal transition, M1 or M2 transition, and fibroblast-myofibroblast transition, respectively. We isolated small EVs from cell line culture media using size-exclusion chromatography and characterized the size and concentration using nanoparticle tracking analysis. We used nanoplasmonic EV sensing (nPLEX) chips for single EV analysis. We used the Cy7 channel for all EV detection, the AF555 channel for cell-specific EVs, and the AF647 channel for IPF markers. We then analyzed cell-specific EV counts and marker intensities in single EVs.
Results: For EVs from Beas-2B epithelial cells, the rate of EpCAM+/Fibronectin+ EVs was significantly higher in the IPF model than in control. For EVs from MRC5 fibroblast cells, CD44+/Fibronectin+ and CD44+/Col1A1+ EVs were increased in the IPF model. For THP-1 EVs, CD45+/CD80+ was elevated in EVs derived from M1 macrophages, while CD45+/Arg1+ was increased in M2 macrophages' EVs.
Summary/Conclusion: This study evaluated IPF marker levels in the cell-specific EVs using the 3-channel single EV analysis. We will apply the assay to human BALF samples and establish a correlation of IPF marker levels between cell-specific EVs and cells in lung tissues.
Funding: This study is supported by Basic Science Research Program (NRF-2021R1A6A3A14039686) of the National Research Foundation of Korea (NRF).

PT12.11 | Plasmon-enhanced biosensing of tumor-derived extracellular vesicles in breast cancer

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Introduction: Molecular analysis of extracellular vesicles (EVs) represents a new paradigm for early cancer detection and treatment monitoring through liquid biopsies. In particular, single EV analyses are important to understand the heterogeneity of EVs, identify the cellular origins of individual EVs, and detect key biomarkers in the subpopulations. Here, we introduce a nanoplasmonic sensing platform for single EV analysis. Specifically, we used a low-cost plasmonic substrate that can be fabricated on a wafer scale to amplify EVs' fluorescence signals without the need for additional chemical or enzymatic signal amplification processes.
Methods: We isolated EVs from breast cancer cell lines with different molecular subtypes using size exclusion chromatography and measured the sizes and concentrations by nanoparticle tracking analysis. The isolated EVs were fluorescently labeled by using tetrafluorophenyl with Alexa Fluor 555. The labeled EVs were captured on the plasmonic substrate functionalized by SH-PEG-COOH with EDC/NHS activation. We labeled the captured EVs with QUAD cancer markers (MUC1, EGFR, EpCAM, and HER2) for multi-channel single EV analysis.
Results: We showed that the approach could significantly improve the EV detection sensitivity and enable multi-channel single EV analysis. We applied the assay to detect tumor-derived EVs using the QUAD marker signature and interrogating molecular subtypes. We also showed molecular profiles of tumor-derived EVs present similar molecular patterns to originating cells by flow cytometry.
Summary/Conclusion: The plasmon-enhanced sensing enables sensitive, multiplexed EV analysis through a simple assay procedure. The multiplexed single EV analysis could improve the detection accuracy for early cancer detection and treatment monitoring.
Funding: A sponsored research agreement between Canon and Massachusetts General Hospital (agreement # 2020A013232).
Keywords: nanoplasmonics, multiplexing, breast cancer, sensing

PT12.12 | Multiparametric analysis of single HIV virions using combinatorial single-molecule fluorescence in-situ hybridization (smFISH) and single-particle interferometric reflectance imaging sensor (SP-IRIS)

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Introduction: Enveloped viruses, with a lipid bilayer and host components, can be seen as a type of EV hijacked by the viral life cycle to further viral replication. We and others have shown that HIV displays EV surface markers, including tetraspanins CD63 and CD81. Individual HIV virions could be distinguished from the EV background, however, using the size and surface marker analysis afforded by the SP-IRIS technique in conjunction with fluorescent anti-HIV gp120 antibody. Here, we report an improvement on SP-IRIS analysis of HIV, using smFISH to fluorescently label HIV genomic RNA (gRNA) and allow discrimination between infectious (containing gRNA) and non-infectious (lacking gRNA) virions.
Methods: Virions and/or host EVs were concentrated from the conditioned medium of chronically-infected (HIV-1, BaL strain) and uninfected PM1 cells using differential ultracentrifugation. Samples were UV-inactivated before incubation on SP-IRIS chips. An AF488-conjugated anti-gp120 antibody was used as a fluorescent probe. smFISH-AF647 probes were designed against the HIV-1 BaL gRNA. SP-IRIS was done using ExoView R100 and tetraspanin capture chips from Unchained Labs (former: NanoView).
Results: SP-IRIS detected an smFISH-AF647+ population of gp120/CD81+ particles around 100–120 nm diameter, likely infectious HIV-1 virions. This smFISH-AF647+ population was not seen in control EVs isolated from uninfected cells. Use of non-HIV-specific smFISH-AF647 probes also did not result in smFISH-AF647+ signal.
Summary/Conclusion: The detected particles are likely to be mature, infectious HIV virions carrying HIV gRNA. In ongoing studies, we will phenotype these particles for additional surface proteins and determine if different smFISH probe sets might be used to detect diverse HIV strains. The technique could also be applied to image highly abundant RNAs in host EVs.
Funding: This work was funded by the ION-ARPA initiative.
Keywords: SP-IRIS, smFISH, HIV, virion, enveloped virus, rna detection, extracellular vesicles

PT12.14 | Deformation analysis of liposarcoma extracellular vesicles for mechanical properties estimation

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Introduction: Biomechanical analysis of single extracellular vesicles (EVs) can provide label-free insights to morphological structure, biomarkers, and potential targets for therapeutics. However, reliable and quantitative measurements on the mechanical properties for single EVs are scarce as these are compliant, nanoscale particles with limited tools for mechanical property measurements. Conventional methods use nano-indentation with instruments such as an atomic force microscopy, which lack high-throughput and are limited to a small number of EVs. EV isolation and separation methods use mechanical forces on the entire EV in centrifugation and filtration. Here, we report on a flow-based method complemented by transmission electron microscopy (TEM) imaging to quantify the change in the shape of EVs due to the mechanical forces acting on the entire EV. We show that the well-established thin shell theory can be used to quantify the EV deformation and provide an estimate of the elastic modulus.
Methods: Cells from Human liposarcoma (LPS) cell line Lipo246 were cultured in serum-free media for 48 h for EV production and LPS cell-conditioned media (LCCM) collection. LCCM was centrifuged at 2000g for 20 mins to remove cellular debris and the supernatant was microfiltered with a PVDF membrane filter (pore size 220 nm). Nanotracking analysis and TEM characterized EVs in pre- and post-filtration LCCM. TEM image analysis with ImageJ quantified EV shape and size for calculating EV deformation due to microfiltration. Thin shell theory was used to calculate elastic modulus (E).
Results: TEM images (N∼ 400) of microfiltered LCCM confirmed particles in 10–953 nm range as observed by NTA.The E of EVs yielded $E = 0.89 \pm 0.09$ MPa (mean ± SE) for small EVs (sEVs; 30–150 nm) and $E = 0.95 \pm 0.13$ MPa (mean ± SE) for large EVs (lEVs; >150 nm). E for individual EVs shows that the sEVs E ranges from 0.1-10 MPa while lEVs range from 0.5-2 MPa.
Summary/Conclusion: This is the first report on the mechanical property estimation of LPS-derived EVs. This study uses a flow-based methodology to provide a high throughput, whole EV deformation analysis method for estimating statistically significant mechanical properties as function of EV size.
Funding: This work was supported by the National Institutes of Health under grant number DOD CDMRP Grant CA210874.
Keywords: liposarcoma, extracellular vesicles, elasticity modulus, TEM

PT13: Cancer Biomarkers 1

Location: Hall 4A

16:45 - 18:45

PT13.01 | Rhabdomyosarcoma-derived extracellular vesicles contain clinical characteristic-specific protein expression patterns

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Introduction: Rhabdomyosarcoma (RMS) is the most frequent pediatric soft tissue sarcoma. It is classified into 2 primary histological subtypes: alveolar (ARMS) or embryonal (ERMS), and by presence (FP) or absence (FN) of a PAX3/7-FOXO1 gene fusion. Novel biomarkers are needed for improved and less invasive diagnosis. Extracellular vesicles (EVs) are present in many biological fluids and play key roles in intercellular communication and the pathophysiology of tumorigenesis and metastasis. This study aimed to identify correlations between RMS-derived EV protein cargo and clinical characteristics.
Methods: We isolated and analyzed EVs from 4 RMS cell lines (RH4, RH18, RH30, RD) in triplicate, in accordance with MISEV 2018. Cells were grown to 70% confluency and EV-depleted FBS media was conditioned for 48 hours, then concentrated (120mL to < 10mL). EVs were isolated through differential ultracentrifugation. EV quality and characterization was performed by Western blot, transmission electron microscopy and nanoparticle tracking analysis. EV protein cargo was evaluated by LC-MS/MS. Proteomics, compared to Vesiclepedia, and gene ontology was examined using FunRich (v 3.1.3). Protein interaction networks were generated using the stringApp within Cytoscape (v 3.9.1).
Results: We identified 1,657 RMS-derived EV proteins. We also identified protein patterns correlating with PAX3/7-FOXO1 fusion status, histological subtype and TP53 mutation status. ARMS EV proteins were associated with collagen fibril organization, cell communication, DNA replication, and striated muscle development, whereas ERMS EV proteins were linked to cell-matrix

adhesion, extracellular matrix organization, and binding various ligands. FP EV contained proteins involved in protein binding, fatty acid metabolism, and DNA damage repair regulation. FN EV proteins were associated with cytoskeleton organization, positive regulation of cell migration, satellite cell maintenance in skeletal muscle regeneration, non-canonical Wnt signaling, and negative regulation of angiogenesis.

Summary/Conclusion: RMS cells secrete EVs with unique protein cargo based on clinical characteristics, demonstrating a potential liquid biopsy method for diagnostics or surveillance. Further validation of these expression patterns in patient-derived biological fluids is necessary prior to clinical translation.

Funding: This study was funded by scholarships granted to P. R. Quaglietta by the Canadian Institutes of Health Research (Canadian Graduate Scholarship-Masters) and The Hospital for Sick Children (SickKids RESTRACOMP Graduate Award), and support from the Research Institute, The Hospital for Sick Children to R. M. Baertschiger.

Keywords: pediatric cancer, rhabdomyosarcoma, diagnosis, liquid biopsy, biomarker

PT13.02 | Extracellular vesicles in aqueous humor correlate with ocular tumor features

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Introduction: For retinoblastoma (RB), tumor biopsy is contraindicated, necessitating non-tissue biomarker discovery. Extracellular vesicles (EVs) are secretory vesicles carrying bioactive molecules that can be phenotyped using validated tetraspanin markers, making them promising biomarker candidates. Aqueous humor (AH) is a liquid biopsy source of tumor information and EVs have been newly established in AH, but relationships between EV characteristics and RB clinical features are unknown. Here, we profiled EVs in RB AH and performed a retrospective case review of our patient cohort to explore clinical correlations.

Methods: 37 AH samples from 18 RB eyes with varying International Intraocular Retinoblastoma Classification (IIRC) classifications were analyzed. Samples were taken from 10 treatment naïve (Tn) eyes and 27 during treatment (Tx). Tn AH was drawn from one A, one B, six D, and two E eyes by the IIRC group; among them, six eyes were enucleated and four salvaged. 10μL of unprocessed AH underwent Single Particle-Interferometric Reflectance Imaging Sensor (Exoview R100) analysis for fluorescent-based particle count and immunophenotyping of tetraspanin expression (CD63/81/9). Counts were converted to percentages for co-expression analyses. The analysis utilized Mann-Whitney U, ANOVA, and Tukey's tests.

Results: Comparing the 10 Tn samples and 27 Tx samples, a higher percentage of CD63/81+ EVs was found in Tn eyes (16.3 $\pm$ 11.6% vs. 5.49 $\pm$ 3.67%, P = 0.0009). Mono-CD63+ EVs enriched Tx AH (43.5 $\pm$ 14.7% vs. 28.8 $\pm$ 9.38%, P = 0.0073), consistent with recent literature. CD63/81+ EVs were significantly higher in the six enucleated eyes than the four salvaged eyes by count (9.7 $\times$ 10E4 $\pm$ 2.08 $\times$ 10E5 vs. 7.68 $\times$ 10E2 $\pm$ 8.49 $\times$ 10E2, P = 0.0381), and marginally higher by percentage (22.5 $\pm$ 11.3% vs. 7.10 $\pm$ 1.41%, P = 0.0667). By IIRC, CD63/81+ EVs were highest in the two Group E eyes compared to the six Group D eyes by count (2.75 $\times$ 10E5 $\pm$ 3.40 $\times$ 10E5 vs. 5.95 $\times$ 10E3 $\pm$ 8.16 $\times$ 10E3, P = 0.0006), and the two Group A+B eyes by count (2.75 $\times$ 10E5 $\pm$ 3.40 $\times$ 10E5 vs. 2.73 $\times$ 10E2 $\pm$ 2.59 $\times$ 10E2, P = 0.0096) and percentage (32.1 $\pm$ 7.98% vs 7.79 $\pm$ 0.02%, P = 0.0187).

Summary/Conclusion: CD63/81+ EVs populate AH from most advanced Group E eyes when compared to less advanced RB eyes by IIRC grouping, suggesting the utility of these EVs in RB classification. Because CD63/81+ EVs also dominate Tn and enucleated AH, they may be tumor-associated; examination of their cargo could uncover vital RB biomarkers.

Funding: Knight Templar Eye Foundation TRSI RCDA from Children's Hospital Los Angeles.

Keywords: aqueous humor, liquid biopsy, small extracellular vesicle, tetraspanin, single vesicle analysis

PT13.03 | Molecular Characterization of Sialylated Extracellular Vesicle Cargos for Human Breast Cancer Subtyping

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Introduction: Breast cancer (BC) is a highly heterogeneous disease with various subtypes that differ in pathological features, prognosis, and therapeutic responses. Current tissue-based molecular subtyping presents several disadvantages including invasive nature, a snapshot of tumor heterogeneity, and inability to monitor cancer evolution and subtype interconversion. While tumor-shed extracellular vesicles (EVs) have been shown to play important roles in cancer and glycans are major components of EVs, little is known about the structures of EV glycosylated cargos and their diagnostic value in BC. Here, we characterized the sialylated molecules in EVs derived from different BC subtypes to define subtype-specific EV glycosylation.

Methods: BC cells representing 4 different subtypes (Luminal A, Luminal B, HER2-enriched, Triple Negative) were cultured in the presence of azido-modified sialic acid biosynthetic precursor. EVs were isolated from the conditioned media using a differential ultracentrifugation approach and characterized by Nanoparticle Tracking Analysis, transmission electron microscopy, and Western blot (WB) with a panel of EV markers. Surface azidosialic acid in parent cells and EVs were further tagged by biotin via copper-free click chemistry. Immunofluerecence (IF), WB, and Norther Blot (NB) were performed to evaluate labelled sialoglycans, sialoglycoproteins, and glycoRNAs, respectively.
Results: IF results showed that azidosialic acid was efficiently incorporated into cellular nascent sialylatedglycoconjugates, mainly located at cell surface and the Golgi complex. A large range of tagged cell-surface sialoglycoproteins demonstrated marked difference across BC subtypes and were correlated with BC aggressiveness. Small RNAs bearing sialyloglycans were detected on the cell surface. Collected EVs, mainly between 50–150 nm, harbored membrane-encapsulated vesicular structure and presented classical EV markers. Importantly, they were heavily sialylated and retained similar surface sialoglycoprotein patterns as their parent cells.
Summary/Conclusion: EVs are enriched with BC subtype-specific silaylated molecules, with the potential in the discovery of novel glycosylated biomarkers to improve BC stratification and diagnosis in a non-invasive way.
Keywords: breast cancer, extracellular vesicles, glycosylation

PT13.04 | Size-exclusion chromatography and data-independent acquisition mass spectrometry improve proteome profiling of extracellular vesicles from plasma and serum of melanoma patients

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Introduction: Extracellular vesicles (EVs) are important players in melanoma progression, but their use as clinical biomarkers has been limited by the difficulty of profiling blood-derived EV proteins with high depth of coverage, the requirement for large input amounts, and complex protocols.
Methods: Here, we provide a streamlined and reproducible experimental workflow to identify plasma- and serum- derived EV proteins of healthy donors and melanoma patients using minimal amounts of sample input. SEC-DIA-MS couples size-exclusion chromatography to EV concentration and deep-proteomic profiling using data-independent acquisition.
Results: From as little as 200 μl of plasma per patient in a cohort of three healthy donors and six melanoma patients, we identified and quantified 2'311 EV-associated proteins, achieving a 2.8-fold increase in depth compared to previously published melanoma studies. To compare the EV-proteome to unenriched blood, we employed an automated workflow to deplete the 14 most abundant proteins from plasma and serum and thereby approximately doubled protein group identifications versus native plasma and serum. The EV proteome diverged from corresponding unenriched plasma and serum, and unlike the latter, separated healthy donor and melanoma patient samples. Furthermore, known melanoma markers such as MCAM, TNC, and TGFBI were upregulated in melanoma plasma-derived EV but not in depleted melanoma plasma samples, highlighting the specific information contained in EVs. Overall, EVs were significantly enriched in intact membrane proteins and proteins related to SNARE protein interactions and T cell biology.
Summary/Conclusion: Taken together, we demonstrated the increased sensitivity of an EV-based proteomic workflow for biomarker discovery in plasma and serum. The ease of automating and scaling up such an approach enables generalized application to larger cohorts from melanoma and other indications.
Keywords: extracellular vesicle, EV, exosome, melanoma, proteomics, mass spectrometry, liquid biopsies, plasma, serum, size-exclusion chromatography, SEC, biomarker

PT13.05 | Extracellular vesicles-associated GPRC5C and EPS8 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 discover non-invasive

biomarkers for early detection before PDAC reaches the incurable stage. The main objective of this study is the identification and detection of PDAC-specific EVs in patient serum.
Methods: We purified circulating EV proteins from PDAC patients' serum using our novel EV purification methods and carried out proteomics analysis of these EV proteins. After the selection of the candidate for PDAC biomarkers, biomarker candidates were validated by immunoblotting for EVs from PDAC patient blood samples using ultracentrifugation.
Results: Proteomics analysis identified over 500 proteins in each pooled sample (stage II, III, IV, and healthy donors). To narrow down candidate proteins as PDAC biomarkers, we selected the detected exclusively in the PDAC but not non-PDAC. Finally, EV-associated GPRC5C and EPS8 were selected for PDAC biomarkers. GPRC5C or EPS8 positive EVs were significantly more abundant in PDAC patient serum than healthy controls. Furthermore, the combination of EV-associated GPRC5C and EPS8 had high precision with an area under the curve values of 0.922 and 0.946 for distinguishing early-stage PDAC patients from healthy controls in the two cohorts. Moreover, we analyzed 30 samples from 10 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 and recurrence samples.
Summary/Conclusion: These results indicated that GPRC5C- or EPS8-positive EVs were biomarkers that have the potential to detect stage I early pancreatic cancer and small recurrent tumors detected by computed tomography. In addition, we are analyzing EPS8 and GPRC5C in serum EVs in patients with risk factors for pancreatic cancer, such as diabetes, chronic pancreatitis, and IPMN, and conducting follow-up studies to evaluate the risk of developing pancreatic cancer.
Funding: This work was supported by Project for Cancer Research and Therapeutic Evolution (P-PROMOTE) from the Japan Agency for Medical Research and Development (AMED) (No. 22ama221405h0001) and CREST from the Japan Science and Technology Agency (No. JPMJCR19H1).
Keywords: early diagnosis, liquid biopsy, pancreatic ductal adenocarcinoma, proteomic analysis

PT13.06 | Identification of cancer specific exosomal biomarkers to aid in diagnosis of cancer

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Introduction: The limitations of taking serial tumor biopsies are well known, thus many labs are developing liquid biopsies which may allow for repeated plasma-based tumor analysis. Here we aim to identify a panel of unique proteins, which could be used as a screening or diagnostic tool to identify the presence of cancer using enriched exosomes as analytical material.
Methods: To identify novel biomarkers, we performed proteomics analysis on exosomes enriched from a cohort of patients (n = 125) consisting of 25 plasmas each from colorectal (CRC), breast, non-small cell lung cancer (NSCLC), ovarian cancer, and normal patients. Exosome fractions were enriched using size exclusion chromatography and proprietary loading and elution buffers to increase exosomal protein recovery, while minimizing contamination with abundant serum proteins. Each enriched exosomal plasma sample was lysed and subjected to DIA mass spec analysis to identify and quantitate as many proteins from each sample as possible.
Results: Using this method, protein IDs ranged from 1337 to 1387 for the four tumor types, with proteins significantly ($p < 0.05$) and substantially (>2-fold change) up or down regulated compared to normal plasma ranging from 114 in breast cancer to 142, 124 and 204 differentially expressed proteins in CRC, NSCLC and ovarian cancer respectively. These cancer exosome associated proteins represent a broad range of proteins from both tumor exosomes as well as proteins representing 'host' response. In depth bioinformatics analysis is underway, with a goal of developing smaller panels of predictive protein fingerprints which will be tested and validated on additional larger cancer sample sets.
Summary/Conclusion: These initial data are encouraging in that differentially regulated proteins are unique and not typical oncoproteins suggesting that tumor specific exosome based panels can be identified and developed in the clinical space to support better patient care.
Keywords: mass spectrometry, proteomics

PT13.07 | Exploration of EVs membrane proteins as novel salivary biomarkers for early detection of OSCC

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Introduction: Oral squamous cell carcinoma (OSCC) is one of the most malignant diseases with high prevalence in Taiwan, frequently results in poor prognosis and mortality because of delay diagnosis, tumor recurrence or metastasis. Therefore, early

detection and disease monitoring are very important for disease management in OSCC. However, there are still no biomarkers currently approved by official health agents in the endemic areas for aiding OSCC management, and the membrane fraction of salivary EVs is worth to be comprehensively analyzed for biomarker discovery and verification because of their stabilities via clinical detection.

Methods: We optimized our strategy for EVs isolation, reduced operating time from 8 hours to 2 hours, as well as increased total protein amounts recovery (in EVs fractions) from 10 μg to 60 μg (about 4.5 × 10^10~1.3 × 10^11 particles) via 1 mL of saliva sample chromatographed using home-made Sepharose 2B column. The isolated EVs from saliva samples with different level of oral lesions malignancies (normal, low-risk precancerous, high-risk precancerous, and OSCC cases) were determined using NTA, TEM, and specific EVs markers (CD9, CD63, and CD81) following detected for iTRAQ-labeling quantitative proteomics analysis using 2D-LC-MS/MS analysis.

Results: A total of 703 proteins were quantified in salivary EVs. Among these proteins, 86% and 10% have been annotated as EVs proteins and vesicle membrane proteins, respectively. It benefits finding membrane biomarkers on EVs for oral cancer detection/monitoring. Since protein profiles are much different between salivary EVs from OSCC and non-OSCC patients, we also demonstrated that viability of cancer cells can be decreased via precancerous EVs stimulation; and cancer's EVs treatment increased viability of precancerous cells.

Summary/Conclusion: in summary, we discovered salivary EVs membrane protein biomarkers for OSCC detection/monitoring. Hopefully, these markers will be verified, and their detection reagents will be further developed soon.

Funding: We expect that the success of verification of EVs membrane biomarkers will improve early detecting and monitoring of OSCC, and investigation of salivary EVs-mediated mechanisms in OSCC progress will provide a new insight into development of novel treatment strategy.

Keywords: oral cancer, extracellular vesicles (EVs), membrane protein, salivary biomarker, disease monitoring, quantitative proteomics.

PT13.08 | S100A9 protein from plasma derived EV as a predictor of treatment response in CRCLM

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Introduction: Colorectal cancer is the 3rd most common type of cancer and second leading cause of cancer death. The primary cause of death in colorectal cancer is due to metastasis with liver being the dominant metastatic site for colorectal cancer (CRC). Liver metastases present as two major histopathological growth patterns (HGP) 1. Desmoplastic Histological growth pattern (D-HGP): Cancer cells are separated from hepatocytes by a desmoplastic ring, and it is angiogenic driven while 2. Replacement HGP (R-HGP): cancer cells infiltrate the normal liver, and co-opt pre-existing blood vessels. CRCLM patients treated with chemotherapy and Bevacizumab have a worse five-year overall survival when their lesions employ R-HGP instead of D-HGP. Thus, precisely defining the type of metastatic lesion that a cancer patient has is critical in selecting the appropriate treatment options.

Methods: We have developed EV based signature using blood-based biomarkers (Liquid Biopsy), which focuses on the protein cargo within extracellular vesicles (EVs) to stratify patients into D-HGP or R-HGP and guide a personalised treatment plan for CRCLM patients. We have collected plasma from 19 Chemonaive patients, isolated Extracellular vesicles using ultracentrifugation method, and characterised EV abiding to MISEV2022 guidelines. We performed Mass spectrometry in all the patient and analysis the data by performing GSEA for pathway and T-sample test for biomarker detection. We used IHC for biomarker validation in CRCLM tissue, ExoView for biomarker localisation on EV and ELISA for EV biomarker validation.

Results: GSEA analysis showed, all the pathways upregulated in R-HGPs is either associated with cell proliferation or migration while D-HGP showed increased adaptive immune response and B cell activation. Further, T sample test on the HGPs showed 22 differentially expressed significant gene and Unsupervised Principal component analysis showed clear segregation of both the HGPs suggesting potential signature. Interestingly, S100A9 protein was the top signature observed and the expression of the S100A9 in the EV correlated with the tumor tissue thus showing significant differences in expression at the tumor liver interphase in co-opting lesions only. Further we observed that S100A9 proteins on the tissue were expressed by the macrophage and neutrophils which has been suggested to be involved in the inhibition of T-Cell response.

Summary/Conclusion: Our findings indicate that S100A9 protein from Plasma derived Extracellular Vesicles as a predictor of treatment response in CRCLM.

Funding: MEDTEQ Innovation for Health and Fonds de recherche du Quebec Sante.

PT13.10 | Analysis of single EVs for studying treatment effects on non-small lung cancer cells

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Introduction: Precision cancer medicine with tyrosine kinase inhibitors (TKIs) towards a mutated epidermal growth factor receptor (EGFR) has improved the outcome for non-small cell lung cancer (NSCLC) patients. Yet, treatment responses are heterogeneous, and there is a need for non-invasive monitoring using liquid biopsies. Researchers have proposed extracellular vesicles (EVs) as means for non-invasive liquid biopsies, but due to the heterogeneity of EVs, it is not always easy to decipher biomarker signatures in the proteomic landscape of EV surfaces. Here we address this using single EV analysis of protein surface markers in the context of EGFR-TKI treatments of NSCLC cells.
Methods: EVs harvested from cell culture media of the EGFR-mutant NSCLC cell line H1975 cell line 48h post-treatment with erlotinib (10μM), osimertinib, (0.1 μM), cisplatin (12.5 or 25 μM) or mock-treatment. EVs were isolated from media by centrifugation followed by a capturing by size exclusion chromatography (qEVoriginal 70nm Columns) followed by pooling and concentration of fractions giving EV sizes of 30–300 nm. We characterized the EVs following the MISEV 2018 guidelines using nanoparticle tracking (for size and amount) and western blotting for analyses of EV markers (CD9, TSG101, and CD73). Furthermore, five surface markers (CD9, CD81, EGFR, HER2, and PD-L1) were stained with fluorescently labeled antibodies and imaged with an inverted microscope (Axio Observer 7). Individual EVs were analyzed based on their expression levels, distribution, and colocalization of the selected markers.
Results: Comparing the results in the EVs harvested from cells refractory to the treatment (erlotinib) with EVs harvested from cells responsive to the treatment (osimertinib) revealed different expression levels of PD-L1, EGFR, and HER2. The set of colocalized markers also changed between the samples. The number of PD-L1 molecules per vesicle decreased, while the number of PD-L1 positive EVs increased post-osimertinib treatment. These results differed from the results in the cisplatin-treated sample, which remained like the untreated sample.
Summary/Conclusion: Our results show that through single EV analysis, it is possible to distinguish the surface expression and colocalization levels of EGFR, HER-2, and PD-L1 after treatment with the two different EGFR-TKIs. It would not have been feasible with a bulk technique. This highlights the roll of single EV studies when investigating the suitability of EV analysis for cancer treatment monitoring.
Funding: Erling Persson Family Foundation, the Swedish Research Council (2016–05051), the Swedish Cancer Society (CAN 2018/597 and CAN2021/1469), the Stockholm Cancer Society (221212 and 221383), VR (2018-06228), and Stockholm County Council (909121, 750032 FoUI-966345).
Keywords: fluorescence microscopy, single EV analysis, immunostaining, non-small cell lung cancer, EGFR-TKIs

PT13.11 | Exploration of transmembrane proteins in extracellular vesicles derived from liver metastatic cancer model show markers of organotropism

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Introduction: Tumour-derived extracellular vesicles (TEVs) play an important role in cell-to-cell communication; they transfer biomolecules that contribute to malignant transformation, preparation of the pre-metastatic niche and are essential in TEV organotropism. Transmembrane proteins such as integrins ITGB5 and ITGA6 play a role in cell adhesion, signaling and are particularly implicated in organotropism to the liver and lung. Other transmembrane proteins such as prominin-1 are used as markers of cancer stem cells (CSC) and are known to be found in colorectal cancer cells. The aim of our study was to determine whether there was EV enrichment for transmembrane proteins implicated in metastasis using two cancer models with organotropism to the liver.
Methods: Colorectal (CRC) (HT29) and uveal melanoma (UM) cell lines (MP41, MP46, and OMM 2.5) were cultured until 80% confluency was reached. Then, the cell culture medium was replaced by an EV-depleted supplemented medium. After 24h, the

medium was collected and spun to remove cell debris and apoptotic bodies. Then, the medium was concentrated using 100 kDa Amicon ultrafiltration units. 50 μL of diluted medium per cell line was added on top of a microarray chip coated with capture antibodies (CD9, CD63, and CD81). Fluorescent antibodies CD133 (APC), ITGA6 (Alexa-488), and ITGB5 (PE) were added according to the manufacturer's protocol and measured in the Exoview 200.

Results: EVs derived from UM liver metastasis cell line OMM 2.5 and liver metastatic CRC HT29 cells had significantly higher expression of prominin-1 (CD133) across the three different capture probes (CD9, CD63, CD81) with 35% and 36% of the total averaged counts compared to MP41 and MP46 cells (6% and 3%). All studied cell lines EVs showed a low % count for ITGB5., while ITGA6 was found in all cell lines EVs (59%-93% total averaged counts).

Summary/Conclusion: Transmembrane proteins that have been implicated in site-specific metastasis were found in EVs from liver metastatic cells. Interestingly, stem cell marker CD133 was enriched in liver metastatic cell line-derived EVs. This result has potential implications for the understanding the communication between cancer cells and their microenvironment during organotropic metastasis.

Funding: New Frontiers Research Fund.

Keywords: transmembrane proteins, liver metastatic, colorectal cancer, uveal melanoma, cancer stem cells, tumour-derived extracellular vesicles

PT13.12 | EV sialylation in epithelial ovarian cancer as potential diagnostic biomarker

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Introduction: Epithelial ovarian cancer (EOC) is usually diagnosed at later stages with 5 year survival rate less than 30%. There is a need for better diagnostic markers. Glycosylation changes is a universal phenomenon of cancer cells and could be targeted for potential biomarker discovery. Glycans are strongly associated to integrins (ITG) and tetraspanins (e.g. CD9, CD63, CD81) stability and function, and their interaction partners are dysregulated in tumorigenic processes. CD63 is a marker for exosomes and glycans are major constituents of extracellular vesicles (EV). EVs carry an array of cargo which includes integrins.

Methods: EVs were purified from OC002 cell line using Exo-spin SEC columns (CGS, UK). Purified EV glycovariants were screened with the use of integrin and tetraspanin specific antibodies. The most promising ITG and CD63 glycovariants (STn, WGA, UEA) were detected from 10 EOC and 5 benign ovarian cyst fluid samples on microtiter wells using antibodies or lectins conjugated on europium nanoparticles. Total ITG and CD63 immunoassays were also performed. Finally, the best performing glycovariants were clinically evaluated on the whole cohort of 77 ovarian cyst fluid samples. This study was approved by the ethics committee in Gothenburg and informed consent was obtained from all individual participants included in the study.

Results: STn glycovariant of both ITGα3 and CD63 was detected in EVs purified from EOC cell line and performed better than corresponding protein epitope-based immunoassays, ITGα3-IA and CD63-IA respectively in clinical samples. Combined ITGα3 based assays (ITGα3-IA + ITGα3-STn) detected 49 out of 55 malignant and borderline cases without detecting any of the 22 benign or healthy cysts.

Summary/Conclusion: Our findings indicate the potential diagnostic application of STn glycovariants of ITGα3 and CD63 along with total ITGα3-IA, which could help reduce the unnecessary surgeries. The results encourage studying further the potential use of these novel EV based assays to detect EOC at earlier clinical stages.

Funding: N/A.

Keywords: integrin, CD63, glycosylation, cancer, diagnostic

PT13.13 | Tumor-Derived Extracellular Vesicles Predict Disease Recurrence in Oligometastatic Castration-Sensitive Prostate Cancer Treated with Stereotactic Ablative Radiotherapy: Analysis of the ORIOLE trial

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Introduction: Stereotatic ablative radiation therapy (SABR) can prolong survival in oligometastatic prostate cancer (omPC) patients. However, predictive tools to identify those who will benefit from SABR are necessary. Our group was the first to

demonstrate that blood levels of prostate cancer-derived extracellular vesicles (ProstEVs) correlate with tumor burden and predict disease progression in omPC after SABR. Herein, we conducted a blinded validation study using blood samples from the ORIOLE randomized phase 2 clinical trial.

Methods: Plasma samples from 46 omPC patients from the Baltimore ORIOLE trial: a 2:1 ratio randomization to SABR vs observation. Baseline PSMA+ ProstEV levels were measured by standardized and calibrated nanoscale flow cytometry using fluorescent PSMA antibodies. Median ProstEV levels was used as cut-off for low and high levels. Kaplan-Meier curves and Cox regression models were used to determine the association of ProstEV levels with clinical outcomes [PSA progression-free survival (psaPFS) and metastasis-free survival (MFS)].

Results: No association was observed between number of metastatic lesions or baseline PSA and blood ProstEV levels. MFS for patients treated with SABR was 29.6 months. MFS for patients treated with SABR with high and low ProstEV levels was 11.1 months and 36 months, respectively (Hazard Ratio: 2.85; 95% CI, 1.01-7.48; P = 0.02). The psaPFS for patients treated with SABR arm was 11.9 months. psaPFS for patients with high and low ProstEV levels was 5.9 months and 24.3 months, respectively (HR: 2.44; 95% CI, 1.00-5.94; P = 0.03).

Summary/Conclusion: ProstEVs is the first blood biomarker of tumor burden that can predict the risk of disease recurrence in omPC patients treated with SABR. While biomarker-guided trials are warranted, our validation study strengthens the clinical value of ProstEVs for personalized radiation therapy.

PT13.15 | EBC exosomes: A novel biomarker source for lung cancer

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Introduction: Being the leading cause of worldwide mortalities, the burden of early diagnosis of lung cancer is the utmost requirement of the present research. Due to the invasive nature of Blood and Biopsy samples, noninvasive sample sources such as EBC can be screened for early predictive lung cancer biomarkers. EBC, originating from the lower part of the respiratory tract can be a promising source of biomarkers that reflects lung cancer pathogenesis. Akin to blood and tissue, EBC consists of a colossal array of molecules such as lipids, proteins, nucleic acids, cell metabolites, and tiny intercellular messengers called exosomes. These nanoparticles can be screened as lung cancer biomarkers based on the discrepancy in their size, composition, concentrations, and their existence.

Methods: EBC samples were collected from three study groups: non-smokers (NSE), smokers (SME), and lung cancer (LCE) followed by exosome isolation. DLS, NTA, and TEM were performed to evaluate exosomal properties such as size, concentration, and morphology. Exosomal protein was quantified by using the Bradford assay.

Results: We successfully isolated EBC exosomes using a commercially available kit with slight modifications per the requirement. DLS (NSE 61.08 ± 24.12 nm, SME 94.45 ± 26.45 nm, LCE 126.4 ± 20.55 nm) and NTA (NSE 91.6 ± 10.46 nm, SME 129.2 ± 10.91nm, LCE138.2 ± 2.86 nm) results confirm the size range of EBC exosomes (30-150 nm). We found significant differences (*P 0.05) among NSE and LCE exosome size as per NTA results. TEM images validate the EBC exosome morphology. Isolated EBC exosomes are quite quantifiable (NSE 2.6E+09 ± 2.64E+08 particles/ml, SME 4.15E+09 ± 1.26E+09 particles/ml, LCE 7.22E+09 ± 2.79E+09 particles/ml) and exosomal protein (NSE758 ± 252.7 mg/mL, SME1903 ± 633.2 mg/mL, LCE1090 ± 133.9 mg/mL) can be used for downstream processing.

Summary/Conclusion: Being novel and non-invasive in nature EBC exosomes can be used to screen biomarkers for LC diagnosis, prognosis as well as therapeutics.

Funding: Indian Council of Medical Research, India.
Keywords: exhaled breath condensate, exosomes, lung cancer, protein, novel, biomarker source

PT14: Biomarkers from DNA to RNA

Location: Hall 4A

16:45 - 18:45

PT14.01 | A simple and available measurement of onco-exosome dsDNA to protein ratio as a potential tumor marker

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Introduction: Exosomes have great potential as new biomarkers in liquid biopsy. However, due to the limitations of exosome extraction and component analysis procedures, further clinical applications of exosomes are hampered. Carcinoembryonic antigen (CEA) is a commonly used broad-spectrum tumor marker that is strongly expressed in a variety of malignancies.
Methods: In this study, CEA+ exosomes were directly separated from serum using immunomagnetic beads, and the nucleic acid to protein ultraviolet absorption ratio (NPr) of CEA+ exosomes was determined.
Results: It was found that the NPr of CEA+ exosomes in tumor group was higher than that of healthy group. We further analyzed the exosome-derived nucleic acid components using fluorescent staining and found that the concentration ratio of double-stranded DNA to protein (dsDPr) in CEA+ exosomes was also significantly different between the two groups, with a sensitivity of 100% and a specificity of 41.67% for the diagnosis of pan-cancer. The AUC of dsDPr combined with NPr was 0.87 and the ACU of dsDPr combined with CA242 could reach 0.94, showing good diagnostic performance for pan-cancer.
Summary/Conclusion: This study demonstrates that the dsDPr of CEA+ exosomes can effectively distinguish exosomes derived from tumor patients and healthy individuals, which can be employed as a simple and cost-effective non-invasive screening technology to assist tumor diagnosis.

PT14.02 | Extracellular vesicles and their long DNA in the context of ovarian cancer diagnostics

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Introduction: One of the biggest challenges associated with ovarian cancer is that there are currently no diagnostic tests available to predict recurrence and chemoresistance. If ovarian cancer recurs after treatment, it is often incurable and treatment involves chemotherapy to prolong good quality of life and alleviate symptoms. A downward spiral occurs for subsequent chemotherapy treatments, where increasing chemoresistance results in shorter treatment-free intervals and cumulative toxicity. Our project will explore the use of DNA in extracellular vesicles as a diagnostic test for ovarian cancer chemoresistance.
Methods: Extracellular vesicles from the conditioned media of SKOV-3, OVCAR-3, and MET-5a normal and ovarian cancer cell lines were isolated through size exclusion chromatography (SEC). The SEC fractions were analysed for particle concentration and size using Nanoparticle Tracking Analysis (NTA), while relative protein quantification was measured using the bicinchoninic acid assay. Fractions were combined into a particle rich pool and a protein rich pool, then DNA extracted using the DNeasy Blood and Tissue kit. This resulted in EV DNA (from particle rich fractions) and cell-free DNA (from protein rich fractions) for each cell line. The size and quantity of the EV DNA and cell-free DNA was confirmed using Tapestation and Qubit. Real-time PCR was used to confirm the presence of DNA mutations in EV DNA, cell-free DNA and cells.
Results: The DNA from particle rich (EV DNA) and protein rich (cell-free DNA) SEC fractions of conditioned media was successfully isolated. Tape station data confirmed the size of EV DNA is longer (1300 bp) compared to cell-free DNA, where the latter is shorter and fragmented into approximately 150 bp increments (187, 390, 587 and 785 bp). Using PCR targeting the ENC1 DNA mutation, it was shown that this mutation was only present in ovarian cancer cells SKOV-3 and their EV DNA, while absent for cell-free DNA.
Summary/Conclusion: Our results suggests that EV DNA mutations (such as ENC1) more accurately reflect the ovarian cancer cell, compared to cell-free DNA. Additionally, the long length of EV DNA may be beneficial in the diagnostics field, through better characterization of DNA mutations using long read sequencing techniques. These advantages of EV DNA have potential for improving diagnostics in ovarian cancer detection.
Funding: NHMRC, MRFF.

PT14.03 | Liposarcoma EVs show size-based upregulation of MDM2 DNA across EV-size distributions

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Introduction: Dedifferentiated liposarcoma (DDLPS) is among the most common sarcoma subtypes. In addition to growing to massive size before detection, DDLPS can also present or recur as synchronous multifocal lesions and are characterized by

ultimately lethal multicentric recurrence in approximately 60% of patients, resulting in an overall survival rate of 10% at 10 years. At the molecular level, practically all DDLPS are characterized by MDM2 gene amplification. Recent studies have shown a possible role for extracellular vesicle (EV) cargoes in cancer initiation, progression and metastasis. Major cargoes in LPS EVs include MDM2 DNA which has been described to play an important role inducing tumour progression and metastasis. Our past work has shown upregulation of MDM2 DNA within EVs from DDLPS patient serum as well as from DDLPS cell lines. Given the distinct pathways for the biogenesis and secretion of EVs of varied size, it is hypothesized that MDM2 DNA expression is EV size dependent.

Methods: Cells from Human liposarcoma (LPS) cell line Lipo246 were cultured in a serum-free medium for 48 h for EVs production and LPS cell-conditioned media (LCCM) collection. LCCM was centrifuged at 2000g for 20 mins to remove cellular debris and the supernatant was filtered first with 200 nm (pore size) PVDF membrane filter followed by 100 nm (pore size) PDVF filter. The quality of isolated particles at each filtration step was assessed through TEM and Nanotracking analysis. EV MDM2 DNA was analyzed using real-time PCR and results were normalized to GAPDH.

Results: The pre-filtered LCCM showed the highest upregulation in MDM2 DNA by 3.4x over the control case. 2.4x MDM2 DNA upregulation was observed in LCCM with EVs< 200 nm, while the LCCM with EVs< 100 nm gave an upregulation of 1.9x.

Summary/Conclusion: Our study represents the first investigation of EV size dependence on MDM2 gene expression and the initial findings suggest that the EV size is related to the MDM2 DNA upregulation in LPS-derived EVs. Future experiments will quantify the number of MDM2 molecules as a function of EV size.

Funding: This work was supported by the National Institutes of Health under grant number DOD CDMRP Grant CA210874.

Keywords: liposarcoma, MDM2 DNA, biomarker, extracellular vesicles

PT14.04 | Integrative analysis of whole proteome and transcriptome of cancer-derived extracellular vesicles confirms results from single EV RNA-Seq

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Introduction: Extracellular Vesicles (EVs) are membrane covered particles of heterogeneous size and cargo. While small EVs (S-EVs) have been widely investigated, little is known about Large EVs (L-EVs). The paucity of studies comparing protein cargo of L- and S-EVs, the absence of studies focused on protein coding RNA and integrative analyses to compare the protein and transcript expression in different EV fractions, prompted us to perform mass spectrometry to profile EVs from different cancer cell models. The prostate cancer model was selected for additional complementary RNA-Seq to enable an integrative, multi-analyte approach of three EV populations in this model.

Methods: LC-MS/MS, SWATH-MS,, RNA-seq, vFC, TRPS, TEM, immunoblotting, single-EV RNA-seq, UC, and Iodixanol gradient.

Results: We identified novel marker candidates for L- and S-EVs, some of which were present both at the protein and transcript level. The correlation between protein and protein-coding mRNA was higher in L- than S-EVs. Additionally, the protein cargo differed among L- and S-EVs more than the transcript, suggesting that the identity of the EV populations is defined by their proteins more than by their mRNA cargo. Pathway analysis demonstrated that L-EVs are enriched in mRNAs and proteins involved in essential metabolic activities including mitochondrial functions. GSEA of the most abundant transcripts confirmed enrichment of mitochondrial function in L-EVs versus S-EVs, and this finding was corroborated in single EVs. The proteins/RNAs enriched in prostate cancer cell-derived L-EVs were also identified in L-EVs from the plasma of patients with metastatic prostate cancer by a SWATH proteomic assay. L-EV signature, but not the S-EV signature correlated with progression to metastasis.

Summary/Conclusion: The integrated L-EV proteomic and transcriptomic signature enabled distinction between benign and localized prostate cancer, as well as between localized cancer and metastatic castration-resistant cancer.
Funding: R01CA218526 NIH/NCI and R01CA234557 NIH/NCI.

PT14.06 | Identification of suitable internal control mRNAs and miRNAs in bovine milk small extracellular vesicles for normalization in quantitative real-time polymerase chain reaction

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Introduction: The amount of RNAs varies depending on tissues, physio-pathological status, and age of the host. Therefore, in quantitative real-time polymerase chain reaction (qPCR), stable internal control genes must be chosen for normalization to minimize experimental error, but not identified yet in bovine milk small extracellular vesicles (sEVs). The study aimed to identify suitable internal control mRNAs and miRNAs in bovine milk sEVs for normalization in qPCR analysis.
Methods: For identification of stable mRNAs and miRNAs in bovine milk sEVs, microarray analyses were carried out using milk from 32 Holstein-Friesian cattle. qPCR analysis was performed by using newly collected and isolated milk sEVs from 12 cattle. Further, the cycle threshold (Ct) value from qPCR analysis was evaluated by using four algorithms such as geNorm, normFinder, ΔCt, and BestKeeper algorithms. The final ranking of candidate internal control mRNAs and miRNAs were determined using RefFinder analysis.
Results: Microarray analyses identified a total of 43,713 mRNAs and 1,420 miRNAs in bovine milk sEVs. Further, a total of 17 mRNAs and five miRNAs were selected from the microarray data on the basis of their lowest coefficient variation value. qPCR analysis quantified and detected eight mRNAs out of 17 mRNAs and five miRNAs in bovine milk sEVs. Finally, the RefFinder demonstrated that TUB, ETFDH, and ACTB mRNAs and hsa-miR-27b-3p and bta-miR-29a miRNAs were highly stable in bovine milk sEVs.
Summary/Conclusion: The current study suggests that use of identified internal control mRNAs and miRNAs may improve the reliability, accuracy, and effective guideline for normalization in qPCR analysis in bovine milk sEVs.
Funding: JSPS KAKENHI grant (21H02357 and 22F22097), Morinaga Foundation for Health and Nutrition, Hirose Foundation, Kobayashi Foundation, and Yanmar Environmental Sustainability Support Association, Japan.
Keywords: bovine milk, internal control mRNAs and miRNAs, normalization, qPCR, small extracellular vesicles

PT14.07 | Proteomic and transcriptomic analysis of multiple myeloma plasma and bone marrow extracellular vesicles

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Introduction: Multiple myeloma (MM) is a haematological malignancy characterised by the clonal expansion of plasma cells in the bone marrow (BM). Complex interaction between malignant plasma cells and the BM-microenvironment plays an important role in disease progression, and includes intercellular communication mediated by extracellular vesicles (EVs) through the transfer of active protein and RNA between cells. A better understanding of the MM-EV cargo is important for the identification of new therapeutic targets and disease biomarkers. The aim of this study is a comprehensive multi-omics analysis of control and MM Peripheral blood (PB) and BM-derived EVs.
Methods: Ethical approval was acquired through the Mater Misericordiae University hospital human research ethics board. PB and BM were collected from MM patients (n = 24) and healthy volunteers (n = 17) following informed consent, EVs were isolated from plasma samples using ultracentrifugation (UC) and density gradient-UC. EVs were characterised using TEM, NTA and western blot analysis following MISEV2018 guidelines. Mass spectrometry and miRNA-seq were performed to compare EV-packaged protein and miRNA.
Results: Results reveal differential PB and BM-EV protein and miRNA expression between the patient and control groups. Targets identified include a clinically relevant protein within MM BM_EVs related to bone metastasis, but not previously associated with MM. Furthermore, several pro-tumorigenic proteins are upregulated in both MM PB and BM EVs highlighting the ability of MM PB EVs to reflect the BM environment. In addition, numerous miRNAs are significantly upregulated in MM PB compared to controls.

Summary/Conclusion: This study reveals that MM-derived EVs are characteristically different from normal control EVs with unique proteomic and transcriptomic profile. Moreover, circulating PB-EVs have potential as non-invasive liquid biopsy for routine cancer monitoring.
Funding: This work was funding by the Irish Cancer Society.

PT14.08 | Comparative studies on glioblastoma multiform patient tissue and serum extracellular vesicles for the expression of protein and RNAs by using a Au biochip

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Introduction: The liquid biopsy approach holds clear advantages over traditional tissue biopsies. Compared to tissue analysis, EVs in biofluids become an interesting source for noninvasive collection and dynamic reflection of tumor progression for novel assays. Glioblastoma multiform (GBM) cancer-derived extracellular vesicles (EVs) are regarded to have a significant function in most steps of cancer progression. A comparative analysis of GBM EVs from both tissues and serum will provide knowledge on the co-expression of up/down-regulated biomarkers for GBM diagnosis. Our studies demonstrate the ability to capture and detect single EVs on an in-house developed Au-based biochip that aims to discover GBM biomarkers for cancer diagnosis.
Methods: A Au biochip was used for the detection of protein, and RNA biomarkers present in single EVs. EVs were isolated using Tangential Flow Filtration (TFF) and Tunable resistive pulse sensing was used to measure the EVs number. Total internal reflection fluorescence microscopy (TIRFM) was used to image protein and RNA expression in EVs. RNAs were chosen from GBM cells/EVs sequencing analysis. Lipoproteins were also identified and characterized by specific detection antibodies.
Results: The isolated EVs from tissues and serum samples of the same GBM patient were detected with protein and RNA through Au biochip EV capture technique. EVs purified from tissue samples have significantly higher protein expression than those of serum. However, no significant difference was observed in the miRNA and mRNA expressions of both tissue and serum EVs. Hence, this Aubiochip technique differentiates the biomarker expression by capturing the single EVs isolated from different sources (tissues/serum) and helps in identifying potential non-invasive testing for GBM diagnosis.
Summary/Conclusion: Aubiochip enables protein and RNA detection from single EVs isolated from two different sources of the same GBM patient. This technique paves way for a cheap and rapid diagnostic kit for GBM patients.
Funding: NIH UG3/UH3TR002884 and U18TR003807.
Keywords: biomarkers, glioblastoma, extracellular vesicles, miRNA, mRNA

PT14.09 | Long RNAs in urinary extracellular vesicles before and after radical prostatectomy

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Introduction: Guideline-recommended PSA screening for early detection of prostate cancer (PC) lacks specificity, especially for clinically significant (CS) disease, resulting in over-diagnosis or over-treatment of low-grade PC, and exploration of additional non-invasive biomarkers to improve CSPC prediction both at diagnosis and treatment decisions. Small extracellular vesicles (EVs) contain molecular constituents, such as RNA transcriptome, derived from their parent tumors and can be profiled from biofluids such as urine for these biomarkers.
Methods: To identify transcripts originating from PC for biofluid-based test development, we analyzed long RNAs in urinary EVs associated with CSPC. EVs were isolated using our clinical ExoLution™ platform from urine collected from prostate cancer patients with CS disease (≥GG2, the majority was GG3 or higher with extracapsular extension) pre- and post-radical prostatectomy (RP), respectively. Over 60 RNA sequencing libraries were generated to profile long coding and non-coding RNAs in whole transcriptome, whole exome, and a prostate cancer gene panel, respectively.
Results: High-quality QC metrics showed over 17,000 protein coding and long noncoding RNAs. About 456 differentially expressed long RNAs were found to be associated with CSPC from the matched pre- vs post-RP urine samples, many of which have been implicated in PC. Further demonstrating biomarker specificity, the top 20 prostate-specific genes predicted by the tissue GTEx database show higher abundance in pre- vs post-RP, while the top 10 bladder-specific genes, top 10 kidney-specific genes,

and house-keeping genes exhibit little changes. Moreover, the top 3 pre-RP upregulated pathways are shown to be associated with androgen response.

Summary/Conclusion: This gene-set provides a blue-print for CSPC RNA biomarker targets in urine EVs and forms a foundation for robust biomarker discoveries, advancing the next generation of liquid biopsy-based approaches in PC management.

Funding: Exosome Diagnostics, a Bio-Techne Brand.

Keywords: clinically significant prostate cancer, radical prostatectomy, long RNAs, urinary EVs, biomarkers, RNA-seq, transcriptome

PT14.12 | Highly sensitive EGFRvIII detection in circulating extracellular vesicle RNA of glioma patients

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Introduction: Liquid biopsy offers an attractive platform for noninvasive tumor diagnosis, prognostication, and prediction of glioblastoma clinical outcomes. Prior studies report that 30% to 50% of GBM lesions characterized by EGFR amplification also harbor the EGFRvIII mutation. In this study, we investigate the clinical feasibility of liquid biopsy assessment of the EGFRvIII mutation as a diagnostic and predictive blood-based biomarker in patients with glioma. The cost effectiveness and safety of our proposed method provides an alternative strategy to repetitive, invasive tissue-based brain biopsies.

Methods: A novel digital droplet PCR (ddPCR) assay for high GC content amplicons was developed and optimized for sensitive detection of EGFRvIII in tumor tissue and circulating extracellular vesicle RNA (EV RNA) isolated from the plasma of patients with glioma.

Results: Our optimized qPCR assay detected EGFRvIII mRNA in 81% [95% confidence interval (CI), 68%–94%] of EGFR-amplified glioma tumor tissue, indicating a higher than previously reported prevalence of EGFRvIII in glioma. Using the optimized ddPCR assay in discovery and blinded validation cohorts, we detected EGFRvIII mutation in 73% (95% CI, 64%–82%) of patients with a specificity of 98% (95% CI, 87%–100%), compared with qPCR tumor tissue analysis. In addition, upon longitudinal monitoring in 4 patients, we report detection of EGFRvIII in the plasma of patients with different clinical outcomes, rising with tumor progression, and decreasing in response to treatment.

Summary/Conclusion: This study demonstrates the feasibility of detecting a canonical tumor mutation, EGFRvIII in plasma using a highly sensitive and specific ddPCR assay. Our extracellular vesicle RNA-based ddPCR method suggests the potential for serial, longitudinal assessment of tumor mutational burden even in the context of intratumoral heterogeneity. We also show a higher than previously reported EGFRvIII prevalence in glioma tumor tissue. Several features of the assay are favorable for clinical implementation for detection and monitoring of EGFRvIII-positive tumors.

Keywords: glioma, EGFRvIII, EV RNA, biomarekrs

PT14.13 | Urinary extracellular vesicles for predicting therapy responses from muscle invasive bladder cancer patients

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Introduction: The molecular mechanisms of muscle invasive bladder cancer (MIBC) phenotypes and their progression are poorly understood. Current MIBC patients with residual tumor or node positive disease have only 25% 5-year disease-free survival after treatment of radical cystectomy and pelvic lymph node dissection (RC-PLND). Even though, the benefit of neoadjuvant chemotherapy (NAC) is underutilized to overcome the complication and morbidity from RC-PLND, due to the lack of robust response prediction. Extracellular vesicles, particularly nano-sized exosomes, have gained tremendous attention in bladder cancer therapy and diagnosis recently. The ability to pinpoint tumor markers enriched from exosomes could greatly aid our understanding of molecular mechanisms driving the initiation, progression, and malignancy of bladder cancer.

Methods: Our recent studies also found the unique bladder cancer biomarkers derived from urinary exosomes with improved specificity and sensitivity for detecting bladder cancer. In this presentation, we will report a deep machine learning bioinformatic approach for multi-omic molecular information extraction from exosome subtypes,enabling precisely definition of MIBC for therapy response prediction, which is building upon our developed NanoPoms capture-release exosome isolation approach. The prepared exosomes are highly specific to enrich tumor markers in multi-tissue fluids, including urine and patient plasma, which

is unique biomarker sources for studying multi-omic profiles. Mapping the multi-omic profiles from exosome subtypes across multiple body fluids has not been explored, yet is an emerging approach for reliably and precisely modeling MIBC tumor biology.
Results: The developed machine learning bioinformatic approach for spatial multi-omic exosome analysis overcomes the challenges caused by data heterogeneity and complexity, and enables simultaneously elucidation of tissue-specific and cross-tissue cancer biomarkers from NGS total RNA sequencing data. The patient urine samples from healthy, non invasive stage, MIBC, and after therapeutic treatment were tested by Nanopom exosome preparation and NGS total RNA seq, which exhibited unique differentiating profile for patient therapy selection.
Summary/Conclusion: This study would provide a platform to develop accurate and reliable prognostic and therapeutic predictors as the fingerprint to drive individual patient therapy selection and improve clinical and functional outcomes. Instead of relying on one single genomic or cluster of genomic markers, this technology would allow for a multi-tissue multi-omic modelling of a MIBC patient's cancer biology.
Funding: NIH 1R35GM133794 UFHCC GU pilot.

PT14.14 | Substrate variation in cell culture conditions alters EV generation without changing RNA content

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Introduction: Tumor microenvironment stroma is an important contributor to the physiopathology of cancer and has been shown to shape tumor stiffness. While much of our understanding about cancer comes from cell culture studies in two dimensions (2D), it is appreciated that 3D culture systems better recapitulate in vivo aspects of tumors. It is known that substrate stiffness can alter cells at multiple levels, including gene expression, yet little has been done to investigate the impact of substrate stiffness on EV biogenesis and cargo.
Methods: In the present study, we wanted to explore if different cell culture conditions would affect RNA cargo of tumor derived EVs, with a specific focus on non-coding RNA (ncRNA). Further, we explored the impact of culture conditions on EV size and number. For this, cell lines generated from ascites fluid of patients with metastatic PDAC were grown in 2D and 3D, as well as on hydrogels with defined mechanical stiffness levels (0.5 kPa, 2kPA and 8kPa).
Results: Analysis of total RNA content of EVs isolated from 2D and 3D conditions showed an enrichment of more than 50% of repeat ncRNAs, specifically LINE, SINE and ERV elements, when compared with parental cells grown in the same conditions. While cells cultured in 3D produced more EVs than 2D cell culture conditions (~ 2-fold more), vesicle size was not impacted (around 250nm). Interestingly, PDAC cells grown on less stiff surfaces (0.5 kPa) released at least 40-fold more EVs compared with 2D. However, substrate stiffness did not affect the levels of repeat RNAs detected in vesicle cargo, which were maintained across all conditions.
Summary/Conclusion: Overall, our data shows that cells grown under non-adherent conditions and in more elastic hydrogels present an increase in EV production, without affecting size and RNA content. Nonetheless, secreted EVs are much more enriched in repeats ncRNA when compared with their cells of origin, which can represent a novel and specific class of biomarkers in EVs from patients with PDAC.

PT14.15 | Selective enrichment of plasma cell-free messenger RNA in cancer-associated extracellular vesicles

Hyun Ji Kim[1]; Matthew j Rames[2]; Florian Goncalves[2]; charles Ward Kirschbaum[2]; Breeshey Roskams-Hieter[2]; Josephine Briand[2]; Aaron Doe[2]; Joseph Estabrook[2]; Josiah Wagner[2]; Emek Demir[1]; Gordon Mills[1]; Thuy M. Ngo[1]
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Introduction: Extracellular vesicles (EVs) have been shown as key mediators of extracellular small RNA transport. However, carriers of cell-free messenger RNA (cf-mRNA) in human biofluid and their association with cancer remain poorly understood. Here, we performed a transcriptomic analysis of size-fractionated plasma from lung cancer, liver cancer, multiple myeloma, and healthy donors.
Methods: We fractionate plasma into vesicular and non-vesicular carriers using a size exclusion column. We developed a strategy to purify and sequence ultra-low amounts of cf-mRNA from vesicular and non-vesicular subpopulations with the implementation of RNA spike-ins to control for technical variability and to normalize for intrinsic drastic differences in the amount of cf-mRNA carried in each plasma fraction.

Results: Morphology and size distribution analysis showed the successful separation of medium and small EVs and non-vesicular carriers. We found that the majority of cf-mRNA was enriched and protected in EVs with remarkable stability in RNase-rich environments. We observed specific enrichment patterns of cancer-associated cf-mRNA in each vesicular and non-vesicular subpopulation. The EV-enriched differentiating genes were associated with specific biological pathways, such as immune systems, liver function, and toxic substance regulation in lung cancer, liver cancer, and multiple myeloma, respectively.
Summary/Conclusion: Extracellular vesicles are the major carriers of cf-mRNA in plasma. Subpopulations of the vesicles are enriched for distinct sets of genes associated with multiple cancer types. Our results suggest that dissecting the complexity of EVs subpopulations illuminates their biological significance and offers a promising liquid biopsy approach.
Funding: Research in Ngo lab was supported by the Cancer Early Detection Advanced Research (CEDAR) Center at OHSU Knight Cancer Institute (Full 2020-1289), Cancer Research UK/OHSU Project Award (C63763/A27122 to T.T.M.N.), the OCTRI CTSA grant (UL1TR000128), the Kuni Foundation, the Department of Defense (W81XWH2110853 to T.T.M.N.) and the Susan G. Komen Foundation (CCR21663959 to T.T.M.N.).

PT15: EV Sizing and Counting

Location: Hall 4A

16:45 - 18:45

PT15.01 |

Yuichiro Iwamoto[1]; Benjamin Salmon[2]; Miu Tamamitsu[3]; Yusuke Yoshioka[4]; Alexander Krull[2]; Sadao Ota[5]
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Introduction: Large-scale optical analysis of extracellular vesicle(EVs) properties is essential to understand the characteristics of EV populations and holds potential to realize EV-based clinical diagnosis in practice.However, no method simultaneously satisfies the requirements as an ideal EV analyzer: enough sensitivity for detecting individual nanoparticles(< 100 nm) with sizing capability, fluorescence sensitivity, a high throughput and a large scalability.Here we realized a high throughput (96,000 events/sec) and sensitive (< 40 nm) nanoparticle analyzer by integrating a unsupervised learning-based denoising method for detecting weak scattering detection with a home-built optofluidic set-up.
Methods: We performed all experiments using a home-built optical and fluidic analyzer system. First, we measured the scattering of polystyrene particles of known refractive index and size to evaluate the performance of our system. Next, we demonstrated large-scale EV analysis. Scattering and fluorescence of antibody-stained EVs of HCT(human undifferentiated thyroid cancer cell) were measured.
Results: Using the analyzer developed, we demonstrated the detection of polystyrene particles (< 40 nm) at a detection throughput of 98,000 particles/s.We show that applying a machine learning-based denoising method significantly improves the measurement system's sensitivity. Experiments with polystyrene particles show that this method accurately removed noise and detected nanoparticles even though it does not require training data-set. Finally, we performed an experiment of detecting EVs which were labeled with CD9 and CD147 with a detection throughput of 5,000 particles/s. As a result of analyzing 100,000 particle detections in total, 85% of the detected EVs were observed to be CD9-positive, and 10% of them were CD9/147 double positive.
Summary/Conclusion: We developed a high throughput and high sensitivity nanoparticle analyzer by combining a machine learning-based denoising method and an optofluidic system for detecting weak scattering.Furthermore, we demonstrated large-scale EV analysis using EVs labeled with CD9 and CD147.Our system potentially becomes a powerful tool for large-scale individual EV analysis.
Funding: This work was sup-ported by JST, CREST grant number JPMJCR19H1, Japan.
Keywords: flow cytometry, deep learning

PT15.03 | Linearity in exosome concentration measurement by nanoparticle tracking analysis (NTA)

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Introduction: EV concentration measurement results are routinely reported. For NTA analysis many exosome samples need to be diluted before measurement. Naturally, the degree of required dilution needs to be known and that is coupled to trade-off's in measurement performance. One issue is the linearity of the measurement results. That is how do determine the range of concentrations for which results are sufficiently reliable.
Methods: Lyophilized exosomes were obtained from Abcam (Lyophilized MCF7 Exosome Standards, lot GR3401014-1). These are derived from MCF7 a human cancer cell line and characterized by Abcam. Exosomes were reconstituted following manufacturer instructions, and diluted in Dulbecco's phosphate buffered saline (dPBS). NTA was performed with a HORIBA ViewSizer 3000 multi-laser NTA instrument. Multiple lasers were used to ensure analysis of all EV sizes in the sample. Laser power settings were 210 mW blue, 12 mW green, 8 mW red and camera gain was 30 dB.
Results: A single aliquot of reconstituted exosomes was diluted to varying degrees and the concentration of each resulting sample was determined by NTA. Dilutions ranged from 30,000 times to 1000 times and the resulting measured concentrations ranged from ~5e6 p/mL to 6e7 p/mL. The lowest measured concentrations showed distortion due to background particle concentration while the highest measured concentration showed an overly low concentration.
Summary/Conclusion: A linearity study is important to determine the acceptable range of measured concentrations. There is a trade-off between linearity (underreporting concentration) and uncertainty/measurement duration (measuring too few particles).
Keywords: concentration, NTA, nanoparticle tracking analysis, viewsizer, uncertainty, error, linearity

PT15.04 | Quantification and deep multiparametric analysis of circulating extracellular vesicles by spectral flow cytometry

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Introduction: Extracellular vesicles (EVs) have excellent potential to become biomarkers for diagnosis, prognosis, or monitoring of disease. They can be found in cell culture supernatants as well as in bio fluids such as blood, urine, tears and mother milk. They carry proteins, nucleic acids, and other cellular components and play an important role in the communication between cells.
Flow cytometry is one of the most used techniques for EVs analysis and quantification, however, since the EV size is close to the electronic noise of common conventional flow cytometers, classical procedures which are usually applied for cells, cannot be applied for EVs. Therefore, the analysis of such small particles is still challenging.
Methods: Moving in this technological field, we have developed a twenty-two color multiparametric panel for in-depth immunophenotyping and quantification of circulating EVs from human plasma, using both lineage and activation / exhaustion markers, through the new generation spectral flow cytometry. Of note, sample collection, processing and analysis have been performed following MISEV (Minimal Information for Studies of Extracellular Vesicles) recommendations.
Results: We were able to identify EVs ranging between 100 nm and 900 nm by exploiting the detection of the Side Scatter (SSC) on the violet laser, instead of the conventional blue one. The spectral approach minimized spreading error and spectral overlay thus allowing the simultaneous identification of several EV subpopulations. In addition to the most common EV subsets, such as erythrocyte-derived EVs, platelet-derived EVs, leukocyte-derived EVs and endothelial-derived EVs, we observed EV subsets carrying only activation and exhaustion markers.
Moreover, we observed that most of the extracellular vesicles exhibited only one antigen on their surface probably due to their small size. Indeed, the unsupervised analysis performed on platelet-, neutrophil- and endothelial-derived EVs, showed only up to 5% of the EVs carrying two, three or four antigens together on their surface.
Summary/Conclusion: Overall, these data highlight the importance of a spectral flow cytometry approach for deciphering the high heterogeneity of the circulating plasma derived EV thus paving the way for a routine use of high dimensional fluorescent antibody panels to study circulating EVs under different pathophysiological conditions.
Keywords: spectral flow cytometry

PT15.05 | Optimization of single EV imaging flow cytometry for quantification of extracellular vesicle subsets

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Introduction: Extracellular vesicles (EVs) play a key role in many physiological and pathophysiological processes and hold great potential for therapeutic and diagnostic use. Due to the small size of EVs and their heterogeneity, it remains a general challenge in the EV field to define and set up reliable methods that facilitate accurate and specific quantification of total EVs and EV subsets, particularly in relatively impure EV preparations. Previously, we have optimized single EV Imaging Flow Cytometry (IFCM) for detection and phenotyping of EVs and EV subsets using the Amnis ImageStreamX platform (Görgens et al, JEV 2019, Tertel et al Cytometry PartA 2020). Here, by using an Amnis Cellstream instrument, we established a robust and improved workflow for accurate quantification of subsets of fluorescently labelled EVs.
Methods: EVs were prepared by ultrafiltration and size exclusion chromatography from conditioned media derived from wild-type HEK293 cells and from HEK293 cells engineered to produce fluorescently tagged EVs (CD63-mNeonGreen). EVs derived from CD63 knockout cell lines were used for control purposes. All EV preparations were characterized by nanoparticle tracking analysis (NTA) and multiplex bead-based EV flow cytometry.
Results: By using fluorescently tagged EVs as reference material we defined and optimized various parameters related to sample preparation, IFCM acquisition and data analysis on the Amnis Cellstream platform for the detection of single sEVs. By combining this approach with fluorescent antibody-labelling and a focus on improving dim signal detection, we demonstrate that Cellstream IFCM facilitates accurate quantification of subsets of fluorescently labelled EVs in differently pure samples, thereby also enabling robust quantification of engineered versus non-engineered EVs for quality assessment and quality control of produced EV batches. Side by side comparison of ImageStreamX and Cellstream platforms following fluorescence calibration revealed overall comparable sensitivity and quantification capabilities of both platforms. Furthermore, we evaluated and titrated different buffer compositions for sample preparation and dilution and report optimized conditions resulting in drastically reduced sample loss compared to PBS alone.
Summary/Conclusion: We provide a robust and improved IFCM workflow applicable for accurate quantification of total EVs and subsets of fluorescently labelled EVs in EV containing samples, independent of purity. We propose that such versatile IFCM based assays will improve our ability to rigorously and reproducibly quantify EV subsets, assess EV heterogeneity, and identify disease-specific biomarkers.
Keywords: EV flow cytometry flow cytometry imaging flow cytometry

PT15.06 | Comparative analysis of particle size and concentration using nanoscale flow cytometry and microfluidic resistive pulse sensing

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Introduction: Extracellular vesicles (EVs) are heterogenous in origin, abundance and function, which leads to multiple opportunities to explore their biological roles and clinical value. However, the lack of reproducibility in EV research has impeded translation of discoveries into the clinic. Traceable and reproducible EV measurement is a prerequisite for credible EV research. We previously demonstrated the utility of standardized and calibrated nanoscale flow cytometry (nFC) for reproducible particle sizing and enumeration from biofluids. Herein, we conducted a reproducibility study comparing nFC and microfluidic resistive pulse sensing (MRPS) for measuring synthetic beads and biofluid-derived particles.
Methods: NIST-traceable silica and polystyrene beads with a size range of 180 to 1,300 nm, cell culture supernatants, platelet-free plasma and cell-free urine were analyzed by nFC (Apogee A60 Micro Plus, A60MP, Apogee Flow Systems) and MRPS (nCS1, Spectradyne). A60MP was calibrated using Rosetta calibration beads (Exometry). Statistical analysis was performed to evaluate the reproducibility of particle sizing and enumeration.

Results: Both nCS1 and A60MP provided similar size resolution of bead populations in both polydisperse and monodisperse condition and similar concentration measurements in monodisperse condition (1.1-fold change; P = 0.9). nCS1 and A60MP could also provide similar size ranges (190 – 2000 nm) of particles from all biological samples. However, nCS1 measured higher particle concentrations from conditioned medium and urine samples (4.2-fold and 2.8-fold change; P = 0.2 and P = 0.1 respectively) whereas A60MP measured greater particle concentrations from plasma samples (3.4-fold change; P = 0.7).
Summary/Conclusion: In conclusions, both nFC and MRPS can provide compatible size resolution of nanoparticles and biological particles. Differential particle measurement suggests imprecise diameter approximation of particles from nFC using the scatter-diameter relationship (Mie theory).

PT15.07 | Repeatability of classical and novel urinary extracellular vesicle isolation and quantitation methods

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Introduction: Reliability of quantitative urinary extracellular vesicle (uEV) assessment methods should be evaluated using healthy subject samples before test results can be applied to screen for pathological conditions, including cancer. Our goal was to estimate the short-term precision that a lab may expect to obtain when analyzing naïve chemically untreated subpopulations of uEVs isolated by classical differential velocity centrifugation (DC) or novel silicon carbide precipitation (SiC-P) techniques.
Methods: Varying size uEVs were isolated in parallel from 6 technical replicates of 34 ml raw or pre-processed (0.45 μm vacuum-filtered) healthy consented adult human morning urine samples by DC at 800 × g (10 min), 2K × g (0.5 h), 12K × g (1 h) and 100K × g (1 h) at 4°C, followed by 12K (uMV) or 100K (uEXO) pellet resuspension in 55 μL or 100 μL PBS-25 mM Trehalose buffer, and an optional 0.45 μm spin-filtration (SF) step of PBS-diluted final analyte solutions. uEXO2 nanoparticle fraction was captured from 800 × g (10 min) pre-cleared pH 8.6-adjusted urine on 3 ml 1000 grit size SiC slurry for 0.5 h and eluted in 3 ml PBS (pH 6.0) for 15 min followed by 1 μm gravity filtration and 0.45 μm SF steps. We fitted a random-effects nested ANOVA model to determine the extent and relative contribution of procedural (sample processing, (sub)sampling) & instrumental (between-run, within-run) errors to the total technical variability of uEV concentration and size measurement process by nanoparticle tracking analysis. Closeness of agreement between measured variables was expressed as coefficient of variation (CV%).
Results: There was a strong linear relationship between raw urine volume input and uEV yield. Sample pre-processing caused a 30% to 40% loss of uEVs. Intra-individual bimonthly variations (CVI) of uMV counts were ~1.6-fold higher than CVI of uEXO (41.1% vs 26.3%). CVI of uMV and uEXO size was comparable (7.6% vs. 7.8%). CV of uEV sedimentation and collection process was 7.3% & 2.4% for concentration and size variables, respectively. The corresponding estimates for samples generated by the SiC-P method were 17.5% & 2.2%. 6 min long run-to-run variability of DC-derived uEXO and uMV sample concentration reads was 2.35% & 2.9%, and only 0.75% & 1.9% for size reads. Runs analyzing uEXO2 counts were less precise (4.6%). Due to minor drift, within-run repeatability was 6.2%, 7% or 6% for uMV, uEXO or uEXO2 concentration and 1.8%, 2% or 1.5% for their size.
Summary/Conclusion: With the estimated < 10.5% combined analytical CVA for uEV concentration and < 3.5% CVA for size measurements, only DC met the desired allowable limit of analytical performance (CVA< 0.5 × CVI) goals, favoring it over the novel more rapid SiC-P uEV separation method.
Keywords: repeatability, precision, ultracentrifugation, precipitation, rigor, analytical variability, performance, NTA

PT15.08 | Sorting subsets of extracellular vesicles by nano-flow cytometry

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Introduction: Extracellular vesicles (EVs) are umbrella term to cover the whole lipid bilayer membrane particles. Classically, exosomes or ectosomes with canonical EV marker protein such as tetraspanins, i.e. CD63, are main subsets of EVs but recent single EV detection technologies such as single EV imaging and cytometry have been revealed the complex and heterogeneous nature of EVs with different size and molecular composition. Although it is postulated that EV subsets have different functionality, the research has been hampered due to the lack of efficient EV subset sorting technology. In this study, we developed the efficient sorting procedure of EV with different antigen-positive, DNA-positive, and different size by nano-flow cytometry.

Methods: CD63/GFP-containing EVs were isolated by size exclusion chromatography. Fluorescent dye-labeling methods were applied with CFSE or Far Red dyes for total EVs, anti-CD147 for CD147-positive EVs, and PicoGreen for DNA-containing EVs. EV subsets were sorted by optimized multicolor nano-flow cytometry with sorter, CytoFLEX SRT.
Results: We established the EV sorting by nano-flow cytometry according to different size of CD63/GFP EVs with diverse parameters. Comparing with different flow rate, low flow rate (10,000 events/sec) showed the considerable sorting of single CD63/GFP EV from other EVs. Moreover, we applied the different sorting modes including single, purity, and enrich but there is no significant difference of sorting purity at optimal flow rate. Further, we mixed the CFSE- and Far Red- labeled EVs with one-to-one ratio and then each subpopulation was sorted again. Sorter effectively isolated each fluorescent-positive population without contamination of other population. We further applied EV sorting of CD147-positive and -negative subpopulation by antibody labeling and we established the sorting of DNA-positive subset among total EVs from Docetaxel-treated cells.
Summary/Conclusion: In this study, we isolated the EV subsets by nano-flow cytometry. This approach illustrates the potential of nano-flow cytometry coupled sorter is unique method to isolate the specific subset of EVs.
Keywords: exosome, sorting, nano-flow cytometry, DNA

PT15.09 | Comparison of analytical performance of NTA, DLS and SEC for evaluating particulate size of sEV preparations and application of SEC for characterizing particulate impurities

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Introduction: For the therapeutic applications of small extracellular vesicles (sEVs), the size distribution of particulate content should be evaluated for the quality control. Although many methods to analyze the size of EVs have been reported, there has been a few discussions on what method and condition are suitable for size measurement, or how to identify particulate impurities in EV formulation. Here, we evaluate the performance of several size measurement methods and applied the methodology to characterize particles in EV preparations.
Methods: Dilution series of 60 nm and 200 nm polystyrene size standards were used as analytes to evaluate accuracy and precision of the particle size measurement by DLS, NTA and SEC with multiangle light scattering (SEC-MALS). EVs were isolated from A549 cell culture medium by ultrafiltration (UF), phosphatidylserine affinity beads and size exclusion gravity column. For characterization of particulate contents in EV preparations, the EV sample prepared by UF were size-dependently fractionated by SEC and each fraction was submitted to proteomic analysis using LC-MS/MS.
Results: When evaluating the 60 nm polystyrene particles, significant increases in mean diameter were observed along with the decrease in particle concentration in DLS and NTA measurement. It was considered that particle concentration is critical analytical condition in measurement of particle size using these methods. For the EV samples, SEC-MALS showed higher selectivity for evaluating particle size distribution. As a result of proteomic analysis, it was found that the crude EV sample included three bioparticle species: microvesicle, exosome and lipoprotein.
Summary/Conclusion: We evaluated the analytical performance of size measurement methods and conducted the characterization of the particulate contents in the EV samples. These results would be useful for characterization and quality control of EV therapeutic development.
Funding: This work was supported by AMED under Grant JP22mk0101218.
Keywords: size measurement, proteome analysis, DLS, NTA, SEC-MALS

PT15.10 | Simplified visualization of extracellular vesicle preparations by silicon nanomembrane-enabled microfluidic devices

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Introduction: Developing and characterizing methods for separation and concentration of extracellular vesicles (EVs) remain an area of significant interest. EV applications increasingly require isolated EVs of high purity and yield to perform basic biological, diagnostic, and especially therapeutic analyses. An ability to characterize EVs by simple and rapid means before, during, and after isolation steps would benefit understanding of both currently used methods and development of new methods. However, assessing EV preparations for EV size, concentration, and presence/absence of molecular cargo currently requires multiple dedicated instruments and practiced knowledge of these systems' procedures and pitfalls. We aim to bridge this gap by

developing a user-friendly EV visualization procedure for determining concentration and detection of molecular markers within EV preparations.
Methods: Here, we report on our assessment of a microfluidic device capable of rapid visualization of EVs using simple pipet-driven loading and on-membrane EV capture and imaging. This device, named the μSiM-EV, was assessed for its ability to catch and visualize EVs by a number of microscopy techniques. On-membrane immunofluorescence was used to detect EV surface proteins, while comparisons to nanoparticle tracking analysis was used for assessing EV concentration.
Results: We assess nanoporous silicon nitride membranes, which are incorporated into the μSiM-EV device, for their ability to capture small- and medium-sized EVs. We showcase the ability to visualize EVs when captured on-membrane by optical and electron microscopy. We further compare nanoparticle tracking analysis to the μSiM-EV for particle concentration determination.
Summary/Conclusion: The μSiM-EV offers ease of use for rapid EV visualization during separation and concentration procedures. Future work will focus on expanding the variety and number of EV preparations from different sources that are analyzed via the μSiM-EV procedure.
Funding: NIH Grant No. NIGMS 2-R44-GM137651-02 to SiMPore Inc.
Keywords: EV characterization, single EV analysis, microfluidics, microscopy

PT15.11 | Water-soluble extracellular vesicle probes based on conjugated oligoelectrolytes

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Introduction: We developed a series of transmembrane conjugated oligoelectrolytes (COEs) with tunable optical emissions from the UV to the near IR to address the false-positive problem when detecting nanometer-sized extracellular vesicles (EVs) by flow cytometry. The amphiphilic molecular framework of COEs is defined by a linear conjugated structure and cationic charged groups at each terminal site. Consequently, COEs have excellent water solubility and the absence of nanoaggregates at concentrations up to 50 μM, and unbound COE dyes can be readily removed through ultrafiltration. These properties enable unambiguous and simple detection of COE-labeled small EVs using flow cytometry with negligible background signals. We also demonstrated the time-lapsed tracking of small EV uptake into mammalian cells and the endogenous small EV labeling using COEs. Briefly, COEs provide a class of membrane-targeting dyes that behave as biomimetics of the lipid bilayer and a general and practical labeling strategy for nanosized EVs.
Methods: N/A
Results: N/A
Summary/Conclusion: N/A

PT15.13 | An optimized, high-throughput method for the measurement of nanoparticle size distribution in clinical samples using transmission electron microscopy

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Introduction: The smallest of the biologically important nanoparticles, high-density lipoproteins (HDL, 5–12nm diameter) are too small for most particle sizing methods. Negative-stain transmission electron microscopy (NS-TEM) allows direct visualization and precise size quantification of individual particles down to this size range.
Methods: HDL were isolated from 26 plasma samples, negatively stained (2% uranyl formate), and micrographs were obtained using a JEOL 1230 model EM at 40k magnification, 120 kV with a bottom-mounted Ceta camera (4k × 4k pixels). Particle size was analyzed with ImageJ using an in-house automation script with different circularity, roundness, and aspect ratio cutoff combinations and compared to manual selection. Particle sample sizes from 1 to 5,000 were selected randomly 100 times from a population of 43,494 particles in one representative sample, and the sample size required for deviation from population mean by 1%, 5%, and 10% within 95% of the time was simulated. The coefficient of variation (CV) across 15 images per sample was calculated and compared to batch dynamic light scattering (DLS).
Results: The optimal parameters of circularity < 0.5, roundness < 0.5, and aspect ratio > 1.5 yielded 78% particle recovery and 87% correct rate compared to manual particle selection, in a fraction of the time. A sample size of at least 3,000, 120, and 50

particles resulted in a mean particle size within 1%, 5%, and 10% deviation from the mean population particle size 95% of the time. Inter-run CVs of 0.34% vs. 2.8% for size standards, and 3.503% vs. 10.43% for biological samples were obtained for the NS-TEM method vs. batch DLS, respectively.

Summary/Conclusion: Our optimized method using NS-TEM followed by automated image analysis is reproducible and amenable to particle sizing of HDL in large numbers of clinical samples due to its accuracy and high-throughput.

Funding: This project was funded by NIH Grants R01AG062240, R01GM147545, UH3CA241694.

Keywords: nanoparticles, high-density lipoprotein, electron microscopy

PT15.14 | Optimization of Nanoimaging and nanoflow cytometry-based characterization of single extracellular vesicles

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Introduction: The single vesicle characterization has been an emerging technology for early detection for various disease, although we still face the challenge for interpreting the data with different protocols or devices among institutions. In this work, we attempted to develop optimal extracellular vesicle (EV) sample preparation for two single EV analysis technologies: NanoFCM for flow-based EV characterization, and NanoImager for stochastic optical reconstruction microscopy (STORM)-based EV characterization at a single vesicle level.

Methods: EVs derived from the HCT116 human colorectal cell line were labeled with fluorophore-conjugated antibodies against two tetraspanin markers (Alexa 488-conjugated anti-human CD9 and Alexa 647-conjugated anti-human CD81). Amicon filters (100 kDa cut off) pre-treated with PBS (0.1% BSA, double filtered) and size exclusion column (qEV single) were applied to remove free dye. The protocol using Amicon filters was optimized to reduce EV loss and BSA contamination. Nanoparticle Tracking Analysis was carried out to determine and compare the EV concentration. EV samples were prepared by the dilution methods, Amicon filter or qEV column, followed by NanoFCM and NanoImager analysis to evaluate the particle size, fluorescent intensity and populations of CD9+ and CD81+ EVs.

Results: When the dilution factor increasing from 50 to 400, the percentage of CD9+/CD81+ EVs increased from 2% to 5% as determined by NanoFCM. Removal of excess fluorescent dye by Amicon filtering improved the reproducibility of the percentage of CD9+/CD81+ EVs to ~10 %, with minimal contamination of BSA-derived particles. Similar results were obtained using qEV-based free dye removal, and CD9+/CD81+ EVs population reached to ~9%. The population of CD9+/CD81+ EVs by NanoImager after Amicon filtration was 23%, much higher than the result from NanoFCM, reflecting the difference in sensitivity of detecting CD9+/CD81+ EVs using two instruments. The population of CD9+/CD81+ EVs after the qEV-based dye removal was 2%, possibly due to the dilution of EVs after the fractionation.

Summary/Conclusion: Our results show that florescence-based characterization of tetraspanin positivity in EVs were successfully conducted at a single vesicle level using NanoImager and NanoFCM. It also provides different results based on the dye removal steps and different detection systems. Optimization and standardization of the sample preparation and thresholding are necessary to generate reproducible analysis of EVs at a single particle level using these instruments.

PT16: Immunity, Autoimmunity & Inflammation #1

Location: Hall 4A

16:45 - 18:45

PT16.02 | Antigen-loaded extracellular vesicles to regulate adaptive immune responses and drive immunological tolerance

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Introduction: Autoimmune diseases are driven by adaptive immune responses that are inappropriately directed towards an autoantigen. Tolerogenic vaccines capable of sequestering immune responses to autoantigens pose a potential treatment without

the drawbacks of broad immunosuppression. However, development of such therapies has been limited by the inability to deliver and present adequate antigen without co-stimulation. As many extracellular vesicles (EVs) are immunologically inert and can load a variety of cargos, we developed an EV platform for the co-delivery of antigens and immunoregulatory cues.
Methods: EVs were isolated from HEK293SF-3F6 cells using an iodixanol density gradient and characterized in accordance with MISEV 2018 guidelines. Azide-functionalized EVs were generated by adding Ac4ManNAz to the culture media. HEK cells expressing BASP1-OVA were generated using a PEI MAX transfection reagent. Ovalbumin (OVA) was functionalized with AF488 and DBCO via NHS/TFP ester chemistry. Tolerance induction was assessed using in vitro and in vivo models involving bone marrow dendritic cells (BMDCs) or DC2.4s and OT-I and OT-II T cells.
Results: We first demonstrated that HEK EVs can be loaded with the model antigen OVA via both an exogenous metabolic glycoengineering approach and an endogenous producer cell engineering approach. Furthermore, we demonstrated that OVA-loaded HEK-EVs induce antigen presentation without activating the innate immune system of DCs. Treating mice with engineered EVs after adoptive transfer of OT-I and OT-II cells, followed by OVA + LPS challenge, indicated that EV treatment suppressed T cell proliferation and shifted OT-II cells towards a regulatory phenotype.
Summary/Conclusion: These results indicate that EVs may be capable of serving as a tolerance-inducing vaccine platform. Future work aims to load multiple immunosuppressive cues into EVs and demonstrate that treatment with engineered HEK EVs can downregulate the adaptive immune response in an experimental autoimmune encephalitis model.
Funding: NSF FMSG 2036809 NIH ITED T32 DK101003 NSF GRFP 2022305770.
Keywords: autoimmunity, immune tolerance, metabolic glycoengineering, immune regulation

PT16.03 | Complementing keratinocyte-originating exosomes

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Introduction: Paracrine effects via exosome emerged as the primary mechanisms of cell-cell crosstalk at the wound site. Resolution of wound inflammation relies on successful crosstalk between keratinocytes that must re-epithelialize, and the blood-borne wound-site macrophage, which must mount and timely resolve inflammation. We tested the hypothesis that keratinocyte-originated exosomes (EXO-k) are specifically directed to inflammatory macrophages for the resolution of wound inflammation.
Methods: Murine EXO-k were genetically labeled with GFP reporter using tissue nanotransfection and isolated using differential ultracentrifugation followed by immunomagnetic separation. Isolated wound-edge (WE) EXO-k was characterized per MISEV 2018 guidelines and reported in EV-track (EV 220292; EV-metric score 100%). Protein cargo on EXO-k surface was detected, validated, and quantified using LC-MS/MS, dSTORM imaging, and flow cytometry. The cellular source of Complement Factor I (CFI) was determined following transfection with either KRT14 or Lyz2 promoter-driven CFI plasmids with an "in frame" reporter. N-glycans compositions from EXO-k were analyzed by capillary electrophoresis-mass spectrometry (CE-MS). The Glycoproteome of EXO-k was generated using LC-MS/MS.
Results: In non-diabetic WE, EXO-k were selectively taken up by inflammatory macrophages. CE-MS identified 51 unique N-glycan compositions of which 7 glycans including mannose were exclusively present in WE- EXO-k. CFI is known to be released by macrophages for opsonization, and also by keratinocytes for complement activation. dSTORM imaging identified that 58% and 7% of CFI on EXO-k were of keratinocytes and macrophage origin respectively. Uptake assay showed that unlike EXO-k isolated from m+/db WE, uptake of diabetic EXO-k was significantly compromised by inflammatory macrophages. Glycoproteomics analysis of EXO-k isolated from m+/db and diabetic db/db WE tissue identified a total of 584 glycopeptides. A comparative analysis revealed that glycoprotein CFI was present in significantly lower abundance in diabetic EXO-k. Glycosylation mapping of the 3 glycosylation sites of CFI showed significant fucosylation in diabetic EXO-k. A sialylation profile analysis of CF1 revealed that the percentage of triply sialylated glycoforms is significantly lower in EXO-k under diabetic conditions (* $p < 0.05$).
Summary/Conclusion: This work provides a novel insight into the pathophysiological mechanism of diabetic EXO-k that results in compromised crosstalk between WE keratinocytes and macrophages. Hyperglycemia dysregulates exosome surface glyco modification of EXO-k. These perturbations compromise crosstalk between keratinocytes and wound macrophages complicating the resolution of wound inflammation.
Funding: NIH R56DK129592 to SG.
Keywords: keratinocyte-originated exosomes, resolution of inflammation, complement factor I, macrophages

PT16.04 | EVs show tropism to CD20+ B cells and monocytes in blood ex vivo

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Introduction: Exogenous EVs from different sources may interact with different cell types when introduced into a complex organism for therapeutic purposes. However, the biodistribution of EVs in larger animals is still obscure. Following up on a recent study reporting the association of EVs with peripheral blood mononuclear cell (PBMC) subtypes in vivo (Driedonks et al, JExBio 2022), we used an ex vivo blood system to interrogate EV cell tropism and interaction mechanisms.
Methods: Expi293F cells were transfected with pLenti-palmGRET, and EVs were separated from culture medium by tangential flow filtration and size-exclusion chromatography, then characterized by Western blotting, flow NanoAnalyzer, single particle interferometric reflectance imaging sensing and transmission electron microscopy. Whole blood samples from Macaca nemestrina were incubated with palmGRET EVs for 10–30 min at 37°C. PBMCs and CD20+ B cells were separated for confocal imaging analysis. EV-spiked PBMCs were also phenotyped by flow cytometry, tested for cell viability, and treated with trypsin for 15–30 min.
Results: palmGRET EVs bound to different PBMC subtypes with different affinities, with the greatest association with CD20+ B cells, CD14+ monocytes, and CD14dim monocytes. palmGRET signal decreased with exposure to trypsin, suggesting that at least some EVs remain at the cell surface or in trypsin-accessible compartments. Cell viability and EV integrity were not altered by trypsin treatment. Confocal imaging shows that EVs are internalized by CD20+ B cells within the first hour of exposure, with the diffusion of palmGRET fluorescence in unlabeled cells.
Summary/Conclusion: palmGRET EVs spiked into ex vivo macaque blood show preferential tropism to CD20+ B cells, CD14+ monocytes, and CD14dim monocytes.
Funding: São Paulo Research Foundation [2022/04146-9], Michael J. Fox Foundation for Parkinson's Research [00900821]; National Institute of Allergy and Infectious Diseases [AI144997]; NIH Office of the Director [U42OD013117]; National Institute on Drug Abuse [DA047807]; National Cancer Institute [CA241694]; National Institute of Mental Health [MH118164].
Keywords: B cells, monocyte, EV uptake

PT16.05 | Exploring extracellular vesicles as biomarkers in models of inflammatory arthritis

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Introduction: Extracellular vesicles (EVs) have been demonstrated to be important in the pathogenesis of rheumatoid arthritis (RA). For example, platelet-EVs are elevated in RA and drive pathology. We were interested in characterising endogenous EVs released during acute and chronic inflammation, specifically EVs that have the potential to predict acute arthritic flares and RA-associated comorbidities, such as cardiovascular dysfunction.
Methods: We utilised two murine models of inflammatory arthritis: i) the K/BxN F1 model, which spontaneously develops chronic arthritis at 4 weeks of age and left ventricular (LV) diastolic dysfunction (from 6 weeks) and ii) the serum-transfer induced arthritis model (STIA), an acute and self-resolving phenotype, representing the flare and remission of RA. 1mL whole blood was collected in EDTA-coated tubes, assessed using a haematology analyser and flow cytometry. Plasma EVs were isolated from whole blood using centrifugation with an initial 200xg spin for 20min at RT, then: 4,000xg 15min, 13,000xg 2min, pelleting EVs at 20,000xg 30 min, all at 4°C and stored at -80°C. EVs were analysed using imaging flow cytometry, nanoparticle tracking and western blotting.
Results: In the STIA model, no changes in total EV concentration or size were observed during peak inflammation or resolution. A specific subset of neutrophil (Ly6G+CD11b+) EVs were elevated at day 8 and returned to baseline by day 12, providing an early indication of resolution. In the chronic model of arthritis, a correlation was observed between increased platelets, platelet (CD41+) EVs and the appearance of LV diastolic dysfunction.

Summary/Conclusion: Neutrophil-EVs could be explored as biomarkers for drug-free remission in patients as they are an early indicator of resolution of arthritis. Platelet-EVs could also be monitored for predicting cardiovascular dysfunction in RA patients, this is important as RA patients have an increased risk of developing heart disease.
Funding: Funding: Versus Arthritis, grant number: 21941.
Keywords: rheumatoid arthritis, heart dysfunction, biomarkers, extracellular vesicles, platelet-EVs

PT16.06 | Extracellular vesicle-associated microRNAs activate macrophages through the SIRT1/NF-κB pathway in cell senescence

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Introduction: In this study, we report that SEN EVs can induce transcription of pro-inflammatory cytokine genes in macrophages via downregulating SIRT1. We then evaluate several miRNAs that target the mRNA of SIRT1. Our results suggest that EV-associated miR-30b-5p reduces the levels of SIRT1 in recipient cells. In mouse aging and cell senescence processes, miR-30b-5p in EVs increases in a senescence degree-dependent fashion.
Methods: Cell lines and cell culture An in vitro SEN EV functional assay was established using L929 cell lines that were cultured for 45 to 50 generations, and hyper SEN (h-SEN) cell lines were used etoposide-induced SEN cells. Non-senescent (control, CON) cells were used for comparison representing young cells. Raw 264.7 cells were used as the macrophage model. All cells were purchased from ATCC and cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% (V/V) fetal bovine serum (FBS) or EV-free FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. EV-free FBS was prepared by ultracentrifugationat 120,000 × g for 12 h at 4°C and filtration of the FBS supernatant with a 0.22 μm PVDF filter. Cells were cultured at 37°C in a humidified incubator containing 5% CO2 and tested negative for mycoplasma infection every week.
Senescence was confirmed in the SEN and h-SEN models using the ratio of phospho-H2A.X to β-Actin, a DNA damage marker, and SASP levels.
Results: 1. SEN EVs induce macrophage immune responses 2. SEN EVs regulate SIRT1 and induce canonical NF-κB activation 3. miR-30b-5p level in cells and EVs are correlated with aging 4. Levels of EV miR-30b-5p increased significantly with age in mice
Summary/Conclusion: In conclusion, cellular senescence is a state of permanent cell-cycle arrest, the causes of which are incompletely understood. Learning more about the cellular and molecular contributors to senescence will facilitate new approaches to treating senescence-related phenomena and diseases. Our work here reveals an unappreciated relationship between innate immune responses and senescence, establishing a link between cells and different cell types that involves cellular EVs transfer of miRNAs. These results give new insights into the inflammatory process in SASP and therefore heighten our understanding of the accurate regulation of senescence. SEN EV-induced inflammatory responses of macrophages may also be persistent: the macrophages will not be cleared by immune cells, since these SEN cells, possibly influenced chronically by released SEN EVs, are in a state of immune escape. The sustained action of SEN EVs may thus assist in transforming the macrophage into a promoter of aging and disease.
Keywords: aging, EVs, Sirt1, miRNAs, NF-kB

PT16.07 | Extracellular vesicles as a mediator and biomarker of telomere attrition-induced aging and human diseases

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Introduction: Telomere attrition is a hallmark of aging and associated with various age-related diseases. Recently, studies showed that telomeric repeats were detected in extracellular vesicles (EVs). Given the central role of EVs in mediating intercellular communication, we set out to investigate how telomere attrition alters circulating EV level and its cargo content, which in turn might contribute to aging and age-related diseases.
Methods: Plasma EVs were isolated from murine models with short telomeres, human healthy aging and disease cohort using ExoQuick precipitation method. MISEV2018 compliant EV characterization was performed. We have established the protocol to detect and quantify telomeric DNA cargo in different EV populations. Protein profiling was further analyzed by mass spectrometry.

Results: We have observed consistent alteration of circulating EV level, along with telomeric DNA cargo in both mouse and human plasma samples with short telomeres. Thus, circulating EV level, along with telomeric DNA cargo could serve as a marker for telomere attrition. Our preliminary data suggested that plasma EVs derived from mice with critically short telomeres displayed detrimental effects on recipient cells. The analysis of proteomic data is still ongoing.
Summary/Conclusion: Our studies revealed a close connection of EV, telomere attrition, and aging. In addition, we illustrated an EV mediated non-canonical mechanism underlying telomere attrition in aging and human diseases.
Funding: NIH Intramural Research.

PT16.08 | In vitro validation of Low-Intensity Pulsed Ultrasound (LIPUS) as an approach to enhance the release of microvesicles in Ulcerative Colitis patients

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Introduction: Ulcerative colitis is a gastrointestinal inflammatory disease whose diagnosis is based on a multidisciplinary invasive approach, which has an unmet need for new circulating biomarkers that can help an early diagnosis. Lately, Extracellular vesicles(EV) have been identified as promising biomarkers in several intestinal inflammatory pathologies, carrying peptides, miRNAs, lipids, and proteins. LIPUS was recently demonstrated to promote the release of Evs from cells non-invasively. We hypothesise that LIPUS can be used to locally stimulate UC patients damaged tissue microenvironment inducing the release of specific factors from these cells.
Methods: Mucosal fibroblasts and Endothelial Cells, and Peripheral Blood Mononuclear Cells (PBMC). The cell viability after the LIPUS stimulus was tested using MTT assay at 60', 120' and 24h at 38kHz in an intensity range from 0 to 250 mW/cm2 for 180s. In cell-stimulated supernatant, pro-inflammatory cytokine levels were evaluated by ELISA assay. In parallel, the EVs are isolated from a conditioned growth medium, employing centrifugation sequential and quantified by Nanoparticle tracking analysis. Acute and chronic colitis model was induced in C57BL/6N mice by administration of dextran sodium sulfate, respectively 2 and 2.5% ad libitum in their drinking water for seven days (acute) and three cycles of 7 days of DDS spaced out by three cycles 7days of H20. The colitis was scored daily, evaluating the body weight loss, bleeding and stool consistency. The mice received the LIPUS stimulus during the active phase of colitis. The blood was collected to isolate the plasmatic EVs through the employment of Amicon® filter. Subsequently, the EVs were quantified, and were analysed to identify the miRNAs associated with the inflammatory state through an RNA sequencing of isolated EVs compared with miRNA already correlated with UC.
Results: The parallel stimulations of mucosal cells allowed us to demonstrate that LIPUS is safe at 38 kHz and at intensities lower than 200 mW/cm2. Interestingly, at 150 mW/cm2 of intensity, the LIPUS decrease the IL-8. Furthermore, LIPUS at 150 mW/cm2 displayed the maximum release of EVs after 120' by stimulation in both UC and healthy-derived cells but not on PBMC. Consisting in promising in vitro results, we evaluated the effectiveness of LIPUS by measuring EVs concentration in acute and chronic models at I = 0, 150 and 375 mW/cm2, both 2 and 24 h after treatment, observing a positive trend can be seen when comparing mice treated at I = 0 mW/cm2. In contrast, after 24h, the levels dropped, likely indicating that EVs release is transitory. Similarly, in the chronic colitis model, a progressive increase in EVs was observed after 2 h of LIPUS but not after 24 h. Finally, the anti-inflammatory effect of LIPUS was assessed by the positive reduction of KC in mice stimulated at 150 mW/cm2.
Summary/Conclusion: Overall, these data support the hypothesis of LIPUS as a disruptive liquid technology in UC.
Keywords: LIPUS UC EVS

PT16.09 | Mesenchymal stem cell-derived extracellular vesicles ameliorate experimental autoimmune uveoretinitis in mice via suppressing autoreactive T cell infiltration

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Introduction: Accumulating evidence shows that extracellular vesicles (EVs) produced by mesenchymal stem/stromal cells (MSCs) exert immunomodulatory effects in several preclinical models. Previously, we also demonstrated that MSC-derived EVs (MSC-EVs) prevent the onset of autoimmune disease in three murine models: experimental autoimmune uveoretinitis (EAU), type 1 diabetes and Sjogren's syndrome. In addition, we found that 3D microcarrier culture conditions increased the expression levels of immunomodulators in MSC-EVs such as TGF-β1, let-7b and TSG-6, enhancing the immunomodulatory function of MSC-EVs. Building on these findings, we herein further investigated if MSC-EVs produced under 3D microcarrier culture conditions (3D MSC-EVs) can be used to treat autoimmune uveitis after EAU onset.
Methods: To examine the therapeutic potency of MSC-EVs in the management of EAU after disease onset, MSC-EVs were systemically infused into EAU mice on day 14 after EAU induction and the severity of EAU in mice was assessed on day 28. To examine if MSC-EVs can directly suppress the infiltration of retina-specific autoreactive T cells, an adoptive transfer model of EAU was induced in mice with or without MSC-EV treatment and T cell infiltration in the eyes and disease progression were evaluated in recipient mice. Also, in vitro chemotaxis assays were used to demonstrate the inhibitory effect of MSC-EVs on T cell migration.
Results: We found that MSC-EV treatment after EAU onset suppressed the disease progression in EAU mice and 3D MSC-EVs were more effective than those from control monolayer cultures. Also, 3D MSC-EVs effectively inhibited the migration of retinal-antigen reactive T cells toward the eyes in an adoptive transfer model of EAU, preventing the development of EAU in recipients. Mechanistically, we found that 3D MSC-EVs directly suppressed the MAPK/ERK pathway in activated T cells, inhibiting the migration of T cells.
Summary/Conclusion: Collectively, our results demonstrated that MSC-EVs directly inhibit the infiltration of retinal-antigen reactive T cells toward the eyes, thereby halting the disease progression in EUA mice. The data strongly support the therapeutic potency of MSC-EVs for the treatment of autoimmune uveitis.

PT16.10 | Proinflammatory stress activates neutral sphingomyelinase 2 based generation of ceramide-enriched β cell extracellular vesicles (EVs)

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Introduction: β cell EVs may act as paracrine effectors in islet health. However, mechanisms linking β cell stress to changes in EVs and whether activation of these pathways can impact diabetes remain unexplored. In other cells, neutral sphingomyelinase 2 (nSMase2) induces ceramide-dependent EV formation and release. We hypothesized that β cell inflammatory stress engages ceramide-dependent EV formation pathways, and that increased ceramide-enriched EVs could then impact surrounding β cells.
Methods: We treated INS-1 β cells with 24 hrs of 5 ng/mL IL1β. nSMase 2 and ceramide expression were quantified with immunoblot and/or flow cytometry and EVs were isolated using ultracentrifugation or tetraspanin (CD9, CD63, CD81) bead-based pulldown.
Results: IL1β increased β cell nSMase2 and ceramide expression in concert. Direct nSMase2 activation with 24 hrs of Caffeic acid phenethyl ester also increased ceramide staining. Both treatments also increased β cell EV ceramide staining. In contrast, nSMase2 knockdown reduced IL1β induction of cellular and EV ceramide staining. Human islets exhibited similar increases in cellular nSMase2 and ceramide staining after 24-hr cytokine mix treatment (IL1β, 10ng/mL TNFα, and 100ng/mL IFNγ). To test potential for reversal, cells were treated with IL1β +/- the GLP-1 agonist Exendin-4. Exendin-4 also reduced IL1β induction of cellular nSMase2 and cellular and EV ceramide staining. To test for β cell EVs transfer to surrounding cells, we generated INS-1 cells with GFP tagged CD9. Flow cytometry staining in mCherry+ INS-1 cells after 24-hr coculture with CD9-GFP cells showed that cocultured cells exhibited increased double-positive fluorescence, suggesting CD9-GFP+ EV transfer. This was enhanced by 24-hr IL1β treatment.
Summary/Conclusion: Our findings suggest that nSMase2 activity regulates β cell EV subpopulations under conditions of inflammatory stress. Future work will interrogate impacts on other EV cargo and physiologic impacts of EV transfer.
Keywords: EV, diabetes, beta cell, ceramide, lipid, inflammation

PT16.11 | Using designer EVs as host-directed innate immunotherapies to control primary human neutrophil fate and function

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Introduction: Polymorphonuclear neutrophils (PMNs) are the most abundant white blood cell in circulation and respond to stress within minutes. We identified a key transition state (A0) during PMN activation that determines mobilization of the antimicrobial primary granules. The first fate of PMNs (A1), defined as HDAC11low (protein) and MALAT1low (lncRNA), has intracellular granules and are adept at clearing bacterial pathogens P. aeruginosa and S. aureus. The second fate (A2), HDAC11hi and MALAT1hi, puts granules to the surface of the cell and promotes clearance of viral pathogens such as influenza A, RSV, and SARS-CoV-2.
Since PMNs are highly pinocytic, we hypothesized that engineered EVs could control the transition of A0 PMNs to the A1 or A2 fates and thus promote clearance of bacterial or viral pathogens, respectively.
Methods: We used the designer EV toolkit (DEVkit), a modular cloning toolkit developed in our lab, to generate all EVs, which were purified by differential ultracentrifugation. Structure, size, concentration, contents and contamination were evaluated by electron microscopy, Nanosight NS300, western blot, luminescence and qRT-PCR.
Results: We validated A0 PMNs could use EVs with EGFP and nluc protein and mRNA. PMNs treated with HDAC11 or MALAT1 siRNA EVs differentiated into A1 PMNs and doubled bacterial pathogen clearance compared to no EV and scramble siRNA EV control conditions. HDAC11 or MALAT1 EVs caused conversion of A0 PMNs to the A2 fate, which then completely removed all viruses within 24 hours compared to the control groups that could only clear 30–60% of IAV or SARS-CoV-2 in 24 hours.
Summary/Conclusion: Taken together, engineered EVs can control the fate and function of PMNs to clear bacterial and viral pathogens relevant in many inflammatory diseases Furthermore, this is another proof of concept of the utility of the DEVkit. We are very excited to unveil it via Addgene for broad use by the EV community to enable standardization and customization of a portion of the engineering process.
Funding: Emory I3 Program, R01HL159058, CFF TIROUV19A0.
Keywords: designer EVs, neutrophils, immunotherapy

PT17: Cancer Microenvironment 1

Location: Hall 4A

16:45 - 18:45

PT17.01 | A tale of two (or more) tumors: differential, but detrimental, effects of glioblastoma extracellular vesicles (EVs) on normal human brain cells

Mary Wang[1]; Arin Graner[1]; Bryne Knowels[2]; Charlotte McRae[3]; Petr Paucek[1]; Steven Ojemann[4]; Kevin Lillehei[4]; Xiaoli Yu[1]; Michael W. Graner[5]
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Introduction: Glioblastomas (GBMs) are dreadful brain tumors with abysmal survival outcomes. GBM EVs dramatically affect brain cells constituting the tumor microenvironment (TME), largely astrocytes due to their relatively high brain mass proportion. EVs from different patient-derived GBM spheroid lines and organoids induced differential transcriptomic, metabolomic, secretomic, and proteomic effects on cultured astrocytes and brain tissue slices as GBM EV recipients. The net outcome of brain cell differential changes nonetheless converges on increased tumorigenicity.
Methods: GBM spheroids, organoids, and brain slices were derived from neurosurgical patients, all following written informed consent (COMIRB #13–3007). Astrocytes were purchased. EVs were isolated from conditioned serum-free culture media by ultrafiltration, differential (ultra)centrifugation, and SEC. EVs were characterized by NTA, TEM, ExoView, biochemical markers, and proteomics. Astrocytes/brain tissues were treated with GBM EVs for 24hrs before downstream analyses.
Results: EVs from different GBMs induced brain cells to alter secretomes with pro-inflammatory or TME-modifying (proteolytic) effects. Astrocyte responses ranged from anti-viral gene/protein expression and cytokine release to altered ERK1/2 signaling pathways. Certain GBM EVs promoted increased metabolism in astrocytes/brain slices. Astrocytes treated with GBM EVs formed colonies in soft agar, and conditioned media from GBM EV-treated cells increased GBM cell growth.
Summary/Conclusion: Astrocytes and brain slices treated with EVs from different GBMs underwent non-identical changes in various 'omics readouts and other assays, indicating "personalized" tumor-specific GBM EV effects on the TME. This raises concern regarding "model" systems. Nonetheless, the net downstream impacts from the differential cellular and TME effects still led to increased tumorigenic capacities for the different GBMs.
Funding: US NIH 4R33MH118174; CU Neurosurgery.
Keywords: glioblastoma, extracellular vesicles, tumor microenvironment, spheroids, organoids

PT17.03 | Extracellular vesicles (EVs) derived from KRAS/LKB1 co-mutated Non Small Cell Lung Cancer sustain an inflammatory-like phenotype in fibroblasts

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Introduction: Non-Small Cell Lung Cancers (NSCLCs) with LKB1/KRAS co-mutation (KL) show a very aggressive clinical behavior. Since Extracellular vesicles (EVs) are key mediators of intercellular communication, we hypothesize that EVs derived from KL NSCLCs (KL-EVs) are particularly proficient in the modulation of the tumor microenvironment (TME) toward a phenotype supportive to lung cancer progression.
Methods: We isolated by ultracentrifuge EVs from 48h-serum free conditioned medium of H1299 NSCLC cell line (WT-EVs) and the KRAS G12C/LKB1 DEL isogenic clone (KL-EVs). EVs were characterized by NTA (Nanosight NS300) and EVs-associated markers (CD9, CD81, CD63) by Flow Cytometry and Western Blot. In vitro EVs-functional studies were performed on CCD-19Lu and CAF154hTERT normal and immortalized lung fibroblast cell lines. Impact on fibroblast phenotype was assessed by gene expression analysis (RT-qPCR) and ELISA. CBioportal dataset was used for in silico analyses. In vivo experiments were carried out by subcutaneous injection of NSCLC cells in SCID mice.
Results: KRAS/LKB1 co-mutation induced a significant increase in EVs released by H1299 (1.70 fold increase). However, KL-EVs had lower protein content than WT-EVs (2.1 fold-decrease). EVs size was not affected by the mutational status. In vitro data of the effects of EVs on TME demonstrated that KL-EVs induced a significant increase in the expression and release of the pro-inflammatory cytokine IL-6 in normal and in immortalized cancer associated fibroblast, suggesting a modulation toward an inflammatory-like phenotype. Of note, α-SMA, marker of myofibroblast phenotype, was not affected by the exposure to EVs. An increase of IL-6 expression level in LKB1 MUT compared to WT tumor samples was confirmed in Patient Derived Xenografts (PDXs) in vivo and in silico analyses (cBioportal, CPTAC series). Moreover, the peri-tumoral injection of KL-EVs in WT-H1299 xenografts induced the establishment of a TME closely resembling that of KL-H1299 xenograft.
Summary/Conclusion: KRAS/LKB1 co-mutation impacts EVs release and phenotype. Our results support the hypothesis of an active role of EVs in the TME remodeling, in particular fibroblasts, in KL NSCLC.
Funding: Italian Association for Cancer Research (AIRC).
Keywords: lung cancer, tumor microenvironment, IL-6

PT17.04 | Investigating pancreatic cancer-derived extracellular vesicle signaling in the tumor microenvironment

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Introduction: In many solid tumors, including pancreatic ductal adenocarcinoma (PDAC), the secretion of extracellular vesicles (EVs) has been shown to support tumor progression, chemotherapeutic resistance, and metastasis. Importantly, PDAC tumors are composed of up to 80% non-tumor cells and an extracellular matrix, yet no studies have been performed to determine whether PDAC EVs are imported preferentially by specific non-tumor cell types.
Methods: We performed in vitro live imaging studies with PKH67 labeled PDAC EVs isolated via size exclusion chromatography and validated utilizing three independent techniques (electron microscopy, immunoblotting for classical EV markers, and nanoparticle tracking analysis). All imaging experiments were performed with dye only negative controls as well as samples kept at 4C during the duration of imaging. Further, we have generated human PDAC and mouse KrasG12D Trp53R172H driven (KPC) PDAC cell lines to express an extracellular particle reporter labeling the inner leaflet of cell membranes with GFP-nLuc, known as PalmGRET. We developed flow cytometry and immunofluorescence panels to assess PDAC EV import by CAFs, endothelial cells, macrophages, B cells, and T cells in developed tumors. Utilizing immunofluorescence staining for a panel of phenotypic markers, we will assess the functional importance of EV internalization.
Results: From in vitro studies, we found that cancer-associated fibroblasts (CAFs) import more PDAC EVs than normal pancreas epithelial, endothelial, or PDAC cells. We are currently using the PalmGRET KPC cells to characterize recipient cells of the PDAC EVs in an immunocompetent orthotopic PDAC mouse model.
Summary/Conclusion: Based on in vitro studies, PDAC EV trafficking may be preferential to CAFs. To improve our understanding of this signaling, we aim to define the recipient cells of PDAC EVs and evaluate the functional importance of this

internalization. Future studies will begin to determine how EV mRNA cargoes are important for recipient cell phenotypic and functional changes. This study is the first to ascertain the fate of PDAC EVs within the tumor microenvironment in a physiologically relevant model.

Keywords: Pancreatic cancer, extracellular vesicles, tumor microenvironment

PT17.06 | Pancreatic cancer cell derived extracellular vesicles educate macrophages towards an immunosuppressive phenotype

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive cancers with a majority of patients presenting with metastatic disease at diagnosis. Extracellular vesicles (EVs) released by cancer cells have emerged as key mediators of intracellular signaling, communication, and immune modulation within the tumor microenvironment (TME). Tumor-associated macrophages (TAMs) are crucial components of TME that influence tumor growth and metastasis. The present study is focused on determining the role of PDAC-derived EVs in altering macrophage phenotype, function and metabolism.
Methods: EVs were isolated from the conditioned medium that was used to culture one established pancreatic cancer cell (PANC-1), one PDX cell line (PPCL68) and a normal (non-tumorigenic) immortalized pancreatic epithelial cell line (hTERT-HPNE), using size exclusion chromatography. The EVs were extensively characterized using nanoparticle tracking (NTA), cryo-electron microscopy, and immunoblot analysis as per ISEV guidelines. Macrophages were generated from the bone marrow cells of C57BL6 mice by cultured in vitro in macrophage colony stimulating factor (M-CSF) containing medium. Macrophages were co-cultured with isolated PDAC or normal cell derived EVs. Flow cytometry and metabolomic analyses were performed to determine the effect of EVs on macrophage phenotype, function(in vitro and in vivo) and metabolism.
Results: Our results show that PDAC cell derived EVs were taken up by the macrophage in a time-dependent manner that resulted in an immunosuppressive phenotype characterized by higher expression of CD206 and PD-L1 and higher secretion of immunosuppressive cytokines including TGF-beta, IL-10 and GM-CSF. Functionally, PDAC-EV treated macrophages were able to suppress proliferation of CD8 T cells under in-vitro and in-vivo conditions. Metabolically, cancer EV-treated macrophages showed accumulation of immunosuppressive metabolites (5-methylthioadenosine, lactic acid, and kynurenate).
Summary/Conclusion: We present novel findings suggesting that PDAC cell derived EVs are immunomodulatory as these influence macrophage function that might abet tumor progression and metastasis. Estimation of precise mechanisms of EV mediated immune modulation of macrophages is ongoing.

PT17.09 | The impact of extracellular vesicles derived from lung adenocarcinoma cells on CAF activation

Jessica Angelina Trejo Vazquez; Cathie Garnis
BC Cancer Research Centre, Vancouver, Canada

Introduction: Lung cancer is the leading cause of cancer deaths worldwide, largely due to metastasis. Its main subtype is lung adenocarcinoma (LAC). Communication between cancer cells and cells within the tumor microenvironment, such as fibroblasts, can promote metastasis. This interaction can be mediated by extracellular vesicles (EVs) transferring bioactive cargo. Thus, we hypothesize that fibroblasts can be activated into cancer associated fibroblasts (CAFs) by LAC-derived EVs through specific pathways.
Methods: For EV isolation, H2073 and H2228 LAC cells lines were cultured with EV-depleted FBS. The conditioned media was centrifuged at three different slow spins to remove non-EV debris. Two cycles were performed in the ultracentrifuge to wash and pellet the EVs. Finally, Nanoparticle Tracking Analysis was used for quantification and size characterization and EV associated markers were assessed by western blot (WB). For lung fibroblast activation, WI-38 cells were treated with 1xPBS, TGFB (10 ng/ul) or EVs (5.6×1013) every 24 hours. Protein (for WB) and RNA (for gene expression profiling) were harvested after 96 hours.
Results: Most vesicles produced by both cell lines were between 50 and 250 nm in diameter. Fibroblasts treated with TGFB or EVs had an increase in CAF markers (PDGFRβ and α-SMA), especially in the EV group compared to the PBS treatment group. The activated fibroblasts shared many differentially expressed genes in the TGFB and EV treatments; however, there were specific genes only deregulated in the EV-treated group, indicating a unique pathway to CAF activation.
Summary/Conclusion: LAC EVs have a role in differentiating fibroblasts to CAFs and may do so using an unconventional pathway. Further understanding of the genes distinct to the EV-treated fibroblasts could lead to the identification of novel targets

involved in CAF activation, and thus aid in the development of novel therapeutics that could improve patient outcomes by reducing their metastatic contribution.

PT17.10 | The influence of extracellular vesicles (EVs) on immune cells in pancreatic ductal adenocarcinoma (PDAC)

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Introduction: Pancreatic Ductal Adenocarcinoma (PDAC) is a devastating disease characterized by a dense stroma and an immunosuppressive tumor microenvironment. Our lab has shown that PDAC-derived extracellular vesicles (EVs) play a significant role in tumor growth and metastasis. Furthermore, MUC1 seems to be an important marker for these EVs, associated with their activity and influence on tumor growth. Additionally, EVs are involved in processes of immune evasion. Determining which immune cell populations are most impacted is essential to improving our understanding of the role of EVs in PDAC. We hypothesize that EVs lead to phenotypic, transcriptional, and functional changes in immune cells resulting in a decreased anti-tumor immune response.
Methods: Methods: EVs were isolated from cell culture supernatant according to widely accepted ultracentrifugation protocols. Briefly, cells were cultured in EV-free media for 46 h, followed by EV-isolation (1. 2,000 g / 20 min / 4°C – 2. 15,000 g / 45 min / 4°C – 3. Two rounds: 110,000 g / 2 h / 4°C (SW28 Rotor; Beckman Coulter)).
Our in vivo study to investigate EV-effects on different immune cell populations has two distinct phases. A 3-week "Pre-Education Phase", with injection of either MUC1+ EVs or MUC1KO EVs is followed by orthotopic implantation of tumor cells in the pancreas. Blood and spleen were harvested for immune cell profiling with Flow Cytometry, staining for a wide range of immune cells.
Results: Most immune cells preferably took up MUC1+ EVs; however, macrophages and dendritic cells took up greater proportions of EVs. EV education led to differential gene expression in the liver. Treatment with MUC1+ EVs downregulated B cell-related processes; differentiation, proliferation, signaling activity and antibody-production were decreased. Immune cell profiling showed that MUC1+ EVs reduced percentages and numbers of NK cells and macrophages in the blood. Additionally, CD8+ T cell frequencies were increased in the spleen of MUC1+ EV treated mice compared to MUC1KO EVs after three weeks.
Summary/Conclusion: We have shown that EVs induce phenotypic and transcriptional changes in immune cell populations, and alter immune cell frequencies in vivo. Ongoing research in our lab involves proteomic analysis of patient-derived EVs as well as the analysis of functional differences in EV-treated immune cells.

PT17.11 | The role of Nidogen 1 in small extracellular vesicle-mediated immune tolerance in hepatocellular carcinoma and its therapeutic potential

Kyu won Lee[1]; Zhijie Xiao[1]; Zhixian Chen[2]; Enyu Huang[1]; Liwei Lu[1]; Judy WP YAM[3]; Xiaowen Mao[4]
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Introduction: Nidogen 1 (NID1), a glycoprotein in extracellular matrix, forms an extensive network in tumor microenvironment (TME). Our previous study reported the role of NID1 in small extracellular vesicles (sEVs)-induced hepatocellular carcinoma (HCC) metastasis. However, if it is involved in the immune modulation of tumor is not well studied, and its role in onco-immunology of HCC remains unknown.
Methods: Tumor Immune Estimation Resource 2.0 (TIMER2.0) database was used to analyze the correlation of NID1 expression and the immune cell infiltration in HCC. NID1 was knocked down in murine HCC cell line 1753p53-/-cMyc and sEVs derived from non-target control (shCtl) and NID1 knockdown (shNID1) cells were collected by ultracentrifugation and subjected to in vitro and in vivo models. Multiplex immunohistochemistry was performed to demonstrate the correlation of NID1 and TME factors in patient tissues. GEPIA survival analysis was performed to study the correlation of NID1 expression and patient prognosis.
Results: Conventional dendritic cells (cDCs), which is mostly correlated with NID1 in TIMER2.0 was selected to study the impact of NID1 in the modulation of immune-tolerant TME. More CD11b+ DCs were infiltrated in xenograft derived from 1753p53-/-cMyc shCtl cells compared to those formed by shNID1 cells. In addition, sEVs derived from 1753p53-/-cMyc shCtl cells inhibited the maturation of bone marrow-derived dendritic cells (BMDCs) while sEVs derived from shNID1 cells lost this function. Education with 1753p53-/-cMyc shCtl sEVs significantly increased the percentage of CD11b+ DC population in the liver of mice, but such phenomenon was not seen in shNID1 sEV educated mice. HCC tumor tissue with high expression of

NID1 is correlated with higher infiltration of cDCs and lower infiltration of CD8+ T cells. Co-consideration of NID1 expression and cDC infiltration but not evaluated by the individual factor can predict the overall survival of HCC patients.
Summary/Conclusion: The result of the present study revealed a novel mechanism of sEV-NID1 in modulation of the liver local dendritic cells to provide a favoring microenvironment for tumor cells to survive. NID1 could be a promising target against immune-suppressing TME and prognostic indicator in HCC.
Funding: This project was supported by the Young Researcher Support Scheme of State Key Laboratory of Liver Research (The University of Hong Kong) [Project no. SKLLR/YRSS/2022-01].
Keywords: hepatocellular carcinoma, nidogen 1, small extracellular vesicles, conventional dendritic cell, prognostic marker

Friday Poster Presentations

PF01: Model Systems, Production and Characterization

Location: Hall 4A

16:00 - 18:00

PF01.01 | Highly sensitive innovative ELISA technique for profiling of histological markers on EVs

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Introduction: A liquid biopsy is a promising tool for non-invasive cancer diagnosis, tumor profiling, evaluation of therapy response and post-treatment follow-up. Extracellular vesicles are promising for liquid biopsy tests since they are enriched with biomarkers reflecting the composition of the cell of origin.
Majority of EV based liquid biopsy tests are based on analysis of RNA and DNA markers, however the gold standard of tumor profiling is analysis of protein markers on tumor cells. Detection of commonly used histological markers on EVs could provide more complete information about tumor phenotype and find the shorter root to the clinics. However, common ELISA which allows high throughput screening of protein markers is not enough sensitive to detect pathological markers on EVs isolated from clinical samples. We have developed innovative highly sensitive 3D ELISA techniques allowing profiling of low represented protein markers on EVs. The technique was validated by analysis of putative cancer stem cell markers CD166, CD117 and CD44 and EpCAM and onco-marker CA125 on EVs isolated from cell cultures and clinical samples.
Methods: EVs were isolated from HEK293t and SK-OV-3 cell culture supernatants and urine of ovarian cancer patients and cancer-free individuals by differential centrifugation (500 g for 10 min, 3500 g for 25 min in an A-4-81 rotor at 20°C, EV size cutoff is 620 nm) followed by 100 kDa ultrafiltration. EV concentration was measured with NTA.
Results: The sensitivity of the 3D ELISA technique was estimated for the mesenchymal markers CD9, CD81, CD166, CD44, CD117 and EpCam and the onco-marker CA125. The detection limit (LOD) for CD9+/CD81+ EVs is 2 orders of magnitude higher than LOD of commercially available EV ELISA kits. The high sensitivity of the 3D ELISA technique allowed the detection of tumor markers on EVs isolated from the urine of patients with ovarian cancer.
Summary/Conclusion: The highly sensitive 3D ELISA platform allows the profiling of histological and onco-markers on EVs isolated from cell cultures and body fluids.
Keywords: histological markers, highly sensitive protein detection

PF01.02 | A CD73 enzymatic assay as a quality control for manufacturing and storage of EV therapeutics from diverse sources

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Introduction: Extracellular vesicles (EVs) show high potential as novel therapeutics. Human stem cell derived EVs are employed as standalone therapies, whereas EVs derived from milk represent promising drug delivery vehicles. A clinical translation demands for robust methods for EV quality control to verify integrity and functionality after manufacturing and storage and to adjust dosages. CD73 is a membrane-anchored 5´-Ectonucleotidase present on human stem cells, and we were able to demonstrate its presence in bovine milk EVs. Thus, we implemented a rapid, easy to use assay verifying integrity of CD73+ EVs based on CD73 enzymatic activity.
Methods: CD73 enzyme activity of EVs was assessed based on AMP consumption using a commercial luminescence based system. The assay was optimized for EV buffer compatibility, EV concentration and miniaturized to 384 well format. Engineered EVs were prepared either lacking or displaying transgenic CD73 on the surface for assay validation. Recombinant CD73 and pharmacological CD73 inhibitors were used as additional controls. The optimized assay was then used to quantify CD73 activity for EV samples from different manufacturing processes and storage conditions.
Results: EVs were tested for their presence (Mesenchymal stem cell EVs, bovine milk EVs) or absence (HEK293T cell EVs) of CD73 by proteomics and AMP consumption was confirmed to correlate with their CD73 status. Using this assay revealed that storing EVs from scale manufacturing in different industrial dry forms did not substantially change their CD73 activity, whereas pre-treatment for deliberate protein denaturation resulted in a loss of activity.
Summary/Conclusion: Using the developed CD73 activity assay we were able to quantitatively assess the suitability of different EV manufacturing steps and storage in industrial dry forms. Thereby this assay provides a simple and robust QC for pharmaceutical EV manufacturing from both, human primary cells as well as industrial milk.
Funding: This work was supported by the following grants: EVTT (European Union, EFRE/IWB 20102-F1900731-KZP), EV-TT BPro (County of Salzburg, WISS2025, P1812596), and CONSONANT (County of Salzburg, WISS2025, F2200397-KZP).

PF01.03 | Optimisation of 3D models of ovarian cancer for extracellular vesicles studies

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Introduction: Ovarian cancer (OC) is the most common gynaecological malignancy and globally it is the eighth commonly diagnosed cancer in females. Studies have implicated liquid biopsies like Extracellular Vesicles (EVs) could be used as a potential biomarker for multiple diseases including OC. Studies indicate culturing cells in 3D system can better mimic the cellular microenvironment than the 2D monolayers. Therefore, this study aimed to optimise and establish a standard technique for developing 3D models, as well as to isolate and characterise the EVs and cells from these models.
Methods: OC cell lines used in this study - SKOV-3 and OVCAR-3 were cultured as a 2D monolayers and were also encapsulated using Gelatin methacryloyl hydrogels at the seeding density of 50,000 cells per 20ul gel. Cultured for nine days, multiple assays were performed on 3D cells to observe the spheroid formation inside the hydrogel, which were later characterised histologically. The EVs were isolated from the cell-conditioned media using differential centrifugation and characterised using Nanoparticle Tracking Analysis, Transmission Electron Microscopy, and ExoView®. qPCR was performed to analyse the expression of the different miRNA associated with chemoresistance in ovarian cancer isolated from EVs and spheroids.
Results: Based on the initial experiments performed in this project, on cell-laden hydrogels, we observed the ability of OC cells to grow, proliferate, and aggregate inside the hydrogel, thus characterising this as an ideal 3D model. The structures of these spheroids were further validated histologically. Furthermore, we were able to successfully isolate and characterise EVs from

these 3D models based on the size, morphology, and surface markers. Lastly using qPCR, we were able to observe miRNAs differentially expressed within spheroids and EVs.

Summary/Conclusion: Results indicated that the encapsulated cells were able to grow and proliferate into spheroids and were also used for harvesting the different populations of EVs. Therefore, characterising this as an optimised 3D model that can be used for extracellular vesicles studies. Furthermore, this in vitro model could be used for understanding and developing therapeutics for ovarian cancer.

PF01.04 | Design, synthesis and evaluation of novel exosome inhibitors

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Introduction: Exosomes play important roles in the tumor process. The tumor-derived or tumor macrophage-derived exosomes could promote tumor growth, immunosuppression and cancer cell dissemination. Therefore, inhibiting exosome release has therapeutic potential in cancer. However, most existing exosome inhibitors could either interrupt exosome biogenesis or block exosome release with high cytotoxicity, which may lead to undesired adverse effects on normal cells and thus limit their translation to anti-cancer therapy. In this study, we redesigned a cytotoxic RAB27A inhibitor through chemical modification for developing novel exosome inhibitors with low cytotoxicity.

Methods: Candidate compounds were synthesized by aldol reaction. The cytotoxicity of these compounds was evaluated by MTT assay on RAW264.7 cells. The exosome inhibitory potency of these compounds was evaluated on RAW264.7 cells, BMM cells, MDA-MB-231 cells and 4T1 cells through exosome isolation (ExoQuick-TC, SBI) and exosome quantitation (FluoroCet Exosome Quantitation Kit, SBI).

Results: The nitro group of the RAB27A inhibitor was replaced by other functional groups to reduce cytotoxicity and their chemical structures were confirmed by LC-MS and NMR. The results showed that six candidates exhibit low cytotoxicity, among which LJ271, LJ272 and LJ245 showed strong exosome inhibitory potency on RAW264.7 cells, BMM cells, MDA-MB-231 cells and 4T1 cells.

Summary/Conclusion: The results indicated that LJ271, LJ272 and LJ245 exhibited low cytotoxicity and exosome inhibitory potency on macrophage and breast cancer cells. Based on this, the drug candidates will be studied whether they could inhibit the macrophage-derived or tumor-derived exosome-mediated modulation of the tumor microenvironment.

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Keywords: exosome inhibitors, cancers, drug development

PF01.05 | High-throughput EV purification from biological fluids for diagnostic application

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Introduction: Extracellular vesicles (EV) enriched from liquid biopsies such as blood have great potential as a source for biomarkers. Lipid encapsulation protects the cargo from degradation and allows the EV to be transported throughout the body and across physiological barriers. High-throughput EV purification combined with high-performance omics technologies are crucial for predictive and prognostic applications in non-invasive manner. Here we focused on implementation of high-performance proteomic analysis from low amount of plasma and serum purified by size exclusion chromatography (SEC) approach compatible with high throughput.
Methods: SEC and differential ultracentrifugation (UC) were applied as EV enrichment methods from plasma and serum followed by quality control (QC) assessment with NanoFCM, Western blotting, Transmission electron microscopy (TEM) and high-performance mass spectrometry-based proteomics analysis
Results: SEC first elution fraction (fraction 1) and EV purified by UC were showing highest enrichment in EV and EV markers compared to other elution fractions or direct measurement of undepleted and depleted serum samples. Principal component analysis shows separation of samples enriched by UC and SEC fraction 1 from other SEC fractions, plasma, and serum, as well as highest EV markers enrichment.
Summary/Conclusion: Early implementation of high-performance mass spectrometry based proteomic analysis as a part of EV QC influenced the selection of SEC as method of choice for EV purification from low volume serum and plasma samples. We envision that optimized combination of efficient EV enrichment from limited sample volume. followed by high sensitivity proteomic analysis will be useful for the variety of applications in diagnostic field.
Keywords: high-performance mass spectrometry-based proteomic analysis, high-throughput EV purification

PF01.06 | Characterizing the effects of ganglioside GM1 on extracellular vesicle biogenesis and phenotype

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Introduction: Gangliosides are glycosphingolipids most abundantly found in the central nervous system and at the plasma membrane. Our previous work has demonstrated that ganglioside GM1 increases the secretion of extracellular vesicles (EVs) in mouse neuronal cells. Here we seek to investigate the mechanism of ganglioside-driven EV biogenesis and to characterize GM1 effects on EV phenotype.
Methods: Cells stained with a fluorescent lipophilic dye that labels cell membranes were incubated with GM1 for 22 hours. EVs secreted in the medium were analyzed by imaging flow cytometry or fluorometry directly from cleared cell culture supernatants or after EV isolation via size exclusion chromatography. Characterization of EVs size and protein markers was obtained using nanoparticle tracking analysis, single-particle interferometric reflectance imaging sensing (SP-IRIS) and immunoblotting. siRNA silencing, specific pharmacological inhibitors and immunoblotting were used to determine the involvement of specific EV biogenesis pathways in GM1-mediated increase of EV secretion.
Results: Incubation with GM1 increased EV secretion in various cell types including neural (iPSC-derived neurons, N2a), epithelial (HeLa) and mesenchymal (patient-derived fibroblasts) cells. Strikingly, GM1 does not significantly alter the size of EVs but modulates their membrane markers, distinctly altering the tetraspanin profile of EVs from immortalized cell lines and to a lesser degree EVs from primary cells. Surprisingly, GM1 administration does not alter the abundance of EV biogenesis proteins such as HRS, TSG 101 or Alix nor is GM1-driven EV biogenesis abrogated by siRNA-induced depletion of HRS or inhibition of neutral sphingomyelinase or ROCK.
Summary/Conclusion: We have discovered a novel role for ganglioside GM1 in EV secretion that might be independent from canonical EV biogenesis pathways and affects the secretion of specific EV subpopulations.
Funding: Funded by the Canadian Institute of Health Research and GlycoNet.
Keywords: gangliosides, EV biogenesis, GM1, EV subtypes

PF01.07 | Recombinant human peptide growth factors increase the secretion of sEVs in mesenchymal stem cell

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Introduction: Regenerative Medicine aims to achieve treatments that accelerate the different stages of the healing process in both humans and animals, and the manipulation of the growth factors (GF) composition can improve or modify the repair and remodelling process of injured tissues, as well as providing more aesthetic and functional scars. The present study aimed to evaluate the effect of recombinant human platelet-derived growth factor (PDGF-BB) and/or combined with endothelial vascular growth factor (VEGF165) on adipose canine mesenchymal/stromal stem cells (AD-MSC) and their secretome.
Methods: AD-MSCs were isolated and characterized, cultured and the conditioned media were collected under conditions without SFB for isolation of exosomes by ultrafiltration and ultracentrifugation. Samples were quantified resulting in higher yield in exosomes of cells treated for 24 hours using 10ng/mL of each GF or their combination. GF were biologically tested in Rowett nude rats, where 3μg/mL of GF were applied alone or combined in wounds on the animals' backs, and after 7 days the healing evolution was evaluated macroscopically and histopathologically.
Results: GFs were obtained from purified media by heparin affinity chromatography and characterized by ELISA and Western blot, and their biological activity were tested in vitro and in vivo in a rat model. Mesenchymal stem cells were obtained, characterized, cultured and treated with GF and exosomes were isolated from conditioned media. The present study demonstrated for the first time the potential of rhPDGF-BB and/or rhVEGF165 supplementation in the healing of skin wounds in seven days, stimulating angiogenesis, fibroblast reaction and re-epithelialization, but a long-term evaluation is necessary. The treatment with both factors combined improved the therapeutic profile of adipose mesenchymal stem cells by stimulating cell migration and exosome secretion.
Summary/Conclusion: Together, the data support future investigations into the effect of GF supplementation in vitro, on cellular and molecular content of the mesenchymal stem cell secretome and in vivo, in a safe and reproducible model, opening a new perspective for a cell-free therapeutic approach for wound healing.
Funding: Coordination for Improvement of Higher Education Personnel (CAPES), National Council for Scientific and Technological Development (CNPq), The State of São Paulo Research Foundation (FAPESP No. 2016/05311-2).
Keywords: wound healing, PDGF-BB, VEGF, mesenchymal stem cells, Exosomes

PF01.08 | Modeling cancer cell-derived extracellular vesicles using liposomes: A synthetic model to determine the impact of EV characteristics on cellular uptake

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Introduction: Extracellular vesicles (EVs) play a crucial role in disseminating cancer to distant organs, communicating with the tumor microenvironment (ME) through cellular uptake by recipient cells. EV parameters influencing cellular uptake include surface proteins, lipid profile, and physicochemical properties such as size and zeta potential. However, EVs are heterogeneous, making it difficult to study single EV variables. Moreover, EV isolation is a lengthy and laborious process with an extremely low yield. Liposomes are synthetic vesicles that share properties with EVs, such as a lipid bilayer and the capability to encapsulate biomolecules. Our aim was to develop a synthetic system to mimic EVs by producing "EV-like liposomes".
Methods: Our approach was to "progressively mimic" cancer EVs. To do this, we aimed to: 1) characterize the physicochemical and lipidomic profile of EVs isolated from a panel of cancer cells; 2) produce EV-like liposomes through microfluidics; and 3) assess their uptake by ME cells in vitro and 4) in vivo. 1) EVs were isolated using ultracentrifugation (UC) and characterized using dynamic light scattering, nanoparticle tracking analysis, and transmission electron microscopy (TEM). Lipids were extracted for high-throughput lipidomics by high-resolution 'shotgun' mass spectrometry. 2) EV-like liposome formulations were produced using the periodic disturbance mixer (nanoprecipitation). 3) Cellular uptake of labeled EV-like liposomes was assessed in human hepatocytes and fibroblasts through microscopy and Incucyte. Finally, we assessed the biodistribution of EV-like liposomes in SCID mice after tail vein injection.
Results: Using surface response methodology, we created a model to predict size and zeta potential. We produced liposomes ranging in zeta potential (0-~-30 mV) and size (100~200 nm), mimicking the characterized cancer EVs. TEM demonstrated EV-like morphology. We identified four lipid species that segregated EV subpopulations. Our data confirmed uptake of EV-like liposomes by ME cells within 12 hours. Modulating size, zeta potential and lipid profile had significant impact on cellular internalization. In-vivo imaging indicated high accumulation of EV-like liposomes in the kidney and liver, with no indication of organ damage.
Summary/Conclusion: We describe a novel approach using synthetic biology to produce EV-like liposomes. Our data show that liposomes are a feasible tool to study EV variables individually, thereby addressing the high heterogeneity of biological samples and producing high liposome yield. This model is therefore valuable to accelerate research on the contribution of EVs in disease, such as cancer metastasis.

Funding: New Frontiers in Research Fund Exploration grant (to JVB).
Keywords: EVs, metastasis, liposomes, biomedical engineering, synthetic EVs

PF01.09 | Cell membrane models via supported lipid bilayer formation from extracellular vesicles

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Introduction: Extracellular vesicles (EVs) share the same membrane composition as their parent cells, which not only makes them useful biomarkers in disease detection, but also offers excellent potential for cell membrane models in the form of supported lipid bilayers (SLBs). Such model interfaces can serve in fundamental studies of nanoscale interactions at the cell membrane and also feed into the design of effective biosensors for use in the detection of analytes such as cancerous cells. In this work, the optimal conditions for the formation of extracellular vesicle derived supported lipid bilayers (EVSLBs) are studied, including temperature and ionic strength.
Methods: The EVs were isolated from Human Umbilical Mesenchymal Stem Cell Culture Media (HUMSCCM). The media underwent centrifugal ultrafiltration using a 10kDa NMWCO filter, followed by size exclusion chromatography using a 35 nm pore size column with agarose resin and 500μL sample loading. The resulting supernatant underwent further centrifugal ultrafiltration using a 3kDa NMWCO filter. The EVs were characterised using nanoparticle tracking analysis (NTA) and Western Blot. SLB formation was monitored and characterised via Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D) and liquid atomic force microscopy (AFM). An acoustic based, label free instrument, the QCM-D allows for nanoscale events to be monitored in real time, whilst liquid AFM provides valuable topographic data.
Results: QCM-D data indicates that the presence of divalent cations and higher temperatures lead to higher adsorption of EVs to the sensor surface, and comparably lower material loss in vesicle rupture and rinse stages. When the conditions were repeated in liquid AFM studies, the data shows confirmed QCM-D results, showing improved EVSLB coverage.
Summary/Conclusion: Higher temperatures and divalent cation presence is in important in promoting the critical vesicle concentrations required for EV adsorption, vesicle rupture and thus EVSLB formation.
Keywords: QCM-D, liquid AFM, supported lipid bilayer

PF01.12 | Characterization of extracellular vesicles derived from electrically stimulated human neural organoids

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Introduction: Understanding the reparative potential of extracellular vesicles (EVs) is critical for developing novel EV-based diagnostics and therapeutics for diseases such as multiple sclerosis (MS). Recent evidence suggests that neuronal EVs exit the central nervous system and may modulate distal cells, including lymph nodes and other immune organs. We hypothesized that the cargo and biocorona of neuron-derived EVs have the capacity to exert both neuroprotective and immunomodulatory effects, thus we sought to characterize EVs released from human induced pluripotent stem cell (iPSC)-derived neural organoids in response to electrical field stimulation modeled on known deep brain stimulation paradigms.
Methods: Microglia were differentiated from skin fibroblasts-derived iPSCs using the STEMdiff kits. Neural organoids were obtained by co-culturing matured microglia with human neural aggregates. Organoids were electrically stimulated on the c-PACE EM platform (C-Pace Navigator software) for automated in-well electrode-based electrical field stimulation. We optimized EV extraction methods using ultracentrifugation (UC), tangential flow filtration (TFF), and size exclusion chromatography (SEC), and characterized the number, concentration, and size of EVs by NanoSight. Tetraspanins (CD9, CD63, and CD81) on EV membranes were measured by flow cytometry and ZetaView. miRNA isolated from stimulated EVs was analyzed by miRNAseq and regulatory network analysis was conducted using miRWALK and gene ontology profiling. We also analyzed the protective capability of EVs using a cellular interleukin-1 receptor (IL1R) reporter assay.
Results: EV yield and total miRNA yield from SEC-isolated EVs were ~100 fold higher compared to UC and TFF methods. The number of EVs recovered from stimulated brain organoids was 4–20 fold higher compared to unstimulated cultures. The size of EVs released from the neural organoids increased with the stimulation time. Electrical field stimulation induced changes in expression of 102 EV-associated miRNAs. Regulatory network analysis revealed a highly significant network comprised of 17 miRNAs, which was further associated with regulatory control of 54 target genes involved in inflammation, cell survival, translation, and synaptic development. Preliminary data from the IL1R reporter assay showed suppression of IL1 activity in cells treated with electrical stimulation-derived EVs compared to EVs from unstimulated neural organoids.

Summary/Conclusion: Our data demonstrate a working scheme for isolating electrical stimulation-induced EVs from neural organoids for cargo analysis. Future work will focus on evaluating the neuroprotective and immunomodulatory capacity of these EVs and associated miRNAs.
Funding: Mayo Clinic Center for MS and Autoimmune Neurology and the Sol Goldman Foundation.

PF01.13 | Towards reliable extracellular vesicle reference material

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Introduction: The first annual meeting of the International Society for Extracellular Vesicles (ISEV) identified the need to develop standards for clinical certification and manufacturing of EV-based diagnostic kits and therapies. The National Institute of Standards and Technology (NIST) has been actively developing techniques to measure the integrity and properties of EVs. This presentation describes the work at NIST to characterize candidate EV reference materials produced from cancer cell lines from commercial sources.
Methods: Five candidate EV reference materials were obtained from the ATCC: LnCAP, PC3 prostate cancer cells, HCT colorectal cancer cells, A549 lung cancer, and hTERT immortalized mesenchymal stem cells. EV size distributions were evaluated at NIST via nanoparticle tracking analysis (NTA), asymmetrical flow field-flow fractionation (AF4), and resistive pulse sensing. EV morphology was evaluated using Cryo-EM. EV-relevant proteins were identified using data-independent acquisition mass spectrometry (DIA-MS). EV-specific small RNA (mRNA and microRNA (miRNA)) was identified by small RNA sequencing (RNAseq) platform from two facilities with LnCAP prostate cancer EVs as a candidate. Illumina MiSeq platform was utilized at Facility 1 and Illumina NovaSeq 6000 was utilized at Facility 2.
Results: Different EV characterization methods yielded different EV concentrations and different particle size distributions. AF4 resulted in the highest yield and narrow particle size distribution while NTA yielded EV size range between 50 nm to 200 nm for all samples. DIA-MS data returned approximately 2100, 2160, 600, 1600, and 2700 global proteins/EV samples for A549, HCT, hTERT, LnCAP, and PC3, respectively. RNAseq data revealed that 103 genic mRNAs were identified in at least one sample in Facility 1 whereas 375 genic mRNAs were present in samples from Facility 2 with thirty-three common genic mRNAs from both facilities. 694 miRNAs were expressed in at least 1 sample from Facility 1 whereas 740 miRNAs from Facility 2 and 485 common miRNAs were identified.
Summary/Conclusion: Proteomic profiling provides information on the level of tetraspanin expression and the likely difference in tetraspanin expression between cancer derived EVs and normal EVs. Small RNA sequencing allows for the capture of a complete range of small RNA and miRNA species in the candidate EV sample by read depth. Because the development of reference materials for EVs is challenging, large interlaboratory collaborative studies are essential. The work at NIST in collaboration with other organizations will continue to characterize the concentration, size distribution, protein, and nucleic acid content of the EV preparations for their suitability as fit-for-purpose reference materials to aid quality assurance in clinical applications.

PF02: Metabolism and Metabolic Disease

Location: Hall 4A

16:00 - 18:00

PF02.01 | Metabolites of human sweat evs are associated with blood glucose levels

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Introduction: Human body has its own ways of communication which are still to be fully discovered, and one of those is the sweat. Lately sweat has been a subject of interest as a non-invasive tool to monitor physiological changes in healthy and disease conditions, because of its components i.e., analytes, ions, and extracellular vesicles (EVs), and easy accessibility.
Methods: A pilot study* with 11 healthy individuals was conducted, where sweat was induced, at rest, by heat exposure and collected using clinical grade absorbent patches along with monitoring of blood glucose levels. By our novel method, sweat EVs were isolated from patches and enriched by ultracentrifugation and analyzed by LC-MS.
*Ethical License EETMK:199/2016
*Clinical trials Registration: NCT02855905
Results: A targeted metabolomics analysis was performed using the isolated sweat EVs, in which 41 metabolites were examined. Based on samples quality control (QC) and Blank, 24 metabolites were produced at an acceptable range for detection by LC-MS. Upon testing the association between the metabolite concentration and blood glucose by applying Spearman's rank association, a significance was revealed.
Summary/Conclusion: Our study provides first evidence of the metabolic composition of sweat EVs and proposes the use of metabolites as a novel approach to study the physiological responses of the human body to the health-related problems, hence offering a novel strategy for monitoring health and well-being.
Keywords: diabetes, diseases, biomarker

PF02.02 | Application of liver-specific extracellular vesicles to track changes in drug clearance in patients with metabolic associated fatty liver disease

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Introduction: Hepatic metabolism by cytochrome P450 (CYP) 3A4 is a primary determinant of exposure for >50% of drugs. Drug metabolising enzymes, including CYP3A4, have been detected in extracellular vesicles (EVs) isolated from blood. Metabolic associated fatty liver disease (MAFLD) causes steatosis (SS) and inflammatory injury (NASH), and alters CYP expression. Given the major role of CYP3A4 in drug clearance, reduced expression places MAFLD patients at higher risk of toxicity. We aimed to characterise CYP3A4 expression in circulating liver derived EVs to track changes in drug clearance in MAFLD patients.
Methods: EVs were isolated from plasma of subjects with SS (n = 10) or NASH (n = 5) and from healthy controls (n = 13) by a two-step protocol using size exclusion chromatography followed by anti-ASGR1 immunoprecipitation (IP). EVs morphology and abundance was characterised by TEM and NTA. The abundance of global EV, liver specific EV and co-isolation markers was quantified using targeted LCMS proteomics. Differences in CYP3A4 expression in ASGR1+ EVs isolated from control, SS and NASH subjects was used to predict changes in drug exposure in patients with MAFLD.
Results: Anti-ASGR1 IP successfully captured plasma EVs of hepatic origin. A significant decreasing trend in CYP3A4 expression was observed in ASGR1+ EVs with increasing MAFLD severity (p = 0.049) and predicted changes in exposure to clinically relevant drugs.
Summary/Conclusion: Liver derived EVs accurately reflect the impact of MAFLD on hepatic CYP3A4 expression and represent a valuable liquid biopsy platform to understand clinically relevant changes in drug exposure.
Keywords: drug exposure, tissue-specific isolation

PF02.03 | Integrated genomics and proteomics analysis uncovers the regulatory role of miR-10b targets in normal and diabetic human limbal epithelial cells

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Introduction: MicroRNA-10b (miR-10b) is up-regulated in the limbus vs. central human cornea and also in diabetic (DM) vs. normal (N) limbus, which may contribute to limbal cell function and diabetic corneal disease, respectively. Our purpose was to identify miR-10b target genes/proteins using integrated genomic and proteomic analyses to elucidate its role in the normal and diabetic limbus.
Methods: Human autopsy N and DM corneas were procured by the National Disease Research Interchange (NDRI) in Optisol medium. In vitro experiments were performed with stem cell-enriched human N and DM primary limbal epithelial cells (LECs). MiRNA transfection was performed using Lipofectamine RNAiMAX. Normal LECs were transfected with miR-10b mimic (M), or its mimic control (MC). At day 3 post-transfection, cells were harvested for total RNA isolation using Trizol for next-generation RNA sequencing (RNA-seq). A matching set of transfected LECs was collected and cell lysates were prepared for proteomics analysis
Results: Sets of 661 mRNAs (FC of ± 2, 305 up- and 356 down-regulated) and 144 proteins (FC of ± 1.5, 72 up- and 72 down-regulated) were identified as differentially expressed in miR-10bM vs. MC with adjusted $p < 0.05$ using RNA-seq and proteomics analyses, respectively. Combined proteomics and genomics analysis revealed a total of 17 overlapping genes as potential targets of miR-10b, all considered putative or validated targets in the literature. Additionally, pathway analysis of proteomics and transcriptomics data revealed significant changes in multiple signaling pathways such as cell cycle, DNA repair and replication, Wnt, and TP53 signaling. Overexpression of miR-10b in primary N and DM LECs significantly decreased expression levels of target proteins GCLM, CMPK1, Lig1, and TPM4 by western blot and immunostaining. In addition, down-regulation of miR-10b in DM organ-cultured corneas using miR-10b inhibitor increased expression levels of TPM4 and Lig1 involved in DNA repair and replication by immunostaining
Summary/Conclusion: In conclusion, by using integrated genomic and proteomic analyses of cultured N and DM LECs, potential key target genes of miR-10b were revealed as well as the underlying pathways affecting human LEC function. Further analysis of promising targets may improve our understanding of the molecular mechanisms underlying diabetic corneal alterations and wound healing
Funding: NIH grants R01 EY025377 and EY029829 (Saghizadeh), Cedars-Sinai Board of Governors Regenerative Medicine Institute, and Department of Biomedical Sciences.
Keywords: exosome, cornea, diabetes, wound healing

PF02.05 | Islet β-cells shed DOC2B-laden EVs via sorting signals present in DOC2B protein

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Introduction: Loss of β-cell function occurs early in type 1 diabetes (T1D). C-peptide, the clinically standard biomarker, is insufficient to detect early pre-type 1 diabetes-related β-cell dysfunction. Our recent publication demonstrates that circulating levels of exocytosis regulatory protein double C2-containing protein β (DOC2B) abundance is closely related to β-cell health and function. However, the mechanisms by which β-cells export DOC2B into the circulation remain unknown. We hypothesize that DOC2B is uniquely sorted into β-cell extracellular vesicles (EVs). Indeed, preliminary data show DOC2B is detected in human plasma EVs, and that DOC2B-laden EVs can stem from β-cells. Because DOC2B is a broadly expressed protein, we questioned whether DOC2B-laden EVs are predominantly released by β-cells, relative to that of other cell types known to express DOC2B protein.
Methods: Cell/debris-free conditioned media from DOC2B biosensor treated or untreated INS-1 832/13 β-cells, L6 myotubes, HEPG2 cells, and SH-SY-5Y cells were used to isolate EVs via size exclusion chromatography. EVs were characterized using transmission electron microscopy, nanoparticle tracking analysis, and immunoblots for DOC2B and EV marker proteins.
Results: Biochemical analyses reveal that DOC2B largely exists as luminal cargo in β-cell-derived EVs and is largely absent from EVs released by liver, brain, and skeletal muscle cells. Mechanistically, the research has delineated that the structural domain enabling DOC2B protein sorting into β-cell-derived EVs is that which encompasses the tandem C2 domains at the C-terminus of DOC2B.
Summary/Conclusion: Taken together, these data indicate that DOC2B in circulation could originate from DOC2B-laden EVs released from β-cells, and that the mechanism by which DOC2B is sorted into those EVs involves one or both C2 domains of the DOC2B protein.
Funding: National Institute of Diabetes and Digestive and Kidney Diseases (DK067912, DK112917, and DK102233), the Wanek Family Project to Cure Type 1 Diabetes at the City of Hope (D.C.T.), and Ford Foundation Pre-doctoral Fellowship (D.E.).
Keywords: Islet β-cells, DOC2B, diabetes

PF02.07 | Study of the biodistribution in vivo of hepatocytes-released extracellular vesicles in mouse model for metabolic syndrome progression

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Introduction: Metabolic syndrome (MetS) groups different diseases such as obesity, hypertension, insulin resistance and dyslipidemia. A combination of these diseases predisposes to more severe complications in the metabolism and to cardiovascular diseases. It is known that the liver has an important role in the establishment and progression of obesity and MetS. However, the mechanisms that disseminate the disease throughout the body are not completely understood. Here is where extracellular vesicles (EVs) could play an important role. Hepatic EVs (HepEVs) are active metabolic vesicles that can extend the hepatocyte activities outside the liver to other target organs as a response to different stimuli. In this sense, in MetS they could be part of the vectors that propagate the disease. We hypothesize that, as in other diseases, in MetS EVs can have different biodistribution, pattern of secretion and cargo.
Methods: To follow and study HepEVs in MetS, we have generated in house by crossing the genetically modified mice that express a membrane-bound Green Fluorescent Protein (mGFP) only in the hepatocytes due to liver specific-driven recombination. We induce obesity and liver damage by feeding mice with high fat diet (40 kcal% fat or 60 kcal% fat). Liver damage is controlled by following the levels of transaminases in serum over time. We use CytoFLEX technology to measure the levels of HepEVs in serum at different time points. Biodistribution and cargo of EVs is studied through histology and immunoblotting.
Results: We were able to induce obesity to mice by feeding them with high fat diet. In serum samples from mGFP expressing mice, we are able to detect an average of 2.3E6 green particles/mL from a total of 2.5E9 particles/mL.
Summary/Conclusion: Our in vivo model is a proper system to study the secretion of HepEVs in the progression of metabolic syndrome.
Funding: Science and Innovation Spanish Ministry Reference (PID2021-125104OB-I00).
Keywords: metabolic syndrome, extracellular vesicles, mouse model, cytometry

PF02.08 | The morphology, compositions and therapeutic differences between white, brown and beige adipose tissue-derived extracellular vesicles: a comparative study in treating multiple diseases in mice

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Introduction: Adipose tissue-derived extracellular vesicles (ATEVs) have been shown to play an important role in various physiological and pathological processes. While knowledge on EVs from the white adipose tissue (WEVs) and the brown adipose tissue (BEVs) is available, EVs from the beige adipose tissue (BeEVs), browning from the white adipose tissue, is restricted from understanding. Here, we present a comparative study to investigate BeEVs with WEVs and BEVs in morphology, compositions, and therapeutic efficiency in multiple diseases.
Methods: Three methods of inducing browning, including time-restricted fasting, exercise, and cold exposure, were performed in mice. Liberase digestion followed by differential centrifugation was used to extract ATEVs. Western blot, nanoparticle tracking analysis, and transmission electron microscope were applied for characterization of ATEVs. Proteomic analysis and cryo-electron microscopy were further performed. Therapeutic application of ATEVs in type 2 diabetes, skin wound healing, hair regeneration, and glioma models was conducted in mice.
Results: The beige adipose tissue was obtained effectively by cold exposure and ATEVs were successfully extracted. All ATEVs were positive for CD9, CD63 and CD81 with particle size ranging from 100 to 250 nm and showed typical shape of EVs with no significant difference among groups. The unique proteome of BeEVs was identified and the levels of mitochondria-related proteins were the main feature distinguished from WEVs and BEVs. Importantly, ATEVs revealed context-dependent effects in treating type 2 diabetes, skin wound healing, hair regeneration and glioma tumor models in vivo.

Summary/Conclusion: BeEVs are a novel subtype of ATEVs with typical morphology and unique protein composition. In a variety of disease conditions, exogenous supplementation of ATEVs can be considered as a convenient and efficient therapeutic approach, which holds promise in future translation.
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Keywords: adipose tissue, extracellular vesicles, brown adipose, white adipose, beige adipose, type 2 diabetes, wound healing

PF03: Cancer EV Pathogenesis

Location: Hall 4A

16:00 - 18:00

PF03.01 | The cholesterol metabolite, 27-hydroxycholesterol impairs lysosomal function, leading to increased secretion of cancer promoting extracellular vesicles

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Introduction: Breast cancer is highly prevalent among women in the United States and elevated circulating cholesterol has been associated with its onset and metastasis. Extracellular vesicles (EVs) have been strongly implicated as factors contributing to breast cancer metastasis. Interestingly, 27HC, a cholesterol metabolite, increases EV secretion in myeloid immune cells and these EVs promote tumor growth and metastasis in murine models of breast cancer. Given that these EVs are involved in cancer progression, it is imperative that we understand how their biogenesis and secretion are regulated.
Methods: Primary macrophage and RAW 264.7 cell EVs were isolated using ExoQuick Kit (SBI Biosciences). EVs were characterized using Nanoparticle Tracking Analysis and Transmission Electron Microscopy. The biogenesis and secretion mechanism was studied using qPCR, Immunofluorescence and Western blot analysis.
Results: Immunofluorescence microscopy revealed that 27HC increases size of CD63+ multivesicular bodies (MVBs) but not EEA1+ endosomes. Expression of ESCRT (Endosomal Sorting Complex Required for Transport) genes were unchanged on 27HC treatment. However, the size of LAMP1+ lysosomes increases, along with an increase in pH. Treatment of cells with BafilomycinA1, a lysosomal proton pump inhibitor phenocopied these 27HC mediated effects. In addition, 27HC decreases levels of ISG- and NEDDylated proteins in cells; modifications that have been previously implicated to alter EV secretion.
Summary/Conclusion: 27HC increases EV secretion by impairing lysosomal integrity and skewing MVBs away from lysosomal degradation and towards secretion as EVs. Collectively, our studies reveal novel mechanisms by which EV secretion is modulated by 27HC. Furthermore, this axis represents a novel avenue for the treatment of breast cancer.
Funding: Department of Defense BRCP Era of Hope Scholar Award (BC200206), and NIH-NCI (R01CA234025).
Keywords: breast cancer, immune cells, EV biogenesis

PF03.02 | Elucidating the role of Activated Leukocyte Cell Adhesion Molecule (ALCAM) in the biogenesis and function of tumor extracellular vesicles

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Introduction: Activated Leukocyte Cell Adhesion Molecule (ALCAM) is a widely expressed transmembrane adhesion protein that regulates cell motility and metastasis, especially in bladder cancer. Given the important roles of extracellular vesicles (EVs) in tumorigenesis, we asked whether ALCAM might also impact EV biogenesis or function.
Methods: To study the effects of ALCAM on cancer cells, we generated ALCAM-deficient HT1080 fibrosarcoma and UMUC3 bladder tumor cell lines using CRISPR-Cas9. EVs were isolated from control and ALCAM knockout cell culture supernatants by serial ultracentrifugation to generate 10,000g and 100,000g pellets. Nanoparticle tracking (ZetaView), western blots, and single EV flow cytometry with dimensional reduction analysis after staining with di-8-ANEPPS were performed.
Results: Although no differences were observed in EV counts between HT1080 WT and ALCAM deficient cells, we observed an increase in EV counts by nanoparticle tracking in both the 10,000g and 100,000g pellets of UMUC3 ALCAM-deficient bladder tumor cells compared with controls. By western blot, we observed reduced amounts of CD9 and Syntenin, with relatively preserved levels of Alix and TSG101 in the lysates of 100,000g pellets of UMUC3 ALCAM-deficient cells. Flow cytometry identified differences in EV subpopulations between control and ALCAM-deficient cell lines.
Summary/Conclusion: This work identifies changes in EV number and protein cargos in a bladder tumor cell line in the absence of ALCAM expression. This finding suggests that ALCAM may regulate EV biogenesis and/or cargo loading in cancer cells.
Funding: NIH R01CA249424 (HHP).

PF03.04 | Regulation of extracellular vesicle biogenesis in pancreatic ductal adenocarcinoma by MUC1 through its interaction with ALIX

Kristine V. Hoagstrom[1]; Ying Huang[2]; Heather Jensen-Smith[2]; Michael A. Hollingsworth[2]
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Introduction: Pancreatic ductal adenocarcinoma (PDAC), a highly aggressive disease with poor prognosis, lacks early detection methods. Recent studies highlight the value of extracellular vesicles (EVs) in PDAC diagnostics and treatments. Overexpression and selective enrichment of MUC1, a type I transmembrane protein, has been found in tumor EVs, correlating with hyperplasia, migration, and invasion. Additionally, ALIX, a cytoplasmic adapter and ESCRT-associated protein, demonstrates critical mediation of EV biogenesis. Our unpublished data reveals significant reduction in EV secretion upon knock-out of MUC1 in multiple pancreatic cancer cell lines. This study aims to elucidate the nature of the MUC1-ALIX interaction and its function in EV biogenesis regulation.
Methods: EVs were isolated from conditioned media of pancreatic cancer cell lines, purified by centrifugation then ultracentrifugation at 110,000g for 2 hours in a Beckman SW28 Swinging Bucket rotor, and characterized by transmission electron microscopy (TEM), nanoparticle tracking analysis, and immunoblotting. MUC1-ALIX association was analyzed by immunofluorescence microscopy (IFM), fluorescence resonance energy transfer (FRET), and co-immunoprecipitation (CoIP).
Results: IFM data illustrated colocalization between the C-terminal subunit of MUC1 (MUC1.CT) and ALIX. FRET studies with CFP-labeled MUC1 and YFP-labeled ALIX showed decreased YFP intensity with photobleaching concomitant with increased CFP intensity, demonstrating a MUC1-ALIX association. CoIP analyses using MUC1.CT and ALIX mAbs revealed an impact of knocking out MUC1 or ALIX on the protein content of EVs. Together these results implicate a role for the MUC1-ALIX interaction in regulating EV biogenesis and packaging.
Summary/Conclusion: This study demonstrates a clear MUC1-ALIX interaction and that its disruption impacts EV biogenesis. The results lay the groundwork for ascertaining MUC1's role in modulating protein content in tumor EVs and the influence of this process on the progression of PDAC.
Funding: Acquired Resistance to Therapy Network (ARTNet) 1U54CA274329-01. Structural Biology and Molecular Biophysics (SBMB) Training program at UNMC from the Department of Education Graduate Assistance in Areas of National Need (GAANN) program fellowship.

PF03.06 | Annexin A2 in tumor-derived extracellular vesicles: Molecular contributions in metastatic triple negative breast cancer

Rucha Trivedi; Jamboor K. Vishwanatha
University of North Texas Health Science Center, Fort Worth, USA

Introduction: Tumor-derived extracellular vesicles (TEVs) are highly implicated in tissue-specific metastasis. Additionally, TEVs interacts with the distant microenvironment to shape a pre-metastatic niche (PMN) for homing the tumor cells. Annexin A2 (AnxA2) is a plasma and endosomal membrane-associated protein. Its high levels have been correlated with poor distant metastasis-free survival and poor overall survival in triple negative breast cancer (TNBC) patients. It is also abundantly present in TEVs and recruits TEV-associated cargo such as proteins and microRNAs. Our lab reported that in vivo education with AnxA2 depleted EVs led to reduced TNBC metastasis to lungs and brain suggesting a key role in the formation of a PMN. While the

presence of AnxA2 in EVs has been reported, its contribution in the formation and development of PMN via EVs is still unexplored. We aim to evaluate the implications of AnxA2 in EVs and elucidate the mechanisms promoting TNBC metastasis.
Methods: We used RNA interference-mediated gene silencing to stably downregulate AnxA2 in organotropic TNBC cell lines derived from the parent MDA MB 231 cells, LM2 and BrM2. Differential ultracentrifugation was used to isolate EVs from cell culture supernatant and size analysis was done using Nanoparticle Tracking Analyzer. Biological characterization was done in concordance with MISEV 2018 guidelines using immunoblotting. Additionally, the EVs will be subjected to quantitative proteomic analysis to identify differentially expressed proteins upon loss of AnxA2. We will further carry out transcriptome profiling of the AnxA2 depleted TNBC cells and -derived EVs to identify the differentially expressed genes.
Results: Upon depletion of AnxA2 protein, we observed a significant effect of AnxA2 depletion on its physiological role in plasmin generation. We observed a size distribution of the isolated EVs between 30–300 nm. Using immunoblotting we confirmed reduced levels of AnxA2 in EVs derived from AnxA2 depleted TNBC cells. We verified their purity using EV enriched markers - ESCRT, Heat shock proteins and tetraspanins such as CD81, CD9, CD63 and confirmed the absence of negative markers - GM130, calnexin and cytochrome c. Interestingly, we observed a reduced yield of EVs with AnxA2 depletion indicating a potential effect on EV biogenesis and release.
Summary/Conclusion: The role of AnxA2 in TEVs biogenesis, release and selective cargo loading will lead to potential identification and understanding of the novel secretory and EV protein that may act as a functional regulator in promoting advanced metastasis in TNBC.
Funding: This research is supported by the NCI of National Institute of Health under Award Number R01CA220273 (JKV), awarded to Dr. Jamboor K. Vishwanatha.
Keywords: Annexin A2, TNBC, extracellular vesicles.

PF03.07 | Dual role of ULK1 in small extracellular vesicles biogenesis and tumorigenesis in hepatocellular carcinoma

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[1]The University of Hong Kong, Hong Kong, Hong Kong; [2]The University of Hong Kong, Hong Kong, USA

Introduction: Our previous study suggested secretion of tumor-derived small extracellular vesicles (T-sEVs) elevate significantly in advanced hepatocellular carcinoma (HCC), but the exact mechanism remains unelucidated. Here, we aim to investigate key mediators involved in the biogenesis of HCC-derived sEVs and their clinical significance.
Methods: Bead-based Amplified Luminescent Proximity Homogenous Assay (ALPHA) was performed on the conditioned medium of HCC cells after treatment of the Selleckchem kinase inhibitor library. Candidate inhibitors that displayed the strongest suppression effect on sEV secretion were identified. The targeted kinase was knocked down in HCC cells and their sEV were collected through ultracentrifugation for mass spectrometry analysis. Collected sEVs were functionally characterized by in vitro assays and animal models.
Results: MRT68921, an ULK1 inhibitor, was found to be significantly involved in the inhibition of CD63+ and CD9+ sEV biogenesis through ALPHA. The involvement of ULK1 was corroborated by the significant reduction in sEV secretion by stable ULK1 knockdown cells compared to its respective control group established in metastatic MHCC97L cells. Mass spectrometry revealed a drastic shift in the protein cargos of sEV collected from ULK1 knockdown cells, notably a decreased level of sEV-derived AE2. Functionally, the inducing ability of MHCC97L sEV on HCC proliferation and motility was significantly dampened compared to sEV collected from ULK1 knockdown cells. Intracellularly, AE2 was observed to be co-localized with ULK1 whereas its expression was significantly upregulated in the endosomal fraction upon overexpression of ULK1, suggesting its regulatory role in sEV biogenesis and protein cargo sorting in HCC cells.
Summary/Conclusion: This study unveils a bidirectional stimulation of ULK1 in terms of HCC-sEV biogenesis and oncogenic signaling through sEV-AE2 axis. This study also provides insights into targeting ULK1 as a new therapeutic strategy for HCC.
Keywords: Hepatocellular carcinoma, small extracellular vesicles, EV secretion, ULK1, AE2

PF03.08 | A novel interaction of CD47 with filamin A identified using single vesicle imaging and mass spectrometry analyses of extracellular vesicle released from T lymphoblast and prostate carcinoma cells

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Introduction: CD47 is a ubiquitously expressed membrane protein that functions as a receptor for thrombospondin-1 and the counter receptor for signal regulatory protein-α on phagocytes. CD47 is widely expressed on extracellular vesicles (EV), and identifies a distinct population of EV from those that express the traditional EV markers.
Methods: Mass-spectrometry, immunoprecipitation-western blotting (IP/WB), flow cytometry, confocal image analyses and single particle interferometric reflectance imaging sensing (SP-IRIS) via ExoView® imaging platform were used.
Results: Targeted mass spectrometry analysis of proteins captured on anti-CD47 beads from wild type (WT) but not CD47- mutant Jurkat T lymphoblast cells identified filamin A as a novel interacting protein partner of CD47. Filamin A, YBX1, and the CD47 cytoplasmic adapter ubiquilin-1 were uniquely present in EV secreted from WT but not CD47- mutant Jurkat cells. Specific association of CD47 with filamin A in cells was further confirmed from WT and CD47- mutant Jurkat and PC3 prostate carcinoma cells using IP/WB. SP-IRIS analysis showed less filamin A and $\alpha 4\beta 1$ integrin sort into EV in CD47- Jurkat and PC3 cells. 3D visualization via Z-stack images showed colocalization of filamin A with CD47 on Jurkat and PC3 WT cells and altered filamin A localization in the respective CD47- mutants. Our findings suggest that CD47 indirectly binds to filamin A via ubiquilin-1 and/or β Integrin. Filamin A may thereby play an important role in CD47-dependent sorting of protein cargoes into specific subsets of EV.
Summary/Conclusion: CD47 and ubiquilin-1 interact with filamin A, which is known to interact with the cytoplasmic domain of $\beta 1$ integrins to regulate integrin function. Less filamin A and $\alpha 4\beta 1$ integrin sort into EV in the absence of CD47, suggesting that CD47 promotes filamin A and integrin sorting into EV, mediated through ubiquilin1.
Funding: The Intramural Research Program of the NCI Center for Cancer Research.
Keywords: CD47, Ubiquilin1

PF03.09 | Acute chemotherapy exposure alters the miRNA expression profile in extracellular vesicles released from medulloblastoma cells

Rosa Mistica C. Ignacio[1]; Helen Forgham[2]; Zerong Ma[3]; Sze Wing Wong[3]; Anya Jensen[3]; Chelsea Mayoh[4]; Maria Kavallaris[5]; Joshua McCarroll[5]
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Introduction: Medulloblastoma (MB) is a deadly childhood brain tumour classified into 4 molecular-based subgroups (WNT, SHH, group 3, group 4) all with varying survival rates. Understanding mechanisms that promote tumour growth are needed to develop therapeutic strategies to increase patient survival. Extracellular Vesicles (EVs) are used by cells to communicate with other cells by transfer of molecular cargo such as miRNA, which regulate cell function. Exposure to chemical stressors can alter miRNA expression in EVs secreted by cancer cells to promote survival in recipient cells. No studies have examined effects of chemotherapy on EV-miRNA expression in MB cells. Hypothesis: Chemotherapy will alter miRNA expression in EVs secreted by MB cells.
Methods: SHH (DAOY) and group 3 (D283) MB cells were treated with or without cisplatin for 3h. Forty-eight hours later EVs were isolated using size exclusion-filtration, ultracentrifugation, and precipitation and characterised using standard techniques. EV-miRNA was isolated and small RNA-Seq performed. EVs isolated from cisplatin treated-and control (no drug) cells were added onto recipient MB cells and clonogenic and medullosphere growth measured.
Results: Cisplatin induced a significant release of EVs from DAOY and D283 cells compared to controls. EV-miRNA expression was altered in cells exposed to cisplatin. miR-449a and miR-1275 (tumour suppressors) were significantly increased in DAOY-EVs but downregulated in D283-EVs compared to controls. In support of these findings, EVs isolated from cisplatin-treated DAOY cells when added to recipient cells decreased clonogenic growth, and did not effect medullosphere growth. In contrast, EVs isolated from cisplatin-treated D283 cells increased clonogenic and medullosphere growth.
Summary/Conclusion: Cisplatin exposure has differential effects on EV-miRNA expression in MB cells from different subgroups. Future studies are warranted to understand the role of EV-miRNAs in regulating MB growth.
Funding: This work was funded by Cancer Australia / Kids Cancer Project (Application #: 1184840) and The Ross Trust Foundation.
Keywords: extracellular vesicles, medulloblastoma, microRNA, cancer chemotherapy

PF03.11 | Hypoxic extracellular vesicles drive oncogenic transformation in normal mammary epithelium, in vivo

Irene Bertolini[1]; Michela Perego[1]; Andrew Milcarek[2]; Jagadish Ghosh[1]; Dario Altieri[2]
[1]*The Wistar Institute, Philadelphia, USA;* [2]*The Wistar Institute, USA*

Introduction: Extracellular vesicles (EV) have emerging roles in cell-to-cell communication. Cancer cells produce higher levels of EV compared to normal cells, and this response is augmented in hypoxia. Intra-tumoral hypoxia is a common occurrence in breast cancer, correlating with increased risk of metastasis and poor prognosis. We have demonstrated that EV produced by cancer cells in hypoxia induce oncogenic changes in normal epithelial cells in vitro. Therefore, we have now examined the effect of hypoxic-EV in vivo.
Methods: We isolated EV from four different mouse mammary carcinoma cell lines (two metastatic and two non-metastatic) in hypoxic (EV-HYP) or normoxic (EV-NORM) conditions (1% or 5% O2 for 24 h). EV were isolated using differential centrifugation followed by SEC (Izon). EV characterization was carried out according to MISEV guidelines. EV were quantified after each isolation using ZetaView. 6 × 10^9 EV were injected in the abdominal mammary gland of C57BL/6 mice and sample tissues were harvested after 3–6 and 18 weeks.
Results: Our data show that EV released by invasive breast cancer cells in hypoxic conditions disrupt the differentiation hierarchy of the normal mammary epithelium, with expansion of stem and luminal progenitor cells, and induction of atypical ductal hyperplasia, and intra-epithelial neoplasia. Moreover, EV-HYP induce sustained angiogenesis and resistance to cell death. Mechanistically, we demonstrate that EV released by metastatic cell lines in hypoxic condition contain HIF1α, which is required for downstream responses of aberrant mammary epithelium developmental morphogenesis.
Summary/Conclusion: Invasive breast cancer cells in hypoxic condition produce HIF1α-containing EV. These EV can induce critical steps of mammary epithelium transformation and sustained angiogenesis, in vivo. Taken together, these results identify a novel pathway of local breast cancer recurrences.
Keywords: breast cancer, hypoxia, HIF1a, angiogenesis, TME

PF04: Therapy Adipose and MSC EVs 1

Location: Hall 4A

16:00 - 18:00

PF04.01 | Reversal of lethal dose of radiation damage by mesenchymal stromal cell derived extracellular vesicles

Sicheng Wen[1]; Elaine Papa[2]; Mark Dooner[2]; Mandy Pereira[2]; Michael Del Tatto[2]; Peter Quesenberry[2]
[1]*Rhode Island Hospital/ Brown University, providence, USA;* [2]*Rhode Island Hospital, Providence, USA*

Introduction: The current therapies for patients exposed to a lethal dose of radiation are limited. We have previously shown that human mesenchymal stromal cell (MSC) derived vesicles would completely or partially reverse the bone marrow injury elicited by exposure to mild or moderate radiation in vitro or in vivo.
Methods: We now evaluated the effect of MSC-EVs on the reversal of radiation damage after 950 cGy of whole-body lethal irradiation. Mice received an IV infusion of 1 × 109 hMSC EVs daily for three days, after 24 hours of irradiation.
Results: The MSC-EV untreated mice were dead between 12–27 days post-radiation, but MSC-EV treated mice maintained a 70% survival rate in 120 days post-radiation. The peripheral blood counts significantly reduced, at ten days after radiation with/without EV treatment and recovered after three months of EV treatment although WBCs were still lower than normal control, suggesting that MSC-EV treatment could significantly extend the survival rate of mice and enhance the recovery of blood cells after exposure to lethal radiation. We further evaluated the 30-marrow stem cell-related gene expression in WBMCs from these mice with/without EV treatment at 14 and 120 days post-exposed radiation by real-time PCR analysis. We identified the differential expression genes (DEGs) between radiation with/without EV treatment and healthy controls according to the cutoff values of $|\log2FC| \geq 1.0$ and p-values < 0.05 (t-test), and the 14 DEGs were accordingly identified between groups. Within the 14 DEGs, the gene expression of CXCL12, CD90, and Nestin were upregulated, and another 10 genes including SATB1, RUNX1, PECAM, PBX1, CKIT, CD34, CD33, MPO, SPI1, and S100A9 were downregulated in mice 14 days post radiation with/without EV treatment compared to the non-radiation control mice. However, the alterations of gene expression in these genes were rescued to normal levels at day 120 post-EV treatment. No DEGs were found between mice with and without EV treatment at 14 days post-radiation except the gene CD3E was upregulated by radiation but came down to normal on day 14 with EV treatment. We didn't

observe DEGs between the non-radiation control mice group and the radiation with 120 days post EV treatment. This suggests that the change of gene profile induced by radiation is not rescued by EV treatment at day 14 post-radiation but significantly recovered at day 120 post-radiation.

Summary/Conclusion: Our data suggest that EV treatment could significantly extend the survival rate of mice and help bone marrow cells recover from radiation injury.

Funding: NIH P20GM119943.

Keywords: mesenchymal stromal cell, extracellular vesicles, radiation

PF04.02 | Comparison of exosomal microRNA of subcutaneous and epidural adipose tissue-derived mesenchymal stem cell and the application of nerve damage diseases

Soo-Eun Sung[1]; Min-Soo Seo[2]; Young-In Kim[3]; Kyung-Ku Kang[1]; Joo-Hee choi[1]; Si-Joon Lee[4]; Min-Kyoung Sung[1]; Seung-Yun Yang[5]; Gun-Woo Lee[6]

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Introduction: Subcutaneous fat is commonly used for isolating adipose tissue-derived mesenchymal stem cells (AD-MSC). Spinal epidural fat is adipose tissue gained from posterior decompression surgery of the lumbar spine. Although epidural fat is more difficult to separate than subcutaneous fat tissue, it has different properties comparing subcutaneous fat. Epidural fat is known to play a role in buffering, sliding, and protecting spinal function. Removing epidural fat during surgery causes several problems such as post-laminectomy syndrome. In this study, exosomes were isolated by culturing subcutaneous and epidural adipose tissue-derived stem cells, and exosomal miRNAs were analyzed by small RNA sequencing.

Methods: Stem cell exosomes were isolated by centrifuging, tangential flow filtration, and a 0.2 μm syringe filter. To confirm exosome characteristics, we used TEM, FACS, and NTA. Exosomal miRNAs were analyzed using small RNA sequencing. Genes targeted by MicroRNA analyzed biological process functions using DAVID.

Results: We confirmed that several miRNAs were regulated differently between subcutaneous AD-MSCs and epidural AD-MSCs. In the epidural exosomes, the most up-regulated miRNAs were hsa-miR-122-5p, hsa-miR-299-5p, and hsa-miR-361-3p. These miRNAs targeted many genes and we list up to 5,499 genes. In the biological function results, 113 genes were analyzed to be related to the development of the nervous system and expressed the highest statistical significance. Based on the results of these studies, human epidural AD-MSC-derived exosomes were administered to spinal cord injury rats to confirm the effect of restoring motor function and reducing the inflammatory response.

Summary/Conclusion: Taken together, these data suggest that stem cell exosomes derived from human epidural adipose tissue have a nerve regeneration effect on nerve damage diseases.

Funding: This research was supported by the grant from the National Research Foundation of Korea Ministry of Science and ICT (Grant number: 2022R1C1C1005410).

Keywords: epidural fat, mesenchymal stem cells, exosomes, neurogenesis

PF04.04 | Characterization of Extracellular Vesicles and soluble factors from IL-1β primed Mesenchymal Stromal Cells and their therapeutic potential in Traumatic Hemorrhagic Shock

Guillaume VALADE[1]; Claire LANGLE[2]; Marine DETADDEO[2]; Bileyle LORENZINI[3]; Clement DEVAUTOUR[2]; Marion GROSBOT[4]; Muriel NIVET[4]; Sylvie GOULINET[3]; Philippe MAUDUIT[3]; Marina TROUILLAS[4]; Sebastien BANZET[4]; Juliette PELTZER[4]

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Introduction: Traumatic Hemorrhagic shock can quickly induce death by bleeding or later by organ failure. The administration of IL-1beta primed mesenchymal stromal cells (MSCIL) modulates the inflammatory response and reduces organ dysfunction. We explore the therapeutic potential of concentrated fractions of MSC-derived conditioned medium (CM) +/- enriched in extracellular vesicles (EV) and soluble factors. These products, are easier to store than MSC and immediately available for emergencies. Our aim is first, to characterize these fractions and second, to explore their immunomodulatory potential and determine the best therapeutic agent.

Methods: CM are obtained after 72h of human bone marrow MSCIL secretion, from a pool of 5 donors and from each donor separately. Two priming conditions were evaluated : IL-1beta at 1 and 5 ng/mL. The CM are purified/concentrated by Tangential

Flow Filtration (TFF) with two different filters. Two enrichments are evaluate, depending on filtration conditions: i) purified EV fraction, and ii) the association of EVs and soluble factors. Their characterization are performed with Nano Tracking Analysis (NTA), and MACSPlex Exosome kit® (Miltenyi). Their immunomodulatory properties are evaluated in vitro by a monocyte anti-inflammatory assay and a mixed leucocyte reaction.
Results: Our preliminary NTA results found almost twice as much particles in the fraction containing EV and soluble factors compared to the EV fraction. Moreover, an anti-inflammatory activity and an immunosuppressive effect are obtained with both fractions. No difference in efficiency is observed between the priming conditions.
Summary/Conclusion: These first results show that the tendency tends to improve the anti-inflammatory and immunosuppressive activities of CM-enriched in MSC-EV and MSC-EV with soluble factors. The molecular characterization of both fractions is ongoing. The best product selected in vitro will be evaluated in an in vivo hemorrhagic shock model.
Keywords: traumatic hemorrhagic shock, extracellular vesicles, mesenchymal stromal cells, tangential flow filtration, priming, characterization, therapy, immunomodulation

PF04.06 | Human adipose-derived extracellular vesicles (AdEVs) increase proinflammatory markers in renal and endothelial cells: an in vitro preliminary study

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Introduction: During obesity, white adipose tissue (WAT), undergoes hypertrophic and hyperplastic changes by differentiation of preadipocytes into adipocytes, which increase the expression of PPARγ, FASN, FABP4, and adiponectin genes. These adipokines play a key role in obesity progression and in cell communication. WAT also causes a chronic inflammatory state that modifies the gene expression and secretome, including releasing of adipose-derived extracellular vesicles (AdEVs) that carry a specific cargo thought to modify different signaling pathways in target cells. Our aim was to evaluate the effect of AdEVs in renal and endothelial cells and its inflammatory phenotype.
Methods: Human SW872 preadipocytes and differentiated adipocytes were cultured and characterized by optical microscopy and Oil-Red-O stain. EVs from both preadipocytes and adipocytes were isolated by ultracentrifugation and characterized by nanoparticle tracking analysis, electron microscopy and w-blot. AdEVs ($1 \times 10\hat{}3$) were added to renal HCD and endothelial EaHy926 cells for 24 hours. Expression of adipogenic differentiation genes in preadipocytes, adipocytes and their EVs, as well as the expression of inflammatory markers (IL-6 and IL-1B) were perfomed by RT-qPCR.
Results: SW872 differentiated cells showed a classical adipocyte morphology and important accumulation of lipid-droplets. Isolated AdEVs have a donut shape morphology and size (50 - 150 nm) in accordance to the MISEV2018 guidelines, including also the EVs markers CD9 and Tsg101. A higher EVs concentration released from preadipocytes compared to adipocytes was observed ($5.21 \times 10\hat{}10$ vs $0.44 \times 10\hat{}10$ particles/mL, $p \leq 0.05$). The analysis of relative gene expression ($2\hat{}-\Delta\Delta CT$), showed that adipocytes increase FAPB4 and adiponectin and decrease in PPARγ and FASN gene expression, with respect to preadipocytes ($p \leq 0.05$). EVs from preadipocytes and adipocytes present similar relative expression of adiponectin, PPARγ and FASN than their parental cells. Finally, both renal or endothelial cells treated with AdEVs express higher levels of IL-6 and IL-1B that untreated cells ($p \leq 0.05$).
Summary/Conclusion: EVs released from SW872 adipocytes and preadipocytes have similar gene expression as their parental cells. Treatment of both renal and endothelial cells with AdEVs showed an increase in inflammatory markers, as IL-6 and IL-1B. These preliminary results reveals novel insights about the impact of AdEVs and its cargo in cell-cell communication, and further support novel roles of AdEVs in the obesity pathophysiology.
Funding: ANID-FONDECYT 1212006 (CAC), 3200646 (ATC); CONICYT-FONDEQUIP EQM150023 (CAC) & CETREN.

PF04.07 | Development of a 3D multicellular model for the study of ADSC-EVs in the context of autologous fat grafting

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Introduction: Autologous fat grafting (AFG) is a favourable surgical option for oncologic breast reconstruction. However, retention rates are variable, limiting AFGs clinical utility. Enriching grafts with EVs from adipose-derived stem cells (ADSCs) may

promote AFG retention by modulating the tissue microenvironment to promote vasculogenesis and a favourable inflammatory state. Our previous work has shown that ADSC-EVs promote HUVEC tube formation and modulate macrophages towards anti-inflammatory phenotypes in single cell systems. This study aims to develop multicellular 3D models that are more representative of a tissue microenvironment and assess the function of ADSC-EVs within these systems.
Methods: Following written informed consent and ethical approval, lipoaspirate was collected from 3 patients undergoing AFG in Wellington. ADSCs were isolated enzymatically and cultured. ADSC-EVs or Dummy EVs (DEVs) were isolated from media using SEC (qEV35, Izon) and characterised by TRPS, TEM, and Western blotting. Monocytes were isolated from two healthy volunteers and cultured towards M0 macrophages. Macrophages were lifted and stained with CellTracker Deep Red and HUVECs were stained with Calcein AM before being added to Matrigel coated wells. ADSC-EVs were stained with PKH26 and added to cultures. Models were imaged by confocal microscopy to assess tube formation, cell association, and EV uptake.
Results: ADSC-EV uptake was visualised in both cell types. HUVEC tube formation increased relative to DEVs; including increased number of junctions (1.74 ± 0.48, $p = 0.0034$), junction density (1.76 ± 0.49, $p = 0.0037$), and vessel percentage area (1.22 ± 0.13, $p = 0.0026$). The number of macrophages associating with HUVECs increased following ADSC-EV treatment relative to DEV (1.21 ± 0.20, $p = 0.0246$).
Summary/Conclusion: ADSC-EVs increased tube formation and cell association and this work provided a base model for continuing to study the potential therapeutic role of ADSC-EVs in the context of AFG.

PF04.08 | Neuroprotective and neuro-regenerative effect of extracellular vesicles derived from human Wharton's jelly stem cells (hWJ-MSC-EVs) after spinal cord injury (SCI)

Leila Noori[1]; Francesco Cappello[2]; Somayeh Arabzadeh[3]; Yousef Mohamadi[4]; Sina Mojaverrostami[5]; Mohammad Akbari[5]; Gholamreza Hassanzadeh[5]
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Introduction: In the early stages of CNS disorders including spinal cord injury (SCI), neuroinflammation causes to activation of the astrocytes, overexpression of glial fibrillary acidic protein (GFAP) and lead to formation of glial scar which are considered as one of the main obstacles in the regeneration of spinal cord tissue (1). Further, neural progenitor cells (NPCs), located in ependymal layer around the central canal of spinal cord, effectively contributed to tissue repairment after injury and trophic factors increase the Nestin, NPC marker, expression (2). Death of neurons is also a destructive outcome of CNS injuries which leads to functional impairment (3). Extracellular vesicles (EVs), heterogeneous bilayer nanovesicles containing growth factors, lipids, nucleic acids and proteins, derived from mesenchymal stem cells (MSCs-EVs) showed neuroprotective properties via crossing BBB and providing growth factors, support neuronal survival and conserving from apoptosis during neurodegeneration (4). Intrathecal delivery of EVs ensures their distribution around the injured site (5). The damaged BBB is permeable within 1 h till 5 days after SCI with a peak in 24 h post-injury (6). We investigated potential neuroprotective and neuro-regenerative effects of human Wharton's jelly mesenchymal stem cells derived extracellular vesicles (hWJ-MSC-EVs) on injured spinal cord tissue one week after SCI in rats.
Methods: We applied three intrathecal different doses (1, 2 and 3 μg) of human Wharton's jelly mesenchymal stem cells derived extracellular vesicles (hWJ-MSC-EVs) 24 h after injury in a compressive SCI rat model. Immunohistochemistry (IHC) was done to measure the GFAP and Nestin expressions. Cell death was studied by TUNEL assay.
Results: Our finding showed that intrathecally administrated hWJ-MSC-EVs attenuated GFAP expression in all treatment groups both in gray and white matter of epicenter, although this reduction was significantly valuable in mid and high doses groups. Nestin expression remarkably improved with mid and high doses of WJ-MSCs-EVs. Moreover, it reduced neuronal death more significantly in mid and high doses and saved higher number of typical neurons in ventral horn of spinal cord tissue in those groups.
Summary/Conclusion: We came to the conclusion that hWJ-MSC-EVs are promising neuroprotective tools to modulate the astrocytes activity and neuronal cell death as well as potential neuro-regenerative agents to stimulate NPCs after SCI in rats.
Funding: This work was funded by Tehran university of medical sciences.
Keywords: neurodegeneration, neuro-regeneration, extracellular vesicles, mesenchymal stem cells

PF05: Kidney and Binary EVs 2

Location: Hall 4A

16:00 - 18:00

PF05.01 | Large extracellular vesicles in idiopathic nephrotic syndrome

Robert Myette[1]; Janusz Feber[1]; Pavel Geier[1]; Christopher Kennedy[2]; Dylan Burger[2]
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Introduction: Idiopathic Nephrotic Syndrome (iNS) is one of the most common causes of kidney injury in children. There has been an increased focus on reactive oxygen species (ROS) in podocytes as drivers of proteinuric disease. Our lab and others have suggested that large extracellular vesicles (LEVs) may have utility as biomarkers of podocyte injury. The aim of our study was to investigate the potential of urinary LEVs as biomarkers in iNS, and to characterize LEV release using cultured podocytes exposed to toxins in vitro.
Methods: We analyzed urine samples from a prospective cohort enrolling children 1–18y with iNS. Podocyte specific LEV were quantified using flow cytometry and nanoparticle tracking (NTA). Human immortalized podocytes (hPod) were used in vitro. Puromycin aminonucleoside (PAN; 25 ug/mL; 24 hours) and lipopolysaccharide (LPS; 25 ug/mL; 24 hours) were used as podocyte toxins.
Results: In paired relapse and remission samples from 14 patients the median [IQR] podocyte LEVs were significantly lower in remission (0 LEVs/mmol of Cr [0, $14.7 \times 10\hat{}3$]) vs. nephrosis $22.8 \times 10\hat{}3$ LEVs/mmol of Cr [IQR $1.11 \times 10\hat{}3$, $74.6 \times 10\hat{}3$] ($p < 0.01$). Urine Albumin to creatinine ratio was positively associated with elevated LEVs ($p = 0.01$). In cultured hPods, PAN treatment resulted in a 2.5-fold increase in hPod LEVs ($p = 0.03$) while LPS caused a 3.5-fold increase ($p = 0.0004$). This was abrogated with inhibitors of ROS generation (N-acetyl cysteine,4-OH-Tempol and MITO-Tempo).
Summary/Conclusion: In summary, LEV appear to serve as a novel indicator of iNS relapse and their levels can differentiate disease status. hPods show similar characteristics when treated with common podocyte toxins, while protective antioxidant strategies also reduce LEV release.
Funding: Canadian Institutes of Health Research, Kidney Foundation of Canada, Kidney Research Scientist Core Education and National Training Program.
Keywords: kidney, nephrotic syndrome, podocyte, biomarker

PF05.02 | Isolation, characterization, and preservation of extracellular vesicles in human urine samples to facilitate self-collection

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Introduction: Urinary extracellular vesicles (EVs) are the potential source of biomarkers to detect urogenital tract diseases, systemic neurological disorders, and cancer types. Despite significant advances, the issue of EV preservation has yet to be extensively studied. This is of particular concern for at-home self-collection, and multi-site urinary sample collections are challenging for large-scale recruitment, leading to variability in the time between collection and processing. Here, we developed and evaluated a novel non-lytic formulation (UAS™) to preserve EVs to enable self-sampling of urine samples.
Methods: First-void urine (FVU) samples were collected from healthy male and female donors using Colli-Pee® FV-5040 devices (Novosanis) to generate male-pooled and female-pooled urine samples, respectively. Firstly, we compared a commercial EV extraction kit and an in-house ultrafiltration (UF) method using Western blot analysis for TSG101 protein. In one of the studies, EVs extracted from male-pooled urine samples using UF method were characterized for size and concentration using nanoparticle tracking analysis and Western blot analysis (CD9 and TSG101 proteins). The extracted EVs were spiked into female-pooled urine samples (which lacked endogenous exosomal markers) with and without UAS™ preservative. Samples were then held at room temperature (RT) for 14 days and analyzed for EV content. In another study, FVU samples from healthy male donors were collected in Colli-Pee® devices pre-filled with UAS™ preservative, held at RT for up to 14 days, and analyzed for EV proteins (CD9 and TSG101) and RNA (GAPDH) content.
Results: EVs prepared using UF method showed efficient detection of TSG101 protein while the commercial extraction kit failed to do so. Male FVU samples showed consistent and higher recovery of EV protein markers relative to female FVU samples. Unpreserved spiked urine sample showed reduction of EV protein markers, when held at RT for 14 days; unlike UAS™ preserved sample, which showed no significant change under similar storage conditions. EV proteins and RNA content were found to be preserved in the urine samples collected in Colli-Pee® devices pre-filled with UAS™ preservative and stored at RT for 14 days.

Summary/Conclusion: Our studies demonstrate that there are gender-specific and extraction method-based differences in the expression and detection of EV markers, highlighting their heterogeneity in urine samples. Our results highlight the need for urine preservation and demonstrate UAS™ based preservation of urinary EVs and their cargo, which could enable at-home self-collection-based solutions.
Keywords: urine, preservation, oncology, extracellular vesicles, self-collection, liquid biopsy, exosomes

PF05.03 | Modelling INS disease subtypes stratification based on a surface EV biomarkers signature

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Introduction: Idiopathic Nephrotic Syndrome (INS) is a paediatric glomerular disease characterized by immune dysfunction and lipid dysregulation. INS frequently responds to corticosteroids, showing possible adverse effects. Biofluid-extracellular vesicles (EVs) may represent a source of INS diagnostic biomarkers.
Methods: EV surface characterization was analyzed on urine and serum from 80 patients compared to control children (approved informed consent). EV structure, size, and concentration were measured by NTA, TEM, and STORM analysis; expression of EV surface proteins was analyzed by beads-based cytometry; profiling of lipids was realized by GC on ultracentrifuged EVs.
Results: Urine-EVs showed typical cup-shaped structure and different tetraspanins spatial distribution, related to the disease. NTA analysis showed a higher urine-EVs number in INS children, that positively correlated with urinary creatinine levels. A positive correlation was also found between CD9/CD63/CD81 expression and pathological proteinuria levels. Principal component analysis clearly separated patients in active phase of disease from remission. Unsupervised clustering study revealed a superimposable pattern between different disease stages and clearly distinguished INS patients from controls, mainly based on immune/platelet/cell adhesion markers. A cluster of four markers (CD24, HLA-DR, CD142, SSEA-4) separated patients in the active phase from remission by univariate analysis. The concomitant serum EVs evaluation allowed the generation of a biofluid matrix protein profile able to better distinguish different disease subtypes. Urine-EVs lipid profile showed an unbalance in saturated and polyunsaturated fatty acids, as well as a significant decrease of omega-3/omega-6 ratio, associated with an inflammatory and pro-oxidant milieu in INS.
Summary/Conclusion: Biofluid-EV surface signature, by representing immune dysfunction and inflammatory processes, may complement the stratification of different INS subtypes.
Keywords: extracellular vesicles, nephrotic syndrome, immune dysfunction, lipid dysregulation

PF05.04 | Diagnostic application of urinary extracellular vesicles in chronic kidney disease

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Introduction: Kidney fibrosis is the histological manifestation of irreversible CKD. We hypothesize protein cargo of urinary extracellular vesicles (uEVs) is differentially expressed in kidney fibrosis.
Methods: Study approved by ethics committee and informed consent obtained. 29–47.5ml urine collected from 3 people without kidney fibrosis and 4 people with 40–90% kidney fibrosis. Urine spun at 650xG for 10mins and supernatants treated with protease inhibitor prior to -80°C storage. Urine thawed at 37°C for 15mins. Samples spun at 2000xG for 20mins and supernatant filtered through 30μm filter. Supernatant concentrated to 500μl via centrifugation at 3500xG for 40mins in 15ml centrifugal filter units with 100,000Da cut-off regenerated cellulose membrane. Samples then washed in 50nm filtered PBS and spun at 20000xG for

30mins. uEV pellet resuspended in 500ul filtered PBS. Centrifugation steps completed at 4°C in a fixed angle rotor on maximum brake. uEVs confirmed with electron microscopy and proteomics. 50ul uEVs stained for antibodies against nephron segment markers (podocin, CD90, CD31, CD45, CD10, CD13, Tamm Horsfall Protein, CD227) and interleukin receptors (CD126, CD217, CD25, CD121a, CD121b) for 30mins. 400nm polystyrene NIST traceable size standards were added as counting beads. Samples analysed on Cytek Aurora with Enhanced Small Particle module.

Results: Mean uEV concentration was 4.03 × 10^8 uEVs/ml from fibrotic kidney and 7.53 × 10^8 uEVs/ml from non-fibrotic urine ($p = 0.65$). Interleukin 2 receptor, CD25 was expressed on mean 2.84 × 10^6 uEVs/ml in fibrotic kidney urine vs. 4.97 × 10^5 uEVs/ml in non-fibrotic urine; $p < 0.001$. Tubular epithelial markers (CD10, CD13, CD227) were the most highly expressed nephron compartment markers. Ratio of uEVs expressing CD121a (interleukin 1 receptor type 1): CD121b (decoy interleukin 1 receptor type 2) was higher in fibrotic kidney urine (mean 1.86 vs. 0.37, $p = 0.04$).

Summary/Conclusion: Majority of kidney uEVs are of tubular origin. There is an emerging uEV signature that can be used to distinguish kidney fibrosis.

PF05.05 | Optical biomarkers of radiation therapy in canine urinary extracellular vesicles characterized by label-free multimodal multiphoton microscopy

Jaena Park[1]; Katerina Baxter[2]; Sarah Samuels[2]; Edita Aksamitiene[1]; Marina Marjanovic[1]; Eric Chaney[2]; Kimberly Selting[2]; Stephen A. Boppart[1]

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Introduction: Radiation therapy is commonly used for treating many types of solid tumors. The radiation affects the metabolism in cells which subsequently alters the concentration of autofluorescent biomolecules. These changes can be identified by analyzing their autofluorescence by label-free multimodal multiphoton microscopy. Because extracellular vesicles (EVs) reflect the metabolic state of their parent cells, this phenomenon could be used to determine the efficacy or dosimetry of radiation therapy. In this study, we investigated the effects of non-lethal radiation on the optical signatures of live canine tumor cells, their EVs, and urinary EVs (uEV) of tumor-bearing dogs.

Methods: Large EVs were isolated using differential velocity centrifugation at 4°C from either control or linear accelerator irradiated canine transitional cell carcinoma (TCC) cell-conditioned serum-free media, or from the clean-catch urine collected from both healthy and radiotherapy-treated urogenital cancer-bearing dogs under an IACUC-approved protocol. Samples were precleared at 800 × g for 10 min and 2,000 × g for 30 min. 34 ml of supernatant was spun in a swinging bucket rotor at 12,000 × g for 1 h. The resulting pellet was resuspended in 100 μl of PBS supplemented with 25 mM trehalose. Concentration and size of EVs was measured by NTA. Fresh EVs were imaged with a custom-built multimodal microscope capable of detecting two- and three-photon autofluorescence, and second and third harmonic generation.

Results: EVs isolated from the media of irradiated TCC cells and EVs from urine of radiotherapy-treated dogs had a greater number of EVs that showed stronger autofluorescence from FAD and NAD(P)H. Furthermore, we were able to distinguish between radiotherapy-treated dogs and untreated control animals by combining optical characteristics of uEVs, such as the optical redox ratio, the number of uEVs, and the intensity of the third harmonic generation. Lastly, the cumulative radiation dose given to the dogs was reflected in the optical properties of the uEVs.

Summary/Conclusion: The effect of non-lethal radiation treatment on the metabolites of canine TCC cell-derived EVs and uEVs was measured. The irradiation dose was reflected in the label-free optical characteristics of metabolites in EVs. This study demonstrated the potential of using uEVs and label-free multimodal multiphoton microscopy to develop a noninvasive, label-free method for dosimetry following radiotherapy in pre-clinical animal models.

Keywords: transitional cell carcinoma, bladder cancer, prostate cancer, dog urine, radiotherapy, linear accelerator, dosimetry, nonlinear optics, two photon autofluorescence, three photon autofluorescence, second harmonic generation, third harmonic generation

PF05.06 | Analysis of extracellular vesicles in urines from prostate cancer patients

Martin E. van Royen[1]; Natasja Dits[2]; Gerald Verhaegh[3]; Volkert van Steijn[4]; Irene Bijnsdorp[5]; Elena S. Martens-Uzunova[2]; Connie Jimenez[6]; Jack Schalken[3]; Guido W. Jenster[2]

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Introduction: Urinary extracellular vesicles (uEVs) are a promising source of biomarkers for detection of prostate cancer (PCa). As a proof of concept for detection of PCa EV surface markers, we quantified uEVs in a large cohort of urines from PCa patients and analyzed CD9/CD63 status of individual uEVs and compared outcome with clinical information.
Methods: Clinical urine samples were collected from men after Digital Rectal Examination (DRE) and stored at -80˚C. EVs in minimally processed urines of men without PCa, with indolent PCa and with significant, aggressive PCa were analyzed in three independent pre-validation and a validation cohort. We used two complementary assays that allow high-throughput quantification and characterization of (individual) EVs. Time Resolved-Fluorescence Immuno Assay (TR-FIA) measures in bulk surface marker levels of captured EVs. Immune-EVQuant assay uses a unique in-gel EV immobilization and fluorescent labeling to identify EV subpopulations based on their surface markers. EV concentrations and CD9/CD63 EV marker status were related to relevant clinical information (serum and urinary PSA, age at diagnosis and prostate volume).
Results: A positive correlation is found between uEV concentration and uPSA, indicating that more prostate fluid in the urine is accompanied with a larger release of prostate EVs. However, total uEV concentration, uEV marker (CD9/CD63) status of individual markers in TR-FIA analysis and combined in EVQuant did not consistently provided enrichment in urines of men with PCa. Interestingly, when corrected uPSA levels as measure for prostatic fluid in the urine samples, several marker combinations in uEVs were consistently higher for men with PCa in both assays. This indicates uPSA as strong correction factor for uEV analysis, reducing the large variability of the measured markers among urine samples.
Summary/Conclusion: The complementary TR-FIA and EVQuant assays have shown to be able to rapidly quantify uEVs in minimally processed clinical urine samples, and characterize their marker status with high sensitivity and in high throughput. However, EV markers CD9 and CD63 alone do not show diagnostic value. Only after correction for prostatic fluid levels in urine, several (CD9/CD63) marker combinations correlate with presence of (significant) PCa.
Funding: Supported by IMMPROVE Alpe d'HuZes grant of the Dutch Cancer Society (EMCR2015-8022).
Keywords: urinary EVs, prostate cancer, biomarkers, CD9, CD63

PF05.07 | Proteomic analysis of urinary extracellular vesicles indicates breast cancer subtypes for non-invasive screening: Pilot study

Rassanee Bissanum[1]; Nilobon Jeanmard[2]; Hutcha Sriplung[3]; Sawanya Charoenlappanit[4]; Sittiruk Roytrakul[5]; Raphatphorn Navakanitworakul[6]
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Introduction: Breast cancer (BC) is a complex heterogeneous disease which is a leading cause of death cancer mortality in Thai women and worldwide. The higher mortality rate may be caused from the poorer screening and treatment practices that limited in low and middle-income countries. Thus, the aim of our study is to identify potential proteins found in urinary extracellular vesicles (uEVs) for non-invasive screening detection that can differentiate healthy women from breast cancer patients with different subtypes.
Methods: Urine samples were collected from BC patients (n = 47) with Luminal A, Luminal B, HER2, TNBC and healthy controls (n = 29). uEVs were isolated by using differential ultracentrifugation and characterized by Western blotting and transmission electron microscope. We performed a proteomic profiling of uEVs through LC-MS/MS. Differentially expressed proteins (DEPs) among BC subtypes were analyzed. The DEPs with significantly up-regulated (Fold change >4; p-value< 0.05) were selected as inputs for Gene Ontology and pathway enrichment analysis using Metascape.
Results: A heat map and a principal component analysis presented a different pattern of uEVs protein expression in each BC subtypes (normalized with healthy controls). We identified 752, 154, 619, and 587 unique exosomal proteins in Luminal A, Luminal B, HER2, and TNBC, respectively. The function analysis of unique exosomal proteins and DEPs of each subtype were mainly enriched in various cancer related pathways.
Summary/Conclusion: Our data provide unique uEV proteins that specific to each subtype of BC, hence, these proteins would be potential biomarkers for breast cancer screening. However, validation with larger cohort of breast cancer subtypes is necessary to address in the further study.
Keywords: breast cancer, urinary extracellular vesicles, proteomic analysis, molecular subtyping

PF05.08 | Comparative proteomic profiling of urinary extracellular vesicles and urine distinguishes between breast cancer patients and healthy women

Nilobon Jeanmard[1]; Rassanee Bissanum[2]; Hutcha Sriplung[3]; Sawanya Charoenlappanit[4]; Sittiruk Roytrakul[5]; Raphatphorn Navakanitworakul[6]
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Introduction: Urinary extracellular vesicles (uEVs) emerged as a non-invasive approach to reflect the biological conditions of the producing cells, and allow us to better understand cancer progression. Herein, we aims to quantify concentration and investigate protein profiling of uEVs in BC patients compared to healthy controls (CT).
Methods: Urine samples were collected from 29 CT and 47 BC patients. uEVs were isolated by using differential ultracentrifugation, and were then characterized by Western blotting and transmission electron microscopy. Nanoparticle tracking analysis (NTA) were used to measure the concentration and size distribution of urine particles and uEVs. The proteomic profiling of the uEVs was facilitated through LC-MS/MS.
Results: Our finding showed that the uEV concentration was not significantly different between the assessed groups. The proteomic analysis revealed 15,473 and 11,278 proteins in BC and CT groups, respectively. Furthermore, heat map analysis showed differential protein expression while a principal component analysis highlighted two clusters. The volcano plotting indicated 259 significantly differentially expressed proteins (DEPs) which were 155 up- and 104 down-regulated proteins regulated proteins in uEVs deriving from BC patients compared with CT. Remarkably, the up-regulated DEPs contained proteins such as periostin, the ATPase family AAA domain-containing protein 2 (ATAD2), and the breast carcinoma-amplified sequence 4 (BCAS4); proteins that are highly expressed in breast tumors and BC cell lines and that, along with Ki-67, often act as a marker of cell proliferation. Moreover, BC-derived uEVs enriched protein in pathways related to cancer progression (i.e., cell proliferation, cell survival, cell cycle, cell migration, carbohydrate metabolism, and angiogenesis).
Summary/Conclusion: Consequently, our study has discovered numerous uEV proteins which differential expression between BC patients and CT patients, providing additional evidence for screening biomarker discovery in BC.
Keywords: breast cancer, proteomic profiling, urinary extracellular vesicles, urine

PF06: Outreach and Knowledge Synthesis

Location: Hall 4A

16:00 - 18:00

PF06.01 | Integrating analytical technologies for extracellular vesicle research

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Introduction: Research on extracellular vesicles (EVs) and particles (EPs) continues to expand, constantly pushing against analytical barriers. Heterogeneity of EVs/EPs and their inner complexity render traditional approaches insufficient to fully map, characterize and understand their landscapes across biological systems. While multiple technologies have evolved to address this gap, integrated broad-spectrum assemblies of required instrumentation remain rare. Here we describe an institutional initiative, the Center for Applied Nanomedicine (CAN), as a comprehensive instrumental pipeline for EV/EP studies at the Research Institute of the McGill University Health Centre (RI-MUHC) in Montreal, Canada.

Methods: The instrumentation forming the CAN platform includes nanoparticle tracking analysis (NTA), micro-fluidic resistive pulse sensing (MRPS), nano-flow cytometry (nFC), imaging flow cytometry (iFC), chip-based fluorescent imaging (ChipFI), fluorescence-activated vesicle sorting (FAVS) and super-resolution microscopy (single-molecule localization microscopy; SMLM). Funding was provided by Canada Foundation for Innovation (CFI) and instruments were integrated with RIMUHC Technology Platforms.
Results: The CAN analytical pipeline includes all steps of EV/EP preparation, analysis and downstream applications. A preparation suite is equipped with isolation and purification equipment (Ultracentrifugation, SEC columns), and a profiling suite features an array of orthogonal NTA and MRPS profilers (NS300, PMX120, nCS1) allowing for enumeration, sizing and zetapotential measurement. Molecular maps of individual EV/EPs can be subsequently built using nFC (CytoFLEX), iFC (ImageStream) and ChipFI (ExoView R200) instruments, with further validation through super-resolution microscopy (Nanoimager), electron microscopy and other technologies. Finally, EV/EP sorting capacity is being actively developed at CAN (CytoFLEX SRT). This would allow to further focus on EV/EP subsets of interest, characterize them, and better elucidate their functions and involvement in various physiological/pathological contexts.
Summary/Conclusion: Integrated instrumental platforms, like CAN, incorporating versatile analytical workflows may offer unique insights into complexity of EV/EP landscapes in health and disease.
Funding: Canada Foundation for Innovation (CFI).
Keywords: extracellular vesicles, extracellular particles, EVs, EPs, nanoFlow, FAVS, NTA, MRPS, Super-resolution microscopy, Single-molecule localization microscopy, SMLM, Nanoimager, EV/EP sorting, NS300, PMX120, nCS1, ExoView, CytoFLEX, CytoFLEX SRT, ImageStream

PF06.02 | Towards rigor and standardization of bacterial extracellular vesicle research

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Introduction: Gram-negative and Gram-positive bacteria release nanometer-sized membrane vesicles enriched with bioactive proteins, lipids, nucleic acids, metabolites and virulence factors into the extracellular environment. The International Society for Extracellular Vesicles (ISEV) has previously issued consensus guidelines on EV (Théry et al., JEV, 2018). While the general principles of these MISEV guidelines are applicable, research on bacterial extracellular vesicles (BEV) encounters specific challenges and requires tailored recommendations to ensure rigor and standardization.
Methods: We conducted a systematic analysis following the PRISMA guidelines of all manuscripts (n = 885), published between 2015 and 2021, preparing and analysing BEV from biological samples. We submitted experimental parameters to the EV-TRACK knowledgebase (http://evtrack.org). Publications that included multiple sample types or EV preparation protocols were divided into multiple entries, resulting in over 3000 experiments. To assess current practice and rigor in BEV experiments, we performed an in-depth analysis of recorded experimental parameters related to sample type, EV separation and characterization methods.
Results: We revealed a high diversity in nomenclature, study aim, species of origin, sample types, separation methods, characterization methods, and storage protocols used for BEV research. Cell culture supernatant was mostly used as BEV source, while only in few experiments BEV were harvested from body fluids. Different separation methods enrich for various BEV subtypes with diverse composition and purity. In most of the experiments (differential) (ultra)centrifugation was included in the separation protocol, but with variable parameters and in combination with different techniques. Characterization of BEV was often limited to particle analysis or not provided. Based on this analysis, we identified BEV-specific experimental parameters for inclusion and centralized the knowledge on BEV biology and methodology in the EV-TRACK knowledgebase to support data queries and coach BEV researchers.
Summary/Conclusion: The high heterogeneity in BEV research revealed by this analysis demonstrates the need for experimental and reporting guidelines to facilitate interpretation and reproducibility of BEV experiments.
Funding: This work was supported by FWO, ERC, and Ghent University.
Keywords: extracellular vesicles, bacteria, outer membrane vesicles, systematic review, rigor, standardization, databases

PF06.03 | Evolution of the extracellular vesicle club (EVClub) and video archiving: education, networking, and post-publication peer review

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Introduction: The "extracellular vesicle club", or EVClub, is an education, networking, and post-publication peer review suite consisting of a weekly virtual journal club event, regular social media and email communications, and video archiving. Established in March, 2020, as many laboratories around the world began to be shut down by the COVID-19 pandemic, EVClub continued to amass followers even after the lifting of COVID restrictions.
Methods: EVClub uses a combination of video conferencing software, data collection by survey, email and social media marketing, and video archiving on YouTube and other platforms.
Results: EVClub achieved its initial objective of bringing EV researchers together during the COVID pandemic restrictions of 2020 and 2021 but has continued to serve the community. Today, EVClub has >4000 email subscribers and >3000 YouTube subscribers. Although average attendance at the live events has declined with the lifting of COVID restrictions, from >120 participants per session (2020-2021) to just under 80 (2022-2023), views of video archives have increased strongly, to more than 100,000 total. In addition to scientific presentations, EVClub features collaborations with allied societies including the Student Network on EVs and SOCRATES, and videos advertising national or regional EV societies and the ISEV journals, JEV and JExBio. Not everything has "worked": for example, a straight-to-video format called "EVTrailers" was first offered in 2022 but received only one submission. Plans for the future of EVClub and its relationship with ISEV will be presented here, including ideas for deeper integration with society journals and rigor initiatives.
Summary/Conclusion: The EVClub will continue to serve the EV community. Feedback and suggestions are encouraged.

PF06.04 | Extracellular RNA (exRNA) analysis of human biofluids derived extracellular particles using different computational tools

Tzu-Yi Chen[1]; Edgar Gonzalez-Kozlova[2]; Taliah Solemani[2]; John Fullard[2]; Susmita Sahoo[2]; Natasha Kyprianou[2]; Alex Charney[2]; Ash Tewari[2]; Panos Roussos[2]; Carlos Cordon-Cardo[2]; Gustavo Stolovitzki[3]; Navneet Dogra[2]
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Introduction: Extracellular RNA (exRNA), identified as part of the extracellular canopy comprised of microvesicles, exosomes, and lipoproteins, has been shown to play a role in cellular crosstalk and pathogenesis. Nevertheless, the differences in short-read aligners and computational tools used in analyses have shown to be correlated with large outcome variability despite similar exRNA experimental setups.
Methods: In this study, we have analyzed ~100 exRNA samples derived from four biofluids (urine, serum, tissue, and cell culture medium) of various disease types (prostate and liver cancer, Alzheimer's, and Parkinson's disease) via three different combinations of isolation methods (UC, UC-DG, SEC, nanoDLD) and library preparations. To address this, we implemented the most common short-read aligners (Bowtie, Bowtie2, BWA, STAR, exceRpt) and downstream bioinformatic tools. Downstream computational analysis and visualizations were done in R using dream, edgeR, ggplot2 packages.
Results: We observed over 80 percent of the unused reads belong to multimapping loci. However, after applying read rescue, approximately 50 percent of which align with intergenic regions. Our results indicate that exRNA is potentially enriched in more non-coding RNA (primarily Y RNA) than previously assumed. These alignment results are compared based on the percent of aligned reads, base mismatches, and run time
Summary/Conclusion: In summary, we present a rigorous comparison of exRNA computational analysis using samples derived from various biofluids, isolation methods, and disease types. Ultimately, we aim to highlight these computational tools' existing bias and ambiguity while providing a reproducible analysis workflow for characterizing exRNA. Overall, we show key considerations for exRNA analyses from various biofluids-derived extracellular particles, as no single tool meets all requirements.
Keywords: exRNA alignment, RNA alignment, smRNAseq, RNAseq, aligners, sequencing

PF07: Cancer Biomarkers 2

Location: Hall 4A

16:00 - 18:00

PF07.01 | Identification of the early diagnostic protein markers for pancreatic cancer using a unique isolation technology of extracellular vesicles

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is the fourth most common cause of cancer-related deaths worldwide. Most PDAC is diagnosed at advanced stages or metastasis, as there are no typical early symptoms. In addition, the low sensitivity and specificity of CA19-9, a biomarker currently available for PDAC, contribute to the late diagnosis of this deadly disease. Biomarkers for early diagnosis of PDAC are highly needed. We recently developed a unique immunoprecipitation method called EViSTEP that utilizes a chelating-based reagent and CD9 and CD63 antibodies that isolates extracellular vesicles (EVs) from body fluids with high purity and high yield. In this study, we sought to identify candidate proteins for early diagnosis of PDAC through proteomic analysis using EViSTEP. We evaluated our method with the most common EVs isolation method, ultracentrifugation (UC), for comparison.
Methods: We prepared EVs from 1000μl healthy control (HC) (n = 15) and PDAC serums (Stage I-IV, n = 19) using EViSTEP and UC. The resulting EVs were digested with trypsin and applied to proteomics on Orbitrap Exploris 480 by data-independent acquisition. Volcano plot analysis was performed to narrow down the PDAC-specific proteins.
Results: EViSTEPTM identified about 5898 proteins while the UC identified about 4189 proteins. The expression intensity of EV markers including CD9, CD63, CD81, TSG101, and ALIX was also higher using EViSTEP. Analyzing a volcano plot to detect differentially expressed proteins in EVs between HC and PDAC, we identified 767 candidate proteins of EVs for PDAC with EViSTEP (p-value < 0.001, fold change ≥ 4). In contrast, only 49 candidate proteins were identified with UC method. Further analysis of these PDAC-specific candidate proteins identified dozens of proteins that are closely related to PDAC.
Summary/Conclusion: EVs proteomics analysis utilizing EViSTEP resulted in higher identification of total proteins, EVs protein markers, and PDAC-specific proteins compared to UC in all cases. The dozens of PDAC-specific candidate proteins identified by EViSTEP have the potential to be valuable for the early diagnosis of PDAC.

PF07.02 | A multiple myeloma plasma extracellular vesicle protein signature for monitoring patient response to daratumumab

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Introduction: Extracellular vesicles (EVs) represent promising non-invasive biomarkers that may aid in the diagnosis and risk-stratification of multiple myeloma (MM). Daratumumab (DARA) is a CD38 antibody approved for treatment of MM, and despite the anti-tumour effects of DARA, the majority of patients eventually relapse. One mechanism of DARA resistance is the upregulation of complement inhibitory proteins CD55 and CD59, and the upregulation of programmed death ligand 1 (PD-L1) which binds to PD-1 on T-cells inducing apoptosis. Our recent published data reveals that EVs from peripheral blood plasma (PB) and bone marrow aspirates (BM) from patients treated with DARA contain EV markers as well as MM-associated CD38, CD55 and CD59, and PD-L1. CD55, CD59 and CD147 were elevated in MM PB EVs relative to healthy PB EVs. In addition, EV PD-L1 levels are associated with patient response to DARA (Brennan et al. 2022, PMID: 36359760). The aim of this study was to expand on these markers within PB EVs to develop an EV protein signature as a non-invasive liquid biopsy for monitoring patient response to DARA
Methods: 61 MM patients treated with DARA and 12 heathy donors participated in the study. Participation was voluntarily, and written informed consent was obtained from all subjects. EV isolation was performed on platelet free PB and BM samples by differential centrifugation and density gradient ultracentrifugation, and were analysed by flow cytometry and mass spectrometry.

Results: Mass spectrometry analysis on a subset of samples revealed that several adhesion proteins are elevated in MM PB EVs relative to healthy PB EVs, and are associated with a poor response to DARA. Furthermore, numerous immune cell markers are elevated in MM PB EVs relative to healthy PB EVs, and are associated with a positive DARA response.
Summary/Conclusion: Overall, this study identifies an EV protein signature with potential as non-invasive liquid biopsy to complement or replace invasive Bone Marrow sampling for monitoring patient response to DARA.
Funding: Funding is acknowledged from the UCD Wellcome Institutional Strategic Support Fund, which was financed jointly by University College Dublin and the SFI-HRB-Wellcome Biomedical Research Partnership (ref. 204844/Z/16/Z). Furthermore, we received grants from Holms Mindelegat (20034), The Region of Southern Denmark (Region Syddanmarks Forksningspulje 2019 19/12124/A233), and the Dagmar Marshalls Foundation (500020), and a private donation from Lars-Erik Houmann Christensen.
Keywords: daratumumab, extracellular vesicles, plasma, bone marrow, multiple myeloma

PF07.03 | Circulating extracellular vesicles drive the metabolic reprogramming in acute myeloid leukemic cells

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Introduction: Acute myeloid leukemia (AML) is an aggressive disease for which less invasive tools are needed to explore disease dynamics and the metabolic state of leukemic stem cells (LSCs). Indeed, metabolic reprogramming is considered one of the main hallmark for AML. In this regard, Extracellular Vesicles (EVs) represent not only a promising tool for liquid biopsy but may reveal a key role in energy metabolism for AML. Along with EV profiling from peripheral blood (PB) versus bone marrow (BM) plasma, we explored the putative metabolic role of EVs on LSC-like cells.
Methods: PB was collected from AML patients at diagnosis (n = 50) and healthy donors (HD, n = 12). EVs were mainly purified from platelet-free PB or BM plasma of AML patients by size-exclusion chromatography (SEC) followed by ultrafiltration and counted by Nanoparticles Tracking Analysis. MACSPlex Exosome Kit was used for screening surface EV markers. Transmission electron microscopy and western blot analysis were also performed. Energy cell metabolism was explored by SCENITH (Single Cell ENergetic metabolism by profilIng Translation inHibition) in LSC-like cells after co-culture with EVs using flow-cytometry.
Results: We developed a study exploiting both circulating EVs and LSC-like cells from AML patients for metabolic exploration. We firstly profiled the surface proteins of isolated EVs to detect their cell origins and to provide a reference map for clinical prognosis. Thus, we observed that AML EVs showed an increase in their size and were mainly enriched in CD44 compared to HD EVs. Also, the depletion in the expression of selected EV markers (such as CD8, CD29, CD31) was associated with high-risk AML disease. interestingly, paired AML BM EVs appeared mostly derived from immune cells. In parallel, we revealed that AML LSC-like cells were highly dependent on glucose and used glycolysis. However, after co-culture with PB EVs, we discovered that EVs enhanced the glutahione content and mitochondrial dependence of AML LSC-like cells suggesting their role in metabolic rewiring.
Summary/Conclusion: Overall, in our work we may identify novel prognostic biomarkers exploiting EV-based liquid biopsy in AML. Of note, we provide evidence for metabolic switch driven by circulating EVs in leukemia.
Funding: The work is funded by Fondazione Umberto Veronesi, the Italian Ministry of Health (RC-2022-2773296) and Bologna AIL.

PF07.04 | Profiling of exosome markers and cargo proteins isolated from various cancer cell line supernatant

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Introduction: The study of exosomes has attracted worldwide research due to its ability to modulate cellular activities, especially in the progression of cancer. Exosomes are membrane vesicles that can deliver lipids, proteins, and nucleic acids from one cell to another through membrane fusion, providing a unique form of cell-to-cell communication. Exosomes have been isolated from

bodily fluids including blood, saliva, urine, milk, and cell supernatant using various isolation methods. Our goal for this study was to examine protein content in exosomes isolated from various cancer cell line supernatant.
Methods: To this end, we at MilliporeSigma, the U.S. and Canada Life Science business of Merck KGaA, Darmstadt, Germany, developed MILLIPLEX® immunoassays to detect cell signaling proteins as well as the exosome markers (CD9, CD63 and CD81), using Luminex® xMAP® technology which allows multiplex detection of proteins in a small volume of sample. Exosomes were isolated from various cancer derived cell lines, including A549 (lung), MCF-7 (breast), COLO 201 (colon), PANC-1 (pancreas) and AsPC-1 (pancreas), using Qiagen exoEasy Maxi kit according to the kit protocol.
Results: The most abundant cell signaling proteins found in these isolated exosomes were ERK and p70S6K, which are important kinases for mitogenesis. Less abundant cell signaling proteins included Ras, JNK and p38, which are also part of the MAPK signaling pathway. In addition, all the isolated exosomes were enriched in CD63 marker, whereas both CD9 and CD81 were less abundant, with highest expression in exosomes isolated from MCF-7 supernatant. As a control, all analytes tested were expressed at various levels in all cancer cell line lysates.
Summary/Conclusion: Using MILLIPLEX® multiplex immunoassays, our data supports research findings that exosomes contain mitogenic signaling components that may enhance progression of cancer.
Funding: salary from MilliporeSigma company.
Keywords: cancer cell supernatant, multiplexing immunoassays, profiling exosome cargo proteins

PF07.05 | A liquid biopsy assay for ultra-sensitive detection of IDH1R132H mutations in plasma from glioma patients using digital PCR (dPCR)

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Introduction: Gliomas are the most common primary malignant brain tumors in adults. About 80% of low-grade gliomas exhibit a diagnostic R132H mutation in the IDH1 gene. This mutation also identifies patients responsive to cytotoxic treatments leading to improved survival. The IDH1R132H dPCR assay described here offers a minimally invasive option for disease diagnosis, treatment stratification and monitoring.
Methods: Our assay improves the detection of IDH1R132H mutations in plasma by examining a combination of tumor cell-free DNA (cfDNA) and tumor RNA (exoRNA) from small extracellular vesicles. The assay suppresses the amplification of wild-type IDH1 targets released from healthy tissues into plasma using allele-specific blockers followed by the selective amplification of IDH1R132H targets via ARMS-dPCR. cfDNA and exoRNA were prepared from 2 mL plasma using the ExoLution™ platform (ExosomeDx, Waltham, MA) and the assay was optimized for the detection of single-copy targets. We also compared dPCR on the Bio-Rad ddPCR and Qiagen's QIAcuity™ dPCR instruments, measured the limit of detection using synthetic targets spiked into normal plasma, and assessed performance in a cohort of 10 clinical samples with known IDH1 mutation status per an IDH1 SNAPSHOT assay.
Results: The assay detected IDH1R132H in clinical samples with 100% sensitivity and 100% specificity. The limit of detection was 5 or 10 copies for the Bio-Rad and the Qiagen instruments, respectively (5-10 mutant copies in 18,000 WT copies, 0.06%-0.03% mutant allele fraction). These results resemble the sensitivity of IDH1R132H detection in cerebrospinal fluid, making plasma a promising biofluid for diagnostics.
Summary/Conclusion: Once validated, our assay for ultra-sensitive detection of rare IDH1R132H mutations in plasma from patients with low-grade gliomas could provide a minimally invasive method for the diagnosis and assessment of gliomas.
Funding: This work was supported by NIH Grant 5U01CA230697.
Keywords: glioma, IDH1, digital PCR, plasma, rare-mutation detection, biomarkers, cancer, small extracellular vesicles

PF07.06 | Mutated proteome analysis of circulating extracellular vesicles enabled sensitive and effective liquid biopsy for renal cell cancer

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Introduction: Cancer cell-derived EVs are considered to have a great potential as biomarker carriers. Since no effective biomarker is available for diagnosis of clear cell renal cell carcinoma (ccRCC) in clinical use, we aimed to develop a novel liquid biopsy method for ccRCC by targeting mutated proteins encapsulated in circulating EVs.

Methods: The ccRCC and matched normal tissues were collected from 11 patients who received partial or radical nephrectomy at the University of Tokyo Hospital. Whole exome sequence analysis was performed using tissue samples to construct personalized amino acid sequence databases containing somatic mutations (neo-sequences) by the R package, Neoantimon. The tissues were then analyzed by Orbitrap Fusion Lumos-FAIMS Pro LC/MS system to identify mutated proteins. We also constructed the multiple reaction monitoring (MRM)-based absolute quantification method for detection of the ccRCC mutated protein panel and analyzed EVs isolated from plasma samples collected from each patient before and 2–4 months after surgery.
Results: Whole exome sequence analysis of cancer tissues and matched normal samples identified 63.5 nonsynonymous and 16.0 frameshift mutations per sample on average. Subsequent mutated proteome analysis identified 11,417 proteins (FDR < 0.01), in which, importantly, 3 mutated proteins were included. Further absolute quantification measurement of the mutated protein panel for pre/post operative plasma EV samples showed that drastic reduction or complete disappearance of EV mutated proteins were observed in all post-operative cases.
Summary/Conclusion: Cancer-specific mutated proteins were detectable in plasma or urine EVs, suggesting that our circulating mutated protein-based liquid biopsy could serve as an effective tool for diagnosis of kidney cancer detection or monitoring.

PF07.09 | Circulating extracellular vesicles in the identification of immune checkpoint associated with lung cancer

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Introduction: Immunotherapy has been one of the significant advances in recent years for the treatment of advanced tumors. This work aims to detect and block ICPs in Non-small cell lung cancer (NSCLC) patients. We recently developed a new methodology that identifies several ICPs at the surface of EVs in single-liquid biopsies. First, patients treated by immunotherapy will be profiled to track the emergence of resistance due to ICP overexpression. Having identified novel EV-derived ICP candidates, we will determine if EVs can be used for immune checkpoint blockade.
Methods: EVs were isolated from plasma by ultracentrifugation method and characterized (NTA, western blotting, and DLS). Ethical approval was obtained from B707201422832;2021/212. ICP will be identified via an analysis of EV surface makers using the MAGPIX platform. Endothelial EVs were electroporated with siRNA targeting programmed death ligand 1 (PD-L1) to obtain ICP blockade. Effects on pro-tumoral properties were assessed through several functional tests (proliferation, migration, adhesion, survival, invasion) and in vivo using the TC1-xenograft mice model.
Results: As a proof of concept, we designed our assay to detect 6 ICP-EV in the bloodstream of patients (LAG-3, PD-1, PD-L1, TIM-3, TIGIT, VISTA). We will use this technology to monitor the ICP-EV profile in patients undergoing immunotherapeutic treatment at three different time points (before treatment, after 3 and 6 months of treatment). We have studied the effects of blocking PD-L1 in cancer cells. We confirmed PD-L1 inhibition in the targeted cells with the CARGO-EV treatment. PD-L1 downregulation affects pro-tumoral properties of lung cancer models in vitro and in vivo.
Summary/Conclusion: We present a new method for circulating ICP-EV characterization in lung cancer patients. This study also shows that modified endothelial EV impairs tumor growth in the TC-1 lung cancer model.
Funding: This work is supported by ULiege, CHU, and Walloon Region.
Keywords: immunotherapy, lung cancer, biomarkers

PF07.11 | Early detection of colorectal cancer with extracellular vesicles associated markers (CD151-CD63) in human serum by europium-nanoparticle (EuNP)-based assay

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Introduction: Colorectal cancer (CRC) is one of the most common cancers globally. Of concern, CRC is increasing most rapidly in in the younger population. Early detection can improve the chance for curative treatment. Current non-invasive techniques or circulating biomarkers are unspecific for use in screening or early detection, including CEA and CA 19-9. Of note, specific

glycoconjugates (sugar-based molecules) may enhance detection of known biomarkers. Such glycoconjugates are thought to be associated with the development and progression of CRC. The aim of this study was to investigate specific glycoconjugates in the serum for potential early indication of CRC.

Methods: The technical approach is based on the use of fluorescent nanoparticles to detect specific glycoconjugates (sugar-based molecules) associated with extracellular vesicles (EVs) directly from patients' sera. EVs are isolated with SEC (qEV, Izon) from cell culture and sera were immobilized using monoclonal antibodies specific to CD63 and CD151 and detected with the use of antibodies coated onto europium-doped nanoparticles. The most promising subpopulations of EVs expressing tetraspanins (CD9, CD63, CD81, and CD151) found from the cell culture were clinically evaluated with a panel of serum samples including early-stage CRC patients (n = 31), benign condition (n = 22), and healthy control (n = 18).

Results: The majority of CRC cell lines expressed tetraspanin sub-populations and also glycovariants of integrins and conventional tumor markers (CA19-9, CEA). A subpopulation of CD151 in combination with CD63 (CD151CD63) was found to be significantly ($p = 0.000054$) elevated in CRC and benign patients and was able to significantly discriminate ($p = 0.00011$) between healthy controls and CRC patients.

Summary/Conclusion: The use of nanoparticles for the detection of colorectal cancer-associated EVs (tetraspanins) in human serum holds promise as a tool for the early detection and diagnosis of the disease.

PF07.13 | Early detection of colorectal cancer with extracellular vesicles associated markers (CD151-CD63) in human serum by europium-nanoparticle (EuNP)-based assay

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Introduction: Colorectal cancer is a type of cancer that affects the colon and rectum. It is the third most common cancer in men and the second most common cancer in women. Early detection of colorectal cancer can significantly improve the chances for a successful treatment. Our technical approach is based on the use of fluorescent nanoparticles to detect specific glycoconjugates (sugar-based molecules) associated with extracellular vesicles directly from patient serum. These glycoconjugates are thought to be associated with the development and progression of colorectal cancer, and their presence in the serum may be an early indication of the presence of the disease.

Methods: EVs isolated with SEC (qEV, Izon) from cell culture and serum were immobilized using monoclonal antibodies specific to CD63 and CD151 and detected with the use of antibodies coated onto europium-doped nanoparticles. The most promising subpopulations of EVs expressing tetraspanins (CD9, CD63, CD81, and CD151) found from the cell culture were clinically evaluated with a panel of serum samples including early-stage CRC patients (n = 31), benign condition (n = 22), and healthy control (n = 18).

Results: The majority of CRC cell lines expressed tetraspanin sub-populations and also glycovariants of integrins and conventional tumor markers (CA19-9, CEA). A subpopulation of CD151 in combination with CD63 (CD151CD63) was found to be significantly ($p = 0.000054$) elevated in CRC and benign patients and was able to significantly discriminate ($p = 0.00011$) between healthy controls and CRC patients.

Summary/Conclusion: The use of nanoparticles for the detection of colorectal cancer-associated EVs (tetraspanins) in human serum holds promise as a tool for the early detection and diagnosis of the disease.

PF07.14 | High-throughput enrichment and analysis of Extracellular Vesicles for the detection of Prostate Cancer

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Introduction: Prostate Cancer (PCa) is the most diagnosed urologic cancer. Existing PCa tests have their own set of limitations. Thus, new methods are needed to diagnose PCa from benign conditions. The aim of this study was to explore whether the enrichment of extracellular vesicles (EVs) by a high throughput method (FastEV™) could improve access to PCa biomarkers in combination with a simple nanoparticle-aided time-resolved fluorescence immunoassay (TRFIA).
Methods: The EV- and protein enriched (PE)- fractions from the serum of PCa and benign prostate hyperplasia (BPH) patients were isolated with FastEV workflow using six different isolation conditions. The optimal conditions for isolation were determined by analyzing the EV- and PE-fractions for the enrichment of different protein and glycan biomarkers. Among six FastEV conditions, three were selected for EV enrichment for individual PCa (n = 30) and benign (n = 30) serum samples. This study was conducted following the guidelines of Helsinki Declaration. However, the separated EV- and PE- fractions were passively immobilized on microtiter wells and the biomarkers were detected using europium doped nanoparticles (polystyrene beads doped with ~30,000 Eu3+ chelates) conjugated with either antibodies or lectins.
Results: We have found differential enrichment of several protein markers (e.g., PSMA, CA15-3, ITGA4) and glycan signatures (MGL and WGA lectin binding) when the EV and PE- fractions were compared. Initial testing with pooled samples showed over 10-times enrichment of some biomarkers, such as PSMA, to the EV fraction of PCa in comparison to BPH. From the individual patient samples, we found that the lectin MGL could significantly discriminate PCa compared to BPH ($p = 0.036$).
Summary/Conclusion: This study suggests that a high-throughput isolation with FastEV™ in combing with TRFIA may be used to identify PCa patients from clinically challenging BPH conditions. However, larger cohorts of samples with optimized assay conditions need to be conducted to confirm the results.
Keywords: high-throughput isolation (FastEV), prostate cancer, TRFIA, biomarker, lectin, PSMA

PF08: Eye and Liver EVs

Location: Hall 4A

16:00 - 18:00

PF08.02 | Retina targeting CRISPR-Cas9 system encapsulated in exosome

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Introduction: The wide heterogeneity of genetic causes reside in retinitis pigmentosa(RP). Most common form of disease is eys shut homolog(EYS) autosomal recessive mutation. zebrafish can be used as an ideal model to study eye disease. The zebrafish retina resembles human retina demonstrating similar arrangement of cells. Zebrafish has similar ocular development in humans and vertebrates.
EYS mutation in zebrafish can address RP more critically, needs Crucial egineering. Here we present knock-out system with exosomes. To avoide immune responses as Large size vector of CRISPR-Cas9 in vivo and establish tissue specific knock out, we electroporated CRISPR-Cas9 system in exosome. Exosomes extracted from tissue-specific have different surface protein homologous to origin cell, which makes possible cell-to-cell communication, and does not induce immune responses.
We established delivering Cas9 RNP system encapsulating into exosome for retina tissue specific knockout model
Methods: For retinal pigment epithelial cell-secreted exosome isolation, exosome-depleted FBS was prepared by ultracentrifugation at 100,000g, 4°C for 20 hours. retinal pigment epithelial cells(RPE) were cultured in DMEM containing exosome-depleted FBS. Then, exosomes were isolated from the culture media using differential centrifugation. The exosomal protein was quantified by BCA assay and the presence of exosome was determined by western blot analysis. Cas9 proteins were and dgRNA were thoroughly combined to create Cas9 RNP complexes. Then, RNP complexes were loaded onto exosome using electroporation. Exosome RNP complexes were added into RPE culture medium to investigate the delivery of exosomal RNP. After incubation, the effectiveness of gene targeting was confirmed by western blot. For tissue targeting, exosome RNP complexes were injected into retina tissue were collected for further gene targeting analysis.
Results: We characterized the RPE-derived exosomes expressing exosome specific markers but not the Golgi apparatus-associated protein. The delivery of RNP targeted retina tissue resulted in tissue specific null phenotype. Targeting EYS gene with RNP encapsulated to exosome identified as reasonable model with higher effiency.Additionally EYS knock out caused a abnormal stricture in retina
Summary/Conclusion: In this study, we established Retinitis Pigmentosa disease models with RNP-based CRISPR-Cas9. RNP encalsulated in exosomes secreted from RPE and transported to retina successfully
Funding: This work has been done by Molecular & Medical Genomics lab of Ji Eun Lee in Sungkyunkwan University and supported by the National Research Foundation of Korea government's MSIP (2021R1A4A2001389 and 2021R1A2C3004572 to J.E.L).

PF08.03 | Isolation and characterization of ocular EVs obtained from human conjunctival epithelial and stromal cells

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Introduction: The conjunctiva is the mucous membrane that provides mechanical, sensory, and immune protection for the ocular surface. The aim of this study is to characterize and compare EVs obtained from conjunctival cells and from adipose tissue (AT) mesenchymal stromal cells (MSCs).
Methods: EVs were obtained from 48 h secretome of 3 cell types: the human conjunctival epithelial cell line IM-HConEpiC, conjunctival MSCs (Conj-MSCs) isolated from cadaveric donor tissue, and AT-MSCs obtained from lipoaspirates, with ethical approval. EVs were isolated by differential centrifugation and ultracentrifugation to generate 2K, 10K, and 100K EV fractions. The EV subpopulations in each fraction were analyzed by nanoflow cytometry using a Beckman Coulter CytoFLEX LX flow cytometer. The levels of the EV markers CD9, CD63, CD81 and CD147 were compared by EV-Bead Conjugated Flow Cytometry. 100K EV fractions were analyzed by atomic force microscopy to study EV morphology, and their protein cargo were studied by mass spectrometry. The effect of MSC-EVs on cell proliferation and oxidative stress was tested on IM-HConEpiC.
Results: The majority of EVs had a violet side scatter (VSSC) in the range of 80–110nm polystyrene beads (180-500nm EVs by Rosetta system analyses), while the proportion of large EVs varied between the cells. All markers were present in the EV fractions. CD63 and CD81 were enriched in the 100K fractions relative to the 2K and 10K fractions. Mass spectrometry results showed the presence of a variety of proteins in the three cell types EVs, with some of them being especially relevant in ocular surface pathophysiology, such as thrombospondin 1 or IL-23 receptor. MSC-EVs significantly reduced oxidative stress on IM-HConEpiC.
Summary/Conclusion: Human conjunctival EVs showed differences depending on cell origin (epithelial or stromal). Conj-MSC-EVs are similar to AT-MSC-EVs and both showed antioxidant effects on IM-HConEpiC. These results suggest an interesting potential therapeutic effect of Conj-MSCs derived EVs.
Funding: This work was supported by Ministerio de Ciencia, Innovación y Universidades (MCIU), Agencia Estatal de Investigación (AEI) and Fondo Europeo de Desarrollo Regional (FEDER), Grant number RTI2018–094071-B-C21. LG-P was funded by the Postdoctoral contracts 2017 call (Universidad de Valladolid). IR-C was supported by the "Ministerio de Ciencia e Innovación" PRE2019-089985 predoctoral contract.
Keywords: ocular surface, EV subpopulations, nanoflow cytometry

PF08.05 | Human bone marrow mesenchymal stem cells (hBM-MSCs) cultured in 3D environments produced extracellular vesicles (EVs) with increased nerve regenerative properties in wounded corneas

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Introduction: Painful ocular surface sensitivity provoked by damaged corneal nerves necessitates prompt action for restoration of nerve innervation in the cornea. However, the lack of effective treatments created imminent demand for novel therapies. We have shown that EVs can trigger in vitro nerve regeneration. In this study, we compared in vivo nerve regenerative capacity of EVs obtained from hBM-MSCs cultured in two environments (i.e., 2D monolayer flasks and 3D bioreactors.).
Methods: Cells were cultured in 2D monolayer flasks and 3D bioreactors. Condition media was ultracentrifuged to obtain 2D and 3D-EVs. NanoSight and ExoView analyses were utilized to characterize EVs. The corneal nerve injury model on BL6 mice was established by a 2 mm debridement on their corneas. Three 25 μL subconjunctival injections of PBS, 2D, and 3D-EVs were performed on days 0, 2, and 6. Eyeballs were collected from euthanized animals on day 12 for cornea dissection. Confocal microscopy was used to image nerve regeneration on β3-tubulin-stained corneas. Neurolucida analysis was used to evaluate nerve regeneration.
Results: NanoSight and ExoView analyses revealed increased EV production (28-fold) and higher expression of exosome markers (CD63, CD81, and CD91) in the 3D vs. 2D cultures. Size distributions of 3D and 2D-EVs were roughly the same with a significant shift towards more heterogeneity in 3D vs. 2D-EV phenotypes. Utilizing Neurolucida, nerve regeneration induced by 3D-EVs was

evaluated to be 40.62 ± 26.8 mm (almost doubled compared to 2D-EVs and PBS control injections.) Using fluorescein staining, wound closure at 24 h was measured to be 91.7% and 89.9% for 3D and 2D-EVs, respectively compared to 88.2% for the PBS control group.

Summary/Conclusion: Compared to 2D monolayers, 3D cultures significantly enhanced EV production quantity and quality with improved nerve regenerative capacity. Understanding the nerve regeneration mechanisms of EVs will help us develop novel and more robust therapeutics for corneal nerve interventions.

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Keywords: 3D-bioreactor, 3D-culture, in vivo corneal wound healing, corneal nerve regeneration, ocular sensitivity

PF08.06 | Plasma extracellular vesicles as mediators of liver fibrosis in alpha-1 antitrypsin deficiency

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Introduction: Alpha-1 antitrypsin deficiency (AATD) is characterized by reduced circulating levels of α-1 antitrypsin. The common mutation responsible for this genetic disease is the Z allele which is associated with accumulation of α -1 antitrypsin in the hepatocytes. The consequences are deficiency of circulating α-1 antitrypsin and a toxic gain of function in hepatocytes. Affected individuals develop early onset lung inflammation and liver fibrosis. Despite the role of hepatic accumulation of Z α-1 antitrypsin, little is known about the contribution of the extra hepatic signals released from inflamed lungs to dissemination of the liver disease in AATD. Here we investigate the contribution of lung derived neutrophil elastase (NE)-rich EVs as signaling molecules in AATD liver disease.

Methods: To confirm that AATD lung inflammation generates NE-EVs, we treated AATD mice intranasally with either saline or LPS at 40ug/mouse and isolated EV from the Bronchoalveolar Lavage (BAL) and serum. Size and concentration of EV were quantified using Nano sight analysis. Purity and morphology of EV were assessed by Western blot, and electron microscopy. We also isolated plasma EVs from AATD and normal individuals and characterized the concentration and cargo of plasma NE-rich EVs. Later to study the association between NE-EVs and AATD liver disease, we administered NE-EVs intravenously to AATD mice and characterized the liver tissues and compared them with the liver tissues from AATD mice with and without lung disease. We used immune histochemistry, flow cytometry, and RNA-seq to characterize the livers.

Results: Our results indicated that AATD individuals and AATD mice have more circulating NE-rich EVs with a modified pro-inflammatory cargo. We also observed that NE-rich EVs can activate hepatic stellate cells (HSC) in vitro. Our results also indicate that AATD lung inflammation results in infiltration of immune cells within the AATD liver, aggravating liver disease in AATD mice model. Administration of NE-rich EVs also induces infiltration of immune cells within the AATD liver and activates liver immune cells. Furthermore, activation of liver immune cells mediated by NE-rich EVs contribute to AATD liver fibrogenesis by activation of HSC.

Summary/Conclusion: EVs are small membrane-bound vesicles serving as natural carriers of signaling molecules to promote cellular and organ crosstalk. Systemic inflammation and immune complication are mechanisms involved in liver diseases. We determined the contribution of NE-rich EVs released from AATD inflamed lung to AATD liver disease. This findings will potentially provide a rational basis of novel diagnostic and therapeutic strategies that target EVs as a new contributor to liver disease in AATD as well as other inflammatory disorders.

Funding: Alpha-1 Foundation.

PF08.07 | Stem cell-derived engineered intrinsic nanovesicles ameliorate acute liver failure by concurrently orchestrating immunity and regenerative efficacy

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Introduction: Acute liver failure (ALF) is orphan disease that is hard to expect and does not have drugs. The only therapy is liver transplantation. To provide therapeutic options for ALF patients, we developed SBI-102 which are mesenchymal stem cell (MSC) derived extracellular vesicles (EVs) expressing SIRPα (signal regulatory protein alpha) protein on their membrane. We expected that regenerative effect of MSC and enhancing efferocytosis by blocking CD47 would synergy in ALF.

Methods: Protein, particle number, and SIRPa expression were confirmed. Two representative ALF models were used, LPS/D-galN (LPS 10ug/kg and D-galactosamine 700mg/kg) and acetaminophen (APAP, 300mg/kg). SBI-102 were intravenously administered and therapeutic indexes were assessed.
Results: ALF induced liver tissue showed increased expression level of CD47, and APAP treated mouse hepatocytes also over-expressed CD47. SBI-102 exhibited higher CD47 binding effects to APAP treated hepatocytes, and pre-blocking with anti-CD47 antibody significantly reduced binding activity. The therapeutic effect of SBI-102 showed prolonged survival and liver restoring effect against LPS/D-galN induced ALF. Biochemical parameters and pro-inflammatory cytokines (IL-6, TNF-α) were remarkably decreased and kidney, representative complication occurring tissue, structures were normal. Furthermore, SBI-102 reduced liver damage and apoptotic scores in liver tissue when we applied APAP induced ALF model which has pathologically high similarity with human ALF. Also, when we analyzed liver single cell with flow cytometry, SBI-102 alleviated infiltration of pro-inflammatory monocytes, but promoted pro-regenerative monocytes.
Summary/Conclusion: We confirmed that SBI-102 bound to CD47, effectively induced phagocytosis of damaged liver tissue and suppressed inflammation. Liver immune-environmental change promoted regeneration in liver. Collectively, these data provided sufficient support for SBI-102 as a promising ALF therapy.
Keywords: acute liver failure, mesenchymal stem cell, regeneration, SIRPa, CD47

PF08.08 | Extracellular vesicles from pan PPAR agonist-stimulated induced mesenchymal stem cells alleviate liver fibrosis in mice

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Introduction: Liver fibrosis is a chronic liver disease that causes critical health problem associated with high mortality and morbidity. The pathogenesis of liver fibrosis occurs as consequences of multiple factors, making development of an effective therapeutics difficult. As an alternative, mesenchymal stem cells (MSCs) have been applied as a promising therapeutic agent, which possibly result from their paracrine activities. Recent cell and preclinical studies have showed that the extracellular vesicles from MSCs (MSC-EVs) have potential for mitigating various diseases, mostly due to their immune-regulatory, anti-inflammatory, anti-apoptotic, and tissue-regenerative potential. Pan peroxisome proliferator-activated receptor (pan PPAR) agonist has been known for its anti-fibrotic and anti-inflammatory function.
Methods: Extracellular vesicles from pan PPAR agonist-primed iMSCs (pan PPAR-iMSC-EVs) were characterized, and proteomic analysis was performed. Whether pan PPAR-iMSC-EVs can alleviate liver fibrosis was investigated using a liver fibrosis mouse model induced by thioacetamide (TAA). The anti-fibrotic functions of pan PPAR-iMSC-EVs in inhibiting liver fibrosis progression was investigated using liver tissues, human primary hepatic stellate cells, and human primary hepatocytes.
Results: We investigated whether pan PPAR-iMSC-EVs can inhibit the liver fibrosis. TAA-induced liver fibrosis mouse model and relevant cell studies were used. Pan PPAR-iMSC-EVs significantly decreased the expression of pro-fibrogenesis markers and SMAD signaling pathway in fibrotic liver tissues from TAA-induced mice, as well as in TGF-β1-stimulated primary hepatic stellate cells. In addition, increased expression of epithelial-mesenchymal transition (EMT)-related markers by TGF-β1 was remarkably inhibited by pan PPAR-iMSC-EVs in primary hepatocytes.
Summary/Conclusion: Taken together, these findings indicate that pan PPAR-iMSC-EVs has potential to become a cell-free nanotherapeutic agent for liver fibrosis.
Keywords: extracellular vesicle, induced mesenchymal stem cell, pan PPAR agonist, liver fibrosis

PF09: EV Biogenesis and Bioengineering

Location: Hall 4A

16:00 - 18:00

PF09.01 | Identification of a novel consensus motif for selective sorting of circular RNAs into extracellular vesicles

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Introduction: Circular RNAs (circRNAs) are recently appreciated class of regulatory RNAs that are found to be enriched into extracellular vesicles (EVs). Since circRNAs are resistant to exonucleases owing to their closed end shape, it was assumed that cells passively package these into EVs to get rid of them. However, recent work has shown that not all circRNAs are equally likely to be packaged into EVs making us wonder about the mechanism of selective sorting of circRNA cargo for loading into EVs. Although several consensus motifs in microRNA cargo have been explored, the identification of consensus motifs in circRNAs is just beginning to be explored. Here we report our findings of identification of novel consensus motif that is found in circRNAs that are enriched in EVs.
Methods: EVs were isolated and characterized from the culture supernatants of DLD-1 cells as per MISEV guidelines. Total RNA was isolated from these EVs and cells. We performed RNase R Treatment on part of the isolated RNA to enrich circRNAs from both cellular and EV fractions. Libraries were prepared and sent for sequencing. Linear RNA candidates were identified by mapping to human reference genome and circRNAs were identified by the presence of unique back spliced junctions (BSJ) and the data was cross-referenced with existing databases like CircInteractome. The raw data was analyzed for differential enrichment of linear and circRNAs into EVs.
Results: We first looked for reported consensus motif in literature (Zhang et al., RNA biology, 2019) and found it to be absent in about 70% of enriched circRNAs. Interestingly, motif analysis using our data revealed a similarly purine-rich but a novel motif. Importantly, this new motif was found only in 10% of linear RNAs that are enriched in EVs implying that it is exclusive to circRNAs. Similarly, the motif enriched in linear RNAs in EVs was absent in two thirds of circRNAs in EVs.
Summary/Conclusion: Identified motifs seem to be specific for linear or circular RNAs despite originating from the same gene sequence. Future work includes exploring whether the motif enriched in circRNAs preferentially localizes to the unique BSJ of circRNAs.

PF09.03 | Production and biological activity of red blood cell (RBC)-derived extracellular vesicles loaded with a STING agonist

Karine Sénéchal; Marie Châlons-Cottavoz; Diana Piedrahita; Laura Vidault; Nathalie Guerin; Florian Tavernier; Aurore Cleret; Fanny Gallix; Gaëlle Campello; Julie Charro-Perez; Baptiste Martinon; Anaïs Belledant; Safia Arous; Emmanuelle Dufour; Françoise Horand; Karine Charton
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Introduction: Numerous active cargoes require a new drug delivery system (DDS) to improve their therapeutic index. RBC-derived extracellular vesicles (RBCEVs) represent a promising novel DDS. Their intrinsic properties (small size, biocompatibility, natural targeting to immune cells and ease of manufacturing) constitute advantages.
This study aimed to evaluate the feasibility of producing functional cargo-loaded RBCEVs (ERYCEV) from RBCs already loaded using the ERYCAPS® process. Given delivery challenges, STING agonist (STINGa) was selected to demonstrate the potential of the ERYCEV platform.
Methods: STINGa (ADU-S100) encapsulated inside RBCs using hypotonic dialysis encapsulation (ERYCAPS® process) and empty processed RBCs were subjected to starvation and purified by size exclusion chromatography and Amicon 100kD to produce ERYCEV-STINGa and ERYCEV respectively. The yield and size of the resulting RBCEVs were analyzed by nanoparticle tracking analysis. RBCEV markers (Alix, TSG101, CD81, CD235a, CD47 and PS) were determined by western blot or FACS. In vitro RBCEVs uptake by various cell types was assessed using PKH67 or pHrodo labelling. STING activation pathway was evaluated in vitro by THP1-DualTM cells and cytokine release assays.
Results: After purification, the produced RBCEVs are sphere shaped bilayer vesicles with a mean size of 108 nm and 32 μg/mL mean STINGa concentration in ERYCEV-STINGa. Common luminal and surface markers of RBC and EVs were successfully detected. THP-1 and EMT6 cells were able to uptake ERYCEV and THP1-derived macrophages phagocytosed ERYCEV-STINGa. STING pathway was activated by ERYCEV-STINGa and led to IFN-β production.
Summary/Conclusion: Functional STINGa-loaded RBCEVs were produced following the starvation of RBCs pre-loaded using the ERYCAPS® process. This first in-vitro proof of concept supports the development of the ERYCEV platform including further investigation of in vivo pharmacologic properties and additional cargo loading options.

PF09.04 | Biomanufacturing of RNA-containing extracellular vesicles via a hollow fiber bioreactor

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Introduction: Extracellular vesicles (EVs) have emerged as a promising strategy to deliver effector molecules for intercellular signaling. Current approaches for EV production typically rely on 2D cell culture system due to lack of a scalable biomanufacturing platform. However, the low EV production yield from 2D cell culture remains a challenge. In this study, we used commercial hollow fiber bioreactors, which allow cells to grow to high density under 3D-like conditions, to produce high yield of RNA-containing EVs without serum contamination.
Methods: We investigated the production yield and the characteristics of small EVs (sEVs) purified from DLD-1 cells and hTERT-MSCs via commercial hollow fiber bioreactors. sEVs were collected by iodixanol density gradient ultracentrifugation and characterized by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and Western blot analysis. In addition, we evaluated the expression levels of small non-coding RNAs (ncRNAs) in sEVs after treatment with RNase and with or without detergent.
Results: The number of sEVs purified from bioreactors was increased approximately 20-fold compared with that purified from an equivalent volume of conditioned medium from cells cultured in 2D. DLD-1 and hTERT-MSC derived sEVs from hollow fiber bioreactor displayed the expected round shape and size (50-200 nm in diameter) along with EV marker proteins, including TSG101 and CD63. Small ncRNAs in sEVs collected from bioreactors, including U6, miR-100, miR-125b, and let-7a, were resistant to treatment with RNase only, but digested after treatment with RNase and detergent.
Summary/Conclusion: Our results demonstrate that hollow fiber bioreactors can enhance the production of RNA-containing EVs from cells while also preserving the integrity. We are planning to use hollow fiber bioreactors to isolate engineered EVs for developing EV therapeutics
Funding: NSF MCB-2036809, NIH P01CA229123.
Keywords: biomanufacturing, hollow fiber bioreactor, RNA-containing extracellular vesicles

PF09.05 | New perspectives in the removal of protein corona: host define peptides as useful tools in EV surface engineering

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Introduction: Host defense membrane active peptides (HDPs) play important roles as part of the innate immune system from antimicrobial activities, through biofilm inhibition, immunomodulation, tissue repair to wound healing. Some of the HDPs are overexpressed at infection sites where they could disrupt the lipid bilayer of EVs that are either mammalian or bacterial origin, where the former interaction could help molecule distribution in wound healing while the latter could hinder spread of important components of the cell-cell communication in bacterial biofilms. In our initial investigations (Singh et al. 2020), we have observed that some peptides have only minor disrupting effect, but they could directly interact with the surface of the vesicles, removing the surface adsorbed protein corona from them. This phenomenon could potentially be exploited to manipulate protein corona of EVs and other nanoparitcles enabling separation of proteins from different origins. To explore both the potential in vivo role of this phenomenon, and also to progress towards better surface engineering components for EV protein corona modulation, here a set of HDPs were selected and studied with a model system, red blood cell-derived vesicles (REVs) to gain an overview on their interactions and to reach a broader understanding on how they could be used to manipulate EV content.
Methods: The interaction of HDPs with REVs were investigated using flow-linear dichroism, circular and infrared spectrocopy, microscale resistive pulse sensing, microscale thermophoresis, transmission electron microscopy and proteomics analysis. The experimental parameters were submitted to EV-TRACK knowledgebase (EV220311).
Results: Based on these results, HDPs can be categorized based on their action mechanism, some of them removes the surface proteins of vesicles at lower concentrations, or can disrupt the vesicles forming lamellar bilayer structures, or can penetrate without disrupting the membrane. Importantly, the proteomic results enabled us to identify fifteen proteins as external protein corona members.
Summary/Conclusion: The removal of protein corona enables the separation of proteins from different origin: Those adsorbed on the surface, from those located inside the EVs. It is hoped that these results will aid the use of HDPs and related membrane active peptides in surface engineering of EVs and other biological nanoparticles.

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Keywords: protein corona, membrane active peptides, proteomics, membrane biophysics

PF09.06 | Matrix-bound vesicles as tissue-specific extracellular vesicle models for biosensor development

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Introduction: Heart disease remains the leading cause of death in Puerto Rico and the US. There are limited options for monitoring cardiovascular diseases, and current monitoring methods involve high radiation exposure, which has a grade of invasiveness and other side effects. Endothelial extracellular vesicles (end-EVs, CD144+ -endothelial cell marker-) are new biomarkers associated with the prognostic and diagnostic of cardiovascular diseases. We need an end-EV model to develop a selective biosensor; however, end-EVs are found in body fluids in combination with other EVs and biomolecules as a heterogenous population hampering its use as an EV model. Matrix-bound vesicles (MBVs), EVs embedded within the decellularized extracellular matrix (dECM), are a new class of tissue-specific EVs. We hypothesize that MBVs isolated from endothelial tissues (end-MBVs) will be CD144+, playing a pivotal role as an EV model in developing end-EV biosensors. We seek to build an EV biosensor for the selective detection of end-EVs CD144+.
Methods: To accomplish this, we first must i) derive end-dECM from porcine endothelium, ii) isolate end-MBVs, and iii) characterize them as CD144+ to use them as an EV model for biosensor construction. We have developed a method based on sodium deoxycholate to decellularize endothelium–ECM. The MBVs were isolated using dECM solubilization via KCl solution. Then, the MBVs were quantified and characterized using methods validated for exosomes (i.e., nanotracking analysis -NTA-, Exocet, microRNA staining). Finally, an immunoblotting technique (dot blot) was used to detect CD144 in the isolated end-MBVs.
Results: The decellularization protocol developed yielded 20–30 mg of end-dECM. Using a 48 hours long-lasting solubilization, the KCl solution could extract the MBVs embedded within the end-dECM, which were further purified using a qEV column. The MBVs particle size was about 120 nm, characterized using NTA. The MBV concentration was quantified as 10^8 EVs/ uL. The immunoblotting test identified the endothelial protein marker CD144 in the MBVs
Summary/Conclusion: This project provides evidence of the isolation of CD144+ MBVs that will be used as a target model for engineering the endothelial-EV biosensor.
Funding: We acknowledge the startup funding support provided to Dr. Mora-Navarro by the CAWT under NSF grant OIA-1849243.
Keywords: MBVs, dECM

PF09.07 | Exogenous loading of nucleic acids into extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are important intercellular communicators that might be used as delivery vehicles for macromolecular drugs. Yet, challenges remain in manufacturing EV-based drug carriers. Presently, a versatile and efficient drug loading strategy, especially for hydrophilic macromolecular drugs, such as nucleic acids, is still missing. In this work, various exogenous loading strategies were compared head-to-head in terms of loading efficiency and vesicle integrity to find the optimal one.
Methods: EVs were isolated by ultracentrifugation from 3D cultures of mesenchymal stem cells (MSCs). A small (69 nts) RNA aptameric probe and fluorescently labeled small interfering RNA (21 bp) were used as model compounds and were subjected to different exogenous loading methods (e.g., extrusion, electroporation and vesicle hybridization). Unloaded RNA aptamer was removed by degradation with RNase, and loading efficiencies were subsequently analyzed by fluorescence read-out or nano flow

cytometry (nanoFCM). Physicochemical characteristics of MSC-EVs, as well as the biological activity of two membrane enzymes, CD73 and DPP4, were assessed after loading.
Results: Most of the investigated loading methods did not lead to efficient incorporation of the RNA aptamer in EVs, except for the hybridization-based approach. However, nanoFCM experiments revealed that loading efficiencies of commercially available EV hybridization reagents still remained relatively low (< 5 %). Further, we showed that the enzymatic activity of the MSC-EV membrane proteins was downregulated after the hybridization process and that the polydispersity increased, suggesting the formation of aggregates.
Summary/Conclusion: Efficient loading of nucleic acids into EVs remains challenging, with the hybridization-based approach being the most promising one. However, novel formulations with minimal impact on the EV structural and functional characteristics need to be developed.
Funding: This work was supported by the ETH Zurich – Open ETH project SKINTEGRITY.CH.
Keywords: extracellular vesicles, drug delivery, drug loading, method comparison, hybridization, nucleic acids, gene therapy

PF09.08 | Engineered exosomes expressing ICAM-1: A promising targeted delivery system for T cell modifications

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Introduction: Exosomes as a type of extracellular vesicles (EVs) are natural nano-carriers that possess the required crucial features of an ideal biomolecular delivery system. However, using unmodified exosomes may have some limitations such as low accumulation in target sites. Studies have established that most of these hurdles can be overcome by engineering exosomes against different cell surface markers.
Methods: In this study, the newly designed fusion protein of ICAM-1/LAMP2b was initially modeled, and its stability and binding affinity to interact with LFA-1 were assessed by the MD simulation and the Z-dock server, respectively. HEK293T cells were then stably transduced by a lentiviral vector encoding ICAM-1/LAMP2b. The purified exosomes were characterized, and their interaction with recombinant LFA-1 was studied by ELISA and western blot analysis. The uptake of targeted and non-targeted exosomes was also evaluated by imaging and flow cytometry. Lastly, to assess the ability of targeted exosomes to be applied as a safe carrier, pAAVS1-puro-DNR plasmids were encapsulated into exosomes by electroporation and GFP expression in T cells was checked by imaging and flow cytometry.
Results: The bioinformatics study indicated the acceptable binding affinity of ICAM-1 for LFA-1. Then, the HEKT 293 cell line was successfully modified permanently by a lentiviral vector to express ICAM-1 on the surface of the derived exosomes. TEM imaging, DLS, and western blotting confirmed the intact nature of purified exosomes. Furthermore, the ELISA and western blot tests established the binding affinity of targeted exosomes for recombinant LFA-1 with a significant difference from non-targeted exosomes (1.81 OD difference). Furthermore, flow cytometry results revealed noteworthy differences in the binding of LFA-1-positive (54.7%), non-targeted exosomes (18.7%), and targeted exosomes to LFA-1-negative cells (23.2%). Finally, flow cytometry indicated that 19.5 % of T cells were GFP positive after treating them by loaded targeted exosomes.
Summary/Conclusion: Engineered exosomes expressing ICAM-1/LAMP2b fusion protein on their surfaces were produced and isolated. The targeted exosomes were able to efficiently interact with T cells as their recipient cells. These engineered exosomes can be utilized as an ideal targeted delivery system to transfer various biomolecules to T cells, facilitating immunotherapies or other cell-based treatments.
Keywords: lentiviral vector, lymphocyte function-associated antigen-1, intercellular adhesion molecule-1, targeted exosomes

PF09.09 | Development of exogenous loading platforms for functional targeted EV-mediated siRNA therapeutics delivery

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Introduction: The greatest challenge for clinical application of siRNAs is effective, nonimmunogenic, and tissue-specific delivery. Due to the intrinsic capability of extracellular vesicles (EVs) to transfer cargo between cells, they have been leveraged to deliver several types of payloads. Multiple EV-siRNA loading methods exist, however, there is still room to improve the robustness,

efficiency, and scalability of most loading methods. The objective of this study was to develop exogenous EV loading platforms for luminal and surface siRNA loading, aiming for process and loading efficiency, scalability, and EV-mediated potency in vitro and in vivo.
Methods: Targeted engineered EVs were generated by transient transfection of CAP cells. EVs were purified using TFF and CaptoCore700. Modified siRNA was conjugated with Cy5 at the 5' end and (where relevant) either with teg-tocopherol or teg-cholesterol at the 3' end of the sense strand. Electroporation and co-incubation loading conditions were optimised using Design of Experiment (DoE). Loaded samples were further purified to remove unloaded siRNA. EVs were quantified using NTA, siRNA using Spectramax, and in vitro potency using qPCR.
Results: High loading efficiency was achieved using electroporation (100-200 siRNA molecules per EV) and co-incubation (1000-3000 siRNA molecules per EV). Both methods were scalable, and the process efficiency was improved 60-fold due to DoE parameter optimisation. For co-incubation, siRNA with various conjugations were successfully loaded and showed in vitro potency. Loading by electroporation was enhanced by an active endosomal escape strategy for functional cargo delivery. Improved EV-mediated delivery was observed when compared to gymnotic control.
Summary/Conclusion: Two exogenous loading platforms were successfully developed for EV-mediated siRNA delivery and tested in vitro and in vivo. This paves the way for therapeutic delivery of siRNA using EVs.
Funding: Evox Therapeutics.
Keywords: siRNA, exosomes, EV-mediated delivery, exogenous loading, electroporation, co-incubation

PF09.10 | Therapeutic promises of cell-derived vesicles (CDVs) as mRNA delivery platform

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Introduction: Cell-derived vesicles (CDVs) produced by serial extrusion of diverse human cells are emerging as novel delivery vehicles due to their superior biocompatibility and excellent cellular uptake and tissue penetration capabilities. Here, we present an alternative approach for mRNA delivery using CDVs to overcome the immunogenicity and toxicity concerns of synthetic vehicles used in mRNA therapeutics. In addition, the therapeutic potential of mRNA-carrying CDVs was evaluated by examining key characteristics in vitro and in vivo.
Methods: mRNAs were complexed with CDVs via positively-charged lipids. Fluorescent-labeled mRNAs or reporter mRNAs such as luciferase and EGFP were used to determine the potential of CDVs for mRNA delivery. Flow cytometry and luciferase reporter assay estimated mRNA uptake and protein expression level. The in vivo delivery and biodistribution of mRNA-loaded CDVs were assessed by IVIS imaging. Furthermore, acute toxicity and inflammatory response were evaluated by assessing a hematology profile and multiplex cytokine/chemokine panel. All the animal experiments were conducted in comparison with lipid nanoparticles (LNP).
Results: In this study, we first established a method of loading mRNAs to CDVs using cationic lipids. The mRNA-loaded CDVs showed a uniform size distribution with higher than 90 % loading efficiency. We observed that CDVs facilitated the delivery of mRNAs to target cells, resulting in robust protein expression. Additionally, we assessed the biodistribution and toxicity of mRNA-loaded CDVs in mice. The hematology and cytokine analyses did not detect noticeable alterations in CDVs, whereas multiple immunogenic responses were evident in LNP.
Summary/Conclusion: This study demonstrated the potential of CDVs as an mRNA delivery carrier with high loading efficiency and in vivo safety. This finding will facilitate the development of more efficient mRNA-based therapeutics for cancer and other debilitating diseases.

PF09.12 | Extracellular vesicles(EVs)-loaded photodegradable hydrogel microparticles for on-demand release generated by droplet microfluidics

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Introduction: Mesenchymal stem cells (MSCs)-derived extracellular vesicles (EVs) are known to provide therapeutic benefits. However, EVs are rapidly diluted by the circulation of bodily fluids when injected into the body, so hydrogels are emerging as carriers of EVs for improvement of the delivery efficiency to the target. Photodegradable hydrogels have the advantage of being

able to control the release of EVs by adjusting hydrogel degradation through UV light irradiation. However, a high dose of UV exposure is needed for the decomposition of bulk hydrogels and the release of EVs, resulting in damage to both therapeutic targets and EVs. So, the microparticle-type hydrogel is required for the optimal delivery of EVs.
Methods: The EVs were isolated from MSCs culture media by size-based ultrafiltration and characterized using western blot, TEM, and NTA. We developed a droplet microfluidic device for the creation of EVs-loaded photodegradable hydrogel microparticles. The microfluidic device consists of three inlets, resistance channels, a serpentine channel, and an outlet. Prepolymer solution, a crosslinking agent with EVs, and oil are injected into each inlet. After the EVs-loaded hydrogel droplets are generated at the junction, the serpentine channel allows for effective solution mixing within each droplet. These droplets are harvested from the outlet.
Results: The photodegradable hydrogel microparticle-loaded EVs has a consistent diameter of 100 μm with nanometer-scaled pores, which can enhance the EVs retention. They were degraded and all the loaded EVs were successfully conveyed to a target within 7 minutes. After UV irradiation, the microparticle-type hydrogel transferred EVs to cells more efficiently than the bulk hydrogel under the same UV conditions, increasing the cell proliferation rate.
Summary/Conclusion: Our proposed photodegradable hydrogel microparticle-loaded EVs can be used for the improvement of clinical outcomes by getting around the drawbacks of traditional therapeutic EVs delivery.
Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2021R1C1C2007646, No. 2021R1A2C3011254, and No. 2020M3A9I4039045).
Keywords: extracellular vesicles, hydrogel microparticles, droplet microfluidics

PF09.13 | Encapsulating CRISPR/Cas9 system into extracellular vesicles by protein S-palmitoylation

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Introduction: The CRISPR-Cas9 system is a promising technology that revolutionized genome editing applications for the potential treatment of a variety of genetic diseases. However, efficiently delivering the CRISPR-Cas9 components to the target organ or cell remains a significant challenge. Our study aimed to encapsulate CRISPR/Cas9 protein into extracellular vesicles (EVs) by protein S-palmitoylation, which is the reversible addition of fatty acids to the cysteine residues of proteins. It is an important post-translational modification that regulates multiple aspects of protein function, including the localization to membranes, intracellular trafficking, protein interactions, protein stability, and protein conformation. In this study, we demonstrate that S-palmitoylation proteins were preferentially encapsulated into EVs.
Methods: We obtained the peptide sequence from the N-terminus of a few membrane-related proteins, which is the ideal substrate for S-acyltransferase, the enzyme that catalyzes S-palmitoylation. We fused those peptides onto the N-terminus of both eGFP and SpCas9, which promoted the palmitoylation and encapsulation of those proteins into EVs. The encapsulation efficiency of palmitoylated eGFP was verified by flow cytometry and western blotting. Meanwhile, the packaging efficiency of SpCas9 was verified in reporter cells.
Results: The palmitoylation modification permitted 87.2% of eGFP entry into EVs. The N-terminus palmitoylation of SpCas9 did not affect its activity and was successfully encapsulated into EVs. EVs coated with VSV-G encapsulating palmitoylated SpCas9/sgRNA complex restored 12.3% mCherry expression from the Ai9 reporter cells.
Summary/Conclusion: Our study provides a novel approach to encapsulating CRISPR/Cas9-sgRNA complex into EVs. This may open an effective avenue for using EVs as vehicles to deliver CRISPR/Cas9 for genome editing.

PF09.14 | Function of the MBsome as an innovative mRNA carrier

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Introduction: The midbody some (MBsome), once thought to be a remnant of cell division, is now known to play a key role in cytokinesis. It was thought that the MBsome is either released into the extracellular space or autophagically degraded by one of its daughter cells. However, recent studies have revealed that MBsomes can be maintained by cells even after cell division is complete, and that they accumulate in the cytoplasm and regulate cell proliferation and survival through integrin and epidermal growth factor receptor-dependent pathways.
Methods: we examined the ability of MBsomes to act as carriers of mRNAs, a novel function that has not been studied.
Results: We found that MBsomes isolated from human lung cancer and stem cells via sucrose cushion ultracentrifugation were 300–400 nm in size and stable for up to 4 days when stored at 4°C. In addition, we confirmed successful expression of the EGFP protein following incubation of the isolated MBsomes with the EGFP mRNA at room temperature.

Summary/Conclusion: These results suggest that MBsomes have the potential to serve as mRNA carriers and therapeutic agents capable of delivering a gene-of-interest.
Funding: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2022R1A2C100780111).
Keywords: MBsome, mRNA carrier, sucrose cushion ultracentrifugation

PF09.16 | Does EV biomolecular corona impact surface engineering?

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Introduction: One strategy to improve EV targeting properties for drug delivery includes EV surface engineering with tissue-specific peptides or protein ligands. This procedure requires the incubation of EVs with the ligand, followed by washing cycles. On the other hand, it has recently been reported EVs immersed in a biofluid containing biomolecules can adsorb proteins on their surface. This phenomenon leads to the formation of a so-called Biomolecular Corona (BC). While BC role and composition in natural biofluids have started to be investigated, its formation and eventual role in EV surface engineering by biomolecules has not been yet studied. We will introduce the problem and present a first investigation of this kind on Red Blood Cell (RBC)-derived EVs engineered with the monoclonal antibody Cetuximab (CTX), the target ligand of EGFR.
Methods: MISEV 2018 compliant EVs, separated from healthy donors' RBCs, were reacted with CTX under conditions leading to the formation of covalent bond (chemisorption, through click chemistry) or weaker non-covalent interactions (physisorption). Both EV sets were evaluated for physicochemical properties, molecular recognition performances, and in vitro cellular uptake.
Results: Both EV sets showed a comparable amount of bound CTX. Surprisingly, the EV set functionalized by CTX physisorption showed the same affinity for EGFR as the EV set functionalized by chemisorption at Surface Plasmon Resonance and microarray assay. Nevertheless, only the chemisorbed EV set showed improved uptake ability by EGFR-positive cells in physiological conditions compared to physisorbed EV set and native EVs.
Summary/Conclusion: CTX physisorbs and chemisorbs with the same efficiency onto EV surface.
CTX physisorbed on EVs bears the same binding affinity of covalently bound CTX towards EGFR however it is not able improve EV uptake in vitro. These findings add new perspectives and approaches to the study of EV biomolecular coronas.
Funding: 2019-08 to 2022-08 | GRANT_NUMBER: 2017E3A2NR_004, Development of a biotechnological nanoparticle platform for the delivery of antitumor therapies using Patient Derived-Organoid library of Breast Cancer, Ministero dell'Istruzione, dell'Università e della Ricerca (Roma, IT).
Keywords: extracellular vesicles, EV corona, cetuximab, EV surface engineering

PF10: EV Separation from Blood

Location: Hall 4A

16:00 - 18:00

PF10.01 | "Immunoaffinity method for isolating blood neuron-derived extracellular vesicles ExoSORT"

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Introduction: Blood extracellular vesicles contain lipids, proteins, and nucleic acids that image, to a certain extent, their cell of origin. Immunoaffinity Isolation of EV subpopulations based on cell-specific surface proteins can provide a liquid biopsy platform with increased specificity.
Methods: An optimized immunoaffinity isolation procedure called ExoSORT was used to screen over 70 antibodies against neuron-specific surface markers. We identified that combining antibodies against GAP43 and NLGN3 provides increased specificity toward neuronal-derived EVs (NDEs). Antibodies against oligodendrocyte and liver-specific EV markers were also identified.
Results: ExoSORT ability to isolate EVs was demonstrated according to MISEV—including electron-microscopy, nanoparticle-tracking-analysis, western-blot, ELISA, FACS analysis for EV markers (CD63, CD9, CD81, and FLOT1), and negative markers (Calnexin, Albumin, and ApoA), and by unbiased proteomic analysis. ExoSORT specificity toward NDEs was demonstrated in comparison to isotype control antibody (IgG) and EV-depleted plasma by measuring neuronal-specific proteins (SYP, NFL, ENO2, proBDNF, and RGMa) and mRNA (NRGN, ENO2, NEFL, and HCRT). Enrichment was also demonstrated by unbiased proteomic analysis and RNAseq. ExoSORT efficiency was shown by spike-in recovery experiments with EVs isolated from IPS-derived neurons and HEK293 cells genetically engineered to express the neuronal markers. ExoSORT precision (CV< 16%) was demonstrated by repeating the isolation over nine independent experiments.
ExoSORT diagnostic potential is demonstrated by measuring alpha-synuclein aSYN and TDP43 in plasma samples from 30 Parkinson's, 25 Lewy-Body Dementia, and 30 Multiple-System Atrophy patients than in 45 healthy controls. Significant differences in aSYN could identify synucleinopathies with over 80% specificity and sensitivity. In addition, NDE-associated TDP43 was significantly elevated in plasma samples from ALS patients.
Summary/Conclusion: ExoSORT is an optimized immunoaffinity EV isolation method that works well for NDEs and potentially for EVs from additional cell types. It has the potential to serve as a novel biomarker platform with increased cell specificity.
Keywords: biomarkers, immunoaffinity, cell-specific

PF10.02 | Removal of lipoproteins from UC- and SEC-treated plasma samples with a ten-minute process via magnetic nanoparticles towards high purity extracellular vesicles

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Introduction: Lipoprotein (LP) in plasma is five-order of magnitude more than that of EV, rendering high purity EV challenging. Much progress has been made with a sequential size- and density-based process, e.g. size exclusion chromatography (SEC) followed by density gradient (DG). The two-step process is required due to the EV-fractions from SEC still contain substantial EV-sized LP, however, DG requires ultracentrifugation (UC) hence time-consuming. Further, stand-alone UC process to isolate EV would co-isolate LP with similar density. Available LP removal reagents mostly use antibody with some success. This study presents a glycan-based approach for high affinity LP removal resulting in purified EV from SEC-or UC-treated plasma samples.
Methods: LipoMin (Reliance Biosciences) with functional magnetic beads was loaded into UC- or SEC-treated samples. After mixing for 10 minutes for LP capture, the supernatant containing purified EV was collected while on a magnetic separator. The purified EV sample was characterized with ApoA1, ApoB and CD81 sandwich ELISA, as well as TEM, NTA and Western blot (WB)
Results: TEM images of UC-LipoMin- and SEC-LipoMin-treated samples showed characteristic cup-shaped and intact EV morphology. NTA results showed particles concentration and distribution comparable with that of SEC-DG-treated samples. WB results revealed significant removal of both ApoA1 and ApoB for both UC-LipoMin- and SEC-LipoMin-treated expressions while substantially preserving EV flotillin and CD81 signatures. Sandwich ELISA results showed SEC-LipoMin-treated ApoB expression decreased by 72% compared to that without LipoMin treatment while CD81 signal is essentially unchanged (within 8%). WB of LipoMin-treated colorectal cancer (CRC) and Alzheimer's disease (AD) plasma samples verified the corresponding EV and clinical markers (CDX2 & CD66a for CRC; ptau217 for AD) are clearly expressed.
Summary/Conclusion: This novel approach requires 10-mins processing for removal of lipoproteins in UC- or SEC-treated plasma samples. LipoMin-treated samples showed robust EV and clinical expressions and should be viable for clinical studies.
Funding: Reliance Biosciences, Taiwan.

PF10.03 | Filtration produces vesicle-like particles beyond a critical concentration of platelets in blood plasma

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Introduction: Processing blood by double centrifugation (2 × 2500g) is common but yields plasma that still contains ~10e5-10e6 platelets/mL, which may affect downstream EV analyses. Remaining platelets can be removed by filtration without affecting EV concentrations. Since a filtration procedure requires additional time, we investigated whether platelet-free plasma can also be prepared by combining single centrifugation and filtration.
Methods: EDTA blood from healthy controls was centrifuged at (1) 2 × 2500g for 15 minutes (no brake; plasma collected to 10 mm above the buffy coat/cell pellet), (2) 1 × 2500g, and (3) 1 × 5000g. Plasma was then filtered using 0.8 um polycarbonate track-edged filters to remove remaining platelets. Concentrations of platelets and EVs (>150 nm; CD61+, CD61+/CD62p+, CD45+, CD235a+) were measured in filtered and unfiltered plasma by calibrated flow cytometry (Cytek Northern Lights and Apogee A60-Micro).
Results: Plasma contained ~10e7 platelets/mL after single centrifugation and ~10e5 platelets/mL after double centrifugation. Filtration removed >98% of remaining platelets for all protocols. However, after single centrifugation, the removal of platelets using filtration led to a 2-fold increase in the concentration of CD61-EVs and an 11-fold increase in CD61/CD62p-EVs, the latter likely derived from activated platelets. The maximum concentration of remaining platelets that can be removed by filtration without generating CD61-EVs was 2 × 10e6 platelets/mL. A linear regression analysis showed that on average 2.5 CD61-EVs are generated for each removed platelet above this platelet concentration. The concentration of CD45-EVs and CD235a-EVs was not affected by filtration.
Summary/Conclusion: The maximum concentration of platelets that can be removed by filtration without generating CD61-EVs within the detection range of the used flow cytometers is 2 × 10e6 platelets/mL plasma, and therefore we recommend to measure the platelet concentration in plasma before filtration.
Funding: This study was funded by Health Holland (AQrate project). EvdP receives funding from the Dutch Research Council (grant number VIDI 19724).
Keywords: blood plasma, extracellular vesicles, filtration, platelet removal

PF10.04 | Non-invasive characterization of prostate cancer by immunoaffinity-based capture of plasma-derived tumor extracellular vesicles

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Introduction: Tumor-derived extracellular vesicles (tdEVs) are promising biomarkers for cancer liquid biopsy. In metastatic prostate cancer, we found that high blood levels of prostate cancer derived EVs (ProstEVs) are associated with reduced levels of tumor-reactive CD8 T cells and rapid disease progression. Our clinical findings support an immunosuppressive function of tdEVs. Thus, there is a need to characterize the molecular cargo of ProstEVs to develop novel therapies that can restore antitumor immunity and improve patient outcome. To that end, we developed a highly specific and efficient method to capture ProstEVs directly from patient blood leading to the first characterization of the ProstEV molecular cargo in prostate cancer.
Methods: Blood was drawn from healthy men and prostate cancer patients following IRB approval. ProstEV-negative platelet-free plasma samples were spiked with 5.0 × 107 EVs/mL of cell line-derived PSMA+-ProstEVs for optimization. ProstEV concentrations were measured using microfluidic resistive pulse sensing and nanoscale flow cytometry. ProstEVs were captured by magnetic bead-based immunoaffinity. Capture efficiency and specificity was analyzed with PSMA antibody and isotype control. Protein content of captured ProstEVs was analyzed by western blotting and mass spectrometry.
Results: PSMA Ab-Mg beads successfully captured 91, 99, 99% of PSMA+ EVs from spiked-plasma samples (N = 3). PSMA-Mg beads also captured PSMA+ EVs (4.3 × 108, 7.7 × 107, 8.3 × 107 EVs/mL) from high-risk prostate cancer patient plasma samples (N = 3). EVs captured by PSMA-Mg beads showed the presence of prostate cancer-associated proteins (PSMA and AMACR), but not from isotype-Mg beads. Proteomic profiling is ongoing and original data will be presented at the annual meeting.

Summary/Conclusion: In conclusion we developed a simple and efficient method to capture tdEVs in prostate cancer blood with high specificity. This work will serve as methodological framework for a new wave of liquid biomarker studies in prostate cancer.

PF10.05 | Evaluation of different blood plasma sEV enrichment methodologies for miRNA next-generation sequencing

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Introduction: Analysis of small extracellular vesicles (sEV) (30-200nm) miRNA is critical in understanding the cascade of cellular signalling pathways in diseases. Next-generation sequencing provides comprehensive miRNA profiles across biological fluids. However, selecting the best sEV miRNA isolation technique for next-generation sequencing is challenging (1). Here, we compare combinations of three sEV miRNA isolation techniques for miRNA next-generation sequencing.
Methods: Blood plasma from dairy cows (n = 4) with similar genetic and physical characteristics were utilized to isolate sEV. Ultracentrifugation (UC) (100,000 x g for 2 hours at 4°C), size-exclusion chromatography (SEC) (qEV 70nm) and ultrafiltration (UF) were used to design four methodological approaches (UC + SEC, SEC + UC, SEC + UF and UC + SEC + UF). All sEV samples were characterized using western blot markers (exosome: Flot-1, CD81, CD9; non-exosome: BSA), Nano-particle tracking analysis (NTA) and transmission electron microscopy (TEM). sEV miRNAs were isolated using TRIzol and miRNeasy mini kit. Novaseq S1 platform was used for single end 100bp sequencing. Read counts were mapped using unitas and the differential expression was performed using edgeR.
Results: All four methods yielded more than 1700 of miRNAs in each and differential miRNA expression demonstrated a distinct separation of miRNA profiles to the control plasma miRNA sample. The PCA determined a clear separation of expression profiles of miRNAs from all four methodological approaches to the control blood plasma circulating miRNA. However, UC+SEC yielded a higher number of sEV (characterized) which generated reliable sEV miRNA next-generation miRNA sequence results.
Summary/Conclusion: Four methodological approaches yielded a satisfactory level of sEV miRNAs, however, the selection of the best sEV miRNA isolation method depends on the downstream miRNA characterization technique. UC + SEC is the most precise and accurate bovine blood plasma sEV miRNA isolation method for next-generation sequencing.
Keywords: sEV, miRNA, ultracentrifugation, size-exclusion chromatography, ultrafiltration

PF10.06 | High purity EV isolation from plasma in ten minutes with a minimum of 50 uL sample

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Introduction: Although many methods have been proposed, high purity and rapid isolation of EV from plasma have been challenging. For example, some polymer-based methods, e.g. ExoQuick, would result in increased viscosity of the sample mixture raising the uncertainty in processing repeatability. Contaminants would also be co-precipitated along with target EVs in some methods. This study presents an optimized polymer-based approach (ExoRich, Reliance Biosciences) enabling high purity and rapid isolation of EV from minimal amount of plasma sample. The method* is based on a two-part polymer recipe with simple centrifugation to isolate EV pellet.
Methods: ExoRich was added to multiple plasma samples with volume from 50 uL to 250 uL. After well-mixed by pipetting, the mixture was centrifuged at 1000 g for 10 minutes at 4-degrees C. The supernatant was discarded and the pelleted EV was resuspended in PBS. The ExoRich-treated samples were compared to that isolation from ultracentrifugation, SEC and a commercial EV isolation kit. Purified EV samples were characterized by TEM, NTA, nanoFCM and Western blot (WB).
Results: TEM images show characteristic cup-shaped features and intact EV morphology. NTA results of ExoRich-treated sample show a distinct and clean peak at 69 nm with medium at 83 nm. Three repeated ExoRich treatments of the same plasma sample showed particle variation within 5%. WB revealed clear signal in EV markers (flotillin & CD81). Comparison of WB between exosomal fraction from SEC and ExoRich-treated sample showed excellent agreement in exosomal protein expressions. Tests to minimize sample volume with 250 uL, 125 uL and 50 uL of plasma revealed only 50 uL is needed for consistent sandwich ELISA expression of CD81. Clinical sample of 50 uL was processed which verified that this low sample volume enabled consistent result with that from 250 uL sample volume.
Summary/Conclusion: The new method enabled high purity isolation of EV in as low as 50 uL plasma in 10 mins. The approach should be suitable for clinical studies when plasma availability is challenging.
*Provisional application
Funding: Reliance Biosciences, Taiwan.

PF10.07 | Single step microfluidic extraction of platelet-free plasma for extracellular vesicle isolation

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Introduction: Circulating extracellular vesicles (EVs) in blood are promising biomarkers widely studied in diseases due to their disease-associated cargoes. Current EV isolation methods are laborious and prone to contaminations from residual platelets or cell lysis after centrifugation. Herein, we report a high throughput (~10 mins/per mL of whole blood) microfluidic device (ExoArc) for single-step automated plasma extraction from whole blood. With a size cut-off of 500 nm, ExoArc-isolated plasma is completely platelet-free and highly enriched in EVs for direct microRNA (miRNA) profiling in clinical diagnostics.
Methods: The ExoArc is a 2-inlet-2-outlet arcuated microchannel. Under the influence of Dean vortices and size-based particle migration effects, plasma and small EVs (50 – 500 nm) are eluted as "EV-rich" platelet-free plasma (PFP) into the target outlet while platelet fragments (PFs, 0.5 – 1 μm), platelets (2 – 4 μm), and blood cells (> 5 μm) are sorted into the waste outlet. PFP can be further processed using size exclusion chromatography (SEC) to isolate EVs.
Results: A significant reduction (> 99%) of platelets and PFs was observed in ExoArc-isolated PFP as compared to 2-step centrifugation (1500g + 2500g) based on flow cytometry analysis. miRNA profiling (qPCR) also showed ~3 fold lower platelet-associated miRNAs in ExoArc-isolated PFP. Combinatory ExoArc+SEC had ~10x higher EV yield (CD9+ EVs by NTA) as compared to ultracentrifugation (UC). ExoView analysis further showed that ExoArc+SEC isolated ~50% less platelet-derived EVs than UC, thus confirming minimal platelet contamination. Finally, we demonstrated miRNA profiling of ExoArc-processed PFP from lung cancer and type 2 diabetes mellitus patients as a potential clinical workflow.
Summary/Conclusion: ExoArc is a low-cost label-free microfluidic technology for gentle EV isolation from blood. It enables automated single-step PFP extraction and helps to simplify and standardize EV isolation for clinical EV diagnostics.
Keywords: EV isolation, microfluidic

PF10.08 | Efficient isolation of biologically active extracellular vesicles using low amount of plasma from blood cancer patients

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Introduction: The role of Extracellular Vehicles (EVs) in orchestrating the complex interplay between tumor cells and the inflammatory and senescent microenvironment is poorly understood. New method to obtain EVs from low amount of blood are urgently needed. Here, based on a novel and customized flow field fractionation (FFF) method, we isolated EVs from low amount of plasma from patients with Polycythemia Vera (PV). PV is a clonal disorder of hemopoietic stem cell characterized by chronic inflammation. Functional characterization of EVs in PV with a special focus on the inflammatory and senescence pathways is still elusive.
Methods: To isolate EVs, platelet-free plasma samples (40 μl/subject) were collected from PV patients (n = 3) and healthy donors (HD; n = 3). Plasma was size-fractionated with FFF method. EV-enriched fractions were concentrated by ultrafiltration

(100 kDa ultrafilters) and characterized by transmission electron microscopy, Tunable Resistive Pulse Sensing, western blotting (according to MISEV 2018), and MACSPlex. Then, we functionally investigated in vitro effects of EVs on senescence phenotype (β-Galactosidase expression) and intracytoplasmic production ability of Interleukin-1β (IL-1β) and IL-6 in primary normal circulating mononuclear cells by Flow Cytometry.
Results: The isolated EVs from PV patients were 95–117 nm in size, spherical, and expressed EV-specific markers including CD9, CD63, CD81, FLOT-1 and TSG101. Western blotting ruled out also the presence of contaminants, such as albumin. Interestingly, PV-EVs were enriched in immune, tumor, and senescence markers including CD25, CD24, ROR1, CD29, and β-Galactosidase. Based on EV functionality, EV fractions from both PV patients or HD promoted the senescent phenotype, but reduced the intracytoplasmic production of IL-6 by CD14+ monocytes. Notably, no effects were observed for IL-1β.
Summary/Conclusion: The newly customized method was convenient (20 minutes isolation time) and effective in the isolation of biologically active EVs directly from plasma of blood cancer patients to cast light on the role of EVs in senescence and inflammation.
Funding: Italian Ministry of Research through the PhD scholarship (PNRR PON project-University of Bologna), the FIN-RER Bottom up project and Italian Ministry of Health, RC-2022- 2773402 project.
Keywords: senescence, inflammation, blood cancer, flow field fractionation

PF10.10 | Enrichment of extracellular vesicles from human plasma with molecular net matrix-coated magnetic beads (EXO-NET®)

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Introduction: Isolating extracellular vesicles (EVs) from human plasma can be challenging. EVs are at low concentration in plasma compared with, e.g., lipoprotein particles, which further overlap in size and density with EVs. To separate EVs, methods such as differential centrifugation, ultrafiltration, size exclusion chromatography (SEC), and combinations thereof are time-consuming and unsuitable for clinical settings. There is an urgent need for a fast, robust and simple method for enriching vesicles in a one-step process for downstream analysis. Here we present proteomics data of evaluation plasma-derived EVs isolated using an affinity matrix magnetic nanoparticle technology (EXO-NET, INOVIQ Ltd).
Methods: EVs were isolated from 200 μL of healthy plasma pool using 15 μl EXO-NET following the manufacturer's instructions. On-bead lysis with RIPA buffer was used to recover EV-associated proteins. Desalted peptides were analyzed by LC tandem mass spectrometry (LC-MS/MS) on LumosETD (Thermo Fisher Scientific). MS/MS spectra were searched with Mascot 2.8.0 via PD2.4 against the UP5640_H.sapiens database. The data analysis was performed based on DAVID Knowledgebase (v2022q3).
Results: 318 unique proteins were identified (protein threshold 95%), and around 200 proteins were identified as commonly EV-associated proteins, including CD9, HSPA8, HSP90, ENO1, Annexin 2, 14-3-3 family members, and PLP. 200 proteins were identified as liver and lipoprotein-specific, followed by proteins specific to blood cells, such as platelets. Interestingly, around 30 proteins are putatively CNS-specific. The yield of CD9-positive EVs was similar to that obtained with commercial SEC columns; however, less ApoB100 (low-density lipoprotein marker) and ApoA1 (high-density lipoprotein marker) were observed in the EXO-NET output.
Summary/Conclusion: The EXO-NET platform may provide a simple and rapid enrichment EVs from human plasma, especially for clinical settings, offering minimal co-isolation of lipoproteins and a one-step process.
Funding: The work is supported by the National Institute of Mental Health (NIMH; R21/R33MH118164).
Keywords: extracellular vesicles, lipoproteins, plasma, proteomics

PF10.11 | Stability evaluation of plasma exosomal RNA stored at ambient temperature collected on Norgen, Streck and EDTA tubes

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Introduction: Several blood collection devices are available that can stabilize cell-free RNA (cf-RNA) and extracellular vesicles (EVs) in plasma at room temperature, which include Streck's RNA Complete BCT tubes and Norgen's cf-DNA/cf-RNA

preservative tubes. Amongst the cargo carried in EVs, RNA is the most instable components. In the current study, we assessed the performance of EDTA tubes, Streck's RNA Complete BCT tubes and Norgen's cf-DNA/cf-RNA preservative tubes to stabilize intact exosomes as well as exosomal RNA at room temperature up to 14 days.
Methods: Blood from five donors was collected in EDTA, Streck, and Norgen tubes and were stored at room temperature for 0, 5, 7 and 14 days. Hemoglobin concentration was estimated using NanoDrop to determine hemolysis. Intact exosomes from all tubes were purified using Norgen's Plasma/Serum Exosome Purification Kits and were characterized and visualized on the NanoSight LM10 instrument. Furthermore, the exosomes were processed to purify exosomal RNA using Norgen's Exosomal RNA Isolation Kit, and small RNA library was constructed from all purified exosomal RNA using Norgen's Small RNA Library Prep Kit for Illumina. Libraries were quantified and sequenced using Illumina platform.
Results: NanoSight data revealed that the number of intact exosomes were consistent from day 0 to day 14 for plasma collected using Norgen's tubes, while the number of exosomes increased for EDTA as well as Streck tubes. The hemoglobin concentration also showed an increase in concentration from day 0 to day 14 for Streck and EDTA tubes, however, it remained constant for Norgen tubes. The average miRNA count for Norgen and Streck tubes was consistent for 14 days and 7 days respectively, while on the other hand the miRNA count for EDTA tube showed a sharp decline on day 5 and remained low up to 14 days.
Summary/Conclusion: Norgen's cf-RNA/cf-DNA preservative tubes are able to stabilize intact exosomes and exosomal RNA with high quality and for a relatively higher period as compared to Streck and EDTA tubes without any sign of hemolysis.
Funding: Norgen Biotek Corp. funded the study.
Keywords: exosomes, cf-RNA/cf-DNA preservative tubes, small RNA sequencing, plasma

PF11: Neurogenerative Disease and EVs

Location: Hall 4A

16:00 - 18:00

PF11.01 | Cerebrospinal fluid (CSF)-derived exosomes from patients with parkinson's disease induce parkinson-like symptoms and pathology in mice

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Introduction: Parkinson's disease (PD) is characterized by the gradual appearance of intraneuronal, Lewy aggregates (Lewy bodies), which are primarily composed of misfolded α- synuclein (α-syn), resulting in cytotoxicity and neural death.
Methods: death. To examine whether aggregated α-syn can actively spread from the nasal cavity to the brain via exosomes and initiate pathological aggregate in the brain, we isolated exosomes from patients CSF- PD patients or non-PD patients.
Results: We found that intra nasal delivery of CSF-derived exosomes from PD patients induce impairments in motor behavior, hyposmia, elevated anxiety and gained less weight.
Summary/Conclusion: These data suggest that exosomes are involved in the propagating α-syn aggregation and in the initiating of PD-like symptoms.
Funding: NA.
Keywords: parkinson's disease, CSF

PF11.02 | In vivo neuronal and glial internalization of peripheral extracellular vesicles from Alzheimer's patients: a potential mechanism underlying the pathogenesis of Alzheimer's disease?

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Introduction: Extracellular vesicles (EVs) are produced by all cell types. EVs play an important role in the intercellular communications by transferring their functional cargo to nearby or distant recipient cells via the biofluids. Interestingly, their cargo depends on the physiopathological state of the donor cells. We hypothesized that peripheral EVs (pEVs) could pass through the blood-brain barrier (BBB) and then diffuse into the brain.

Methods: For this, pEVs from healthy and AD patient serums were enriched, characterized, and then labelled with a fluorescent probe. Their diffusion through the bEnd.3 endothelial cells was studied on an vitro Transwell model. Then, labeled pEVs were microinjected into the blood circulation of 2-days post-fertilization transgenic Danio rerio.
Results: The passage of pEVs though the bEnd.3 was first confirmed, and the internalization of pEVs by neurons in co-culture was validated by flow cytometry and immunohistochemistry. Then, by using the green fluorescent protein (GFP) expressing the blood vessel-specific endothelial cells Tg(flk1:EGFP) line, we observed the distribution of the healthy and AD pEVs into the brain-blood vessels of Danio rerio. The biodistribution of pEVs into larvae was monitored 1h and 24h post-injection by confocal microscopy. We observed that the diffusion of pEVs into the cerebral areas increased with time. The important colocalization of pEVs with endothelial cells suggest that these vesicles notably reach the brain through the BBB. Moreover, with the Tg(huc:EGFP) and Tg(gfap:EGFP) lines, two transgenic models which, respectively, show the neuron- and glial-specific expression of GFP, we demonstrated that pEVs were engulfed by both cell types.
Summary/Conclusion: These results support our hypothesis and bring up new knowledge about the fate of pEVs after their passage through the brain barriers. Also, it raises new questions about the putative role of pEVs in AD pathogenesis, especially when patients suffer from peripheral inflammatory or metabolic disorders.
Funding: Research Chair Louise & André Charron on Alzheimer's disease, Fondation Armand-Frappier.
Keywords: extracellular vesicles, blood-brain barrier, alzheimer's disease

PF11.03 | Characterization of hiPSC-neuronal derived extracellular vesicle lipids in Alzheimer's disease

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Introduction: Alzheimer's disease (AD) is the most common cause of dementia with tens of millions of victims worldwide. Mechanisms of progression are poorly understood and there are no effective therapies. There are few reliable CSF and imaging biomarkers for early detection or monitoring of progression, but none involving blood. Extracellular vesicles (EVs) are membrane derived particles containing lipids, proteins and RNA species. Since brain derived EVs are found in peripheral circulation they are a potential source of biomarkers for AD. Based on evidence that lipids play a significant role in maintenance of cellular homeostasis and data that lipid metabolism is altered in brain, cerebrospinal fluid and blood of AD patients, circulating EVs may be a useful biomarker for AD.
Methods: Induced pluripotent stem cells (iPSCs) from people with AD and controls were differentiated into glutamatergic neurons. Size exclusion chromatography (SEC) using qEV columns was performed for EVs isolation from culture's supernatant and characterized by Nanoparticle Tracking Analysis & further characterization was performed by transmission electron microscopy and Heatmap of Single Particle Interferometric Reflectance Imagin Sensing (SP-IRIS) platform for surface markers (CD9, CD63, CD81). Comprehensive lipid profiling of EVs was conducted on a TripleTOF 5600 high resolution mass spectrometer to quantify & annotate lipids carried in these EVs.
Results: The identification and quantification of 326 lipid features were performed on EVs isolated from neuronal cultures of 4 AD patients and 5 healthy controls. Compared to controls, EVs isolated from AD neuronal cultures had lower levels of phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine.
Summary/Conclusion: EV lipidomic analysis could potentially facilitate the AD biomarker discovery in an inexpensive and efficient approach, by using samples from patients with diverse clinical manifestations.
Funding: This work was funded by The Richman Family Precision Medicine Center of Excellence in Alzheimer's disease at Johns Hopkins.

PF11.04 | The possible role of IRSp53 I-BAR-derived extracellular vesicles for carrying Alzheimer's Disease related cargo

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Introduction: The I-BAR domain membrane deformation protein, Insulin receptor substrate 53 kDa (IRSp53) senses and induce plasma membrane protrusions including filopodia and spines. This I-BAR domain-induced filopodium can be a source of extracellular vesicles (EVs), which is called protrusion-derived EVs. Previous proteomic and correlation studies implicated the potential involvement of IRSp53 in Alzheimer's disease. Furthermore, recent research has devoted attention to EVs, particularly exosomes acting as carriers of amyloid beta for their spreading in Alzheimer's Disease(AD).
Methods: EVs were harvested from HEK293 cells expressing IRSp53 I-BAR and amyloid precursor protein(APP) that carries human AD mutation by sequential centrifugation. The EVs are further analysed by Nanoparticle Tracking Analysis (NTA) and Western blotting for their content according to MISEV 2018 guidelines.
Results: The overexpression of both proteins together significantly increased the number of larger EVs than the control cells (Student T-test, $p < 0.05$). The Western blotting indicated the cleaved products of APP, β-CTFs, on the EVs when IRSp53 I-BAR was expressed. Furthermore, amyloid precursor protein and IRSp53 was co-localized in filopodia.
Summary/Conclusion: Our data suggest that IRSp53-dependent vesicles could carry AD-related cargo such as β-CTFs. These results shed light on the possible role of IRSp53-derived EVs in AD for further investigation on the EVs.
Keywords: IRSp53, I-BAR domain, APP

PF11.05 | Investigating the impact of n-3 PUFA supplementation on plasma EV signature in relation to cognitive function in aged individuals

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Introduction: Plasma-derived extracellular vesicles (PLEVs) have garnered a lot of interest in recent years due to their dual potential as prognostic and diagnostic biomarkers for a range of pathologies. Exploiting this duality is of particular interest in the field of age-related cognitive alteration for which there are currently no robust biomarkers that can predict the status of cognitive health in order to allow early therapeutic intervention. Such interventions may include supplementation with n-3 polyunsaturated fatty acids (PUFAs) which has been associated with improved cognitive health. Therefore, we aim to uncover whether there is a common PLEV signature associated with cognitive alteration and whether this is modified in response to supplementation with n-3 PUFAs.
Methods: A double-blinded, randomized and placebo-controlled study consisting of 120 otherwise healthy individuals aged between 60–70 years old underwent cognitive evaluation (CANTAB) before and following a 12-month supplementation with omega-3 FAs. Plasma samples were collected before and after dietary intervention and PLEVs were subsequently enriched using size-exclusion chromatography. NanoFCM, western immunoblotting and TEM were employed in order to characterise the phenotype of PLEVs. Gas-chromatography mass spectrometry was used to profile the fatty acid content of PLEVs.
Results: Nutritional intervention was associated with a significant improvement in short-term memory ($p = 0.0043$) and increased circulating levels of docosahexaenoic acid (DHA). Assessment of PLEVs by NanoFCM revealed that the average size of PLEVs was between 80–90 nm. Overall, CD9 was found to be the most abundant tetraspanin expressed by PLEVs compared with CD81 and CD63. Our initial results indicate successful detection of several FA species including DHA, docosapentaenoic acid (DPA) n-3 and DPA n-6.
Summary/Conclusion: Further comparisons will be made between placebo and treatment groups in order to uncover if PLEVs are altered in relation to cognitive status and whether there is a change in the level of FAs. In depth characterisation of PLEVs may offer insight into the mechanism of action of PUFAs on cognitive health.
Funding: Chair Région Nouvelle-Aquitaine.
Keywords: fatty acid, ageing, lipid, cognitive decline

PF11.06 | Human iPSC-NSC-derived EV therapy in late middle age improves cognitive function with modulation of brain inflammation and autophagy

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Introduction: Cognitive impairment in late middle age is associated with low-level chronic neuroinflammation and reduced autophagy in the brain. Hence, biologics capable of alleviating neuroinflammation and enhancing autophagy are beneficial as anti-aging interventions.
This study investigated the efficacy of intranasal (IN) administration of extracellular vesicles (EVs), isolated through chromatographic methods from cultures of human induced pluripotent stem cell (hiPSC)-derived neural stem cells (NSCs), in 18-month-old C57BL/6 mice to alleviate cognitive dysfunction in old age.
Methods: Mice received IN administration of hiPSC-NSC-EVs (75 × 109 EVs) or the vehicle (biweekly for a month). A month later, animals were tested for cognitive function via behavioral tests. Animals were euthanized when they were 21 months old, and brain tissues were processed to quantify neuroinflammation and autophagy.
Results: A series of objects-based behavioral tests revealed that aged animals treated with the vehicle displayed cognitive impairments for discerning minor changes in the environment, novel object recognition, and pattern separation. In contrast, aged animals receiving hiPSC-NSC-EVs displayed improved cognitive function in all tests. Brain tissue analysis revealed that hiPSC-NSC-EV treatment reduced neuroinflammation in the hippocampus, which was evidenced by reduced microglial clusters, the extent of activated microglia presenting CD68 and NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasomes, astrocyte hypertrophy, concentrations of mediators and end products of NLRP3 inflammasomes, and multiple proinflammatory cytokines. The hippocampus of mice receiving hiPSC-NSC-EVs also exhibited increased concentrations of several autophagy-related proteins.
Summary/Conclusion: IN administration of hiPSC-NSC-EVs is a novel therapeutic approach for maintaining better cognitive function in old age through the modulation of neuroinflammation and autophagy.
Funding: Supported by a grant from the National Institute for Aging (1R01AG075440-01 to A.K.S.).
Keywords: aging, autophagy, NLRP3 inflammasomes, neural stem cell-derived EVs, intranasal administration of EVs, neuroinflammation, cognitive dysfunction

PF11.07 | Characterizing extracellular vesicles in Niemann Pick Disease Type C

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Introduction: Niemann Pick Disease Type C (NPC), also known as Childhood Alzheimer's, is a rare neurodegenerative disease. NPC is caused by endolysosomal disruptions driven by abnormal cholesterol storage. Extracellular vesicles (EVs) can be formed through the endolysosomal pathway, and have been implicated in neurodegeneration. However, whether EVs are affected by NPC is unknown. Here, we isolated and characterized EVs from both cerebrospinal fluid (CSF) and fibroblasts of NPC patients.
Methods: NPC and control CSF (n = 12) and dermal fibroblasts (n = 8) were obtained from Oregon Health & Science University, Rush University, and Coriell Institute. CSF was ultrafiltered and probed for EV markers with immunoblots. EVs were enriched from CSF and cell conditioned media using ultrafiltration (Millipore 100kD) and size exclusion chromatography (Izon 35nm). Fractionated EVs were validated using immunoblots, fluorescent nanoparticle tracking analysis (fNTA), and transmission electron microscopy. fNTA, vesicle flow cytometry (VFC), and multiplexed bead-based flow cytometry (MBFC, Miltenyi Biotec) were then used to characterize EV populations. Groups were compared using Student's t-test or Mann-Whitney.
Results: Immunoblots show NPC CSF contains significantly more Flotillin 1 and CD81 protein compared to controls ($p = 0.041$, $p = 0.015$). Similarly, fNTA and VFC show NPC fibroblasts release more 100nm-sized EVs than controls ($p = 0.004$). MBFC shows NPC EV surface proteins differ from controls in both CSF and cell culture media, including enrichment of proteins implicated in NPC pathology.
Summary/Conclusion: Our results indicate that human NPC CSF and cell culture media contain more EVs compared to controls, and that proteins on NPC EVs are distinct from those on control EVs. Current work is investigating whether these increases result from altered EV biogenesis or uptake, and further identifying alterations to NPC EV cargo. Ultimately, this work demonstrates how EVs are altered in NPC, a devastating neurodegenerative disease that has no cure.
Keywords: niemann pick disease Type C, extracellular vesicles, neurodegeneration

PF11.08 | Puerariae Lobatae Radix derived exosomes overcome blood brain barrier for plant miRNAs delivery to brain for Parkinson's disease treatment

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Introduction: The treatment of Parkinson's disease (PD) is hindered by the complex pathology and the blood brain barrier (BBB), which forms a big obstacle for the delivery of most drugs into brain. Recently, exosomes are emerging as promising nanoplatforms in treating brain disorders with enhanced efficacy in transferring therapeutic nucleic acids through BBB to regulate the brain pathological process.
Methods: Herein, the medicinal plant, Puerariae Lobatae Radix derived exosomes (Pu-Exos) were prepared and characterized for the contained small molecules, nucleic acids, proteins and lipids qualitatively and quantitatively. Further, Pu-Exos were modified by the functional target ligand (named as Pu-Exos-RVG) and investigated for their capabilities in BBB penetration, miRNAs delivery and therapeutic potential in PD treatment both in vitro and in vivo.
Results: And for the first time, we demonstrate that Pu-Exos-RVG are efficient in delivering nerve-regulating plant microRNAs with efficacy in modulating the mitochondrial dysfunction of SH-SY5Y cells through intervening ubiquitin and PINK1-Parkin mediated mitophagy and recovering the function of mitochondrial respiratory chain, as well as reversing the deficiency in ATP production and apoptosis. In vivo, Pu-Exos-RVG are shown for the efficiency in delivering the incorporated plant microRNAs to brain lesion site that significantly affords neuroprotective effects in reducing DA neurons loss, promoting neural function recovery and improving movement coordination/psychiatric symptoms.
Summary/Conclusion: Pu-Exos-RVG were shown as a promising nanoplatform with excellent efficacy in delivering functional plant miRNAs to brain with obvious therapeutic capacity for PD treatment that possesses great potential in treating brain neuron degenerative diseases.
Funding: The study was supported by National Key Research and Development Program of China (2022YFC3501904, 2021YFC1712805), Zhejiang province com monweal projects (LG F22H280001), the Key Project at Central Government Level (2060302), and the Macau Science and Technology Development Fund, Macau Special Administrative Region, China.
Keywords: parkinson's disease, blood brain barrier, Puerariae Lobatae Radix derived exosomes, mitochondrial dysfunction, plant microRNAs

PF11.09 | Proteomic comparison between CSF samples and CSF-derived EVs from patients with Alzheimer's disease, Parkinson's disease and Lewy Body dementia

Inge Mertens[1]; Yael Hirschberg[1]; Kurt Boonen[2]; Sebastiaan Engelborghs[3]; Yannick Vermeiren[4]; Inge Mertens[1]
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Introduction: Extracellular vesicles (EVs) have recently been considered as a potential biomarker source for a variety of diseases, including neurodegenerative disorders. EVs are important mediators of intercellular communication due to their capacity to transfer genetic material, lipids and proteins. By means of their communication role, interesting biomarkers are often enriched in EVs compared to total biofluid. In this study, this hypothesis is tested as untargeted proteomics is performed on both CSF samples and CSF-derived EV samples from the same cohort with different dementia diseases.
Methods: CSF samples were collected from 297 patients (with Alzheimer's disease (AD), Parkinson's disease (PD), PD with cognitive deficit (PDCD), PD with dementia (PDD), Lewy Body dementia (LBD)) and healthy controls (HC). Furthermore, EVs were obtained from 500μL of the same CSF samples, isolated by SmartSEC HT. Total protein quantification was measured using absorbance at 280nm for CSF samples, and by fluorescence (NanoOrangeTM) for CSF-derived EV samples. For both types of sample, LC-MS/MS based proteomics analyses were performed.
Results: Total protein quantification showed multiple significant differences between all groups, both in CSF samples and in CSF-derived EV samples. In both types of sample, PDCD group showed the highest protein concentration after normalization. In regard to LC-MS/MS results, a first analysis resulted in a total of 3036 identified proteins (1564 protein groups) for the CSF samples, and 6627 proteins (3360 protein groups) for the CSF-derived EV samples. The current analyses show four statistically differentially expressed proteins between CSF-derived EV samples from HC and AD, AD and PD, LBD and PDCD. By the time of ISEV2023, also CSF samples will be analysed and a comparison will be drawn between both types of sample.
Summary/Conclusion: As more in-depth proteomic analyses will be performed by ISEV2023, a definite conclusion will be given at the conference. For now, already differences in total protein concentration were observed between the different disease groups and LC-MS/MS of CSF-derived EV samples resulted in four statistically differentially expressed proteins.
Keywords: dementia, proteomics, mass spectrometry

PF12: EV Labels and Imaging

Location: Hall 4A

16:00 - 18:00

PF12.01 | Shining light into the complexity of extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are lipid bilayer-delimited nanoparticles released by all cells. Given their potential for diagnostic and therapeutic applications, numerous approaches have been utilised for labelling EVs to assess their biodistribution, cell uptake and trafficking, as well as to determine their size and abundance in a biofluid. Specific and complete labelling of EVs is complicated by their heterogeneity and the unavoidable presence of non-vesicular material in EV preparations. These limitations generate a high level of false positive signal, often leading to inaccurate conclusions. There is a need for more robust labelling techniques.
In this work we developed and established different techniques to label EVs – including a novel lipophilic dye, ExoriaTM, and a scalable approach to fluorescently engineer EVs – that overcome current limitations.
Methods: EVs from engineered or naïve human embryonic kidney (HEK) 293 cells were produced using Exopharm capabilities, including Hexocollect for collection and LEAP for purification. A range of analytical techniques such as Asymmetric Flow-Field Flow Fractionation, flow cytometry and Western blotting were performed to characterize the labeled EVs.
Results: In-house experiments have shown that Exoria is less prone to forming dye aggregates and that Exoria-labelled EVs showed an increased florescence signal than all controls, with a reduced background signal as opposed to other commercially available dyes.
Using Exopharm's manufacturing capabilities, we have demonstrated the scale-up production of fluorescently engineered EVs from HEK293 cells. Over 90% of LEAP-purified engineered EVs were mGL positive and exhibited stable fluorescence.
Summary/Conclusion: Both techniques enable successful tracking of EV cell uptake as well as assay development, calibration and validation. The study also highlights the importance of fluorescently labelling EVs to comprehensively analyse their properties.

PF12.02 | In vivo detection and quantification of phosphatidylserine positive extracellular vesicles

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Introduction: Phosphatidylserine (PS) is a phospholipid normally retained on the inner leaflet of cellular membranes. However, it has been shown to be exposed on the surface of apoptotic cells, activated platelets and importantly also on extracellular vesicles (EV). EV detection based on PS, using for example fluorescent Annexin V, has been widely established in vitro, but Ca2+-dependence prohibits its application in vivo. In contrast, Milk fat globule-EGF factor 8 (MFG-E8) represents a Ca2+-independent PS-binding protein. Previously, we have used fluorescent MFG-E8, or its C1-domain, to detect EV-associated cells in vivo by imaging flow cytometry (IFC). The aim of the present study was to extend the application of C1-reagents to bind and detect circulating PS+ EVs in serum in vivo.
Methods: For in vivo labelling of PS+ EVs, C57BL/6 mice were intravenously injected with 50 μg of fluorescent labelled mC1-multimers. Serum EVs were isolated by size exclusion chromatography (SEC) using qEV35 columns (Izon) and EV containing fractions were confirmed by nanoparticle tracking analysis, bicinchoninic acid assay and western blotting. Following isolation, EVs were analyzed by IFC (ImageStreamTM) and dSTORM microscopy (ONI).
Results: EVs were bound by the PS-specific mC1-multimer in vivo and the label remained intact following their isolation from serum, as assessed by IFC. This analysis revealed that the majority of endogenous circulating serum EVs were PS+ (>90%). Furthermore, co-incubation of differentially labelled EVs did not show dye transfer between EVs after isolation via SEC.
Summary/Conclusion: We could demonstrate a novel method for detection of PS+ EVs in vivo, which enables the study of endogenous EVs without the need for introduction of reporter genes. Moreover, we showed that mC1-multimer labelled EVs can be used for distinct downstream analyses, including quantitative measurements and characterization of surface molecules.
Funding: This project was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research foundation) – Project-ID 210592381 - SFB 1054 B03 to TB and SFB 1954 Z02 to JK.
Keywords: extracellular vesicles, phosphatiylserine, dSTORM, imaging flow cytometry, MFG-E8, lactadherin

PF12.03 | A transgenic reporter mouse line reveals exosome mediated muscle-bone crosstalk

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Introduction: Sarcopenia and osteoporosis are musculoskeletal disorders that often develop in parallel. Emerging evidence suggests that skeletal muscle (SM) can regulate bone through mechanical and endocrinal interactions. Exosomes (EXOs) are efficient carriers for material transfer between cells. Previous research found that the SM-EXOs and their cargo are involved in interorgan crosstalk under physiological and pathological states. However, the contribution of SM-EXOs to muscle-bone crosstalk remains underexplored.

Methods: We generated a transgenic mouse line (SM-CD63GFP) expressing the GFP-tagged CD63 (a general exosome marker) specifically in SM driven by the human alpha-skeletal actin promoter after tamoxifen induction. The IVIS Spectrum imaging and Confocal microscopy were used to examine the GFP signal in various organs. Immunostaining was used to assess the colocalization of GFP with CD63 in SM and bone cells, respectively.

Results: As revealed by IVIS Spectrum imaging and Confocal microscopy with immunostaining, abundant GFP signal was observed in SM of the SM-CD63GFP mice, most of which was colocalized with CD63, suggesting the specific expression of GFP-tagged CD63 in SM. Interestingly, GFP signal was also observed and colocalized with CD63 in bone and bone marrow cells of SM-CD63GFP mice, indicating the intraosseously transfer of SM-EXOs. Confocal microscopy with immunostaining further revealed the colocalization of GFP with Cathepsin K (an osteoclast marker) or Runx2 (an osteoblast marker), indicating the transfer of SM-EXOs to osteoclast and osteoblast.

Summary/Conclusion: Our study has established a transgenic reporter mouse line for in vivo tracking of the SM-EXOs, which warrants further study on the potential contribution of SM-EXOs to muscle-bone crosstalk.

Funding: Theme-based Research Scheme (T12-201/20-R) and General Research Fund (12136616 and 12103519) of the Research Grants Council of Hong Kong SAR. The 2020 Guangdong Provincial Science and Technology Innovation Strategy Special Fund (Guangdong-Hong Kong-Macau Joint Lab, No: 2020B1212030006), Interdisciplinary Research Clusters Matching Scheme of Hong Kong Baptist University (RC-IRCs/17-18/02).

Keywords: reporter mouse, exosomes, skeletal muscle, bone

PF12.04 | Tracking big and small extracellular vesicles via near-infrared bioluminescence resonance energy transfer

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Introduction: Accurate and precise tracking nano-sized extracellular vesicles (EVs) remain as a major challenge in EV research in vivo due to the inverse correlation between signal intensity and penetration depth. We previous developed a multi-resolution, bioluminescence resonance energy transfer (BRET)-based PalmGRET reporter with pan-EV labeling ability, which emits signals at blue-to-green spectra (~450-520nm). In an effort to increase in vivo imaging capability of EV subpopulations including small EV (sEV; < 200 nm), big EV (bEV; 200–1,000 nm), and large EV (lEV; > 1,000 nm), we developed a near-infrared (NIR)-BRET EV reporter, PalmSORET.

Methods: We molecularly fused palmitoylation signal of growth cone associated protein 43 to the N-terminus of iRFP-Rluc8.6-535SG (Nishihara and colleagues) to create PalmSORET. PalmSORET emits bioluminescence (BL) and BRET-excited fluorescence (BRET-FL) for dual reporter outputs. The palmitoylation signal enables EV inner membrane labeling through

S-palmitoylation of cysteine which minimizes PalmSORET from being exposed to the EV surface, thereby mitigating enzymatic degradation and/or surface ligand-receptor hindrance. PalmSORET catalyzes BBlue2.3 substrate to yield peak BL and BRET-FL signals at 413 nm and 713 nm (via Soret band excitation of the iRFP713), respectively.
Results: Upon stable expression in HEK293T cell (293T-PalmSORET), PalmSORET localized to the membranes and enabled tracking of EVs under super-resolution at near-infrared range (> 700 nm). PalmSORET labels the inner membrane of both bEV and sEV without significantly affecting their respective sizes. In addition, bEV/sEV-PalmSORET emitted robust BL (413/ 10 nm) and BRET-FL (700/ 50 nm) signals with a positive correlation to EV amount (R2 value > 0.98) and high sensitivity (100 ng EV), which could be rapidly detected in vitro with 1 sec of integration time.
Summary/Conclusion: We successfully established a NIR-BRET EV reporter, PalmSORET, with pan-EV labeling and multi-resolution imaging functions. Efforts are currently underway in applying this powerful and sensitive imaging system to track bEVs and sEVs in vitro and in vivo.
Funding: National Science and Technology Council (NSTC) grants NSTC 111-2628-B-001-022 (C.P.L.), Academia Sinica Innovative Materials and Analysis Technology Exploration (i-MATE) Program AS-iMATE-107-33 (C.P.L.), Institute of Atomic and Molecular Sciences grants IAMS 30-08 (C.P.L.), and Academia Sinica Career Development Award 109-M04 (C.P.L.).
Keywords: small EV, big EV, near-infrared bioluminescence resonance energy transfer, Palmitoylation

PF12.05 | Detection of tumor-derived extracellular vesicle interactions with immune cells is dependent on EV-labelling methods

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Introduction: Cell-cell communication within the complex tumor microenvironment is critical to cancer progression. Tumor-derived extracellular vesicles (TD-EVs) are key players in this process. They can interact with immune cells and modulate their activity, either suppressing or activating the immune system. Understanding the interactions between TD-EVs and immune cells is essential for understanding immune modulation by cancer cells. Fluorescent labelling of TD-EVs is a method of choice to study such interaction.
Methods: This work aims to determine the impact of EV labelling methods on the detection of EV interaction and capture by the different immune cell types within human Peripheral Blood Mononuclear Cells (PBMCs), analyzed by imaging flow cytometry and multicolor spectral flow cytometry. EVs released by the triple-negative breast carcinoma cell line MDA-MB-231 were labeled either with the lipophilic dye MemGlow-488 (MG-488), with Carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE), or through expression of a MyrPalm-superFolder GFP (sfGFP) that incorporates into EVs during their biogenesis using a genetically engineered cell line.
Results: Our results showed that these different labeling strategies, although analyzed with the same techniques, led to diverging results. While MG-488-labelled EVs incorporate in all cell types, CFSE-labelled EVs are restricted to a minor subset of cells and sfGFP-labelled EVs are mainly detected in CD14+ monocytes which are the main uptakers of EVs and other particles, regardless of the labeling method. Moreover, MG-488-labeled liposomes behaved similarly to MG-488 EVs, highlighting the predominant role of the labelling strategy on the visualization and analysis of TD-EVs uptake by immune cell types.
Summary/Conclusion: Consequently, the use of different EV labeling methods has to be considered as they can provide complementary information on various types of EV-cell interaction and EV fate.
Funding: INSERM, Institut Curie, ANR, INCa, ARC, USA NCI, EU H2020 Erasmus and MSCA-ITN programs.

PF12.06 | Investigation into commercially available EV membrane dyes to improve EV purity analysis in complex biofluids

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Introduction: Single extracellular vesicle (EV) characterization often utilizes fluorescent labelling of proteins, lipids, nucleic acids, or structural components. Generic EV labelling, unbiased by specific protein markers, could improve EV identification and quantification, particularly in complex biofluids. The aim of this work is to investigate different qualities displayed by

commercially available products to help guide EV researchers toward optimal reagents based on their EV source and labelling requirements.

Methods: EVs were obtained from bioreactors growing SW620 and SW480 cell lines, isolated by ultrafiltration and size exclusion chromatography (SEC). EV presence was confirmed by Western Blot, TEM and nano-flow cytometry (nFCM). Purified very low-density lipoproteins (VLDL) and LDL as well as EV depleted plasma were used to generate complex particle samples with known EV quantities. Labelling of particles (EV or non-EV) by 12 reagents was determined by nFCM.

Results: Memglow and Cell mask dyes showed efficient labelling of SEC isolated cell line derived EVs with over 90% of >40nm EVs labelled with high intensity fluorescence compared to classical options such as PKH and CFSE. However, incubation with purified VLDL showed lack of specificity for EVs for one of these leading dye options. Dyes which labelled EV populations with high specificity included ExoBrite and Cell Tracker Deep Red. Combining anti-tetraspanin labelling and dye labelling allowed for verification of EV identity and calculation of binding efficiency.

Summary/Conclusion: Here, we assess market available dyes, demonstrating their efficacy, specificity, and ease of use to provide EV researchers a tool kit of dyes suitable for multiple EV characterisation and purity analysis techniques. Dyes from two manufacturers demonstrated superior properties: good EV integration and simple, robust labelling protocols. However, lipoprotein cross-reactivity makes one a poor choice in complex biofluids.

Funding: N/A.

Keywords: Flow cytometry, Membrane dye, LDL

PF12.07 | Retroviral transduction and production of palm-GRET labelled extracellular vesicles using bone marrow erived MSC in a 3D hollow fiber bioreactor

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Introduction: Various methods have been applied to the transfer of genes into MSC. The generation of stable MSC transfectants is hampered by the limited number of passages MSC can undergo before they start to differentiate and difficulty in performing at scale. Current data suggests that mesenchymal stem cells, when seeded into a 3-D hollow fiber bioreactor show little proliferation and may be maintained in culture and produce EVs continuously for extended periods of time. Transient transfection under these conditions could result in a usefully stable MSC transfectant. To this end a retroviral transfection of bone marrow MSC using a hollow fiber bioreactor was performed.

Methods: Bone marrow MSC from ATCC were expanded to 5 × 10e7 cells using DMEM/10% FBS and 6 T300 flasks. Retrovirus encoding for green fluorescent protein was produced in culture. A FiberCell C2025D 20 kd MWCO polysufone cartridge with 450cm2 of area and a 2.8 ml volume was seeded as follows. 5 × 10e7 cells in a volume of 5 mls was attached to one side-port and 4.5 × 10e9 retrovirus in a volume of 4 mls was attached to the opposite side port. Equal volume of cell/virus mixture was flushed into each syringe and then injected into the ECS of the cartridge with the excess volume flowing through the fibers into the medium reservoir, concentrating the cells and the virus together. After 24 hours the ECS was drained and the medium replaced with basal DMEM (no serum). Harvests were performed at 24 hour intervals.

Results: 1) Isolated EVs from the first 16 days of harvest collection show strong Nluc signal and approximately 5–12% of all detected particles in those EV samples were GFP positive, indicating release of the GFP-Nanoluc fusion reporter proteins via EVs by MSC transfectants.

2) The particle count of EVs produced by palmGRET MSCs declined from 1.4E+10 particles/mL in the first harvest to 3.9E+06 particles/mL measured in the last harvest (day 27). These data strongly suggest that EV production was drastically reduced after 2 weeks of MSC transduction in HFBR

3) It was not possible to directly determine transduction efficiency under these conditions.

Summary/Conclusion: A hollow fiber bioreactor can reduce the volume required to perform transductions by 100X, and utilizes a closed, cGMP compatible format. Overall, these promising preliminary data warrant further optimization and refinement of the transduction protocol, particularly by modifying the viral titer, selection strategy, and length of the experiment. Cell viability assays will also be performed to determine whether EV concentration declined as a result of decreased cell viability or if other factors are at play. Increasing scale by a factor of 100x or more is possible using existing systems.

Funding: na.

Keywords: retroviral transduction, palm-GRET, hollow fiber

PF12.11 | Controlling the biochemistry of liposome formation and cargo encapsulation during in-situ liquid-phase TEM studies, while enabling real-time visualization

Hans Radhoe
employee, Delft, Netherlands

Introduction: The characterization of liposomes/polymersomes typically involves a combinatorial approach. Resolving the details with nanometer resolution usually requires one of three TEM techniques: staining, freeze−fracture or cryoTEM. Staining is a fast process but it can alter the sample structure and composition. The freeze fracture processing preserves the sample and allows for the analysis of fine structural details, but is time-intensive and can result in morphological changes and sample fusion. Cryo-TEM is a direct imaging and enables high resolution but the liposomes are removed from the liquid environment making a dynamic and functional analysis of the structures impossible. As a consequence not only the systematic errors are introduced but the process prevents the possibility of enabling high-throughput studies.
Methods: We introduce our technology for in-situ Liquid Phase Transmission Electron Microscope (TEM) studies which is an add on and a plug-and-play system. The latter relies on a Microfluidic Lab on a Chip device as a smart sample carrier the Nano Cell which allows to flow the biological sample in its native liquid environment inside the TEM. The Microfluidic Lab on a Chip device contains an integrated set of biasing electrodes or an integrated microheater i.e. to perform liquid heating experiments. Furthermore the Nano Cell contains an integrated microheater and/or electrodes. This enables comprehensive in-situ studies on liposomes/polymersomes and pharmaceutical research as a function of temperature/biasing/pressure/flow rate.
Results: As a result the user can study the structure/size/morphology/composition and visualize real-time dynamics with high resolution. As a reference we have reported single particle analysis of proteins with 4.5 angstrom resolution while being in liquid The different stimuli e.g. temperature/pressure/flow/pH and the mass transport are controlled simultaneously enabling the user to optimize the biochemistry on the fly. We have shown that these capabilities allow to visualize in-situ the synthesis of liposomes/polymersomes. Furthermore the user can visualize the exact moment when a cargo molecule gets encapsulated by the liposome providing a unique opportunity to optimize the drug delivery vehicle before using it for e.g. cancer immunotherapy purposes. Moreover the system enables HT studies.
Summary/Conclusion: Our development provides the unique possibility to visualize biological processes in real time with high resolution, while the bio sample is in its native environment (as a function of different stimuli). This opens up several possibilities for pharmaceutical applications of liposomes/polymersomes and other EVs e.g. disease biomarkers drug delivery vehicles or vaccines and fundamental studies in biophysics and biochemistry. We believe that this technique will become a very powerful complementary technique to cryo EM and other techniques conventionally used within EVs research.

PF13: EV Heterogeneity 1

Location: Hall 4A

16:00 - 18:00

PF13.01 | Extracellular vesicle mediated lymphatic communication between the primary tumor and sentinel lymph node

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Introduction: Metastasis is the primary cause of cancer mortality contributing about 90% of all cancer-related deaths. Although cancer therapies have been developed, few have proven successful for treating cancer metastasis. Communication between the primary breast tumor and the sentinel draining lymph node (SLN) has long been suspected of influencing the systemic metastatic process at distant sites. Extracellular vesicles (EVs) derived from the tumor have been hypothesized to influence the SLN. We hypothesize that specific contents of tumor EV subpopulations act directly at the SLN to modulate the immune system to facilitate immunosuppression and accelerate distant metastases formation.
Methods: We have developed a syngeneic BALB/c mouse model to sensitize the SLN to the emerging breast cancer tumor. Specifically, we deliver EV subpopulations derived from the 4T1 TNBC cell line (2K and 100K) using differential ultracentrifugation and density gradient centrifugation and then packaged into hydrogel nanoparticles to the SLN. Two days later we challenge the animal with 4T1 tumor cells. After 14 days, we sacrifice the mice, measure the tumor burden, and analyze the proteomics of the isolated tumor interstitial fluid EVs. We performed IHC on the distant metastasis for immune cell markers such as F4/80, CD206, Ly6G/C, CD11b, and Arginase-1 to determine the immune cell populations present.

Results: Pilot-scale experiments showed lymph-node priming with EV subpopulations demonstrates an induction of tumor growth and metastasis from the 2K EVs treatment compared to a suppression of metastasis by the 100K treated group. Differential H&E staining of 2K metastasis versus 100K metastasis indicates a generalized immune suppression profile following 2K treatment (F4/80+/CD206+). Whereas the 100K EV treatment group had a significantly lower tumor and metastatic burden compared to control with fewer immune cell infiltrate. Overall, a tumor EV subpopulation specific vaccine offers a completely new approach to reverse the tumor immunosuppression of the SLN and suppress distant metastasis formation.

Summary/Conclusion: The expected outcomes of the study would be a targeted metastasis EV based therapy for more aggressive triple negative breast cancer (TNBC), a subtype that metastasizes with greater frequency compared to other breast cancer types. Early detection of identified panel of metastatic markers would act as a diagnostic key to avoid the primary stage cancer to get invasive. This proposal can provide knowledge about the full repertoire of molecular information mediated by EVs that enter the tumor IF to in turn be carried in the lymph highway, new basic knowledge about the different roles played by different classes of EV to modulate the SLN, and the identification of non-PD-L1 EV-associated molecules that act on the antigen processing immune cell machinery of the SLN to trigger distant immunosuppression that favors metastatic colonization.

PF13.02 | Colorectal cancer EV ID-card: characterization of vesicles extracted from different sources in CRC patients

Sarah Tassinari[1]; Federica Collino[2]; Benedetta Bussolati[3]; Edoardo D'Angelo[4]; Marco Agostini[5]; Federico Caicci[6]; Jacopo Burrello[7]; Alessandro Musso[8]; Giuseppe Giraudo[9]; Marco Ettore Allaix[10]; Giorgio Maria Saracco[9]; Mario Morino[9]; Paola Cassoni[11]

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Introduction: Colorectal cancer (CRC) is the third most frequently diagnosed cancer in men and the second in women. In this study we aimed to characterize EVs from different sources in CRC patients.

Methods: EVs have been extracted from 110 fresh biopsies or 48 decellularized tissues (tumor and coupled 10-cm far normal tissue) and from 16 plasma samples, following the WMA Declaration of Helsinki. EVs were extracted from biopsies after enzymatic digestion and sequential centrifugations and analyzed by NTA, TEM, super-resolution microscopy and bead-based cytofluorimetric analysis (FACS).

Results: NTA and TEM confirmed the presence of intact EVs in our preparations. The number of extracted EVs was higher in fresh biopsies compared to decellularized one. Tetraspanins expression was confirmed with super-resolution microscopy and FACS, being CD63 the most expressed. The surface marker analysis showed different EV-related information in the different sources analyzed.

Comparing EVs extracted from fresh tumor vs normal biopsy, we observed alteration of markers related to the cellular microenvironment and tumor phenotype, such as markers of angiogenesis (CD31, CD105), platelet activation (CD41b, CD42a and CD62p), and epithelial-to-mesenchymal transition (SSEA-4, CD146). Analysis of EVs extracted from decellularized tissue showed a different markers distribution related to tumor stage, and a significant increase of inflammatory/immunological molecules in the normal tissue surrounding the tumor of metastatic patients. Selected tissue markers were confirmed to be modulated in plasma of CRC patients.

Summary/Conclusion: In conclusion, fresh biopsies contain EVs, exposing the heterogeneity of tumor and infiltrating cell composition (tumor cells, endothelial cells, platelets), their interactions and the resulting tumor state. At variance, EVs from decellularized tissues, entrapped in the extracellular matrix, may better embody the microenvironment alterations more prominent in the tumor surrounding tissue.

Keywords: cancer, colon cancer, biomarker, biopsy, decellularized tissue, tumor microenvironment

PF13.03 | Exploring the extracellular vesicles landscape in pancreatic cancer

Gaelle M. Vanderstichelen[1]; Florian Müller[2]; Claire Remacle[3]; Ingrid Struman[4]
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Introduction: Studying extracellular vesicles (EVs) in the tumor microenvironment (TME) remains challenging because one needs help to distinguish EVs between cell origins easily. In this project, we studied surface proteins in EVs in an in-vitro and in-vivo model of pancreatic cancer.
The objective is to characterize the different EV populations and select specific EV populations based on their surface markers, determine their role in pancreas cancer pathogenicity, and conduct longitudinal studies to assess the evolution of the EVs landscape during pancreatic cancer progression.
Methods: EVs were isolated by ultracentrifugation and characterized by nanoparticle tracking analysis, dynamic light scattering, and Western blot. The surface marker profile for the exosomes isolated from different cell models or that compose the TME in liquid biopsy was obtained using the Luminex MAGPIX (Luminex Corp., Texas, USA). This multiplex bead methodology allows the detection of up to 50 surface markers simultaneously.
We also generated murine pancreatic cell lines carrying a CD9-HA TAG that we could detect on EVs. These cells were injected into mice to follow the tagged EVs during pancreatic cancer progression. The other EVs originating from other cells that compose the TME were also analyzed.
Results: We showed that EVs isolated from different cell lines expressed different markers and that the EVs showed the same pattern of expression for the cell markers of the cells of origin. Hence, we found similar markers specific to the cell of origin, and we established a distinct signature for different EVs that compose the TME. We also demonstrated that the MAGPIX protocol could detect EV surface markers in mice plasma samples.
Summary/Conclusion: We showed that EVs isolated from different cell types expressed distinct markers and that MagPix could detect specifically surface markers on EVs in vitro and plasma samples.
Funding: Fonds de la Recherche Scientifique (F.R.S.-FNRS), ULiège.
Keywords: Biomarkers, MAGPIX, pancreatic cancer

PF13.05 | Functional and omics profiling of circulating extracellular vesicles subtypes in colorectal cancer

Luis Arteaga-Blanco; Dan A. Dixon
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Introduction: Colorectal cancer (CRC) is in the top 10 cancers most prevalent worldwide, affecting both men and women. Current research on tumor-derived extracellular vesicles (EVs) indicate that EVs play an important role in cellular communication through the transport of bioactive molecules to recipient cells and thus potentially modulate cancer progression via multiple pathways.
Methods: Here, we isolated and characterized circulating large EVs (LEV) and small EVs (SEV) released in the plasma from the APCMin/+ CRC mouse model and from patients with late-stage CRC (stage 3 and stage 4). EVs were separated by multiple ultracentrifugation steps and characterized by physical and biochemical properties.
Results: NTA and SEM showed two populations of vesicles with sizes over 200 nm and under 150 nm in diameter. The markers CD63 and CD81 were detected by WB in the preparations indicating the presence of EVs in our samples. Pellets enriched with LEV and SEV from APCMin/+ mice and late-stage CRC patients showed 4- and 3-fold higher particle concentrations, respectively, as compared with vesicles from normal mice and healthy donors. Proteomes from mice-derived EVs were characterized by LC–MS/MS. Our protocol enabled 126 and 11 protein groups for LEV and SEV, respectively, to be identified and quantified. Differences on miRNA content is ongoing and will be discussed. Moreover, we studied the impact of mice-derived EVs on the modulation of the pro-tumorigenic properties of normal colonic myofibroblasts. Our results showed that EV subtypes from tumor-bearing mice promoted migration in CCD-18Co fibroblasts.
Summary/Conclusion: These initial findings provide relevant information for further studies to explore the role of tumor-derived EVs and their ability to modulate CRC progression. We believe that continued in-depth analysis and omics characterization of these EVs from CRC mouse models and patients may be useful to identify molecules associated with tumor development and candidate biomarkers for CRC diagnosis and prognosis.
Keywords: colorectal cancer, cellular communication, colon fibroblast, extracellular vesicles

PF13.06 | Heterogeneity and uptake of extracellular vesicles affected by oncogenic RAS and RAF

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Introduction: Extracellular vesicles (EVs) are nano-sized vehicles equipping with diverse molecular components for the intercellular communication. In particular, it has been reported that the release of various EV subtypes are dynamically affected by oncogenic transformation, resulting in different functionality and uptake efficiency. In this study, we applied the nano-flow cytometry, quantitative proteomics, and high-throughput uptake screening of EV subtypes affected by mutant KRAS and BRAF.
Methods: EVs were isolated from wildtype (Caco2), mutant BRAF (HT29, WiDr), and mutant KRAS (HCT116, LoVo) colorectal cancer cells by size exclusion chromatograph. EVs were labeled with fluorescent labeled antibody (CD147, CD44, CD63, CD9, CD81) and their subtypes were analyzed by nano-flow cytometry. Proteomes of EVs were analyzed by Orbitrap Eclipse Tribrid Mass Spectrometer. EV uptake and their subcellular trafficking (cytosol, nucleus, lysosome) in recipient cells was measured by Lionheart FX automated microscope.
Results: We found increased CD147- and CD63-positive EV subtypes in mutant BRAF HT29 and WiDr cells. However, exosomal CD63-positive EVs were downregulated in mutant KRAS HCT116 and LoVo cells. CD9- and CD81-positive EVs are upregulated in both mutant RAS/RAF cells than wildtype cells. Moreover, mutant RAS/RAF cell-derived EV showed differential uptake and subcellular trafficking efficiency in the cytosol, lysosome, and nucleus. Quantitative EV surface proteomes showed the overexpression of specific integrins (ITGB4) and heparan sulfate proteoglycans (GPC1) in mutant RAS/RAF EVs relating in their increased EV uptake.
Summary/Conclusion: In this study, we discovered the heterogeneity of EVs and their differential EV uptake efficiency according to the oncogenic transformation. Our study shed light the new landscape of EV subtype-depend EV uptake and thus this knowledge can be used as a novel drug delivery platform in efficient transfer of therapeutic cargo from lysosome degradation.

PF13.07 | Spatial EV analysis by EV sheet unveils the location EV heterogeneity in cancer patients

Akira Yokoi[1]; Kosuke Yoshida[2]; Masami Kitagawa[2]; Hiroaki Kajiyama[1]; Yusuke Yamamoto[3]; Takao Yasui[2]
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Introduction: Extracellular vesicles (EVs) including exosomes are recognized as promising targets of disease mechanisms. However, one of the limitations of EV analysis is the requirement for a certain volume of bio-fluids. In addition, the location EV heterogeneity in cancer patients is largely unknown.
Methods: We developed a unique nanofiber-based sheet, named EV sheet for resolving these challenges. EV sheets successfully captured small-EVs (sEVs) from around 10 micro litters of bio-fluids and enabled to analyze proteins or RNAs which are included in sEVs.
Results: By attaching the EV sheet to the moistened organs in vivo, the sEVs from a tiny amount of ascites were collected and miRNA sequencing for those sEVs successfully worked. In the ovarian cancer mouse model, the EV sheet revealed that cancer-related miRNAs were detected from the very early phase when the mice did not have apparent ascites. EV sheet analysis in ovarian cancer patients, it is revealed that the tumor surface sEVs had distinct profiles from whole ascites. Furthermore, the direct EV sheet attaching method during the surgery of ovarian cancer patients identified the location-based unique sEV miRNA profile, and it can contribute to revealing unknown mechanisms of cancer progression. Comparing advanced-stage cases, the trajectory analysis revealed that the pattern of connection was different in patients with localized disease. Regarding biomarker application, the EV sheet has innovative properties that allow sEVs to be stored for one week in a dry condition. Tumor-derived sEV miRNAs on tumor surfaces were also detectable in serum, urine, or saliva those sEVs were captured by EV sheet, and reflected patient conditions.
Summary/Conclusion: EV sheet analysis can provide a whole new concept that the tiny amount of ascites-sEVs from ovarian cancer patients had location heterogeneity, and can create a new biomarker strategy, contributing to cancer diagnosis, staging evaluation, and therapy planning.
Funding: The Fusion Oriented Research for disruptive Science and Technology (FOREST; JPMJFR204J) from Japan Science and Technology Agency (JST) and the Project for Cancer Research and Therapeutic Evolution (P-PROMOTE) grant number: 22ama221407h0001 from the Japan Agency for Medical Research and Development (AMED).

PF14: Preeclampsia, Reproduction and Development

Location: Hall 4A

16:00 - 18:00

PF14.01 | Placental trophoblasts-derived extracellular vesicles as potential "Universal" tools for development of novel strategies for chronic inflammatory diseases

Nell Hirt[1]; Enzo Manchon[2]; Jean-David Bouaziz[3]; Safaa Saker-Delay[4]; Nabila Jabrane-Ferrat[5]; Reem Al-Daccak[6]
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Introduction: Human placental trophoblasts by their immune/regenerative/reparative advantages could present an optimal allogeneic source for development of therapeutic strategies. They orchestrate key steps of fetal developmental and continuous dialogue with the mother, by secreting extracellular vesicles (TEVs) to the maternal circulation and implantation bed. Within this setting, we explored the potential of allogeneic placental trophoblasts and their small TEVs as tools to lessen chronic inflammation by determining their impact on major actors of inflammation monocytes/macrophages.
Methods: Human placental trophoblasts were isolated from first-trimester placenta samples obtained, prior written informed consent in agreement with the guidelines of the Declaration of Helsinki, from healthy women going through elective termination of pregnancy. Small TEVs (50-150 nm) were obtained by successive ultracentrifugation and characterized by proteins (EV-enriched and non-EV-enriched) and particles analyses (NTA and high-resolution flow cytometry).
Results: Primary trophoblasts and TEVs directed the activation and differentiation of freshly-isolated classical monocytes towards anti-inflammatory profile imminent to IL4/IL13-macrophages. TEVs also fine-tuned inflammatory LPS/IFNg-macrophages towards anti-inflammatory IL4/IL13-macrophages, evidenced by increased expression of CD163/CD206 and phagocytic capacity, decreased inflammatory TNFa and increased anti-inflammatory IL10 cytokines production, and modified mitochondrial fitness (increased mitochondrial potential and decreased ROS production). TEVs also fine-tuned lipid signature of inflammatory (COX1/5-LOX-derived) monocytes/macrophages towards an anti-inflammatory (15-12-LOX/CYP450-derived) one.
Summary/Conclusion: Our findings reveal that TEVs have high potential to regulate chronic inflammation and open remarkable prospects for "Universal" tools that can be used to develop novel strategies for chronic inflammatory diseases.
Funding: French society of dermatology, Fondation Avenir, INSERM funding.
Keywords: trophoblast, strategies for chronic inflammatory diseases

PF14.02 | The role of placental macrophage extracellular vesicles in normal physiology and infection

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Introduction: Bacterial and viral infections of the placenta are linked to inflammation and adverse effects in pregnancy,. Hofbauer cells (HBCs) are fetal-origin placental macrophages, present throughout gestation, and permissive to bacterial and viral infection. This study aimed to characterise HBCs and HBC EVs under resting conditions, and following exposure to viral and bacterial pathogen associated molecular patterns; poly(I:C) and lipopolysaccharide (LPS).
Methods: HBCs were isolated from human placentas and cultured for 24h in the presence and absence of poly(I:C) and LPS. EVs were isolated by SEC and characterised by NTA and TEM. Cellular and EV protein was extracted and labelled with TMT reagents for quantitative proteomics. Labelled peptides were fractionated, subjected to LC-MS/MS, and data analysed using R.
Results: A total of 5982 proteins were found in HBCs and 169 in HBC EVs after accounting for batch corrections. A large fraction of proteins was significantly differentially abundant between control cells and cells treated with poly(I:C) and LPS, indicating a switch towards a pro-inflammatory phenotype. 28 proteins were differentially abundant (6 down and 22 up) in LPS-treated HBC EVs, and 39 (3 down and 36 up) in PIC-treated compared to control HBC EVs. Of these, 13 were commonly upregulated in LPS and PIC EVs, and were involved in clathrin-dependent endocytosis, Wnt5A signalling, and other pathways. The majority of up/down-regulated proteins in EVs were not changed in cells, suggesting specific shuttling of cargo to EVs. Sex-associated differences were observed in the cells but were not observable in EVs. Ongoing experiments are investigating the functional effect of HBC EVs on placental trophoblast.

Summary/Conclusion: We provide a novel understanding of the phenotype of HBCs and HBC EVs in normal physiology and infection, which could benefit the development of interventions for congenital infections.
Funding: National Institute for Child Health (NICHD) of the National Institutes of Health (NIH) under award number R01HD093801.
Keywords: placental macrophages, Hofbauer cells, placenta, infection

PF14.03 | Adipocyte-derived small extracellular vesicles may induce metabolic changes in post-implantation embryos

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Introduction: Obesity among women of childbearing age can alter fetal development and increase the risk of metabolic disease in the offspring. The mechanisms underlying the adverse effects of maternal obesity on post-implantation embryonic development before the establishment of the placental circulation remain poorly understood. We previously demonstrated that adipocyte-derived small extracellular vesicles (ad-sEVs) reflect maternal adipose tissue dysfunction. For example, ad-sEVs microRNA from pregnant women target PTEN and STAT3 and are associated with increased baby adiposity. We hypothesize that ad-sEVs from individuals with adipose tissue dysfunction (i.e. obesity and diabetes) will alter metabolic pathways during early embryonic development.
Methods: Cultured murine embryos at E8.5 were exposed to 3 μg/mL of visceral adipose tissue ad-sEVs from patients with obesity and insulin resistance (OIR; n = 5), or with obesity and diabetes (OD; n = 5). After 24 hours, embryo growth was measured, and development parameters were recorded using an adapted validated scoring system. Embryos, yolk sacs, and ad-sEVs were isolated for RNASeq.
Results: The embryos exposed to ad-sEVs from OIR, and OD had similar head size (1.14 $\pm$ 0.2mm; 1.07 $\pm$ 0.2mm), dorsal length (11.9 $\pm$ 1.6mm; 11.01 $\pm$ 2.3mm), and yolk-sac diameter (5.01 $\pm$ 0.15mm; 4.5 $\pm$ 0.7mm), when compared to controls (1.25 $\pm$ 0.2mm, 12.8 $\pm$ 3.4mm, 5.3 $\pm$ 0.5mm; $p>0.05$). In addition, developmental scores for heart development, embryo flexion, yolk sac circulation, and somite numbers were similar in all embryos. RNAseq from embryos and yolk sacs, in addition to small RNAseq from ad-sEVs is currently being performed.
Summary/Conclusion: As expected, a 24-hour exposure of post-implantation murine embryos to ad-sEVs from OIR and OD does not significantly alter morphology and developmental scores. However, we do expect the imminent molecular analyses will define that ad-sEVs microRNAs target genes in the embryo and yolk sacs. This analysis will identify changes in key pathways (i.e., IGF2 and PPAR-γ) that alter embryonic metabolism increasing the risk for metabolic disease in the offspring.
Funding: Children's National Research Institute, Center of Genetic Medicine - Collaborative Pilot Award.

PF14.04 | Extracellular vesicles in fetal circulation mediates metabolic changes in gestational diabetes pregnancies

Valeska Ormazabal[1]; Soumyalekshmi Nair[2]; Dominic Guanzon[2]; Andrew Lai[3]; Alexis Salas[4]; Felipe Zuniga[5]; Katherin Scholz-Romero[6]; David McIntyre[7]; Martha Lappas[8]; Carlos Salomon[9]
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Introduction: Changes in the levels and bioactivity of extracellular vesicles (EVs) have been well characterized in maternal circulation in normal and gestational diabetes (GDM) pregnancies, however, the little is known about the profile of EVs in fetal

circulation. The aims of this study were to establish the EV miRNA profile in fetal circulation from GDM and their effect on metabolic cell reprogramming.

Methods: EVs were isolated from cord-blood from women with normal tolerance glucose (NGT, n = 35) and GDM (n = 40) at the time of delivery. The miRNA content of EV was determined using a small RNA sequencing. Ingenuity pathway analysis (IPA) was used to determine the targeting and biological functions of EV miRNAs. Hepatic cells (HepG2) were used to establish the effect of EVs on cell metabolism and glycolysis.

Results: The levels of circulating EVs were significantly higher in GDM compared to NGT ($p < 0.05$). Linear regression analysis identified a positive association between fetal length, maternal BMI and the levels of circulating EVs in GDM, while no significant association was observed for NGT. Analysis of the miRNA within EVs identified a range of miRNAs differentially expressed between NGT and GDM samples. Principal component analysis reveals a separation of the groups based on their miRNA profile. Gene Ontology analysis shows that the miRNAs identified in EVs regulate genes involved in insulin secretion in response to glucose stimulus, insulin receptor signalling, and glucose homeostasis. Interestingly, EV from GDM regulates genes associated with glycolysis on HepG2 cells.

Summary/Conclusion: We suggest that diabetic envroment associated with GDM regulates the miRNA profile within circulating EVs in fetal circulation leading to changes of the metabolic function of target cells.

Funding: Fondecyt Initiation Project 11190522, NHMRC 1195451.

Keywords: fetal metabolism, gestational diabetes, miRNA

PF14.05 | Placental cells export insulin resistance associated miRNAs in extracellular vesicles via specific miRNA-protein interactions in gestational diabetes

Carlos Salomon[1]; Soumyalekshmi Nair[2]; Dominic Guanzon[2]; Nanthini jayabalan[3]; Andrew Lai[4]; Katherin Scholz-Romero[3]; Flavio Carrion[5]; Martha Lappas[6]

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Introduction: Gestational diabetes mellitus (GDM) is the hyperglycemia that develops during pregnancy and leads to short-term and long-term compilations to mother and fetus. Placenta is central to regulation of maternal metabolism during pregnancy and placenta-derived small extracellular vesicles (sEVs) are critical mediators of cross-talk between placenta and metabolic tissues. We have recently demonstrated that GDM is associated with selective enrichment of miRNAs in sEVs released from placenta. The aim of the present study is to identify the RNA binding proteins (RBPs) responsible for selective enrichment of miRNAs in sEVs in placental cells.

Methods: Biotin labelled miRNA probes for miRNAs hsa-miR-1246, hsa-miR-150-5p, hsa-miR-486-5p and hsa-miR-1285-5p was used to capture RBPs from trophoblast cells RBPs specifically interacting with the miRNAs were identified using mass spectrometric analysis. The proteins identified to be specifically interacting with the miRNAs were knockdown using siRNA and the miRNA packaging in sEVs were analyzed using real-time PCR. The binding of the RBPs to the miRNAs were confirmed by RNA immunoprecipitation (RIP).

Results: We identified that a unique repertoire of proteins (CPSF6, CWF19L1, DISC3, ELF4B, FASTKD2, GRSF1, HDLBP, HNRNPH2, PTBP3 and YBX3) specifically interacting with the biotinylated miRNAs. Knockdown of YBX3 protein in cells selectively reduced the expression of miR-1246 in sEVs. Interestingly, when GRSF1, HNRNPH2 and FASTKD2 were knockdown, an increase in expression of miR-1246 in sEVs was identified. In addition, knockdown of HDLBP and DISC3 selectively decreased the expression of miR-150-5p in sEVs. Further, RIP confirmed the binding of miR-1246 and miR-150-5p to YBX3 and HDLBP respectively.

Summary/Conclusion: This data provides new insights into the mechanism of selective packaging of miRNAs in sEVs mediated by RNA binding proteins and their association to regulation of insulin sensitivity in GDM.

Funding: National Health and Medical Research Council (NHMRC, 1114013).

Keywords: gestational diabetes mellitus, placenta, insulin resistance, miRNAs

PF14.06 | RECK in placental small extracellular vesicles; a possible origin and diagnostic of severe preeclampsia

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Introduction: Severe preeclampsia (sPE) is a pregnancy syndrome characterized by placental dysfunction and a generalized antiangiogenic state, associated to maternal hypertension and multisystemic damage. The number of placental derived small extracellular vesicles, recognized by PLAP expression (sEV PLAP) were found increased in maternal plasma (MP) of sPE compared to normal pregnancies. It is proposed that sEV PLAP are involved in sPE development. We evaluate the expression of RECK, a protein whit antiangiogenic effects and related to development of sPE, in sEV PLAP in the first trimester MP of mother that subsequently developed sPE and its angiogenesis effects.
Methods: sEV PLAP from MP of sPE and normal pregnancies at 1° trimester, were isolated by ultracentrifugation and characterized by common features. The expression of RECK was measured by ELISA and immunoblotting. We determined the role of sEV PLAP in endothelial cells, the uptake of stained sEV, tube formation assay, and expression of angiogenic markers.
Results: sEV from sPE and normal pregnancies present similar particle size, morphology, and exosome markers. The number of sEV PLAP are increased in sPE vs normal pregnancies. The expression levels of RECK are increased in sPE in relation to normal pregnancies. No differences in the uptake of sEV were observed. However, the sEV derived from sPE result in antiangiogenic effects.
Summary/Conclusion: RECK expression and number of sEV PLAP are increased in first trimester MP from pregnancies that subsequently develop sPE. We demonstrate a role of RECK on sEV PLAP in the angiogenic process. This suggest that RECK in sEV play key roles in the origin and development of sPE and emerge as predictor biomarker in sEV PLAP.
Funding: RECK expression and number of sEV PLAP are increased in first trimester MP from pregnancies that subsequently develop sPE. We demonstrate a role of RECK on sEV PLAP in the angiogenic process. This suggest that RECK in sEV play key roles in the origin and development of sPE and emerge as predictor biomarker in sEV PLAP.
Keywords: preeclampsia, RECK, placental sEV, biomarker

PF14.07 | Comparison of five isolation methods using commercially available kits to enrich extracellular vesicles from human seminal fluid

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Introduction: Extracellular vesicles (EVs) are nanosized membrane-enclosed compartments that serve as cell-to-cell communication messengers. Seminal plasma, the non-cellular liquid component of semen, supports sperm development and function, and contains EVs derived from male reproductive glands. As such, seminal fluid EV (sfEV) could potentially serve as novel biomarkers for male infertility and reproductive success. Currently, there is no 'one-size-fits-all' standardized technique for EV isolation. Here, we compared 5 commercially available size, filtration, charge and affinity-based kits for the enrichment of sfEV. We excluded ultracentrifugation due to its association with significant EV mechanical damage and protein contamination.
Methods: Excess semen donated to the CReATe Fertility Centre Biobank was used. sfEVs were isolated from pooled samples (n = 4) using 5 different isolation kits. For isolated EV characterization we used nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). For quantitative analysis we used flow cytometry.
Results: The TaKaRa EV isolation kit resulted in increased yield and purity, and also had an optimal size distribution, when compared to other kits. NTA displayed an average concentration of 1.44e+0.9 +/- 1.14e+0.8 particles/mL and 85% of EVs had diameters ranging from of 100–250nm. Five reference gates were used to detect EVs by size through flow cytometry; 76% of events were in the 100–500nm range vs ~12% using other kits. TEM confirmed spherical EVs sized between 100 to 250 nm, and importantly, showed less non-EV fragment contamination. Thus, our data suggested that TaKaRa kit isolated purer sfEV than other methods.
Summary/Conclusion: Our study provides insight into optimal sfEV enrichment methods that are compatible with a limited sample volume, such as semen, and doesn't require special equipment. The TaKaRa kit had superior yield, purity and optimal size distribution than the other four kits studied.

PF15: EV Seperational Technology 2

Location: Hall 4A

16:00 - 18:00

PF15.01 | Automated, high-throughput immunomagnetic isolation of extracellular vesicles from biofluids

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Introduction: Traditional extracellular vesicle (EV) isolation methods such as differential ultracentrifugation (UC) are time consuming, difficult to implement in clinical settings, and cannot be automated. We have recently developed immunomagnetic methods (EasySep™) to isolate EVs based on the tetraspanins or user-defined surface markers. In this study, we have developed fully automated EV enrichment protocols on the RoboSep™-16 platform, which can process up to 16 samples with volumes ranging from 0.5 - 2 mL.
Methods: EVs were either labelled directly with a tetraspanin (CD9, CD63, and/or CD81)-specific antibody cocktail or indirectly with a PE-conjugated antibody, followed by a PE-specific antibody cocktail. The labeled EVs were bound to magnetic particles and separated from unwanted EVs using an EasySep™ magnet. Automated isolation was performed as follows: guided by the on-screen prompts, plasma, EasySep™ reagents, tips, tubes, and wash solutions were loaded onto RoboSep™-16; the instrument automatically labeled EVs with antibody cocktails and particles, and transferred the sample to an EasySep™ magnet on the instrument deck for magnetic separations. EVs isolated manually or via RoboSep™-16 were analyzed by western blot to assess recovery.
Results: With EasySep™, EV subtypes from human and non-human (e.g. mouse) species were isolated in less than 30 minutes. The manual EasySep™ pan-EV kit recovered 1- to 2-fold more EVs than UC (n = 6). The fully automated RoboSep™-16 pan-EV and CD63+ EV protocols recovered 89 - 98% EVs with lower albumin contamination relative to the manual protocols (n = 6 for pan-EV; n = 6 for CD63+ EV). A full capacity run of 16 CD63+ EV enrichments took approximately 1 hour and 40 minutes with variance in recovery of 9.3%.
Summary/Conclusion: EasySep™ allows for fast and easy immunomagnetic enrichment of EVs. Fully automated EV isolation on RoboSep™-16 further reduces impurities and user variability, offering practical solutions for clinical laboratories using EVs as biomarkers of disease.
Funding: N/A.
Keywords: EV separation, immunomagnetic isolation, automation, high-throughput

PF15.02 | Pan-specific, affinity isolation of small Extracellular Vesicles from minimally pre-treated biological fluids by membrane sensing peptides

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Introduction: Affinity based systems for isolation of Extracellular Vesicles (EVs) from complex biosamples are commonly plagued by poor recovery and often requires a pre-concentration step in the analytical workflow. Here we present a pan-specific isolation method of small EVs (sEVs) from minimally pretreated biological fluids (serum, plasma and urine) based on membrane sensing peptides (MSP) as general EV binders, providing efficient EV recovery and minimum co-isolation of contaminants.
Methods: Magnetic agarose beads (40 micrometer diameter) are modified by MSPs using conventional His-tag chemistry. 100 microliters of bead suspension are incubated for 1 hour in 1 mL urine (following 2 clearing steps of 15 min at 3000g) or 50 microliters of serum or platelet free plasma (EDTA, Heparin, Citrate) diluted 1:10 in PBS. Intact EVs are released by a mild treatment with a saline buffer for subsequent characterization by microscopy (AFM, TEM), Nanoparticle Tracking Analysis and Western Blotting (WB) of EV external and luminal markers. Common contaminants like uromodulin for urine and albumin and

lipoproteins (ApoA, ApoB, ApoE) were also checked by WB. Following isolation, EVs are subjected to immunophenotyping by ExoView and nanoFCM platforms and RNA extraction and RT-PCR analysis.
Results: Highly efficient isolation of sEVs was observed from both serum/plasma and from urine, with minimal recovery of lipoproteins and undetectable albumin contamination for blood derivatives and uromodulin for urine. Electron microscopy confirmed the recovery of EVs with an average diameter in the 80–110 nm range. EVs were captured propaedeutic to the RT-PCR analysis of specific miRNA and lncRNA and immunophenotyping by single-vesicle techniques. The protocols were compared to reported standard procedures using antibody modified beads, ultracentrifugation (UC), ultrafiltration (UF), size-exclusion chromatography (SEC).
Summary/Conclusion: We developed highly efficient, easy, timesaving and robust protocols for capture-and-release of sEVs from biological fluids. We envision that the integration of MSP as pan-selective molecular tools on different platforms (beads, resins, microplates) may find broad application in blood and urine analytical workflows.
Funding: EIC funded project MARVEL. Grant agreement ID: 951768.
Keywords: affinity isolation, blood workflow, urinary EVs

PF15.03 | Tumor derived extracellular vesicles enrichment using a novel immunoaffinity magnetic bead-based matrix

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Introduction: Despite emerging evidence of the surveillance and diagnostic potential of tumor-derived extracellular vesicles (TEX), current extracellular vesicle (EV) isolation tools do not differentiate between tumor and non-tumoral EVs. SubB2M 1, a site-directed mutation of the B subunit of the subtilase cytotoxin (Sub2B), recognizes the tumor-associated sialic acid, N-Glycolylneuraminic acid (Neu5Gc). The aim of this study was to evaluate the utility of SubB2M immobilized on paramagnetic nanoparticles (TEXO-NET) to isolate a Neu5Gc enriched subpopulation of tumor-derived EVs for diagnostic application.
Methods: SubB2M was covalently bound to paramagnetic nanobeads (~140 nm diameter) using an amine-reactive crosslinker. EV were isolated from both breast cancer plasma and normal human plasma using TEXO-NET, EXO-NET (a pan EV capture matrix) and two other commercial EV isolation kits. Captured EVs were quantified using Nanoparticle Tracking Analysis (ZetaView, Particle Metrix). Cancer known miRNAs and protein content of captured EVs were assessed by RT-PCR and Western blotting.
Results: ZetaView analysis showed TEXO-NET captured a significantly higher number of particles ~83% from breast cancer plasma compared to other two competitor kits which captured ~66.5% and 68%. RT-PCR analysis also demonstrated higher abundancy of tumor-known miRNAs, miR1229 and Let7d, from TEXO-NET-captured EVs compared to other two kits. This data was supported by protein analysis data demonstrating a distinct protein profile for TEXO-NET-captured EVs compared to EXO-NET-captured EVs.
Summary/Conclusion: The data obtained confirm that TEXO-NET captures a subpopulation of EVs that is enriched in tumor-specific biomarkers which can potentially be used for identification of diagnostics biomarkers of tumor onset, progression, triage to treatment, and treatment response.
Keywords: extracellular vesicles, tumour derived EV, TEXO-NET, EV isolation, cancer biomarker

PF15.04 | High-throughput isolation and enrichment of extracellular vesicles using an immunoaffinity magnetic bead-based matrix

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Introduction: Extracellular vesicles (EVs) including exosomes have great potential for both diagnostic and therapeutic applications. The lack of standardized methods for efficient and high-throughput isolation and analysis of EVs, however, has limited their use and application for the clinical practice. Here we present a novel bead-based immunoaffinity system (EXO-NET®) that captures a highly enriched subpopulation of EV. EXO-NET is 3-D monoclonal antibody matrix constructed on magnetic bead-based immunoaffinity for isolation and enrichment of EVs.
Methods: EVs were isolated from human pooled plasma (500 μl) using EXO-NET (300 μl) and 3 others commercial EV isolation kits according to manufacturers' instructions. Nanoparticle tracking analysis (NTA, ZetaView) was used to analyze the particle size and number. The protein content of captured EVs was characterized by mass spectrometry and Western blotting. RT-qPCR was used to evaluate and quantify mRNA (GAPDH and OAZI) and microRNA cargo (miR-16, let-7a, miR-21) of isolated EVs from EXO-NET and other commercial kits.
Results: NTA established that EXO-NET captured a greater proportion (>75%) of EVs (50-130 nm size) from plasma when compared to other comparable commercial kits (< 65%). Western blotting analysis demonstrated that EXO-NET not only outperformed other kits in term of enrichment of EVs marker (flotillin) but also reduced commonly co-isolated contaminant protein (ApoB). Mass spectrometry analysis indicated that contaminant albumin peptide intensity was 3-fold higher in other isolation kits compared to EXO-NET. In addition, the total peptide intensity of known EV associated markers was 6 to10-fold higher in EXO-NET than other kits. At the transcriptomic level, qPCR analysis showed that EXO-NET had higher yield and recovery (4-8-fold) of both mRNAs and microRNAs compared to other commercial kits.
Summary/Conclusion: The EXO-NET is a novel method for the rapid, efficient, and scalable enrichment and purification of EVs that reduces contaminants which may confound downstream analysis.
Keywords: EXO-NET, extracellular vesicles, EV isolation, EV enrichment

PF15.06 | Fabrication of nanopillar-based EV sorting device via mixed lithography

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Introduction: Nanopillar structures have been utilized as a separating or sorting structure for biomolecules. For sorting of EV with the size of 50 to 200 nm, it is needed, a stable fabrication of the nanometer-sized structures.
Methods: We employed semiconductor-based fabrication methods like electron-beam lithography (EBL), photolithography, and reactive ion etching (RIE). For the fabrication of nanopillar array, we started with the thin film stack of 100 nm silicon-oxide (SiO2) hard mask layer and 400 nm low-pressure chemical vapor deposition (LPCVD) amorphous silicon (a-Si) layer upon 300 nm thermal oxide layer on 6 inch silicon wafer. By EBL, the array of nanohole patterns were generated, which was transferred to SiO2 hard mask by RIE. For the patterning of microstructure, large scale micropatterns were generated by photolithography, which was transferred to the SiO2 hard mask by RIE. Accordingly, the SiO2 hard mask layer has both EBL and photolithography patterns together, which was transferred to a-Si layer by the second RIE. On top of the patterned a-Si layer, tetraethyl orthosilicate (TEOS) oxide and plasma-enhanced chemical vapor deposition (PECVD) oxide were back-filled followed by being planarized by chemical-mechanical polishing (CMP). Vent-hole structures were generated in the oxide layer. By using the a-Si layer as sacrificial materials, we released the a-Si by XeF2 gas phase etching to convert the a-Si nanochannels to nanofluidic channels. To seal the vent holes, 2 micrometer PECVD oxide was deposited.
Results: By designing an optimized jig structure, we introduced EV sample into the nanopillar-based sorting device. The device has one inlet and five outlets. From HEK293 cell-lines, EV was isolated by ultracentrifuge process. Nano tracking analysis (NTS) shows 100–200 nm size particles. The HEK293-driven EV was sorted into five different ports. RNA and protein analysis were under investigation.
Summary/Conclusion: We present a method to fabricate nanopillar-based EV sorting device. HEK293-drivien EV was sorted into five different sub-groups. Our method provide an EV sorting process based on physical interactions between EV and nanopillar array under continuous flow.
Keywords: EV sorting, semiconductor, HEK293, nanopillar, sacrificial process

PF15.07 | Rapid isolation of extracellular vesicles by a portable microstructured electrochemical fluidic device

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Introduction: Extracellular vesicles (EVs) have recently emerged as versatile therapeutic agents, biomarkers, and potential drug carriers. Despite the growing interest in developing EVs for therapeutic and diagnostic usage, progress in this field is partly hindered by the lack of efficient isolation techniques. This work describes an electrochemically controlled device enabling the rapid capture and release of EVs, from various human biofluids and wounded skin tissue originating from healthy (WT) and diabetic mice.
Methods: The device consists of a fluidic channel where EVs are immobilized by affinity interactions and controllably released by applying voltage. The median diameter and concentration of the isolated EVs were determined by nanoparticle tracking analysis and electron microscopy. Their protein composition was assessed by western blot and proteomics. A principal component analysis was used to reflect the variation between protein abundances of EV populations of diabetic and WT mice.
Results: The investigated biosamples (i.e. serum, urine, cell culture supernatant, plasma) were injected into the fluidic channel and EVs were purified using two different antibody coatings. Isolated EVs displayed differences in their size distribution and concentration, indicating that the device could be further used for an estimation of the abundance of EV subpopulations, simply by changing the type of the attached antibody. EVs recovered from wounds in both WT and diabetic mice showed different protein composition. In particular, an increase in mitochondrial proteins in the EVs from wounds isolated from diabetic vs. healthy mice was observed.
Summary/Conclusion: Our electrochemical device was used to isolate EVs from a number of physiological fluids. The selective isolation of different EV subpopulations could, in principle, be easily performed for more in-depth vesicle characterization and diagnostic use, as suggested by the EVs wound data.
Funding: This work was supported by the ETH Zurich – Open ETH project SKINTEGRITY.CH, the Swiss National Science Foundation (grant 31003B-189364 to S.W.).
Keywords: extracellular vesicles, immunoaffinity, electrochemical device, carbon microfibers, biosample, purification, preconcentration

PF15.08 | Reproducible automated isolation of extracellular vesicles using high-performance liquid chromatography (HPLC) for clinical applications

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Introduction: The current method of isolating extracellular vesicles, such as ultracentrifugation, can be time-consuming, requiring specialised equipment, labour intensive, and quality control can be challenging. Here we describe the development of a reproducible, automated method to isolate extracellular vesicles using high-performance liquid chromatography (HPLC) that can be applied to routine pathology laboratories.

Methods: A modular HPLC system with a fraction collector coupled with either a multimodal chromatography or size-exclusion column was used for this study. To optimise the HPLC method, replicates of fetal bovine serum or human serum were processed (200 μL), with multiple elution fractions collected from each starting sample. EVs containing fractions were selected based on Nanoparticle Tracking Analysis (NTA), Western blots, and electron microscopy. Reproducibility was calculated based on NTA and protein quantification data. To further demonstrate this isolation platform, the isolated EVs from human plasma were subjected to next-generation sequencing (NGS) and mass-spectrometry proteomic analysis. Finally, to show the real-world clinical utility of this automated isolation method, we isolated EVs and investigated the proteomic and miRNA profile of EVs from patients with ovarian cancer (n = 36), colorectal cancer (n = 26), endometrial cancer (n = 20), lung cancer (n = 30), brain metastasis (n = 36), and controls. control. A pooled plasma sample was also isolated intersperse throughout as a quality control.
Results: A highly reproducible method of isolating extracellular vesicles from bovine and human plasma was achieved. Fractions containing EVs were confirmed. The average inter CV for among the technical replicate was below 10% for the particle mean (3.75 %), mode (7.68 %) and protein concentration (8.45 %). This suggests that this method is reproducible with low technical variations. In addition, using the optimised automated method from one biological sample, most of the total vesicles can be purified into only one tube. Furthermore, the purity of the isolated vesicles was comparable to current isolation techniques and can be used for downstream analyses such as NGS and proteomics. Using PCA analysis on the proteomic and miRNA data, we demonstrated that we could identify cancer-type-specific plasma EVs using our HPLC system.
Summary/Conclusion: Using automated systems to isolate extracellular vesicles could have important implications in diagnostics, where high throughput and reproducibility is essential.
Keywords: isolation, size exclusion chromatography

PF15.09 | Nanoscale optical trapping of single extracellular vesicles using plasmonic cavities

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Introduction: Extracellular vesicles are heterogeneous particles released by cells and comprise a bonafide means for cellular communication. In addition to EVs, recently new kinds of extracellular particles known as exomeres and supermeres have been discovered. The heterogeneity of extracellular vesicles and particles (EVPs) have been identified as a key factor limiting an enhanced understanding of EVPs. Most of the EV analysis techniques such as western-blotting work at the EV population level and unable to analyze EVs on an individual particle level. Recently, laser trapping Raman spectroscopy (LTRS) has become an emerging approach for EV analysis. In this approach, EVs are trapped within a laser focus using an optical tweezer and the Raman signal is acquired, which provides information on the nature of the biomolecules present in EVs such as proteins, lipids, and nucleic acids. This approach could be harnessed to discriminate EVs from contaminating lipoproteins.
However, LTRS are not applicable for analyzing nanosized EVs because the nanosized EVs are too small to be trapped in an optical tweezer due to the diffraction limit of light. In this work, we report a novel approach for trapping nanoscale EVPs by coupling light to optimized gold nanostructures that generate stable optical force due to the excitation of plasmonic waves. We report the stable trapping of nanoscale EVs using the plasmonic cavities for the first time.
Methods: The plasmonic cavities for trapping EVs were fabricated by using focused ion billing machine to mill nanoscale features on a 100 nm thick gold film, and packaged into a microfluidic chip. Experimental testing of stable EV trapping at the plasmonic cavities was performed using commercially available EVs obtained from Creative Diagnostics. The EV solution was injected into the microfluidic chip containing the plasmonic cavities. One of the plasmonic cavities was illuminated with a laser beam to trap the EV at the plasmonic cavity. The EV was released by turning off the laser beam. To confirm the size of the trapped EV, we applied a low frequency alternating current field to pattern the trapped EV and image with an SEM.
Results: Our results show that nanoscale EVs can be trapped with a low optical power of 2 mW using the plasmonic cavity. In comparison with the conventional laser tweezers, the trapping would have required about 70 to 100 mW optical power. Furthermore, our results show that the EVs can be reversibly released from the trap by turning off the laser power or moving the laser away from the plasmonic cavities.
Summary/Conclusion: We have reported a novel nanotweezer approach based on plasmonic cavities for the stable trapping of nanoscale EVs, which are too small to be trapped and analyzed using the conventional laser tweezer. Our proposed plasmonic cavity nanotweezer system paves the way for tether-free stable trapping of nanoscale EVs and Raman spectroscopy of trapped EVs to understand their heterogeneity.
Keywords: laser tweezers, nanoplasmonics

PF16: Cancer Microenvironment 2

Location: Hall 4A

16:00 - 18:00

PF16.02 | Extracellular vesicles promote cancer associated fibroblast activation in oral cancer

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Introduction: Oral cancer (OC) is the most common form of head and neck cancer and has a survival rate of just ~50% over 5 years. New treatment strategies are sorely needed to improve patient survival for which we must uncover underlying mechanisms in tumorigenesis.
The role of the tumor microenvironment (TME) is increasingly shown to be crucial in tumor progression and metastasis. One of the main constituents of the TME, cancer associated fibroblasts (CAFs), play a key role in influencing the biological behavior of tumors. Multiple mechanisms contribute to CAF activation such as TGFβ signaling, but our knowledge of the role of extracellular vesicles (EVs) in CAF activation is limited in OC. Assessing the impact of oral cancer derived EVs on CAF activation could better our understanding of OC and lead to novel treatments.
Methods: EVs were isolated from OC cell lines (CAL27, SCC9, SCC25) using differential centrifugation. Nanoparticle tracking analysis was used for EV quantification and size characterization, and western blots to confirm the presence of EV protein markers. Oral fibroblasts (OF) were co-cultured with enriched EVs, TGFb or PBS over 72 hours in order to assess activation. Flow cytometry was used to evaluate the difference in CAF markers. RNA collected from the fibroblasts was extracted and the transcriptome was sequenced.
Results: Flow cytometry of the OF revealed that EVs derived from all three OC cell lines show upregulated CAF markers. RNA sequencing also revealed two distinct CAF marker signatures depending on whether the CAFs were activated through EVs or TGFβ, potentially indicating different mechanisms of activation.
Summary/Conclusion: Taken together, our results reveal the ability of OC derived EVs to activate fibroblasts into CAFs via a mechanism distinct from canonical TGFβ activation. Gaining an understanding of the interplay between EVs and stromal cells such as CAFs could lead to further insights into OC tumorigenesis and potential novel therapeutics.

PF16.04 | SERPINA3 and LCN2 exerts the osteoblastic and tumor-suppressive functions in prostate cancer

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Introduction: Prostate cancer (PCa) exhibits two primary features: the frequent occurrence of osteogenic bone metastases and a favorable survival rate. PCa boasts a 10-year survival rate of 45%, even for patients with metastatic disease, which may be due to the characteristics of osteoblastic bone metastasis. In this study, we hypothesized that understanding the molecular mechanisms of osteoblastic bone metastasis could clarify the pathogenesis of PCa.
Methods: To assess the impact of osteoblasts on PCa cells, we employed a horizontal co-culture model with osteoblasts (OBs) and either osteolytic PCa (LPCa) or osteoblastic PCa (BPCa). RNA sequencing of these PCa cells revealed SERPINA3 and LCN2 as key players in BPCa. To evaluate the effect of SERPINA3 and LCN2 on osteogenesis, we generated an overexpression model using LPCa cells. Furthermore, we collected conditioned medium from SERPINA3- or LCN2-overexpressing HEK293T cells, administered the medium to BPCa cells, and evaluated their roles in BPCa itself. We also examined the feasibility of SERPINA3 and LCN2 as biomarkers using TCGA data from PCa patients.
Results: In a co-culture of OBs and BPCa cells, SERPINA3 and LCN2 were significantly upregulated in BPCa via OB-derived extracellular vesicles, while they were not in the co-culture of OBs and LPCa cells. In both the co-culture system and mouse xenograft experiments with intracaudal injection, enhanced expression of SERPINA3 and LCN2 in PCa led to osteogenesis.

Additionally, the addition of SERPINA3 or LCN2 to BPCa cells significantly suppressed proliferative potential. Retrospective analysis also confirmed that high expression levels of SERPINA3 and LCN2 were significantly correlated with a better prognosis.
Summary/Conclusion: Our results may partially explain why the prognosis for PCa forming osteoblastic bone metastasis is relatively better compared to osteolytic bone metastasis.
Funding: JSPS KAKENHI grant numbers 21H02721, 19K07652, and the Jikei University Research Fund for Graduate Students.
Keywords: prostate cancer, osteoblastic bone metastasis

PF16.05 | Osteoclasts educated by prostate cancer cells regulate osteoblast-activity via communication networks with extracellular vesicles

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Introduction: Prostate to bone cancer metastases induce mixed lesions containing areas of bone destruction and formation that are directed by osteoclasts (OCs) and osteoblasts (OBs), respectively. OCs play an essential role in the tumor invasion areas even in osteogenic bone metastases of prostate cancer (PCa). OC-derived extracellular vesicles (EVs) are reported to regulate OB activity in normal bone homeostasis; however, there is no study investigating the role of EVs from OC educated by PCa cells in the tumor bone microenvironment.
Methods: We prepared four types of OCs and EVs from these cells; OCs differentiated from RAW264.7 cells (OC cells), OC cells co-cultured with normal prostate epithelial cells (OCN cells), osteolytic PCa cells (OCP cells), and osteoblastic PCa cells (OCC cells). OC differentiation was induced in the presence of RANKL. EVs were purified from culture supernatant using ultracentrifugation.□To observe the changes in PCa-educated OCs, we investigated the response to denosumab (anti-RANKL antibody) and signaling pathways in OCP and OCC cells. Further, to reveal the function of EVs from PCa-educated OCs, we added EVs to mineralizing OBs (MC3T3-E1). The expression levels of OB marker genes such as ALP and BGLAP, which are stably expressed in MC3T3-E1, were compared by qPCR and ALP staining was conducted. Next-generation sequencing (NGS) was performed to identify EV-delivered miRNAs regulating OB activity.
Results: OCP and OCC cells showed denosumab resistance. Some signaling pathways, which are reported to promote OC differentiation independent of the RANKL pathway, were increased. Moreover, OCP and OCC cell-derived EVs significantly inhibited OB activity; these EVs down-regulated OB marker genes. NGS identified some candidate miRNAs as EV components regulating OB activity.
Summary/Conclusion: We report the role of EVs derived from OCs educated by cancer cells for the first time. OCs educated in the tumor invasion areas may release EVs, inhibiting OB activity and leading to further bone destruction.
Funding: This work was supported by Project for Cancer Research and Therapeutic Evolution (P-CREATE) grant number: JP20cm0106402 (to T.O.) from the Japan Agency for Medical Research and Development (AMED).

PF16.06 | Small extracellular vesicles from neutrophils treated with 27-hydroxycholesterol promote epithelial to myesnchymal transition (EMT) and stemness in breast cancer cells

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Introduction: Breast cancer is the second leading cause of cancer-related death in women, continuing to provide rationale for studies to increase our mechanistic understanding and pharmacologic development. Elevated cholesterol is associated with poor prognosis; although likely multifactorial, the mechanisms by which cholesterol impacts breast cancer are still being elucidated.

We have found that the primary metabolite, 27-hydroxycholesterol (27HC) induces secretion of small extracellular vesicles (sEVs) from primary neutrophils. Importantly, these 27HC induced sEVs were found to promote tumor growth and metastasis in murine models of breast cancer. We hypothesized that 27HC results in altered cargo within sEVs which results in functional changes within target cells, the ultimate consequence of which being their increased metastatic potential.
Methods: sEVs isolation and characterization are described in PMID: 33959755. Briefly, the sEVs isolation was performed using an ExoQick kit [SBI]; characterized for size by NTA and morphology by transmission electron microscopy. Common sEVs markers were detected by flow cytometry, and metabolic profililing by SLAM microscopy.
Results: Using a non-biased miRNA-seq approach we found that sEVs from 27HC-treated neutrophils had an altered miRNA signature. Several altered miRNAs were implicated in the WNT pathway. Our subsequent work found that mammary cancer cells do take up sEVs, and adopt a mesenchymal and stem cell phenotype after treatment with 27HC-sEVs. Our data to date indicate that this is likely due to modulating the canonical WNT signaling pathway.
Summary/Conclusion: 27HC increases metastasis in part through its effects on neutrophils, by releasing sEVs carrying pro-tumor microRNA to cancer cells promoting EMT and stemness, and thus their ability to migrate, invade and metastasize.
Funding: Supported by NIH (ERN: R01CA234025) Department of Defense (ERN: BCRP Era of Hope Award), and Beckman Institute for Advanced Science and Technology (NK).

PF16.07 | Tumor-derived large extracellular vesicles in Drosophila

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Introduction: Tumor-derived extracellular vesicles (EVs) play important roles in communication between tumor cells and immune cells, which elicits anti- and pro-tumor immune responses depending on the context. Considering the powerful genetic tools available in Drosophila and the transparency of the larvae, Drosophila could be a desirable in vivo model for studying the mechanisms underlying the production of EVs from tumor cells and the processing of EVs in immune cells, as well as the physiological roles of the EV-mediated communications. However, it is unknown whether Drosophila tumors also produce EVs that play a role in communication with immune cells.
Methods: We employed a well-characterized Drosophila epithelial tumor model to address whether Drosophila tumors produce EVs. Live imaging techniques allowed us to trace tumor-derived large EVs in live animals and observe the production of large EVs from tumors. Moreover, we could biochemically enrich tumor-derived large EVs and transplant them into either wild-type or genetically modified larvae to investigate their physiological roles (Track ID: EV140287).
Results: We found that the production of large EVs (larger than 1 micrometer) from tumors is a conserved process in Drosophila. Interestingly, the injection of large EV fractions was sufficient to induce a systemic immune response in a manner dependent on Drosophila immune cells hemocytes. cGAS-STING signaling was elevated in tumors, and STING knockdown in tumors suppressed the production of large EVs. Interestingly, injection of large EV fractions also increased cGAS-STING signaling in hemocytes, and STING knockdown in hemocytes suppressed the large EV-induced systemic immune response.
Summary/Conclusion: Our study visualizes how cGAS-STING signaling propagates via the generation of tumor-derived large EVs to induce a systemic immune response and establishes Drosophila as an animal model for studying the biology of tumor-derived large EVs. Furthermore, we elucidate the conserved role of cGAS-STING signaling in controlling the production of large EVs from tumor cells.
Keywords: tumor, large extracellular vesicle, drosophila and systemic immune response

PF16.08 | Tissue hypoxia modulates the lipidomic profile of small extracellular vesicles from head and neck squamous cell carcinoma cells

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Introduction: Tissue hypoxia, present in solid tumors, including head and neck squamous cell carcinomas (HNSCCs), alters molecular and functional activity of cancer cells. Our previous experiments demonstrated that hypoxia increased the release of tumor-derived small extracellular vesicles (sEVs) and influenced their proteomic profile. In this study we aimed to characterize the lipidomic profile of sEVs released from cells cultured in normoxic and hypoxic conditions.
Methods: HNSCC cells (PCI-30) and normal control cells (HaCaT keratinocytes) were exposed to 21 % (normoxia) and 1 % (hypoxia) oxygen supply. sEVs were isolated from supernatants using size exclusion chromatography (SEC) and characterized by

nanoparticle tracking analysis, electron microscopy, immunoblotting, and high-resolution mass spectrometry. Gene expression levels based on RNA-seq data from HNSCC patients and clinical characteristics were obtained from the Cancer Genome Atlas (TCGA). Expression profiles of lipidomic signatures were compared between a total of 522 cases of primary HNSCC and 44 normal control samples.
Results: Isolated sEVs ranged in size from 125–135 nm and carried CD63 and CD9 but not Grp94. We detected 6176 lipids and glycerolipids were among the most abundant lipid classes in sEVs. We found almost 1000 lipids exclusively carried by tumor-derived sEVs in comparison to normal sEVs from keratinocytes. Hypoxia triggered a major switch of the lipid profile with ~1000 lipids being exclusively detected in hypoxia-derived sEVs and 706 lipids being significantly upregulated under hypoxic conditions. In terms of numbers, fatty acids were the most significantly hypoxia-induced lipid class, in terms of abundance the most significant changes were observed for glycerophospholipids. This data was validated on the transcriptome level using the TCGA HNSCC cohort. The expression levels of genes involved in the biosynthesis of glycerophospholipids significantly correlated with hypoxia-, angiogenesis- as well as sEV secretion-related genes in the TCGA HNSCC cohort. Also, the expression levels of genes involved in catabolism of glycerophospholipids significantly correlated with improved survival.
Summary/Conclusion: Lipid profiles in HNSCC-derived sEVs are characterized by remarkable plasticity and are modulated by environmental factors such as hypoxia. Thus, sEV-associated lipids may emerge as clinical biomarkers for tumor progression or tissue hypoxia in HNSCC.
Funding: MB/M/48(79)# to A.G./Medical University of Warsaw.

PF16.09 | Selective export of miR-100 and miR-125b from colorectal cancer cells

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Introduction: We previously showed that miR-100 and miR-125b are selectively exported from KRAS mutant colorectal cancer cells. We also generated a Cetuximab resistant cell line (CC-CR) that dramatically overexpresses and selectively secretes miR-100 and miR-125b. These two miRNAs are encoded within an intron of the long noncoding RNA MIR100HG. Initial analysis suggested that miR-100 and miR-125b target negative regulators of Wnt signaling. Here, we used RNA sequencing, bioinformatic analyses, and knockout cell lines to identify the full range of mRNA targets for miR-100 and miR-125b.
Methods: NAseq was performed on Cetuximab resistant CC-CR cells, Cetuximab sensistive CC cells, and cells lacking miR-100, miR-125b, or MIR100HG. We used bioinformatic approaches and miRNA prediction algorithms to identify putative mRNA targets for miR-100 and miR-125b. To better identify such targets, we also performed immunoprecipitation of Ago2 complexes and conducted small and long RNAseq. The combined data sets allowed for the identification of mRNA targets for miR-100 and miR-125b. Validation of these targets is being performed using luciferase assays in which the 3' UTR sequences from predicted targets are fused to luciferase and the extent of silencing determined in transfected cells. Testing of the ability of these miRNAs to undergo transfer from donor to recipient cells is being performed using Transwell assays.
Results: miR-100 and miR-125b are selectively exported from colorectal cancer cells and can be transferred to recipient cells. The use of knockout recipient cells to validate transfer of these miRNAs has shown the level of mIR-100 and miR-125b can be increased from 70-150-fold in Transwell assays. RNAseq and Ago2 immunoprecipitation experiments allowed us to develop extensive lists of mRNA targets for miR-100 and miR-125 including IGF2BP2, CGN, and RASGRP3. Direct testing of these targets is underway.
Summary/Conclusion: miR-100 and miR-125b target many mRNAs that are involved in colorectal cancer progression and metastasis, through both cell-autonomous and non-cell-autonomous function. How these miRNAs are transferred between cells, whether by extracellular vesicles or other mechanisms is under active investigation.
Funding: Funding for this work is PO1CA229123.
Keywords: miRNA

PF16.11 | Radiation-Induced extracellular vesicle secretion alters intercellular communication in fibroblasts

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Introduction: Although primary tumors are managed through a combination of surgery, radiotherapy, and chemotherapy, triple-negative breast cancer (TNBC) patients experience relatively high rates of recurrence after treatment. The role of intercellular communication in this process is not well understood. Recent studies have shown that ionizing radiation (IR) activates several systemic biological responses, which largely depend on interactions between healthy and injured cells. Radiation-induced

bystander effects (RIBE) are secondary effects that develop in non-irradiated cells as a result of complex intercellular signals sent by irradiated cells. Therefore, we hypothesized that extracellular vesicles (EVs)—membrane-delimited structures containing all major classes of biomolecules—function as mediators of RIBE, leading to TNBC progression. In this study, the microenvironmental consequences of radiation-induced EV secretion were examined. This work represents a critical step toward determining how cell-cell crosstalk after IR contributes to breast cancer recurrence.

Methods: Human fibroblasts were used to model the key cell type in the wound healing response. Fibroblasts were irradiated to a dose of 10 Gy. We used nanoparticle tracking analysis (NTA) to characterize EV secretion 48 hours post-IR in control and irradiated cells. We also determined the ability of irradiated fibroblast-derived EVs to alter the phenotype of recipient cells. We examined fibroblast morphology and cytoskeletal dynamics following EV treatment through visualization and quantification of actin fiber reorganization. Furthermore, we used western blot and immunofluorescence analysis to analyze fibroblast activation and shift to a cancer-associated phenotype. Finally, we utilized mass spectrometry to evaluate the differentially regulated proteins in EVs after IR.

Results: IR enhances EV secretion but does not alter the size of the EVs. Additionally, the treatment of fibroblasts with EVs from irradiated cells led to morphology changes and a re-distribution of F-actin. This trend follows changes observed in directly irradiated cells. Our findings suggest the ability of irradiated cells to induce bystander effects through EVs. Finally, mass spectrometry results suggest differential packing of key EV proteins after IR causes EV-induced phenotypic shifts.

Summary/Conclusion: Our results establish changes arising from interactions between irradiated and non-irradiated cells through the transfer of EVs, suggesting a connection to local and systemic RIBE. Notably, EVs derived from irradiated fibroblasts resulted in morphological and phenotypic changes in unirradiated fibroblasts. This work will further our understanding of EV-mediated communication after radiotherapy and may lead to novel therapeutic strategies for preventing TNBC recurrence.

PF16.12 | Cancer cells release A to I edited RNA repeat elements into extracellular vesicles for reprogramming of the tumor microenvironment

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Introduction: Ewing sarcoma (EwS) is a highly aggressive cancer and the second most common malignant bone tumor in children and young adults with high propensity for metastasis. Patients with metastasis have a poor long-term outcome. Novel targeted therapeutic strategies that are more efficacious and less toxic are therefore desperately needed. Intercellular communication within the tumor microenvironment (TME) is emerging as a crucial mechanism for cancer cells to establish immunosuppressive and cancer-permissive environment. Extracellular vesicles (EVs) offer a candidate mechanism as they are actively released by tumor cells and enriched with proteins and RNAs to communicate with other cells in the TME.

Methods: For EV purification, Conditioned medium (CM) was subjected to sequential centrifugation at 2000 g for 10 min and 10,000 g for 20 min. CM was then concentrated using the Tangential Flow Filtration Easy columns, passed through 0.22 μm filter, diluted with equal volumes of PBS and subjected to ultracentrifugation (UC) at 100,000 g for 4hrs. EV pellets were then re-suspended in 3 ml of PBS and pelleted again by UC. Purified EVs were dissolved in 500 μl PBS. EVs were quantified using Nanoparticle Tracking Analysis. Purified EVs were tested for presence of exosome markers (CD63, CD9, and CD81) using western blot and using R-PLEX Human CD63/CD81/CD9 (EV) Antibody Set.

Results: In our recent study involving whole transcriptome RNA sequencing, it was found that EVs secreted by EwS cell lines as well as those detected in the plasma of EwS patients are selectively enriched with Adenosine to Inosine (A to I) edited RNAs. A high proportion of these A-to-I edited transcripts are derived from diverse long and short interspersed retrotransposon elements (LINEs and SINEs), human endogenous retroviral elements (HERVs) and pericentromeric genomic regions, where their abundance in plasma was associated with metastatic progression. A to I conversion is catalyzed by the ADAR1 enzyme. We therefore performed ADAR1 knock-down (KD) in EwS cells, which accumulated cellular levels of these repeat RNAs in donor cells and limited their packaging into EVs. Moreover, we observed a significant reduction of pro inflammatory response in target cells treated with EwS ADAR1 KD EVs compared to the wild-type cells derived EVs. Notably, ADAR1 KD in EwS cells decreased the potential of their EV mediated monocytes differentiation and T-cell activation and priming.

Summary/Conclusion: These results suggest that EwS cells secrete EVs enriched with A to I edited RNAs derived from various repeat elements to target the non-tumor host cells, including stromal fibroblasts, monocytes, T cells for dampening and escaping the immune response against them.

Keywords: ewing sarcoma, extracellular vesicles, repeat RNAs, tumor microenvironment, reprogramming

PF17: Cancer Microenvironment 3

Location: Hall 4A

16:00 - 18:00

PF17.01 | Dissecting the multiomics atlas of extracellular vesicles in Parkinson's disease

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Introduction: Parkinson's disease (PD) is a neurodegenerative disorder that currently suffers from diagnosis at late stages. As currently there is no cure for PD, the pressing need of the hour is the discovery of molecular markers that would be potent in diagnosing the disease at its early stages. Therefore, a biomarker with interconnecting links between the proteome and miRNAs may be imperative in PD. With the proven link of exosomes in the progression of neurodegenerative diseases, these nanovesicles have extensive potential in finding novel biomarkers for PD. To date, integrated omics-based profiling, establishing the links amongst proteomic and miRNomic of the exosomes has not been worked upon in PD. Our current work focuses on this area for opening up new avenues in the mechanistic details of this intricate neurodegenerative disease.
Methods: Salivary and blood-derived EVs from PD patients and healthy cohorts were isolated by chemical precipitation followed by antibody-based validation through CD63, flotillin, and CD9 (universal surface marker) and confirmed neuronal origin by CD171. These nanovesicles were also characterized by an Electron microscope and via NTA. Additionally, miRNA and protein were purified from these different cohorts. Finally, miRNA analysis and proteomic analysis were performed via Illumina and Mass Spectrometry-based platforms.
Results: We have correlated the multi-omics data from the exosomal set of PD-diseased and healthy cohorts. We were able to find some significant differences between PD patients and healthy controls. The miRNA data shows some unique miRNAs, solely expressed in PD disease. Additionally, proteomics data also highlights some possible biomarkers for PD. The implications of these results will be discussed.
Summary/Conclusion: This study highlights potential molecular biomarkers via extensive pathways analysis and interconnecting pathways between proteomes, and miRNaome respectively. These markers could later also be identified in the biofluids directly.
Keywords: extracellular vesicles, parkinson's disease, biomarker, proteomics, miRNAs

PF17.02 | Investigation of diagnostic biomarker for Fukuyama congenital muscular dystrophy in serum-delivered EVs-miRNA

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Introduction: Fukuyama congenital muscular dystrophy (FCMD) is a congenital muscular dystrophy characterized by central nervous system abnormalities and ocular involvement, and is an autosomal recessive genetic disorder. In recent years, myomiR, a skeletal muscle-specific microRNA (miRNA) referred to as a biomarker for muscular dystrophies such as Duchenne-type, has been reported in the blood. However, there have been few reports on biomarkers, including miRNAs, that are specific to FCMD. In this study, we aimed to identify FCMD-specific miRNA markers by collecting extracellular vesicles (EVs) from serum samples of FCMD patients and healthy controls and performing miRNA analysis on these samples.
Methods: Serum samples were collected from 12 FCMD patients and 8 healthy controls with informed consent. EVs were collected from 1 ml of serum using magnetic particles conjugated with anti-CD9 and anti-CD63 antibodies. miRNA was extracted from the recovered EVs using the mirVana miRNA Isolation Kit. Small RNA-seq was performed on samples from 4 FCMD patients and 2 healthy controls. The remaining samples were subjected to qPCR targeting a total of 19 genes, including miRNAs that were highly expressed by small RNA-seq and the myomiRs miR-206, miR-1, and miR-133a, for validation.
Results: As a result of small RNA-seq, 153 miRNAs whose expression was found to be upregulated in FCMD patients were extracted. These included the myomiRs miR-206 and miR-133a. As a result of qPCR validation for 19 genes, the expression of

level miR-26 was upregulated in FCMD patients compared to healthy controls (2.28-fold, 2-ddCt method), and the difference was statistically significant. ($p < 0.001$, U test).
Summary/Conclusion: Our study demonstrates that miRNA analysis on EVs collected by immunoprecipitation can identify miRNAs whose expression levels are specifically increased in FCMD patients. This result suggests that the immunoprecipitation method is effective for miRNA analysis and may pave the way for the development of a novel FCMD diagnosis.
Funding: This study was funded by H.U. Group Reseach Institute G.K.
Keywords: miRNA, immunoprecipitation, muscular dystrophy, small RNA-seq, myomiR

PF17.03 | Neuronal-enriched extracellular vesicle miR-182 and miR-486 expression display opposing directionality in non-suicidal and suicidal individuals with Major Depressive Disorder

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Introduction: Suicide is one of the top ten causes of death in the US. For several reasons, it has been difficult to identify biological markers of the underlying process or predictive measures of suicide: (1) underlying neurobiology of suicidal behavior (SB) is still poorly understood; and (2) peripheral blood measures may not adequately assess the brain state preceding SB. The aim of this pilot study was to determine whether alterations in miRNA expression in neuronal-enriched extracellular vesicles (NEEV) could serve as biological markers of SB in individuals with Major Depressive Disorder (MDD).
Methods: This study was approved by the Western Institutional Review Board and carried out in accordance with the Declaration of Helsinki. Subjects gave informed consent. Healthy comparisons (HC = 18); non-SB (MDD- = 18) and SB (MDD+ = 18) were matched for age, sex, and percent body fat. Total EVs were isolated from plasma with a polymer-based kit; and NEEV immunocaptured with a biotinylated neuronal adhesion marker antibody. NEEV were characterized with flow cytometry and immunoblot analysis to validate EV-positive markers, and microfluidic resistive pulse sensing to determine NEEV size and particle concentration. miRNA was isolated from NEEV with a small miRNA kit, purity verified with a bioanalyzer, and sent for Next Generation Sequencing. For statistical analysis, data were log-transformed and non-parametric Kruskal-Wallis and Dunn's tests were performed. Gene Set Enrichment Analysis (GSEA) was performed with the miRWalk software to identify genes and relevant biological pathways (BP). EV-TRACK ID EV210507.
Results: MDD+ versus MDD- differed on 1) miR-182-5p ($H = 9.16$, $p = 0.01$; MDD+ lower than MDD- (Cohen's d ($d = 1.04$); and 2) miR-486-3p ($H = 9.16$, $p = 0.01$; MDD+ higher than MDD- ($d = 0.99$). HC did not differ between MDD+ nor MDD- for either miRNA. For each miRNA, GSEA revealed 120 genes and 15 BP (miR-182-5p) and 322 genes and 26 BP (miR-486-3p). Bioinformatics analyses identified several shared pathways with both miRNAs including FC gamma receptor signaling involved in phagocytosis and viral process.
Summary/Conclusion: MDD+ versus MDD- differed on 2 miRNAs. Findings provide evidence that dysregulation of NEEV miRNAs could offer possible molecular targets for predicting SB in individuals with MDD.
Funding: This work was supported by the William K. Warren Foundation, the National Institute of Mental Health (R01MH123652 to JS, K99MH126950 to LFH) and the National Institute of General Medical Sciences Center Grant Award (P20GM121312).
Keywords: neuronal-enriched extracellular vesicles, suicide, major depressive disorder, hsa.miR.182.5p, hsa.miR.486.3p

PF17.04 | Extracellular vesicle has-miR-200a-3p and has-miR-29a-3p derived from in vitro endometriosis model reduce mitochondrial apoptosis by the autocrine effect

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Introduction: Endometriosis is a condition where tissue similar to the lining of the womb starts to grow in other places. The pathogenesis of endometriosis has not been fully elucidated yet. Extracellular vesicles (EV) are secreted by living cells and contain functionally active proteins, mRNA and microRNA, rendering them important mediators of intercellular communication. In this study, we hypothesized that EV miRNA may play a role in the pathogenesis of endometriosis.
Methods: We collected eutopic endometrial tissues and blood serum from the patients with (EMS-EM) and without (CTL-EM) endometriosis. The study was performed after approval from the IRB of Gangnam Severance Hospital. Extracellular vesicles

were extracted in media supernatant of cultured EMS-EM or CTL-EM cells by using Exoquick-TC kit. EMS-EM cells were incubated with each EV for 24h. We investigated apoptosis using CCK8, Flow cytometry, WB, JC-1 dye. To find related miRNAs, we performed miRNA array in EMS-EM and CTL-EM cell EV and confirmed these miRNAs in blood serum EV by qRT-PCR.
Results: CCK-8 and Flow cytometry results showed that EMS-EV treatment reduces apoptosis. WB results showed that EMS-EV reduces apoptosis through PI3K/AKT signaling. JC-1 dye was significantly increased, mitochondrial apoptosis signal pathway was regulated in EMS-EV treatment. MiRNA array results confirmed 12 miRNAs were upregulated and 4 miRNAs were down regulated in EMS-EV. Has-miR-200a-3p was upregulated and Has-miR-29a-3p was downregulated in EMS-Serum-EV.
Summary/Conclusion: We demonstrated anti-apoptotic effects of EMS-EV through PI3K/AKT pathway in vitro endometriosis model. And we confirmed mitochondrial downstream mechanism BAX-Bcl2 pathway. It was reported that has-miR-29a-3p induced apoptosis and has-miR-200a-3p was associated with inhibiting apoptosis via Bax-Bcl2 pathway in some diseases. But it was not reported in endometriosis. Therefore, these observations may provide the main mechanism of EV related pathology and novel candidates that may serve as diagnostic biomarkers in endometriosis.
Funding: This work was supported by the National Research Foundation of Korea (NRF)(2020R1I1A1A0106783711).
Keywords: endometriosis, apoptosis, mitochondria, extracellular vesicle, pathogenesis

PF17.05 | Impact of acquiring cancer driver gene mutations for EV-miRNA profiles derived from pancreatic epithelial cells

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Introduction: Circulating miRNAs are one of the promising biomarkers for cancer detection in the early stage. However, the alterations of extracellular vesicle miRNA (EV-miRNA) profiles released from epithelial cells in the carcinogenic process have not yet been fully understood. This study aimed to compare EV-miRNA profiles among pancreatic epithelial cells with a variety of drive gene mutations.
Methods: We established a culture method of mouse pancreatic ductal progenitor cells (mPP) by modifying mature hepatocyte's chemical direct reprogramming technology (Cell Stem Cell 20:41, 2017). We introduced each driver gene mutation such as KRAS(G12D) and the loss of CDKN2A, TP53, SMAD4 into mPP by CRISPR-Cas9. Small EVs (sEVs) released from each cell line were collected with ultracentrifugation. The quality of sEVs was confirmed by nanoparticle tracking analysis, transmission electron microscopy, and immunoblotting of EV-positive and -negative marker proteins (CD9 and CANX).
Results: Acquiring KRAS and TP53 mutations significantly enhanced the cell proliferation. sEV particle counts released from mPP lines were increased by the introduction of KRAS(G12D) and the deletion of TP53. miRNA-seq analysis revealed that the levels of miR-155-5p, known as circulating biomarkers of pancreatic ductal carcinoma, in sEV were increased by KRAS(G12D).
Summary/Conclusion: Extracellular miR-155-5p levels could be a biomarker of KRAS mutation in pancreatic ductal cells. In vitro culture techniques of organ progenitor cells and CRISPR-Cas9 can be useful for elucidating the molecular mechanisms of the alteration of circulating miRNA profiles in cancer patients.

PF17.06 | miR-150-5p in plasma extracellular vesicles is associated to Gleason score in prostate cancer patients

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Introduction: Prostate cancer is the fifth most common cause of death in men. PSA levels in the blood are the gold standard for diagnosing prostate cancer (PCa), but they are highly unspecific. Liquid biopsies allow us to detect miRNAs within extracellular vesicles (EVs) in biological fluids such as blood or urine. Differential expression of miRNAs has been linked to cancer development. The purpose of this study is to determine the presence of miRNAs plasma Evs from PCa patients. We isolated EVs using aqueous two-phase separation (ATPS) and identified EV markers using nano-scale cytometry. We discovered that the expression of miR-150-5p in plasma EVs differed according to Gleason score.

Methods: To extract EVs from PCa cell lines (LNCaP, PC3, DU145, BPH-1, NHPrE and BHPrE), two rounds of ultracentrifugation were performed. We used electron microscopy to examine the size and shape of EVs, as well as the EV markers TSG101, CD63, and CD9 by western blot. Based on Gleason score, human plasma samples were split into three categories. Every group had 7–8 samples and 12 healthy donor samples. All samples were obtained with proper informed consent and the approval of the Sunnybrook Research Institute's ethics committee. The ATPS and Qiagen's Exo RNeasy Midi kit were used to isolate EVs from 500uL of plasma. Nano-scale cytometry was used to detecting canonical EV biomarkers. RT-qPCR was used to detect the presence of miRNAs using Taqman MicroRNA Reverse Transcription kits and Taqman Small RNA probes for each miRNA. RNU6B acted as a normalizer.
Results: We confirm the presence of CD9, CD63, and TSG101 in LNCaP and PC3 EVs from PCa cell lines. TEM images show the presence of EVs with traditional shapes and sizes in LNCaP, PC3, and DU145 samples. The presence of CD63, CD81, and CD9 in all EV samples from PCa cell lines and EVs from plasma samples was revealed by nanoscale cytometry, with CD9 being the most prevalent in plasma EVs. RT-qPCR revealed that MiR-150-5p was considerably higher in EVs from LNCaP and BPH-1. The miR150-5p expression was found to be inversely proportional to Gleason score in PCa samples with a high Gleason score >4+3.
Summary/Conclusion: In most of the cell lines EVs we found overexpression of the miRNAs. This study demonstrates the detection of EVs and miRNAs in 500uL of plasma using ATPS and the presence of tetraspanins using nano-scale cytometry. The miR-150-5p levels in plasma vesicles are inversely proportional to Gleason score, suggesting possible use as an indicator of more advanced PCa.
Keywords: biomarker, EVs, prostate cancer, PCa, miRNAs, ATPS, nano-scale flow cytometry

PF17.08 | Drug-resistant extracellular vesicles predict tumor response in TNBC patients receiving neoadjuvant chemotherapy

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Introduction: Predicting tumor response after neoadjuvant chemotherapy (NAC) is critical for predicting prognosis and deciding the treatment strategy in patients with breast cancer (BC); however, there are no reliable blood biomarkers that can assess tumor responses effectively. Therefore, we aimed to validate the clinical feasibility of miRNA and protein markers in extracellular vesicles (EV) collected for predicting tumor response during NAC.
Methods: Drug-resistant clones were generated from three triple-negative breast cancer (TNBC) cell lines. Profiling of drug-resistant TNBC identified potential drug resistance-related biomarkers. We isolated tumor-derived EVs and validated that drug-resistant biomarkers were also significant in EVs released from drug-resistant tumor cells. The putative drug-resistant EV markers were validated in plasma samples from 72 BC patients, including 42 individuals showing no tumor response and 30 individuals showing a complete response.
Results: Compared with wild-type EVs and drug-resistant EVs, 5 EV miRNAs (miR-125b, miR-146a, miR-484, miR-1246, and miR-1260b) and 3 EV membrane proteins (MDR1, MRP1, and BCRP) were confirmed as biomarkers contributing to the acquisition of drug resistance. The optimal combination of drug-resistant EV markers represented the best performance to differentiate tumor response. We also analyzed The GEO datasets to identify target genes of EV mRNAs related to drug resistance. The miRNA-target gene networks correlated highly with cell mitosis, metabolism, drug transport, and immune response.
Summary/Conclusion: Our study suggests that drug-resistant EV markers effectively predict tumor response, which can be clinically applicable. Moreover, drug-resistant EV markers seem to increase with repeated drug treatment in the tumor EV population. This approach allows real-time monitoring of drug-resistant EV marker alterations potentially sensitive to targeted therapy or associated with treatment resistance in patients with BC during NAC.
Funding: This study was supported by a Severance Hospital Research fund for Clinical Excellence (C-2022-0018) and the National Research Foundation of Korea Grants (2021R1I1A1A01051594 and 2022R1F1A1074605).
Keywords: TNBC, neoadjuvant chemotherapy, liquid biopsy, predictive biomarker, extracellular vesicles, microRNA, EV protein

PF17.09 | Plasma extracellular vesicle biosignatures for methamphetamine use disorders

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Introduction: The long-term health effects of methamphetamine (MA) use include cognitive impairments, anxiety, and depression, which can persist during recovery and are associated with poor treatment outcomes. Thus, biosignatures based on objectively quantifiable blood biomarkers that relate to clinical features of MA use disorders could be used clinically to monitor recovery from addiction.
Methods: Multiplexed bead-based assays (Miltenyi) were performed on plasma extracellular vesicles (EVs) from humans with active MA use (MA-ACT, n = 10) and controls (CTL, n = 10). EV subtypes were prioritized based on an F-statistic ranking, large effect size, and area under the curve (AUC > 0.75) for classifying MA-ACT vs. CTL. The normalized median fluorescence intensity (nMFI) values for prioritized EV subtypes were correlated to measures of i) MA use characteristics, ii) neuropsychiatric function, and iii) markers of inflammation and CNS injury. Next, the expression levels of 7 plasma EV miRNAs that are relevant to MA-ACT were correlated to the aforementioned measures. Data analyzed by Pearson's correlations with false discovery rate corrections.
Results: Plasma EVs positive for inflammatory markers significantly correlated to measures of craving in MA-ACT as well as anxiety and memory impairments in MA-ACT and CTL participants. Five EV miRNAs also significantly correlated to clinical features of MA use disorders including frequency of use, lifetime exposure, anxiety, memory, and pain. Plasma EV expression levels for 3 of these miRNAs also significantly correlated to ICAM-1, S100β, and/or neurofilament in MA-ACT. Relevant to these findings the predicted targets of the five miRNAs identified pathways associated with neuroinflammation, neuroplasticity, and neurodegeneration, which contribute to behavioral alterations that occur with MA dependency.
Summary/Conclusion: These studies demonstrate the potential utility of plasma EVs to serve as metrics of recovery by relating EV markers and their miRNA cargo to clinical features of MA use disorders.
Keywords: addiction, miRNAs, neuropsychiatric function, methamphetamine use disorders, inflammation

PF17.10 | Establishing extracellular vesicle-based miR-375 as a liquid biopsy for neuroendocrine neoplasms (NENs)

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Introduction: Neuroendocrine neoplasms (NENs) are a constellation of rare cancers arising from neuroendocrine cells of many different anatomical sites (lungs, pancreas, etc.). miR-375 has been reported to be overexpressed in NEN tissues compared to non-NEN tissues, suggesting its strong promise as a universal biomarker for NENs. Peripheral expression of miR-375 has not been demonstrated before and for the first time we show its expression in extracellular vesicles (EVs) isolated from NEN patient plasma samples.
Methods: EVs were isolated using aqueous two-phase separation (ATPS) method from plasma samples of NEN patients and healthy volunteers. Classic EV biomarkers (CD9, CD63, CD81) were measured with nanoscale flow cytometry (nFC). Multiple RNA isolation methods (Trizol, RNeasy, exoRNeasy) were tested to determine RNA content of isolated EVs. RNA length was analyzed using Bioanalyzer and miR-375 expression was measured with RT-qPCR.
Results: Classic EV biomarkers were detected on plasma EVs isolated using ATPS. exoRNeasy kit was found to be the most effective method for RNA isolation from plasma EVs. Length of plasma EV RNA was determined to be between 25 – 200 nt. miR-375 expression was enriched in NEN patient samples compared to healthy control and statistically significant.
Summary/Conclusion: Given that miR-375 is known to be associated with NEN tissues, we found that it is also a plasma biomarker supporting its use as a promising liquid biopsy target for NENs.

PF17.12 | Human neuronal-enriched extracellular vesicle microRNA profile in individuals diagnosed with COVID-19

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Introduction: SARS-CoV-2 can enter the nervous system via various routes and cause CNS infection symptoms including fatigue, depression, and anxiety. We hypothesized that alteration in inflammation-associated extracellular vesicle (EV) micro(mi)RNA expression in neuronally enriched extracellular vesicles (NEEVs) due to COVID-19 would be associated with modification of brain functions related to COVID-19 post-recovery symptoms. To test our hypothesis, NEEVs from COVID-19 positive and negative individuals were analyzed for miRNA expression.
Methods: The study was approved by the Western Institutional Review Board, performed in accordance with the Declaration of Helsinki, and participants provided informed consent. Blood from COVID-19-negative (n = 14) and -positive (n = 62) patients were collected. Study participants were matched on age, sex, and mood/anxiety symptoms. EVs were isolated from plasma using the precipitation method and were subsequently subjected to NEEVs enrichment using magnetic streptavidin beads immunocapture against the neural adhesion marker. NEEVs were confirmed by flow cytometry and western blot; size and concentration were determined by microfluidic resistive pulse sensing. NEEV small RNAs were purified and profiled by small RNA sequencing. Differential gene expression analysis based on the negative binomial distribution (DESeq2) was used for statistical analysis; significant differences were determined by FDR-adjusted p-value (q) < 0.05.
Results: Compared to COVID-19-negative subjects, COVID-19-positive subjects NEEVs exhibited higher miR-205-5p (q < 0.001, d = 1.37), miR-203a-3p (q = 0.003, d = 0.94), miR-203b-5p (q = 0.005, d = 1.04), and miR-103a-3p (q = 0.026, d = 0.68) expressions and lower miR-16-5p (q = 0.006, d = 1.18), and miR-92a-3p (q = 0.006, d = 1.11). In addition, COVID-19 positive subjects who had fatigue symptoms exhibited higher miR-203a-3p (p = 0.006, d = 0.66) and miR-205-5p (p = 0.031, d = 0.50) than those without fatigue symptoms.
Summary/Conclusion: COVID-19 alters NEEV miRNA expression levels that could potentially be related to impaired brain function. Further studies are required to elucidate the molecular mechanism of altered NEEV miRNA levels as contributors to long-term neural and symptom changes linked to COVID-19.
Funding: This work was supported by The William K. Warren Foundation, and the National Institute of General Medical Sciences Center Grant Award (P20GM121312).
Keywords: neuronal enriched extracellular vesicles, COVID-19, microRNA, depression

PF18: Plant EVs

Location: Hall 4A

16:00 - 18:00

PF18.01 | Comparisons of mammalian-derived EVs with plant cell suspension derived EVs in terms of isolation and characterization

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Introduction: Cells secrete differential-sized vesicles called "Extracellular vesicles " to communicate with neighbor cells and the environment. It has been extensively revealed that EVs especially small EVs exhibit cellular identity in protein, lipid, and nucleic acid manner. Extracellular vesicles derived from various plant sources (PDEVs) has been started in the late 60s with carrot culture. Although extracellular vesicles derived from mammalian cells (MDEVs) have a higher rate of EV studies than PDEVs, there is increasing interest in PDEVs. Nowadays the isolation of PDEV from a wide variety of plant sources has been studied. Isolation of PDEVs from a plant cell suspension system instead market-derived products provides more accurate results in both characterization and functionality experiments. In this comparison study, PDEVs were isolated from Vitis vinifera suspension medium (VVSM) and MDEVs from Neural Progenitor Cells (NPCs).
Methods: MDEVs were isolated from the collected, serum-free medium of NPCs by mixing ultracentrifugation and ultrafiltration protocols. On the other hand, PDEVs were isolated from VVSM by using ultrafiltration. Nanoparticle Tracking Analysis (NTA) was used to quantify EVs and determine their size distribution. The atomic force microscopy technique was preferred to exhibit EVs in nanoscale imaging. LC/MS proteomic analysis was used to identify EV cargo proteins. Fatty acid profiles (FAME) of PDEVs and MDEVs were investigated by GC-MS. The peaks were identified by the Sherlock software. Differences between MDEVs and PDEVs were analyzed by the Student t-test and P< 0,05 was considered statistically significant.
Results: The size of PDEVs was close to 150 nm while the size of MDEVs was 100 nm. AFM images verified NTA results. AFM Images of MDEVs showed that the mean size of MDEVs was 180 ± 45 nm. Moreover, PDEVs showed a similar size to NTA which is 150 ± 20 nm. LC/MS proteomic analysis showed that origin cell features are mostly passed to the EVs. Furthermore, FAME profiling determined that PDEVs had more saturated fatty acids (45%) than MDEVs (15% and 39%). The mono-unsaturated fatty acid content of MDEVs (59% and 27%) was higher than PDEVs (13,3%).

Summary/Conclusion: In conclusion, both mammalian and plant suspension cell-derived EVs had similar EV sizes and quantities. However, their membrane-bound fatty acid pattern was different. This difference gives EVs stability and membrane rigidity and will open a new scientific window that intensively needs to be investigated.
Funding: This study is supported by TUBITAK (2211/C) and Yeditepe University.
Keywords: FAME, PDEVs, MDEVs

PF18.02 | Surface modification of grapefruit-derived extracellular vesicles for targetable drug delivery carrier

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Introduction: Recently, membrane-modified mammalian exosome is considered as a novel nanovesicle carrier for targeted drug delivery owing to their biocompatibility, biodistribution and low immune response. There are, however, still several challenging issues with mammalian exosomes including stability and homogeneity as well as large-scale manufacturing. To address these challenges, artificial extracellular vesicles(EVs) like nanovesicles obtained from cell membranes or liposomes from various lipids have been studied for targeted drug delivery systems, but most of them do not meet the demands of efficacy, efficiency, and less side effects. We studied on the feasibility of plant-derived extracellular vesicles(pEVs) as targeted drug delivery carriers replacing mammalian cell exosomes and liposomes, since pEVs are non-toxic and stable nanovesicles that can be easily internalized into mammalian cells. Moreover, pEV is suitable for mass-production with high yield and stability.
Methods: Grapefruit was used as an origin of pEVs isolated by ultracentrifugation and size exclusion chromatography. Their sizes, concentrations and shapes were characterized using DLS(Dynamic Light Scattering), NTA(Nanoparticle Tracking Analysis), and Cryo-EM(Cryogenic Electron Microscopy), Confocal microscopy. To impart targeting ability to pEVs, a functionalizable lipid moiety with maleimide group for drug targeting was inserted in the membrane of pEVs.
Results: The diameter of pEVs from grapefruit was 175 ± 12 nm and the concentrations were 1.96 × 1012 particles/ml obtained from 3 ml juice of a grapefruit. The size distribution of pEV was maintained stable at 4°C for 4 weeks. Cellular uptake of pEV was studied using U87MG and hCMEC/D3 cell line. The DiO labelled pEVs was visualized in the inside of cells by confocal microscopy.
To impart targeting ability to pEVs, a functionalizable lipid moiety with maleimide group for drug targeting was inserted in the membrane of pEVs. The moiety at pEV membrane was confirmed by colocalized fluorescence of DiO and Cy5-labelled lipids. The targeting function can be easily augmented to the moiety with click chemistry. The cellular (hCMEC/D3 and U87MG) uptake of pEV whose surface was attached by aptamer were observed using of DiO dye by confocal microscopy. The result confirmed that aptamer enhances selective cellular uptake of pEV by the increase of fluorescence intensity.
Summary/Conclusion: We expect the functionalization of pEV membrane would be helpful to provide low-cost target drug delivery carriers with no less efficacy than mammalian exosomes or liposomes with high stability and mass productivity.
Keywords: Plant-derived Extracellular Vesicle, targeted drug delivery, drug carrier

PF18.04 | Curcuma contains small lipid-derived microvesicles which complex curcumin and participate in macrophage polarization

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Introduction: It has been demonstrated that plants contain microvesicles with anti-inflammatory properties which participate in intestinal tissue renewal process and modulation of gut microbiota. Here we determined whether curcuma (Curcuma longa L.) roots also contain microvesicles with immunomodulation properties on macrophages
Methods: As we did not know the physicochemical properties of curcuma microvesicles (CuMVs), the use of commercial kits has been banned and the method of differential centrifugations/ultracentrifugations and filtrations at 0.45 um was preferred

to isolate CuMVs. CuMV pellet was characterized by transmission electron microscopy, Zetasizer, and metabolomics. THP-1 macrophages were treated with different concentrations of CuMVs (1 to 5 ug/ml). ROS production was detected by FACS.
Results: CuMVs were heterogeneous in size (50–250nm). Interestingly, they carried curcumin, the main active molecule in curcuma, and its derivatives. At low concentration, CuMVs induced ROS production in THP-1 macrophages and consequently, their polarization into a population of anti-inflammatory macrophages (decreased CD86 expression and increased CD163 expression). However, these CD163+ macrophages retained their anti-tumor IL-10 and TGF-beta secretory properties. In addition, CuMVs stimulated the expression of anti-bacterial cytokines (Il1-beta, Il-6, TNF-alpha).
Summary/Conclusion: By affecting the ROS levels of macrophages, curcuma vesicles prevent the passage of M2 into tumor-associated macrophages (TAM) and thus reduce the pro-tumor effect of M2. CuMVs are involved in the anti-inflammatory properties of turmeric because they carry curcumin. It is therefore important that the industrial processes developed to commercialize curcuma keep high concentrations of CuMVs as they participate in the passage of curcumin, which is insoluble in aqueous solution, across cell membranes
Keywords: inflammation, macrophages, curcuma-derived microvesicles

PF18.05 | Hovenia dulcis Thunb decoction derived EV contains active micro-RNA and can alleviate cellular damage caused by alcohol stress

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Introduction: By previous exploration, Extracellular vesicles (EVs) derived from plants have been proved to have great bioactivity. However, there is no specific study on whether Evs can maintain their morphology and bioactivity and exert biological effects under special circumstances. Hovenia dulcis Thunb is a plant with medicinal value, and the discovery of its effects originated in ancient China. In this study, we explore those questions, to study whether the Hovenia dulcis Thunb still exists Evs with normal morphology and bioactivity after being decocting at high temperature, and whether the decocted halelns-EVs can play an active role in the treatment of alcoholic liver disease (ALD).
Methods: After decocting Hovenia dulcis Thunb at 100°C for 30 minutes, Evs were isolated by ultracentrifugation from decoction. Evs were identified and characterized by TEM, NTA and BCA. The differential expression of micro-RNA in Hovenia dulcis Thunb-derived exosome-like nanovesicles (HALELNs) were identified by RNA sequencing, and the constant expression of miRNA in HALELNs were detected by RT-PCR. By confocal, HALELNs labeled with PKH6, were performed to confirm the uptake of HALELNs by hepatocytes. In order to explore the biosafety and therapeutic effect of HALELNs on alcoholic injury, we designed an in vitro cell model of alcohol injury, and then verified the effect of HALELNs on Alpha Mouse Liver 12 cell (AML-12) by CCK-8.
Results: The HALELNs isolated from the decoction still showed vesicles in a bilayer membrane structure with an average diameter of 135nm under TEM. Moreover, Let-7b-5p was stably expressed in HALELNs. HALELNs uptake was enhanced in alcohol-treated AML-12 cells compared with normal AML-12 cells. In mice, HALELNs administered by intraperitoneal injection were mainly enriched in the stomach and intestine after 24 hours. In particular, HALELNs appear to have a therapeutic effect on alcoholic injury. When AML-12 cells were treated with 4μg/mL HALELNs for 48 hours and then treated with 400 mM ethanol, the decrease of cell viability was significantly reduced compared with those treated without HALELNs. Compared to alcohol gavage alone, interval intraperitoneal injection of HALELNs reduced serum glutathione transaminase (AST) concentrations in mice.
Summary/Conclusion: The decoction of Hovenia dulcis Thunnb was found to contain Evs, which maintain normal morphology and bioactivity, after high temperature decoction. Most surprisingly, we found that decoction-Evs still had stably expressed micro-RNAs. Besides, decoction-Evs were indicated to play a positive role in the treatment of alcoholic liver disease (ALD).
Funding: This research was funded by grants from National Natural Science Foundation of China (#81902147 and #82172966).
Keywords: plant-derived Evs, micro-RNA, alcohol stress

PF18.06 | ROS response targeted delivery of rapamycin-carrying plant-animal fusion vesicles in early rejection of heart transplantation

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Introduction: Heart transplantation is the best treatment for end-stage heart failure. Due to cold ischemia-reperfusion injury and transplant rejection during transplantation, which reduces the utilization rate of donor hearts, there is an urgent need for new mechanisms and therapeutic targets to develop effective therapies to treat I/R injury and inhibit rejection in heart transplantation rejection. In this study, we screened plant exosomes with antioxidant damage and anti-inflammatory properties, fused them with IPSC-derived vesicles containing phagocytotropic targets for drug delivery, and adopted click-chemistry to promote delivery of drug delivery systems, providing a new strategy for the treatment of heart transplant rejection
Methods: 1. Characterization of materials by electron microscopy /particle size/ potential
2. Synthesis of ROS responsive Tetraacetated N-azide acetyl-D-mannosamine(ros-n3)
3. Evaluation of ros-n3 targeted modified grafts by imaging in vivo in small animals
4. In vitro, the anti-inflammatory and anti-myocardial oxidative damage effect of FV@RAPA was detected by flow /QPCR/ELISA
5. Construction of mouse heart transplantation model, and caudal vein injection and therapy
6. MASSON/HE/ flow assays the in vivo efficacy of DBCO-FV@RAPA
Results: 1. Tomentose Pummelo Peel derived exosomes have anti-myocardial oxidative damage and anti-inflammatory effects.
2. Targeting macrophages a multifunctional stem cells (IPSCs) vesicles with calreticulin that promote the phagocytosis and efferocytosis of macrophages.
3. IPSCs vesicles with calreticulin that promote phagocytosis, fused with Tomentose Pummelo Peel derived exosomes, and loaded with rapamycin (FV@RAPA). In vitro, the FV@RAPA not only maintain the bioactivity of TEVs, RAPA and IPSC membranes, but also can promote uptake of apoptotic cardiomyocytes by macrophages under the action of calreticulin, thus promoting the efferocytosis of macrophages.
4. In a mouse xenograft heart model, ros-n3 labeling at the transplanted heart site promoted more targeted delivery of DBCO-FV@RAPA, thereby alleviating ischemia-reperfusion injury to cardiomyocytes and inhibiting early macrophage immune activation.
Summary/Conclusion: In this study, we first constructed ROS-responsive tetraacetyl N-acetylazide-D-mannoamine, which can mark the transplanted heart site and promote more targeted delivery of DBCO-FV@RAPA, thereby alleviating ischemia-reperfusion injury and inhibiting early macrophage immune activation to achieve therapeutic effect.
Funding: This research was supported by National Key R&D Program of China (No. 2022YFA1104900, China.
Keywords: early rejection of heart transplantation, CALR, fusion vesicles, ROS response biological orthonormal chemistry

PF18.07 | Isolation of Centella asiatica-derived specific extracellular vesicles and their potential benefits for skin improvement

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Introduction: Plant-derived extracellular vesicles (PDEVs) have gained the interest of many experts in fields such as cosmetic and therapeutic materials. It has been known for a long time that Centella asiatica extracts exhibit lots of biological activity such as anti-aging, anti-oxidant, collagen enhancement, and moisturizing. In order to isolate EVs from Centella asiatica, it is necessary to develop a new method because the widely used method for the isolation of EVs, such as ultracentrifugation, precipitation, and filtration, have shown a low yield and difficulty in mass production.
Methods: Here, we developed an EV extraction method to effectively separate the specific EVs from Fresh Centella asiatica. First, total EVs from fresh Centella asiatica (CEVs) were extracted by using tangential flow filtration (TFF). Specific EVs from fresh Centella asiatica (spCEVs) were sequentially isolated by using the microfluidic chip with specific target-biotinylated streptavidin beads. Then, the biological properties of CDEVs and spCDEVs were evaluated in comparison with EVs from commercialized Centrella asiatica extracts (exCEVs).

Results: Our results showed that spCEVs were isolated at higher concentrations and had faster separation time than the other three different conventional methods. In addition, the spCEVs had better uptake efficiency to the target cells than CEVs and exCEVs, resulting in the promotion of skin regeneration. Also, spCEVs showed significant improvement in skin hydration.
Summary/Conclusion: These results provide a foundation for the development of plant-derived therapeutic agents as well as the potential industrial applications of various PDEVs.
Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No.2021R1A2C3011254), Korea Medical Device Development Fund grant funded by the Korea government (MSIT,MOTIE, MOHW,MFDS) (Project Number: 1711139115, KMDF_PR_20210527_0008), and Korea Environment Industry & Technology Institute (KEITI) through Aquatic Ecosystem Conservation Research Program, funded by Korea Ministry of Environment (MOE) (2020003030007).

PF18.08 | Plant exosome-like nanoparticles: building up novel animal-free anti-inflammatory therapeutic agents for drug delivery

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Introduction: Plant exosome-like nanoparticles (ELNs) have recently been shown to have a very promising therapeutic potential to alleviate inflammation in different pathologies. They have similar physical and chemical properties to extracellular vesicles (EVs) derived from mammary cells. Hence, they may be a revolutionary therapy because (1) large quantities of EVs are required for in vivo applications, and different methods with less production costs and time are therefore needed, (2) their plant origin facilitates transferring these therapeutic agents into clinical practice and (3) they can be classified as vegan, which will make users have a better perception of the final product. Moreover, it is possible to bioengineer them for drug delivery.
Methods: ELNs' isolation methods from different vegetal sources have been assayed: 1) fresh leaves from plant tea (Camellia sinensis), 2) fresh ginger rhizome roots (Zingiber officinale) and 3) fresh red cabbage (Brassica oleracea). Large-scale size exclusion chromatography (SEC) has been optimized and its efficiency compared with an ultracentrifugation isolation method. ELNs were characterized by NTA and TEM. PKH67 staining (with relevant negative controls) was used to test internalization by HUVECs. Flow cytometry analysis of the expression of ICAM-1 and VCAM-1 on inflammed HUVECs treated with ELNs have been performed.
Results: Efficient methods for ELNs isolation from different plant sources have been optimized. Preliminary in vitro experiments suggest internalization and an anti-inflammatory effect of the ELNs in human endothelial cells.
Summary/Conclusion: ELNs from plant leaves and roots can be isolated with great yield. These ELNs constitute novel candidates for therapeutic drug development due to their anti-inflammatory effect.
Funding: Talent Program,Comunidad Autónoma de Madrid (2019-T1/IND-13794); Ministry of Science (PID2021-126274OB-I00).
Keywords: exosome-like nanoparticles, plant, anti-inflammatory

PF18.09 | Plant-derived Extracellular Vesicles enhances drug delivery with radiation mitigation/sensitivity effects

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Introduction: We focus on the exploration of plant kingdom in order to identify both new sources and delivery systems of therapeutics. For instance, plant produce plant-derived extracellular vesicles (pEVs) that can be found in their paramural space. Similar to animal-derived exosomes, these pEVs exhibit low immunological risk and good bioavailability but also offer many more resources of raw materials, cost efficient production and do not harbor zoonotic or human pathogens. Interestingly, these pEVs display two features of relevance for drug discovery and development: (1) they carry pre-encapsulated natural cargos that could be readily available targets for the screening of new therapeutic compounds and (2) they can be loaded with synthetic cargo to overcome current limitations of animal-derived nanoparticles drug delivery systems. So in overall pEVs can not only act as a certain type of drug, but also work as a cargo vesicle to deliver drug. Our lab targeted on exploring pEVs which potentially have high contents of anti-oxidants by sorting of many types of plant. Some of them potentially show radio protective effects on human cells and some of them show radio sensitivity effects. And the meantime, we explored new methodologies to encapsulate drug into selected pEVs. Both radio protector drug and anti-cancer drug are tested to be encapsulated. In this case, pEVs potentially generate benefits if a combination of chemotherapy and radiation therapy induced for cancer treatment, by enhancing

chemotherapy working as a anti-cancer drug delivery cargo and at the mean time, reducing the side effects of radiation therapy since pEVs's radiation protective characteristic.
Methods: Isolation: step centrifugation; sucrose gradient; Size-exclusion Chromatography (SEC) Characteristics: NTA/SEM/TEM/Fluorescence Microscopy. Biology tests: MTT test/ clonegenic test.
Results: Spinach, tumeric, clove, duckweeds, grape, green olive, black olive etc… Many plants were tested. Olive potentially generate radio sensitivity on human cancer cells while no significant effects on normal human cells. Grapes generate radio protectivity on human normal cells, while no significant effects on human cancer cells.
Summary/Conclusion: Because of the minimal cytotoxicity of each plant EVs, also variation of radiation effects on cancer and normal human cells in different types of pEVs, pEVs have strong potential benefits with loading certain types of drug to meet some special treatment requirement, such as if a combination of radiation therapy and chemotherapy is required.

PF18.10 | Plant exosomes fused with engineered mesenchymal stem cell-derived nanovesicles for synergistic therapy of autoimmune skin disorders

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Introduction: Among autoimmune diseases, psoriasis and atopic dermatitis are two major skin disorders becoming the major issues threatening public health with increasing prevalence. However, Existing therapeutics for autoimmune skin diseases remain problematic due to low efficacy, severe side effects, and difficulties to reach target tissues. The shortcomings of mainstay treatments and surging cases of autoimmune skin disorders across the world meant safer and more effective therapeutic strategies were now urgently needed. we proposed a new approach to prepare engineered hybrid EVs by fusing plant-derived EVs and engineered MSCs-derived nanovesicles to take advantage of both types of nanovesicles and to generate personalized delivery nanovectors.
Methods: We first investigated the literature as well as Chinese medicine prescriptions and determined ten promising tissues from edible plants to isolate plant-derived extracellular vesicles (PLEVs) by differential ultracentrifugation. We assessed the quality of PLEVs by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), lipidomic analysis, miRNA sequencing, and analysis of their protein composition. We first screened out grapefruit-derived exosomes (GEVs) with anti-inflammatory and antioxidant effects, but they lacked immunosuppressive function. we then encapsulated CX5461 into GEVs by electroporation. By GEO data analysis, we found that chemokine CCL20 is elevated in the lesions of autoimmune diseases, so nanovesicles carrying CCR6 may target inflamed tissues by interacting with regional CCL20. However, genetic modification of EVs remains a big challenge in plant cells when compared to animal cells. Therefore, we proposed to fuse bioengineered cell membrane vesicles with the membrane of GEVs. Gingiva-derived□mesenchymal□stem□ cells (GMSCs) were selected for genetic engineering due to their significant immunosuppressive effect compared to other MSCs. Subsequently, we prepared CX5461-loaded fusion vesicles (FV@CX5461) by hybriding GEVs with CCR6 enriched GMSCs membrane-based nanovesicles (CCR6-NVs) by extrusion.
Results: 1. Grapefruit-derived exosomes had anti-inflammatory and antioxidant effects but lacked immunosuppressive function in vitro.
2. The anti-oxidative, anti-proliferative, anti-inflammatory and immunosuppressive effects of FV@CX5461 were better than GEVs, CX5461, or CCR6-NVs alone in vivo and in vitro.
3. In IMQ-induced psoriasis mice and DNCB-induced AD mice FV@CX5461 exhibited excellent immunomodulatory capabilities, including calm down Th17 cell activation, inhibition of intracellular ROS in macrophages, and induce Treg cell infiltration.
Summary/Conclusion: A nanotherapeutic drug delivery strategy is developed using fusion nanovesicles derived from plant and animal cells with high clinical potential.

PF19: EVs and the Central Nervous System

Location: Hall 4A

16:00 - 18:00

PF19.01 | Extracellular Vesicles from human induced pluripotent stem cell-derived astrocytes display antiinflammatory and neuroprotective properties

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Introduction: Astrocytes play multiple roles, including the maintenance of the blood-brain barrier, neuron-glia communication, neurotransmitter recycling, regulating immune response, and promoting repair after injury or disease. Hence, it is believed that extracellular vesicles (EVs) released by astrocytes have neuroprotective and antiinflammatory properties. This study examined the ability of ADEVs, purified through chromatographic methods from cultures of human induced pluripotent stem cell (hiPSC)-derived astrocytes (iAstrocytes), to mediate antiinflammatory effects on lipopolysaccharide (LPS) stimulated mouse macrophages and hiPSC-derived microglia (iMicroglia) and neuroprotective effects against amyloid-beta (Aβ) induced neurotoxicity.
Methods: hiPSCs were first differentiated into neural stem cells and then into astrocytes through sequential inhibition of SMAD, TGFb, and BMP signaling. Differentiated astrocytes were characterized with specific markers, and the spent media from astrocyte cultures were used to isolate ADEVs. The ADEVs were next characterized and tested for their antiinflammatory and neuroprotective properties.
Results: ADEVs had a mean size of 91.4 nm and were positive for CD63, CD81, and CD9. LPS stimulation resulted in increased release of IL-6 by mouse macrophages and IL-1b by iMicroglia. However, the addition of ADEVs (at 40–80 billion doses) significantly reduced the release of these proinflammatory cytokines by LPS-stimulated macrophages and iMicroglia. The addition of Aβ to mature human neuronal cultures led to reduced depolarization of mitochondrial membrane potential and significant neurodegeneration. However, ADEV administration to Aβ-treated neuronal cultures rescued mitochondrial membrane depolarization and reduced the extent of neurodegeneration.
Summary/Conclusion: ADEVs isolated from iAstrocytes have potent antiinflammatory and neuroprotective properties and hence have the potential for treating neurodegenerative diseases.
Funding: Supported by a grant from the National Institute for Aging (1RF1AG074256-01A1 to A.K.S.).
Keywords: astrocyte derived extracellular vesicles, neuroprotection, anti-inflammation, neural stem cells

PF19.02 | Microglial exosmoes loading miR-223 promote remyelination by accelerating recruitment of myeloid-derived suppressor cells

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Introduction: Remyelination is a critical aspect after spinal cord injury, and the immune microenvironment at the injury is crucial for remyelination. The main objective of this study was to clarify that microglia cell lineage exosomes overexpressing miR-223 can more effectively promote remyelination in mice after demyelination, and to seek the underlying mechanisms
Methods: We applied RNA-seq to analyze the levels of miRNA and mRNA in the spinal cords of healthy and demyelinated mice, and verified that the levels of miR-223 were higher in the spinal cords of demyelinated mice than in healthy mice by RT-qPCR. Lentiviral transfection and ultra-high speed centrifugation were used to extract miR-223 overexpressed and normally expressed BV2-derived exosomes, which were identified by electron microscopy, particle size analysis, WB and qPCR. Mice were divided into four groups:miR-223+BV2-Exo(OE), miR-223control-Exo(NC), Vehicle and Sham. Transcriptomics and proteomics identified downstream signaling and binding proteins of miR-223. Flow analysis was performed to investigate the recruitment of myeloid suppressor cells in spinal cords and peripheral blood of spleen; WB and q-PCR were performed to detect iNOS, CD86, Arg1 and CD206 to investigate the polarization of microglia in spinal cord; EC staining was performed to observe the morphology of spinal cord injury centers, and immunofluorescence was used to statistically analyze the polarization of microglia and oligodendrocytes.
Results: In demyelinated mice, miR-223 levels were elevated. EC and immunofluorescence staining showed increased regenerative myelin and more MBP in the OE group compared to other groups. Flow analysis showed that MDSCs were 8.99% ± 1.4% higher in peripheral blood of spleen and 28.1% ± 2.3% higher in spinal cord tissue in the OE group compared with the control group, and the differences were statistically significant; WB and q-PCR showed that M2-type microglia were 32% ± 2.8% more and M1-type microglia were 18% ± 2.4% less in the OE group compared with the control group, and the differences were Immunofluorescence showed that the number of 0PCs in spinal cord tissue increased by 24% ± 2.1% and the proportion of mature

OLs increased by 37% ± 3.0 % in the OE group compared with the control group. Mass spectrometry analysis showed that miR-223 activated LRP1 via the Shc1/PI3K/Akt pathway, thereby promoting recruitment differentiation of MDSCs and polarization of microglia to the M2 type.
Summary/Conclusion: Our study shows that miR-223+BV2-EXO activates LRP1, promotes the recruitment of myeloid suppressor cells (MDSCs) and the polarization of microglia to M2 type to improve the immune microenvironment at the site of injury through the Shc1/PI3K/Akt pathway, and promotes the proliferation and differentiation of OPCs into mature oligodendrocytes (OLs) to promote remyelination. It was demonstrated that miR-223+BV2-EXO could be a new potential therapeutic approach.

PF19.03 | Polarized release of EVs from brain microvascular endothelial cells is coupled to EV:leukocyte binding

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Introduction: Our lab previously showed EVs from brain microvascular endothelial cells (BMECs) contain the tight junction protein CLN-5, can bind to leukocytes in vitro and, during neuroinflammation, varied types of circulating leukocytes acquire CLN-5. This led us to consider EV-mediated acquisition of CLN-5 might be coupled to leukocyte transendothelial migration (TEM) via a zipper mechanism. An extension of this hypothesis is that BMEC-EVs are released in a polarized fashion, wherein EVs derived from the apical plasma membrane would be released luminally, while EVs originating from the basolateral plasma membrane would be discharged abluminally. This would allow apical-derived EVs to more efficiently interact with blood-born cells, and basolateral-derived EVs to target elements in the vascular adventitia or parenchyma.
Methods: Apical vs basolateral membranes of BMECs grown on a Transwell filter were differentially labeled with fluorescent dyes. EVs isolated from top and bottom chambers by differential ultracentrifugation were assessed by flow cytometry, NTA & transmission electron microscopy. For TEM experiments, splenocytes were added to the top chamber and collected from both chambers at 24 hours.
Results: EVs from either chamber were mostly labeled with that dye associated with the respective membrane surface. This was the case for all-sized EVs assessed. Significantly, multivesicular bodies largely contained dye derived from only one membrane surface, suggesting that exosomes may remain segregated from their incipient state till release. Transmigrated leukocytes of all subtypes analyzed preferentially acquired apical-derived EVs.
Summary/Conclusion: BMEC-EVs are released in a polar manner in vitro and may be predestined for interaction with specific targets. Future investigation of the proteomic cargo of apical vs basolateral-derived EVs will highlight distinctive roles of EVs in neuroinflammation. Analogous experiments are being conducted in a flow-based system to better recapitulate the in vivo circulatory environment.
Funding: This work was funded by NIH R0-1 NS099855-02 and NIH R21NS113593-01.
Keywords: neuroinflammation, blood-brain barrier, leukocytes, transendothelial migration

PF19.04 | SASP mediated by sEV as senomorphic target

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Introduction: Cells have the capacity to modulate the microenvironment through secreted molecules and factors like interleukins, cytokines, chemokines, extracellular matrix proteins, etc. Pathological process like cancer or natural process like ageing can modify this microenvironment. The ageing modifications are related with the increasing number of senescent cells. Cellular senescence is a process characterized by a cell cycle arrest, an increased β-galactosidase activity and a secretome that acquires a specific phenotype. This senescence-associated secretory phenotype known as SASP leads the microenvironment to a more pro-inflammatory state triggering with time age-related diseases. SASP has the capacity of paracrine senescence transmission. Small extracellular vesicles (sEV) are an important part of SASP. The regulation of the sEV biogenesis has a high potential to develop senomorphics, drugs that modulate SASP, to treat age-related diseases such as type II diabetes, cardiovascular diseases among others.

Objetive. Find a proteomic signature of the SASP mediated by sEV to reveal pathways associated with the SASP senescence transmission through sEV.
Methods: In this study we knock-down in mesenchymal stem cells RELA or RAB27A, genes implicated in the paracrine senescence and sEV biogenesis respectively, using CRISPR-Cas9 methodology. We compared the paracrine senescence transmission through sEV in these cells with proliferation and β-galactosidase activity assays after the treatment with senescent or non-senescent sEV. Finally, we perform the shot-gun technique Tandem Mass Tag (TMT) Systems (10-plex) to identify, quantify and compare the proteome of senescent cells with the knock-down senescent ones.
Results: The paracrine senescence transmission in the RELA and RAB27A knock-downs was inhibited. The quantitative and comparative proteomic analysis identified 4099 proteins, which of 25 were differentially regulated by the SASP mediated by sEV. These proteins are involved in the Golgi traffic and network.
Summary/Conclusion: This study provides evidence that Golgi traffic and transport are involved in the SASP mediated by sEV. This data will be useful to design new therapeutic strategies or support the actual ones against age-related diseases.
Funding: JFL was funded by Proof-of-concept from ProteoRed-ISCIII (PPC2020) and Xunta de Galicia, Grant Number ED481D-2021-020 and MINECO (RYC2021-032567-I) for the funding and the InTalent program from UDC-Inditex for the research grant. The proteomic analysis was performed in the Proteomics Unit of Complutense University of Madrid, a member of ProteoRed and is supported by grant PT17/0019, of the PE I+D+i 2013- 2016, funded by ISCIII and ERDF. Or Grant PRB3 (IPT17/0019 - ISCIII-SGEFI / ER. MCA. received a grant from the Spanish National Health Institute Carlos III (PI20/00497).
Keywords: cellular senescence, SASP, senomorphics, omics

PF19.06 | Increased signal transduction mediated by extracellular vesicles

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Introduction: The role of extracellular vesicles (EVs) in several oncological and neurological diseases is widely studied. Their use in clinical practice is still delayed due to some difficulties in tailoring their cargo and tissue tropism. Their use as a delivery vector for several types of molecules could be a very interesting tool to exploit in several pathological conditions. In our system, we loaded EVs with IL4 (IL4 EVs) and used them to mediate microglia phenotype shift towards an activated state.
Methods: We engineered a murine microglia cell line modified with a lentivirus overexpressing IL4, or with CRISPR Cas9 to display IL4R tagged with eGFP. We observed that the use of IL4 EVs elicits a faster and more potent effect on recipient microglia when compared to a similar concentration of recombinant IL4 (rIL4), administered in soluble form.
Results: EVs use endocytosis as a general mechanism to enter recipient cells and release part of their cargo by endosomal escape in the cell cytosol. So, we studied EVs internalization by electron microscopy and immunofluorescence, measuring the colocalization of specific organelles belonging to the endocytic pathway. We hypothesized that the difference in the signaling efficiency between the treatment could be related to their different interaction with IL4R, in terms of cellular localization and receptor engagement. We finally studied in live imaging receptor clusters formation mediated by short-term administration of IL4 EVs and rIL4. Our experiments indicate that IL4 EVs and rIL4 have different effects on recipient cells. In our model, IL4 EVs change microglia phenotype after 3h post-administration. The kinetic of the process mediated by EVs is faster than the one mediated by rIL4. Such difference suggests that the process, or some component of it, is different between IL4 EVs and rIL4. Receptor clustering on plasma and endosomal membranes can mediate signal boosting, so cluster formation mediated by the two treatments can be different.
Summary/Conclusion: In our system, it is clear that EVs stabilize receptor clustering formation keeping their number stable, with respect to rIL4, and increasing receptor quantity over time. Although EV use in clinical practice is distant, we think that understanding EVs signaling mechanisms and biology is a key point in moving forward the research on their use as a delivery tool. The advantage given by their use in vitro, if translated in vivo, could be greatly exploited and open a very new way of delivering therapeutic molecules.
Keywords: uptake, internalization, signaling, receptor clustering

PF19.07 | Self-assembled blood-brain barrier (BBB) spheroids as a model of EV transcytosis

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Introduction: While biodistribution studies have long suggested that EVs cross the BBB, little is known about how or which EVs cross this barrier. To this end, both a representative model including multiple cell types to best recapitulate the in vivo BBB and a comparison of EV types will be necessary to better understand this mechanism. Here we apply BBB spheroids, wherein brain microvascular endothelial cells, pericytes, and astrocytes spontaneously form an intricate BBB-like structure, as a high-throughput model to explore the extent to which brain metastasis associated EVs cross the BBB.
Methods: Spheroids were prepared by combining brain endothelial cells (hCMEC/D3) with primary human pericytes and astrocytes (1:1:1) in 96 well spheroid microplates and allowing to assemble for at least 1 week. EVs were collected from brain-metastasizing breast cancer cells (231-Br), breast cancer cells (MDA-MB-231), and control HEK293T cells via ultracentrifugation, labeled with carboxy-fluorescein succinimidyl ester (CFSE) and separated from dye via size-exclusion chromatography. After incubation with EVs, spheroids were fixed and labeled for imaging via confocal microscopy.
Results: Spheroids assembled into appropriate layers with endothelial cells forming an external layer, having limited permeability to fluorescent albumin and expressing tight junction proteins, and pericytes and astrocytes forming the core. EVs could be identified in spheroids at 6 and 24 h with increasing uptake. Similarly, EV puncta were visible after incubation at ≥ 4*10^10 p/mL with more puncta for high concentrations. EVs penetrated the endothelial layer to different extent across EV types.
Summary/Conclusion: Here we introduce a new high-throughput BBB spheroid model for screening of BBB crossing of various EV types, showing that both increased concentration and exposure time increase internalization. While all EV types showed uptake, we were able to detect differences that may be important to discerning a mechanism of transcytosis.
Funding: The research was supported by the NIH by Award Number F31NS120590 (RRM).
Keywords: blood-brain barrier, spheroid

PF19.08 | Mammalian neuronal exophers – who are they and what do they do?

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Introduction: Proteostasis dysfunction and accumulation of protein aggregates occur under many cellular stress conditions. Cellular mechanisms that remove misfolded proteins and damaged organelles have mostly been studied in the context of cell-autonomous housekeeping mechanisms, such as the autophagy-lysosomal pathway. Exophers are new cellular structures, first discovered in C. elegans light-touch neurons in 2017, and hypothesized to be a heretofore unknown component of neuronal proteostasis. It has not been known whether they exist in higher-order organisms and what roles they might serve if they do. We discovered exophers in mammalian neurons of mice and humans.
Methods: Exophers were analyzed in multiple cell culture systems and brain sections using light and fluorescence microscopy. They were recognized morphologically and by the absence of a nucleus.
Results: Similar to nematode exophers, mammalian neuronal exophers vary widely in size from ~1.5 μM in diameter to structures that are as large as the originating cell itself. Accordingly, they can be substantially larger than those in C. elegans. They contain multiple types of cellular organelles and cytoskeletal proteins. Exopher number increases adaptively in response to cell stress but falls sharply under over-stress conditions. In addition to their adaptive role in the removal of unwanted cellular material and protein aggregates, innate exophers were observed that presumably assist in sharing resources among neighbor cells.
Summary/Conclusion: Exophers are newly discovered extracellular vesicles that exist not only in lower organisms but also in mammals, including humans. There appear to be two types of exophers – innate and adaptive – that participate in the transfer and removal of cell content throughout the neuron life and may be important in both health and disease.
Funding: The work was supported by NIH/NIA grants R01AG050721 and RF1AG054000.
Keywords: exopher, neuron, glia, non-autonomous, proteotoxicity, clearance, intercellular

PF19.09 | Specificity and abundance of nervous system-origin markers of extracellular vesicles

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Introduction: Establishing the specificity of EV surface proteins opens new opportunities for studies of otherwise inaccessible tissues, such as the central nervous system (CNS). Capturing and detecting brain-origin EVs that leak or are transported into peripheral samples is a type of 'liquid biopsy.' Most EVs in peripheral biofluids are irrelevant to brain disease, but one

might boost signal above noise by enriching cell/organ-specific EVs with immunocapture methods. Although numerous studies have attempted to harvest EVs of neuronal origin from peripheral samples, EVs from other CNS cells have been relatively under-studied. Here, single-particle interferometric imaging sensing (SP-IRIS) and other platforms were used to capture and phenotype EVs from several CNS cell types.

Methods: EVs were separated from cell cultures representing oligodendrocytes (HOG), astrocytes (U-87 MG), microglia (SV40), and neurons (SH-SY5Y), as well as differentiated iPSCs using ultracentrifugation followed by size exclusion chromatography and ultrafiltration. Characterization was done with Western blot, nanoflow cytometry, and transmission electron microscopy. SP-IRIS was performed by capturing with CD9, CD63, and CD81 antibodies and visualized by fluorescent imaging with tetraspanins (all cell lines); myelin oligodendrocyte glycoprotein (MOG); neural cell adhesion molecule (CD56); transmembrane protein 119 (TMEM119); and disialoganglioside (GD2). Super-resolution microscopy was done with tetraspanin labeling. CNS-specific antibody-coated magnetic beads were used to compare the presence and abundance of surface markers via flow cytometry.

Results: Approximately half of EVs derived from microglia, astrocytes, and oligodendrocytes were triple-positive for CD9, CD81, and CD63, while only 9% of neuronal EVs expressed all three tetraspanins. Additionally, 84% of microglial and 94% of neuronal EVs were positive for CD81, while astrocytes and oligodendrocytes had higher percentages of CD63+ EVs, at 91% and 82%, respectively. However, putative cell-specific markers were displayed at a much lower abundance than tetraspanins. Some markers, such as oligodendrocyte proteins MOG, were not detected on EVs.

Summary/Conclusion: Biological barriers and low expression of cell-specific markers are challenges for diagnostic utility of CNS EVs that may need to be overcome by implementing multi-target capture strategies.

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Keywords: extracellular vesicles, SP-IRIS, super-resolution microscopy, iPSCs, central nervous system

PF19.10 | A Novel marker for brain derived extracellular vesicles isolated from blood

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Introduction: Neurodegenerative disease is diagnosed after brain function is irreversible. Therefore, diagnosing at incipient disease and predicting the disease stage are imperative for prevention and effective treatment. Unfortunately, early detection of brain disease progression and onset remains challenging because brain-specific molecules secreted from the brain are difficult to capture into the blood.

Brain-specific proteins present in the EV membrane were analyzed, and among them, APLP1, which had the highest expression level on brain, was selected as a marker for isolating brain-derived EVs in blood. APLP1 is brain specific and is expressed throughout the brain. Above all, APLP+ EVs can be isolated from a variety of sources, including biofluids.

Methods: To enrich for brain derived EVs, rabbit anti-APLP1 was biotinylated. NTA. Nanoparticle tracking analysis (NTA), TEM, Westernblot, qPCR were used for the characterization of EVs. Mass Spectrometry Analysis and Small RNA sequencing of EVs were performed for the proteomics and miRNA transcriptoms, respectively.

Results: It was confirmed that APLP1 was more brain specific than L1CAM, previously reported as a marker of neuronally derived extracellular vesicles. In addition, the expression of neuronal markers and brain disease-related proteins including Alzheimer's disease (AD) and Parkinson's Disease (PD) was high in APLP1+EV obtained from human plasma EV. This is to verify that the APLP1+EV present in the blood is a brain-derived EV. Additionally, we verified the potential of captured EV by APLP1 from human plasma through analysis of small RNA sequencing. The small RNA expression patterns of APLP1+EV and APLP1-EV were clearly distinguished, and it was confirmed that the putative targets of miRNA in APLP1+EV were significantly and highly expressed in the brain. In addition, the function of the putative target of miRNAs with low expression among miRNAs in APLP1+EV was significantly analyzed with axon guidance related to brain function.

Summary/Conclusion: Our results demonstrate that APLP1 is a suitable novel biomarker for isolating brain-specific- EVs. Furthermore, our results suggest usefulness as a diagnostic platform for early diagnosis of neurodegenerative diseases through a combination of APLP1 and another specific neuronal marker.

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Keywords: alzheimer's disease, APLP1, biomarker, brain, extracellular vesicle, L1CAM, early diagnosis of brain disease

PF19.11 | Extracellular vesicles derived from the choroid plexus trigger the differentiation of neural stem cells

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Introduction: A carpet of beating cilia transports cerebrospinal fluid (CSF) through the brain ventricles. CSF contains extracellular vesicles (EV), secreted by the choroid plexi that reside in the ventricles. Along their path, EVs pass by neural stem cells (NSC). We study the content of the EVs, their path of movement and their interaction with cells in vitro and in ventricular explants.
Methods: EVs were isolated from the murine choroid plexus secretome and from mouse embryonic fibroblast (MEF) by differential centrifugation and where indicated by flotation on an iodixanol density gradients. EVs were characterized by EM, Western blotting and NTA. EVs were co-cultured with murine NSCs isolated from the niche of the lateral and the third ventricles. Fluorescently labelled lipid particles were applied to the NSCs or ventricular explant.
Results: A brief treatment of EVs from choroid plexus but not from MEFs induced NSC to differentiate. The EVs converted the round NSCs to cells that extended long processes that contacted nearby, alike-shaped cells and expressed genes characteristic for neurons and astrocytes.LC-MS/MS showed that the differentiation-inducing EVs were enriched for membrane and membrane-associated proteins involved in cell differentiation, membrane trafficking and membrane organization. EVs MEF had little effect on the NSCs and did not show an enrichment of such proteins. Fluorescently labelled EVs associated with the NSCs. When applied to the ventricular explants, such particles were transported by beating cilia bundles along the ventricular wall.
Summary/Conclusion: We show, for the first time, that EVs originating from the choroid plexus induce neural stem cell differentiation. In particular, such EVs evoke profound morphological changes that lead to the multicellular network formation. This may represent an initial step in NSC differentiation to astrocytes and neurons. Potentially the choroid plexus EVs can trigger NSC differentiation after the brain injury.
Funding: This work was supported by the Max Plank Society and Deutsche Forschungsgemeinschaft (SFB1286).

PF19.13 | Serial immunocapture of P2Y12+/TMEM119+ microglia derived extracellular vesicles reveals distinct activation states in normal and inflamed neonatal mouse brain

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Introduction: Microglia are brain resident macrophages that once activated by neuroinflammatory signals undergo changes in morphology and surface marker expression. Little is known about microglia-derived extracellular vesicles (M-EVs) in the neonatal brain during sepsis. Herein, we separated and characterized M-EVs in a neonatal sepsis model and evaluated their neuroinflammatory cargo.
Methods: Animals: Brains of 9-day-old C57BL/6 mice were harvested from normal pups or pups with sepsis and brain neuroinflammation confirmed by high expression of inflammatory markers IL1β and NLRP3 (WB), and high density of ameboid IBA1+ microglia (immunofluorescence).
M-EVs: Brains were minced, digested with dispase, differentially centrifuged, and filtered (300g/1200g/100,000g). M-EVs were enriched by serial immunocapture of canonical microglia receptors with a biotin-streptavidin platform:
- EVs were incubated with P2Y12 antibody and biotin-tagged secondary antibody
- Streptavidin coated agarose beads were added
- After washes, P2Y12+EVs were released by acid catalysis
- Incubation steps were repeated using TMEM119 antibody, and P2Y12+/TMEM119+ EVs were separated.
M-EVs were assessed for size (NTA), morphology (TEM), and CD63, TSG101, Histone-H3, IL1β and NLRP3 protein expression (WB). Immunogold labelling was performed to confirm capture of P2Y12+/ TMEM119+ M-EVs (TEM).
Results: M-EVs from control and inflamed brains had similar size distribution and were CD63+/TSG101+ and Histone-H3-. Immunogold labeling showed P2Y12 and TMEM119 localization on the M-EV extracellular domain. M-EVs from inflamed brains had higher P2Y12 ($p = 0.03$), NLRP3 ($p = 0.002$) and IL1β ($p = 0.03$), and lower TMEM119 ($p = 0.03$) protein levels compared to control M-EVs.
Summary/Conclusion: Our method was effective in separating M-EVs from normal and inflamed neonatal brains. In an experimental model of neonatal sepsis, M-EVs have a different signature during neuroinflammation.
Funding: SickKids Foundation.

Poster Presentation

PS01: Therapy Adipose and MSC-Derived EVs

Location: Hall 4A

16:50 - 18:50

PS01.02 | Mesenchymal stem cell aggregation-released extracellular vesicles induce CD31+EMCN+ vessels in skin regeneration and improve diabetic wound healing

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Introduction: Type 2 diabetes (T2D) has become a major global health problem and a leading cause for non-healing wounds. We have previously developed the mesenchymal stem cell (MSC)-derived cell aggregates (CA) for regenerative application, which provide an efficacious therapy to promote diabetic wound healing but the mechanisms are not fully understood. Notably, the regenerative effect of CA is suppressed by pharmacological inhibition of release of extracellular vesicles (EVs). Here, we aimed to explore the characteristics, therapeutic potential and underlying mechanisms of CA-EVs treating T2D skin wounds.
Methods: EVs were isolated based on differential centrifugation from the media supernatant of cultured CA induced by umbilical cord-derived MSCs (UCMSCs). CA-EVs were characterized by NTA, TEM and western blot analysis, and protein lysates of CAs and CA-EVs were subjected to LC-MS/MS analysis. Scratch assay, tube formation assay and immunofluorescence staining of CD31+EMCN+ vessels were performed to investigate the effects of CA-EVs on angiogenesis in vitro, and CA-EVs were applied to skin wounds of db/db mice to evaluate therapeutic effects in vivo.
Results: Compared with single UCMSCs, CA produced significantly increased amount of EVs with diameters peaking at 150–250 nm. Proteomic analysis indicated that aggregation induced release of EVs enriched with a set of pro-angiogenic functional proteins. In vitro experiments showed that CA-EVs significantly rescued high glucose-induced impairment of the migration, tube formation and CD31+EMCN+ vessel formation of endothelial cells. In vivo data demonstrated that CA-EVs improved diabetic wound healing and enhanced angiogenesis of regeneration-relevant CD31+EMCN+ vessels in skin, which were attributed to upregulation of Notch signaling.
Summary/Conclusion: Collectively, these results add to the current knowledge of EVs derived from MSC-CA, and help establish feasible strategies to benefit wound healing under diabetic condition.
Funding: National Natural Science Foundation of China, 81930025 (YJ); National Natural Science Foundation of China, 82170988 (FJ).
Keywords: extracellular vesicles, mesenchymal stem cell, wound healing, diabetes, regeneration, angiogenesis

PS01.03 | Neurogenic potential of mesenchymal stem cells derived exosomes

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Introduction: Exosomes are the indicator of tissue health. Mesenchymal Stem Cell (MSCs) derived exosomes (MSC-Exo) are one of the most popular therapeutically active extracellular vesicles. Though extensive work has been done to evaluate the immunomodulatory and anti-fibrotic effects of MSC-Exo, there is limited information about its neurogenic potential. Here, we showed that MSC-Exo promotes neurite growth and multilineage differentiation of neurons.
Methods: MSC cells were routinely cultured in MEM supplemented with HPL. Exosomes were prepared in MEM+ 2% HPL. Exosomes were prepared using ultra centrifugation and were characterized using a nano-tracker for the size and zeta potential. The neurogenic potential of exosomes was evaluated on neuroblastoma cells SH-SY5Y by evaluating i) neurite length, ii) Immunofluorescence (ß3 tubulin staining), iii) real-time -PCR (RT-PCR) and iv) immunoblot

Results: Throughout the experiment, neurotrophic growth factor (NGF) is used as a positive control. We observed MSC-Exo showed faster and better neurite growth compared to NGF and negative control (differentiation media only). We observed faster growth and migration of neuronal cells in the presence of MSC-Exo than in NGF. Significantly increased expression of neuronal differentiation and multilineage neurogenic markers were observed in MSC-Exo but lacks in NGF.
Summary/Conclusion: NGF is an FDA-approved drug for treating corneal diseases. Our study showed that MSC-Exo is a better pro-neurogenic factor than NGF. Also, MSC-Exo were more efficient in the multilineage differentiation of neurons than NGF. In conclusion, we can say that MSC-Exo can be an advance and next-generation therapy not only for nerve growth but also for the pathologies associated with neuronal degeneration.
Funding: None.
Keywords: neurogenic, multilineage, exosomes

PS01.04 | Mesenchymal stem cell-derived EV carries different vascular and skin regeneration depending on the cell culture environment

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Introduction: In recent decades, extracellular vesicles (EV) have been studied in various fields and have been evaluated for their wide utilization and high value. These studies found that EVs have cell-specific cargo proteins, lipids, and are substances that have functions that reflect the characteristics of cells. In particular, EV derived from stem cells have been reported to have stem cell characteristics, and they function similar to stem cells as cell free materials. Therefore, EVs secreted according to the culture environment of stem cells produce different results.
Methods: Mesenchymal stem cells were cultured using different culture media and then EVs were separated by tangential flow filtration (TFF) system from conditioned media. Size distribution and particle number of EVs were measured by using Zetaview and confirmed by expression of EV markers (CD9, CD63, and CD81) by western blot. Isolated EVs were treated on human umbilical vein endothelial cells (HUVECs), HaCaT cell (human epithelial keratinocyte), dermal fibroblast and epidermal keratinocyte to evaluate vascular and skin regeneration.
Results: We confirmed that EVs secreted from cells cultured with serum free serum free chemically defined medium (CDM) have remarkable skin regeneration ability and vascular regeneration ability. Through the experimental results, compared to MSC-derived EV secreted from the medium containing FBS, we found that HUVEC cells and HaCaT cells of the MSC-derived EV treatment group from CDM showed higher angiogenesis index and wound area recovery rates, respectively. In addition, normal human skin fibroblasts (NHDF) and epidermal keratinocytes were treated with each EV to confirm that genes (Involucrin, Loricrin) and proteins (Hyaluronic acid, pro-collagen) related to skin barrier, moisturizing and anti-aging were highly expressed MSC-derived EV isolated from CDM.
Summary/Conclusion: The application of serum free chemically defined medium is a method of obtaining MSC-derived EVs with the ability to regenerate stem cells while maintaining the characteristics of stem cells in cell culture. By cultivating cells in animal-derived components free, it is possible to improve the quality of the stem cell culture environment and obtain 'human stem cell-derived EV' that completely exclude animal-derived EV. Ultimately, this study shows that CDM lead to the only stem cell-derived EVs you want to get.
Keywords: CellCor EXO CD, serum free chemically defined media, Stem cell

PS01.05 | Separation of mesenchymal stem cell-derived extracellular vesicles using chitin magnetic beads functionalized with engineered CBD-intein-lactadherin C1C2 fusion protein

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Introduction: Extracellular vesicles (EVs) released from mesenchymal stem cells (MSCs) are membrane-bound vesicles that mediated the intercellular communication between cells through transporting active biological cargoes, such as proteins, as lipids, and RNAs. However, the most commonly used techniques for EV isolation (e.g., differential ultracentrifugation and hydrophilic polymer precipitation) have disadvantages regarding feasibility and purity. Low purity and retention of polymer can interfere with further downstream analysis. Here, we developed a novel method for the separation of MSC-derived EVs using chitin magnetic particles bound with chitin-binding domain (CBD) fusion with self-cleaving intein tag and lactadherin C1C2 which has high affinity to phosphatidylserine (PS) exposed on EV membranes.

Methods: The gene of human lactadherin C1C2 was amplified by polymerase chain reaction and cloned into the N-terminal of intein and CBD from pTXB1vector, then expressed in E. coli. Prior to thiol-induced cleavage of thioester bond between the intein tag and lactadherin C1C2, cell conditioned medium (CCM) containing EVs was mixed with chitin magnetic beads for 1hr at 4oC to allow the binding between lactadherin C1C2 and PS on EVs membranes. Then, lactadherin C1C2-EVs were isolated from the beads by the cleavage buffer containing 50 mM dithiothreitol.
Results: Our approach yielded high purity and high specificity of EVs better than those obtained using the classical methods. Relevant EV biomarkers (i.e., CD63, CD9, CD81, Tsg 101, and Alix) were detectable by the Western blot. The size and charge of EVs were determined respectively to be 111.21 ± 11 nm and −14.47 ± 2.2 mV, which are within the size ranges of exosomes and microvesicles.
Summary/Conclusion: The specific binding affinity between lactadherin C1C2 and PS-expressing EVs was harnessed to effectively capture EVs from MSC CCM using chitin magnetic beads tethered with CBD-intein-lactadherin C1C2 fusion protein.
Funding: N/A.
Keywords: Mesenchymal stem cell, chitin magnetic bead, chitin binding domain, intein, lactadherin C1C2, phosphatidylserine

PS01.06 | CSF extracellular vesicles and particles derived from adipocytes: potential role in spreading microRNAs associated with neuroinflammation

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Introduction: Interactions between the peripheral organs, such as adipose tissue, and brain have been widely described. In this context, a growing body of evidence indicates that adipose tissue dysfunction, such as in aging process, can be involved with obesity-induced susceptibility to tissue injury even in distant sites, as well as the role of adipocytes-derived extracellular vesicles and particles (EVPs) as a potential mechanism for the spread of bioactive molecules has been raised. We hypothesized that CSF EVPs derived from adipocytes can be involved to the cerebral aging process via their microRNA cargo. Our aim was to study the impact of aging process on microRNA profiles of CSF EVPs derived from adipocytes (FABP4+), performing an in silico prediction of their downstream signaling effects.
Methods: Fatty acid-binding protein 4 (FABP4) was used as a marker for adipocyte-derived EVPs that were isolated from CSF of young adult and aged Wistar rats (3 and 21 months old) using the commercially available kit. microRNA isolation and microarray expression analysis were performed. Canonical pathways, Disease & Functions, and Upstream Regulator analyses were performed using IPA-Qiagen.
Results: The analysis revealed that 78 miRNAs were differentially expressed between groups ($p < 0.05$; fold change $\geq |1.1|$), of which 30 miRNAs were up-regulated and 48 were down-regulated in adipocyte-derived EVPs obtained in CSF from aged animals compared to young adults. Interestingly, the "Neuroinflammation Signaling Pathway" was listed as a significant canonical pathway (z-score = 2,425). MiRNAs from adipocyte-derived EVPs obtained in CSF reduced by aging, such as miR-1-3p, miR-24-3p, miR-3065-5p, miR-16-5p, miR-17-5p, can target key molecules of neuroinflammation. For instance, the downregulation of miR-17-5p that targets C-X-C motif chemokine ligand 8 (CXCL8) and tumor necrosis factor (TNF) can be related at least in part to higher levels of these cytokines in brain areas during aging process.
Summary/Conclusion: miRNA signature in CSF adipocyte-derived EVPs may be involved with susceptibility to neuroinflammation conditions and consequently with deleterious effects of obesity in aging process.
Funding: This study was financed in part by the CNPq (Dr. I.R. Siqueira - 307980/2018-9) and CAPES (Dr. Laura Reck Cechinel - Finance Code 001, # 88881.189257/2018-01).

PS01.07 | The therapeutic effects of small extracellular vesicles derived from klotho-overexpressed mesenchymal stem cells on acute kidney injury

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Introduction: Acute kidney injury (AKI), defined by a rapid deterioration of renal function, is a common complication in hospitalized patients with no effective therapy. Among the recent therapeutic options, mesenchymal stem cells (MSCs) are

considered as promising strategy in animal studies and clinical trials, but their clinical application still faces many challenges. Small extracellular vesicles (sEV) have more advantages to overcome the limitation of stem cells in clinical application. Klotho is an antiaging substance with pleiotropic actions including regulation of anti-inflammation and damage repairing. This study aimed to investigate the protective effects of sEV derived from klotho-overexpressed MSCs (klotho-sEV) on AKI.

Methods: Klotho-overexpressed MSCs were prepared and sEV were isolated using anion exchange chromatography, and their characteristics were determined using NTA/TEM/WB. FCM was performed to confirm sEV absorption and reno-protective role in HK2 cells. An AKI mouse model was induced and sEV were injected into glycerol-induced rhabdomyolysis mice, and renal injury was evaluated using the serum creatinine, urea nitrogen, renal pathology and acute tubular necrosis score. The expression of inflammatory and injury markers was performed by PCR.

Results: We found that sEV from klotho-overexpressed MSCs expressed abundant klotho, and expressed the common sEV characteristics with the size, shape and exosome markers (CD9/CD63/CD81/ALIX/TSG101). The sEV could be taken in by HK2 cells in a time-dependent manner. In addition, klotho-sEV markedly reduced myoglobin-induced HK2 cell apoptosis. In vivo, klotho-sEV had stronger functional improvement of accelerated renal recovery, decreasing serum creatinine, urea nitrogen and tubular necrosis score, stimulating tubular cell proliferation, reducing the expression of inflammatory (IL-1beta/IL-6/ TNF-α) and injury markers (NGAL/ PAI/ SOX9) compared to sEV.

Summary/Conclusion: Klotho-sEV could be a novel cell-free strategy for the treatment of AKI.

PS01.08 | Do microRNAs play a role in the therapeutic potential of small extracellular vesicles (sEV) from Wharton's jelly mesenchymal stromal cells

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Introduction: Preterm birth is the leading cause of childhood morbidity and mortality. Preterm birth often results in neurological complications such as premature white matter injury (WMI). WMI leads to lifelong disabilities. There is no cure for premature WMI. Mesenchymal stroma cell-derived small extracellular vesicles (MSC-sEV) show promising results as a therapeutic agent for neurological injuries. MSC-sEV carry microRNAs (miRNAs), predicted to target mRNAs encoding for proteins belonging to signaling pathways of premature WMI. We hypothesize that miRNAs, released by MSC-sEV upon uptake in their target cells, have a key function in the observed beneficial effects from MSC-sEV.

Methods: sEV were purified from Wharton's jelly MSC by ultracentrifugation followed by size exclusion chromatography and characterized according to morphology, protein content, size, and zeta potential. The miRNA content was measured by qPCR. A luciferase assay and a DROSHA knock-down of the MSC were established to evaluate the regulatory activity of sEV miRNA. The regulatory potential of the sEV was validated in cell differentiation and cell death in vitro assays.

Results: The samples were positive for sEV markers CD81, CD63, and CD9, and contained miRNAs being involved in WMI, such as hsa-miR-22-3p, hsa-miR-21-5p, hsa-miR-27b-3p, and the hsa-let-7 family. sEV significantly reduced the luciferase signal in a luciferase assay with a vector containing a 3'UTR of TP53 and TAOK1, genes involved in WMI, indicating an inhibitory effect of sEV miRNA. sEV enhanced differentiation in the oligodendrocyte lineage. After oxygen-glucose deprivation, sEV reduced apoptotic markers in neuroblastoma cells. sEV from WJ MSC with DROSHA knock-down had less miRNA cargo and a lower effect on the in vitro assays than sEV from untreated cells.

Summary/Conclusion: Our results show the functionality of the miRNA cargo in the therapeutic effect of MSC-sEV in preclinical WMI. To confirm the in vitro results, in vivo experiments in a model of premature WMI are ongoing.

Keywords: white matter injury, mesenchymal stromal cells, small extracellular vesicles, therapeutic potential, microRNA

PS01.09 | Metabolomics of extracellular vesicle-containing human adipose-derived stem/stromal cell secretome

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Introduction: Secretome, and the extracellular vesicles (EVs) it contains, has been progressively recognized as mediating most physiologic and therapeutic effects of mesenchymal stem/stromal cells. Many studies have characterized various proteomic and miRNA components of MSC secretome and its EVs but relatively little is known about its metabolome, and its role in the activity of secretome and its EVs.
Methods: We compared the metabolome of human adipose-derived stem/stromal cell (ASC) secretome derived from expanded ASC of two healthy donors and control media (n = 4 per donor), using global metabolomics profiling with a Thermo Q-Exactive Orbitrap mass spectrometer with Dionex UHPLC and autosampler. Samples were analyzed in positive (POS) and negative (NEG) heated electrospray ionization with mass resolution of 35,000 at m/z 200 as separate injections. Separation was achieved on an ACE 18-pfp 100 × 2.1 mm, 2 μm column with mobile phases A 0.1% formic acid in water and B acetonitrile with flow rate 350 μL/min and column temperature 25°C. MZmine (freeware) was used to identify features, deisotope, align features and fill gaps. Adducts and complexes were removed.
Results: PCA plots of both modes of all metabolites revealed distinct separation of the secretomes from control media (74 and 68% of total variance explained by PC1+PC2 in POS and NEG mode respectively); the secretome groups were substantially overlaid in the POS mode but separated in PC2 in NEG mode. PLS-DA plots showed separated plots in both modes in PCA2 (observed statistic 0.99 in both modes). Thus, these two ASC secretomes share many common metabolites, and also display inter-donor differences.
Summary/Conclusion: These data provide a foundation for mechanistic insight into activities of MSC-S and MSC-EV that influence metabolic processes, and also may assist in defining donor variability and batch-to-batch consistency in the production of MSC-S and MSC-EV for clinical therapeutics.

Poster Presentations

PS02: Neurodegenerative Diseases and EVs 2

Location: Hall 4A

16:50 - 18:50

PS02.01=OWP2.05 | nSMase2 inhibition reduces tau propagation in Alzheimer's Disease mouse models

meixiang huang[1]; Barbara Slusher[2]; Carolyn Tallon[3]; benjamin Bell[2]; Xiaolei Zhu[2]; Medhinee Malvankar[2]; Ajit Thomas[2]; Angela Rubin[3]; Arindom Pal[2]; Kristen Hollinger[2]; Erden Eren[2]; Carlos J. Nogueras-Ortiz[2]; Rangaramanujam Kannan[2]; Rana Rais[2]; Norman Haughey[2]; Dimitrios Kapogiannis[3]
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Introduction: Mounting evidence correlates the propagation of hyperphosphorylated tau (pTau) along synaptically connected networks in the brain with progressive cognitive decline in Alzheimer's Disease (AD). Recent findings have highlighted extracellular vesicle (EV)s in enabling transcellular transmission of pathological tau and identified the partial inhibition of EV biogenesis via small-molecule inhibitors of nSMase2 as a potential therapeutic avenue. However, there are no suitable compounds for clinical development so far.
Methods: Through high-throughput screening and subsequent chemistry, our lab identified PDDC, a highly selective and potent nSMase2 inhibitor with excellent brain penetration and oral bioavailability. To characterize the potential therapeutic effect of PDDC in vivo, we administered PDDC-containing chow to both PS19 transgenic mice and to wild-type mice stereotaxically injected with an AAV vector encoding for P301L mutant human tau into their hippocampus (AAV-hTau seeded model). After chronic dosing, we quantified tau levels in the hippocampus of PS19 mice and the contralateral dentate gyrus (DG) of the AAV-hTau mice. Neuronally-derived EVs (NEV)s from plasma were isolated via immunocapture against L1CAM/CD171. Intact NEVs were used to determine particle concentration and diameter using nanoparticle tracking analysis (NTA). Total plasma EVs isolated via Size Exclusion Chromatography were subjected to flow cytometry analysis (FCA) with labeling for p262Tau and b-III-tubulin.
Results: PS19 mice exhibited robust elevation of multiple ceramide species and enhanced brain nSMase2 enzymatic activity, both of which were normalized by PDDC treatment. PS19 mice treated with PDDC had significantly reduced total tau and pTau, reduced gliosis, protected synapses, and increased neuronal counts. Plasma NEVs of treated mice were fewer in number, greater in size, and had lower p181-Tau levels than the untreated group; FCA confirmed the decrease of NEVs carrying p262-Tau at the single EV level. Similarly, the AAV-hTau-seeded mice treated with PDDC had reduced tau staining intensity in the contralateral DG.

Summary/Conclusion: Data in two AD models using PDDC provides strong preclinical support for using nSMase2 inhibition as a therapeutic strategy to slow tau propagation in AD.
Funding: NIH R01 AG063831, R01 AG059799, P30 MH075673, R25GM109441 (Hopkins PREP), Tau Consortium (T-PEP-18-579974C), the Maryland Innovation Initiative award (135726), and the Intramural Research Program of the National Institute on Aging, NIH.
Keywords: alzheimer's disease, EV, nSMase2, tau

PS02.02=OWP2.04 | Selenoprotein P regulates extracellular vesicle secretion from neurodegenerative microglia in an animal model of Alzheimer's disease

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Introduction: Microglia are primary innate immune cells in the central nervous system and display a neurodegenerative phenotype (MGnD) in response to amyloid plaque deposition in human and mouse models of Alzheimer's disease (AD). We previously reported that MGnD hyper-secrete extracellular vesicles (EVs) compared to homeostatic microglia in APPN-L-GF knock-in mouse model of AD. We recently identified selenoprotein P (Sepp1), a secreted heparin-binding glycoprotein, as a potential regulator of EV and IL-1B secretion from microglia under a pro-inflammatory stimulus. We hypothesize that silencing of Sepp1 suppresses EV secretion in plaque associated MGnD microglia in vivo.
Methods: In this study, we employed the small interfering RNA (siRNA) to determine the effects of silencing Sepp1 on EV secretion. We monitored EV secretion from BV-2 cells, a murine microglial cell line, constitutively expressing tdTomato-CD63 EV reporter molecule with or without downregulation of Sepp1 by Nanoimager, which can detect the tdTomato signal at a single molecule level. To track EVs secreted by microglia in vivo, a microglia-specific lentivirus expressing mEmerald-CD9 (mEm-CD9) reporter molecule was co-injected into the hippocampus of aged APPN-L-GF mice with lentivirus expressing Sepp1 or scramble shRNA and mCherry. Mice were euthanized at 2 weeks post injection and immunostained for galectin-3 (Mac2, MGnD marker), RFP (mCherry), GFP (mEm-CD9) and fluorostyrylbenzene (FSB, amyloid plaque). The images of mEm-CD9+ voxels (EV particles) in the proximity of Mac2+/RFP+/GFP+ microglia were captured by Lightning super-resolution confocal microscopy and the number of EV particles were quantified after 3D surface rendering of EV particles using IMARIS software.
Results: Under pro-inflammatory stimulation of BV-2 cells with lipopolysaccharide followed by ATP for rapid EV secretion, we observed a reduction in the loading of tdTomato-CD63+ molecules to EVs secreted in three independent Sepp1-shRNA clones. At seven months of age, we mostly detected mEm-CD9+ microglia as Mac2+ MGnD surrounding amyloid plaques in APPN-L-GF mice. Lentiviral shRNA-induced Sepp1 silencing reduces the EV secretion (mEm-CD9+ voxels) from mEm-CD9+/Mac2+/RFP+ microglia compared to the scramble shRNA-transduced mEm-CD9+/Mac2+/RFP+ microglia in the APPN-L-GF mouse brain.
Summary/Conclusion: These data demonstrate that in vivo silencing of Sepp1 suppresses EV secretion from MGnD microglia. Since activated microglia secrete neurotoxic and pathogenic molecules, our data suggest Sepp1 as a potential target for ameliorating microglia-mediated disease progression in neurodegenerative conditions including AD.
Keywords: microglia, alzheimer's disease, selenoprotein P

PS02.03=OWP2.02 | Blood-based neuronal and astrocyte extracellular vesicles biomarkers identify treatment responders for Alzheimer's disease in Down Syndrome

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Introduction: Individuals with Down Syndrome (DS) show AD neuropathology and cognitive impairment at an accelerated rate as they age. There are limited resources for treating AD in DS patients (DSAD). Using biobanked plasma samples from a previously completed AD/DS clinical trial of anti-inflammatory treatment, we aimed to characterize AD neuropathology in blood-based small extracellular vesicles (EVs) over time and as a function of treatment to develop a precision medicine approach for use in DS trials. We hypothesized that changes in amyloid β (Aβ) peptides, total tau (taut), Nf-L, and GFAP in neuronal and astrocyte EVs (NDEVs/ADEVs) might reflect treatment response in DSAD individuals.

Methods: We used archived plasma samples (n = 138; with 106 non-demented and 32 demented subjects) from the previously completed Phase 3 clinical trial, Vitamin E in Aged Persons with Down Syndrome (NCT00056329). Small EVs were isolated from baseline and 36-month plasma of DSAD individuals treated with Vitamin E or placebo. NDEVs and ADEVs were enriched with magnetic immunocapture using neuronal and astrocyte-specific proteins CD171 and GLAST, respectively and fluorescence-activated cell sorting. NDEVs and ADEVs were characterized for size, integrity and homogeneity as per the guidelines of the International Society of Extracellular Vesicles using nanoFCM, electron microscopy, immunoblots, and ELISA. AD marker proteins Aβ40–42, taut, Nf-L and GFAP were quantified by SIMOA assays in native plasma, NDEVs and ADEVs of DSAD individuals in treatment vs. control group.
Results: Blood-based NDEVs and ADEVs demonstrated the expected size, shape, and distribution of small EVs i.e. exosomes. Immunoblots and ELISA showed the presence of small EVs marker proteins. Various AD biomarkers were significantly altered in NDEs and ADEs of the vitamin E-treated DSAD group compared to the control.
Summary/Conclusion: Changes in AD protein markers can serve as a biomarker of treatment response in DSAD individuals.
Funding: INCLUDE-NIH R01AG073979.

PS02.04=OWP2.01 | Differential extracellular vesicle gene expression in Next Generation Sequencing targeted panels as potential platforms for biomarker discovery in Parkinson's disease

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Introduction: Parkinson's disease (PD) is the second most common and fastest growing neurodegenerative disease globally. Neuronal extracellular vesicles are in low abundance in hematopoietic-rich plasma after crossing the blood-brain barrier; however, targeted profiling of brain-derived transcripts can help resolve rare isoforms and enrich their signal.
Methods: To that end, we performed transcriptome (WTS), exome (WES), and brain-specific exosomal RNA profiling on 20 PD and 20 healthy control plasma samples. Following RNA-Seq analysis, DEx analysis was conducted alongside Boruta feature selection to see how each enrichment platform influenced the resolution of gene expression and isoform detection.
Results: We observed tremendous neuronal gene expression, orders of magnitude higher in the brain panel over WES or WTS. Over 278 genes, more than a quarter of the brain panel, was undetectable in WTS but shown in the targeted panel. Additionally, Unique Molecular Indexing (UMI) confirmed more unique RNA transcripts profiled as capture enrichment became more specific. Furthermore, the targeted brain panel revealed splice variant events that were not identified with WTS or WES. DEx analysis identified several novel potential biomarkers between PD and healthy controls in all three platforms. Remarkably, extracellular vesicles from PD patients showed significant dysregulation in gene pathways related to locomotory and walking behavior compared to the healthy controls using the targeted brain panel only.
Summary/Conclusion: High TPM expression and UMI count shown in the brain panel contributed to the retention of rare isoforms not seen in the broader panels. Our most targeted platform contributed to high sensitivity and unique differential expression patterns not seen with the other sequencing libraries. This finding will be evaluated in a larger clinical cohort.
Keywords: parkinson's disease, NGS, differential expression, target enrichment

PS03: EVs and Viral Infection

Location: Hall 4A

16:50 - 18:50

PS03.01=OWP1.04 | Presence of small infectious extracellular vesicles from a chronically infected T-cell line

Sebastian Molnar[1]; Sebastian M. Molnar[1]; Lindsay Wieczoreck[2]; Yuriy Kim[3]; Mark Santos[4]; Aurelio Lorico[4]; Victoria Polonis[5]; Fatah Kashanchi[3]
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Medicine, Turo University, Henderson, NV, USA; [5]Military HIV Research Program, Walter Reed Army Institute of Research, Silver Spring, MD, USA

Introduction: In 2021, 1.5 million people acquired Human Immunodeficiency Virus-1 (HIV), and an estimated 37.7 million people were living with HIV worldwide. Despite extensive efforts, successful vaccines and functional cures have yet to be developed, highlighting the need for further viral characterization. One challenge to our understanding of HIV-1 biology is the heterogeneity of virions and extracellular vesicles (EVs) and particles (EVPs) in biological fluids (i.e., bodily and cell culture). Here, we utilized a latently infected T-cell line as a model to investigate the size range, content diversity, and infectivity of EVPs released in cell culture supernatants and identify smaller than previously known infectious EVPs.
Methods: HIV-1 latently infected J1.1LAV T-cells were cultured for four days, then cell culture conditioned medium was collected. Secreted EVPs were separated by differential ultracentrifugation (DUC) to generate the following fractions (Frac): Frac-A (2000g force, 20 min); Frac-B (10,000g force, 45 min); Frac-C (100,000g force, 90 min); Frac-D (167,000g force, 3-hours); Frac-E (167,000 g force, 18-hours). The fractions were characterized for size, viral markers by WB and dSTORM, and HIV-1 infectivity and blocking.
Results: We observed a heterogeneous particle size distribution for all fractions. Frac-C had a modal size of 132 nm (similar to HIV), whereas Frac-E had a modal size of 15 nm. WB analysis indicated diverse distribution of the EVs and HIV markers amongst the fractions, with Frac-E being positive for all of them. We also observed the colocalization of EVs and HIV markers on the same particles by dSTORM. Functional analysis revealed that small-sized EVPs in Frac-E were infectious and sensitive to neutralizing antibodies.
Summary/Conclusion: Utilizing the DUC approach, we obtained small infectious (sub-100 nm) Frac-E EVPs that share EVs and HIV markers.
Funding: This work was supported by a cooperative agreement (W81XWH-18-2-0400) between the Henry M. Jackson Foundation for the Advancement of Military Medicine Inc., and the US Department of Defense (DoD).
Keywords: HIV, small extracellular vesicles and particles (sEVPs), NTA, direct stochastic optical reconstruction microscopy (dSTORM)

PS03.02=OWP2.10 | Reduction in dengue virus serotype 2 (DENV-2) replication induced by small extracellular vesicles (sEVs) produced by EA.hy 926 endothelial cells

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Introduction: DENV infection can cause endothelial cell (EC) hyperpermeability and vascular leakage. Small EV of infected EC (sEVIC) may participate in the viral spread and regulate EC response to DENV. Still, there is no information on sEVIC cargo and its effect on non-infected EC. This work aimed to characterize the sEVIC and evaluate their impact on polarized EC.
Methods: EA.hy 926 were infected with DENV-2, MOI 1. The virus was removed, and serum-free medium was added. Cell viability (Resazurin, LDH) and viral infection (IFI and PCR for DENV E protein) were confirmed. After 48h post-infection, sEV were isolated (ultracentrifugation) and characterized by NTA, DLS, Western blot, LC/MS/MS, and small RNA sequencing. For function assay, sEVIC were pretreated with neutralizing antibody 4G2 or UV exposed after pretreatment. Later, polarized EC were exposed to the different sEV for 24h and then infected. TEER and permeability (Dextran-blue 2KDa) were measured at different times, and IFI for ICAM, E-sele, and actin were made along with qPCR for DENV detection. sEV non-infected cells (sEVNIC) were used as control.
Results: EC infection induced a high concentration of EV (2×109) with low ALIX expression. Compared to EVNIC, EVIC had 129 increased proteins (mainly of immune response -IR-), 206 were downregulated (cellular adhesion (CA) and developmental processes), and a viral NS5 peptide was found along with sncRNA like YRNA and miRs that mainly regulate CA and IR genes. Interestingly, miRs found exclusively in EVIC may have a proviral effect. Functionally, EVIC induced ICAM and E-sele expression and a protective effect in the polarized EC that maintained a stable TEER (45Ω) and reduced viral replication.
Summary/Conclusion: EVIC transported DENV elements and sncRNA that may regulate viral response. Our model suggests that EVIC induced a protection response during infection, reducing viral replication and activating the EC in the first 24h.
Funding: MinCiencias – U Bosque: Grant 130884467149, Contract 431–2020.
Keywords: DENV, sEVs, endothelial cells, viral replication

PS04: Cardiovascular EVs 2

Location: Hall 4A

16:50 - 18:50

PS04.01=OWP2.08 | Cardiomyocytes-derived EVs for the treatment of cardiac fibrosis in hypertension and COVID-19-derived cardiac damage

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Introduction: Cardiac fibrosis, found in most cardiovascular diseases, including COVID-19 patients, is the result of an erroneous hyperactivation of fibroblasts. This dysfunction evokes excessive fibroblast cell proliferation and extracellular matrix (ECM) accumulation, decreasing cardiac function and eventually causing death. It is known that cardiomyocytes (CM) communicate and affect the surrounding cells through extracellular vesicles (EVs). Therefore, CM-derived EVs may be a promising cell-free system for fibrosis treatment.
Methods: A defined conjunct of chemicals was used to improve human CM culture and ensure a high collection of EVs. Terminal differentiation, as well as senescence markers emergence, were delayed in comparison to the predetermined culture medium without apparent malignant alteration. EVs were isolated by ultracentrifugation, and their characteristics (morphology, particle number, membrane markers, and internalization) were analyzed in accordance with MISEV2018 guidelines. Finally, their effect on fibrosis was tested.
Results: EV secretion and their characteristics were unaffected in chemically-treated CM. Interestingly, CM-derived EVs were specifically internalized by cardiac fibroblasts compared to other corporal fibroblasts, while no apparent differences were observed in mesenchymal stem cell-derived EVs. Treatment of EVs on TGFβ-activated cardiac fibroblasts showed a decrease of fibroblast activation markers at mRNA and protein levels. Furthermore, ECM secretion was also reduced. Consequently, intracardiac injection of EVs reduced the fibrotic area and induced angiogenesis, which translated to an improved cardiac function in a hypertension mouse model. Because the EV content was comprised of not only anti-fibrotic but also anti-inflammatory microRNAs, their use in COVID-19-like infection is also being studied. To date, the anti-inflammatory effect of EVs on macrophages activated by SARS-CoV2 Spike protein was corroborated by cytokine secretion. Correspondent animal experiments are being processed to elucidate the anti-inflammatory and further anti-fibrotic protection effect in SARS-CoV2 infection-like mouse model.
Summary/Conclusion: Our findings indicate that, due to the anti-fibrotic effects and the specificity of the EV cargo, the use of EVs derived from CM is a promising treatment for several types of cardiac fibrosis. In addition, their study may help to understand the biological meaning of CM-derived EVs in the cardiac microenvironment.
Funding: AMED.
Keywords: EVs, cardiomyocytes-derived EVs, cardiac fibrosis, EV therapy, specific internalization

PS04.02=OWP2.07 | Cell-secreted extracellular matrix nanoparticles restore endothelial barrier function

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Introduction: Nanoscale mediators secreted from mesenchymal stromal cells (MSCs) were shown to be efficacious in animal models of tissue injury that disrupts vascular function. However, recent studies highlight the heterogeneity of nanoscale mediators, including extracellular vesicles (EVs) and non-vesicular extracellular particles (NVEPs). Restoration of endothelial barrier function requires the activation of extracellular matrix (ECM) signaling. Thus, we tested the hypothesis that an ECM-containing nanoscale fraction is essential for the restoration of vascular permeability upon tissue injury.

Methods: The crude fraction was isolated by following EV-TRACK ID EV150007 from mouse MSCs. We used immunoaffinity-based approaches along with Triton-X sensitivity assay and nanoparticle tracking analysis to quantify the subpopulations that contain fibronectin (FN). FN+ fractions were pulled down via FN antibody-functionalized magnetic nanoparticles (~8 nm) for characterization by transmission electron microscopy (TEM). The fractionated nanoscale subpopulations were delivered to mice 4 h after treatment with lipopolysaccharide (LPS), and the mice were analyzed for edema and vascular permeability in the lungs.
Results: Our results show that MSC-secreted nanoscale mediators consist of Triton-X sensitive CD63+FN- (40%) and Triton-X resistant CD63-FN+ (33%) subpopulations, while the CD63+FN+ population is less than 5%. TEM analysis confirms that the FN+ fraction is non-vesicular with a single particle size of ~30 nm and is more irregular in shape than EVs. We show that FN+ NVEPs are essential for the restoration of endothelial barrier function after LPS-induced lung injury.
Summary/Conclusion: This study reveals the importance of FN+ NVEPs as a novel nanoscale mediator to restore vascular integrity in response to tissue injury. Future studies will investigate the biogenesis mechanisms of ECM-presenting NVEPs and mechanisms of action by which FN+ NVEPs restore endothelial barrier function.
Funding: This work is supported by NIH Grant No. R01-HL141255 (to J.-W.S.).
Keywords: non-vesicular extracellular particles, extracellular matrix, fibronectin

PS04.03=OWP2.09 | Extracellular vesicles from ischemic heart disease (IHD) patients demonstrate enhanced procoagulatory activity

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Introduction: Extracellular vesicles (EVs) carry unique repertoires of biologically active cargo that hold promising novel biomarkers for cardiovascular diseases (CVDs). However, it is unclear how the number, location, cellular origin, and size of these EVs within the circulation can influence CVDs such as ischemic heart disease (IHD). The current study compares these novel markers in arterial and venous blood in subjects undergoing routine coronary angiography analysis for IHD. EVs were then characterized from those presenting with and without IHD.
Methods: Arterial and venous blood from IHD and control patients was collected from the Cardiac Catheterization Lab at the Royal Berkshire Hospital after obtaining informed consent for the project. This research was approved by the University of Reading Research Ethics Committee and the Human Research Authority (REC 20/NW/0263). Blood was centrifuged at 1500 x g for 15 minutes at room temperature (RT) to generate platelet-poor plasma followed by 13,000 x g for 2 minutes at RT to isolate platelet-free plasma (PFP). EV fractions were isolated from 500μL of PFP by size exclusion chromatography. EVs were analyzed by Nanoparticle Tracking Analysis, flow cytometry to characterize number, size and cellular origin and a thrombin generation assay was used to assess procoagulatory activity of the isolated circulating EVs.
Results: Coagulatory activity of EVs isolated from IHD patients was significantly higher in IHD patients compared to controls. There were higher numbers of endothelial-derived EVs in arterial blood compared with venous blood. Linear regression models revealed that plasma triacylglycerol concentration and age independently predicted circulating EV numbers in IHD patients, although numbers of EVs were not significantly different in the two groups.
Summary/Conclusion: Although numbers of EVs in IHD patients were not elevated, EVs in IHD patients had greater procoagulant activity, highlighting a potential important role for EVs in IHD.
Funding: This project was funded by the Joint Academic Board between the Royal Berkshire NHS Foundation Trust and the University of Reading.

PS05: EV Cargo Characterization II

Location: Hall 4A

16:50 - 18:50

PS05.01=OWP1.06 | Simultaneous size-based fractionation and surface marker detection of extracellular vesicles in a one-step workflow

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Introduction: Extracellular vesicles (EVs) are increasingly studied as diagnostic and prognostic biomarkers. Typically, the analysis of EV-associated biomarkers requires their separation from biofluids, which warrants larger input volumes, leads to longer turn-over times, and increases technical variability. To overcome these issues we aimed to develop a one-step workflow for simultaneous size-based fractionation and biomarker detection.
Methods: We coupled a multi-angle light scattering detector (MALS) and a fluorescent light detector (FLD) in-line with the asymmetrical flow field-flow fractionation (AF4) equipment. We first optimized the AF4-MALS-FLD parameters using recombinant EVs (rEVs) including spacer (350 μm), membrane (10 kDa regenerated cellulose), running buffer (PBS + 0.02% NaN3), detector flow, and cross flow profile. Next, we evaluated the performance of the AF4-MALS-FLD set-up using diverse biofluids including cell culture medium, urine and blood plasma. Fractions were collected and quality was controlled using complementary characterization methods in compliance with MISEV2018 guidelines. Relevant experimental parameters were submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV210212).
Results: We were able to analyze EV surface markers CD9, CD63 and CD81, and cancer biomarkers HER2, EpCAM and PSMA in pre-purified EV preparations from different breast and prostate cancer cell lines. Analysis of increasing numbers of EVs revealed a linear correlation with the number of particles as measured by NTA (4E+9 – 2E+10 particles). Furthermore, the AF4-MALS-FLD workflow allowed to detect EVs in samples with increasing complexity as cell culture supernatant, urine and blood plasma. Finally, we validated the one-step workflow by confirming the presence of PSMA-positive EVs in urine from prostate cancer patients (n = 25), and by demonstrating the presence of EpCAM- or HER2-positive EVs in blood plasma enabling discrimination of breast cancer patients (n = 15) from healthy donors (n = 15).
Summary/Conclusion: We have successfully optimized and validated the AF4-MALS-FLD workflow, that allows simultaneous size-based fractionation and surface marker characterization in a fast, reproducible and sensitive manner.
Funding: This work was supported by UGent, FWO, CRIG, and KOTK (the Flemish cancer society).

PS07: EV Heterogeneity

Location: Hall 4A

16:50 - 18:50

PS07.01=OWP1.05 | Differential protein expression in immune cell-derived EVs isolated at different densities

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Introduction: Small EVs (sEVs) are the most studied EV subpopulation, but less is known about other subpopulations e.g. large EVs (lEVs). Consequently, specific subpopulation markers are missing. Therefore the aim of our study was to compare the differential protein expression between EVs of different sizes and densities.
Methods: HMC-1 (mast cells) and THP-1 (monocytes) were cultured in EV-depleted cell culture media. The lEVs (16,500xg, 20min) and sEVs (118,000xg 2.5h) were isolated by differential ultracentrifugation and purified by a density cushion. Low density EVs (LD; ~1.05-1.12 g/mL) and high density EVs (HD; ~1.12-1.15 g/mL) were collected from both lEV and sEV.
Purity, morphology, and yield of EVs were determined by nanoparticle tracking analysis (NTA), protein measurement and transmission electron microscopy (TEM). The proteome of the different samples was analysed with quantitative mass spectrometry (TMT-LC-MS/MS).

Results: TEM and NTA showed different size and concentration of the different EV subpopulations (lEV LD, lEV HD, sEV LD and sEV HD). In total 3972 and 4735 proteins were quantified in HMC-1 and THP-1, respectively. lEV and sEV were well separated, however, the separation between the LD and HD samples was more pronounced for the THP-1 EVs than for the HMC-1 EVs. Several protein groups were enriched, confirming our previous finding. Among these groups we found that mitochondrial proteins were enriched in lEV LD. Namely, TIM/TOM complex, MICOS, ATP5 proteins. KIF proteins were enriched in the lEV HD samples. Tetraspanins, ESCRT, Syndecans and Syndecan binding protein were enriched in the HMC-1 sEV, additionally these proteins were enriched in the THP-1 sEV LD. ADAM proteins were enriched in the sEV HD.
Summary/Conclusion: This study identified several protein groups differently enriched in lEV and sEV, which validate previous findings. In addition the proteome of EVs isolated at different densities had significant differences.
Funding: Swedish Heart Lung Foundation; Emil and Wera Cornell Foundation; Lars Hierta Memorial Foundation.
Keywords: EV subtypes, small EVs, large EV, size, density, TMT-LC-MS/MS

PS08: EVs in Neurological Disorders and Injury

Location: Hall 4A

16:50 - 18:50

PS08.01=OWP2.06 | iPSC-derived sEVs rejuvenate senescent blood-brain barrier to protect against ischemic stroke in aged mice

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Introduction: Blood–brain barrier (BBB) breakdown after ischemic stroke exacerbates brain injury and BBB senescence can cause severe neurological deficits in the aged ischemic stroke population. Recent evidence reveals that inducible pluripotent stem cell-derived small extracellular vesicles (iPSC-sEVs) possess the phenomenal antisenescence capability. However, whether iPSC-sEVs can rejuvenate BBB senescence to improve stroke outcomes in aged mice remains unknown.
Methods: Aged mice were treated with iPSC-sEVs for 2 months, and transient middle cerebral artery occlusion (MCAO) was conducted. BBB senescence, BBB leakage, infarct volume, immune cell infiltration, neuroinflammation, neural death, and sensorimotor functions were detected. Next, D-gal was utilized to induce BBB senescence, and oxygen and glucose deprivation (OGD) was performed. BBB senescence and BBB leakage were further evaluated in vitro. Mechanistically, proteomics analysis of iPSC-sEVs was performed to explore the bioactive factors. eNOS inhibitor, AKT1 and CALM inhibitors were used to verify the mechanism.
Results: In aged mice long-term treatment with iPSC-sEVs alleviated aging-induced BBB senescence. In aged stroke mice, iPSC-sEVs significantly mitigated BBB integrity damage, reduced the following infiltration of peripheral leukocytes, and decreased the release of pro-inflammatory factors from the leukocytes, which ultimately inhibited neuronal death and improved neurofunctional recovery. Mechanism studies showed that iPSC-sEVs could activate the endothelial nitric oxide synthase (eNOS) and up-regulate sirtuin 1 (Sirt1) in senescent endothelial cells. Blocking the activation of eNOS abolished iPSC-sEV- mediated rejuvenation of BBB senescence and the protection of BBB integrity. Proteomics results demonstrated that iPSC- sEVs were enriched with bioactive factors including AKT serine/threonine kinase 1 (AKT1) and calmodulin (CALM) to activate the eNOS–Sirt1 axis. Further investigation showed that AKT1 and CALM inhibitors blocked iPSC-sEV-afforded activation of the eNOS–Sirt1 axis in senescent endothelial cells.
Summary/Conclusion: iPSC-sEVs can protect against ischemic stroke in aged mice by rejuvenating BBB senescence, partially, through delivering AKT1 and CALM to activate the eNOS–Sirt1 axis.
Funding: This study was funded by the National Natural Science Foundation of China under Grant Nos. 82071371, 82201543, and 81871833, National Postdoctoral Program for Innovative Talent under Grant No. BX20220356, China Postdoctoral Science Foundation under Grant No. 2022M723562, Natural Science Foundation of Hunan Province under Grant No. 2022JJ40828, Natural Science Foundation of Changsha city under Grant No. kq2202377, Young Foundation of Xiangya Hospital under Grant No. 2021Q01.
Keywords: induced pluripotent stem cell-derived small extracellular vesicles (iPSC-sEVs), blood–brain barrier (BBB), cell senescence, ischemic stroke, aging

PS09: Preeclampsia, Reproduction and Development

Location: Hall 4A

16:50 - 18:50

PS09.01=OWP2.11 | Placental extracellular vesicle-vasorin in preeclampsia associated cardiac dysfunction

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Introduction: Women with preeclampsia (PE) are at increased risk of cardiovascular disease (CVD) during pregnancy and postpartum. The mechanisms of heightened risk for CVD are poorly understood. Consequently, approaches for prevention and treatment are unknown. Preliminary data indicates that the protein vasorin (VASN) was highly down regulated in extracellular vesicles (EV) isolated from patients with severe PE (sPE-EV), as compared to normotensive-EV (NTP-EV). VASN has been implicated in regulating calcium homeostasis and calcium dynamics in cardiomyocytes. Therefore, we hypothesized that the down regulation of VASN protein in EV in sPE may play a role in PE-associated cardiac dysfunction via altered regulation of intracellular calcium dynamics.
Methods: EV from pregnant women with sPE (n = 15) and NTP (n = 15) were separated from plasma using a precipitation-based kit. We used an unbiased proteomic approach to compare the EV protein cargo profile from women with sPE and NTP using a tandem mass spectrometry approach. In adult murine cardiomyocytes (mCM), calcium dynamics were assessed using fura-2AM imaging in an Ion Optics instrument and VASN and calcium sensor proteins were quantified by western blot.
Results: We verified VASN down regulation in maternal plasma EV, and in placental tissue in sPE as compared to NTP. We also found that the levels of stromal interaction molecule 1 (STIM 1) were decreased in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) and in mCM treated with sPE-EV. Treatment of contractile mCM with sPE-EV that have decreased VASN content inhibited levels of STIM 1 while mCM isolated from pregnant mice injected with AD-sFLT-1 (a model of PE) exhibited altered dynamics of calcium signaling as compared to mCM from untreated pregnant mice (UT). Likewise, treatment of hiPSC-CM results in the decrease of STIM1, phospholamban (PLN) and sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) levels. We also found that in CM isolated from AD-hsFLT-1 injected mice had elevated peak Ca2+ ratio when compared to mCM from UT.
Summary/Conclusion: Our data indicate that levels of VASN, a TGF-beta signaling inhibitor, in EV correlate with the effects of EV on expression levels of proteins with roles in Ca2+ signaling and alterations of Ca2+ dynamics in CM. We speculate that that loss of VASN in sPE-EV contribute to the mechanisms of pathological cardiac remodeling, by decreased inhibition of deleterious TGF-beta signaling.
Funding: This study was supported by seed funds from the Department of Anesthesiology and Perioperative Medicine (D.E.B.) and a REINVENT grant (S.M. and T.J).
Keywords: preeclampsia, cardiac dysfunction, extracellular vesicles, vasorin, calcium signalling

PS09.02=OWP2.12 | Detection of uterine lumen-derived extracellular vesicles in bovine plasma in vivo

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Introduction: Embryo-maternal communication is essential for pregnancy establishment. Extracellular vesicles (EVs) are significant molecules in intercellular communication required during early pregnancy. The EVs can directly or indirectly trigger an endocrine response. However, at this stage of pregnancy, the uterine epithelium has a stable adhesion that could alter the exchange of molecules. Accordingly, the present study aims to investigate whether stained EVs leave the uterine environment.

Methods: The experiment used six Nellore heifers. The heifers received an intrauterine infusion of either PBS+PKH26 (control group) or follicular fluid (FF)-derived EVs, isolated by ultracentrifugation twice at 120000xg and stained with PKH26 (treatment group). To isolate EVs, plasma samples were taken from the jugular vein at pre-determined periods (10 min, 30 min, 1 h, and 3h). EVs were isolated by size exclusion chromatography (Izon qEV35) and evaluated by nanoparticle tracking analysis for particle size and concentration and flow cytometry for positive events for PKH26. Controls were performed to discriminate other nanoparticles in flow cytometry, and samples were labeled with CD9-FITC and CD63-FITC. Data were compared by ANOVA followed by Tukey.
Results: Evs from the treatment group had higher concentration and particle size than those from the control group ($P < 0.05$). Considering only the treatment group, size and concentration increased over time ($P < 0.05$). The PKH26-positive events differed by time and group, although the number of events was relatively small ($P < 0.05$). The detection of EVs in the systemic circulation was relatively rapid, peaking at 30 min, and after a longer period, the detection decreased, suggesting that the effect and migration of EVs are timely. The detection of PKH26 in the control animals could also represent the autofluorescence of particles, one of the limitations of the present experiment; however, the values in the treatment group were always numerically higher when subtracted from the negative samples.
Summary/Conclusion: Although this experiment has some pitfalls, our data suggest that stained EVs leave the uterine environment based on their detection in the bloodstream and elicit an endocrine response in as short as 30 minutes.
Funding: FAPESP #2022/01235-0; #2021/06645-0; #22/01505-8; #2015/21829-9.
Keywords: reproduction, pregnancy, extracellular vesicles

PS10: Lung EVs

Location: Hall 4A

16:50 - 18:50

PS10.01=OWP1.01 | Differential expression of bacterial small RNAs in severe bronchiolitis

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Introduction: Bronchiolitis is a heterogenous condition with respiratory bacterial taxa as risk factors for a spectrum of outcomes. Bacteria produce small RNAs (sRNAs) carried via extracellular vesicles (EVs) that can alter human gene expression, similar to human microRNAs. We aimed to define sRNA reference libraries for four important respiratory bacterial species and then determine their association with bronchiolitis severity.
Methods: We used 574 samples from a multicenter, prospective cohort study of US infants hospitalized with bronchiolitis. Nasal swabs collected at the time of hospitalization were sequenced for sRNAs (MiSeq; Illumina). To define the bacterial EV libraries, we isolated EVs from Haemophilus influenzae (Hi), Moraxella catarrhalis (Mc), M. nonliquefaciens (Mn), and Streptococcus pneumoniae (Sp) cultures via precipitation and size exclusion chromatography. Bacterial cell and EV RNA were extracted for sRNA-sequencing (MiSeq; Illumina).
Results: The median age at hospitalization was 4 months (IQR = 4.1); 41% female; 45% White, 23% Black, and 29% Hispanic. Intubation (n = 18) and/or positive pressure ventilation (n = 15) represented higher bronchiolitis severity (total n = 28). We identified reference sRNA libraries from bacterial cells and EVs: 286 sRNAs from Hi (64%), 272 from Mc (57%), 49 from Mn (100%), and 159 from Sp (87%). Of these, 20 sRNAs were significantly different between bronchiolitis severity groups ($p < 0.05$, fold change>|1.1|). Higher severity-associated sRNAs were linked to endocytic processes, whereas lower severity-associated sRNAs were linked to NFkB pathway activation.
Summary/Conclusion: Bacterial sRNAs differ between higher and lower bronchiolitis severity and may be involved in relevant biological processes during acute illness. These data provide new insights into bacteria-host signaling in bronchiolitis, particularly through interspecies RNA communication.
Keywords: bacteria, bronchiolitis, small RNAs

PS10.02=OWP1.02 | Extracellular vesicle mediated cytokine secretion in chronic airway disease

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Introduction: The cytokine interleukin-33 (IL-33) is a driver of chronic airway diseases such as asthma and chronic obstructive pulmonary disease (COPD). This cytokine lacks a signal peptide and is tightly sequestered in the cell nucleus where classically it was thought to be passively released and bioactive upon necrotic cell death, although the exact release mechanism is still unknown. We recently described a chronic disease-associated spliced isoform of IL-33 expressed in airway epithelial cells that can be found secreted as membrane-bound cargo on extracellular vesicles (EVs). This IL-33 isoform lacks exons 3–4, is unable to localize to the nucleus and instead can be found throughout the cytosol where it can then be tonically secreted into the extracellular environment.
Methods: Using both chemical inhibition and genetic depletion approaches, this secretion phenomenon was inhibited by the nSmase2 inhibition (GW4869 and genetic knockdown) in both in vitro and in vivo models. Subsequent proteomic, biochemical and imaging analyses reveal that in this non-canonical secretion mechanism, IL-33 interacts directly with the chaperone HSP70 to facilitate EV binding and associated cellular secretion.
Results: In an IL-33 dependent airway mouse model, co-administering GW4869 with Alternaria allergen significantly reduced airway inflammation compared to Alternaria treatment alone. This result, coupled with our human biospecimen observations, point to nSmase2 and HSP70 dependent EV biogenesis pathway as a potential mechanism for IL-33 is secretion in chronic airway inflammation.
Summary/Conclusion: This work highlights a recruitment pathway for EV-bound signaling cargo and reveals unique therapeutic targets for chronic airway diseases.
Keywords: IL-33 HSP70 nSmase2 GW4869 inflammation

PS11: EVs from Microorganisms 2

Location: Hall 4A

16:50 - 18:50

PS11.01=OWP1.03 | Acidovorax temperans outer membrane vesicles promote lung inflammation and tumorigenesis

Haneen Abu-Freih[1]; Shani Cohen[1]; Bibek Bhatta[2]; Etti Azulay[3]; Ishai Luz[4]; Elena Voronov[1]; Isana Veksler-Lublinsky[1]; Tomer Cooks[4]
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Introduction: Dysbiosis is a common feature of solid tumors, however whether this dysbiosis directly contributes to tumor development is largely unknown. We previously characterized the lung cancer microbiome where the Gram-negative Acidovorax temperans was found to be enriched in tumors of smokers and patients harboring TP53 mutations. We showed that A. temperans exposure accelerated tumor development and burden through infiltration of proinflammatory cells in the lungs. Herein, we investigated the involvement of outer membrane vesicles (OMVs) shed by Acidovorax temperans in driving inflammatory dynamics and tumorigenesis.
Methods: OMVs were extracted using standard serial centrifugations and filtrations followed by a sucrose gradient to get rid of flagella and non-vesicular elements. The purified samples were characterized using NTA, electron microscopy and ImageStream flow cytometer microscope. RNA from the OMVs was isolated using miRNeasy kit (Qiagen) and was prepared for RNAseq using the NEBNext Multiplex Small RNA Library Prep (Illumina).
Results: Our results suggest that A. temperans OMVs are taken up by A549 lung cancer cells and that A. temperans OMVs facilitate a strong pro-inflammatory response also in A549 and THP-1 macrophages. In addition, macrophages exposed to A. temperans OMVs overexpress SIRPα which is associated with tumor cell immune escape and tumor progression. Notably, the levels of CD47, the receptor recognizing SIRPα, were elevated in the cancer cells after the OMVs treatment. We also optimized an in vivo protocol where A. temperans OMVs were introduced into the lungs of mice by intranasal administration. Intranasal administration of A. temperans OMVs lead to an increased secretion of proinflammatory cytokines. We used OMVs RNAseq specifically focusing on short RNAs (sRNA) and identified a unique signature of RNA species including various fragmented tRNAs.
Summary/Conclusion: OMVs shed by Acidovorax temperans promoted inflammatory signaling in lung carcinoma cells and elevated CD47 expression on tumor cells and SIRPα levels on macrophages. We currently investigate the capacity of these OMVs to drive lung tumorigenesis and study the molecular cargo shipped between the pathogen and cells of the host.
Funding: This study is funded by the Israel Science Fund (ISF) grant #: 1178/20.
Keywords: outer-membrane-vesicles, lung cancer, microbiome

PS12: EVs in Cancer Progression

Location: Hall 4A

16:50 - 18:50

PS12.01=OWP1.10 | RNA modification levels determine the tumor promoting effect of 5'tRF-GlyGCC in colorectal cancer Te-EVs

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Introduction: We have previously shown that 5'tRF-GlyGCC encapsulated in colon cancer EVs acts in a tumor-promoting manner by promoting the production of inflammatory cytokines via TLR8 in macrophages. However, it is not clear how 5'tRF-GlyGCC regulate TLR8 reactivity in macrophages. Since RNA modifications have been reported to modulate TLR8 reactivity, we focused on RNA modifications on 5'tRF-GlyGCC to determine the mechanism of action of tumor EVs on macrophages.
Methods: Clinical specimens: The colon cancer specimens were obtained from patients undergone primary resection at the Osaka Medical and Pharmaceutical University, Japan. Written informed consent was obtained from each patient, and the study was approved by the ethics review board of the Osaka Medical and Pharmaceutical University.
Isolation of tumor tissue-derived EVs: Tissue-immersed medium was centrifuged at 2,000 g, for 30 min, and the collected supernatants were subjected to the ultracentrifuge method (100,000 g x 2) for recovery of tissue-exudative EVs (Te-EVs). The size and concentration of EVs were determined using qNano.
Isolation of 5'tRF-GlyGCC from Te-EVs: EV-RNA were isolated from Te-EVs by using miRNeasy kit. 5'tRF-GlyGCC were collected from EV-RNA by Dynabeads magnetic beads conjugated with anti-sense oligo targeting 5'tRF-GlyGCC.
RNA modification analysis of EV-RNAs: Quantitative analysis of RNA modification levels was performed by UHPLC-UniSpray-MS/MS using 10 ng of EV-RNA sample.
Results: UHPLC-MS/MS analysis identified tumor characteristic RNA modification landscape in tumor Te-EVs. Among the modifiers reported to contribute to TLR8 reactivity, m6A levels were decreased in tumor Te-EVs compared to normal Te-EVs. The 5'tRF-GlyGCC in tumor Te-EVs also showed a significant decrease in m6A content as well. The reduction of m6A levels in tumor Te-EVs by the recombinant protein ALKBH5, an m6A demethylase, promoted inflammatory cytokine production in macrophages.
Summary/Conclusion: The reduction of m6A modification level on 5'tRF-GlyGCC in tumor Te-EVs, was found to act in a tumor-promoting manner by modulating the reactivity of TLR8 in macrophages. To our knowledge, this is the first report showing that dysregulation of RNA modifications in EV-RNA functions as a tumor-promoting factor in colorectal cancer.

PS13: EV Production for Therapeutics

Location: Hall 4A

16:50 - 18:50

PS13.01=OWP1.09 | Multi-omic landscaping of mesenchymal stem cell-derived extracellular vesicles generated from xeno-free culture condition

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Introduction: Mesenchymal stem cell-derived extracellular vesicle (MSC-EVs) are a bright star in regenerative medicine. A growing body of evidence has demonstrated the effectiveness of MSC-EVs in animal models of renal disease and cardiovascular disease by virtue of their cargo. However, before clinical translation, there is a need to optimize MSC culture condition without any substrate of animal origin for EV harvest. In the present study, we aimed to identify the optimal xeno-free culture medium

for the clinical-grade production of MSC-EVs and employed multi-omic analysis for the molecular and functional profiling of these vesicles.
Methods: MSCs were isolated from human umbilical cords and cultured with xeno-free media until passages 6 (P6) to evaluate differentiation potential, cell doubling time, metabolic activity, apoptosis and cellular senescence. EVs were prepared from the culture supernatants by differential centrifugation (2000g, 4°C, 30min; 13500g, 4°C, 30min; 100000g, 4°C, 2h) and were further purified via size exclusion chromatography. The purified EVs were characterized by transmission electron microscopy, nanoparticle tracking analysis, NanoFCM and western blotting analysis of exosomal markers. MSC-EVs from four donors were subjected to multiomics analysis, including transcriptomics, proteomics, metabolomics and lipidomics.
Results: First, we analyzed the influence of different xeno-free media on biological properties of MSCs and found that human platelet lysate (HPL)-supplemented media support the isolation and long-term proliferation of MSCs. Compared to FBS-based media, HPL augmented proliferation and metabolic activity of MSCs while improving apoptosis and senescence, suggesting that HPL is an efficient alternative to FBS for MSC culture. Next, we purified the EVs from FBS or HPL-cultured MSCs and verified them according to the MISEV2018 guideline. Nonsignificant differences in protein markers, size, and morphology were found between these two vesicles. Interestingly, multiomics analysis clarified that differences in the composition of MSC-EVs under FBS and HPL culture conditions were mainly focused on proteins and miRNAs but not mRNAs, lncRNAs, cirRNAs, hydrophilic metabolites and lipids. However, more than 80% of differential miRNAs and 90% of differential proteins were not highly expressed in HPL-produced MSC-EVs, indicating that the therapeutic potential of MSC-EVs may not be affected by HPL. GO enrichment analysis of the highly expressed miRNAs showed they are associated with regeneration and development, immune regulation, and extracellular matrix composition. Moreover, principal component analysis revealed a similar molecular content of MSC-EVs from different donors.
Summary/Conclusion: HPL-based culture condition well-maintained the molecular composition and therapeutic potential of MSC-EVs and could serve as an efficient xeno-free alternative for the production of clinical-grade MSC-EVs.

PS14: EV Therapy

Location: Hall 4A

16:50 - 18:50

PS14.01=OWP2.03 | Extracellular vesicles from iPSC-derived hindbrain organoids in Alzheimer's disease

Cristina Zivko[1]; Olesia Gololobova[2]; Ram Sagar[3]; Ariadni Xydia[4]; Kenneth W. Witwer[5]; Constantine Lyketsos[4]; Vasiliki Machairaki[1]
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Introduction: Alzheimer's disease (AD) is a neurodegenerative disorder and the most common cause of dementia. Gradual deterioration of brain function eventually leads to death. Almost all AD patients eventually suffer from neuropsychiatric symptoms (NPS; e.g., agitation, depression) whose emergence correlates with dysfunctional serotonergic systems. Our aim is to generate hindbrain spheroids containing serotonergic neurons using induced Pluripotent Stem Cells (iPSCs) from healthy volunteers or AD patients with and without NPS. The spheroids can be used to study AD, NPS, and to evaluate patient individual differences in disease progression and response to pharmacologic therapies.
Methods: iPSCs were differentiated into hindbrain spheroids. The presence of serotonergic neurons was confirmed by quantitative RT-PCR, flow cytometry, and detection of serotonin in the extracellular environment. Extracellular vesicles (EVs) were isolated from cell culture supernatants by differential centrifugation followed by size exclusion chromatography. EV presence was confirmed by nanoflow cytometry measuring particle yield and size distribution. EVs were further characterized by single particle interferometric reflectance imaging to detect markers such as CD9 and CD81, as well as a neural cell adhesion molecule (NCAM). EV morphology was validated by transmission electron cryomicroscopy.
Results: Hindbrain spheroids containing serotonergic neurons were successfully generated from iPSCs of healthy volunteers (n = 3) and AD patients (n = 3). EVs were consistently isolated, purified and characterized from all samples.
Summary/Conclusion: It is possible to generate hindbrain serotonin neuron spheroids, and associated EVs, from iPSCs of individuals, laying foundation for a precision medicine approach to study AD, NPS, and to predict response to specific pharmacologic therapies.

PS02: Neurodegenerative Diseases and EVs 2

Location: Hall 4A

16:50 - 18:50

PS02.05 | Biomarker analysis in CNS-originating EVs improves the differential diagnosis of Parkinson's disease and multiple system atrophy

Hash Brown Taha[1]; Simon Hornung[2]; Suman Dutta[3]; Leony Fenwick[4]; Otmane Lahgui[5]; Karl E. Biggs[6]; Carter Lantz[7]; Kathryn Howe[3]; Nour Elabed[3]; Irish Del Rosario[8]; Darice Y. Wong[9]; Aline Duarte Folle[8]; Daniela Markovic[10]; Jose-Alberto Palma[11]; Un J. kang[12]; Roy N. Alcalay[13]; Miriam Sklerov[14]; Horacio Kaufmann[11]; Nadia Stefanova[15]; Brent L. Fogel[16]; Jeff M. M. Bronstein[17]; Joseph A. Loo[7]; Beate Ritz[18]; Gal Bitan[19]

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Introduction: CNS-originating EVs cross the blood-brain barrier and circulate in the blood thus providing a rich source of minimally invasive biomarkers for CNS disorders. Differential diagnosis of Parkinson's disease (PD) and multiple system atrophy (MSA) is challenging due to symptom overlap, especially in the early stages. Current diagnostic tests do not achieve high sensitivity or specificity. Because α-synuclein (α-syn) deposition occurs in neurons in PD and oligodendrocytes in MSA, we reasoned that comparing the concentration of α-syn in neuronal (nEVs) and oligodendroglial EVs (oEVs) could help distinguish between these diseases. Indeed, the total α-syn concentration together with the oEV:nEV α-syn ratio separated MSA from PD with high sensitivity and specificity. Here, we tested whether pS129-α-syn, a pathological form of α-syn, as well as tau, and neurofilament light (NfL) would improve the model's diagnostic accuracy.

Methods: CNS-originating EVs were isolated using a 2-step process from remaining samples in the cohort originally used for measurement of α-syn. EV enrichment was validated using TRPS, western blots, FACS analysis, and TEM. pS129-α-syn was measured using an in-house electrochemiluminescence ELISA (ECLIA). Tau and NfL were measured using commercial ECLIA kits. Multinomial logistic regression was used for ROC analyses.

Results: pS129-α-syn increased significantly in the order healthy control (HC) < PD < MSA in oEVs only. The addition of oEV-pS129-α-syn to the statistical model increased the separation between these groups. Total tau was significantly lower in nEVs and oEVs in MSA compared to HC and PD but did not increase the model's diagnostic accuracy. NfL was statistically significantly reduced in PD. The inclusion of α-syn in nEVs, oEV:nEV α-syn ratio, pS129-α-syn in oEVs, and total EV concentration into the prediction model achieved an accuracy of ~80% for separating PD from HC, ~99% for MSA and HC, and ~94% for PD and MSA.

Summary/Conclusion: A blood-based biomarker panel can achieve high separation of HC, PD, and MSA.

Funding: Multiple system atrophy coalition (20170367 & 2017-10-007), California Department of Public Health (18-10926), Michael J. Fox Foundation for Parkinson's Research (17990 & 18303), CurePSP (665-2019-07), National Ataxia Foundation (20201551), Cure Sanfilippo Foundation (20215318), Parkinson/Parkinson Alliance, The Alzheimer's Association, Weston Brain Institute & Alzheimer's Research UK Biomarker Across Neurodegenerative Diseases (BAND 3, 17990; PI Dr. GB) NIH/NIEHS (ES10544; PI Dr. BR) NIH (R35GM145286 & S10RR028893) & US Department of Energy (DE-FC02-02ER63421; PI Dr. JAL) Ruth L. Kirschtein National Research Service Award Program (GM007185; PI Dr. CL) NIH P50AG16573 & P30AG066519.

Keywords: synucleinopathies, parkinsonism, phosphorylated α-synuclein, tau, neurofilament light

PS02.06 | How different methodological approaches affect the characterization of brain tissue-derived extracellular vesicles and their association with proteopathic Tau seeds in Alzheimer's disease

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Introduction: Misfolding and aggregation of Tau into highly ordered amyloid filaments is characteristic of Alzheimer's disease (AD) and other tauopathies. Tau lesions progressively invade the brain via a prion-like mechanism of self-replication and spreading of diffusible Tau species ('seeds'). Extracellular vesicles (EVs) recently emerged as major vehicles for Tau dissemination (Leroux et al., Mol. Ther. 2021; Ruan et al., Brain 2021). Yet, the extent to which free or EV-associated Tau species contribute to the spatiotemporal spreading of Tau misfolding is unclear. To address these issues, we are using different methodological approaches to isolate and characterize free and vesicle-associated Tau seeds from post-mortem AD brain tissues.
Methods: Post-mortem human brain tissue samples from AD (Braak VI) and non-demented age-matched control subjects were obtained from the NeuroCEB brain bank, in accordance with French bioethics laws. After dissociating the tissues with collagenase, EVs and other extracellular particles were isolated either by size-exclusion chromatography, ultracentrifugation and iodixanol density gradients (Hurwitz et al., J Neurosci Methods 2018), or using an inertial-based microfluidic device (Tay et al., Lab Chip 2021). Extracellular fractions were analyzed by Western blot, nanoparticle tracking analysis, negative staining and immunogold transmission electron microscopy (TEM) and cryo-electron microscopy.
Results: Our preliminary observations suggest that free Tau aggregates frequently co-isolate with brain-derived EVs, which could lead to misinterpretations as to the roles the latter play in AD. We will present the results of our ongoing methodological comparisons at the meeting and discuss their implications.
Summary/Conclusion: We envision that characterizing the diversity of extracellular Tau seeds and their interactions with EVs, with components of the extracellular matrix and with neuronal and glial cells will allow for a deeper understanding of the mechanisms involved in AD progression.
Keywords: EV, tau, alzheimer's disease, prion-like propagation, amyloids, brain tissue

PS02.07 | Differential Pro-inflammatory phenotype of extracellular vesicle cargo amongst hispanic and african american subjects with alzheimer's disease

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Introduction: Detecting toxic proteins, including Aβ and p-tau species within neuronal (NEVs) and astrocyte extracellular vesicles (AEVs) has initiated an ongoing effort to assess the clinical utility of EVs as potential biomarkers for Alzheimer's Disease (AD) and other AD-related dementias (ADRDs). African Americans (AAs) and Hispanics are approximately 1.5 to 2 times more likely to develop AD and ADRD as compared to non-Hispanic Whites (NHW). Specifically, AAs are more likely to have higher levels of chronic stress, inflammation, and vascular related co-morbidities. Here, we assess the proinflammatory phenotype of EV cargo loads of different cellular origins for novel biomarker assessment amongst Hispanic and AA subjects with AD.
Methods: Plasma EVs derived from normal controls and AD subjects (NHW, Hispanics, AAs, n = 20/group) were extracted, precipitated using a polymer-based isolation method (ExoQuick), and enriched against neuronal (L1CAM), astrocyte (GLAST), and microglial (TMEM119) sources using magnetic immunocapture and fluorescence-activated cell sorting (FACS). ONI Nanoimager was used to characterize EVs by size and EV marker profiling was done via ELISA. Concentrations of proinflammatory cargo proteins were quantified using MSD immunoassay (V-PLEX Proinflammatory Panel 1). Patients were stratified based on race, cognitive status, sex, and APOE genotype.
Results: Neuronal and glial EVs were similar in size and distribution. Plasma concentrations of EV marker CD81 were not statistically different between the three EV preparations. Plasma concentrations of IL-12p70 were significantly increased in NEVs derived from demented NHW and Hispanics as compared to AAs. Increased plasma NEV and AEV concentrations of IL-12p70 were positively correlated with APOE4 carrier status. No difference was observed based on sex. No significant difference was observed in MEV concentrations of IL-12p70 based on race, cognitive status, sex, and APOE genotype.
Summary/Conclusion: Plasma NEV concentrations of IL-12p70 may serve as a novel biomarker for AD amongst AAs subjects with at least one ApoEe allele. The biomarker capabilities of additional proinflammatory-related EV cargo proteins will be investigated in a larger sample cohort of normal controls and AD subjects.
Funding: MOSAIC 1K99AG070390-01.
Keywords: extracellular vesicles, alzheimer's disease, biomarkers, inflammation, health disparities

PS02.08 | Intranasal hiPSC-NSC-EV treatment in a model of Alzheimer's disease restrains DAM- and NLRP3-p38 MAPK signaling-related gene expression in Microglia

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Introduction: Disease-associated microglia (DAM) and the associated NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome-mediated p38 mitogen-activated protein kinase (p38 MAPK) signaling contributes to chronic neuroinflammation in Alzheimer's disease (AD). Hence, biologics capable of easing the expression of genes linked to DAM, NLRP3-inflammasome signaling in microglia may restrain the unrelenting neuroinflammation in AD. This study examined the efficacy of intranasally (IN) administered extracellular vesicles (EVs), purified through chromatographic methods from cultures of human induced pluripotent stem cell (hiPSC)-derived neural stem cells (NSCs), for modulating proinflammatory microglia in the 5XFAD mice.
Methods: Mice received IN administration of hiPSC-NSC-EVs (200×109 EVs) or the vehicle (weekly for two weeks). Seventy-two hours later, a cohort of mice was euthanized, fresh microglia were isolated from the brain, and the expression of genes linked to DAM and NLRP3-inflammasome signaling was examined via scRNA-sequencing. Another cohort of mice was euthanized two months post-EV treatment to assess the long-term effects.
Results: Microglia from AD mice receiving hiPSC-NSC-EVs displayed altered transcriptomic profiles compared to AD mice receiving the vehicle. The changes comprised reduced expression of multiple genes linked to DAM, and NLRP3-inflammasome signaling, implying the ability of EVs to restrain neuroinflammation. Such antiinflammatory effects persisted at two months post-EV treatment, evidenced by reduced concentrations of both mediators and end products of NLRP3 inflammasomes. hiPSC-NSC-EV treated AD mice also showed diminished expression of genes and proteins involved in p38 MAPK hyperactivation, which resulted in reduced production of multiple proinflammatory cytokines in the AD brain.
Summary/Conclusion: IN administration of hiPSC-NSC-EVs is an efficient approach for easing DAM-mediated chronic neuroinflammation in AD.
Funding: Supported by a grant from the National Institute for Aging (1RF1AG074256-01A1 to A.K.S.).
Keywords: alzheimer's disease, neuroinflammation, neural stem cells, extracellular vesicles

PS03: EVs and Viral Infection

Location: Hall 4A

16:50 - 18:50

PS03.03 | Small extracellular vesicles (sEVs) from cortical neurons infected with zika virus (ZIKV) transport cargo elements that regulate mitochondrial function and axonal guidance

María Angélica Calderón-Peláez[1]; L. Johanna Madroñero[2]; Jaime E. Castellanos[2]; Myriam Lucía Velandia Romero[2]
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Introduction: Neurons are the main targets for ZIKV infection. This can change mitochondrial morpho-dynamics and induce apoptosis. However, it is unknown if sEVs transport elements that participate in these processes. This work aimed to characterize small EVs from ZIKV-infected neurons (sEVIN) and determine the miRs and proteins that might regulate mitochondrial function.
Methods: Neuron cultures from 1-day-old Balb/C mice purified by 2.5 μM AraC treatment were infected with ZIKV, MOI 0.1 for 1h, then, virus was removed, and cells were kept for 48h in neurobasal medium. Cell viability (Calcein-AM and LDH), and viral infection (IFI and PCR for DENV C protein) were measured. After 48h post-infection, EVs were isolated by ultracentrifugation and characterized by NTA, DLS, Western blot, LC/MS/MS, and small RNA sequencing. Protein files were analyzed,

and enrichment and functional annotation were done (Panther and GORilla). Small RNAs were extracted (mirVana kit) and sequenced. Clean reads were aligned to Mus musculus genome (GRCm39), annotated (miRBase database) and expression levels were estimated.
Results: Neuron infection did not affect cell viability but induced a high production of sEVs (4 × 10^8) that were slightly bigger than sEVs from non-infected neurons (sEVNIN). Protein analysis of sEVs from infected neurons (sEVIN) showed 65 overexpressed proteins regarding sEVNIN, 5 of which were exclusive, including the 14-3-3 protein previously reported as modulator of ZIKV replication. sEVIN preliminary analysis showed 538 total miRs, 11 involved in mitochondrial regulation such as mmu-miR-22-3p, 127, 181a/b-5p, and 25b. Importantly, 9 miRs has been reported as axonal guidance regulators, impacting mitochondrial localization and ATP availability. Additionally, in silico analysis showed 18 miRs that might regulate viral protein function
Summary/Conclusion: sEVs produced by ZIKV-infected neurons carry proteins and miRs that may regulate mitochondrial function and distribution through neurons. These findings could help to explain the aggressive neurodegenerative outcome caused by ZIKV.
Funding: Minciencias-UEB Grant 130884467149, code 431–2020. ISLA Ltda.: Doctoral research grant, 2022.
Keywords: ZIKV infection, neurons, sEVs, mitochondrial dynamics, axonal regulation

PS03.04 | Targeting vector-borne viral diseases with arthropod exosomes, a novel route for transmission blocking vaccines

Hameeda Sultana
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Introduction: Our laboratory is the first to show that arthropod exosomes from medically important vectors such as ticks and mosquitoes transmit pathogens to the vertebrate host. Our recently published studies, not only provides evidence to show that tick/mosquito-borne flaviviruses uses arthropod-derived exosomes for transmission from vector to the mammalian cells but also suggest that infectious exosomes from facilitates viral dissemination within the vertebrate host system.
Methods: Exosomes were isolated from tick and mosquito cells, tick saliva and salivary glands by using density gradient and differential ultracentrifugation methods. In addition to exosome isolation several different functional assays were performed.
Results: Overall, our studies have revealed that arthropod-derived exosomes are important means of communication between the vector and vertebrate host. We have shown that mosquito-borne viruses such as dengue, ZIKA, West Nile virus and tick-borne Langat virus are transmitted from vector to the vertebrate host through arthropod exosomes. These infectious exosomes containing full length viral RNA genomes, and proteins/polyproteins were viable, secured, and highly virulent in all tested conditions such as re-infection kinetics, trans-migration and viral plaque formation assays. We found the first mosquito exosomal enriched marker Tsp29Fb (human CD63 ortholog), that directly interacts with dengue viral envelope protein and facilitate virus transmission. We have recently identified novel tick exosomal cargo molecules in arthropod exosomes that mediates successful blood feeding and tick-borne virus transmission. In addition, our new findings about arthropod exosomal proteins and related mechanisms show their importance in modulation of human cytokine/chemokines at the pathogen-vector-host interface.
Summary/Conclusion: We envision that the transmission strategies used by flaviviruses to exit arthropods via infectious exosomes and to infect human and animal host are the best approaches to develop transmission-blocking vaccines against vector molecules or determinants that facilitate pathogen transmission. We strongly believe that this novel line of investigation is very important in understanding the potential interactions at the cross roads of virus-vector-host to block vector-borne diseases from ticks and mosquitoes.
Funding: This study is supported by funding from National Institute of Allergy and Infectious Diseases (NIAID)/National Institutes of Health (NIH) (Award number R01AI141790 and R01AI141790-05S1 to HS).
Keywords: arthropod exosomes, ticks, mosquitoes, flaviviruses, blood feeding, exosomal cargo, vector-host interactions, vertebrate skin interface, pathogen transmission, vector-borne diseases, transmission-blocking vaccines

PS03.05 | Presence of SARS-CoV-2 viral particles in extracellular vesicles isolated from plasma of patients with COVID-19

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Introduction: The extracellular vesicles (EVs) have been related with the transfer of viral components, contributing to the spread of the virus, such as HIV and HTLV. Our objective is to verify the presence of viral particles and to characterize the SARS-CoV-2 variants in EVs isolated from patients with COVID-19.
Methods: We selected plasma samples fro patients with COVID-19 (N = 20) who were admitted between 03/2021 and 08/2021 to Hospital Sepaco, São Paulo, and samples from healthy individuals (N = 5). We isolated EVs by UC at 100,000 x g for 16 hours and the size and concentrations (particles/mL) were perfomed by NTA. The presence of SARS-CoV-2 viral particles was verified in EVs using the RT-qPCR kit (GeneFinder Kit; OSANG Healthcare) targeting the genes RdRp,Envelope (E) and Nucleocapsid (N). To verify the variants in EVs we used the RT-qPCR molecular 4Plex SC2/VOC (Bio-Manguinhos), which allows the screening of variants Alpha, Beta, Gamma, Delta and Omicron. Positive result was considered Cycle threshold ≤ 40.
Results: The EVs of patients and controls had an average size of 183.7nm and 233.3nm, respectively. Patients with COVID-19 had a higher concentration of EVs (2.3e+10 particles/mL) than the healthy controls (4.0e+08 particles/mL) ($P < 0.05$). RT-qPCR analysis showed amplification in 14 samples of EVs from patients and none of the controls. Gene amplification was detected for RdRp (4 samples), E (5 samples) and N (11 samples) virus particles of SARS-CoV-2. Regarding the screening of variants by RT-qPCR, two samples were able to confirm the Gamma variant.
Summary/Conclusion: EVs can bring a new perspective as potential carriers of SARS-CoV-2 viral particles, contributing to its propagation, making healthy cells more susceptible to infection.
Funding: FAPESP, CAPES and CNPq.

PS03.06 | Extracellular vesicles released from human macrophages mediate anti-SARS-CoV-2 activities in infected monocytes through the modulation of the NF-κB signaling pathway

Luis Arteaga-Blanco[1]; Jairo Temerozo[2]; Lucas Tiné[2]; Rubem Menna-Barreto[2]; Patrícia Bozza[2]; Thiago Moreno[2]; Dumith Chequer Bou-Habib[2]
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Introduction: Infection by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been associated with leukopenia and uncontrolled inflammatory response in patients with severe disease. Evidence suggests a prominent role for circulating monocytes as one of the main sources of uncontrolled levels of the pro-inflammatory mediators in the respiratory tract that cause hyperinflammatory pathology and induction of ARDS in critically ill patients. Previous studies have demonstrated that extracellular vesicles (EVs) secreted by different cellular sources play important roles during SARS-CoV-2 infection, leading to both stimulatory and inhibitory activities. Up-to-date investigations describing the involvement of different EV subtypes LEV (large EVs) and SEV (small EVs) in the pathogenesis of SARS-CoV-2 are limited. In addition, information regarding the role of different EV populations released by human monocyte-derived macrophages (MDM) on the progression or regulation of SARS-CoV-2 infection are not available.
Methods: MDM from healthy donors were cultured in EV-free medium and then EV subtypes were isolated from culture supernatants by differential ultracentrifugation. EVs were characterized by NTA, SEM, and immunoblotting.
Results: Here, we show that MDM-derived EVs were able to inhibit SARS-CoV-2 RNA synthesis/replication in human monocytes. Both EV subtypes, LEV and SEV protected these cells from virus-induced cytopathic and reduced the production of pro-inflammatory mediators. We further demonstrate that MDM-derived EVs prevented the SARS-CoV-2-induced NF-kB activation in monocytes, which is critically involved in the production of inflammatory mediators.
Summary/Conclusion: These findings suggest that MDM-EVs are endowed with immunoregulatory properties that might contribute to the antiviral response against SARS-CoV-2-infected monocytes and expand our knowledge of the regulation and effects of extracellular vesicles during COVID-19 pathogenesis.
Keywords: extracellular vesicles, human macrophages, monocytes, innate immunity, SARS-CoV-2 and host–pathogen interaction

PS03.07 | Extracellular vesicles designed to decoy or compete with spike binding to the ACE2 receptor of SARS-CoV-2 highlight the diversity of Omicron

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Introduction: Extracellular vesicles share multiple mechanistic parallels to viruses including their biogenesis, cellular uptake and trafficking routes. This makes EVs an attractive agent for the development of antiviral applications. We investigated two strategies to interfere with uptake of different SARS-CoV-2 strains by engineered EVs: decoy of viral particles by EVs expressing the Angiotensin- converting- enzyme 2 (ACE2) receptor or competition with host cell receptor binding by EVs displaying the SARS-CoV-2 spike protein.
Methods: EVs were isolated from HEK293T cells expressing different variants of the Spike protein or the hACE2 receptor by ultrafiltration and size exclusion chromatography. EVs were analysed by NTA, Western Blotting, TEM, quantitative single vesicle imaging and super resolution microscopy, and tested for inhibition of viral entry in Vero and Calu-3 cell lines as well as in primary 3D human bronchial explants. The potency of the antiviral EVs was additionally benchmarked against the WHO standards using a pseudotype assay.
Results: Immunestaining with antibodies and sera from Covid positive patients confirmed expression and steric accessibility of the spike or ACE2 proteins on the EV surface. Cotreatment of cells with the SARS-COV2 virus and S-EVs or ACE2 EVs significantly reduced viral entry in all cellular models. While the inhibition was substantially higher with the decoy as compared to the competition strategy for the earlier mutations of the virus (D641G, alpha, beta, delta), the Ace2-EV but not S-EV activity was completely lost for the Omicron clade of SARS-CoV2 as well as SARS-Cov1.
Summary/Conclusion: Our data confirm that SARS-CoV2 infection can be inhibited by engineered EVs but suggests different modes of action in individual variants. This underlines the differential cell uptake route of Omicron which preferentially uses endosomal entry over plasma membrane fusion.
Funding: This work was supported by the following grants: EV-TT BPro (County of Salzburg, WISS2025, P1812596) and EVTT (European Union, EFRE/IWB 20102-F1900731-KZP) and CONSONANT (County of Salzburg, WISS2025, F2200397-KZP).
Keywords: SARS-COV-2 virus, antiviral acitivity, EV engineering, EV uptake

PS03.08 | Prolonged detection of SARS CoV2 RNA in extracellular vesicles present in respiratory tract of RT-PCR negative patients

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Introduction: The prolonged presence of SARS CoV-2 virus found in the respiratory tract especially in patients with underlying co-morbidities. We aim to investigate the presence of virus inside Extracellular vesicles (EV) in patients with active and recovered COVID-19 infection, also with underlying chronic liver diseases.
Methods: SARS CoV2 RT-PCR positive n = 78 {n = 24(66.6%) chronic liver disease (CLD); n = 52 (81.3%) non-liver disease} were studied. SARS CoV2 patients were also followed up on day (d) 7, 14 and 28 posts RT-PCR positivity. EVs were isolated using differential ultracentrifugation from both plasma and respiratory tract followed by detection of SARS CoV2 RNA Transmission was assessed in Vero cells.
Results: In baseline RT-PCR positive patients, SARS-CoV2 RNA inside the EV was present in 64/74 (82%) patients with comparable viral load between nasopharyngeal swab (NP) and EV (mean 1CT – 0.033 ± 0.005 vs. 1CT – 0.029 ± 0.014, p = ns). The sequencing data shows 92–95% similarity in SARS CoV2 in nasal swab and EV isolated SARS Cov2 RNA. On follow-up at day 7, of the 24 patients negative for COVID19, 10 (41%) had persistence of virus in the EV (1CT – 0.028 ± 0.004) and on day 14, 14 of 40 (35%) negative RT-PCR had EVs with SARS CoV2 RNA (1CT – 0.028 ± 0.06). The mean viral load decreased at day7 and day14 in NP from baseline (p = 0.001) but not in EV. SARS-CoV2 RNA otherwise undetectable in plasma was found to be positive in EV in 12.5% of COVID19 positive patients. Interestingly, significantly prolonged and high viral load was found in EV at day 14 in CLD-COVID19 patients compared to COVID19 alone (p = 0.002).
Summary/Conclusion: Identification of SARS-CoV2 RNA in EV, in RT-PCR negative patients indicates persistence of infection for and likely recurrence of the infection. EV associated RNA might play a role in the clinical course of the disease especially in patients with underlying co-morbidities like CLD.
Funding: This project was funded by SERB Power.
Keywords: SARS CoV2, extracellular vesicles

PS04: Cardiovascular EVs 2

Location: Hall 4A

16:50 - 18:50

PS04.04 | Scaling-up the production of human induced pluripotent stem cell-derived extracellular vesicles for next generation heart repair therapeutics

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Introduction: Human-induced pluripotent stem cells (hiPSC) are a promising source of native EV for cardiac regeneration, providing advantages over stem-cell based therapies for repairing adult cardiomyocytes with limited self-renewal after injury. However, large-scale manufacture of clinical grade hiPSC-EV remains a bottleneck for clinical translation. In this study, we developed a scalable bioprocess for hiPSC-EV mass production in stirred-tank bioreactors (STB).
Methods: Briefly, hiPSC were expanded as monolayers in static adherent cultures or as 3D aggregates in STB (200 mL working volume), operated in perfusion (D = 1.3 day-1) with low levels of dissolved O2 (4% O2). EV were isolated from conditioned culture medium by density gradient ultracentrifugation. We investigated the size, morphology, and protein content of EV using electron microscopy, nanoparticle tracking analysis, and western blotting. EV bioactivity was assessed in endothelial cells and hiPSC-derived cardiac fibroblasts. The EV's functional cargo (miRNAs) was assessed using RT-qPCR.
Results: Our results demonstrated a 2.9-fold increase in cell concentration in STB compared to the static culture, which resulted in a 3.1 increase in total particles isolated. hiPSC-EV produced in the STB presented a cup-shaped morphology and were positive for EV markers. Tube formation assays showed increased pro-angiogenic activity for hiPSC-EV produced in STB versus static 2D monolayer culture, and a different miRNA content was found between these EV.
Summary/Conclusion: Overall, our study validated the STB system for large-scale EV production and showed the impact of cell culture on EV composition. Ongoing work aims at scaling the current process for 2 L bioreactors to accelerate clinical development even more.
Funding: FCT PhD fellowships (SFRH/BD/145767/2019; UI/BD/151255/2021); CARDIOPATCH Interreg SUDOE (SOE4/P1/E1063); BRAV3 (H2020-SC1-BHC-874827); iNOVA4Health (UIDB/04462/2020; UIDP/04462/2020); LS4FUTURE (LA/P/0087/2020).

PS04.05 | Genetically engineered vesicles fused with pueraria-derived vesicles for the treatment of ischemia-reperfusion injury in heart transplantation

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Introduction: Heart transplantation is the ultimate and effective treatment for end-stage heart failure or severe coronary heart disease. However, ischemia-reperfusion injury will inevitably occur in donor hearts during transplantation, which seriously affects the postoperative survival rate of heart transplantation.Plant derived vesicle-like nanoparticles from most edible plants have been shown to have no detectable toxicity or immunogenicity. In recent years, some studies have proved that PDVLNs participate in the pathophysiological processes of plant cells themselves, and can resist infection by pathogenic microorganisms, and have the function of protecting plant cell tissues.
Methods: Western Blotting Cells were lysed with radioimmunoprecipitation assay buffer (RIPA) lysis buffer (Thermo Scientific). Cell lysates and purified membrane vesicles were loaded for 10% SDS-PAGE. Primary antibodies for GFP, β-actin,were detected by horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies with an ECL kit (Protein Tech, China).
Quantitative Real-Time PCR
qPCR was performed to determine the expression of genes.
Results: In this study, through small RNA sequencing, we found that different sizes vesicles derived from pueraria may have different effects. Secondly, we found that pueraria-derived vesicles(PNV)have antioxidant stress and anti-inflammatory effects, which can affect macrophage polarization. To be able to enhance its targeting ability and influence macrophage polarization, we

designed a genetically engineered macrophage that overexpresses CSF1(CSF1-RAW NV). We fused pueraria derived vesicles-like nanoparicles with genetically engineered macrophage-derived nanovesicles (Fusion EV) to enhance its ability to target the heart. In vivo, fusion vesicles can reduce I/R injury of transplanted hearts, reduce the expression of related apoptotic proteins, have anti-inflammatory effects, and inhibit the occurrence of acute early immune rejection.

Summary/Conclusion: We screened out one plant-derived vesicle, using the pueraria derived vesicles-like nanoparicles itself rich in active ingredients and MiRNA, etc. To fuse it with genetically engineered macrophage nanovesicle (Fusion EV) to produce a targeted effect, affect macrophage polarization and anti-inflammatory effects.It can successfully reduce the oxidative stress response of cardiomyocytes, inhibit apoptosis of cardiomyocytes, and reduce the expression of Caspase3 and Bax, and reduce reperfusion injury in ischemic transplantation, and the occurrence of early acute immune rejection.

PS04.08 | A Proteo-Transcriptomic analysis of extracellular vesicles derived from patients with hypoplastic left heart syndrome

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Introduction: Hypoplastic left heart syndrome (HLHS) is a severe congenital heart disease with limited treatment options. Newborns with HLHS typically undergo palliative surgery, but complications arising after the procedure have kept postsurgical long-term mortality rates significantly high. Cardiac cell therapy using stem or progenitor cells is an emerging treatment therapy used to address these complications. The mechanism of action for their reparative benefits, however, have yet to be thoroughly studied. The field has increasingly grown to recognize the imperative role of small extracellular vesicles (sEVs) in promoting these benefits. We have previously shown that sEVs from c-kit+ cardiac-derived progenitor cells (CPCs) derived from patients with congenital heart disease house miRNA cargo that are cardioprotective. These studies, however, analyzed sEVs derived from patients with various congenital heart diseases making it difficult to elucidate any specific factors unique to sEVs from HLHS patients. Here, we seek to bridge this gap via a proteo-transcriptomic analysis of sEVs derived from HLHS patients.

Methods: sEV characterization was done following MISEV2018 guidelines. We performed a holistic proteomic and transcriptomic analysis of HLHS CPC-sEVs through LC-MS/MS and total RNA-sequencing. Raw mass-spectrometry files were analyzed using MaxQuant v2.1.3.0 and searched against a Uniprot human database. Quantitation of proteins was performed using LFQ intensities given by MaxQuant. miRNA and RNA reads were aligned to the hg19 reference genome using the STAR aligner tool. Differential enrichment and gene analysis were performed between HLHS CPCs and their respective sEVs using the edgeR and DEP R packages. Multi-omic analysis was done using a multiple co-inertia analysis in R.

Results: Our proteomic analysis reveals an enrichment of angiogenesis-related proteins in HLHS CPC-EVs in comparison to their parent cells with a specific focus in cell-cell junction proteins and extracellular matrix proteins like CSPG2, MMP1, GDN, PTX3, and TSP2. Furthermore, our transcriptomic analysis reveals an enrichment of cardioprotective miRNAs and RNA transcripts affecting RNA translation, angiogenesis, and VEGFA-VEGFR2 and MAPK signaling pathways. Finally, our multi-omic analysis reveals a unique clustering of HLHS sEVs when compared to sEVs derived from patients with other congenital heart diseases (CHD) and mesenchymal stem cells suggesting the formation of unique HLHS sEVs.

Summary/Conclusion: Our current results reinforce the critical role sEVs play in maintaining the reparative benefits found in CPCs and, more generally, other stem cells. We have confirmed that sEVs derived from CPCs house both proteins and miRNAs that are essential to promote angiogenesis, proliferation, and differentiation. Furthermore, our multi-omic analysis suggests that sEVs originating from different CHDs have unique compositions.

PS04.10 | Cardiac extracellular vesicles regulate brain inflammation in heart failure

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Introduction: Although the incidence of heart failure (HF) has declined due to significant advances in treatment and management, increasing evidence shows that cognitive impairment (CI) and central abnormalities are prevalent and affect 35–65% of patients with HF. Neuroinflammation is a common feature in central nervous system disease and is increasingly recognized as a potential mediator of CI. The potential role of extracellular vesicles (EVs) as effective communicators of biological signaling in myocardial dysfunction has been investigated, underscoring EV's significance in cardiac pathophysiology. However, it is still unclear if cardiac EVs mediate cognitive deficits by regulating neuroinflammation in HF.

Methods: EVs were separated and characterized by serial differential ultracentrifugation, NanoSight, TEM and western blotting analyses, respectively following the guidelines described in MISEV2018. In vitro cell cultures, miRNA transfection and qRT-PCR analysis were used; A rodent model of post-MI HF generated by left coronary artery ligation at 6 weeks.
Results: Using a cardiac-specific GFP+ Tg mouse model, we demonstrate the brain distribution and glial uptake of cardiac EVs. We identified miR-21-5p, which was selectively upregulated in cardiac cells under cardiac stress, and then secreted out of cells by EVs, and confirmed that miR-21-5p was enriched in myocardial, in circulating and brain-isolated EVs derived from HF compared to Sham, and that miR-21-5p-enriched circulating EVs or miR-21-5p overexpression in vitro caused pro-inflammatory response in microglia.
Summary/Conclusion: Under cardiac stresses, cardiac-secreted EVs abundant with miRNAs communicate with the brain and are associated with microglial activation, which may be responsible for neuroinflammation and neurotoxicity in the HF state.
Funding: This work was supported by the National Institution of Health Grant R01HL153176 to IHZ/CT; American Heart Association (AHA) Career Development Award (19CDA34520004) to CT.
Keywords: extracellular vesicles, microRNAs, cognitive impairment, heart failure

PS05: EV Cargo Characterization II

Location: Hall 4A

16:50 - 18:50

PS05.02 | Selection of extracellular vesicles-specific DNA aptamers based on immunomagnetic beads and its application in the rapid and simple detection of Lung Cancer

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Introduction: Extracellular vesicles (EVs) are cell-derived vesicles showing various proteins on their membrane surfaces and they have the potential to provide novel biomarkers to diagnose cancer. Aptamers are short single stranded nucleic acid molecules which can bind to the target. At present, there are few reports on direct aptamer screening with EVs as target.
Methods: Here, we applied a newly EVs-SELEX (Systematic Evolution of Ligands by Exponential Enrichment) method to select aptamers against A549-EVs based on immunomagnetic beads.
Results: Through multiple rounds of screening, we have identified one aptamer (Ap3) that can bind to A549-EVs with high affinity and specificity. The Kd of Ap3 is 2.59 ± 3.42 nM. Under different temperatures (4°C, 25°C and 37°C), Ap3 can bind to A549-EVs with the similar affinity.
Then, the detection efficiency of the aptamer for lung cancer (LC) EVs was evaluated by quantitative polymerase chain reaction (qPCR). The detection limitation was 1.6 × 105 particles/mL and a linear range was 1.6 × 105-1.63 × 1011 particles/mL.
Summary/Conclusion: The method would be used in clinic for early diagnosis.
Funding: This study was supported by the National Natural Science Foundation of China (61971216 and 82002242), the Key Research and Development Project of Jiangsu Province (BE2022692 and BE2020768), Nanjing Important Science & Technology Specific Projects (2021-11005), Nanjing Science and Technology Development Plan Project (202205066). The Fellowship of China Postdoctoral Science Foundation (2022M711580).
Keywords: lung cancer, extracellular vesicles, exosomes, aptamer, SELEX, qPCR

PS05.03 | Silicon microchip and electrostatic labelling-based electrical assay for profiling of extracellular vesicles and subpopulations of relevance for cancer

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Introduction: The heterogeneity of extracellular vesicles (EVs) in terms of their of surface proteins means that their immunocapture may result in a bias towards EVs with high expression of the target protein. This may affect the protein profiles of the EVs measured by surface-based bulk sensors. However, non-immunocapture methods need labelling which is mostly limited to fluorescence-based methods. Besides, measuring the relative abundance of EV-subpopulations is becoming vital even for bulk analyses. Here we show a microchip-based electrical method that can analyze EV surface proteins related to lung cancer and EV subpopulations without requiring immunocapture.
Methods: Cell culture medium of non-small cell lung cancer (NSCLC) cells H1975 was used as a source of EVs. The EVs were isolated by size exclusion chromatography (on 70 nm qEVoriginal columns) and characterized for size and amount by nanoparticle tracking analysis and for EV surface CD9 and TSG101 expression by western blot. Streaming current was used for sensing and surface protein analysis of EVs. Capturing was done by either antibodies (biased) or non-specifically (unbiased), followed by surface protein analysis using antibodies conjugated to charge labels. Antibodies targeting CD9, EGFR and CD73 were used. These labels comprised positively charged poly-L-lysine peptides or negatively charged DNA. Single-EV fluorescence microscopy was used to verify the results.
Results: Clear differences in the surface protein profiles were observed when the EVs were captured biasedly and unbiasedly. Biased capture led to higher expression level of the target protein, but the EVs were fewer in number vs. unbiased capture. Further, the possibility to perform correlation analysis between the surface proteins, and carry out sequential profiling of up to three such proteins (CD9, EGFR and CD73) on the captured EVs using the method was also shown.
Summary/Conclusion: Using a combination of single and bulk EV analyses, we show that immunocapture may render bulk analysis less reliable. We then developed an electrical approach for unbiased analysis of EV surface proteins and various EV subpopulations of relevance for lung cancer.
Funding: Erling Persson Family Foundation, Swedish Research Council and Swedish- and Stockholm Cancer Societies, Stockholm County Council.
Keywords: streaming current, charge label, electrical sensor

PS05.04 | Advanced nanoplasmonic technologies multiplexed single EV analysis

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Introduction: Recent studies have suggested the importance of single extracellular vesicle (EV) profiling for early cancer detection, accurate quantification of cell-specific EVs and their cargos for longitudinal monitoring, and better understanding of EV subpopulations and heterogeneity. However, multiplexed molecular characterization of single EVs is technically challenging, mainly due to weak optical signals from EVs in small sizes. My lab has developed advanced nanoplasmonic technologies for reliable and robust multiplexed single EV analysis powered by novel nanostructures and plasmon-enhanced signal amplification.
Methods: We have designed plasmonic nanostructures that exhibit strong resonances and thus significantly amplify EVs' weak optical signals. The nanoplasmonic sensor chips were fabricated on a wafer scale for low-cost, high-throughput chip production. We have characterized and quantified signal amplification of fluorescently labeled EVs and particles in four fluorescence channels. We compared the system's performance with the existing standard EV analysis methods. Finally, we tested the system for cancer detection and longitudinal treatment monitoring using EVs from cell lines and human clinical samples. EVs were isolated by size-exclusion chromatography and characterized according to MISEV2018 guidelines, including nanoparticle analysis tracking, electron micrographs, and western blot analysis.
Results: We demonstrated robust signal enhancements in multiple optical channels. This allows us to significantly improve the detection sensitivity (x1,000) by capturing EVs on our plasmonic sensor chip compared to bead-flow cytometry. More importantly, we could quantify total EVs, cell-specific or marker-specific subpopulations, and protein and RNAs at a single EV level for cancer detection and monitoring. Using the single EV sensing platform, we demonstrated the sensitive detection of ovarian cancer and cholangiocarcinoma from EV analysis in human clinical samples and the potential of EV analysis to evaluate patients' responses to therapy from longitudinal tumor-derived EV quantification.
Summary/Conclusion: The simple, robust, and sensitive multiplexed single EV assay could improve our understanding of EV biology and accelerate clinical translation for cancers and other diseases
Funding: NIH R01GM138779 and R21CA217662.
Keywords: nanoplasmonics, sensing, multiplexing, technology, cancer

PS05.05 | Timegate Raman for monitoring the downstream process of extracellular vesicles purification

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Introduction: The potential clinical use of extracellular vesicles (EVs) will require large scale EV production. For this purpose, a rapid method is needed to assess the EV quality and monitor the purification process. Raman spectroscopy is an excellent candidate for this purpose, as it is able to rapidly evaluate the quality of EV preparations and can be implemented as an efficient, automated process parameter control. Here, we propose the use of a time-gated Raman spectrometer to effectively monitor the downstream chromatography-based process for platelet derived EV purification.
Methods: EVs were purified by ÄKTA Pure 25 Chromatography system (Cytiva) equipped with UV sensor (280nm) and CIM-multus QA 1ml 6 um monolithic Ion exchange chromatography column. Online Raman measurements were performed by timegate Raman spectrometer with ProbePro mini (Timegate Instruments Oy), with a 532 nm pulsed laser. Spectra were recorded continuously in 30 blocks. Raman probe was connected to the chromatography system via ViewCell (Schott, Germany). EV samples were produced from donated platelets provided by Finnish Red Cross Blood Service.
Results: Ion exchange chromatogram shows two main UV absorption peaks: the first, immediately after the sample injection, is associate to the material that does not bind to the ion exchange column. The second peak occurs right after the switch to high salt eluent, and it is associated to the material which interacts with the column via ionic interactions. The two peaks are separated by a washing phase in which no UV absorption is observed. Raman spectra recorded throughout the run, and a principal component analysis on these data revealed three major clusters associated to spectra recorded during the elution of the first and second peak, and the latent phase between the two. The feature of the spectra in these clusters can be associated respectively to non-EV material (first peak), platelet derived EVs (second peak) and running buffer (washing phase in between the peaks).
These result where confirmed also by the level of EV biomarker assessed by western blot.
Summary/Conclusion: Results here presented, indicate that the downstream process of EV purification can be effectively monitored by time-gated Raman spectroscopy. Raman data add considerable value to the UV absorption chromatogram since Raman data are more effective than UV in revealing the biochemical features of the analyte.
Funding: Timegate Instrumet Oy, Finnish Red Cross Blood Service.
Keywords: timegate raman, large scale EV purification

PS05.06 | Raman spectral signatures of plasma-derived extracellular vesicle-enriched isolates support the diagnosis of different cancerous diseases

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Introduction: Spectroscopic analysis of the molecular composition of small extracellular vesicles (sEVs) is a promising but underexplored method for diagnosing cancerous diseases, particularly central nervous system tumors. Using a sufficient number of clinical samples and Raman spectroscopic analyses, we attempt to elucidate the potential role of plasma-derived sEVs in diagnosing seven distinct patient groups.
Methods: The study is conducted in accordance with the Declaration of Helsinki, informed consent forms are collected and the study was approved by national ethics committee. Up to 490 plasma samples will be obtained from seven patient groups (glioblastoma multiforme, meningioma, melanoma and non-melanoma brain metastasis, colorectal tumors, melanoma and a control group). SEV isolation is performed through differential centrifugation. The isolates are characterized by Western Blot, transmission electron microscopy and nanoparticle tracking analysis. Principal Component Analysis–Support Vector Machine algorithm is performed on the Raman spectra for classifications. Classification accuracy, sensitivity, specificity and the Area Under the Curve (AUC) value are used to evaluate the performance of classification.

Results: According to preliminary results, the patient groups are distinguishable with 80–95% sensitivity and 80–90% specificity. AUC scores of 0.82–0.9 suggest excellent classification performance.
Summary/Conclusion: Our findings indicate that Raman spectroscopic analysis of sEV-enriched plasma isolates is a promising strategy for the development of noninvasive, cost-effective methods for the clinical diagnosis of various cancers.
Funding: GINOP-2.2.1–15-2017–00052; ÚNKP-22-3-New National Excellence Program of the Ministry for Innovation and Technology from the source of the National Research, Development and Innovation Fund; University of Szeged, Faculty of Medicine, Szent-Györgyi Albert Research Fund (2021).

PS05.07 | Single extracellular vesicle nanoscopy

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Introduction: Extracellular vesicles (EVs) and their cargo constitute novel biomarkers. However, a significant current roadblock in EV-based diagnostics is the lack of techniques with a wide dynamic range to visualize and rigorously quantify individual EVs within specific EV subpopulations.
Methods: We combined affinity isolation with super-resolution imaging to comprehensively assess individual EVs from human plasma. The analytical protocol was cost-effective and could be easily customized. Our Single Extracellular VEsicle Nanoscopy (SEVEN) assay could be readily used to assess distinct EV subpopulations. In addition to quantifying the number of isolated EVs, SEVEN provided the size, shape, molecular content of specific markers, and overall heterogeneity of EV subpopulations.
Results: We first assessed EVs enriched in abundant tetraspanins (CD9, CD81, CD63) isolated by size-exclusion chromatography from pooled human plasma; the number of detected tetraspanin-enriched EVs positively correlated with sample dilution in a 64-fold range. We further characterized the size, shape, and molecular tetraspanin content (with corresponding heterogeneities) for CD9-, CD63-, and CD81-enriched EVs. Importantly, when SEVEN was directly applied to crude plasma samples, it robustly detected EVs from ~0.1 uL of plasma. Finally, we assessed EVs from the plasma of four pancreatic ductal adenocarcinoma patients with resectable disease. Compared to healthy plasma, their CD9-enriched EVs were smaller with higher curvature; while their IGF1R-enriched EVs were larger, rounder, and contained more tetraspanin molecules. The results suggest a unique pancreatic cancer-enriched EV subpopulation.
Summary/Conclusion: This study provides proof-of-concept for advancing SEVEN into a platform to characterize disease-associated and organ-associated EV subpopulations. We anticipate SEVEN could be implemented in a wide array of different biological contexts since super-resolution imaging is becoming more widely available to the EV research community.
Funding: National Institutes of Health grant UG3/UH3 TR002878; Dorrance Family Research Fund; Board of Governors of the City of Hope, Southwest Food Industries Circle, Circle 1500, Dancing with Chicago Celebrities, Bruce & Lyn Everette, Irell and Manella Graduate School of Biological Sciences at City of Hope. Research reported in this publication included work performed in the City of Hope Analytical Cytometry core supported by the National Cancer Institute of the National Institutes of Health under grant number P30CA033572. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
Keywords: nanoscopy, imaging

PS05.08 | Biochemical characterization of salivary extracellular vesicles by Raman spectroscopy

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Introduction: Saliva is an interesting complex and easily available liquid biopsy and its use in diagnostics is fast increasing as well as the identification of specific salivary biomarkers for several disorders, spanning from neurodegenerative to cancer diseases. Extracellular Vesicles (EVs) are known to be present in saliva, although their low concentration has limited their use in clinics despite the remarkable potentialities. In the present study, we compare the biochemical profile of salivary and blood-derived vesicles to investigate the use of saliva as a valuable source of EVs that could be studied as biomarkers in an easily accessible biofluid.

Methods: EVs were isolated from serum and saliva of 5 healthy volunteers with the same combined protocol that includes 2 steps: size exclusion chromatography and ultracentrifugation. Nanoparticle tracking analysis, western blot and CONAN test were performed for the physico chemical characterization of the obtained EV preparation. Later on, samples were used for the biochemical profiling using a Raman microspectrometer equipped with 532 nm laser.
Results: Using a comparable protocol for the isolation of EVs from both liquid biopsies, salivary EVs showed greater purity in terms of co-isolates (evaluated by nanoparticle tracking analysis and CONAN test). Besides, the use of Raman spectroscopy was not hampered by the limited yield of EVs isolated from saliva, on the contrary we were able to provide a comprehensive characterization of EVs, in a high throughput and repeatable manner. The obtained molecular fingerprint together with the calculated protein-to-lipid and nucleic acid-to-protein ratios allowed us to hypothesize differences in the content of vesicles.
Summary/Conclusion: Raman spectroscopy can represent a turning point in the application of salivary EVs in clinics, taking advantage of the simple method of collection of the liquid biopsy and of the quick, sensitive and label-free biophotonics-based approach.
Funding: This research was funded by the Italian Ministry of Health, Ricerca Corrente 2020–2022.
Keywords: raman spectroscopy, saliva, biophotonics, molecular characterization, biomarkers, liquid biopsies

PS05.09 | The first and direct lipid profile analysis of extracellular vesicles and parental cells under hyper- and normoglycemic conditions using ToF-SIMS

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Introduction: ToF-SIMS (Time of Flight - Secondary Ion Mass Spectrometry) is a non-destructive and direct technique for the analysis of molecules with a mass m < 1000 Da, e.g. lipids. Hyperglycemia (HG) is a state of increased glucose concentration. HG causes metabolic changes in endothelial and β cells and modulates the release of extracellular vesicles (EVs). Here, the aim was to find HG-induced changes in lipid profiles of EVs and cells using ToF-SIMS.
Methods: Immortalized microvascular endothelial cells (TIME) and pancreatic β-cells were cultured under normoglycemic and HG conditions (11 mM and 35 mM of D-glucose). Low-pressure filtration dialysis and ultracentrifugation were used to separate exosomes and ectosomes. TOF-SIMS equipped with the Bi3+ was used, the analysis was performed using a library of characteristic peaks of individual lipid groups.
Results: We compared the levels of fatty acids, phosphatidylcholines, glycosphingolipids, sphingolipids, glycolipids, prenols, and sterols. Significant differences in intensities of characteristic peaks of individual lipids between different EV subpopulations and parental cells were revealed. Experiments showed the enrichment of EVs in specific lipids relative to parental cells. In addition, we showed significant increases in some lipids and decrease in others as HG results.
Summary/Conclusion: Based on our ToF-SIMS data, we can assume that EV subpopulations derived from β-cell and endothelial cell cultures are characterized by changes in the lipid profile as well as relative to parental cells. The external HG environment has a significant impact on the cell lipids and the EV membrane composition.
Funding: The study was funded by "Laboratories of the Young" as part of the "Excellence Initiative – Research University" program at the Jagiellonian University in Kraków. This work was supported by the National Science Center (NCN), grant OPUS 17 to prof. E. Stępień (No. 2019/33/B/NZ3/01004).
Keywords: ToF-SIMS, lipids, EVs, endothelial cells, β-cell

PS05.10 | Capturing and subtyping of extracellular vesicles from plasma using microfluidic magnetic levitation

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Introduction: Extracellular vesicles, in the size of 30–150 nm, can carry molecules including DNA, RNA, and metabolites, which are essential for cell-to-cell communication. Tumor cells are thought to secrete extracellular vesicles into bodily fluids such as plasma to facilitate angiogenesis and metastasis, to make space for migration and proliferation while deactivating tumor suppressors. However, their size at the nanoscale makes the isolation and subtyping extremely challenging. A simple method of isolating and characterizing extracellular vesicles based on protein markers such as CD9, CD63, CD81 would facilitate the research on chemoresistance, metastasis, and disease progression.

Methods: Here, we introduced Exo-Lev, an extracellular vesicle isolation and subtyping method using microfluidic magnetic levitation. In ExoLev, polymer beads of distinct densities, i.e.,1.05 and 1.18 g/mL, act as selective capture surfaces for different sub-types of extracellular vesicles. For this, the beads are first decorated with either anti-CD63 or anti-CD81 antibodies and incubated with filtered plasma samples. Second, the beads mixed with paramagnetic media are fed through the inlet and levitate to a specific equilibrium height within the microfluidic channel of the Exo-Lev. Then, the beads are collected at either top or bottom outlets of the Exo-Lev depending on their density. Third, captured extracellular vesicles are eluted from the collected beads and subjected to downstream analyses.
Results: Beads flowed into the correct output channel with a 100% purity when a flow rate of 7.5 mL/h was applied. According to the Nanoparticle Tracking Analysis (NTA), the size of CD63-positive vesicles captured by Exo-LEV were 59.3 ± 4.7 nm and the size of CD81-positive vesicles captured by Exo-LEV was 86.5 ± 14.0 nm. Total RNA extraction showed that these particles contained biological material (ranging from 0.088 to 0.11 ng/μL).
Summary/Conclusion: In this work, we developed a rapid, cost-effective, and straightforward method to capture and subtype extracellular vesicles from filtered plasma. We anticipate that the ExoLev protocol can be applied to isolate any other subtypes of extracellular vesicles from whole plasma.
Funding: We thank Stanford Department of Radiology for funding our work.

PS05.11 | Novel approaches to acoustic immunosensing of extracellular vesicles

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Introduction: Quartz crystal microbalance with dissipation monitoring (QCM-D) has recently emerged as a powerful alternative for the phenotypic detection of EVs, offering multiple modes of analyte discrimination by frequency and dissipation. In this talk, I will present current activities in my group towards effective interfacing of QCM-D-based approaches for the immunosensing of EVs, including the use of additional electrochemical read-out via impedance spectroscopy (eQCM-D) and nanostructuring the biosensor surface to mirror lateral analyte feature sizes.
Methods: Extracellular vesicles were obtained from human umbilical cord mesenchymal stem cell culture media and isolated using filtration, concentration by centrifugation and size-exclusion chromatography. SEC fractions were assessed by nanoparticle tracking analysis, protein content analysis, western blot analysis and gold immuno-electron microscopy. All QCM-D measurements were carried out using a Q-Sense E4 instrument (Biolin Scientific). For EQCM-D measurements, an electrochemistry module was deployed in tandem with a potentiostat and a three-electrode system. Nanostructuring of the sensor surface was conducted via diblock copolymer self-assembly of polystyrene-block-poly(4-vinylpyridine). Sensor functionalization was carried out using a mixture of SH-PEG (2 kDa)-Biotin and spacer molecule SH-OEG (800 Da)-COOH at a 1:9 mol/mol ratio, followed by exposure to streptavidin (SAv) and immobilization of mouse monoclonal biotinylated anti-CD63 antibodies.
Results: When comparing the various strategies, we found (A) a lowering of the detection limit by a factor of 2–4 when combining QCM-D in tandem with in-situ electrochemical impedance spectroscopy; (B) a higher degree of binding on nanostructured gold islands over flat surfaces, (C) a higher degree of binding when the nanostructured gold islands were dispersed on silica rather than on flat gold, and (D) a higher degree of binding when the nanostructured features were matched to the lateral dimensions of the EVs.
Summary/Conclusion: In summary, we have investigated a range of strategies to enhance the capabilities of acoustic immunosensing of extracellular vesicles, including multimodal read-out using changes in frequency, dissipation and electrochemical properties as well as nanostructuring the biosensor surface. Meanwhile, a limit of detection around 10^7 EV-sized particles / ml can be routinely achieved. Crucially, this analytical platform provides novel opportunities to aid sensor development (e.g. when validating surface functionalization, biomarker recognition or calibrating alternative read-out mechanisms) as well as for quality control.
Funding: We are grateful to EPSRC (EP/L01646X, EP/R035105/1) and MRC (MR/R000328/1).
Keywords: QCM-D, immunosensor, biosensing, electrochemistry, nanofabrication

PS05.12 | Timegate Raman as tool for extracellular vesicles characterization

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Introduction: Raman spectroscopy (RS) is a fast and non-destructive method able to reveal the biochemical composition of complex samples. RS is gaining interest in the extracellular vesicle (EV) field as a quality control tool. Here, we demonstrate the potential application of a timegated (TG) Raman spectrometer in EV characterization. TG spectrometers, in contrast to the traditional ones, suppress fluorescence interferences, thus, improving the signal-to-noise ratio and enabling the characterization of EVs labelled with fluorophores such as green fluorescent protein (GFP). Here, we highlight the capacity of TG-Raman to discriminate between EV preparations with different degrees of purity and between EV obtained from healthy cells and cells infected by adeno-associated viruses. Additionally, we show that TG Raman is effectively able to suppress fluorescence in EVs expressing GFP and allows measuring the Raman spectra of such samples.
Methods: Platelet derived EVs were provided by the Finnish Red Cross Blood Service and purified by ultracentrifugation (UC) and 2xUC for higher purity. EVs-AAV1, EVs-AAV9 and GFP EVs, were purified by differential centrifugation from cell conditioned media derived respectively from HEK293 cells, HEK293 cells infected with adeno-associated virus serotype 2/1 (both GFP positive and negative or 2/9).
Raman measurements were performed by timegate Raman Microprobe (Timegate Instruments Oy), with a 532 nm pulsed laser. EVs were dried on CaF2 substrate prior to measurement. Each sample was measured 30 times.
Results: Raman spectra of platelet derived EVs purified with different protocol show clear differences: preparation with more thorough purification protocol display a reduction in intensity of peaks associated to proteins and amino acids, indicating less contaminating free proteins. In addition, principal component analysis (PCA) cluster the spectra based on the purification method used.
Regarding the EVs, CD(?)9-GFP-EVs, EVs-AAV1 -AAV9, PCA display three major clusters: one associated to EVs-AAV9, one to EV and one to EV-AAV1 which include the GFP positive EV-AAV1.
Summary/Conclusion: These findings indicate that TG Raman is a suitable tool for EV quality control since it discriminates EV preparations based on the degree of purity and distinguishes between healthy EV preparations and preparations infected by AAV. In addition, TG Raman highlights the differences between the diverse AAV found in the EV preparations.
Lastly, TG Raman can overcome the fluorescence interference given by GFP. This allows the acquisition or Raman spectra of GFP-EVs, which display no remarkable difference compared to the unmarked EVs.
Funding: Timegate Instruments Oy Finnish Red Cross Blood Service.
Keywords: raman, timegate raman

PS05.13 | Tetraspanin analysis of plasma Extracellular Vesicles (pEVs) by Interferometric Reflectance Imaging Sensor (SP-IRIS) with and without fractionation

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Introduction: Single particle interferometric reflectance imaging sensing (SP-IRIS) based ExoViewR100 is a platform capable of detecting EV markers without extensive sample preparation and with a minimal sample volume. However, the kit assay protocol recommends purifying the starting sample by size exclusion chromatography (SEC) to eliminate plasma components that interfere with the analysis. The objective of this study was to compare the detection of tetraspanin of pEVs using 1 microL of platelet poor plasma (PPP), 50 microL of PPP for dialysis (PPPdial) as alternative to SEC to remove interference, and finally 150 microL of PPP purified using SEC (PPPsec)
Methods: Whole human blood samples collected in EDTA and centrifuged at 1,500 g for 10' at room temperature (RT) within 30 minutes of collection. Two centrifugations were subsequently carried out at 2,500 g for 10' at RT to obtain PPP. The PPP was dialyzed using a microdialyzer system 500 and a dialysis membrane with a MWCO of 1,000 kDa. The dialysis buffer (120 mL PBS-EDTA) was changed 3 times within 24 hours. A single qEV 35 nm column was used for SEC. Samples were diluted 60 times PPP, 60 times PPPdial and 4 times PPPsec in incubation buffer II respectively. Samples and antibodies were incubated in accordance with the ExoView kit assay protocol.
Results: EVs could be detected using PPP, PPPdial, and SEC fractions. In comparison to PPPsec, background noise detected on the mouse isotype control for PPP was elevated, whereas it was reduced for CD63 (AF647) and CD9 (AF488), but not completely for CD81(AF555) for PPPdial. A close distribution of tetraspanins was seen except for CD81 on both CD41a and CD81 capture spots for the PPPsec. Dialysis followed by SEC did not result in the detection of any tetraspanin on CD41a capture, suggesting that the SEC column may remove preferentially platelet-derived particles. As a result of SEC, the background noise in the isotype control can be reduced. By contrast, when we calculated EV counts based on the dilution factor, we observed a significant decrease in the counts in the PPPsec, particularly for platelets derived particles. As a result of dialysis, the noise on the isotype control was substantially reduced while maintaining a distribution of tetraspanin close to that of PPP.
Summary/Conclusion: In conclusion, dialysis could be an alternative method to SEC to remove plasma component that interfere and it may provide a more native vision of the tetraspanin distribution in pEVs using less sample.

PS05.16 | Identifying leukaemic signatures in blood extracellular vesicles

Sheela Abraham[1]; Tristan Robertson[2]; Christopher Wells[1]; Kathrin Tyryshkin[1]; Jelle Penders[3]; Molly M. Stevens[4]; John Rudan[5]; Eric Bonneil[6]; Pierre Thibault[6]; Xiaoyan Jiang[7]; Mark D. Minden[8]; Isabelle Grenier-Pleau[1]
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Introduction: Intercellular communication events mediated by extracellular vesicles (EVs) are becoming increasingly relevant in cancer research. Although EVs are known to influence haematopoietic stem cell (HSC) proliferation and differentiation, very little is known about how malignant EVs affect both healthy and leukaemic stem cells. This study aims to elucidate the composition and functionality of leukaemic EVs, to understand their role in disease.
Methods: EVs were enriched using a combination of iodixanol density cushion (IDC) and size exclusion chromatography (SEC) from the blood plasma of healthy, chronic myeloid leukaemia (CML) and acute myeloid leukaemia (AML) subjects. Single particle raman spectroscopy and mass spectrometry were performed on leukaemic and healthy EVs to identify novel spectral and proteomic biomarkers for CML and AML. Colony forming cell (CFC) assays were used to assess functional effect of leukaemic EVs on the proliferation and differentiation HSCs.
Results: Results revealed significantly more EVs in the blood plasma of patients with CML and AML compared to healthy controls. Raman spectroscopy identified unique EV profiles in samples from healthy controls, CML and AML patients, suggesting different lipid, nucleic acid and protein content. Machine-learning techniques were used to identify and rank EV proteins quantified by mass spectrometry, identifying potential EV protein biomarkers for leukaemia. Finally, CFC assays displayed an antiproliferative effect of leukaemic EVs on healthy HSCs.
Summary/Conclusion: Our group has identified unique Raman spectral and protein EV signatures that distinguish normal healthy individuals from leukaemia patients. Functional studies using human primary samples have also demonstrated a biological role of leukaemic EVs on HSC function, supporting the possibility of EVs as a therapeutic target in disease.
Funding: Canadian Institutes of Health Research, Canadian Foundation for Innovation, Bickell Foundation.
Keywords: leukaemia, CML, AML

PS06: Musculoskelatal System

Location: Hall 4A

16:50 - 18:50

PS06.04 | Study of the role of microRNAs-encapsulated in extracellular vesicles in osteosarcoma

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Introduction: The tumor microenvironment plays a crucial role in tumor progression and relies on secreted factors, including extracellular vesicles. EVs secreted by cells from the osteosarcoma (OS) microenvironment participate directly in OS growth and invasion. Many studies have found that BMSC-derived EVs can regulate cell proliferation, migration, survival, and OS drug resistance. Studies have highlighted that EVs from adipose-derived stem cells (ASC) decrease OS cell proliferation. Our goal is to determine how ASC-EVs affect OS growth and modify them to improve their therapeutic potential.
Methods: The 3M^3 technology platform consists of a 3-dimensional scaffold-free extracellular matrix (MEC), utilizing differentiated ASC to generate exosomes. EVs were purified by differential ultracentrifugation. EVs and cellular miRNA (miR) content was determined using qRT-PCR miR profiling. Functional tests were performed: scratch migration tests and MTT proliferation tests. Coculture was performed in a transwell system.
Results: To investigate the therapeutic potential of miR encapsulated in the isolated EVs, we perform functional tests on OS cells transfected with the five miRs*. Our results show that the miR* could act as a tumor suppressor (impact migration and

proliferation of OS cells). To investigate the impact on microenvironment cells, we made coculture with miR-transfected OS cells and fibroblasts. Results show that this coculture impacts the migration of fibroblasts. Future work will evaluate the potential of miR-encapsulated EVs on OS cells.
*The names cannot be mentioned now because of patent/publication concerns.
Summary/Conclusion: Overall results show that culturing ASCs in a 3-dimensional scaffold-free extracellular matrix leads to the enrichment of miRs in EVs with potential anti-tumoral activities. Future work will determine if those EVs can be used to treat osteosarcoma.
Funding: The authors declare no conflict of interest. This work has received funding from La Region wallonne and ULiege.
Keywords: microRNA, homing, osteosarcoma

PS06.05 | A homeostatic role for chondrocyte extracellular vesicles in regulating chondrocyte phenotype

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Introduction: Osteoarthritis (OA) is the most prevalent age-related degenerative disorder and severely reduces the quality of life of those affected. Whilst management strategies exist, no cures are currently available. Virtually all joint resident cells generate extracellular vesicles (EVs), which even seed articular cartilage; a structure whose health is central to the initiation and propagation of OA. There are reported changes in the cargo of joint-derived EVs during OA. However, whether EVs play a homeostatic role that is compromised in OA is unknown. Here we investigate how chondrocyte EVs may have a homeostatic role in maintaining chondrogenic gene expression.
Methods: EVs from C28/I2 chondrocytes in monolayers (2D) or pellet cultures (3D) were isolated using differential centrifugation with a final step at 20,000g for 30 minutes at 4°C. EVs were characterised by nano-flow cytometry, transmission electron microscopy and western blotting. Culture media supplemented with insulin-selenium-transferrin (ITS) promoted anabolic gene expression whereas EV-free foetal bovine serum (FBS) decreased it. Secondary EV release and anabolic gene expression was evaluated following treatment of chondrocytes with 2 × 106 EVs from 2D or 3D cultures for 24 hours. Additionally, the uptake of fluorescently labelled EVs was assessed via confocal microscopy following 4 hours of treatment of EVs from 2D or 3D cultures in either FBS or ITS conditions.
Results: Culturing chondrocytes in 3D significantly increased EV generation compared to cells cultured in 2D. EV accumulation in 3D cultures was similar irrespective of culturing in FBS or ITS supplemented media. However, EV release decreased in 2D cultures with FBS compared to those in ITS. Treatment of 2D chondrocytes cultured in FBS with 2D EVs had no effect on anabolic genes; however, treatment with EVs from 3D cultures significantly improved chondrogenic gene expression and promoted secondary EV release. EV uptake was greatest under ITS conditions and irrespective of culture conditions; uptake of EVs was greater from a 3D source compared to 2D.
Summary/Conclusion: These results indicate chondrocyte EVs have an autocrine homeostatic role that maintains chondrocyte phenotype. How this role is perturbed under OA conditions remains the subject of future work.
Funding: Funding provided by the Centre of Osteoarthritis Pathogenesis versus Arthritis/Centre for Innovation and Therapeutic Innovation (CiTI) Doctoral Training Programme (grant number 21621).
Keywords: osteoarthritis, chondrocytes, cartilage

PS06.06 | Amelioration of osteoporosis by peptide-functionalized osteoclast-targeting red blood cell extracellular vesicles delivering microRNA

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Introduction: The upregulation of miR-214 in osteoclasts is considered one of the pathogenic factors of osteoporosis (OP), a disease that currently cannot be cured. Suppressing the level of miR-214 by anti-miR-214 in osteoclastsreverses bone absorption

and provides a possible remedy. Delivery of anti-miR-214 requires a reliable, efficient, and safe vehicle that can specifically target osteoclasts. Red blood cell (RBC) extracellular vesicles (RBCEVs) are a natural-born, nano-sized carrier featuring a low risk of gene transfer (RBCEVs do not contain genomic or mitochondrial DNA) and high availability (RBCs are renewable and can be obtained in large quantities), but lack osteoclast-binding specificity.

Methods: A drug delivery system targeting osteoclasts was constructed by modifying RBCEVs with TRAP affinity peptide and loading miR-214. The effect of miR-214 targeted delivery by RBCEVs on osteoclast-related mRNA and protein was detected using qRT-PCR and western blotting. The targeted effect in vivo was observed using small animal imaging. The therapeutic effect of targeted delivery of anti-miR-214 on osteoporosis in mice was tested by immunohistochemistry and micro-CT. HE staining was used to evaluate the biological safety of the targeted delivery of miR-214 by RBCEVs.

Results: Peptide functionalization displays the osteoclast-binding motif on the surface of RBCEVs. It endows RBCEVs osteoclast-targeting capability to deliver anti-miR-214 to osteoclasts and pre-osteoclasts both in vitro and in vivo. Osteoclast-targeting RBCEVs (OT-RBCEVs) administered through intravenous injection concentrated in the bone skeleton of mice, inhibited the osteoclast activity, promoted the osteoblast activity, and improved the bone density of the osteoporotic mice.

Summary/Conclusion: Altogether, functionalization by the bi-functional fusion peptide proves to be a facile and modular method to modify the surface of RBCEVs and construct cell-targeting delivery vehicles. This work also shows that targeted delivery of anti-miR-214 by OT-RBCEVs is a viable method for OP treatment.

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Keywords: osteoporosis, red blood cells, extracellular vesicles, targeted delivery, anti-miR-214

PS06.07 | Extracellular vesicles from subcutaneous fat-derived mesenchymal stem cells suppress RANKL-induced osteoclast differentiation via miR122-5p

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Introduction: Researchers are increasingly interested in cell therapy using mesenchymal stem cells(MSCs) as an alternative remedy for osteoporosis with fewer side effects. Thus, we isolated and characterized the EVs from human subcutaneous adipose tissue-derived MSCs(hsMSCs), investigated their inhibitory effect on RANKL-induced osteoclast differentiation.

Methods: We collected purified EVs from the supernatant of hsMSCs culture by TFF. Characterization of EVs included typical evaluation of size analysis and concentration of EVs by NTA and morphology analysis by TEM. Osteoclasts were differentiated from BMDMs by RANKL and determined by TRAP staining, F-actin ring formation, and bone resorption assay. Expression of osteoclastogenesis-related genes and activation of MAPKs were determined by RT-PCR and Western blotting, respectively. For miRNA transfection, BMDMs were cultured in miR122-5p mimic or miRNA negative control with Lipofectamine RNAiMAX according to the manufacturer's insructions.

Results: EVs inhibited the RANKL-induced differentiation of BMDMs into osteoclasts in a dose-dependent manner. F-actin ring formation and bone resorption were also reduced by EVs treatment in osteoclast. In addition, EVs decreased the RANKL-induced phosphorylation of p38 and JNK and expression of osteoclastogenesis-related genes in BMDMs treated with RANKL. To elucidate which part of hsMSCs-EVs plays a role in the inhibition of osteoclast differentiation, we performed an Exosomal miRNA array. The results showed that hsa-miR122-5p was present with significantly higher read counts. Overexpression of miR122-5p in BMDMs significantly inhibited RANKL-induced osteoclast differentiation and induced defects in F-actin ring formation and bone resorption. Our results also revealed that RANKL-induced phosphorylation of p38 and JNK and osteoclast-specific genes expression were decreased by miR122-5p transfection, which was consistent with the results of hsMSCs-EVs. The target relationship between miR122-5p found in EVs and osteoclastogenesis-related factors needs further exploration.

Summary/Conclusion: These results suggest that hsMSCs-EVs and miR122-5p found in them inhibit RANKL-induced osteoclast differentiation through downregulation of the same molecular mechanism and could be therapeutic candidates for bone diseases.

Funding: This research was financially supported by a grant from the National Research Foundation of Korea (NRF-2020R1F1A1072045).

PS06.08 | Dipsaci radix derived extracellular vesicle-like nanoparticles alleviate osteoporosis by promoting osteogenic differentiation in BMSCs through the activation of the BMP2/Smads pathway

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Introduction: Dipsaci Radix has been clinically used for strengthening muscles and bones. However, the efficacy of Dipsaci Radix derived extracellular vesicle-like nanoparticles (DREVNs) on relieving osteoporosis in mice models and its underlying mechanism remain poorly understood.
Methods: DREVNs were isolated and characterized from Dipsaci Radix. Using DREVNs to treat bone marrow mesenchymal stem cells (BMSCs) and the postmenopausal osteoporosis (PMOP) mouse models, we analyzed associated genes and poteins of BMSCs and targeting, skeletal structure parameters and proteins of PMOP mouse models.
Results: In vitro experiments showed that DREVNs upregulated the osteogenic-related factors and activate the BMP-2/Smads signalling pathway to promote osteogenic differentiation of BMSCs. In vivo, DREVNs had definite femur and BMSCs targeting. Micro-CT suggested that BMD, BV, BV/TV, BS/TV and Tb.N were significantly increased in the DREVNs treatment group, whereas BS/BV, Tb.Sp., SMI and Tb.pf. were significantly reduced, and the increased levels of BMP2, RUNX2, p-Smad1/5/9 and Smad1/5/9 proteins in femurs reconfirmed the importance of the BMP2/Smads signaling pathway in the progress of the anti-osteoporosis mediated by DREVNs.
Summary/Conclusion: Taken together, our work demonstrates that DREVNs can effectively promote osteogenic differentiation of BMSCs by activating the BMP2/Smads signaling pathway to prevent osteoporotic bone loss in PMOP mouse models.
Funding: DREVNs may represent a new class of nano-drugs for the prevention of PMOP.
Keywords: dipsaci radix, extracellular vesicle-like nanoparticles, osteoporosis, osteogenic differentiation, BMSCs

PS06.09 | Potential use of extracellular vesicle surface markers in human plasma for the diagnosis of osteoarthritis

Jana Bzdilova; Jana Matejova; Lucia Slovinska; Livia K. Fecskeova; Denisa Harvanova; Timea Spakova
Associated Tissue Bank, Faculty of Medicine, P. J. Safarik University in Kosice, Slovakia, Kosice, Slovakia

Introduction: Extracellular vesicles (EVs) represent paracrine factors secreted by almost all cell types. Both, normal and pathological cells are able to release various types of EVs with different physiological properties, functions and compositions. EVs play an important role in intercellular communication, mechanism and tissue repair. Moreover, EVs could help not only in the treatment of diseases but also in their diagnostics. This work aimed to evaluate the potential of EVs used as biomarkers for the diagnosis of osteoarthritis (OA) by comparing the surface markers expression of EVs isolated from platelet-poor plasma (PPP) of healthy donors and patients at different stages of OA. OA is a chronic disease of musculoskeletal system that mainly affects the elderly. Nowadays, there is still no drug for the treatment of OA, patients are either treated symptomatically or in the end-stage of the disease endoprothesis is done on severely damaged joints. For this reason, there is a high interest in trying to find biomarkers through which it would be possible to diagnose OA in the early stages and thus improve the quality of life of affected patients by slowing down the progression of OA.
Methods: Collection of the blood samples was in accordance with ethical approval and realized after obtaining informed consent from healthy volunteers and OA patients. PPP samples were prepared from whole blood. PPP-EVs were separated from 3 groups of donors - healthy control (Kellgren-Lawrence grade (KL) = 0), early stage OA (KL = I-II), end-stage OA (KL = III-IV), and their content was compared and correlated. EVs from PPP were separated by SEC using qEVoriginal ~35nm columns and characterized in terms of their size, yield and surface marker expression by NTA, western blotting and flow cytometry.
Results: Our study confirmed that PPP-EVs were positive to the well-established EVs surface markers CD9, CD63 and CD81 in each donor group, with highest expression of CD81. There were found also significant differences between EVs surface markers of patients and healthy controls correlating with the age of donor (CD31 and ROR1) and early stage of OA (CD45, CD326 and CD56), respectively.
Summary/Conclusion: As potential targets for biomarker discovery, circulating EVs have been extensively under investigation for their capability to predict OA pathology diagnosis. Obtained results suggested potential usage of PPP-EVs surface markers in the OA early diagnosis.

Funding: This publication is the result of the project implementation: OPENMED, ITMS2014+: 313011V455 supported by the Operational Programme Integrated Infrastructure, funded by the ERDF. This research was also supported by the Slovak Research and Development Agency under the contract No. APVV-17-0118.
Keywords: extracellular vesicles, platelet-poor plasma, osteoarthritis, diagnosis

PS07: EV Heterogeneity 2

Location: Hall 4A

16:50 - 18:50

PS07.03 | Characterization of large extracellular vesicles (L-EV) derived from in vitro differentiated regulatory macrophages (Mreg)

Karina S. Zitta[1]; Lars Hummitzsch[2]; Rene Rusch[2]; Katharina Heß[2]; Markus Steinfath[2]; Joachim Cremer[2]; Frank Lichte[3]; Fred Fändrich[2]; Rouven Berndt[2]; Kerstin Parczany[2]; Christopher Schnoor[2]; Martin Albrecht[1]
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Introduction: We have recently shown that human monocyte derived anti-inflammatory macrophages (regulatory macrophages, Mreg) bear pro-angiogenic potential in-vitro (Hummitzsch, L. et al. Stem Cells Int (2019)). Mreg also release large extracellular vesicles (L-EV), which may be involved in the transport of active molecules that participate in cell communication (Paolicelli, R. C. et al., Neuroscience (2018)).
Methods: Mreg were differentiated for 7 days using blood monocytes from healthy donors (N = 9). L-EV populations were enriched from Mreg culture supernatants by differential centrifugation. Characterization of L-EV was performed by cell/particle analysis, brightfield/transmission electron microscopy (TEM) and flow cytometry. Culture media metabolites were analyzed using a blood gas analyzer.
Results: L-EV can be reproducibly isolated from Mreg cultures at day 7 by using 2 standard centrifugation steps. Mreg release about 1.5 L-EV/Mreg into the culture medium. Release of L-EV is negatively correlated with a lactate concentration (6-15mmol/l) and positively correlated with pH (6.8-7.3). The average diameter of Mreg is 13.77 ± 0.87μm (volume: 1.38 ± 0.25pl) whereas L-EV reveal a size of 7.45 ± 0.28μm (volume: 0.22 ± 0.03pl). While Mreg size increased during the differentiation process (t0: 9.95 ± 3.73μm, t7: 13.77 ± 0.87 μm; P< 0.05), L-EV size remained unchained (t1: 7.75 ± 1.3μm, t7: 8.09 ± 1.45μm; P>0.05). Several morphological similarities between Mreg and L-EV were evidenced by TEM, such as numerous intracellular membrane vesicles and pseudopodia-like extensions. A lipid-bilayer structure was demonstrated by the presence of transmembrane anchored proteins specific for EV (CD81:62.3%; CD9:47.5%; CD63:78.7% and LAMP1:54.9%) (Witwer, K. W. et al. J. Extracell. Ves.(2021)).
Summary/Conclusion: L-EV can be reproducibly isolated from Mreg cultures. Their morphological appearance and membrane characteristics suggest that they are derived from Mreg and may possess Mreg-like functions.
Funding: TRIZELL GmBH.
Keywords: large extracellular vesicles (LEV), regulatory macrophages (Mreg)

PS07.04 | Dynamics of extracellular vesicle-mediated crosstalk in 3D tumor models

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Introduction: Evolutionary process of solid tumors highly relies on the extracellular vesicle (EV)-mediated cross-talk between malignant cells and stromal cells in the tumor microenvironment (TME). In this study, we aimed to establish a multicellular three-dimensional (3D) tumor model system for tracking the EV communication network of different tumor tissues under physiological conditions and cytostatic treatments.
Methods: Human ductal carcinoma, melanoma and osteosarcoma models were established via co-culturing the respective tumor cell line (T-47D/A375/MG-63) with MRC-5 fibroblasts and EA.hy926 endothelial cells on flat- or U-bottom plates after staining

with CellTracker dyes (Orange CMTMR, Deep Red, Green CMFDA). To mimic chemotherapeutic stress, low dose doxorubicin were used and the 2D and 3D cultures were imaged daily by a PerkinElmer Operetta High Content Screening Sytem and a Leica SP8 Digital LightSheet microscope, respectively.
Results: We showed that CellTracker dyes can be used for in-cell labelling of EVs, allowing the quantitative monitoring of EV cross-talk, i.e. EV routes between each cell type and in both directions. The three types of tumor models showed differences in their 3D structure, EV cross-talk activity and drug-induced effects as well. We could observe distinct temporal kinetics in the development of the EV communication network in 2D and 3D, also priorities of the investigated EV routes varied between the two co-culture systems.
Summary/Conclusion: The developed 3D model system is suitable for live tracking of EV cross-talk in the TME, which can dinamically change depending on the microenvironmental conditions. Further data will help (i) to identify potential targets of EV-blocking therapies, which may increase the efficacy of chemotherapies, and (ii) to predict the drug-induced changes of the communication activity in different tumor tissues.
Funding: TKP2021-EGA09, Szent-Györgyi Albert Research Fund (University of Szeged), OTKA-K143255.
Keywords: 3D tumor model, EV cross-talk, EV tracking, tumor microenvironment

PS07.05 | Comparison of the release of platelet derived EVs and microRNAs activated by shear stress and different agonists

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Introduction: Platelets are easily activated by external environment because they are very sensitive. The activated platelets release a large amount of EVs, encapsulating microRNAs in it. The platelet derived EVs (pEVs) are also known to have excellent tissue regeneration ability and wound healing effects. In this study, we investigated EVs and microRNAs released from platelets.
Methods: Whole Blood from healthy people was centrifuged at 580 g for 8 minutes, and 2200 g for 20 minutes. Platelet-rich plasma (PRP) was carefully separated. PRP was activated by thrombin, calcium chloride, mixture of thrombin and calcium chloride, and incubated at 37°C for 30 minutes. For physical activation, rotating shear stress of 1.319 Pa was applied for 1 minutes, and incubated at room temperature for 30 minutes. And then, EVs were extracted using a charge-based polymer precipitation.
Results: The amount of EV released from platelets was in the order of thrombin, calcium chloride, and shear stress, whereas that of miRNA per EV was in the order of shear stress, thrombin, calcium chloride, and mixture. These results implied that pEVs released upon platelet activation appeared to have poor internal contents, which might be due to rapid formation of EVs in platelet without sufficiently packaging the contents such as nucleic acids.
Summary/Conclusion: It was found that the amount of EVs and miRNAs were different depending on the activation method. By controlling of the activation method, it may be possible to get EVs containing specific miRNAs having healing effects. This will be applicable in the field of treatment such as cancer treatment further.
Funding: This research was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Korean Government, MSIP (2016R1A5A1010148).
Keywords: platelet-derived exosome, platelet activation, shear stress, agonists

PS07.07 | Large-scale purification of extracellular vesicles by heparin chromatography identifies two EV subclasses

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Introduction: Most conventional extracellular vesicle (EV) purification methods are limited to a smaller scale. We use a tangential-flow filtration and Capto Core 700 (TFF-CaptoCore700) based method for large-scale EV purification and intentionally avoid strong physical force to preserve EV integrity. Separating EV into subclasses that carry unique cargos and markers is essential. This is the prerequisite for any accurate phenotypic tests and even more importantly for EV manufacturing.
Methods: EV purified by TFF-CaptoCore700 was further separated into sub-populations by heparin chromatography. The biophysical properties of EV sub-populations were analyzed using nanoparticle tracking analysis, transmission electron

microscope, and super-resolution microscopy. Protein contents were analyzed using mass spectrometry. Cell intake was analyzed by immunofluorescent assays. ERK phosphorylation was investigated by western blot upon EV treatment.
Results: Two main EV sub-populations were obtained from heparin chromatography. The first does not bind to the heparin column, namely a non-heparin-binding (NHB) fraction. The second binds to the heparin column and can be eluted by higher salt, thus is a heparin-binding (HB) fraction. NHB carries most conventional EV protein markers while HB is enriched in extracellular matrix binding protein and histones. Both NHB and HB can be taken-in by human endothelial cells but only HB induce ERK phosphorylation.
Summary/Conclusion: Heparin chromatography would be an effective novel step to isolate EV subclasses at larger scale and can separate pyrogenic EV from those prepared for clinical applications.
Funding: This work was founded by the NIH under Grant 5R01DE018304 to DPD, RO1ES031635 to JDG, P01CA019014, and R01CA228172 to DPD and JDG. The UNC Proteomics Core Facility is supported in part by NCI Grant (2P30CA016086-45) to the UNC Lineberger Comprehensive Cancer Center.
Keywords: extracellular vesicles, heparin, large-scale purification, tangential flow filtration, Capto Core 700

PS07.08 | Survey of organ-specific small extracellular vesicles protein markers in serum

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Introduction: Extracellular vesicles (EVs) are secreted by all organs and enter teh bloodstream, and theirfore blood EVs, which comprise EVs secreted by blood cells and/or other organs, may reflect physiological and pathological conditions. Given dat blood EVs are extremely heterogeneous, it is challenging to identify teh specific organ of origin. Here, we set out to systematically identify tissue-specific small (s)EV proteins secreted by six tissues (brain, liver, lung, heart, kidney, and fat) in blood serum using proteomic analysis. We identified a number of sEV proteins dat were specific to brain (68), liver (194), lung (39), heart (15), kidney (29), and fat (33). Validation by western blot analysis confirmed teh presence of tissue-specific sEV proteins in sEVs isolated from serum, including brain (DPP6, SYT1, and DNM1L), liver (FABPL, ARG1, and ASGR1/2), lung (SFPTA1), heart (CPT1B), and fat (GDN). Finally, we applied these findings to an aging mouse model and discovered altered levels of these proteins in serum sEVs from old compared to young mice. In sum, we have identified and explored teh traceability of tissue-specific sEV proteins in serum presenting potential non-invasive biomarkers and/or therapeutic targets.
Methods: Tissue dissociation and sEV isolation sEV isolation from serum Nanoparticle tracking analysis Transmission Electron Microscopy Mass spectrometry-based proteomics of EVs Western blot analysis
Results: sEV protein contents reflect teh parent tissue organ of origin Proteome analysis reveals common and unique sEV proteins for each organ Identification of Organ-specific sEV proteins Detection and validation of organ-specific sEV proteins in serum
Summary/Conclusion: Here, we surveyed sEV proteins from mouse organs: brain, liver, lung, heart, kidney, and fat. Given dat organ-specific sEVs retain their protein identity as they travel into the bloodstream, we performed proteomic analysis using sEVs isolated from these tissues and serum. We identified sEV proteins dat were both (me) exclusively or highly specific to a single organ wifin these limited comparisons, and (ii) detectable in serum sEVs, in order to create a protein catalog dat may serve for noninvasive, rapid detection of diagnostic biomarkers of various organ EVs present in the blood.
Funding: NIH.
Keywords: extracellular vesicles, exosomes

PS07.09 | Proteomic analysis of extracellular vesicles identifies Stomatin as a biological marker for acute psychological stress

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Introduction: Psychological stress emerges as a common health burden in the current society due to its highly correlated risk of mental and physical disease outcomes. However, it is still unclear how fast adaptive stress response processes are connected to long-observed changes in organisms. Here, we investigated the profile of circulating extracellular vesicles (EVs) after acute stress (AS) of restraint mice by phenotypic and proteomic analyses.
Methods: Here, we investigated the profile of circulating extracellular vesicles (EVs) after acute stress (AS) of restraint mice by phenotypic and proteomic analyses. And circulating EVs were separated by sequential centrifuged 3,500r for 15 min, 2,500g for 10 min and 16,800 g for 30 min.

Results: We surprisingly discovered that AS-EVs demonstrated significant changes in size distribution and plasma concentration compared to control group (CN) EVs. AS-EVs were further characterized by various differentially expressed proteins (DEPs) in close relationships with biological, metabolic and immune regulations and were functionally important in potentially underlying multiple diseases. Notably, we first identified the lipid raft protein Stomatin as an essential biomarker expressed on the surface of AS-EVs.
Summary/Conclusion: These findings collectively reveal that EVs are a significant function-related liquid biopsy indicator that mediate circulation alterations impinged by psychological stress, while also supporting the idea that psychological stress-associated EV-stomatin can be used as a biomarker for potentially predicting acute stress responses and monitoring psychological status. Our study will pave an avenue for implementing routine plasma EV-based theranostics in the clinic.
Funding: This work is supported by grants from the National Natural Science Foundation of China (82170988 to F.J., 81930025 to Y.J., 32101096 to X.Q., 32000974 to B.S.) and the China Postdoctoral Science Foundation (2019M663986 and BX20190380 to B.S.).
Keywords: psychological stress, extracellular vesicles, proteomic analysis, Stomatin, plasma

PS07.10 | Protein analysis and activity-based protein profiling in human extracellular vesicles

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Introduction: Enyzmatic distrubances of the ubiquitin proteasomal system (UPS) are a hallmark for progressive kidney cell injury. The assessment of UPS enzyme activities is not feasible in archived kidney biopsies from patients. Extracellular vesicles (EVs) provide opportunities in biomarker discovery for diagnosis, prognosis as well as therapy monitoring. Here we focus on the potential of EVs from human podocytes as a source for protein-biochemical assessment of proteostasis disbalances. Podocytes are specialized cells of the kidney blood filter, which are challenged during autoimmune diseases such as membranous nephropathy (MN) resulting in breakdown of the renal filter. In MN the UPS is impaired in podocytes.
Methods: Human cultured podocytes exposed to autoantibodies and isolated podocyte-derived urinary EVs from patients with MN or other kidney diseases were analyzed. From cultured podocytes, EVs were collected in exosome depleted medium and isolated by differential ultracentrifugation. EVs were isolated from healthy control and patient urine with kidney diseases using a combination of liquid size exclusion chromatography and differential ultracentrifugation. The amount of EVs was measured by image stream. Next to total protein abundance, the activity of DUBs and of proteolytic subunits of the proteasome were examined by activity-based protein profiling.
Results: Upon autoimmune injury the amount of podocyte-derived EVs from cultured podocytes and from patient urine increased. Protein biochemical analysis unraveled the presence of mainly inactive enzymes of the UPS within released EVs from cultured podocytes after exposure to autoantibodies. Content of inactive enzymes of the UPS was highly abundant in urinary EVs from patients with MN compared to other kidney diseases.
Summary/Conclusion: Podocyte-derived EVs are a suitable source for protein biochemical analyses of the functional state of the UPS.
Keywords: biomarker, protein, kidney

PS07.11 | Comparative analysis of the hepato-regenerative potential of naïve and bioengineered small extracellular vesicles derived from wharton's Jelly derived-mesenchymal stem cells (MSCs)

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Introduction: Mesenchymal stem cells (MSCs) are multipotent adult stem cells that have gained popularity in regenerative medicine due to their ability to differentiate, immunomodulatory capabilities, and secretion of paracrine mediators such as small extracellular Vesicles (sEVs). These sEVs carry miRNA and mRNA, proteins, and other cargo that help heal damaged or diseased tissues and organs. Several studies have shown the potential role of small EVs cargo, especially the role of miRNAs in hepato-protection and hepatocyte regeneration. Naive sEVs face some of the biggest problems, such as repeatability, scalability, limited cargo encapsulation, and target specificity. Surface modification techniques can be employed to enhance the homing of these sEVs which improves the target specificity of these small extracellular vesicles. The current prospective study aims to develop an EV-based treatment method by developing Bioengineered small Extracellular Vesicles (BioEn-EVs) and assessing their potential applicability in liver disease with a specific focus on hepatic cells.

Methods: Human MSCs were isolated from Wharton's Jelly with due consent forms and characterized according to the International Society of Cellular Therapy (ISCT) guidelines. MSCs were cultured in serum-free media for the isolation of small Extracellular Vesicles (sEVs). sEVs were characterized for size (NTA), morphology (TEM), and surface and cytoplasmic marker profiling (Western blotting). Bioengineering of the surface of sEVs with hepato-specific ligands as well as cargo of sEVs with selective miRNA exhibiting hepatoprotective activity was done. BioEn-EVs were characterized via NTA, TEM, Western blotting, zeta potential, and their cargo content was validated by qRT-PCR. The hepatoprotective and antioxidant effect of naïve and BioEn-EVs was assessed in vitro.
Results: The aggregation of BioEn-EVs was observed with a TEM as compared to naïve sEVs. The diameter and zeta potential of BioEn-EVs were increased and became positive as compared to naïve sEVs. The cellular uptake efficiency of BioEn-EVs were significantly greater extent than naïve sEVs. It was observed that there was a significant ROS scavenging potential and hepato-regenerative potential of BioEn-EVs.
Summary/Conclusion: It is concluded that the Bioengineering of MSC-derived sEVs enhanced their ability to efficiently deliver its content into hepatic cells under stress/injured hepatic cells. Thus, BioEn-EVs are cell-free, promising, and customizable translational approaches to improve the outcomes of patients with liver disease.

PS07.12 | Size matters: Functional differences of small extracellular vesicle subpopulations

Simonides Immanuel van de Wakker[1]; Christian JB Snijders Blok[2]; Julia Bauzá-Martinez[3]; Carla Rios Arceo[4]; Herak Manjikian[2]; Eduard F Willms[5]; Olivier G de Jong[6]; Renee GC Maas[2]; Wei Wu[7]; André Görgens[8]; Samir EL Andaloussi[9]; Joost PG Sluijter[4]; Pieter Vader[10]
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Introduction: Increasing evidence indicates that small extracellular vesicles (sEVs) exist as heterogeneous populations. sEV heterogeneity and thereby reproducibility represents a major challenge in the field, in particular related to the understanding of differences in function. Progenitor cell-derived sEVs have shown great potential to stimulate tissue repair. In this project, functional differences of progenitor cell-derived EV subpopulations were studied to better understand functional EV heterogeneity and to provide new insights that will help with the development of regenerative EV therapeutics.
Methods: Cardiac progenitor cell (CPC)- and mesenchymal stromal cell (MSC)-derived sEVs were purified by binding chromatography, followed by size-exclusion chromatography (SEC) for size-based fractionation of different sEV-subpopulations. sEVs were characterized using western blot, transmission electron microscopy, mass spectrometry and imaging flow cytometry (IFC). Functional differences were studied using multiple cellular assays on various cell types, including AKT phosphorylation, wound healing migration, angiogenesis, target cell EV uptake, fibroblast activation, cardiomyocyte survival and proteasome activity.
Results: SEC was used to separate three distinct subpopulations of CPC and MSC-derived sEVs, which were identified based on differential expression of sEV marker proteins. These sEV subpopulations differed in size, appearance and proteomic composition. Mass spectrometry and IFC analysis confirmed the differences in expression levels of sEV marker proteins. Furthermore, gene ontology cellular component analysis indicated differences in intracellular origin. sEV subpopulations exerted clear functional differences in different recipient cells. Smaller and middle-sized sEV subpopulations were able to stimulate migration, activation and spheroid formation in endothelial cells, but no effect was observed for the larger sEVs. Only middle-sized sEVs were able to stimulate cardiomyocyte survival whereas only the smallest subpopulation presented proteasomal activity. Furthermore, differences in subpopulation uptake were seen in endothelial cells, cardiomyocytes, fibroblasts and macrophages.
Summary/Conclusion: SEC allows for isolation and in-depth study of the functional heterogeneity of sEVs. In our study, we observed the existence of different subpopulations based on size which had a differential composition, origin and biological function. Increasing knowledge of sEV heterogeneity will contribute to a better understanding of the mechanisms of action of sEVs, thereby accelerating translation of EV therapeutics to the clinic.
Funding: Van Herk Foundation, ERC EVICARE (725229), ESC FCIG, MDR Young Talent Incentives Program, NLSEV-MOVE.
Keywords: EV heterogeneity, subpopulations, tissue/cardiac repair

PS08: EVs in Neurological Disorders and Injury

Location: Hall 4A

16:50 - 18:50

PS08.04 | Inducible pluripotent stem cell-derived small extracellular vesicles alleviate microglia senescence to protect against ischemic stroke in aged mice

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Introduction: The polarization of microglia plays an important role in the outcome of ischemic stroke. In the aged population, senescent microglia show a predominant pro-inflammatory phenotype, which leads to worse outcomes in aged ischemic stroke compared to young ischemic stroke. We hypothesized that iPSC-sEVs could alleviate microglia senescence to regulate microglia polarization in aged ischemic stroke.
Methods: iPSC-sEVs were pre-administrated to mice once a week for 2 months. Ischemic stroke operation was performed after the last administration of sEVs followed by the detection of polarized microglia. The infarct volume and behavior tests were examined to assess the protective function of iPSC-sEVs on aged ischemic stroke. Proteomics combined IPA was used to explore the functional proteins carried by iPSC-sEVs.
Results: We showed that treatment with iPSC-sEVs significantly alleviated microglia senescence and inhibited pro-inflammatory activation of microglia both in vivo and in vitro. Furthermore, iPSC-sEVs shifted microglia from pro-inflammatory phenotype to anti-inflammatory phenotype, which reduced the apoptosis of neurons, and improved the outcome of aged stroke mice. Mechanism studies showed that iPSC-sEVs reversed the loss of Rictor and downstream p-AKT (s473) in senescent microglia by transferring TGF-β1, which was involved in the senescence and pro-inflammatory phenotype regulation of microglia. Taken together, our work demonstrates iPSC-sEVs reverse the senescent characteristic of microglia in the aged brains and therefore improve the outcome after stroke.
Summary/Conclusion: This study demonstrated that iPSCs-sEVs improved the inflammatory microenvironment and facilitated the shift of microglia polarization from pro-inflammatory phenotype to anti-inflammatory phenotype through alleviating microglia senescence, therefore protecting neurons from death and improved outcome of aged ischemic stroke. As the anti-ageing nano-vesicles, iPSCs-sEVs provide a novelty therapeutic strategy for aged ischemic stroke and other ageing-associated neurodegenerative disease.
Funding: This study was kindly funded by the National Natural Science Foundation of China (Grant No. 82071371, 82172421 and 82201543).
Keywords: induced pluripotent stem cell-derived small extracellular vesicles, senescent microglia, ischemic stroke, aging

PS08.05 | Extracellular vesicles are critical elements in senescent cell removal by recruiting antigen-presenting cells

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Introduction: Standard-of-care chemoradiaGon limits tumor growth by inducGon of cellular senescence. However, immediate removal of senescent cells (SCs) by immune cells is crucial to avoid SC-mediated cancer relapse. SC persistence promotes recurrence by fostering chronic inflammaGon. The paucity of approved senolyGc drugs highlights the need for more efficient strategies for SC removal. We hypothesized that SC-derived extracellular vesicles (senEVs) promote immune-mediated SC removal.
Methods: To characterize senEVs in a model of oral squamous carcinoma, we isolated them by ultracentrifugaGon and analyzed by nanoparGcle tracking analysis, electron microscopy, immunobloUng and mass proteomics. To study the effect of senEVs on immune response, we suppressed senEV release by expressing Rab35 dominant-negaGve mutant in SCs, which uncoupled senEV release from secreGon of soluble factors. To invesGgate the role of senEVs in cancer recurrence, we challenged mice orthotopically with EV-competent or EV-deficient SCs (generated in vitro by chemotherapy treatment) mixed with limiGng amounts of tumor cells. We evaluated cancer relapse and immune infiltrates using both flow cytometry and single cell RNA sequencing. Importantly, we tracked SCs in Gssues using transgenic markers.

Results: SenEVs inhibited cancer recurrence in vivo. When senEVs are inhibited, we observed a significant decrease of anGgen-presenGng cells (APCs) in senescent Gssues, which may contribute to the observed persistence of SCs. The results of single cell RNA sequencing highlighted changes in APCs in the presence of senEVs. Proteomic analysis revealed several proteins enriched in senEVs compared to those from their non-senescence counterparts and common senEV biomarkers were found in two different oral SC models.
Summary/Conclusion: senEVs have criGcal roles in immune-mediated removal of SCs. Future work will idenGfy the receptor-ligand relaGon between the idenGfied protein markers on senEVs and APCs, which will highlight novel therapeuGc candidates for boosGng immune-mediated removal of SCs.
Funding: NIH Training Grant T32CA254888 CRUK/OHSU A29681.
Keywords: senescence, extracellular vesicles, cancer relapse

PS08.06 | Screening of potential biomarkers for mucopolysaccharidosis III in neuronal extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are released by all cells and contain cargo reflecting the status of the original cell. EVs in the central nervous system (CNS) can cross the blood-brain-barrier into the blood, providing a window into the CNS's biochemistry. Mucopolysaccharidosis III (MPSIII) is a genetic neurodegenerative disease characterized by neurocognitive and neurobehavioral abnormalities due to defects in lysosomal enzymes required for heparan sulfate (HS) catabolism. In addition to the primary storage of HS, lysosomes of patients with MPS III and animal models of the disease accumulate large amounts of amyloid proteins as secondary storage, including α-synuclein (α-syn), tau, and amyloid β-protein (Aβ). In neurodegenerative diseases, autophagy-lysosomal pathway dysfunction leads to increased secretion of these proteins in EVs. Thus, we hypothesized that measuring them in EVs of patients with MPSIII and healthy controls (HC) could lead to sensitive, minimally invasive biomarkers for MPSIII.
Methods: 24 patients with MPSIII (20 MPSIIIA, 3 MPSIIIB, 1 MPSIIIC) and 15 HCs were enrolled. EVs were isolated from plasma by an ExoQuick kit followed by immunoprecipitation of nEVs using an anti-L1CAM antibody. EVs were analyzed using TRPS, western blots, and TEM and nEV enrichment was assessed by flow cytometry. α-Syn, tau and Aβ were measured in nEVs. In addition, IL-10, IL-2Rα, and neurofilament light (NfL) were measured in plasma using ECLIA. Statistical significance was assessed using independent t-tests. Effect sizes were assessed using Hedge's g (Hg).
Results: Patient EVs had significantly higher levels of α-syn compared to HCs with a large effect size (Hg = 1.1). Tau was detected in 23/24 patient samples and 0/15 HC samples. Aβ was found in 3 patient samples only. NfL, a general marker of neurodegeneration, and IL-10 were significantly higher in patients compared to HC with large effect sizes (Hg = 1.2 and 0.9). IL-2Rα did not differ between the groups.
Summary/Conclusion: nEVs α-Syn and tau, and plasma NfL and IL-10 are significantly higher in patients with MPSIII than in HC suggesting that these biomarkers may be useful for monitoring disease progression and assessing treatment effects.
Funding: Cure Sanfilippo Foundation 20215318 (GB) Cure Sanfilippo Foundation (NCT04018755) (LP).
Keywords: biomarker, diagnostics, mucopolysaccharidosis, Sanfilippo syndrome, α-synuclein, tau, amyloid β-protein, neurofilament light, cytokines, lysosomal-storage disorders

PS08.07 | Human neural stem cell extracellular vesicles modulate proinflammatory CCL2 in activated microglia leading to improved functional recovery in a rat reperfusion model of acute ischemic stroke

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Introduction: Acute ischemic stroke (AIS) standard of care is cerebral reperfusion (REP) via clot removal, but only ~16% of patients are eligible, and many live with significant functional deficits after REP. As the field works to improve eligibility, the ideal treatment to improve post-AIS deficits would apply to divergent patient populations - REP and non-REP. Our group has shown that human neural stem cell-derived EVs (NSC EVs) exhibit restorative function in non-REP AIS animal models, but NSC EV has not been tested in a REP AIS model. The goal of this study was to evaluate the therapeutic potential of NSC EV in a rat REP AIS model.
Methods: NSC culture media underwent TFF and chromatography to separate and concentrate NSC EVs followed by NTA for quantification. To assess mechanism of action in vitro, activated microglia were treated with NSC EV, and proinflammatory CCL2 was quantified by ELISA after 24hr. AIS was induced in rats (n = 16/group) via intraluminal filament insertion to the origin of the middle cerebral artery. After 90min, the filament was removed to model REP. NSC EVs (2.7e11 particles/kg) or vehicle was given IV at 6, 24, and 48hr post-AIS. Cylinder (Day 7) and adhesive removal testing (ART, Day 10) evaluated sensorimotor function. Lesion size (Day 3) and cerebral atrophy (Day 91) were also measured.
Results: Study results showed that NSC EV significantly reduced CCL2 in activated microglia. In rats, NSC EV reduced lesion size by 42% on D3, which led to a 10.2% improvement in paretic forelimb use on D7. On D10, NSC EV animals returned to normal function during ART while vehicle animals remained impaired. On D91, there was a 24.2% improvement in cerebral atrophy in NSC EV animals compared to vehicle animals.
Summary/Conclusion: This study demonstrates that NSC EV enhances recovery in a REP AIS model, which may be driven by CCL2 modulation in activated microglia. These results strongly suggest that NSC EV therapy can be applied in multiple types of AIS patient populations.
Funding: NIH R44NS103596.
Keywords: neural stem cell, ischemic stroke

PS08.08 | Dissetting the role of autophagy in metastatic Neuroblastoma

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Introduction: Around 70% of patients with metastatic NB at diagnosis present bone marrow (BM) infiltration, this specific tropism to the BM has not been completely elucidated. Autophagy is a self-degradative process that plays a homeostatic role in normal cells. In cancer cells, it plays a different role based on the context: suppresses and facilitates tumorigenesis. Emerging evidence suggest that, bone metastasis can be supported by MSCs through the creation of metastatic niches and that the dialogue between tumor cells and the surrounding tumor microenvironment may be mediated by exosomes. The aim of this work is to evaluate the role of autophagy in the BM metastatic niche formation through ii) the analysis of autophagy-related proteins into NB cells-derived exosomes; iii) the study of the autophagic pathway in MSCs cells
Methods: Autophagic flux has been evaluated by western blot analysis in 8 NB cell and MSCs isolated from BM of NB patients with/without BM involvement and healthy control (HC-MSCs). Exosomes of NB cell lines have been isolated by high-speed ultracentrifugation. Protein content was analyzed by high-resolution mass spectrometry for the presence of proteins implicated in autophagy. Modification in autophagy flux in MSCs has been evaluated after co-culture with NB-derived exosomes
Results: analysis of basal levels of autophagy revealed that: i) NB cell lines have basal levels of autophagy ii) HC-MSCs have higher basal levels of autophagy than MSCs derived from NB patients; iii) NB-derived exosomes contain proteins implicated in autophagy pathway regulation.
Summary/Conclusion: our study gives new insights into the characterization of the autophagy pathway in NB and in the BM niche. The understanding of this molecular process could help us to develop new therapeutic approaches in patients affected by metastatic NB.
Funding: This work was supported by grants from the Ministero della Salute (GR-2016-02364088 to ADG).
Keywords: exosomes, neuroblastoma, autophagy

PS08.09 | Glioblastoma derived small extracellular vesicles: Nanoparticles for glioma treatment

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Introduction: Glioblastoma (GBM), characterized by a fast growth and invasion into adjacent tissue, is the most aggressive cancer of brain origin. Current protocols that include cytotoxic chemotherapeutic agents effectively treat localized disease, however, these aggressive therapies present side effects due to the high doses being administrated. In order to reduce the therapeutic exposure of the patients, we look for more efficient ways of drug delivery.
Methods: We have isolated and fully characterized small EVs from seven patient derived GBM cell cultures. Besides, we loaded them with two chemotherapeuric drugs (Temozolomide and EPZ015666) by incubation methods and, the amount of drug loaded was measured by high-performance liquid chromatography (HPLC) method. Finally, we test their antiproliferative effect on different cancer cell lines.
Results: Our study indicates that the direct incubation method was the most efficient loading protocol and that a minimum total amount of drug triggers an effect on tumor cells. Moreover, we have seen that GBM derived small EVs, although with lower target specificity, can also induce an effect on cancer cells of different nature.
Summary/Conclusion: GBM derived small EVs represents a promising drug delivery tool that can be further investigated in preclinical studies and be potentially developed in clinical trials for the treatment of GBM.
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Keywords: glioblastoma, small EVs, nanocarriers, chemotherapy, FESEM

PS08.10 | Runner plasma-derived extracellular vesicles reduce neuroinflammation and improve cognitive function in a rat model of Gulf war illness

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Introduction: Gulf war illness (GWI), typified by persistent cognitive dysfunction, is linked to unrelenting neuroinflammation. Physical exercise is known to produce antiinflammatory cellular changes, which are potentially also reflected in the extracellular vesicles (EVs) released by multiple cell types into the circulating blood. This study tested the therapeutic effects of EVs isolated from the plasma of sedentary rats (naive EVs or nEVs) and rats that performed voluntary running (runner EVs or rEVs) in a rat model of GWI.
Methods: nEVs were isolated from the plasma of 3-month-old sedentary rats, whereas rEVs were isolated from the plasma of age-matched rats housed in cages fitted with voluntary running wheels for 28 days. Both nEVs and rEVs were isolated from the plasma through the size-exclusion chromatographic method. The GWI rats were raised through daily exposure of two-month-old rats to GWI-related chemicals pyridostigmine bromide, DEET, and permethrin and 15 min restraint stress for 28 days. Six months later, GWI rats received weekly intranasal nEVs or rEVs (1000 × 109 EVs/week) for 3 weeks or no treatment. The rats were subjected to behavioral studies to examine cognitive function, after which they were euthanized (~3 months after the first dose of EV treatment). Brain tissues were processed for analyzing neuroinflammation.
Results: rEV treatment improved spatial recognition memory and pattern separation in GWI rats, but nEV treatment showed no such improvements. Analysis of hippocampal tissues revealed decreased percentages of microglia displaying CD68 and diminished levels of markers of NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome activation in GWI rats

receiving either nEVs or rEVs. However, the suppression of inflammasome activation was more significant in GWI rats receiving rEVs.
Summary/Conclusion: Intranasal administration of rEVs from the plasma of runners can improve cognitive function in GWI.
Funding: Supported by grants from the NINDS and NIA (1R01NS106907 and 1RF1AG074256-01A1 to A.K.S.) and Texas A&M University School of Medicine.
Keywords: plasma -derived extracellular vesicles, gulf war illness, neuroinflammation, physical exercise

PS08.12 | Bulk and single-particle characterization of Extracellular Vesicles (EVs) in relapsing and secondary progressive Multiple Sclerosis (MS) –Towards the identification of neurodegenerative prognostic markers in MS

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Introduction: Multiple sclerosis (MS), a chronic demyelinating disorder of the central nervous system (CNS), and its cause remains unknown. The most common form of MS, relapsing-remitting MS (RRMS), is characterized by discrete relapses followed by neurological recovery. Most RRMS convert to the secondary progressive form of MS (SPMS), in which accumulation of neurological dysfunction occurs in the absence of relapses. No reliable biomarkers exist to distinguish RRMS from SPMS or inform mechanisms of MS progression over time. EVs have emerged as potential prognostic biomarkers in various neurological disorders and may also provide mechanistic insights into neurodegenerative pathomechanisms.
Methods: Archival serum samples from RRMS and SPMS (n = 5 per group) were processed via tangential flow filtration (TFF) for EV isolation. Microfluidic Resistive Pulse Sensing (MRPS) measured particle size and concentration. EVs were characterized via immunoblotting, Exoview, and Total Internal Reflection Fluorescence (TIRF) microscopy for candidate CNS and EV markers. EV morphology was assessed by Transmission Electron Microscopy (TEM). Quantitative label-free LC-MS/MS proteomics analysis was performed on serum EVs.
Results: TEM of serum EVs demonstrated a cup-shaped morphology. Serum EVs were positive for known EV markers (i.e., CD63, CD81). Bulk analysis of EVs revealed the presence of GFAP+ particles. Single EV analysis via TIRF microscopy showed EVs positive CNS markers such as Tau, beta-amyloid, GFAP, and alpha-synuclein. Proteomics analysis showed proteins related to metabolic processes, protein binding, and cytoskeleton proteins. Cross-validation of Exoview tetraspanin profiles is ongoing.
Summary/Conclusion: In this study, we present an experimental workflow that outlines the enrichment and orthogonal characterization of serum EVs from MS patients for prognostic biomarker discovery. Our preliminary results demonstrate the presence of CNS markers in serum EVs of MS patients, assessed by bulk and single EV analysis—identifying a potential liquid biopsy for neurodegenerative diseases of the CNS.

PS08.13 | Alexander Disease: A Case Study of Erectile Dysfunction and Memory Impairment, role of the unconventional Secretory Pathway in pathomechanism

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Introduction: Alexander disease (AxD) is a rare and fatal neurological disorder that affects the central nervous system. A broad set of symptoms and the presence of Rosenthal fibres characterize AxD. The disorder is caused by mutations in the glial fibrillary acidic protein (GFAP), a protein found in the cells that support and protect nerve cells in the brain. Studies have shown that mutations in GFAP can lead to the formation of abnormal protein aggregates, which contribute to the development of AxD. The specific mutations that cause AxD have been identified, and research is ongoing to understand the underlying mechanisms of the disease.
Methods: Cell culture. COS7 cells were a gift from Prof Michael Schrader, University of Exeter. COS7 cells were grown in T75 flasks in DMEM with the addition of foetal bovine serum (FBS) 10%v/v and penicillin streptomycin 1%v/v. Protein detection using Western Blot. Running buffers were prepared using NuPAGE MES gels. Immunocytochemistry. COS7 cells were grown on coverslips in 6 well plates at a density of 200,000 cells per well and transfected with GFAP wild types and mutants and later

fixed and stained with anti-GFAP primary and fluorescent Alexa secondary ABs. Microscopy & Image analysis. A Leica TCS SP8 microscope was used to obtain Z-stacks at a magnification of 63x.
Results: The patient, a 40-year-old male, presented with a one-week history of anterograde amnesia and apathy and was found to have subclinical corticospinal tract dysfunction. MRI imaging showed progressive volume loss in the medulla, upper cervical spinal cord, and supratentorial brain. Neuropsychological testing indicated persistent deficits in learning and memory likely caused by sub-cortical cognitive impairment. Genetic analysis confirmed a GFAPG301D mutation, and in vitro experiments showed that the mutation resulted in large aggregates and changes in protein conformation or post-translational modifications. The study highlights the need for further research on the link between GFAP mutations and the unconventional secretory pathway in AxD. We utilized the Tunable Resistive Pulse Sensing measurement using a qNanoGold device to analyze the effects of GFAP variants on EVs release. Concentration of EVs were significantly reduced in the case of GFAP AxD-causing variants in comparison to the GFAPwt. AxD mutants also affected size of secreted vesicles in comparison to the GFAPwt, suggesting that AxD-causing mutations may reduce release of EVs from cells and influence size of secreted EVs
Summary/Conclusion: In this study, we present a case of Alexander disease (AxD) characterized by erectile dysfunction and memory impairment as the main clinical symptoms. We provide the first evidence that mutations in the glial fibrillary acidic protein (GFAP) can impair the unconventional secretory pathway.

PS08.14 | Human iPSC-NSC-EV treatment after a repeated closed head injury prevents enduring cognitive dysfunction associated with chronic neuroinflammation

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Introduction: Repeated closed head injuries (rCHIs) typically lead to long-term cognitive deficits linked with chronic neuroinflammation. Thus, biologics capable of restraining the progression of neuroinflammation after rCHI may prevent cognitive impairments. This study examined the ability of intranasally (IN) administered extracellular vesicles (EVs), purified through chromatographic methods from cultures of human induced pluripotent stem cell (hiPSC)-derived neural stem cells (NSCs), to prevent cognitive impairments after rCHI.
Methods: Adult mice subjected to rCHI for three days (one CHI/day) received IN administration of hiPSC-NSC-EVs or the vehicle at 60 minutes (70 × 109 EVs) and seven days (35 × 109 EVs) after the third CHI. Eight months later, the animals were tested for motor function, anxiety-like behavior, and cognitive function, after which the brain tissues were examined for oxidative stress and neuroinflammatory markers.
Results: rCHI mice receiving either vehicle or EVs displayed no motor impairments or anxiety-like behavior. However, in objects-based cognitive tests, vehicle-treated rCHI mice displayed impairments in novel objection recognition, object location memory, and pattern separation. However, rCHI mice that received EVs displayed similar cognitive abilities as naïve control mice. Analysis of the affected cerebral cortical tissues revealed an increased concentration of oxidative stress and NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome-related markers and proinflammatory cytokines interleukin-1 beta (IL-1b), IL-6, tumor necrosis factor-alpha, interferon-gamma, IL17-alpha, and IL13. Notably, in rCHI mice receiving EVs, levels of most of these markers were reduced along with reduced microglial activation and astrocyte hypertrophy.
Summary/Conclusion: IN administration of NSC-EVs is a promising approach for maintaining better cognitive function after rCHI through modulation of neuroinflammation.
Funding: Supported by a grant from the National Institute of Neurological Disorders and Stroke (1R01NS106907 to A.K.S).
Keywords: neural stem cells, extra cellular vesicles, closed head injury, neuroinflammation, cognitive function

PS08.15 | Intranasal hiPSC-NSC-EV treatment 90 minutes after traumatic brain injury inhibits proinflammatory transcriptomic signatures in microglia

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Introduction: Introduction: Acute neuroinflammation after a traumatic brain injury (TBI) activates microglia, resulting in the perpetuation of chronic neuroinflammation linked with cognitive dysfunction.
So, biologics proficient in modulating proinflammatory microglia may prevent chronic neuroinflammation after TBI. This study investigated the ability of intranasally (IN) administered extracellular vesicles (EVs), purified through chromatographic methods from cultures of human induced pluripotent stem cell (hiPSC)-derived neural stem cells (NSCs), for modulating genes related to proinflammatory and antiinflammatory signaling cascades after a TBI.
Methods: Methods: Adult mice received IN administration of hiPSC-NSC-EVs (25 × 109 EVs) or the vehicle 90 minutes after a unilateral moderate controlled cortical impact injury induction. Seventy-two hours later, a cohort of mice was euthanized, microglia were isolated from the injured cerebral hemispheres, and the expression of genes linked to multiple inflammatory signaling pathways was examined through scRNA sequencing.
Results: Results: Microglia from vehicle-treated TBI mice displayed enrichment of genes related to type-1 interferon (IFN)-, IFN-gamma, and interleukin 6 (IL6)-signaling, damage-associated microglia (DAM), and the complement system, involved in innate immune response and various inflammatory processes. Notably, the expression of many of these genes was either normalized to control levels or reduced in TBI mice receiving hiPSC-NSC-EVs. Moreover, the expression of multiple genes linked to antiinflammatory pathways, such as IFN-β, IL4, and IL10 signaling, were either maintained or further enhanced with hiPSC-NSC-EV therapy. hiPSC-NSC-EV treatment also alleviated the upregulation of senescence-related genes after TBI.
Summary/Conclusion: Conclusion: IN administration of hiPSC-NSC-EV treatment after TBI can attenuate the expression of genes involved in type-1 IFN, IFN-gamma, and IL6 signaling, DAM, complement activation, and cellular senescence in microglia.
Funding: Supported by a grant from the National Institute of Neurological Disorders and Stroke (1R01NS106907 to A.K.S.).
Keywords: neural stem cells, extracellular vesicles, traumatic brain injury

PS08.16 | Astrocyte-derived cargo cytokines from extracellular vesicles correlate with anhedonia and pain in Veterans with and without PTSD

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Introduction: Growing evidence suggests inflammation plays a role in trauma-related psychiatric disorders. Studies suggest PTSD is associated with altered immune protein levels. However, little is known about the relationship between central and peripheral inflammation in driving PTSD.
Methods: To specifically probe the relationship between the central nervous system (CNS) and peripheral inflammation, we isolated astrocyte-derived extracellular vesicles (ADEs) from peripheral blood plasma samples from subjects with and without PTSD (N = 32). After vesicle lysis, cargo protein cytokines were quantified using multiplex enzyme-linked immunosorbent assay plates. These cytokines were compared to clinical measures of PTSD using the PCL-5 and PHQ9.
Results: As has been previously demonstrated in the literature, we found that participant plasma correlated with PTSD clinical symptoms measured by PCL-5, using BMI and age as covariates (IL-1b, R = 0.35, p = 0.0062; IL-6, R = 0.30, p = 0.019; TNFa, R = 0.34, p = 0.0071). However, most cytokines from ADEs did not correlate significantly with plasma cytokine levels (ρ ranging from 0.059 - 0.570), except for IL-2 measured from both plasma and ADEs (ρ = 0.498, = 0.170, p =.004). Notably, we detected a significant relationship between anhedonia as measured and ADE IL-1b and IL-2 levels (ρ = 0.493, p< 0.01; ρ = 0.489, p< 0.01).
Summary/Conclusion: Our findings that few plasma cytokines correlate with cytokines isolated from ADEs suggest that ADE cytokines may represent a tissue-specific signature of CNS immune dysregulation. Furthermore, these exploratory findings highlighting the association between ADE cytokine levels and anhedonia underscore that ADE's may hold promise to identify immune dysfunction in neuropsychiatric disorders.
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Keywords: immune, astrocyte, cytokine

PS09: Preeclampsia, Reproduction and Development

Location: Hall 4A

16:50 - 18:50

PS09.03 | Outer-membrane vesicles derived from Akkermansia muciniphila mitigate preeclampsia by promoting placentation through the EGFR–PI3K–AKT pathway

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Introduction: Preeclampsia (PE) is a multisystem disorder with high maternal morbidity and mortality rates. No practical therapeutic approach is available, except for early delivery. Gut dysbiosis is also associated with PE development. However, the host response to microbiome-based therapy for PE remains unknown.
Methods: Previous data showed that the abundance of Akkermansia muciniphila (Am) was lower in patients with PE than in normotensive pregnant women, which was also found using a mouse model of PE.
Results: TPre-supplementation with Am significantly mitigated pre-eclamptic symptoms in the murine model. Am-derived outer-membrane vesicles (AmOVs) entered the placenta and improved the placental pathology in mice with PE. These beneficial effects of AmOVs were mediated by enhanced trophoblast invasion of the spiral artery (SpA) and SpA remodeling through activation of the EGFR–PI3K–AKT signaling pathway.
Summary/Conclusion: Together, our findings indicate the potential benefit of using AmOVs for PE treatment and shed insight into host–microbiota interactions.
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Keywords: outer-membrane vesicles, akkermansia muciniphila, preeclampsia, EGFR–PI3K–AKT pathway

PS09.04 | Uncovering early circulating prognostic markers in pre-eclampsia

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Introduction: Pre-eclampsia (PE) is a serious complication affecting 1 in 12 pregnancies, defined by new-onset hypertension and proteinuria after 20 weeks of gestation. Annually, PE claims the lives of 76,000 mothers and 500,000 babies, making it the world's deadliest pregnancy complication. Early detection of PE is key for appropriate clinical decision-making throughout pregnancy to reduce life threatening complications. Extracellular vesicles (EVs) are important circulating messengers regulating a myriad of biological and pathological processes and may be highly relevant to the pathophysiology of PE, with levels shown to increase as gestation progresses. We hypothesise that EVs circulating in early pregnancy contain markers that may potentially predict occurrence of PE.
Methods: Ethical approval was gained to access the Biological Resource Bank in the Coombe Women and Infants University Hospital Dublin. Early pregnancy (12-15 weeks) plasma samples from healthy women (n = 67) and women who subsequently went on to develop a confirmed diagnosis of PE during their pregnancy (n = 27) were randomly selected from the biobank. Circulating EV particle concentration and size was determined using Nanoparticle Tracking Analysis and flow cytometry. For proteomic analysis, EVs were isolated using IZON qEV single columns and subjected to label-free quantitative proteomics analysis.

Results: We observed a trend towards an increase in particles in the size range of 50–200 nm in the plasma of women who went on to develop PE relative to control patients. Comparative proteomic analysis of EVs revealed that proteins originating from platelets were among the proteins significantly altered between the groups, which we confirmed using ELISA.
Summary/Conclusion: We have found differential levels of circulating vesicles in plasma of expectant mothers who went on to develop PE. Such anomalies identified at this crucial early stage of pregnancy when the placenta becomes definitive and takes over from the yolk sac, may underpin aberrant placental implantation and future pregnancy complications. Our results may provide clinicians with the much-needed tools to identify women at future risk of PE.
Funding: Science Foundation Ireland.
Keywords: early pregnancy, preeclampsia, platelets, placenta, proteomics, NTA, flow cytometry, plasma

PS09.05 | Extracellular vesicles from human preeclamptic placentae increase blood pressure long-term in a rat model

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Introduction: Preeclampsia is a hypertensive disease unique to human pregnancy. Preeclampsia can be divided into early or late onset depending on whether the disease presents prior to or after 34 weeks of gestation, respectively. The two variants of preeclampsia may have different pathogenic mechanisms. Women who have had a preeclamptic pregnancy are at increased risk of early cardiovascular morbidity and mortality. The mechanistic link between preeclampsia and early cardiovascular disease is unknown but the human placenta releases large quantities of extracellular vesicles (EVs) into the maternal blood and we hypothesize that placental EVs may be a mechanistic link.
Methods: Small, large and macro EVs were isolated from normotensive, early or late onset preeclamptic placentae by differential centrifugation and the EVs from each patient combined and injected into spontaneously hypertensive rats (n = 18). Blood pressure was monitored by tail cuff and cardiovascular function by echocardiography for 12 months. Vascular function was assessed by wire myography post mortem.
Results: Systolic blood pressure was significantly increased in the animals receiving early onset preeclamptic EVs (< 0.013), compared to the normotensive EVs, from 3 months post-injection to the end of the monitoring. Whereas, the difference in systolic blood pressure for the late onset preeclamptic EVs was significantly (p< 0.002) increased from one to 9 months post-injection. Body weight, heart rate and stroke volume were not significantly affected by the preeclamptic EVs but wire myography indicated that resistance vessels demonstrated significantly enhanced responsiveness to vasoconstrictors after treatment with preeclamptic EVs.
Summary/Conclusion: Our data show that administration of human placental EVs was well-tolerated by the animals. However, both early and late onset preeclamptic placental EVs cause long-term changes in cardiovascular function with sustained increases in blood pressure which might be explained in part by the heightened responsiveness of resistance blood vessels to vasoconstrictors.
Funding: This work was funded by the Health Research Council of New Zealand and the China Scholarship Council.
Keywords: placenta, preeclampsia, cardiovascular disease

PS09.06 | Changes in the miRNAs contents of placental extracellular vesicles with increasing gestational age may contribute to fetal development and maternal adaptations

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Introduction: Placental extracellular vesicles (pEVs) may be involved in the coordinated regulation of fetal development and maternal adaptation during pregnancy. In this study, we compared the miRNA profiles of pEVs derived from first trimester and term placentae to understand the changes in the miRNA profiles between two gestational ages and whether they may be involved in the regulation of fetal development or the dynamic changes in maternal adaptation to pregnancy.

Methods: Large and small pEVs were collected by differential centrifugation from first trimester and term placentae (n = 5 each). Small RNA was sequenced and significantly differentially abundant miRNAs were identified. Target gene enrichment analysis identified differentially abundant miRNAs that are associated with maternal adaptation and fetal development.
Results: In total there were 823 or 918 miRNAs present in large and small EVs, respectively. Of these miRNAs, 123 were more abundant and 177 were less abundant in term than first trimester large pEVs. In small pEVs, 70 miRNAs were more, and 138 miRNAs were less abundant in first trimester than in term pEVs. GO and KEGG analyses showed that these more or less abundant miRNAs may participate in processes required for maternal vascular adaptation including: angiogenesis, vascular genesis, regulation of blood vessels, endothelial cell migration, and blood vessel morphogenesis. Analysis using Placentacellenrich showed that fetal fibroblasts, decidual perivascular cells, and maternal endothelial cells are enriched for genes that are targets of the differentially abundant miRNAs in both large EVs and small EVs. Analysis using Funrich confirmed the interaction of the differentially abundant miRNAs with these target genes.
Summary/Conclusion: The miRNA content of small and large pEVs changes with increasing gestations. The changes in miRNA content of these EVs may reflect developmental changes in the fetus/placenta and/or regulate fetal development and maternal adaptation during pregnancy.
Keywords: placental extracellular vesicles, miRNAs, fetal development, maternal adaptation

PS09.07 | The role of brain-derived EVs in cognitive impairment associated with gestational omega-3 deficiency

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Introduction: Western diet is associated with a decreased intake of omega-3 polyunsaturated fatty acids (n-3 PUFAs). This is cause for concern as gestational deficiency of n-3 PUFAs is linked with cognitive alterations in both young and adult mice. Moreover, microglia from n-3 PUFA deficient animals display a shift towards an inflammatory phenotype, although the exact mechanisms remain unclear. Therefore, we aim to study the potential role that brain derived EVs (BDEVs) play in this pathway as EVs have been shown to participate in the pathogenesis of several cognitive-related diseases including Alzheimer's disease.
Methods: Male and female C57BL/6 mice were subjected to a chow diet deficient or sufficient with n-3 PUFAs from gestation until 3 months old. The object location memory (OLM) tests was used to assess cognitive function and electrophysiological recordings were made from CA1 neurons. BDEVs were subsequently enriched from entire brain homogenate samples using a protocol of differential centrifugation and density-based sucrose gradient. Nanoscale flow cytometry, western immunoblot and TEM were employed in order to characterise the phenotype and purity of EVs from both conditions. Gas-chromatography mass spectrometry was used to profile the fatty acid content of BDEVs.
Results: Our behavioural assessment revealed that mice subjected to n-3 PUFA deficiency had a decreased OLM performance which was significantly worsened in female mice compared to male mice. Moreover, intrinsic and/or extrinsic excitability properties were altered in pyramidal neurons from the CA1 and correlated to the gender differences observed in OLM. Preliminary analysis revealed that BDEVs are enriched for classical EV markers including CD81, CD9 and CD63. Moreover, a subset of BDEVs expressed the microglial marker, CSF1R, as detected by nanoflow cytometry.
Summary/Conclusion: Further characterisation of n-3 PUFA deficient and sufficient BDEVs will shed light on the function of n-3 PUFAs on overall brain health and may offer novel insight to the apparent gender dependency on cognitive alteration.
Funding: Chair Region Nouvelle-Aquitaine.
Keywords: fatty acid, CNS, cognition, brain, neuroimmunology

PS10: Lung EVs

Location: Hall 4A

16:50 - 18:50

PS10.03 | Preclinical safety and 'omic characterization of an EV-enriched BM-MSC therapeutic for acute respiratory distress syndrome

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Introduction: Many studies have now documented the safety and molecular profile of bone marrow-mesenchymal stem cells and their secretome. Still, safety and molecular content of each therapeutic is unique and must be characterized to support their clinical use. Herein are the results of murine preclinical safety, proteomic and extracellular vesicle (EV) identity studies of a BM-MSC EV enriched biologic currently being evaluated for its clinical safety and efficacy.
Methods: Safety was evaluated in murine models to assess acute tolerability, toxicity, and long term tumorigenicity. IgE levels were measured to assess acute allergic response after either subcutaneous or IV injection. Renal and liver histopathology was performed to assess acute toxicity (7 days). Long term (3 Month) tumorigenicity was determined in NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ(NSG) mice using up to 20x human dose equivalent concentration. Animal studies were performed in ALAAS approved facilities. In molecular characterization studies, the EV content was evaluated using electron microscopy, STED Microscopy, SP-IRIS, and NTA. Proteomic analysis was performed using standard mass spectrophotometric (MS) protein sequencing.
Results: Safety: The therapeutic was deemed safe. No evidence of acute immune response, toxicity, or of tumor induction was observed within the tested time and dose ranges. Blood analyses were within normal ranges after 7 days and after 90 days. Identity: EVs were enriched for CD63+ EVs versus CD9+ or CD81+ EVs. At least 1173 different proteins were identified by MS sequencing of 5 different lots. Protein content was consistent with previously published data sets for MSC EVs.
Summary/Conclusion: These data demonstrate a superior safety profile relative to many traditional small molecule and single protein biologics, consistent with prior BM-MSC therapeutics studies. The complex protein composition indicates a potential multi-molecular, multi-modal mechanism of action for efficacy of this BM-MSC therapeutic.
Funding: Studies were funded by industry company employing authors of this abstract.
Keywords: GLP preclinical safety, In vivo, proteomics, EV therapeutic identity

PS10.04 | The pro-inflammatory role of EVs in chronic lung rejection

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Introduction: At present, lung transplantation (LT) is the treatment of choice for most end-stage lung diseases. Unfortunately, the prognosis is still relatively poor mainly due to chronic lung allograft disease (CLAD). Moreover, no early accurate biomarkers of graft rejection are known, thus we assessed CLAD lung microenvironment by profiling extracellular vesicles (EVs) from bronchoalveolar lavage (BAL) of LT patients.
Methods: We extracted EVs from BAL, then we analysed them for quantity and size by Nanoparticle Tracking Analysis (NTA). After purification by ultracentrifugation with saccharose gradient, we characterized the fractions by western blotting for characteristic markers (ARF6, TSG101, CD63) and electron microscopy. After EV coculture with normal bronchial epithelial cells (HBEpC), we analysed the culture supernatants for cytokines expression.
Results: After co-culture with EV-CLAD, we found up-regulation of secreted GM-CSF, while TNF-alpha, TREM-1, MIP-1, and IL-10/27 were down-expressed together with IL-17a. In cell lysates from EV-CLAD co-cultures, MIF, IL-1-ra and SERPIN were up-regulated, whereas MIP-1, CCL5 and TREM-1 were downregulated.
Summary/Conclusion: Overall, these results suggest a role for EVs in the axis CCL5-CCR-1, which regulates leukocyte migration and monocyte migration. Moreover, the up-regulation of MIF suggests that EVs may be involved in macrophage infiltration, and possibly in tissue repair during CLAD.
Keywords: lung graft rejection, cytokines

PS10.06 | Differential secretion of extracellular vesicle-bound YRNAs in the allergic airway

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Introduction: YRNAs and YRNA fragments (YsRNAs) are small, noncoding RNAs that cells secrete as ribonucleoprotein complexes (RNPs) or in extracellular vesicles (EVs). Cells can increase or decrease EV-bound YRNA/YsRNA secretion levels in response to inflammatory stimuli. However, little is known about how YRNAs are secreted during inflammation in vivo. Previous work from our lab shows that select EV-bound miRNA levels increase in murine bronchoalveolar lavage fluid (BALF) during experimentally induced allergic airway inflammation. In this study, we tested if murine YRNAs (RNY1 and RNY3) and their YsRNAs are differentially secreted in EVs during allergic airway inflammation.
Methods: Whole BALF from mice was treated with RNase, proteinase K, and detergent. qPCR was used to quantify YRNA in the steady state lung. To induce allergic airway inflammation, mice were sensitized and challenged with ovalbumin (ova). EVs were purified by sucrose density-gradient ultracentrifugation from ova-challenged and vehicle-challenged mouse BALF. qPCR was used to quantify YRNA levels in each fraction.
Results: We detected RNY1, RNY3, and their respective fragments in whole BALF and all three sucrose-gradient fractions. Whole BALF YRNAs were RNAse-sensitive in the presence of detergent, but not proteinase K. Additionally, a population of RNY1 fragment was only RNAse sensitive in the presence of proteinase K and detergent. In whole BALF and density gradient purified BALF, RNY1 fragment levels increased in mice with allergic lung inflammation compared to controls.
Summary/Conclusion: Murine BALF contains YRNA, and YRNA is present in BALF EVs. A portion of extracellular RNY1 fragment may be bound to protein in EVs. RNY1 fragment levels increase in BALF EVs during allergic airway inflammation. These data contribute to the growing evidence that YRNAs are selectively secreted and may contribute to cell-cell communication in inflammatory states.
Funding: NIH DP2-HL152426 (HHP).
Keywords: YRNA, extracellular RNA, allergy, inflammation, lung

PS10.09 | The therapeutic effect of small extracellular vesicles from hypoxia-preconditioned, adipose-derived stem cells on pulmonary fibrosis

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Introduction: Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating disease with high mortality rate and the pathogenesis is still not clear. Mesenchymal stem cells (MSCs) with multipotency are capable of regulating inflammation and are one of the promising therapeutic candidates for diverse inflammatory diseases and various tissue repairs. However, stem cell therapy has well-known limitations, such as unwanted immune reactions, low transplantation rate, and potential tumorigenic effects. Small extracellular vesicles (sEVs), which regulate cell-to-cell communication by paracrine effects, could be an alternative to stem cell therapy. Recent studies showed that hypoxia-conditioned MSCs have immunosuppressive and anti-fibrotic effects. In this study, we investigated the effect of EVs secreted from human adipose-derived stem cells (HASCs) during hypoxia condition on IPF.
Methods: sEVs are isolated from hypoxia-conditioned cell culture medium by pre-filtration through a 0.2 μm syringe filter, followed by tangential flow filtration (TFF) system. The sEVs were characterized by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), dynamic light scattering (DLS), flow cytometry, cytokine array, and western blot analysis.
Results: The sEV treatment showed the reduced fibrotic proteins including collagen type 1, fibronectin, and alpha-smooth muscle actin (α-SMA). The intravenous injection and aerosol inhalation of sEVs alleviated fibrosis in bleomycin-induced lung fibrosis mouse models, by histological analysis (H&E staining and trichrome staining) and lung tissue collagen assay.
Summary/Conclusion: Our finding suggests that the administration of hypoxia-preconditioned sEV by inhalation can be an easy and efficient strategy for the treatment of IPF.

PS10.11 | Targeted exosomal delivery of NRF2 siRNA against lung cancer

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Introduction: Small interfering RNA (siRNA) as a potential intervention for cancer is wrought with possibility; however, despite ongoing efforts to identify oncogenic targets and effective delivery vehicles, no siRNA-based therapeutic for lung cancer has reached the clinic. Based on their natural trafficking and targetability, exosomes can overcome many delivery obstacles. Nuclear factor erythroid 2-related factor 2 (NRF2) is aberrantly expressed in many patient lung tumors and is a strong indicator of poor prognosis and resistance to conventional treatment. We report on targeted delivery of siRNA against NRF2 (siNRF2) using bovine colostrum exosomes to inhibit lung cancer in a murine model.
Methods: Exosomes, isolated from bovine colostrum powder by rehydration and ultracentrifugation, with and without functionalization using tumor-targeting ligand folic acid (FA), were complexed with polyethyleneimine (FA-EPM or EPM, respectively) and siNRF2. siNRF2 candidates were screened for efficacy in lung cancer cells in vitro. Exosomal formulations of the lead siNRF2 candidate were tested in vivo against A549 subcutaneous tumors and orthotopic lung tumors produced by inoculation with luciferase-expressing A549-Luc cells in NOD Scid mice.
Results: EPM-siNRF2 decreased expression of NRF2 >85% in vitro in A549 and H1299 lung cancer cell lines. This decrease in NRF2 corresponded to >50% knockdown of downstream targets of NRF2, such as hemeoxidase-1 and significant reduction in cell viability. The FA-EPM delivered siNRF2 given 15 μg intravenously, 3 times per week showed significantly greater inhibition of the orthotopic lung tumors (>80%) than the non-functionalized formulation (>50%); NRF2 reduction was seen only in the tumor, not the adjacent lung. FA-EPM-delivered siSCR (scrambled siRNA) was ineffective.
Summary/Conclusion: FA-functionalized exosomal formulation of siNRF2 resulted in enhanced inhibition of orthotopic lung tumors compared to non-functionalized formulation due to tumor targeting, and was accompanied by downregulation of the target protein, NRF2.
Funding: Supported from 3P Biotechnologies, Inc. and, in part, from Duggan Endowment.
Keywords: colostrum exosomes, siNRF2, lung cancer, tumor targeting

PS10.12 | Targeted oral exosomal delivery of cannabidiol for lung cancer

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Introduction: Cannabidiol (CBD) is a potential anticancer plant bioactive. However, low oral bioavailability and rapid hepatic metabolism limits its application. Bovine colostrum has been discovered to be an abundant source of exosomes and can be used for drug delivery. In this study, we report a targeted oral delivery system for CBD as an effective cancer therapeutic.
Methods: Exosomes were isolated from bovine colostrum powder by rehydration and ultracentrifugation. Exosomes were functionalized with the targeting ligand, folic acid, and loaded with CBD. Exosomes were characterized for size and charge and hallmark proteins and the UPLC was used to determine CBD loading. The antiproliferative and colony formation inhibitory activity of CBD and exosomal CBD (ExoCBD) was tested against drug-sensitive (A549 and H1299) and drug-resistant (A549TR) lung cancer cells. The molecular mechanism was assessed by Western blot and the anti-cancer effects were determined against A549 orthotopic lung tumor xenografts in mice.
Results: Colostrum exosomes exhibited size (107 ± 3 nm), charge (-21 ± 0.7) and carried typical surface proteins. High drug load was observed (~20%) on exosomes and FA-functionalized exosomes. Particle size of ExoCBD (127 nm) and FA-ExoCBD (134 nm) was somewhat increased vs. exosomes. CBD and its exosomal formulations showed dose-dependent antiproliferative

and colony formation inhibitory activities. Orally delivered FA-ExoCBD (≈80%; p< 0.001) showed higher tumor inhibition vs. the free CBD (≈60%; p< 0.01) at half the CBD dose. Mechanistically, CBD demonstrated dose-dependent downregulation of MDR-1, NFκB and oncogenic CB2, and upregulation of the anti-proliferative GPR3 in A549 and A549-TR cells.
Summary/Conclusion: Targeted oral exosomal formulation of CBD inhibits lung cancer via favorable modulation of GPR3, NFκB, oncogenic CB2 and multi-drug resistance (MDR) pathways.
Funding: Supported from 3P Biotechnologies and Duggan Endowment.
Keywords: colostrum exosomes, lung cancer, CBD, oral delivery, tumor targeting

PS11: EVs from Microorganisms 2

Location: Hall 4A

16:50 - 18:50

PS11.02 | Klebsiella pneumoniae-derived small RNAs are delivered into host cells by bacterial extracellular vesicles

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Introduction: Klebsiella pneumoniae is a commensal bacterium that colonizes human mucosal surfaces, the gastrointestinal tract, and the oropharynx. It can cause serious infections, such as pneumonia, urinary tract infections, and liver abscesses, primarily in immunocompromised patients. To date, the mechanisms behind K. pneumoniae-host cell interactions and disease development in immunocompromised patients remain unclear. Recently, some studies indicated bacterial small RNAs may mediate host cell signaling. This study investigated how K. pneumoniae transports small RNAs into host cells.
Methods: LacZ reporter AGS cells were constructed to express LacZ protein by frameshift mutation induced by Cas9-sgRNA complexes upon internalization of specific single guide RNA (sgRNA). K. pneumoniae ATCC 43816 (WT-Kp) was transfected via electroporation with specific sgRNA. LacZ reporter AGS cells were infected with sgRNA-transfected K. pneumoniae (sgRNA-Kp) using transwell insert chambers (MOI = 1:1). Bacterial extracellular vesicles (EVs) were collected using ultracentrifugation and characterized using transmission electron microscopy. Nanosight and qNano were used to measure the size and concentration of bacterial EVs.
Results: Electroporation of sgRNA significantly decreased the mucoid phenotype of K. pneumoniae, and caused a loss of capsules on bacterial surfaces. Although the number of bacterial EVs produced by sgRNA-Kp remained unchanged, the reduction of diameter in EVs isolated from sgRNA-Kp was observed when compared to the EVs derived from WT-Kp by a loss of capsules region. Quantitative PCR analysis detected transfected sgRNAs contained in bacterial EVs. Then, to examine whether transfected sgRNAs are transported and internalized into host cells via bacterial EVs, LacZ reporter AGS cells were infected with sgRNA-Kp. As a result, lacZ positive cells were detected among sgRNA-Kp-infected LacZ reporter AGS cells. Additionally, LacZ-positive cells were also detected by adding purified-bacterial EVs to reporter AGS cells. These results indicate that bacterial EVs transport own sgRNAs into host cells. Our finding show that bacterial EVs derived from K. pneumoniae can deliver the small RNAs into host cells regardless of capsule structure.
Summary/Conclusion: Our findings demonstrate that bacterial small RNAs transported by bacterial EVs may serve a role as the main tool in bacteria-host cell communication.

PS11.03 | Extracellular vesicles of Bacillus cereus: New cues to B. cereus pathogenicity

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Introduction: The Gram-positive spore-forming bacterial pathogen Bacillus cereus which is well-known for its ability to cause food poisoning [1], is gaining prominence as a causal agent of non-GI-related illnesses, including systemic and local infections [2]. With our present research, we show, using in-vitro models, the involvement of B. cereus derived EVs in systemic infections.

Methods: In contrast to EVs of Gram-negative bacteria, EVs in Gram-positive bacteria are far less studied and their role in bacterial physiology and pathogenicity is still elusive. Thus, B. cereus derived EVs were isolated from reference strains and clinical isolates and characterized using resin-embedded transmission electron microscopy, nanoparticle tracking analysis (NTA), FTIR Spectroscopy, and immunoblotting. To gain first insights into EV-mediated in vitro cytotoxicity, bone-marrow-derived macrophages were treated with EVs, and cytokine responses were analyzed. High-resolution imaging was performed to analyze DiO-labeled EV uptake by macrophages using laser scanning confocal microscopy.
Results: Our results demonstrate that EVs represent an important mechanism for the transport of B. cereus virulence factors. Using different microscopy techniques, we investigated the mechanisms of EV- processing by macrophages. Following EV uptake, cytotoxic effects and pro-inflammatory responses were observed in a dose and time-dependent manner in addition to pro-inflammatory responses.
Summary/Conclusion: In this study, we show for the first time that EVs secreted by B. cereus induce a strong pro-inflammatory response in-vitro. Therefore, it is anticipated that this research will provide new ways of understanding the EV contribution to B. cereus pathogenicity and will aid in the development of novel defense mechanisms against pathogenic B. cereus strains.
Funding: This project is partially funded by Hochschuljubiläums Fonds of the City of Vienna (H-409332/2021).

PS11.04 | Extracellular vesicles shaping the colorectal cancer microbiome

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Introduction: The human gut microbiota plays a vital role in the regulation of various physiological processes, and alterations in the composition and function of the system (dysbiosis) are associated with the pathogenesis of colorectal cancer (CRC) 1. Although the causative link between CRC and microbiota is widely investigated, the underlying microbiota-gut interactions are not well understood yet. It is evident that CRC-derived extracellular vesicles (EVs) have an impact on various oncogenesis processes 2, however, their impact on the surrounding microbiota is not clear. Therefore, we hypothesise that EVs could have an impact on the microbiota and contribute to dysbiosis.
Methods: two CRC cell lines (SW480, SW620) were cultured in CELLine AD 1000 bioreactor flasks, and blood was collected from CRC patients and healthy individuals. EVs were isolated from the culture media and blood plasma by size-exclusion chromatography and characterised by nanoparticle flow cytometry (NanoFCM), western blotting, ELISA, and transmission electron microscopy (TEM). The impact of the EVs on the bacterial (MG1655 E. coli (Laboratory strain) and 11G5 E. coli (CRC-associated strain)) phenotypic characteristics (growth curve, biofilm formation) was assessed. Flow cytometry, confocal microscopy, and TEM were performed to assess the interactions between EVs and E. coli strains.
Results: NanoFCM analysis showed a high yield of EVs with characteristic size profiles, and EVs markers detection confirmed the presence of EVs. TEM analysis indicated an interaction between the EVs and E. coli with clear surface binding, and EV treatment had an impact on bacterial phenotypic characteristics; an increase in E. coli growth and a decrease in the ability of the bacteria to form biofilm were shown.
Summary/Conclusion: Overall, EVs appeared to be capable of mediating CRC-microbiome interactions.
Funding: Sheffield Hallam University.
Keywords: colorectal cancer, gut microbiome, dysbiosis

PS11.05 | The good, the bad, and the EV: an investigation into the effect of host colorectal extracellular vesicles (EVs) on probiotic and pathogenic Escherichia coli

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Introduction: Human extracellular vesicles (hEVs) were hypothesised to be possible vehicles for a communication axis from eukaryotic cells to bacteria in the gut.
Methods: Small EVs (sEVs) were isolated from a human colorectal adenocarcinoma cell line, HT29, grown in a CELLine bioreactor for 230 days in Advanced RPMI with EV-depleted FBS. sEVs isolated using differential centrifugation (2,000 × g, 10,000 × g, 100,000 × g), were purified with size exclusion chromatography. The sEVs were characterised by nanoparticle tracking analysis, protein analysis, transmission electron microscopy, and Western blots. Pathogenic enteroinvasive Escherichia coli (EIEC) and probiotic E. coli Nissle were treated with three doses of HT29-EVs: ~2 × 108, ~2 × 109, and ~2 × 1010 EVs/mL, and grown in physiological iron-restricted media (RPMI-1640) or iron-replete media (RPMI + 10 μM FeCl3 (RPMIF)).

Results: In RPMI for each increasing HT29-EV dose, E. coli Nissle growth was increased by ~20%, ~55%, and ~130%; EIEC growth was increased by ~80%, ~40%, and ~580% (plate counts at 8 h). In RPMIF, hEVs did not affect growth of E. coli Nissle or EIEC. Bacteria were treated with CMTPX-Red labelled HT29-EVs in RPMI (no phenol red). Bacteria were counterstained with SYTO9 (green). Fluorescence-activated cell-sorting separated bacteria-only, EV-only, and bacteria+EV populations. Confocal microscopy showed co-location of live, green-stained E. coli Nissle and red fluorescent EVs, indicating a biological interaction. Transcriptomic analysis of E. coli Nissle and EIEC total RNA was performed after treatment with unlabelled HT29-EVs (~2 × 109 EVs/mL) in RPMI and RPMIF. RNA-Seq (Illumina Hi-Seq) showed that genes involved in molybdenum uptake were upregulated, while genes involved in molybdenum cofactor synthesis were downregulated.
Summary/Conclusion: This work provides new insight into the complex host-microbe relationship in the context of health and disease and indicates that hEVs may play an important role.
Funding: This work was supported in part by a grant from Manatū Hauora Health Research Council of New Zealand (HRC) (18/735). Jiwon Hong is funded by the Hugo Charitable Trust. Joni White is funded by a University of Auckland Doctoral Scholarship.
Keywords: human extracellular vesicles, bacteria, interkingdom communication

PS11.07 | Adapting methods from microbiome research to characterize EVP miRNA profiles in human milk

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Introduction: MicroRNAs (miRNAs) carried by extracellular vesicles and particles (EVPs) in breast milk are hypothesized to facilitate maternal-offspring communication. The study of EVP miRNAs is complicated by the zero-inflated nature of the data, which is not addressed by standard methods for the analysis of miRNA expression data. Here, we employ methods developed for the study of ecological and microbiome data to characterize EVP miRNA levels in a pilot study of breast milk samples from the New Hampshire Birth Cohort Study.
Methods: EVP miRNAs were extracted from the supernatant of breast milk samples (n = 54) collected approximately 6 weeks postpartum using the Norgen Urine Exosome RNA Isolation kit. Extraction efficiency was monitored using a synthetic miRNA spike-in from Oryza sativa (osa-miR-414). Eluted miRNAs were further purified using Amicon Ultra Centrifugal Filters and concentrated with a speed vacuum concentrator. 798 miRNAs were profiled using the Nanostring nCounter platform. We characterized and compared the total EVP miRNA transcripts measured in each sample, the number of unique miRNA transcripts present, and sample evenness, calculated using the Shannon Diversity Index, and examined relationships between these measures and maternal pre-pregnancy BMI. Informed consent was provided by all participants.
Results: Total EVP miRNA counts were negatively correlated with sample evenness ($r = -0.37$, $p = 0.006$) and demonstrated no correlation with the number of unique miRNAs detected ($r = 0.05$, $p = 0.722$). The five most abundant miRNAs (hsa-miR4454+hsa-miR-795, hsa-miR-148a-3p, hsa-miR-320e, hsa-miR-4488, and hsa-miR-494-3p) accounted for a larger proportion of total miRNA counts for samples with higher overall miRNA levels. Participants with a pre-pregnancy BMI > 25 kg/m2 had both lower total miRNA counts ($p = 0.018$) and a smaller number of unique miRNAs present ($p = 0.026$) relative to participants with a pre-pregnancy BMI between 18.5 and 25 kg/m2.
Summary/Conclusion: The application of diversity measures developed for ecological and microbiome data may be useful for characterizing EVP miRNA composition. Using these measures, we found that maternal pre-pregnancy weight status may influence the total quantity and diversity of miRNAs in breast milk.
Funding: This work was supported by NIH grants 5P20GM104416, 5UH3OD023275, and 5T32CA134286-13.
Keywords: miRNA, pregnancy

PS11.08 | Fungal cellular communication is mediated by extracellular vesicles

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Introduction: Fungal infections are responsible for over 2 million deaths per year. The diseases caused by Aspergillus spp., Candida spp., and the agents of mycoses such as Paracoccidioides species are among the deadliest mycoses. The risk of fungal

diseases creates the urgent need to broaden the knowledge base regarding their pathophysiology. In this sense, the role of extracellular vesicles (EVs) has been described to convey biological information and participate in the fungus-host interaction process. We hypothesized that fungal EVs work as an additional element in the communication routes regulating fungal responses in intraspecies interaction systems.
Methods: The aim of this study was to address the gene regulation profiles prompted by fungal EVs in intraspecies recipient cells. We sought to analyze the fungal cellular communication mediated by EVs using the fungal pathogens P. brasiliensis, A. fumigatus, and C. albicans using multiple approaches. The EVs isolation were obtained through ultracentrifugation.The size distribution and quantification of EVs isolated were obtained by nanoparticle-tracking analysis.
Results: Our data demonstrated the intraspecies uptake of EVs in pathogenic fungi, such as Candida albicans, Aspergillus fumigatus, and Paracoccidioides brasiliensis, and the effects triggered by EVs in fungal cells. In C. albicans, we evaluated the involvement of EVs in the yeast-to-hypha transition, while in P. brasiliensis and A. fumigatus the function of EVs as stress transducers was investigated. P. brasiliensis and A. fumigatus were exposed to an inhibitor of glycosylation or UV light, respectively. The results demonstrated the role of EVs in regulating the expression of target genes and triggering phenotypic changes. The EVs treatment induced cellular proliferation and boosted the yeast to hyphal transition in C. albicans, while they enhanced stress responsiveness in A. fumigatus and P. brasiliensis, establishing a role for EVs in fungal intraspecies communication.
Summary/Conclusion: Our data demonstrate that fungal EVs mediate cellular communication by regulating the expression of target genes and by controlling cellular proliferation.
Funding: FAPESP, CAPES, CNPq and FAEPA.
Keywords: aspergillus fumigatus, candida albicans, paracoccidioides brasiliensis, EVs

PS11.09 | Genetic and metabolic factors affecting EV-secretion in Bacillus cereus

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Introduction: The food-pathogen Bacillus cereus is a Gram-positive endospore-forming rod causing emesis and diarrhea. Although studies on EVs deriving from gram-positive bacteria are on the rise, the exact secretion mechanisms are still unknown. By using gene deletion mutants of the emetic B. cereus F4810/72, the influence of seven well-known transcription factors and virulence regulators on EV secretion was studied. Furthermore, three different growth media were used to investigate the consequences of external factors influencing EV secretion.
Methods: EVs were isolated from bacterial cultures grown in different media using differential centrifugation. EV numbers and sizes were measured by NTA, bacterial growth was monitored spectrophotometrically, and protein concentration was determined with the aid of a colorimetric assay. The peptide-to-lipid ratio was calculated by means of Fourier-Transform-Infrared (FTIR) spectroscopy. The impact of intrinsic as well as extrinsic factors on EV compositions was analyzed by transmission electron microscopy (TEM), FTIR spectroscopy, and lipidomics.
Results: Our study revealed that global transcriptional regulators, as well as virulence-associated transcription factors, have a significant influence on EV secretion and vesiculogenesis in B. cereus. Furthermore, growth media significantly impacted EV secretion as well as EV composition in a genotypic manner, indicating a tight interplay of intrinsic and extrinsic factors in vesiculogenesis.
Summary/Conclusion: This study shows that the choice of the media considerably affects vesiculogenesis, and thus, establishing suitable culturing conditions is crucial to studying EV secretion dynamics. Furthermore, we show that knock-out mutants are a valuable tool to gain insights into the mechanisms of EV secretion and pave the way for deciphering EV vesiculogenesis in gram-positive bacteria.
Funding: The project is partially funded by the city of Vienna through the HJS program (409332/2021).

PS11.10 | Endothelial exosome plays functional role during rickettsial infection

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Introduction: Rickettsioses are devastating human infections3 that are caused by obligately intracellular bacteria of the genus Rickettsia (R). R are transmitted to human hosts by the bite of an infected tick. Microvascular endothelial cells (MECs) are the primary targets of infection, and edema resulting from EC barrier dysfunction occurs in the brain and lungs in most lethal cases in humans, but the underlying mechanisms remain unclear. The aim of the study is to explore the potential role of Rickettsia (R)-infected, EC-derived exosomes (Exos) during infection.

Methods: Using size-exclusion chromatography (SEC), we purified Exos from conditioned, filtered, bacteria-free media collected from R-infected human dermal MEC (DMECs) (R-ECExos) and plasma of R-infected mice (R-plsExos).
Results: We found that both R-ECExos and R-plsExos induced disruption of both tight junction TJs and adherens junctions AJs, two elemental components of the EC paracellular barrier, and barrier dysfunction in normal recipient brain microvascular ECs (BMECs), depending on exosomal RNA cargos1,2. Deep-seq and stem-loopPCR showed microvasculopathy-related (MVP) microRNAs are selectively enriched in human R-ECExos. In separate studies using a traditional in vitro model and a novel single living-cell biomechanical assay, we demonstrated that ECExos have the capacity to deliver oligonucleotide RNAs to normal recipient BMECs in an RNase-abundant environment; miR23a anti-sense oligonucleotide-enriched ECExos ameliorate R-ECExo-provoked recipient BMEC dysfunction in association with stabilization of ZO-1 in a dose-dependent manner.
Summary/Conclusion: These results suggest that Exo-based therapy could potentially prove to be a promising strategy to improve vascular barrier function during bacterial infection and concomitant inflammation.
Funding: This work was supported by NIH grant R01AI121012 (BG), R21AI137785 (BG), R21AI154211(BG), R03AI142406 (BG), R21AI144328 (BG) and John Sealy Distinguished Chair in Alzheimer's diseases (XF).
Keywords: exosome, extracellular vesicle, endothelial cell, endothelial barrier function, spotted fever group rickettsial infection, intracellular bacterium, fluidic AFM, single living cell study

PS11.11 | Circulating human cell-derived extracellular vesicles harbor bacterial genomic DNA

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Introduction: Reportedly, bacterial information (16S rRNA gene) exists in the blood; however, the mode and source of this bacterial information remains unclear. Therefore, in this study, we aimed to determine the source of bacteria information present in the blood by analyzing human cell-derived EVs (hEVs) recovered from the serum.
Methods: Clinical specimens: Clinical specimens were collected from healthy individuals at Osaka University Hospital. Written informed consent was obtained from each patient, and the study was approved by the Ethics Review Board of the Osaka University Hospital.
Serum EVs isolation: Serum EVs were isolated using different methods following the manufacturer's instructions: ultracentrifugation, qEV columns, Exosome Isolation Kit, and MagCapture Exosome Isolation Kit. Subsequently, EVs were treated with DNase I.
Fecal samples: The fecal swab-immersed medium was centrifuged at 2,000 × g, for 30 min, and the supernatants were filtered through a 0.2 μm syringe filter and subjected to ultracentrifugation for recovery of fecal EVs.
hEVs isolation from serum: Biotinylated mAb (anti-CD9 mAb, anti-CD81 mAb) were incubated with streptavidin magnetic beads. Subsequently, serum filtered with 0.2 μm syringe filter was co-incubated with mAb-coated magnetic beads. The bead-EV complexes were collected using a magnet and subjected to DNA isolation, western blot, and TEM analysis.
16S metagenomic sequencing: V1-V2 region was amplified by PCR and 16S rRNA metagenomic sequencing was performed on a MiSeq platform.
Results: The 16S rRNA gene was detected in serum EVs by various recovery methods. A comparison of the bacterial composition in serum EVs, feces, and fecal EVs revealed that the 16s rRNA gene in serum EVs was similar to that in feces but not to that in fecal EVs. hEVs recovered from the serum revealed the presence of 16S rRNA gene; Firmicutes, Actinobacteria, and Proteobacteria predominantly contributed to the bacterial genomic DNA population in hEVs. Furthermore, bacterial genomic DNA was found in phagocytosed macrophage-released EVs, suggesting these EVs to be a bacterial source in the blood.
Summary/Conclusion: Circulating hEVs harbor bacterial genomic DNA.
Keywords: circulating EVs, blood, bacterial genomic DNA, 16S rRNA gene

PS11.13 | Restrained Staphylococcus aureus physiological activity induced by bovine colostrum exosome and possible application to the antimicrobial agent

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Introduction: Many studies have reported on the antimicrobial agents in bovine colostrum (BC), but that of BC-derived exosomes (BC-Exo) remain largely unknown. Staphylococcus aureus (S. aureus) is a typical opportunistic pathogen. To treat S. aureus infection, antibiotics-based treatment has been used for a long time, however, the frequency of resistant strains has been on the rise; hence, alternative approaches are needed. We demonstrate the antimicrobial activity of BC-Exo against S. aureus, and further investigate its role as a bacteriostatic agent related to the toxin-antitoxin (TA) system.
Methods: BC-Exo isolation from BC provided by Seongan farm was basically used acetic acid treatment, and was centrifuged at 200,000 × g for 1 h at 4°C (beckman coulter, USA). Acquired BC-Exo was confirmed to morphology observation through TEM and cryo-EM. Size and concentration were also measured (iZon, Australia). TSG101 and CD81 were used to identify exosome protein. Purified BC-Exo was used for physiological properties such as growth, hemolysis, bio-film formation, surface morphology through SEM, and ATP quantification. Last, after gDNA extraction from S. aureus, TA protein expression and purification was used to E. coli BL21(DE3). Purified TA protein was treated to S. aureus and BC-Exo, the result was confirmed to SDS-PAGE or native page.
Results: The antimicrobial properties of BC-Exo were identified by the growth inhibition, especially showed bacteriostatic effect by using live/dead assay. Furthermore, hemolysis inhibitory effects and interrupted biofilm formation were also confirmed. Though SEM data, we observed that BC-Exo induced winkled cell surface. Also, BC-Exo reduced ATP production and affected overall phenomenological events in S. aureus. We also demonstrated that TA system was partially related to S. aureus and BC-Exo.
Summary/Conclusion: We demonstrated that BC-Exo can exhibit clear antimicrobial activity against S. aureus. These results suggest that BC-Exo has potential in antibiotic research as a natural substance that can replace chemical antibiotics in the future.
Keywords: bovine colostrum exosome, staphylococcus aureus, antimicrobial effect, bacteriostatic

PS12: EVs in Cancer Progression

Location: Hall 4A

16:50 - 18:50

PS12.02 | Convection and extracellular matrix binding control interstitial transport of extracellular vesicles

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Introduction: Compared to soluble mediators, EVs can traffic a wide range of proteins on their surface including extracellular matrix (ECM) binding proteins, and their large size (~30-150 nm) limits diffusion. We hypothesized that convection from interstitial flow and binding to the extracellular matrix control in vivo transport and spatial distribution
Methods: We isolated EVs from the MCF10 breast cancer progression series (MCF10A, normal; MCF10DCIS, pre-malignant; MCF10CA1, malignant), and examined their transport and binding through a laminin-rich ECM under controlled interstitial flow using computational and experimental models of a microfluidic device. To isolate EVs, each cell line was cultured for 72 h in EV-depleted media. Conditioned media was subjected to increasing centrifugation spins at 4°C (300xg for 10 min, 2,000xg for 15 min, 10,000xg for 30 min) and crudely purified via centrifugation through 150,000 kDA filters (4,000xg for 45min at 4°C). EVs for downstream imaging and analysis were stained with 2 mM CellTrace Far Red and incubated for 2 h at 37°C. Size exclusion chromatography (SEC) was used for final EV purification via qEVOriginal 35nm SEC columns to remove free dye, soluble proteins, and larger vesicles
Results: We demonstrate an increasing presence of laminin-binding integrins $\alpha 3\beta 1$ and $\alpha 6\beta 1$ on the EVs as the malignant potential of the MCF10 cells increased. Transport of the EVs under controlled physiological interstitial flow (0.15-0.75 μm/s) demonstrated that convection, and not diffusion, was the dominant mechanism of transport. Furthermore, binding of the EVs to the ECM enhanced the spatial concentration and gradient, which was mitigated by blocking integrins $\alpha 3\beta 1$ and $\alpha 6\beta 1$
Summary/Conclusion: Our studies demonstrate for the first time that convection and ECM binding are the dominant mechanisms controlling EV interstitial transport and should be considered in the interpretation of EVs as biomarkers and leveraged in nanotherapeutic design
Funding: NSF Graduate Research Fellowship (PAS); ARCS Foundation Scholar Award (PAS); NIH (UH3 HL141800, R01 EB030410, R21 AI161041, R01 CA241666, F31 NS120590); and the Department of Biomedical Engineering and the College of Engineering at the University of California, Davis.
Keywords: biotransport, convection, diffusion, extracellular matrix binding

PS12.03 | Quantitative analysis of ovarian cancer-derived exosome tropism

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Introduction: Exosomes, one of the subtypes of extracellular vesicles, range from 50 to 200 nm in diameter and regulate cell-to-cell communication in the biological and pathological processes. Although exosomes derived from tumors have various functions in cancer progression, resistance, and metastasis through cancer exosome-derived tropism, there is no quantitative information on cancer exosome-derived tropism that will be beneficial to guide cancer therapy by inhibiting exosome release or uptake.
Methods: Using two ovarian cancer cell lines (OVCA4 and OVCA8) that were transfected with the fluorescent protein (mkate2), tropism of cancer cell exosomes was quantified by measuring the release of exosome number from "parent" ovarian cancer cells and determining the uptake of these exosomes amounts into "parent" ovarian cancer cells, 3-D spheroids, and tumors from tumor-bearing mice models.
Results: An OVCA4 cell releases about 50 to 200 exosomes, and single OVCA8 cell secretes about 300 to 560 exosomes. Multifold exosome uptake from ovarian cancer cells into the "parent" ovarian cancer was observed compared to non-cancer cells. In vivo tumor-bearing mice models, most exosomes (200 to 600 million) are home to the "parent" cell tumors.
Summary/Conclusion: Quantification of the release of cancer-derived exosomes and the uptake of the exosomes into their "parent" cancer cells, displayed the target tropism of cancer-derived exosomes. These results will be beneficial for future diagnosis and therapeutic applications.
Funding: This publication's research was partly supported by NIH/NIGMS COBRE grant (P20GM103639).

PS12.04 | Cardiac extracellular vesicles drive tumor growth

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Introduction: Heart failure (HF) is associated with increased incidence of cancer. However, the mechanisms that link heart failure to cancer remain unclear, and specific therapies are limited. We hypothesized that the failing heart secrets small extracellular vesicles (cEVs) that carry and disseminate pro-tumorigenic factors.
Methods: To determine the role of cEVs in tumor growth, we focused on cardiac mesenchymal stromal cells (cMSCs), which play a central role in cardiac repair and remodeling. Using size exclusion chromatography, we isolated cMSCs-EVs from hearts of mice, 10 days after myocardial infarction (MI) and HF or sham-MI. We characterized cEVs by nanoparticle tracking analysis, cryo-electron microscopy, and western blot.
Results: cMSCs after MI secreted twice more EVs than cMSCs from sham-MI. Proteomic analysis revealed a distinct profile of cMSC-EVs after MI and HF. cMSC-EVs from the failing heart harbored more tumor-promoting proteins, cytokines, and microRNA (miR), such as Periostin, Osteopontin, VEGF, IL-6, TNFα, miR 221, miR 21, miR 24 and miR 214. Next, lung cancer cells were inoculated into the hind limb of mice. While MI and HF accelerated tumor growth, EV depletion by GW4869 markedly attenuated this effect (n = 28). In addition, we found that labeled cEVs targeted lung cancer tumors. Moreover, adoptive transfer of cMSC-EVs from failing hearts accelerated tumor growth compared to sham-MI EVs (n = 27). Finally, we found that Spironolactone, a renin-angiotensin-aldosterone inhibitor, mitigated the neoplastic effects of HF, attenuated tumor growth, and reduced the secretion of cMSC-EVs by 26% (n = 38).
Summary/Conclusion: We show, for the first time, that mesenchymal stromal cells from the failing heart secret small EVs that carry neoplastic mediators that target and accelerate tumor growth. Anti-heart failure therapy attenuates the tumor-promoting effects of cardiac extracellular vesicles.
Keywords: heart failure, reverse cardio-oncology, inflammation

PS12.05 | Extracellular vesicles (EVs) from rare cancers: can they teach us anything?

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Introduction: EVs from rare cancers are generally less studied than those of more common cancers. However, such EVs carry information and perform activities relevant to that cancer type, but with implications expandable to other cancers. Here we present EV characterization and activities from two extremely rare cancer cell lines: chordoma and rhabdomyosarcoma. The latter is fleetingly rare–an adult metastatic embryonal tumor (adult eRMS). We analyzed chordoma "small EVs" (exosome-like), and differing EV subtypes from the eRMS line. We performed 'omics analyses on the EVs and recipient cells (tumor cells themselves; osteoblasts as tumor microenvironment [TME] cells).
Methods: Cell lines were derived from patient tumors following written informed consent (COMIRB #13–3007). Human osteoblasts (hOBs) were purchased. EVs were isolated from conditioned EV-free culture media by ultrafiltration, differential (ultra)centrifugation, and SEC. EVs were characterized by NTA, TEM, ExoView, biochemical markers, and proteomics. Cell lines and their secretomes were subjected to shotgun or array-based proteomics.
Results: Proteomic analyses from both tumors' cells and EVs reveal extensive cell surface and extracellular matrix components with strong TME implications. Chordoma EVs promoted cell migration and blocking TGFB on EVs reduced chordoma metabolism. Chordoma EVs altered recipient hOB proteomes with implications again for TGFB and integrin function. Pathway analysis reflects known genetic eRMS cell alterations/mutations (p53, SUFU, DICER1 genes) and EV subsets, with implications in TGBF and integrin family members EV functions.
Summary/Conclusion: Rare cancers are generally understudied; treatment paradigms are often based on utility in other loosely-related cancers. We know of no other EV research in these tumor types; all these data are thus novel and may provide insight into disease pathophysiology. However, both cancers were metastatic to spinal locations; the results here may aid our understanding of other bone or bone-trophic cancers.
Funding: University of Colorado Neurosurgery; CU Cancer Center.
Keywords: chordoma, adult metastatic embryonal rhabdomyosarcoma, osteoblasts, tumor microenvironment, EV subsets, proteomics

PS12.06 | Exosome secretion drives persistent migration of cancer cells along fibers

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Introduction: Exosomes are a type of small extracellular vesicle (EV) that are actively secreted from cells and promote cancer cell motility and metastasis. Previous work has determined that exosome-type small EVs are critical for both directional sensing and cell speed during cancer cell migration in vitro and in vivo. Along with chemical cues, cancer cells also sense and migrate along extracellular matrix (ECM) fibers. The goal of this project is to identify the role of exosomes in driving cancer cell motility along topologically defined nanofibers that mimic in vivo ECM fibers and the role of ECM in that process.
Methods: Small and large EVs were isolated by differential centrifugation. The secretion rate was quantitated using nanoparticle tracking analysis to test the effect of Rab27a expression on exosomes secretion. Random single cell migration and cell migration on the nanofibers were recorded by using a temperature-controlled wide-field microscope. The motility aspect was analyzed by ImageJ.
Results: Here, we show several aspects of cancer cell motility affected by exosome secretion. Cancer cells with a defect of exosome secretion show defects of speed and distance of migration in random single cell migration assay. These findings are complemented by experiments showing that exosome secretion drives directionally persistent migration of cancer cells in a topologically defined nanofiber environment. The speed, total distance, displacement, and directionality of cancer cells on the nanofibers are affected by exosome secretion. The defects of cell migration on the nanofibers are fully rescued by extra ECM protein coating on the nanofibers.
Summary/Conclusion: Overall, we find that dynamic exosome secretion drives directionally persistent migration and quorum sensing behavior of cancer cells.
Funding: R01CA206458, R01CA249684, R01CA249424.
Keywords: cancer cell migration, persistent migration, extracellular matrix, topologically defined nanofibers

PS12.07 | sEVs derived from shCD90/Thy1 breast cancer cells mitigate angiogenesis and cell migration

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Introduction: Angiogenesis is one of the most challenging hallmarks of cancer due to its controversial molecular mechanisms. The CD90/Thy-1 molecule has proven itself as a potential therapeutic target in triple-negative breast cancer, the subtype responsible for the worst treatment and prognosis scenario. Considering the key molecular process mediated by sEV, this report aims to compare the angiogenic potential from sEV-depleted conditioned medium derived from CD90 knockdown and parental cell line, to uncover the key role played by sEV in this molecular mechanism.
Methods: Conditioned media (24h) were obtained from Hs578T triple-negative breast cancer cell lines, both parental (Hs578T/WT) and CD90 knockdown (Hs578T/shCD90). EVs were depleted from the conditioned medium using PEG precipitation technique. We then studied the potential of complete conditioned medium (CM) and EV-depleted CM (dEV-CM) in a scratch wound healing assay using 3T3 fibroblasts and the angiogenesis potential using the embryo quail chorioallantoic membrane (quail-CAM) in vivo assay. The evaluation was complemented with histological techniques to analyze blood vessel and angiogenesis biomarkers. All negative control and sample replicates were done (N = 3).
Results: These data demonstrated that the sEV depletion from the Hs578T/shCD90 CM decreased the migration of 3T3 cells and blocks the angiogenesis in CAM assay, in contrast of the result seen using Hs578T/WT CM. Also, CD90 knockdown has an outstanding anti-angiogenic performance together with sEV depletion.
Summary/Conclusion: These findings evidenced that despite the CM present all molecular factors needed to promote angiogenesis, this mechanism occurs primarily in the sEV presence. Suggesting that sEV is the major intermediary in this tumor progression molecular process.
Funding: Coordination for Improvement of Higher Education Personnel (CAPES), National Council for Scientific and Technological Development (CNPq), The State of São Paulo Research Foundation (FAPESP No. 2016/05311-2).
Keywords: triple-negative breast cancer, target therapy, small extracellular vesicles, angiogenesis, CAM, CD90/Thy1

PS12.10 | Role of serum exosomes in mediating enhanced colorectal cancer metastasis under obesity conditions

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Introduction: The packaging of exosomes may vary under diseased states or under different dietary interventions. Changes of the exosomal contents will lead to different pathological consequences. Under obesity conditions, whether exosomes play a role in the enhanced colorectal cancer (CRC) metastasis remains unknown.
Methods: Obese mouse models were established by high-fat diet (HFD) feeding. Serum exosomes (SExos) were purified from the mouse serum with differential ultracentrifugation method. Protein profiles of SExos were examined by iTRAQ-based proteomic. Bioinformatics analysis was done to investigate the metastatic roles of the differentially expressed exosomal proteins.
Results: In the proteomics study, a total of 2094 proteins were identified in SExos, and 479 of which showed significant difference between the obese and control mice. These differentially expressed proteins (DEPs) interacted with each other in a concert. Furthermore, KEGG enrichment analysis identified some DEPs with pro-metastatic properties, suggesting a role of the serum exosomal proteins in mediating CRC metastasis under obesity conditions.
Summary/Conclusion: Our data suggest that the serum exosomal protein profiles are different under obesity conditions, which may mediate the enhanced CRC metastasis under obesity conditions.
Funding: This work partially supported by FNRA-IG (RC-FNRA-IG/20-21/SCM/01), GDNSF (2021A1515010655), Shenzhen Basic Research Program for Shenzhen Virtual University Park (2021Szvup131), and HMRF (08193596).
Keywords: obesity, exosomal proteins

PS12.11 | Acute myeloid leukemia derived extracellular vesicles promote the inflammatory conversion of healthy hematopoietic progenitors in vivo

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Introduction: Patients with acute myeloid leukemia (AML) suffer frequent relapses and poor treatment outcomes. Acute inflammation in the bone marrow (BM) at diagnosis is propagated by leukemic blasts and contributes to therapy resistance. The source of chronic inflammation after initial remission remains unclear. BM hematopoietic stem and progenitor cells (HSPCs) are long-lived contributors to innate immunity. We hypothesize that AML-derived extracellular vesicles (EV-AML) convert HSPCs into a key source of sustained inflammation in the BM.
Methods: Two genetically different immune competent congenic murine models of AML (C1498 and dox-inducible(i)MLL-AF9) were utilized. EV-AML from C1498 (C1498-EV-AML) and iMLL-AF9 blasts (AF9-EV-AML) were characterized according to MISEV criteria. Gene expression was analyzed by single-cell and bulk RNA sequencing as well as real-time PCR.
Results: In the C1498 AML model, we found elevated inflammatory cytokine secretion and upregulated inflammatory gene expression across HSPC subtypes at a low AML burden in the BM. To test for the involvement of EV-AML, we challenged HSPCs with purified C1498-EV-AML in vitro and found highly upregulated inflammatory gene expression (e.g. Isg15) and chemokine secretion (e.g. Cxcl10) by HSPCs. We independently validated inflammatory signaling utilizing the translationally-relevant AML model, iMLL-AF9, in vivo. In a cell-free approach, we demonstrated that injection of AF9-EV-AML alone can incite inflammatory activation in healthy BM-HSPCs.
Summary/Conclusion: Our results show for the first time that AML-derived EV-AML convert HSPCs to core constituents of the innate immune environment in the leukemic BM. While much recent work is focused on the possibility of redirecting adaptive immunity to treat AML, we propose to target innate immune components in an effort to improve therapeutic outcome in AML.
Funding: Alex's Lemonade Stand Foundation Young Investigator Grant.
Keywords: acute myeloid leukemia, inflammation, niche, hematopoietic stem and progenitor cells, innate immunity

PS12.12 | IL2 induces anticancer immune responses by regulating sEV secretion and PD-L1 expression in cancer cells

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Introduction: Interleukin-2 (IL2) induces anticancer effects by activating immune cells expressing the IL2 receptor complex (IL2R). Interestingly, IL2R is also expressed in cancer cells, such as melanoma cells; however, previous studies are focused on the influence of IL2 on immune cells, not on cancer cells with regard to anticancer immunity. Here, we suggest that IL2 modulates the immunosuppressive tumor microenvironment (TME) by inhibiting sEV release and cancer-derived PD-L1, suggesting a novel effect of IL2 on cancer cells under immune surveillance, in addition to its systemic immunomodulatory efficacy.
Methods: We investigated whether IL2 affects cancer cells to reduce small extracellular vesicle (sEV) release and suppress cellular programmed death ligand 1 (cPD-L1) and exosomal PD-L1 (ePD-L1) expression and in melanoma cells through MAPK/ERK signaling. These anticancer immune responses were observed even in animal models wherein IL2 induces cancer-specific activity by transplantation with IL2-tethered melanoma cells. Notably, the reduction of PD-L1 and Rab27a expression by IL2 was observed in both IL2R-positive lung cancer cell lines and lung cancer cells from human patients.
Results: We show that IL2, identified from a cytokine screening assay, significantly inhibits sEV secretion along with downregulation of cPD-L1 and ePD-L1 levels in melanoma cells and increases their sensitivity to CD8+ T cell-mediated cytotoxicity. Mechanistically, IL2 induces these effects in melanoma cells by activating IL2R-MAPK/ERK signaling. To analyze the direct effects of IL2 on melanoma cells in vivo, a unique mouse model is developed by introducing melanoma cells engineered to express IL2 on their surface via a flexible peptide linker for self-stimulation. In this model, melanoma cell-specific IL2 stimulation strongly inhibited tumor growth and downregulated both the expression of cPD-L1 in tumor tissues and the plasma ePD-L1 levels. IL2R-expressing lung cancer cell lines and clinical samples were employed to confirm the same effects on human samples.
Summary/Conclusion: This study revealed that IL2 enhances immune surveillance by directly reducing both EV secretion and PD-L1 expressions in melanoma cells via the IL2R-MAPK/ERK signaling. This study presents the potential of IL2 as a novel immunoOncology strategy in IL2R-positive cancers.

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Keywords: interleukin-2, PD-L1, sEV, exosomal PD-L1, cancer immunotherapy

PS12.14 | HCC direved exosomes high core-fucosylation elict HCC progression by activtion through PI3K/AKT signalling pathway

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Introduction: Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death and the fifth most common cancer in the world. Recently, exosomes have been focused on tumor diagnostic biomarkers research and transformation. However, the glycosylation modification of exosomes has not been reported in HCC.
Methods: Using western blot and RT-PCR, we measured the expression of proteins and mRNAs of HCC cell models. We detect the core fucosylation of HCC derived exosomes using Lectin-ELISA. Cell proliferation and apoptosis were detected by flow cytometry.
Results: Our previous study found that the level of core fucosylation in HCC patients changed significantly with the progression of the disease, and the level of core fucosylation significantly increased in serum exosomes. Moreover, exosomes derived from serum of HCC patients with high level of core fucosylation can enhance the proliferation and migration of HCC cells, through activing PI3K/AKT signal pathway. The purpose of our study is to detect and evaluate the clinical application of exosomal core-fucosylation level in the early diagnosis of HCC, using the HCC cell model and mice model, as well as the clinical study, to explore the related mechanisms and provide scientific basis for the clinical diagnosis of HCC.
Summary/Conclusion: In our study, we demonstrate exosomal core fucosylation promotes malignant behavior of hepatocellular carcinoma cells through activation of PI3K/AKT signaling pathway.

PS12.15 | LDHA positive EVs activated glioma stem cells by promoting glycolysis and are candidate biomarkers for glioblastoma recurrence

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Introduction: GSCs (Glioma stem cells, GSCs) activation is a hallmark during Glioblastoma (GBM) recurrence and leading to poor outcome of GBM. However, there is no proper biomarker for GSCs activated and GBM recurrence warning at early stages.Here we demonstrated that plasma-derived EVs (pl-EVs) from recurrent GBM patient cohorts could activate GSCs phenotype of glioma cells.
Methods: 265 cases of plasma from recurrent (n = 84) and non-recurrent GBM patients (n = 181) post-surgery and healthy donors (n = 50) were collectted and plasma derived EVs (plEVs) were concentrated.Using proteomics, "pyruvate-glucose transforming" pathway was identified upregulated in the EV cargo and Lactate dehydrogenase A positive EVs (LDHA+EVs) numbers were detected in the plasma from recurrent GBM patients by eco-counter.To study the mechanisms of exomal LDHA, LDHA upregulated giloma cells were constructed to obtain LDHA carried EVs (LDHA-EVs),Glycolysis/glycogenesis activity were evalutaed by ECAR assay and lactate production were evaluated for EV uptaken glioma cells.Given the excellent clinical application prospects of EVs, the biodistributions of them were further evaluated in vivo. Besides,U87-Luciferase (U87-Luc) based

glioma model was established, and LDHA-EVs were administered intraperitoneally continued and tumor fluorescence was detected.Clinically, to determine whether LDHA+ pl-EVs has the potential to predict GBM recurrence, we further compared the amount of LDHA+plEVs in the plasma of patients with recurrent GBM and their MRI-detected images.

Results: Mechanistically, we discovered that LDHA could be transferred via EVs into recipient cells, resulting in further enhancement of glycolysis and ATP production (Warburg effects) to maintain GSCs phenotype. Besides, LDHA-carried EVs (LDHA-EVs) were driven by HIF-1α, also has more obviously homing effects entering to the intracranial tumor sites. Clinically, we found strong correlations of LDHA+plEV numbers and LDHA level in matched patient tumors, and LDHA+plEV was decreased post-operatively and increased with GBM recurrence.

Summary/Conclusion: Our study highlights the effects of increased circulating LDHA+EVs and its induced "Warburg effects" to maintain GSCs phenotypes may an important reason for GBM recurrence. Besides, this finding provides a potential molecular evidence for LDHA+EV-mediated intracellular metabolisms and offers a candidate biomarker for GBM recurrence by liquid biopsy.

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Keywords: LDHA+EVs, GBM recurrence, glioma stem-like cells, warburg effects, EVs homing, liquid biopsy

PS12.16 | miR-92a-1-5p enriched prostate cancer extracellular vesicles regulate osteoclast function

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Introduction: We have previously reported that extracellular vesicles (EVs) derived from osteoblastic, osteoclastic and mixed prostate cancer cells promote osteoclast differentiation and inhibit osteoblast differentiation via transferring miR-92a-1-5p. In the present study, we focused on engineering miR-92a-1-5p into EVs and determining any therapeutic role and mechanism of the engineered EVs.

Methods: A stable prostate cancer cell line (MDA PCa 2b) overexpressing miR-92a-1-5p was constructed by lentivirus, and EVs were isolated by ultracentrifugation. The overexpression of miR-92a-1-5p in both cells and EVs was tested using qPCR. Osteoclast function was evaluated by Trap staining, mRNA expression of osteoclastic markers ctsk and trap, immunolabeling of CTSK and TRAP and microCT using either in vitro and in vivo assays. Target gene of miR-92a-1-5p was proved by a dual-luciferase reporter assay system. siRNAs were designed and used for transient expression in order to determine the role of downstream genes on osteoclast differentiation.

Results: Stable overexpression of miRNA-92a-5p in cell line was associated with increased expression in EVs, as confirmed by qPCR. Further, miR-92a-1-5p enriched EVs promote osteoclast differentiation in vitro by reducing MAPK1 and FoxO1 expression, associated with increased osteoclast function as shown by TRAP-staining and mRNA expression of osteoclast functional genes. siRNA targeting MAPK1 or FoxO1 resulted in similar increase in osteoclast function. In vivo, the miR-92a-1-5p enriched EVs given via i.v. injection promote osteolysis, which was associated with reduced expression of MAPK1 and FoxO1 in bone marrow.

Summary/Conclusion: Our results suggest that the engineered miR-92a-1-5p enriched EVs regulate osteoclast function via reduction of MAPK1 and FoxO1.

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Keywords: miR-92a-1-5p, miR-92a-1-5p enriched EVs, bone diseases, bone metabolism, osteoclast differentiation

PS12.17 | IGF2BP3-EVs impact on Ewing sarcoma migration and may play a role in the intra-tumor heterogeneity

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Introduction: Ewing sarcoma (EWS), a pediatric bone and soft-tissue cancer, is characterized by high high level of intra-tumor heterogeneity and aggressiveness. EWS is defined by the fusion of EWSR1 to the FLI1 gene. Several studies supported the role of EWSR1::FLI in governing EWS heterogeneity but other molecules were also reported to be involved. Here we focused on IGF2BP3, an RNA-binding protein with an oncogenetic role in cancer. Cellular IGF2BP3 was described to be associated with EWS progression when highly expressed. In this study, we investigated whether and how IGF2BP3 is released by EWS cells and whether extracellular IGF2BP3 confers functional variations in recipient EWS cells.
Methods: 11 patient-derived EWS cell lines and experimental models modified for IGF2BP3 expression were used. Elisa assay detected expression of IGF2BP3 in cell supernatants. EVs were extracted from cell culture medium using the ExoQuick or the Ultracentrifugation methods.EVs were characterized using a NanoSight NS300 system. Labelling of EVs was made using the fluorescent dye PKH67. Cells were exposed to 15 ug of EVs derived from cells deprived or not of IGF2BP3 and tested for cell proliferation or migration. Western blotting was used to evaluate EVs markers or signaling mediators. RNAseq and bioinformatic analyses identified the miRNA cargo of IGF2BP3-pos or -neg EVs and associated targets.
Results: IGF2BP3 is released from EWS cells. IGF2BP3 is present in the EVs but not in the vesicles-depleted supernatants. When A673 or TC-71 EWS cells received EVs from the IGF2BP3 knockdown cell lines their migratory capability was significantly decreased compared with when they received IGF2BP3-positive EVs. No difference were observed with respect to proliferation. The uptake of IGF2BP3-positive EVs determined an increase of IGF2BP3 in recipient cells in a dose-dependent manner, indicating that EVs may transfer IGF2BP3 in surrounding cells and modify their behavior. Analysis of miRNA cargo identified a signature of differentially expressed miRNAs. capable to cluster tissue tumor samples with different expression of IGF2BP3. From a mechanistic point of view, the analysis of 11 miRNAs that were found to be in common between two experimental models indicated alterations in the IGF1R_PI3K_Akt pathway in recipient cells.
Summary/Conclusion: We identified in IGF2BP3 a player of Intra-tumour phenotypic heterogeneity, which has been defined as a major determinat of EWS malignancy. IGF2BP3 is secreted via EVs and affects PI3K/Akt signaling in recipient cells, ultimately altering their migratory abilities. This indicates that cells with high expression of this RBP can increase the malignancy of neighbouring cells.
Funding: The research leading to these results has received funding from AIRC under IG 2019—ID. 22805—P.I. Scotlandi Katia.

PS12.18 | Investigating the role of Rab11A in metastatic medulloblastoma pathogenesis

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Introduction: Medulloblastoma is the most common malignant paediatric brain cancer with poorer prognosis related to the onset of metastasis. It has four molecular subgroups; Wingless (WNT), Sonic Hedgehog (SHH), group 3 and group 4, of which group 3 is the most likely to be metastatic and is therefore associated with the poorest prognosis. Increased exosome release is connected with disease progression and metastasis in multiple cancers. Rabs are a family of small GTPases (70 in humans) which regulate vesicle trafficking. Several Rabs are known to regulate exosome biogenesis and secretion, including Rab11A, and may thereby contribute to cancer progression. The role of Rabs in metastatic medulloblastoma is unclear. We aim to explore whether Rabs contribute to the progression of metastatic medulloblastoma through the exosome biogenesis and secretion pathways.
Methods: An analysis of the literature, databases such as ExoCarta.org and the R2: Genomics analysis and visualisation platform was completed to highlight Rab GTPase targets of interest. RT-qPCR was used to assess gene expression of target Rabs across SHH, group 3 and group 4 cell lines. CRISPR-Cas9 technology was used to generate knock out (KO) group 3 medulloblastoma cell lines of key Rab targets. KO cells were imaged using a ZOE fluorescent cell imager. Extracellular vesicles (EVs) were isolated using ultrafiltration followed by size exclusion chromatography using qEV original 70nm Gen 2 columns. Size and concentration of EVs were characterised using the ZetaView and the EV fraction imaged by transition electron microscopy.
Results: Literature and database analysis identified Rab11A as a candidate which may contribute to disease progression in group 3 medulloblastoma. RT-qPCR showed increased gene expression of Rab11A in group 3 and group 4 medulloblastoma patient-derived cell lines. Imaging of Rab11A KO cells showed altered morphology compared to a control cell line.
Summary/Conclusion: Initial findings suggest that Rab11A KO is affecting cellular phenotype. Current and future work aims to continue characterisation of this phenotype to determine the potential role of Rab11A in medulloblastoma pathogenesis through vesicular trafficking and secretion pathways.
Funding: Funded by the BBSRC DTP programme.
Keywords: rab, cancer, medulloblastoma

PS13: EV Production for Therapeutics

Location: Hall 4A

16:50 - 18:50

PS13.02 | Cell spheroid as an exosome production platform

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Introduction: The construction of a spheroid to mimic the human physiological condition is highly desired for drug discovery and clinical applications because conventional monolayer cell culture is inaccurate compared to human physiological condition. However, current spheroid production technologies are limited in ability to control uniformity of diameter and cell number.
Methods: This study presents a method for producing homogenous spheroid using precisely controlled dispensing system incorporating a thermo-sensitive bioink and human adipose derived stem cell. The spheroid can be successfully generated in the core shell structure at a speed of approximately 3,000 spheroids per hour and with a uniform diameter and accurate cell number. Exosomes from cell culture media were isolated by the TFF method.
Results: The spheroids allow dynamic culture without any aggregation and secrete a large amount of biologics such as exosome. The amount of exosomes derived from spheroids increased more than 90 times compared to the amount of exosomes derived from 2D culture cells (ADSC, BM-MSC, NTSC, Fibroblast). The amount of exosomes was measured using NTA(Nanoparticle tracking analysis). In addition, spheroid-derived exosomes showed enhanced skin improvement effect in vitro and in vivo. ADSC-spheroid exosomes increased the proliferation and migration of fibroblast and keratinocyte. Also, these exosomes enhanced collagen1 secretion of fibroblast. In big data analysis (NGs and Proteomics) indicated that these exosomes have proliferative, migratory, and anti-inflammatory effects.
Summary/Conclusion: In summary, these results suggest that spheroid derived exosomes were improved its function and efficacy because of its in vivo similarity. Further, we plan to conduct multi-cell spheroid research to improve its in vivo similarity.
Funding: This research was funded by TIPS(Tech Incubator program for startup) by Korean Goverment (Grant number S3197974, 20105847).
Keywords: spheroid, dynamic culture, exosome

PS13.03 | Production, purification, and characterization of enveloped protein nanocages

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Introduction: Extracellular Vesicles (EVs) are a group of biological nanostructures with diverse physiological functions currently being investigated for clinical applications. Scientists at the Institute of Protein Design recognized the therapeutic value of EVs and have engineered a self-assembling and self-releasing hybrid biological nanostructure known as Enveloped Protein Nanocages (EPNs). These EPNs self-assemble as a protein nanocage, and direct their own release from producer cells inside small vesicles. With the design of this new hybrid EV, scientists have a promising new engineerable EV platform amenable to different uses such as EPN-based vaccines or targeting domains to functionalize the membranes of EPNs. Here we list the current methodology for EPN production, purification, and characterization.
Methods: HEK293T cells were cultured and seeded at 20 million cells onto 15cm TC-treated plates which had previously been coated with 0.2% Gelatin. Cultures were then transiently co-transfected with PEI, and media was changed 4–6h post-transfection. After 48h supernatants (SN) were harvested and filtered through 0.45μM filters. Filtered supernatants were spun at 100,000g for 60 min at 4°C through a 20% PBS-sucrose cushion to concentrate and purify EPN into a pellet from the bulk filtered SN. Cell fractions from each sample were also harvested to check for expression level of non-released EPN proteins. Samples were then characterized using SDS-PAGE and western-blot analysis to probe for expression levels of EPN in the cell fraction, released supernatant fraction, membrane integrity, and displayed transmembrane protein orientation. Samples were also sent to collaborators for imaging via Cryo-EM.
Results: Western blot analysis showed successful EPN production from HEK293T cells and controlled release into the supernatant fractions. Successfully released EPN also displayed trypsin resistance for the interior cage proteins, and trypsin sensitivity for the externally displayed transmembrane proteins.
Summary/Conclusion: EPN modularity, tractability, and versatility create a novel and robust EV platform for therapeutics. Here we presented the current methods used to produce, purify, and characterize EPNs.

PS13.04 | Production, isolation, and characterization of glioblastoma extracellular vesicles using long-term bioreactors

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Introduction: The scalable production of extracellular vesicles (EVs) using conventional in vitro cultures can be challenging due to the large area of cell monolayers and volumes of culture media required. Commercially available EV bioreactors appear to provide one solution to this issue but have not been well-validated for glioblastoma (GBM) EV production.
Methods: GBM cell lines representing different subtypes of the disease, including A172 (neural), BT169 (gliosarcoma), U373 (proneural), U87 (mesenchymal), and U87-EGFRvIII (classical) were inoculated into CELLine AD 1000 bioreactor flasks in complete media and gradually adapted to chemically defined serum replacement CDM-HD with no passaging. Bioreactor cultures were maintained for up to four months with up to three weekly harvests from the cell chamber and one weekly change of the upper media chamber. Large and small EVs were isolated using combinations of ultrafiltration, ultracentrifugation, and size exclusion chromatography depending on the downstream application. EVs were characterized using NTA, TRPS, mass spectrometry proteomics, TEM, western blotting and surface-enhanced Raman spectroscopy, and bioreactor growth surfaces were imaged using SEM.
Results: All GBM cell lines rapidly proliferated and established high density cultures, producing hundreds of total EV preps over their lifetimes with the same incubator footprint and significantly less user time. Bioreactors also produced significantly more EVs per harvest compared to conventional cultures, but with similar size distributions, morphologies, and contents.
Summary/Conclusion: Commercially available bioreactors are a viable option for producing vast amounts of EVs in vitro, with several key advantages compared to conventional cultures. These EVs can be used for a limitless range of downstream applications and are particularly useful in technology development, where large amounts of consistent EVs may be necessary.
Funding: This research was supported by Ohio State's CCE-CURES Accelerator Award and the College of Engineering's LEGACY Postdoctoral Program.
Keywords: bioreactors, extracellular vesicles, SERS

PS13.07 | Biomanufacturing of extracellular vesicles from mesenchymal stromal cells grown in a novel perfusion-based and alternative bioreactors

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Introduction: As the interest and need of extracellular vesicles (EVs) as therapeutic product or as carrier for bioactive cargo rises, so does the necessity of biomanufacturing platform that is cost effective and can generate sufficient material for clinical translation. In this study, we evaluated a novel 3D-printed, scalable, and perfusion-based bioreactor developed by SwRI, along with PBS Biotech Vertical-Wheel® microcarrier based bioreactor for EV production. The objective of the study was to identify and optimize parameters to increase the EV productivity from human bone marrow mesenchymal stem/stromal cells (hBM-MSCs) after growth expansion, with prototypes of different scales of perfusion-based bioreactor as well as the microcarrier-based bioreactor platform.
Methods: The xeno-free RoosterVial-hBM (RoosterBio, MD) were grown in planar as seed train prior to seeding on to the 3D-printed -perfusion bioreactors or microcarriers for suspension culture in PBS Biotech bioreactors. The cells were expanded in xeno-free RoosterNourish-MSC growth medium before EV collection in RoosterCollect-EV (RoosterBio). The process was scaled up from surface area of 250 to2500 cm2 (46 – 460 mL) for the perfusion bioreactors and 100 to 3000 mL for PBS bioreactors. Key process parameters evaluated were seeding density, microcarriers, growth and EV collection duration, and number of media exchanges and monitored in-process by key metabolites with Nova Flex (Nova Biomedical, MA), pH, and particle counts. The harvested conditioned media were analyzed for metabolites and EV productivity with NanoSight through Nanoparticle Tracking Analysis software (Malvern Panalytical, MA).
Results: For the microcarrier-based bioreactor scales of 500 mL and 3000 mL, the optimal microcarrier seeding density and cell growth were achieved when the cell density reached to 2E05 cells/mL and 4E05 cells/mL by the end of growth phase, respectively. The highest EV productivity of 1.2E10 particles/mL achieved at the 3000mL scale. For the 2500cm2 perfusion-based bioreactor, the cell number was estimated at approximately 180E6 at the end of growth phase, with the EV productivity of 3.2E9 particles/mL, which was comparable to similar surface area of suspension culture 500mL scale with EV productivity of 3.0E9 particles/mL.

Summary/Conclusion: The 3D-printed perfusion-based bioreactor can harvest EVs multiple days or even continuously. The highest particle size frequencies were observed 100–200 nm in diameter across all processes. The novel 3D-printed perfusion-based bioreactor, as an alternative, scalable EV biomanufacturing platform, will serve the biotech industry as a cost-effective path in achieving the demand of translational EVs.
Funding: MTEC-19-01-Biomfg-0004 (ESG# MT19007.04).

PS13.08 | Successful development of a scalable and robust process for MSC-EV production

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Introduction: Extracellular Vesicles (EVs) are produced by many cell types, particularly from mesenchymal stromal cells (MSCs). The number of clinical trials investigating EVs from MSCs as a therapeutic agent has been increasing. The goal of this study was to identify and optimize scalable parameters for EV production in a 3D stirred-tank bioreactor.
Methods: Human bone marrow MSCs were expanded in xeno-free RoosterNourish™ medium followed by EV collection in RoosterCollect™-EV medium (RoosterBio) in Ambr®250 bioreactor at 250mL scale. Different microcarriers were screened for optimal MSC attachment and growth. Microcarrier concentrations and cell seeding densities were evaluated to maximize cell yield. Agitation ramping, culture duration and feed strategies were evaluated. The robustness and reproducibility of the process were verified across multiple donors. Optimal parameters were verified at a large scale (15L) in Biostat STR® bioreactor (Sartorius). Critical quality attributes (CQAs) of EVs were evaluated to confirm identity and potency.
Results: Synthemax II was selected as the optimal microcarrier with over 90% of cell attachment and maximal cell yield after 5 days of growth in the bioreactor. Optimized cell seeding density and microcarrier concentration generated over 1 × 1010 particles/mL by day 5 of collection. Agitation ramping maintained homogenous suspension during the culture. Over 90% of the generated particles stained positive with lipid bound membrane dye. Samples also showed positivity for the canonical EV identity tetraspanin markers CD81, CD9 and CD63 in western blot. Potency assays demonstrated the biofunctionality of the generated EVs. The developed process was successfully scaled to 15L to generate a total of over 10 × 1014 particles while maintaining CQAs.
Summary/Conclusion: This study developed an optimized, reproducible, and scalable xeno-free microcarrier-based 3D bioreactor process for MSC-EV production.
Keywords: scale-up, bioreactor, MSCs

PS13.10 | High-Productivity and high-quality exosome production technology by controlling the consistent size of the spheroids

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Introduction: The traditional models of cell culture are two-dimensional (2D) as monolayers, but this has a problem of not mimic the actual natural environment. 3D cell culture techniques have been studied recently because they might provide more accurate environment of human tissues. Among 3D culture methods, spheroids are 3D cell aggregates that can mimic tissues and microtumors, and are in the spotlight because they can be applied to industrial mass production.
Methods: In this study, uniform spheroids were mass-produced by precisely controlling and printing bio-ink mixed with human adipose-derived stem cells (ADSC). The spheroids are formed through static culture for 48 hours within the printed core shell structure. Subsequently, the exosomes from the culture media of the spheroids dynamically cultured for 3 days were purified by the TFF method.
Results: Through the our mass production method, it was confirmed that the production of exosomes of spheroids produced to a predetermined size by controlling the concentration of bio-ink was significantly increased compared to spheroids produced to be non-uniform in size. In addition, there were differences in cell proliferation and regeneration between these two exosomes.
Summary/Conclusion: In conclusion, the technology of maintaining a constant size of spheroids using bio-ink showed high productivity and high quality in exosome production. In short, the exosome production method using this technology can overcome the qualitative and quantitative limitations of the existing exosome production method by producing exosomes from controlled-sized spheroids.
Funding: This research was funded by TIPS(Tech Incubator program for startup) by Korean Goverment (Grant number S3197974, 20105847).
Keywords: spheroid, dynamic culture, exosome

PS13.11 | Optimization of lipid nanoparticle production in a microfluidic device to encapsulate molecular cargo for personalized therapeutics

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Introduction: The advances in droplet microfluidics in the last decade show immense potential to produce lipid nanoparticles (LNPs). The enhanced mixing capabilities of microfluidics allow lipid fragments to recombine as novel therapeutic carriers. Lipid membranes (LM) are considered waste in various protein isolation processes. In contrast, these LM on EVs contain sensitive information on physiological and pathological disorders in humans. The production of recombinant extracellular vesicles (rEVs) using conventional sonication methods suffer from a wide range of size distribution, low reproducibility, and reduced stability of encapsulated molecular cargo. Therefore, we developed a microfluidic approach to control the size of rEVs and the contents of encapsulated molecular payload.
Methods: The lipid components are fed as a dispersed phase on an array of flow-focusing and herringbone microfluidic devices. The continuous high throughput process is optimized by considering hydrodynamic forces, capillary number, geometry, and flow rate ratios ranging from 1:4 - 1:20 μL/min to control size. The device can produce highly homogeneous nanodroplets of sizes including 80, 100, 120, 150, and 200nm. Characterization methodologies include Tunable Resistive Pulse Sensing (TRPS) for size, Total Internal Reflection Fluorescence Microscopy (TIRFM) for analyzing the internal contents of rEVs with molecular beacons.
Results: The microfluidic devices were effectively compared and optimized to produce a monodispersed hydrodynamic diameter of ~120nm. Large-scale production is underway with the herringbone device for volumes required to perform clinical trials. rEVs with sizes over 200nm can also be produced with low polydispersity.
Summary/Conclusion: The novel rEVs carrying advanced therapeutics can escape immunogenic responses and cross the blood-brain barrier to accurately target and cure pathological diseases.
Keywords: recombinant extracellular vesicles, personalized therapeutics, droplet microfluidics

PS13.12 | Scalability of production and activity of human amniotic fluid stem cell extracellular vesicles from 3D hollow fiber bioreactor and 2D culture

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Introduction: Introduction: EV clinical translation is inhibited by limitations in the scale-up of EVs production. Hollow fiber bioreactors (HFBR) support culture of large numbers of cells, at high densities, with concentrated EVs. Culture conditions may affect EV composition and potency. Here we compare the production, potency, identity, and therapeutic potential of EVs collected from cells grown in culture dish (2D) vs a small and medium-sized HFBR (3D).
Methods: Methods: 1 × 10e6 human clonal stem cells from amniotic fluid (hAFSC from consented donors) were seeded in 2D (145cm2), 1.6 × 10e7 hAFSC were seeded on a small cartridge (FiberCell C2025D; 450cm2), and 1.8 × 10e8 hAFSC on medium cartridge (FiberCell C2011; 4,000cm2) with fibronectin coating. All cultures used Chang medium with 20% of ES-FBS, starved for 24hr and then EVs collected. The effect of harvest frequency was tested (8hr, 24hr, 72hr). 2D-EVs and 3D-EVs were compared by Nanosight, potency assay (by WB), identity (by Exoview), and therapeutic effect (in vivo injections in an animal model of chronic kidney disease, Alport Syndrome).
Results: Results: 2D production was ~5.5 × 10e9EV/ml/24hrs while 3D was ~2.8 × 10e10EV/ml (first four 24hrs) and ~4.4 × 10e10EV/ml (two days of hourly harvests). The medium cartridge produced similar concentrations of EVs but at 10X harvest volume indicating linear scalability.
Very little difference in EV concentration was observed during harvest intervals, with a very similar size distribution. This could indicate either significant EV re-uptake or inhibition of EV secretion dependent upon free EV in the supernatant. Both 3D-EVs trapped VEGF in vitro (an in vitro established potency assay) and expressed CD9, CD81, CD63, CD80, CD86, and VEGFR1 as 2D-EVs. 3D-EVs ameliorated proteinuria and histology when injected into Alport mice and also trapped VEGF in vivo as 2D-EVs.
Summary/Conclusion: Summary/Conclusion: 3D-EVs had comparable properties and bio-activity to 2D-EVs, but the HFBR produced 10x to 100X more EVs. Cell culture conditions for hAFSC still need optimization in the HFBR, however, a currently available 1.2m2 cartridge provides a 50X scale-up potential. The HFBR is a closed system that can be cGMP compliant.

In conclusion, the HFBR can produce a sufficient number of EVs to support pre-clinical and clinical applications of EVs with at least similar properties to EVs produced in 2D methods.
Funding: na.
Keywords: 3D, hollow fiber bioreactor, human amniotic fluid stem cells

PS13.13 | Development of an end-to-end scalable purification platform for extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are under investigation as novel therapeutics for a variety of diseases. EVs derived from human mesenchymal stromal cells (hMSC-EVs) constitute most clinical investigations. Despite the growing interest, new methodologies for their purification and characterization have only recently started to be investigated. The goal of this study was to develop a scalable purification platform for hMSC-EVs.
Methods: 15L of EV conditioned media (CM) was generated from adherent hMSC culture in the stirred tank bioreactor and was used for the optimization studies. Clarification filters of various pore sizes, crossflow filters 100–750 kDa molecular weight cut-offs, and chromatography modules were evaluated for purification workflow. Clarified harvest was processed through crossflow filtration (CF) steps for concentration, enzyme treatment for reducing DNA interference, and buffer exchange for chromatography. Turbidity, particle, and protein measurements were used to assess recovery and purity. Particles were confirmed to be lipid-bilayer vesicles by staining positive for fluorescent membrane dye reported on orthogonal techniques and biofunctionality assay was performed.
Results: CM containing $> 1 \times 10\hat{}10$ particles/mL was generated. Clarification of CM using filters $> 3\mu$m achieved $> 40\%$ particle recovery with significant turbidity reduction. CF reduced DNA by 92% mainly due to enzymatic reaction while improving protein removal to $> 1 \times 10\hat{}11$ particles/mg protein. Chromatography module(s) recovered $> 75\%$ of particles with enrichment in purity $> 1 \times 10\hat{}12$ particles/mg protein. The optimized parameters were verified in an end-to-end run.
Summary/Conclusion: The end-to-end platform for EV purification was established with particle recovery of $> 25\%$, particle size distribution between 50–200nm and purity of $> 1 \times 10\hat{}12$ particles/mg protein. These promising results serve as a baseline for a reproducible EV purification process with consistent critical quality attributes.

PS13.15 | Extracellular vesicle production enhancement from mesenchymal stem cells by the extremely low-frequency electromagnetic field

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Introduction: Mesenchymal stem cell (MSC) properties make them useful in many therapeutic applications, but their proliferation risks have significant risks. Due to this, many researchers turned to the MSC-derived extracellular vesicles (EVs), which have the same properties as MSCs but without their shortcomings. However, MSC EV production has yield limitations. Therefore, there is an urgent need to improve MSC EV production. Extremely low-frequency electromagnetic field (ELF-EMF) can induce higher EV production; however, there is no data if this can change EV content and the functionality. Therefore, this study aimed to test ELF-EMF on MSC EV production, content and functionality.
Methods: Our experimental model used an 8–50 hertz ELF-EMF stimulation setup with uniform magnetic field intensity of 0 - 8 mT (militesla). In a series of experiments, MSCs were stimulated with ELF-EMF for 48 hours, followed by EV isolation using ultracentrifugation method. EVs were analysed using nanoparticle tracking assay, Western Blot, transmission electron microscopy, and small RNA sequencing. Additionally, functional tests of ELF-EMF exposed MSC EVs and control EVs were performed on a human primary small airway epithelial cell (HSAEC) line, where cell proliferation was evaluated.
Results: A total of 28 experiments were performed with different testing exposures and conditions. EV secretion was dependent on stimulation frequency with a peak at 20 Hz for MSC, with an average increase in EV by 39% ($p< 0.05$), but not the intensity of the field. No change in EV size or morphology was observed and no significant effect on HSAEC cell proliferation were monitored ($p>0.05$). Sequencing data are currently analysed and will be presented at conference.
Summary/Conclusion: We have shown that ELF-EMF can induce EV production by MSC without significant effect on EV morphology.

Funding: Project No: lzp-2022/1-0373 (https://biomed.lu.lv/project/lzp-2022-1-0373-2/).
Keywords: extremely low frequency electromagnetic field, mesenchymal stem cell secretion enhancement, ELF-EMF exposure on MSC-EV content and functionality, ELF-EMF stimulation setup for EV production

PS13.16 | Production of extracellular vesicles from microsphere encapsulated cells

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Introduction: Mesenchymal stem cells (MSCs) naturally produce therapeutically relevant EVs that have potential to treat many immunological diseases. However, primary MSCs vary extensively between different tissues and donors, have limited replicative potential, and are typically grown in 2D monolayers. Collecting sufficient EVs for a therapeutic treatment is therefore resource-, cost-, and labor-intensive. MSCs differentiated from induced pluripotent stem cells (iPSCs) can provide a reproducible non-invasive source of MSCs that avoids obstacles due to donor-to-donor variability and scarcity of human tissue. This study aims to develop a scalable platform to produce EVs from tissue-specific cell types, using iPSC-derived MSCs as a proof of concept for other cell lines that can also be derived from iPSCs.
Methods: We are optimizing EV production by encapsulating MSCs into GelMA-Cad hydrogel microspheres. GelMA-Cad is a gelatin covalently bonded to N-cadherin to support cell growth by mimicking cell adhesion to the extracellular matrix. Cells are encapsulated using a custom microfluidic device, and the microspheres are cultured in suspension. EVs are collected from the culture media for characterization (NTA, western blot, and TEM) and functional assays.
Results: We have shown that GelMA-Cad supports cell growth in both 2D and 3D culture. By seeding MSCs into hydrogel microspheres, adherent cells can be grown in a pseudo-suspension culture allowing for increased cell density and EV production per volume of culture. EV yield (per cell) and particle size were compared to traditional 2D adherent cultures. Here, we found that MSCs seeded in GelMA-Cad produced a similar yield of small EVs but higher yield of large EVs when grown in pseudo-suspension culture.
Summary/Conclusion: Production of EVs from MSCs embedded in GelMA-Cad leads to higher volumetric productivity than adherent 2D cultures. Future work will expand this platform to other iPSC-derived cell types beyond MSCs.
Funding: NSF MCB-2036809.
Keywords: Hydrogels, Mesenchymal Stem Cells, Induced Pluripotent Stem Cells, Microsphere

PS13.17 | Comparison of EV production efficiency from mouse vs human tumor cell lines

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Introduction: Extracellular Vesicles (EV) are of broad interest as carriers of molecular signatures of tumor progression and cancer treatment response. To study EVs and the nucleic acids, lipids, and proteins that comprise different types of EVs, one commonly used method to generate of EV stocks is production of conditioned cell media (CCM), followed by steps to concentrate and purify EVs. Factors that impact the yield and efficiency of EV production using CCM are not yet fully defined, so we reviewed data from a highly controlled set of 19 EV productions performed in 2022 to identify factors for future consideration or further investigation.
Methods: Cells used in this comparison included 11 mouse and 8 human cell lines. Cells were cultured in serum free media to produce one liter of conditioned media for each cell line. CCM EVs were harvested and isolated by ultrafiltration (100K) followed by size exclusion chromatography. Cell density and viability were evaluated by microscopy at the time of CCM harvest. Duration of the production culture phase was documented in hours, and peak SEC fraction particle size and concentration for this analysis were documented by nanoparticle tracking analysis.
Results: Pairwise comparison of average results in human and mouse samples identified factors related to human vs mouse cellular doubling times as associated with the yield and efficiency of EV production using this CCM method. Human cell lines required an average of 6 more hours than mouse cell lines to grow to a sufficient density to harvest CCM (human: 49.4 vs mouse: 43.3), and resulting CCM samples at the times of harvest produced fewer EVs on average from human cell lines than from mouse cell lines (human: 7.15e10 vs mouse: 1.42e11 particles per mL in 5mL).
Summary/Conclusion: Most human tumor cell lines are known to have longer average doubling times than mouse tumor cell lines. Our results demonstrating lower EV yields and lower cellular densities despite longer average times in culture for human

cells, indicate that EV production from human cell lines might be improved by extending cell culture prior to CCM harvest into a third day, rather than planning to harvest human tumor cell line CCM for EV isolation on the second day of culture.
Funding: NIH, NCI, CCR Intramural Research Program.
Keywords: extracellular vesicles, production, conditioned media

PS13.18 | Considerations and the importance of cell culture conditions that influence stem cells and stem cell-derived extracellular vesicles for regenerative medicine therapies

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Introduction: Naïve extracellular vesicles (EVs) have the potential to revolutionize therapeutic treatments for regenerative medicine on a broad scale. The paracrine factors retained in EVs from parent stem cells have the ability to orchestrate complex physiological changes. The lack of standardization in culture of adherent mesenchymal stem cells has resulted in a diversity of methods for expansion and maintenance. Further, it is unclear how cell culture conditions influence cell expansion, behavior, and EV production. In this work, we analyzed human bone marrow-derived MSCs (hMSCs) and subsequent EV production in various cell culture devices to understand the environmental effects that drive changes in MSC physiology and EV functional performance.
Methods: hMSCs were cultured in xeno-free medium to understand the importance of polymer surfaces, gas-permeable film, surface chemistry, and shear stimuli on hMSC properties and derived EVs. EVs were isolated via tangential flow filtration. EV concentration and size was assessed via Nanoparticle Tracking Analysis, tetraspanin expression was profiled via single particle interferometric reflectance imaging sensor, and biological functionality was assessed via wound healing assay. hMSCs biomarker expression was analyzed via flow cytometry.
Results: Cell culture conditions influenced the expansion and characteristics of hMSCs, which can impact the quantity and quality of EVs produced, supporting the need for process standardization. While material choice and surface treatment are important factors to consider when expanding MSCs, the addition of mild shear stress can enhance EV production with no biological consequences.
Summary/Conclusion: This data suggests that material choice, surface treatment, and environmental stimuli are important factors to consider when developing in vitro cell culture processes, and highlights the impact they can have on the biology and functional performance of naïve EVs for regenerative medicine.

PS13.19 | Development of specialized EV production media using high throughput cell culture and EV characterization capabilities

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Introduction: Extracellular vesicles (EVs) are nano-sized vesicles that are released from a variety of cell types and play a role as messengers of an intercellular network, allowing exchange of cellular components between cells. EVs contain a lipid bilayer that protects proteins, DNA, RNA and lipids from degradation and have the potential to be used as a cell-free therapy for a variety of diseases including cancer, heart disease and inflammation. However, one of the major challenges for EV-based therapeutics is the low productivity of EVs from the producer cells. Here we report the development of serum-free EV production media using high-throughput screening methods.
Methods: We have developed high-throughput cell culture and EV characterization methods to optimize EV production media formulation. A liquid handler allowed us to test more media formulations simultaneously, saving time relative to the manual procedure. Single EVs were characterized with dSTORM-based imaging. The function of the EVs was evaluated using a cell-based assay.
Results: By using high-throughput media screening and EV characterization capabilities, we identified prototype media that promote cell growth and stimulate functional EV secretion. These prototypes were also validated in larger scale culture. We confirmed that EVs purified from the prototype media were functional using the cell-based assay.
Summary/Conclusion: We have demonstrated a platform by which we can implement high-throughput approaches to study media effects on EVs and subsequently optimize media. Our high throughput compatible approach for media development will enable us to develop specialized EV production media for a variety of cell types.
Keywords: extracellular vesicle, exosome, EV therapeutics, culture media development, single EV characterization

PS14: EV Therapy

Location: Hall 4A

16:50 - 18:50

PS14.02 | Development of targeted exosome as plasmid delivery vehicles to HER2-expressing breast cancer cells

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Introduction: Finding a safe gene delivery system is crucial for gene therapy in a broad range of diseases. Exosomes as natural nanocarrier are considered as ideal delivery platforms for gene therapy that can efficiently and selectively deliver their cargo to the target cells. The significant potential of exosomes for being investigated and applied for gene therapy purposes is mainly derived from their high level of biocompatibility and low level of immunogenicity. The potential of using exosome for delivery of functional DNA to target cell should be further investigated.
Methods: HEK293T was engineered to express DARPin against HER2 and targeted exosomes were isolated from modified HEK293T using Exo-spin kit. Purified exosomes were characterized by TEM, zeta sizer and exosomal CD markers. Purified pEGFP-C1 plasmid was introduced to DARPin/Exos using electroporation and termed to pEGFP-C1/Exos. PKH26-labeled pEGFP-C1/Exos were exposed to HER2-positive SKBR3 cell line and exosome uptake was validated by florescent microscopy and GFP expression was analyzed using real-time PCR, florescent microscopy and flow cytometry. Effect of increase in exosome quantity and GFP expression was measured.
Results: In this study, we demonstrated delivery of GFP-encoding plasmid to target cells by exosomes the quantity of pEGFP-C1 plasmid in pEGFP-C1/Exos was measured through Real-time PCR. Transferring of pEGFP-C1/Exos to the target cells and GPF expression in mRNA and protein level proved the sufficient delivery of the plasmids. PKH26 labeled-pEGFP-C1/Exos were uptaken by SKBR3 and expressed GFP. Increase in pEGFP-C1/Exos quantity caused more GFP expression in target cells.
Summary/Conclusion: Exosome as natural, nontoxic, and non-immunogenic carriers have potential for using in gene delivery system in vitro. In this study, through using proper electroporation protocol the 4 kbp GFP-encoding plasmid, pEGFP-C1were loaded into HER2-targeted exosomes successfully and delivered them to the target cells functionally. Based on these results, targeted exosomes may be promising vehicle for cancer therapeutics in the future. Broader assessments of this method using various types of cargoes and target cells can highlight the findings and guarantee their in-clinic applicability for gene delivery and gene therapy purposes.
Keywords: HER2, exosome, gene delivery, gene therapy, breast cancer

PS14.03 | Ultrasound-mediated nanoliposomes as future carriers to deliver miRNAs to cumulus cells during oocyte in vitro maturation

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Introduction: Nowadays, the use of extracellular vesicles (EVs) as drug delivery systems remains challenging, mainly due to the lack of standardized isolation methods, limited drug loading efficiency, and insufficient clinical grade production. In turn, nanoliposomes (NLPs) are lipid carriers that have been efficiently produced in several large-scaled industrial practices, as well as easily characterized by well-established techniques. One can say that EVs can be considered as more complex forms of NLPs due to their biological origin. Therefore, we intended with this study to produce NLPs containing miRNAs usually found in EVs and of interest during oocyte in vitro maturation, in order to design a synthetic vesicle with increased endogenous properties and possible to be scaled-up.
Methods: Unloaded NLPs were produced using cholesterol and commercial phospholipids at a 20:80 ratio. Dispersions were sonicated for 5 s on and 2 s off cycles (50-75 in total) at a 60% amplitude using an ultrasonic tip homogenizer, and then extruded at 12 bar through 100-nm polycarbonate membranes. Samples were characterized in respect to their average mean diameter, size distribution and zeta potential using dynamic light scattering, and to concentration using nanoparticle tracking analysis. Morphology of vesicles was observed using atomic force microscopy. NLPs staining with rhodamine-labeled lipids or PKH26

fluorescent dye was confirmed by flow cytometry. NLPs were incorporated into bovine cells in culture to verify possible uptake mechanisms, which were analyzed using fluorescence microscopy.
Results: Results showed that NLPs were spherical with diameters lower than 100 nm, electrically stable with zeta potential values ranging from -60 and -50 mV, and homogeneous in size with polydispersity index values lower than 0.3. Fluorescence microscopy showed that NLPs appeared to remain in the cytoplasm, around the nuclei of bovine cells.
Summary/Conclusion: Further studies regarding the engineering of NLPs in respect to the amount and transfection of incorporated miRNAs are needed to validate their potential as genetic delivery systems. The hybridization of EVs with NLPs is also proposed to produce endogenous nanocarriers with more flexible membranes, easier to interact with cumulus cells. In addition to that, the NLPs did not present any harming effect to the cells and would possibly allow their use during oocyte in vitro maturation.
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Keywords: lipid carriers, engineered extracellular vesicles, reproduction

PS14.04 | Use of small extracellular vesicles derived from RWP-1 in pancreatic ductal adenocarcinoma treatment

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Introduction: Pancreatic Ductal Adenocarcinoma (PDAC), is the most common aggressive cancer of the pancreas. The standard care of PDAC include tumor resection and chemotherapy, but the lack of early diagnostic and the limited response to the treatment worsens the patient's condition. In order to improve the efficiency of chemotherapy, we look for more efficient ways of drug delivery.
Methods: We isolated and fully characterized small Extracellular Vesicles (EVs) from the RWP-1 cell line. We loaded them with two chemotherapeutic drugs (Temozolomide and EPZ015666) by direct incubation method and, the amount of drug loaded was measured by high-performance liquid chromatography (HPLC). Finally, we tested their antiproliferative effect on different cancer cell lines.
Results: Our study indicates that the direct incubation method was the most efficient loading protocol and that a minimum total amount of drug triggers an effect on tumor cells. Moreover, we have seen that RWP-1 small EVsTMZ, were more efficient than RWP-1 small EVsEPZ015666.
Summary/Conclusion: RWP-1 derived small EVs represent a promising drug delivery tool that can be further investigated in preclinical studies and its combination with PRMT5 inhibitor can be potentially developed in clinical trials for the treatment of PDAC.
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Keywords: pancreatic ductal adenocarcinoma, small EVs, nanocarriers, chemotherapy, FESEM.

PS14.05 | Enhanced antibiotic delivery techniques using outer membrane vesicles

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Introduction: Outer membrane vesicles (OMVs) are spherical proteoliposomes derived from the outer membrane of Gram-negative bacteria that are capable of encapsulating and delivering biomolecules, such as toxins and genetic material. Inspired by

their ability to cross the complex Gram-negative outer membrane, our research focuses on utilizing OMVs as a delivery vehicle for antibiotics. Several passive and active methods have been chosen to test loading optimization. We hypothesize that loading efficiency is based on the hydrophobicity of the antibiotics, and that active methods are more efficient than passive methods.
Methods: OMVs were harvested from the hypervesiculating E. coli JC8031 strain and loaded with ciprofloxacin, norfloxacin, levofloxacin, or moxifloxacin. The two passive loading methods were incubation of free-antibiotics with purified OMVs, and direct incubation, in which free-antibiotics were incubated in the E. coli culture and aOMVs were harvested. The two active loading methods were electroporation and sonication. The encapsulation efficiency was determined by quantifying the antibiotic concentrations before and after loading using UV-vis spectroscopy.
Results: Direct incubation resulted in minimal/no encapsulation of any antibiotic. Of all the loading methods, electroporation provided the highest encapsulation efficiency for all antibiotics except levofloxacin. For levofloxacin, simple mixing with purified OMVs was the most effect method. The active loading methods were more effective than the passive loading methods in encapsulating norfloxacin and ciprofloxacin. Moxifloxacin showed the overall highest encapsulation efficiency, supporting our hypothesis that hydrophobic antibiotics gain entry into OMVs more effectively than hydrophilic ones.
Summary/Conclusion: These results demonstrate that fluoroquinolone antibiotics can be readily incorporated into OMVs to create novel delivery vehicles.
Funding: National Institutes of Health (DE027769).
Keywords: outer membrane vesicles, loading, antibiotics

PS14.06 | Functional and biophysical characterization of wharton's jelly derived small EVs demonstrates stability & efficacy for short term storage at different temperatures

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Introduction: Mesenchymal Stem Cells (MSCs) are the key therapeutic candidate in the field of regenerative medicine and can be derived from various sourcesv(Bone Marrow (BM) and Wharton's Jelly (WJ)). Wharton's Jelly, being a naïve source and posing less ethical concerns is an interesting candidate of choice for MSCs derivation. However, in terms of their translational significance, the point of their storage and transportation poses a major complication due to eventual loss of their viability. Owing to such issues, the derivatives of these stem cells, i.e. Extracellular Vesicles (EVs), especially small EVs (size > 200nm) have secured limelight as novel therapeutic tool. These nano sized vesicles have attracted the interests of scientific and clinical community for their ease of storage, transportation and logistics, making them a strong candidate as a stem cell derived 'off the shelf' product. To delve further into this aspect, we have evaluated the functional and biophysical capabilities of WJ-MSCs derived small EVs to test their durability for translational applications
Methods: Wharton's jelly was collected after obtaining donor consent(IC-SCR/120/21(R)). MSCs were cultured in serum free media for isolation of small EVs using Total Exosome Isolation Kit (Thermofischer). The small EVs were then subjected to short term storage for 1 and 3 months at different temperatures, including RT, 4°C, -20°C, -80°C to assess their shelf life. At each condition, small EVs were characterized via NTA (Size < 200nm), TEM (Cup shaped morphology), Western blotting (Surface marker-CD63;Cytoplasmic marker-ALIX; Negative marker-Calnexin) for their biophysical characteristics, and for the functionality assessment, their effect on the proliferation of keratinocytes cell line (MTT), migration of keratinocytes cell line (Scratch assay), and immunosuppressive activity on PBMCs (MLR) was checked.
Results: It was observed via the biophysical characterization and functional assessments including proliferation, migration, and immunosuppression studies that the small EVs are capable of maintaining their functional capabilities for upto 3 months, when stored at lower temperatures. However for upto 1 month, they are also able to maintain their functionality at a temperature of 4°C
Summary/Conclusion: We can conclude from this study that WJ-MSC-EVs can be stored as an 'off the shelf' product at a common refrigerator temperature for up to 1 month, while for storage up to 3 month, lower temperatures have to be maintained

PS14.07 | Membrane-anchored epidermal growth factor on stem cell exosomes for wound healing

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Introduction: In this study, we found that exosomes with epidermal growth factor (EGF) on their membrane have cell proliferative effects than those of recombinant EGF proteins (rEGF). Furthermore, mesenchymal stem cell exosomes expressing EGF

exhibited regenerative ability compared with either rEGF or MSC exosoms. This suggest that exosome provides the optimal membrane environment for EGF.

Methods: Preparation & characterization of exosome Cells were maintained in DMEM with 10% FBS and 1% antibiotic-antimycotic at 37°C in 5% CO2. The supernatants of transfected HEK293T cells by PEI and transduced ASC52telo cells were centrifuged followed by concentration with tangential flow filtration. The concentrates were ultracentrifuged at 150,000 g for 3 h and were re-suspended in PBS. Exosomes were analyzed by DLS, NTA, and cryo-TEM. Exosomal proteins were analyzed by Western blot. In vitro cell proliferation & migration assay To assess cell proliferating ability of exosomes, Cell Counting Kit-8 assay was performed. Serum-starved cells for 24 h, were incubated with rEGF or exosomes. After 48 h, CCK-8 solution was added and the optical density was measured. To assess the ability of exosomes on cell migration, cells were scratched and incubated with rEGF or exosomes and observed. In vivo wound healing assay 8 mm of wounds were made on the back of Balb/c mice with biopsy punch. PBS, and PBS containing rEGF or exosomes were injected subcutaneously. The tissues were analyzed by H&E staining and IHC.

Results: Exosomes characterization 3 constructs of EGF were prepared. The EGF precursor (preEGF); EGF domain inserted after transmembrane domain of PDGFR (pEGF); lacking prepro domain and juxtamembrane stalks region of preEGF (tEGF). All types of exosomes expressed exosomal markers and EGF. The treatment of ADAM17 induced the loss of the ectodomain only with preEGF. tEGF-Exosomes have the regenerative effect The efficacy of tEGF-Exo was evaluated by Cell Counting Kit-8 and scratch assay. tEGF-Exo treatments significantly increased cell viability and migration of HaCaT and Balb/3T3 cells compared with non-treated group. stEGF-Exosomes promoted migration & proliferation in vitro Stem cell exosomes with tEGF (stEGF-Exo) treatments promoted HaCaT and Balb/3T3 cells proliferation and migration compared with control groups. tEGF-Exo efficiently promoted wound healing in vivo stEGF-Exo significantly enhanced wound closure in vivo. Furthermore, H&E and IHC detection of KI67 showed that stEGF-Exo promoted wound healing.

Summary/Conclusion: The treatment tEGF-Exo promoted cell proliferation compared with rEGF. Further, stEGF-Exo treatment showed effective wound healing. Collectively, exosome enhanced the biologic effect of secretory proteins if they are anchored to exosome membrane. Our study suggests that exosomes can be a promising therapeutic option to overcome limitations of growth factors.

PS14.09 | MicroRNA profiling of royal jelly nanovesicles and its potential role in cell viability and reversing cell apoptosis

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Introduction: MiRNAs are small non-coding RNA molecules that play important regulatory roles in diverse biological processes. Royal jelly, a milky-white substance produced by nurse bees, is the primary food of queen bees and plays a crucial role in their development. However, little is known about the microRNAs (miRNAs) content of royal jelly and their potential functions. Anastasis is the mechanism by which cells can recover from apoptotic lesions and revert to their previous functioning state from the brink of cell death.

Methods: In this study, we isolated nanovesicles from the royal jelly of 60 samples through sequential centrifugation and targeted nanofiltration, and performed high-throughput sequencing to identify and quantify the miRNA content of honeybee Apis mellifera royal jelly extracellular vesicles (RJEVs). RJEVs were visualized through cryogenic transmission electron microscopy, and the size and concentration were evaluated through nanoparticle tracking analysis. To investigate the potential roles of RJEVs in cell viability, RJEVs were supplemented to apoptotic cells induced by ethanol 6% exposure for 30 min, and cell migration and viability were also checked.

Results: We found a total of 27,134 mapped sequences and 29 known mature miRNAs and 17 novel miRNAs. Through bioinformatic analysis, we identified several potential target genes of the miRNAs present in royal jelly, including those involved in developmental processes and the cell differentiation. TUNEL assay showed a significant reduction in the apoptosis percentage when apoptotic cells supplemented with RJEVs when compared with the control no-supplemented group. Moreover, wound healing assay performed on the apoptotic cells showed a rapid healing capacity of RJEVs-supplemented cells when compared with the control no-supplemented group. We observed a significant reduction in the expression of target genes such as FAM131B, ZEB1, COL5A1, TRIB2, YBX3, MAP2, CTNNA1, and ADAMTS9 suggesting that RJEVs may play a role in the regulation of gene expression of cellular motility and cell viability. Moreover, RJEVs reduced the expression of apoptotic genes (CASP3, TP53, BAX and BAK), while significantly increased the expression of anti-apoptotic genes (BCL2 and BCL-XL).

Summary/Conclusion: Our findings provide the first comprehensive analysis of the miRNA content of RJEVs and suggest a potential role for these vesicles in the regulation of gene expression and cell survival as well as augmenting cell resurrection or anastasis.

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Keywords: royal jelly, cell viability, anastasis, apoptosis, cryo-TEM, miRNAome

PS14.10 | Identification of anchor proteins for the BioDroneTM platform

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Introduction: BioDroneTM, developed at MDimune, is a state-of-the-art drug delivery system based on cell-derived vesicles (CDVs) produced by serial extrusion from diverse cell sources. CDVs exhibit physicochemical similarity to extracellular vesicles (EVs) but a tremendous advantage in production scalability over EVs. Furthermore, with genetic engineering of mother cells, CDVs can potentially acquire additional biological features such as targeting and cargo loading capabilities.
Methods: In this study, we identified stable anchor proteins exclusively for HEK-CDV. First, we analyzed the HEK-CDV proteome and selected a set of CDV-specific membrane proteins highly abundant in the CDVs over cells or EVs. Then, these anchor candidates were overexpressed with a tag and GFP in the HEK293 cell. Antibiotic selection and cell sorting established a pool of stable HEK293 cells. After CDV production, the anchor fusion proteins were quantified via GFP ELISA, and their distribution in a single-particle resolution was examined by nanoflow cytometry.
Results: Four HEK-CDV anchors among candidates showed a stable presence in the CDVs, with 30 to 150 molecules per CDV particle and 42 to 66 % of the GFP (+) population among total particles, depending on the anchor proteins. Moreover, their proper topology was confirmed via protease cleavage assay, while their abundance in the CDVs was also confirmed from a large-scale extrusion.
Summary/Conclusion: Together, we present a set of HEK-CDV anchors for the versatile engineering of the BioDroneTM platform. Currently, we are developing various BioDroneTM therapeutics utilizing these anchors.
Keywords: cell-derived vesicle(CDV), BioDrone platform, anchor proteins

PS14.11 | Extracellular vesicles mediated cell-cell communication is required for tissue maintenance and performing RNAi in Planarians

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Introduction: Upon amputation, planarian stem cells (neoblasts) first exhibit body-wide proliferation, followed by mobilization and localized expansion of a subset of cells to the vicinity of the wound. These coordinated steps are critical to blastema formation and suggest an essential role for intercellular communication during wound healing and tissue regeneration. Yet, little is known about the factors affecting the observed cellular biology. Recently, Extracellular Vesicles (EVs) have been shown to play a role in cell-cell communication in multiple systems.
Methods: EVs were isolated from 2 DPA animals by combining Ultracentrifigation and Ultrafiltration methods. NTA was performed on isolated EVs to quantify the particles, and TEM was performed for qualitative analysis. MassSpectrometry was performed to study the EV proteomics profile and the cargo packaged inside them. RNAi was performed to validate the significance of the EV cargo-related genes during planarian regeneration and Homeostasis. Small RNA sequencing was performed to understand the diversity of small RNA species packaged inside the EVs. EVs derived from RNAi animals were transplanted back into healthy animals to understand the role of EVs in planarian RNAi transport.
Results: Proteomics and small RNA sequencing revealed the molecules packaged inside the EVs during planarian regeneration. RNAi on EV biogenesis pathway genes leads to lethality in regenerating and intact animals, suggesting that their pathway is conserved evolutionarily. Perturbing the EV cargo genes by RNAi leads to multiple defects in regenerating planarians, such as lethality, stem cell maintenance, etc. Interestingly, we could also prove that Evs are used as a mode of transport to carry out RNAi-mediated gene knockdowns in planarians. Small RNA profiling of EVs from OVO and UNC RNAi animals showed an array of 20–25 nucleotide gene-specific fragments packaged inside the EVs. Transplanting EVs derived from RNAi animals showed gene-specific RNAi phenotypes in healthy animals.
Summary/Conclusion: Overall, these results reveal EVs essential role in planarian regeneration and Homeostasis. We were also able to find out a novel role of EVs as RNAi transporter in planarians. The latter finding brings out an opportunity for performing transgenics in planarians.
Funding: Stowers Institute and HHMI.
Keywords: stem cells, regeneration, RNAi

PS14.14 | Breast cancer brain metastasis is suppressed by interfering adhesion of cancer EVs to the recipient cells

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Introduction: Cancer cells utilize extracellular vesicles (EVs) to promote cancer pathogenesis, including proliferation and metastasis. Our previous study showed that EVs from brain metastatic breast cancer cells break down the blood-brain barrier (BBB) through the change in actin dynamics and promote brain metastasis in vivo (Nature Communications, 2015). These results indicate a novel mechanism of brain metastasis mediated by EVs that triggers the destruction of BBB. Therefore, we aimed to prevent brain metastasis of breast cancer by a new approach to inhibit EVs-dependent metastatic pathways, especially focusing on interfering adhesion of cancer EVs to the recipient cells.
Methods: For preparing EVs, the cultured medium was filtered with a 0.22-μm and ultracentrifuged at 110,000g for 70 min at 4°C. Then the pellets were resuspended in PBS. BMD2a cells established from MDA-MB-231 cells were used as brain metastatic breast cancer cells, and mice were immunized with these cells-derived EVs (BMD2a-EVs). We obtained some monoclonal antibodies that specifically recognize BMD2a-EVs and performed a screening for the antibodies to inhibit EV uptake. Inhibition of EV uptake by antibodies was assessed by the amount of adhesion of PKH-labeled BMD2a-EVs to the brain vascular endothelial cells.
Results: As the screening result, we obtained two different EVs adhesion-inhibitory antibodies. These antibodies suppressed the BMD2a-EVs-induced BBB destruction in an in vitro BBB model system. To further evaluate the effect on the prevention of BBB disruption in vivo, these antibodies and BMD2a-EVs were co-administered intravenously into Scid mice. The results showed that these antibodies also inhibited BMD2a-EVs-induced BBB destruction in vivo.
Summary/Conclusion: This study provides a novel therapeutic strategy for preventing brain metastasis of breast cancer and other brain metastatic tumors, including lung cancer, by inhibiting adhesion between EVs and recipient cells using antibodies.

PS14.17 | Mitigation of atopic dermatitis by extracellular vesicles derived from interferon-gamma-primed induced mesenchymal stem cells

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Introduction: Extracellular vesicles (EVs) from mesenchymal stem cells (MSCs) are now being recognized a novel therapeutics for various diseases because of their immune-modulatory and anti-inflammatory potential. Atopic dermatitis (AD) is chronic skin disease manifested by pruritus, impaired immunity, and skin tissue destruction. Stimulation of MSCs with inflammatory cytokines such as interferon-γ (IFN-γ) can improve the therapeutic potential of MSC-derived EVs.
Methods: EVs were generated from interferon-γ-stimulated induced mesenchymal stem cells (IFN-γ-iMSC-EVs). The proteome signature of IFN-γ-iMSC-EVs was analyzed. IFN-γ-iMSC-EVs were subcutaneously administered into a mouse model of AD induced by 2,4-dinitrochlorobenzene (DNCB). The repression of AD by IFN-γ-iMSC-EVs was assessed by analyzing clinical features and the expression of molecular markers in AD mice.
Results: IFN-γ-iMSC-EVs was enriched with proteins responsible for regulating interferon activity and inflammatory signaling, as shown by proteome profiling followed by detailed bioinformatic study. The expression of major Th2 receptors (IL-4α/13Rα1/31Rα) was diminished, the their corresponding intercellular signaling molecules also became less active. IFN-γ-iMSC-EVs alleviated clinical symptoms, and blocked infiltration of inflammatory and mast cells in AD skin. In addition, IFN-γ-iMSC-EVs led to a reduction in the expression of thymic stromal lymphopoietin (TSLP), NF-κB activation, and IgE receptors. Further, disintegrated skin barrier by AD was reversed by IFN-γ-iMSC-EVs, which was supported by the upregulation of key factors responsible for epidermal differentiation and lipid synthesis.
Summary/Conclusion: Conclusively, we demonstrated that IFN-γ-iMSC-EVs has potential to become a novel EVs based therapeutics for AD via blocking inflammation/Th2 response as well as promoting skin restoration.
Keywords: extracelluar vesicle, induced mesenchymal stem cell, interferon-gamma, atopic dermatitis