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Extracellular Vesicles and Circulating Nucleic Acids

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Extracellular Vesicles and Circulating Nucleic Acids (*EVCNA*), ISSN XXXX-XXXX (Online), is a peer-reviewed, open-access and continuously online published journal. The journal's full text is available online at www.evcna.com. The journal provides an online platform for the sharing of research data, new methodology, reviews and commentaries in the areas of extracellular vesicles and circulating nucleic acids including DNA, RNA, and miRNA and their therapeutic use. The journal is committed to the rapid publication of original findings that increase our understanding of the molecular and cell biology, biogenesis, and origin of extracellular vesicles and circulating nucleic acids; and their use as biomarkers for the diagnosis, prognostication and surveillance of disease states, and in therapeutics. Manuscripts with clinical relevance are especially encouraged to promote the translation from basic science to clinical applications. The criteria for acceptance are scientific excellence and originality. All works involving the use of animals and human subjects must have been approved by institutional review committees and adhere to accepted international ethical standards.

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Extracellular Vesicles and Circulating Nucleic Acids

Editorial

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Welcome to the journal of *Extracellular Vesicles and Circulating Nucleic Acids*: a new open-access scientific journal

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A wealth of research has emerged over the last five years showing the importance of extracellular vesicles (EVs) as mediators of intercellular communication. EVs range in size from 30 to 400 nm and according to the size have been classified as microvesicles, exosomes, and oncosomes. EVs are released from cells in normal and pathological conditions, into many body fluids such as blood, urine, saliva, cerebral spinal fluid, and milk. EV contents, which include RNA, proteins, and lipids, reflect the state of the cell of origin, such as during metabolic changes and disease. Therefore, EVs have emerged as potential biomarkers. Furthermore, stem cell EVs have now been found to be important in different types of tissue repair. EVs have also been useful for delivery of siRNA, proteins, and other molecules for therapeutic use, and clinical applications of EVs are emerging. At the same time, studies to better understand the cellular mechanism of EV biosynthesis, trafficking, uptake, and release of EV cargoes in cells have facilitated the production, loading, and purification of EVs for therapy.

Besides EVs, cells release nucleic acids into the circulation and other body fluids, and they are potential biomarkers for disease. Cell-free (cf) DNAs have been used for example to monitor tumor progression and heart transplant rejection, while cf-RNA, especially miRNAs, which are highly stable, are useful biomarkers in cancer and neurodegenerative diseases. To facilitate multi-omics analysis of cell-free nucleic acid biomarkers, new techniques such as electrokinetic chip devices and microfluidic systems have been developed to isolate cf-DNA and exosomes from body fluids, respectively. Liquid biopsy employing circulating exosomes and cf-nucleic acids is a non-invasive and safe alternative to tissue biopsy to monitor disease progression and directing therapy.

The Journal *Extracellular Vesicles and Circulating Nucleic Acids* (EVCNA) provides an online platform for sharing of research data, new methodologies, reviews, and commentaries on these exciting areas of research that are moving at a rapid pace, involving partnerships between academia and industry to bring liquid biopsy biomarker assays and exosomal delivery of therapeutic agents to clinical use. At the same time, the journal supports publication of strong basic research which enables development of clinical applications. EVCNA will be published quarterly with additional Special Issues focusing on specific topics. We have assembled an eminent group of international researchers with expertise across the topics covered by the Journal to serve on the Editorial Board. Manuscripts with clinical relevance are especially encouraged to promote the translation from basic science to clinical applications. The criteria for acceptance will be scientific excellence and originality. We also aim to have a quick turnaround time for processing of articles submitted for publication without compromising rigorous peer review.

In this first issue of ECVNA, scheduled to be launched in December 2020, we will publish a report of the highlights of the American Society of Extracellular Microvesicles 2020 meeting that was successfully held as a virtual conference during 17-19 November 2020 due to the COVID-19 pandemic. We will also include abstracts of talks and posters that were presented at the meeting. Our second issue is scheduled to be published in March 2021 with five scientific articles. We would like to invite you to submit papers for future issues of EVCNA (www.evcna.com).

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Extracellular Vesicles and Circulating Nucleic Acids

Conference Report

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A Report on ASEMV2020

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American Society for Exosomes and Microvesicles (ASEMV) held its Annual Meeting, ASEMV2020 on November 16-19, 2020. The precursor meetings of Exosomes & Microvesicles 2011 and 2012 (in Orlando, FL, USA) were among the earliest exosome/extracellular vesicle meetings held in the United States, with many international attendees. The Society coalesced under the ASEMV banner in Orlando in 2013, and has held Annual Meetings ever since. This year's meeting was obviously substantially different, conducted virtually due to the ongoing COVID-19 pandemic. This changed much of the usual meeting dynamic, but the conference maintained its high level of scientific presentations and discussions that are the heart and soul of the Society and its yearly meetings.

Previous ASEMV Meeting sessions consisted of talks with mixed topics that allowed for a broad presentation of information that attendees might have surprisingly found more interesting than they may have ordinarily anticipated. For ASEMV2020, however, the Organizing Committee chose to group talks by topics, sometimes covering two sessions. The rationale was to have all questions pertaining to the entire session covered in one panel discussion period, after all talks for that topic were completed. All speakers were present in the roundtable forum. The audience members entered their questions via a chat function that was enabled during the talks and the discussion. Session moderators compiled questions and directed them to each of the session's speakers accordingly. Attendees could also email questions to speakers, or engage by messaging the community forum. These processes enabled some semblance of the lively scientific dialogue, which is a hallmark of ASEMV Meetings.

ASEMV2020 was hosted online by Designing Events. The Meeting had 181 registrants from 14 countries and 3 sponsors (NanoView Biosciences [gold]; Izon Science [silver]; and Spectradyne Particle Analysis [bronze]). ASEMV2020 spanned four days, comprising three to four sessions and two related discussion periods per

day, plus one poster session. There were also two ASEMV Working Group (WG) Meetings, including the Resource Sharing WG and the newly formed Diet and Nutrition WG. The Meeting was flanked by grants workshops, and ended with an Awards Ceremony with prizes for Young Investigator talks and poster presentations.

ASEMV2020 opened with remarks from ASEMV President, Steve Gould (Johns Hopkins University, US) and ASEMV2020 Organizing Committee Chair, Louise Laurent (University of California San Diego, US), welcoming attendees and describing the new format for this year.

Next came the first of the grants workshops organized by Fatah Kashanchi (George Washington University, US). This featured Program Directors from the US National Institutes of Health (NIH) whose Centers and Institutes provided funding on exosome-related projects.

The grants workshop opened with a presentation from Matthew Young (National Cancer Institute, (NCI), Cancer Biomarkers Research Group, Division of Cancer Prevention) who focused on the utility of extracellular vesicles and particles (EVPs) in cancer detection via liquid biopsy. He covered "hopes and hypes" in the area, noting that as of yet, there are no US Food and Drug Administration (FDA) approved clinical uses for exosomes/extracellular vesicles, although trials for both biomarker and therapeutic applications are underway. He emphasized upon the hope that EVPs could be used for plus/minus cancer detection (cancer *vs.* non-cancer), across various cancer types and stages. This would require rigor in EVP separation methods and biomarker identification, with applicability at either standard clinical lab facilities or in specialized centers. He advertised PAR-20-253, an NCI Funding Opportunity Announcement for innovative research in vesicle separation and characterization, the goal of the PAR is to promote rigor and reproducibility in the field.

Matthew was followed by John Satterlee from the National Institute on Drug Abuse (NIDA), Division of Neuroscience and Behavior, who presented a broad overview of the NIH budget and funding breakdown, then narrowing down to NIDA. In the background of an increase in drug overdose deaths in the US in 2019, he referred to several existing exosome/EV funding opportunities and currently funded projects on neuroscience and neuropathology, associated infectious diseases, and diagnostics where there is overlap with the Extracellular RNA Communications program (see below). John encouraged everyone with an interest in NIH funding opportunities to subscribe to the "NIH Guide to Grants and Contracts" (https://grants.nih.gov/grants/guide/listserv_dev.htm); to contact Program Officers to learn more about particular funding areas; and to study the "ten commandments for preparing a compelling R01 application" that was presented by Fatah Kashanchi in a grants workshop session on the final day of the Meeting.

The session closed with a presentation from Christine Happel (National Center for Advancing Translational Science (NCATS) who is Scientific Program Director of the NIH Common Fund's Extracellular RNA Communications Program (ExRNA) that sponsored the Extracellular RNA Communications Consortium (ERCC). She reported the accomplishments of ERCC's Phase 1, including the massive accumulations of biofluid samples and associated exRNA studies and profiles; analyses of technologies and methodologies for extracellular vesicles (EVs) separation and exRNA identification; and the creation of data analyses and coordination protocols for information processing. ERCC developed a portal for access to these data, protocols, and other resources (e.g., the exRNA Atlas), to be found at exRNA.org (https://exrna.org/). Christine touted a number of landmark papers from the ERCC published in 2019 (Cell journals) that culminated from those data. ERCC Phase 2 will continue research in these areas, with interests in EV and exRNA heterogeneity; improvements in isolation, characterization, identification, and data processing of EVs and exRNAs; and an increased interest in single exosome/EV isolation and cargo determination. The latter, in particular, relates well to an advertised RADx (Rapid Acceleration of Diagnostics) funding announcement,

RFA-OD-20-018, seeking approaches to detect, test, validate, and implement testing for SARS-CoV-2 in the COVID-19 pandemic. She ended with a list of exosome/EV-related funding announcements across NIH Institutes, and commented on the future of vesicles and exRNA in terms of basic biology and translational applications.

The next 2 sessions focused on exosome/EV cargo loading and cargo unloading. Session Chairs were Juan Pablo Tosar (Universidad de la República, Uruguay) and Andrew Leidal (University of California San Francisco, US). Alissa Weaver (Vanderbilt University, US) addressed a controversy in the EV field: is EV-associated Argonaute an artifact or is it selected cargo? She noted that extracellular RNA in blood is prevalent in non-vesicular forms, likely released from necrotic cells, and largely held by RNA-binding proteins (RBPs), including Argonaute 2 (AGO2). Vesicular RNA in blood is less abundant, but is a product of active secretion. These forms may represent different functional capacities, as well. She noted that different cell status (mutations, signaling) result in different RNAs and RBPs that are expressed and localized in cells, and differentially segregated into vesicles. She provided compelling evidence that serum (such as fetal bovine serum) is a source of non-vesicular AGO2 and microRNAs, which further complicates the assessments of AGO2 and miRNAs from cells grown in serum-containing media. Thus, the cell state, mutational and signaling status, and growth conditions all contribute to AGO2 and likely, other RBPs, and their bound RNAs in terms of vesicular or non-vesicular entities.

Leonid Margolis (NIH, National Institute of Child Health and Human Development, US) followed next with a presentation on EV-associated cytokines and their implications for cell-cell communications. This topic highlights broad questions of how exosomes and other EVs are addressed to particular cells, and how the EV cargo affects recipient cells. EVs may play roles in protecting contents and applying appropriate 'shipping addresses'. Leonid pointed out that cytokines are encapsulated within EVs, and are also present on EV surfaces. These operate as systems, and that the cytokine surface *vs.* lumenal localizations are not independent, but reflect changes in parental cell states. Differing soluble *vs.* cytokine-EV states are evident in blood of patients experiencing post-radiation fatigue syndrome and in a form of myocardial infarction, among other pathologic settings. EV-associated cytokines appear functional, and have an impact on recipient cells due to their selective delivery onto cells with appropriate receptors. Improved understanding of cytokine loading and targeting of such EVs could enlighten our concepts of cell-cell communication.

RNAs of various types are described as exosome/EV cargo, begging questions such as: how the RNAs are loaded, are they transferred to recipient cells, and are the RNAs functional in the recipient cells. Olivier de Jong (University Medical Center Utrecht, The Netherlands) reported studies in this area that employed an innovative CRISPR-Cas9 system, which they called CRISPR operated stoplight system for functional intercellular RNA exchange (CROSS-FIRE). It utilizes transfer of single guide RNAs (sgRNAs) that are capable of driving reporter signals in recipient cells in a non-contact system, thus indicating the loading of sgRNA into EVs in donor cells, and functional transfer to recipient cells measured by fluorescent readout. This transfer worked with various donor and recipient cell types. The system uncovered genes involved in endocytosis, extracellular matrix adhesion, and intracellular membrane trafficking that affected RNA transfer via EVs (ITGB1, ROCK1, RAB5, RAB7). For translational development, the system appears more efficient at functional RNA transfer than with lipid nanoparticle-based products, suggesting therapeutic uses of mRNA or siRNA transfer.

In a display of EV cargo (or lack thereof) relevant to disease processes, Laura Ferraiuolo (University of Sheffield, UK) introduced the neurodegenerative disease amyloid lateral sclerosis (ALS). The most frequent mutation in the familial version of ALS (35%) and spontaneous ALS (11%) is in C9ORF72, which can lead to several potential (and overlapping) pathological situations. One potential mechanism is that the C9ORF72 translation product may inappropriately sequester RNAs, thus altering cell function and cell crosstalk.

Laura showed that transfer of EVs from astrocytes, generated from patients with amyloid ALS, to healthy neurons induced neuronal cell death. Scores of small RNA levels were significantly altered in those EVs, including downregulation of miR494, which affects pathways controlling axonal growth cone collapse. Supplementation of miR494 into astrocyte EVs and application of the EVs onto neurons rescue neuron survival, neuronal length, and complexity (increases in nodes and intersections). These studies reveal the potential of therapeutic cargo for use in EV transfer, based on knowledge of the EV-driven pathology.

Crislyn D'Souza-Schorey (University of Notre Dame, US) spoke of microvesicles (MVs), which are extracellular vesicles that bud from cell surfaces, and the loading of pre-miRNA cargo into tumor EVs. She noted the extraordinary heterogeneity of shed vesicles, particularly those released by tumor cells. Her group has identified ARF6, which is involved in endosomal/endocytic recycling; ARF6 activation correlates with increased miRs in MVs. They also found that XPO5 is a binding partner of ARF6; XPO5 plays roles in pre-miR transport from the cell nucleus to the cytoplasm, and is found in tumor MVs, which contains a pre-miR processing machinery. Tumor MVs contain complexes of ARF6/XPO5 and pre-miRs; translocation of XPO5 to the MVs involve a complicated interaction involving CK2, RAN and cytohesion family guanine exchange factors. These routes suggest that the intricate mechanisms for the delivery of cellular cargo into different EV types further display the complexity of these biologic and pathologic processes.

Qin Zhang (Vanderbilt University) finished out the session discussing a recently identified form of extracellular particle, the exomere. Exomeres are non-membranous particles (~35 nm diameter) of unknown biogenesis and no clear biologic function. Researchers previously used a biophysical separation technique called asymmetric-flow field-flow fractionation (AF4) to isolate and identify exomeres; Qin performed high speed ultracentrifugation (UC) on cell culture medium (167K × g; collect supernatant; UC again @ 167K × g; harvest pellet) to obtain similar extracellular particles, which were proteomically distinct from small EVs/exosomes. Exomere-enriched proteins included glycolytic enzymes and AGO RNA binding proteins. Exomeres also contained the EGFR ligand, AREG, which in that format was capable of inducing prolonged EGFR signaling in recipient cells, leading to enhanced tumor organoid growth. Another exomere-enriched protein is the sialyltransferase, STGAL1 (membrane and soluble forms), which is highly enzymatically active in exomere form on recipient cells. Curiously, the SARS-CoV-2 spike protein receptor, ACE2, is displayed on exomeres from some cell lines and can bind the viral spike protein via the S1 receptor binding domain, suggesting that exomeres could serve as decoys against SARS-CoV-2 spike protein-initiated infection.

The final session of the first day of ASEMV2020 was devoted to analytical techniques, and was hosted by Kendal van Keuren-Jensen (Translational Genomics Research Institute, US) and Tijana Jovanovic-Talisman (City of Hope, US). The first speaker was Lisa Meyer (Exosome Diagnostics, US; Germany) who presented an exosome/EV separation and characterization workflow using products from several partner companies. This included a kit-based separation technology, an automated Western blot device in the form of a capillary flow electrophoresis apparatus, and validated primary antibodies against exosome/EV and theoretical non-EV proteins to determine potential contaminants. She showed results confirming the detectability of appropriate target proteins in the correct (EV-containing) fractions from the separation scheme. The system provides scalability, rapid quantification, and good detection limits. They are expanding the antibody repertoire and improving the separation modules, going forward.

Ryan McNamara (University of North Carolina, Chapel Hill, US) introduced a super-resolution microscopy technique called direct stochastic optical reconstruction microscopy (dSTORM). As exosome/EV sizes are below the limits of resolution of conventional light microscopy, more advanced forms of imaging are necessary to accurately discern single EVs and their characteristics, thereby avoiding non-physiologic manipulations that are likely pursued in what might be done for electron microscopy. dSTORM has multi-channel capability that could detect two different membranes dyes within the lens apochromatic correction

limit (< 20 nm), i.e., essentially co-localized. When using labeled tetraspanins, they determined that there is an offset in the overlay of the membrane protein and the membrane dye, which they hypothesized to be the localization of the tetraspanins in lipid raft microdomains. The biophysical characteristics shown were consistent with those found from other methodologies (e.g., nanoparticle tracking analysis, resistive pules sensing, and surface plasmon resonance).

Frederik Verweij (INSERM, France) rounded out the session with a talk on *in vitro* and *in vivo* exosome tracking. He described a novel live cell imaging technique using pH sensitive reporters visualized by total internal reflection fluorescence (TIRF) microscopy. With tagged tetraspanins (CD63, CD81) or other endosomal markers, they could visualize which endosomal sub-compartments fuse with the plasma membrane to release exosomes. They have extended these studies to *in vivo* settings using zebrafish models. The remarkable resolution of their technology allows tracking of release of exosomes, as well as recipient cell uptake throughout the organism; e.g., macrophage and endothelial cell scavenging, and internalizing exosomes released by cells of the yolk syncytial layer. Following the fate of such internalized exosomes, it seems that many exosomes are degraded in the endo-lysosomal system. Further, inhibiting the biogenesis of exosomes in zebrafish embryo caudal vein plexus (CVP) led to reduced growth of that tissue, implying a role for exosomes in trophic support of the CVP.

ASEMV2020 Day 2 began with an EV Function session, chaired by Michael Graner (University of Colorado Anschutz, US) and Leonid Margolis (NIH, US). It started with a joint presentation by Jay Debnath and Andrew Leidal (University of California San Francisco, US) on secretory autophagy and EVs. Jay introduced the biologic processes of autophagy, particularly secretory autophagy, and their relationships to EVs and EV release. Given that knockdowns/knockouts of autophagy components had pleiotropic effects, Drew had developed a technique called proximity-specific biotinylation to identify new targets of autophagy-dependent secretion. This allowed for proteomic identification of > 200 such targets, many of which were exosomal/EV components and RNA-binding proteins, suggesting an intersection between autophagy and exosome protein-related secretion. Additional work showed the dependency of autophagy machinery on secretion of diverse RBPs as well as certain small RNAs (including snoRNAs) in small EVs/exosomes. They believe this represents a sub-routine of the autophagy pathway involving component (LC3) processing and lipidation, "LC3 dependent EV loading and secretion" (LDELS). Drew inhibited lysosomal activity (baflomycin, chloroquine), which revealed a re-direction of endo-lysosomal cargo released as EVs outside the cell in an autophagy-dependent manner, with autophagy proteins, including cargo receptors, prominent in those vesicles. Further genetic studies indicated that inhibition of autophagosome-lysosome fusion enhances cargo receptor release in EVs, in contrast to the requirements for LDELS.

Sarah Andres (Oregon Health and Science University, US) continued the autophagy theme, this time focusing on IGF2PB1/IMP1, an RNA-binding protein whose expression peaks during embryonic development in mammals, and decreases as the organism matures. In the intestine, damage causes upregulation of IGF2PB1/IMP1; this is also seen in GI cancers, where it acts in tumor progression and metastasis. The hypothesis was that IMP1 plays a role in endosomal and autophagic pathways (secretion *vs.* degradation) in colon cancers. IMP1 overexpression increased EV release, and modulated endosomal and autophagy pathways at least in some colon cancer cell lines. Using an enteroid (organoid) culture system (from crypt regions of intestinal epithelium), IMP1 overexpression in these non-transformed cells did not alter EV production, but had some impact on cargo, such as TSG101; these results are an on-going work. Thus, IMP1's influence on EV biology via endosomal and autophagy pathways appears to be cell-type and context dependent.

Inge Zuhorn (University of Groningen, The Netherlands) presented next on exosomal cargo fate upon uptake by recipient cells. Their work originated from challenges that accompanied nanoparticle-based drug delivery to the brain; subsequently leading to EV-based delivery into the brain with functional release of cargo into target cells. Inge presented four hypothetical exosome cargo release scenarios (direct fusion to plasma

membrane; "kiss-and-run" fusion at the ER; endosomal membrane fusion - aka, back fusion; and endosomal rupture). They used an elegant system to dissect these mechanisms, starting with a cell line expressing a GAL3 fluorescent reporter that is detectable upon binding beta-galactoside aggregates, which would be present if endosomes ruptured. Upon exosome incubation, such rupture was not evident. They next used exosomes from cells expressing intralumenal GFP-tagged CD63 that were loaded onto cells expressing a labeled (mCherry) anti-GFP nanobody intracellularly. If exosomes fuse at the plasma membrane, the red nanobody would accumulate there; if exosomes back fuse inside the endosome, the red nanobody would accumulate intracellularly. There would be co-localization with GFP at each of these sites. Their experiments revealed intracellular co-fluorescence, indicating endosomal release; and correlative light and electron microscopy (CLEM) also showed lysosomal localization, suggesting movement through the endo-lysosomal pathway. As these results suggested that protein transfer may occur via exosomes, they attempted treatment of a Huntington's Disease (polyQ, HTT-Q74) murine model. They used their exosome loading protocol to place the HSP40 chaperone homolog DNAJB6 into exosomes. In intrathecally injected transgenic mice, they showed reduction in poly-Q aggregates in mouse brains and spinal cords, indicating functional transfer of the chaperone to reduce aggregates.

Continuing the theme of *in vitro*/*in vivo* work, Nicole Noren Hooten (NIH National Institute on Aging, US) introduced the potential role of EVs in type 2 diabetes mellitus (T2DM). Previous studies found that larger EVs (microparticles) derived from blood cells were elevated in patients with T2DM. Nicole's work examined smaller vesicles (exosome and microvesicle range) in a large longitudinal study was comprised of euglycemic individuals, pre-diabetics, and T2DM patients (NIH HANDLS study). Precipitated plasma EV concentrations were elevated in T2DM patients, and this increased as pre-diabetic patients progressed to full disease. T2DM EVs had lower amounts of insulin signaling proteins and induced inflammatory responses when incubated with monocytes and B cells, thus suggesting that those EVs may promote inflammation in patients. Longitudinal and cross-sectional analysis of EV inflammatory proteins showed that levels of VEGFA were associated with diabetes development in this expansive cohort. They followed this with differential centrifugation of plasma EVs (10K × g and 120K × g fractions). Curiously, the 10K EV fraction from T2DM patients increased endothelial cell migration *vs.* those EVs from euglycemic controls, and those cells showed increased actin rearrangements and lamellipodia formation. This raises the possibility that plasma EVs in T2DM patients may contribute to detrimental vascular changes that are related to the diabetic pathology.

Y. Peng Loh (NIH, National Institute of Child Health and Human Development, US) completed the EV Function session with her group's latest work on exosomal carboxypeptidase E (CPE) in tumor vesicles. CPE mRNA overexpression is correlated with poorer overall survival in patients with hepatocellular carcinoma (HCC), and is generally elevated in serum exosomes of cancer patients as well as in highly malignant cancer cell lines. *In vitro*, exosomes from high metastatic HCC (more malignant) cell lines induce proliferation and invasion in lower malignant HCC cells, and this can be blunted if the exosomes contain shRNA against CPE. Loh *et al.*, transfected CPE shRNA into HEK exosome producer cells to treat high metastatic HCC cells with those exosomes, which reduced proliferation in those cells, perhaps by suppressing CCND1 and MYC. This suggests that CPE is a good cancer biomarker, a good anti-cancer target, and that exosomal delivery of shRNA may be a therapeutic avenue against cancer.

With increasing interest in the roles of EVs in microbiology, the next session featured talks on bacterial EVs, hosted by Meta Kuehn (Duke University, US) and G Marcela Rodriguez (Rutgers University, US). Marcela started the session with a talk on EV (membrane vesicle, MV) production in mycobacteria. Mycobacteria MVs display phospho- and glycolipid content consistent with the plasma cell membrane, but the bacteria is surrounded by a thick cell wall, which makes the MV release somewhat of a mystery. Mycobacterial MV production is regulated by several genes; strains lacking the "vesiculogenesis and immune response regulator" (viR) gene demonstrate enhanced vesicle production. Environmental iron availability also

regulates vesiculation; MV impacts' on host cells frequently involve immune modulation associated with pathogenesis. Searching for common genes involved in conditional upregulation of MV production, they associated the IniBAC operon with increased vesiculation. This encodes dynamin-like proteins (DLPs) necessary for cargo loading and vesicle release, and thus may be targets for therapeutics.

Tatsuo Kurihara (Kyoto University, Japan) also discussed cargo loading into extracellular membrane vesicles (EMVs) from Shewanella vesiculosa strain HM13. The most abundant protein in this bacterium's vesicles is called P49, encoded in a gene cluster that also produces components that are likely to be membrane associated transport systems. Gene disruption analyses of various cluster members led to cellular accumulation or non-vesicular release of P49, and gene deletion of P49 of course deleted the protein entirely. Some of the genes encode cell surface polysaccharides that are also on EMV surfaces,which appears to be required for P49 loading into EMVs. P49 likely binds to those EMV surface polysaccharides. Collectively, this system could be exploited to load foreign proteins into a putative delivery system.

Jean C Lee (Brigham and Women's Hospital, Harvard Medical School, US) followed next with a talk concerning the impact of Staphylococcus aureus (SA) EVs on host macrophage inflammatory responses. SA causes a diverse array of infections due to their varieties of virulence factors, which may serve as cargos in EVs such as pore-forming toxins and small peptide toxins. Macrophages uptake SA EVs via dynamin-dependent mechanisms. SA EVs trigger inflammasome activation via toll-like receptor (TLR) triggering (essentially as a priming step that causes IL6 release), and toxin-mediated inflammasome-driven cleavage of pro-IL1B and pro-IL18 to mature secreted forms. These were dependent on NLRP3 and CASP1 inflammasome components. These outputs are due to the toxin cargo in the SA EVs, which do not affect the TLR-driven IL6 release. The TLR signaling/IL6 release is driven by SA EV surface lipoproteins. Curiously, CASP1 activity was also dependent on lipoproteins, suggesting thereby that lipoproteins modulate the toxin content of EVs, which was verified by mutagenesis. The lipoproteins also modulate EV biogenesis and EV biophysical characteristics. The EVs thus function to protect the cargo and mediate virulence potentially through unmitigated inflammasome activation.

In a very interesting presentation on potential bio-utility of bacterial outer membrane vesicles (OMVs), Allison Z Werner's (National Renewable Energy Laboratory, US) particular project involves generation of green products from biology; in this case, it is the conversion of lignin (e.g., in corn stalks) to usable and sustainable solid products. Lignin is the second most common biopolymer on earth (after cellulose) that remains behind from industrial processes and might be used to develop rigid products rather than form large quantities of burned waste. The material's heterogeneity makes its deconstruction and re-utilization problematic. Pseudomonas putida, KT2440, is a bacterium capable of lignin catabolism as a part of the "biological funneling", which is necessary to obtain simpler molecules for further development. Lignin promotes *P. putida* extracellular secretion in the form of OMVs. The research group characterized and performed exoproteomics on these vesicles over time under different lignin conditions. These included aromatic catabolic enzymes in the beta-ketoadipate pathway. There are many directions for future work in this new area.

Simon R Carding (The Quadram Institute and University of East Anglia, UK) presented information on the roles of Bacteroides thetaiotaomicron(Bt)-derived OMV, in microbiota-host crosstalk in the gastrointestinal tract. Bt is prominent in microbiomes residing at the mucosal interface in the gut. Bt OMVs are abundant and remarkably stable and naturally produced in the GI tract, where they are taken up by gut epithelium by dynamin-mediated endocytosis and macropinocytosis. Intracellular trafficking puts OMVs at the ER, Golgi, endosomal compartments and the nuclear membrane, but can apparently transcytose the epithelial barrier by passing between cells. Bt OMVs biodistribute beyond the GI tract into the liver with lower quantities in kidneys and lung, much like other EVs and nanoparticles. Unlike pathogenic bacterial OMVs, Bt OMVs tend

to promote anti-inflammatory response in intestinal mucosa, probably from regulatory resident dendritic cells. Bt OMVs can also cross mucosal barriers in the lung, suggesting that these OMVs could be used as mucosal delivery vehicles for vaccines.

Hannah McMillan (Duke University, US) discussed bacterial vesicles in inter-kingdom communications with plants. While relatively understudied in plants, such OMVs are known to contain virulence factors, protein secretion components, and plant cell wall-degrading enzymes. Plant responses to bacterial pathogens are at levels of reactive oxygen species, erection of physical barriers, and production of antimicrobial compounds. Bacteria retaliate with the means to block and deactivate these immune responses, leading to further immune escalation from the plant that can include systemic responses and programmed cell death. Hannah investigated the roles of OMVs in these interactions. Using Pseudomonas syringae (Pst, plant pathogen) and fluorescens (Pf, not pathogenic) as OMV sources, she "infected" plant leaves with OMVs. Pst OMVs induced ICS1 protein expression (asso' ciated with salicylate production against the bacteria). Bacterial OMVs produced protective immunity, where plants challenged with bacteria resisted bacterial growth and colonization, even if the non-pathogenic OMVs were used as an initial stressor. Also, both Pst OMVs and OMVs from non-plant pathogens could protect against different pathogenic challenges. The nature of OMV-driven protection at the level of the OMV is currently unknown and is an area of further study.

Blanca Rodriguez (Duke University, US) finished the session by talking about the immunomodulatory impacts of RNA in Staphylococcus aureus extracellular MVs. The RNA content of MVs was originally controversial, but has recently become generally accepted. These are mostly small RNAs; their sorting into MVs can be affected by growth conditions and the RNAs can be transported into host cells. There are many questions in this new area, including, the physical association of RNA with MVs, how RNAs are transmitted to host cells, and do they modulate immunity? It is known that SA nucleic acids are immunomodulatory, driving type I IFN responses, but the mechanism for this SA nucleic acid release and transfer is unclear, with the hypothesis that MVs are involved. Blanca found that exogenous RNA does not bind SA MVs, but does seem to have endogenously associated surface RNA (there is still RNA within the MVs that is extraordinarily stable, even in the presence of proteases and nucleases). The MVs promoted IFNB expression in macrophages; this expression was only mildly reduced if they attempted nuclease degradation prior to incubation with macrophages. MV uptake is dynamin-dependent, as is IFNB induction, suggesting endosomal entrapment where there are nucleic acid sensors (TLRs) that are responsible for most of the IFNB response. Further questions include mechanisms for release of the RNA, which RNA sequences are immune modulatory, and if alternate uptake and trafficking modes are involved.

ASEMV2020 Day 3 started with sessions on the pathology of EVs, moderated by Janusz Rak (McGill University, Canada) and Michael Graner (CU Anschutz, US). The first speaker was Aleks Milosavljevic (Baylor College of Medicine, US) who related the effects of glioma EVs and brain endothelial cells. Glioblastoma (GBM), the deadliest of brain tumors, is a vascular tumor, implying tumor-driven modification of brain endothelial cells; Aleks' group and others asked if EV-mediated angiogenesis differed from growth factor-driven angiogenesis. Using human brain endothelial cells as targets, EVs from GBM8 (stem cell glioma line) enhanced vascularization patterns in the endothelial cells in ways phenotypically similar to growth factors themselves; but which differed considerably at a molecular level involving distinct pathways. They suspected that this was occurring at the post-transcriptome level, and therefore examined the endothelial cell fraction that was deconvoluted from TCGA data. The results showed intersection of *in vitro* and *in vivo* perturbations. Further analyses showed a dominant effect of growth factors over EVs in methylation/epigenetics changes. miR-9-5p emerged from the data as a candidate extracellular RNA mediating angiogenesis upon EV exposure to endothelial cells based on overlapping miR expression data from EVs of several sources and its plasma biomarker status. Additionally, among downregulated gene profiles were miR-9-5p targets, including SOX7, which could be responsible for proliferative effects. These results suggest that EV-mediated angiogenesis,

which has different features from growth factor-driven angiogenesis, may help explain the failure of anti-VEGFA targeting across several clinical trials in GBM.

The theme of detrimental impacts of disease-state EVs on normal cells was continued by Romano Regazzi (University of Lausanne, Switzerland). In the context of Type 1 diabetes, modeled in NOD mice, his group showed that certain microRNA levels (miRs-142-3p; -142-5p; -150; -155) are elevated in pancreatic beta cell islets, and in sorted beta cells, in the pre-diabetic state, and this is not in response to proinflammatory cytokines. However, these miR levels are increased in the infiltrating lymphocytes, begging the question that miRs may be transferred from leukocytes to beta cells. Jurkat cells (model CD4+ T cells) released EVs containing relatively high amounts of miRs-142-3p, -142-5p, and -155, which were indeed transferred to cultured beta cells upon incubation with the T cell EVs. These beta cells increased gene expression levels of several other chemokines in response to T cell EVs, and transfection of the beta cells with the miR mimics promoted the same expression changes. EV exposure also drove apoptotic beta cell death (without affecting other pancreatic cell types), and this was also mimicked by individual miR transfections into beta cells. While non-beta cells also take up lymphocyte EVs, only beta cells exhibited gene expression changes; of note, NF-κB nuclear translocation also occurred in the beta cells, suggesting a global driver of the cyto/chemokine expression changes. Gratifyingly, these effects were replicated in human tissues as well. Transfection of beta cells with anti-miRs apparently successfully prevented the impact of lymphocyte EV-transferred miRs, as this halted the apoptotic effect of the EVs. For an *in vivo* therapeutic attempt, the research group generated a "miR sponge" construct used in beta cell-targeted AAV transfection; this reduced the number of mice progressing with the disease. Continuing work will examine the roles of other non-coding RNAs that may be transferred from lymphocytes to beta cells.

Dennis Steindler (University of North Carolina; University of Florida, US) followed with more examples of exosome/microvesicle (EMV)-driven pathologies. He introduced the concept of adult neural stem cell-driven disease as a proliferative failure (e.g., Alzheimer's and Parkinson's disease) or a proliferative excess (e.g., brain tumors). Focusing on Parkinson's disease, his group has identified human adult neural progenitor (AHNP) cells that are capable of proliferation and differentiate into neurons and glia. The cells are from cases of both idiopathic Parkinson's and gene-identified Parkinson's. These cells release EMVs that bear signature profiles in nanoparticle tracking analysis; in the case of gene-identified Parkinson's, correction of the mutation returns the mutant EMV profile towards normal cell EMV profiles. This holds true for content of EMVs as well. There are interesting overlaps in neurodegenerative diseases between transmission of disease states from cell to cell with near infectious (viral-like) aspects. One hypothesis is that the neural connectome, perhaps via the vagus nerve, is hijacked to put such particles, including EMVs, into the central nervous system.

Moran Amit (MD Anderson Cancer Center, US) further connected the pathologic conditions (e.g., head and neck cancer) that alter normal cell function (neural reprogramming) via exosomes. Until recently, oncologists have viewed nerves involved in cancer as innocent bystanders. We now know that cancer cells can migrate or metastasize along neural tracks, and possibly have a more active role in tumor progression and malignancy. Tumor innervation is often associated with worse outcomes, but little is known about this. Using an *in vitro* ganglion + tumor cell assay, some tumor cells could promote neuritogenesis better than others; a common feature among those cells was the loss of p53, and this held true in murine studies, even in a pre-malignant stage prior to cancer cell/neuron contact. Thus, there should be a secreted component, which appears to be p53-deficient/mutant exosomes. miR-34a, present in p53wt exosomes, restricts neuron growth. In p53 mutant exosomes, in the absence of miR-34a, miR-21 and -324 strongly promote neuritogenesis. Phenotypically, the neurons are mostly sympathetic, have dysregulated pathways in stemness, proliferation, and neural transmission, and may even switch phenotypes. Beta-blockers may inhibit this neuronal-promoted tumor growth.

Tsuneya Ikezu (Boston University, US) told us about EV protein networks from iPSC-derived neural cells and brain tissue of patients with Alzheimer's Disease (AD). High-level proteomic analyses of EVs from iPSC differentiated brain cell types and from brain tissues of patients with AD, mild cognitive impairment, and healthy controls led to specific markers for cell-type specific EVs and could clearly separate the EV types by protein composition. The statistical analyses could also distinguish EV protein subsets between healthy control brain EVs, patients with mild cognitive impairment, and AD patients, including some that might show progressive changes towards AD. Certain proteome modules resembled pathways found in activated astrocytes, including inflammatory processes. This information could form a basis for EV proteomics in liquid biopsy for AD development and monitoring.

Faisal Alibhai (University Health Network, Toronto, Canada) provided insight into a near universal human phenomenon, aging, and the associated changes in circulating EV cargo and function. Parabiosis of murine circulation involving young and old mice rejuvenates multiple organ systems in the old mouse, but the young mouse shows age related defects. Thus, blood circulating factors play roles in aging (and youth), begging the question of the roles of EVs in this. Faisal isolated EVs from young and old murine populations, noting that the particles from old individuals were both fewer and smaller than those from young plasma. Curiously, EV markers (CD63, CD81, TSG101) were more abundant in old plasma, while markers of lipoproteins were more abundant in young plasma, suggesting that the particle differences noted may be skewed in this fashion. In functional studies, peritoneal macrophages were treated with EVs from old and young plasma, and then were stimulated with LPS. Under both circumstances, old EVs decreased IL1B and IL12B expression (and other cytokines) more than young EVs. Old EVs also reduced VEGF-mediated endothelial cell tube formation. The miR content of old EVs targeted numerous pathways that could be dysregulated in aging (inflammation and senescence). In an attempt to reduce aging effects with senolytic therapy (dasatinib and quercetin in this case) in old mice, the treatments reduced miR levels in plasma EVs, and the EVs no longer blocked endothelial cell responses to VEGF. Thus, aging cells manifest senescent phenomena via aged EVs, and some of the effects may be reversed by senolytic therapies (or young EVs).

The last talk of the Pathology session was about the utility of neural stem cell EVs to improve outcomes in a porcine ischemic stroke model, presented by Steven Stice (University of Georgia, US). As mesenchymal stem cells (MSCs, and their EVs) are seeing use in many inflammatory-related neurologic diseases. Steve's group found that neural stem cell (NSC) EVs better promoted an anti-inflammatory immune environment than MSC EVs in a murine stroke model. They chose a pig model as a better representative of human brains than rodent models. In this study, pigs were trained for motor and behavioral function; subjected to ischemic strokes; treated with NSC EVs; monitored by imaging and functional recovery studies; underwent further clinical testing and imaging; and followed finally, by an autopsy. Pigs treated with NSC EVs showed stunning functional recovery compared to surviving untreated pigs. On MRI assessment, the extent of midline shift - a pathology-induced mass effect leading to brain displacement off the midline - is correlated with survival. NSC EV-treated pigs showed reduced midline shift and increase in survival rate. On the pig-adapted Modified Rankin scale (mRS), pigs that received NSC EVs, irrespective of a high or low midline shift, showed reduced mRS scores, indicating less disability. Consistent with reduced inflammatory responses, pigs treated with NSC EVs demonstrated attenuated microglia activation both *in vitro* and *in vivo*.

The final session of Day 3 was devoted to EVs and viruses, and was hosted by Nihal Altan-Bonnet (NIH, National Heart, Lung, and Blood Institute, US) and Steve Gould (Johns Hopkins University, US). It started with James Erickson (George Mason University, US) discussing how to separate EVs from virions in Coronavirus infections. The lab has used several techniques to separate virus (typically HIV) from EVs in virally infected cells, including the use of differential ultracentrifugation with a high-resolution density (iodixanol) gradient ultracentrifugation. They applied this strategy to isolate a betacoronavirus (OC43, BSL2 compatible) from infected cells (lung cancer cell line). Incubating these virus fractions with Vero cells

resulted in OC43 RNA transfer to the recipient cells. Also, viral RNAs were present in EV fractions. Upon noting that lymphopenia is associated with severe SARS-CoV-2 infections, they generated HEK transfectants expressing SARS-CoV-2 spike proteins or multiple viral proteins. EVs from these cells were incubated with T cells; EVs containing multiple viral proteins reduced cell viability, suggesting an EV-based mechanism for viral-induced lymphopenia.

Martin Olivier (McGill University, Canada) then presented the role of EVs where a virus infects another pathogen, in this case, the Leishmania RNA virus and the trypanosome Leishmania. The parasite can be transmitted through the bite of a sandfly, where it infects macrophages and neutrophils (and macrophage engulf the neutrophils).The parasite replicates as amastigotes in the macrophage, which can then transmit the amastigotes to another biting sandfly. Martin's lab has studied how Leishmania hijack macrophage signaling and innate immune responses for propagation. These studies identified the pathogen metalloprotease GP63 as a virulence factor, but it was clustered in small entities in the macrophage. This led to the discovery of GP63 (and other virulence factors) in Leishmania exosomes/EVs, and the exosomes modulated macrophage signaling, inhibiting antimicrobial defenses and inducing inflammatory cell recruitment at the sites of infection (during a blood meal). Recently, a Leishmania RNA virus (LRV) was discovered that might be small enough to fit in Leishmania exosomes, raising the possibility that the virus could be transferred via exosomes. Indeed, the pathogen exosomes contained viral components that were detected biochemically, and electron microscopy showed the presence of the whole virus in about 30% of the exosomes. The virus adds to the skin hyperinflammation upon injection of exosomes, aiding and abetting the infection cycle, but potentially provides a new therapeutic target.

Hameeda Sultana (Old Dominion University, US) brought up another circumstance where host cell exosomes facilitate viral transmission. Zika virus (ZIKV) induces cell death in cortical neurons, with the infections in the differentiated and matured neurons. The infected neurons release exosomes containing viral RNA and proteins, and the exosomes can re-infect naïve cells. Hameeda's group sought to understand the mechanisms behind the enhanced exosome release from infected cells that leads to further viral transmission. Neutral sphingomyelinase 2 (nSMase2; SMPD3), which is involved in numerous points of the uptake, in endosomal trafficking, and in vesicle release pathways, was found to be a logical player. In infected neurons, SMPD3 activity was higher in both the cells and their exosomes; silencing it reduced vesicle release and viral transmission. Thus, it appears that ZIKV induces SMPD3 activity, which leads to greater vesiculation, and thus increased passage of the virus.

In a setting where EVs may be used to actually mitigate viral disease, Heather Branscome (George Mason University) presented data on the use of stem cell EVs in the repair of cellular damage. The scenario involves HIV infection of neurospheres - a novel achievement in itself - as a model of CNS damage such as HIV-associated neurocognitive disorders (HAND), and the use of reparative stem cell EVs in healing cell damage. Stem cell therapies have been touted as a valuable means to treat various diseases, but a more potent activity may reside in stem cell EVs, which have reduced immunogenicity and a more facile handling and storage. She described the stem cell sources (mesenchymal stem cells, MSCs, and iPSCs) and described the tangential flow filtration (TFF) methodology for EV isolation. Stem cell (SC) EV cargo included cytokines (FGF2, VEGF, IL4) that differed between the two stem cell EV sources. The SC EVs could promote cell migration and endothelial cell tube formation, and the EVs were taken up by neurospheres with long half-lives (up to 9 days). In HIV-infected neurospheres, SC EVs reduced p53 (Ser-15) phosphorylation, suggesting a return to the cell cycle, and the EVs also reduced amounts of pro-inflammatory cytokines. Using isolated cell types (neurons, astrocytes, macrophage) exposed to ionizing radiation, SC EV treatment reduced apoptotic induction. The results suggest the ability of SC EVs to protect and repair cell damage even in the face of a brain HIV infection.

The final Virus session speaker was Daniel Pinto (Walter Reed Army Institute of Research, US) who told us of the role of EV in transmission and spread of the HTLV-1 virus. This virus can cause adult T cell leukemia/lymphoma, or a neurocognitive disorder known as HAM/TSP. A prevalent infectious motif is cell-cell contact. During EV preparations using density gradient centrifugation, they noted that viral components could end up in EV fractions that were separated from the virus itself, and from EVs containing virions. This begged the question, which of these are infectious? Curiously, none of them were separately infectious, nor were they infectious if all were combined. However, the treated cells displayed clustering/aggregation phenotypes similar to that seen in bona fide HTLV-1 infection. This perhaps enables the cell-cell contact that is necessary for viral spread. EVs from infected cells enhanced viral infection (measured by viral RNA load in recipient cells), and this could be blocked by antibodies against cell adhesion molecules CD45 and ICAM1 or by treating cells with siRNAs. In an animal model, injecting EVs prior to an infection with HTLV-1 increased the viral load in the blood and numerous target tissues of the mice. Differential centrifugation yielding 2K × g, 10K × g, and 100K × g fractions showed that most of the detrimental effects (and most of the viral components) were in the 2K and 10K fractions, along with differential cytokines (and surface *vs.* internal localizations). Thus, different EV subtypes may have unique effects on cells that ultimately lead to more efficient viral infection and spread.

ASEMV2020 Day 4 started with the beneficial uses and therapeutic effects of EVs, hosted by Saumya Das (Massachusetts General Hospital, US) and Kendall van Keuren-Jensen (TGEN, US). Robert Blelloch (UCSF, US) opened up the session with his group's studies showing how exosomes suppress anti-tumor immune responses. Unsurprisingly, this focused on the immune checkpoint inhibitor PDL1, part of the PD1/PDL1 checkpoint axis. T cell activation can result in expression of PD1 as a means of immune control; cells expressing its ligand PDL1 (often other immune cells, but also cancer cells) can suppress T cell activity as a control against autoimmunity. For instance, as activated T cells infiltrate tumors, tumor PDL1 could bind T cell PD1 to deactivate the T cell effector function, and this is the standard paradigm for use of immune checkpoint inhibitors. Robert's lab believes there may be an important role of PDL1 far earlier in the priming phase of T cell activation. PDL1 is found on tumor-derived exosomes, but not all tumors may localize it there, suggesting that this may be an active process. Their additional data indicate that the vesicles are specifically exosomes and not other EV types. They employed a mouse prostate cancer model (TRAMP-C2) that is resistant to anti-PDL1 antibody (checkpoint inhibitor) therapy. However, the loss of PDL1 or reduced exosome formation (RAB27A or SMPD3 knockout) dramatically reduced tumorigenicity, implying PDL1 as critical to tumor progression; this also involved the immune system via T cell activation. Adding back PDL1-containing exosomes reversed the effects. This was particularly notable in draining lymph nodes, suggesting that T cell priming may be key. PDL1 on plasma exosomes is correlated with a lack of response to anti-PD1 therapy in patients across several cancer types. Their model invokes a tumor cell surface pool of PDL1 (more sensitive to checkpoint inhibition) and an exosome pool of PDL1, which impacts T cell priming in lymph nodes that remains resistant to such therapies.

Ryan Reshke (University of Ottawa, Canada) introduced a method for loading and delivery of siRNA in EVs. While siRNAs are quite effective, the delivery systems beyond liver targets (liver serves as a depot for lipoparticle delivery) can lead to systemic (and liver) toxicity, so better delivery means are needed. The number of small RNAs in EVs remains controversial, but appears to be limited, suggesting a need for efficient loading to improve therapeutic capacity. One novel concept involves the use of pre-miR-451, which is a uniquely short pre-miR hairpin that is too short for processing by DICER and is sliced by AGO2, and is highly enriched in EVs. The short hairpin is responsible for this lack of DICER processing and allows pre-miR-451 preferential EV loading. Other RNAs, such as siRNAs, can also be efficiently loaded, provided they have the miR-451 hairpin, allowing for their enrichment and packaging. Ryan's group demonstrated this in a GFP silencing model, showing that in comparison to liposomal delivery, they could use 10-100-fold less siRNA to achieve silencing. In a translational setting of a murine model, they could knock down

transthyretin (TTR) in the liver, where TTR mutations cause transthyretin amyloidosis. Their system used 100-fold less siRNA than a clinically-approved drug delivery particle. This is scalable (they have used this in non-human primates) and appears to be immunologically safe. They also showed utility in small intestine and kidneys, implying use for targets beyond the liver.

Lance Liotta (George Mason University, US) spoke of how to reverse tumor-induced immune suppression at the level of the sentinel lymph node. He initiated the discussion with a question: how do tumor EVs actually exit the solid mass of the tumor and traverse the extensive extracellular barriers to get into peripheral circulation? They believe that this "open door" is the lymphatic system for interstitial draining. This implies that tumor EVs in blood have likely already circulated through lymph nodes. Using tumor tissue directly, they are able to harvest EVs from the tumor interstitial fluid and separate them by 2K, 10K, and 100K × g fractions. They have also developed a nanoparticle (Nanotrap) that allows for controlled affinity release of cytokines that stimulates migration of immune cells (notably, antigen presenting cells) to remodel the lymph node. The accompanying goal is to have the appropriate tumor EVs (those with antigens for processing and presentation to T cells) enter the now-"hot" lymph nodes. These studies revealed that the 2K vesicles promoted both primary tumor growth as well as metastasis, while the 100K vesicles inhibited this tumorigenicity and progression. This may be related to VEGF content of the 2K fraction, as well as the autophagosome characteristics of that fraction; the roles of extracellular autophagosomes in cancer require further study.

Modification of EVs is another means of improving their delivery capability. Ikuhiko Nakase (Osaka Prefecture University, Japan) showed that one component of EVs as therapeutics is their loading with biofunctional molecules. The other component is targeting EV delivery. EV uptake is thus critical for cargo delivery, and internalization by endocytosis is considered a major pathway for this. However, Ikuhiko's group found that active induction of macropinocytosis (via cancer-related receptors and oncogenic RAS) significantly enhances EV uptake. For instance, stimulation of EGFR enhances EV uptake by several orders of magnitude. Further, they created a modification of the Cathelicidin antimicrobial peptide (CAMP/CAP18), called sCAP18, that enhances cancer cell EV uptake by inducing macropinocytosis. They have also made sCAP12-2, and stearyl-modified versions of these for insertion into EV membranes. Interestingly, target-cell glycosaminoglycans were also required for the macropinocytotic uptake. Loading the EVs with saporin (ribosome inactivator) resulted in efficient cell killing.

In an eye-opening talk, James Patton (Vanderbilt University, US) discussed the roles of EVs in retinal regeneration. In retinal regeneration, the need is for quiescent stem cells to enter the cell cycle and proliferate. Knowing that EVs from KRAS mutant cancer cells can induce KRAS wild type cells to proliferate and invade, James' group pursues EV-driven modes of retina regeneration. A cell type called Muller glia (MG) in the retina is responsible for generating all the retinal cell type upon damage. In zebrafish, this process is active in retinal regeneration; it is far less so for chicks, and even less active in mammals. Using the zebrafish model, they attempted to drive Muller glia proliferation by treating retinas with EVs from a KRAS mutant cell line (DKO1), which resulted in de-differentiation of some MG cells, along with some proliferation. These outcomes prompted a large-scale screen of 59 different types of EVs to measure proliferative responses in zebrafish eyes. They found that a rat glioma cell line, C6, provided active EVs. Further refinement of their EV isolation techniques identified a small EV/exosome fraction that carried the biologic activity. In attempts to boost the this activity, they found that overexpression of IL6 and ASCL1 in the C6 producer line did result in more active exosomes. Going forward, the research group hopes to produce designer exosomes capable of targeting MGs with high payloads of active cargo to promote retinal regeneration.

The final session of the conference was on EVs as biomarkers, conducted by hosts Julie Saugstad (Oregon Health and Science University, US) and Saumya Das (Harvard/MGH US). David Lyden (Weill Cornell

Medicine, US) led the session with his group's extensive work on extracellular vesicle and particle (EVP) cancer biomarkers. The Lyden lab discovered exomeres, non-membranous nanoparticles of ~30nm diameter, which are abundant at pre-metastatic niches; the small sizes of exomeres may contribute to their wide distribution capabilities. These EVPs are among a broad range of extracellularly-released materials possessing biologic content, including dsDNA, which their group found mostly on EV surfaces. Using various separation techniques, including AF4 following differential ultracentrifugation, they categorize EVs into Exo-L (90-120 nm), Exo-S (60-80 nm), and exomeres (< 50 nm), cognizant that there are also larger vesicles. The diversity of the vesicles may relate to the diversity of local and systemic consequences of cancer. The biodistribution of cancer EVs *in vivo* likely relates to surface molecules that interact with other cell surface markers at metastatic organ sites. Their work implies that the organotropism of metastatic tumors may be initiated by the tumor EVs, particularly relating to EV integrin content (which may differ in ratio to the parental cell content). The overall impact of EV-driven cancer biology led them to analyze the proteomes of EVs from > 400 human cancer samples (cell lines, tissue explants and numerous biofluids). The goals were to define common human exosome markers from different source types (tissues, cell lines and biofluids); to identify tumor-specific exosome biomarkers; and to classify primary tumors of unknown origins by exosome proteome signatures. Some notable proteins include HSPA8 and CD9 present in/on many cancer exosomes, but CD63 served mostly as a marker for murine exosomes. The group has many matched samples of tumors, adjacent (and sometimes distant) normal tissues, and plasma, which allow for tissue explant exosome comparisons intra-patient. The proteome profiles may represent biomarkers and therapeutic targets, and the normal tissue serves as a means against drug targets that would affect normal tissue. Pathway analysis derived from pancreatic cancer exosome proteomes found epithelial-mesenchymal transition (EMT), coagulation, and TGFβ signaling as highest ranking. For lung cancer, the pathways were EF2, G2M checkpoint, and MYC targets. Across the 18 cancers in their study, certain proteins stood out as pan-cancer markers such as THBS2, VCAN, SRRT, and TNC. For pancreatic cancer, they identified 50 exosome proteins from patients that were not present in age/sex matched controls. Across cancers, plasma exosomes had sets of cancer proteins, but also sets of immune proteins that were not found in healthy donors.

A new area for EV studies, EVs in cancer related fatigue (CRF), was introduced by Dilorom Sass (NIH, National Institute of Child Health and Human Development, US). In CRF, there is an unusual sense of physical, emotional, or cognitive tiredness related to cancer or cancer treatment. CRF affects 30%-90% of cancer patients receiving therapy, and 30% will continue to experience such fatigue, months or even years post-treatment. Prior work has suggested that a wide variety of cytokines may be involved. Circulating EVs bearing cytokines could be systemic mediators and biomarkers of such inflammation. Dilorom's study searched for associations between levels of fatigue in men after radiation treatment for prostate cancer, determined by questionnaire, and 45 plasma EV-associated immune markers. Hierarchical clustering of the cytokine readouts identified two clusters. A closer analysis found that eotaxin, HSP27, IL3, IP-10, and MIP3 alpha were significantly higher in the EV samples from the fatigued cohort. These studies raise questions in terms of measurements of plasma cytokine values: what fraction is actually measured, and are we overlooking a valuable source of potentially protected cytokines associated with EVs?

Janusz Rak (McGill University, Canada) introduced "leukobiopsy" as a variant of our typical takes on liquid biopsy. The successes of current liquid biopsy, biofluid-based biomarker information relevant to disease states, are significant in some areas, far less so in others. Janusz's group had demonstrated transfer of the oncogenic EGFR variant, EGFRvIII (found in gliomas), from aggressive cancer cells that expressed the protein to more indolent cells that did not express it. This converted the indolent cells into far more aggressive tumors; the presence of EGFRvIII on vesicles in blood suggested that it could be a cancer-specific biomarker. As they extended studies into the RAS oncogene, they noted that cells receiving EVs from RAS mutant cells now possessed RAS protein, RNA, and DNA, implying an extraordinary passage/delivery capacity. Using various assays, the group demonstrated that normal cells could also take up cancer vesicles,

and in certain cases, with observable effects (transient growth in soft agar, presence of micronuclei). From the perspective of the cancer cell, vesiculation and extracellular release of materials is programmed and is a necessary part of the cancer cell's existence. There is another side, however: recipient cells who bind and take up cancer cell EVs, and process the contents. Janusz's lab pursued this in the form of mutant RAS DNA in the blood components of tumor-bearing mice. They were able to find circulating RAS DNA, cell-free and in EVs, but the most abundant depot of the DNA was in white blood cells. Neutrophils in particular seemed to control the amounts of tumor DNA in blood; if those cells are eliminated, circulating tumor DNA and DNA in EVs increased. These data suggest a possible new form of liquid biopsy - leukobiopsy, where leukocytes may harbor the informative characteristics of circulating tumor materials and probed for those tumor entities, be they DNA, RNA, oncoproteins, or oncometabolites.

As introduced earlier by NIH/NIDA's John Satterlee, there is a genuine need for biomarkers for drugs of addiction and substance use disorders. Here, Ursula S. Sandau (Oregon Health and Science University, US) described her work in EVs regarding methamphetamine use and treatment monitoring with EV cargo as a metric for recovery. Methamphetamine use is increasing globally, and can have damaging effects across organ systems, including adverse neuropsychiatric effects. Methamphetamine acts at synaptic dopamine transporters to block dopamine re-uptake, leaving dopamine in the dopaminergic neuronal synapse and stimulating a reward response. The drug also drives neuroinflammatory responses and has multiple implications. Brain microRNAs are altered in response to methamphetamine dosing, and these in turn, can affect proteins implicated in addiction; some of these miRs become diminished in plasma. Certain miRs are also implicated in blood-brain barrier permeability, allowing vesicle release into the blood compartment. In a collaborative study, Ursula's group aimed to characterize plasma EVs in active methamphetamine users, and identify miRs with altered expression in that population. Using clinical data gathered as part of the study, the goal was to follow plasma EV miR changes in the context of clinical and neuropsychological changes in users.They performed vesicle flow cytometry to calculate particle concentrations in the subjects' plasma, and found finding that measures of lifetime exposure showed some correlation to particle quantity. These correlations were maintained when sorting for EVs *vs.* all particles. Purification of EVs and quantification of miRs showed that 20 miRs were significantly increased, and 69 miRs decreased. Following statistical management, they narrowed the numerically relevant miR numbers to six. When correlating these to subjects' clinical features, characteristics of lifetime exposure were significant and included frequency for three of the six miRs. Pathway analysis based on miR targets revealed pathway involved in cardiac, liver, and kidney disease, as well as neurological function. These also correlated with behavioral function related to methamphetamine use. The results of the study suggest that plasma EVs and their miR content may serve as biomarkers in methamphetamine use disorder and may correlate with clinical features.

The final speaker of the session, and of the conference, was Andy Hill (LaTrobe University, Australia), regarding EV-based biomarkers of neurodegenerative disease. Neurodegenerative diseases present with a range of signs and symptoms, and with clinical decline before diagnosis. Causes are also variable, but often involve protein misfolding and deposit in the brain. As diagnosis may involve sophisticated brain imaging techniques not routinely available, the search for blood-based biomarkers is reasonable. EV microRNAs have been sought as potential biomarkers for years, particularly in accessible, minimally-invasive sources, such as blood. However, blood-based biomarkers might not represent the actual pathology of the brain, so Andy's group delved into techniques to isolate vesicles from actual brain tissues (both healthy controls and AD patients). The EV cargo was then compared to the blood (serum) EV cargo they had previously identified as putative AD biomarkers. There were indeed miRs that were found at higher levels in AD brain EVs *vs.* control brain EVs, but also some that were different between serum and brain. Thus, the serum EV miRs were not exactly the same as the brain EV miRs, but could nonetheless be useful. The next step was to compare the miRs predictive of AD with imaging studies. Employing machine learning (Association Rule Mining, ARM), EV miR levels could predict imaging positive (AD) and imaging negative (healthy control)

cases. The brain-serum EV work was extended to Parkinson's Disease (PD), where they found miRs that could distinguish PD stages. They further extended the brain EV work to amyloid lateral sclerosis (ALS) patients, where they identified potential protein and miR biomarkers that relate to ALS pathology. Some of their early work was done in prion disease in mice, which eventually led to brain and blood sampling to identify EV biomarkers that could be used for Creutzfeldt-Jakob disease (CJD) in humans. This work represents a tour de force of investigating disease characteristics of neuropathologies and their relationships to potential blood-based biomarkers.

In the last two portions of the meeting, Fatah Kashanchi (George Mason University, US) held an interactive workshop on grant writing aimed especially at obtaining funding via the US NIH aimed at young (and not so young) investigators. He supplied potential applicants with the perspective of a grant reviewer, and what the reviewers want and expect to see, and he incorporated NIDA's John Satterlee's "ten commandments for preparing a compelling R01 application". He then provided an example of a Specific Aims page and gave rationales for what was stated, why reviewers need and want specific information, and how the applicant should supply that.

The Closing Ceremonies also served as an award platform for young investigators; these included speakers:

Moran Amit (MD Anderson, US)
Frederik Verweij (Institute of Psychiatry and Neuroscience of Paris, France)
Hannah McMillan (Duke University, US)

Poster awards went to

Killian O'Brien (Harvard/MGH, US)
Kathleen Lennon (City of Hope, US)

As the ASEMV and the exosome/EV field have grown over the years, the areas of biology and pathology studied have increased as well. While much research has focused on cancer in the "early years", we see in this conference the breadth of diseases studied, including the basic biology of vesicles in normal tissues, systems, and organisms. Vesicles are now viewed as communicators between and among cells, as well as between hosts, pathogens, symbionts, and kingdoms. The analytical and separation technologies have advanced, while some of the classical techniques continue to maintain their value. Every year brings startling changes, more information, and often more confusion, which one might describe as science in its element.

The ASEMV Annual Meeting for 2021 is already in the planning phase and we hope to meet again in person, and continue to highlight some of the best of exosome/extracellular vesicle science.

ASEMV would like to thank the Organizing Committee for their efforts, and in particular, Roger Alexander for his tireless work in preparing the online format.

Louise Laurent, UC San Diego, ASEMV2020 Organizing Committee Chair
Steve Gould, Johns Hopkins University, ASEMV President
Roger Alexander, Extracellular RNA Communication Consortium
Nihal Altan-Bonnet, NIH NHLBI
Michael Graner, University of Colorado Anschutz
Kendall Jensen, Translational Genomics Research Institute
Fatah Kashanchi, George Mason University
Meta Kuehn, Duke University

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DECLARATIONS

Author's contribution

The author wrote this report based on notes and videos following the ASMV2020 Meeting.

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Extracellular Vesicles and Circulating Nucleic Acids

Meeting Abstracts

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Meeting Abstracts of the American Society for Exosomes and Microvesicles 2020 Annual Meeting

Authors:

Alissa M. Weaver, Ryan P. McNamara, Y Peng Loh, Hannah M. McMillan, Blanca V. Rodriguez,James Erickson, Heather Branscome, Sarah Al Sharif, Ribhav Mishra, Kathleen M Lennon, Amy H. Lee, Jean C. Lee, Bridget Ratitong, Jessica Pullan, Joshua L. Hood, Pooja Khatkar, Janos Zempleni, Yi Zhao, Nicole Comfort, Jordan Pavlic, Nicole Noren Hooten, Sarah F. Andres, Lisa Meyer, Shivani Sharma, Johnny Akers, Laura Perin, Emma Purcell, Dilorom Sass, Tatsuo Kurihara, Shamba Gupta, Ikuhiko Nakase, Maria Cowen, Yuriy Kim, Ryan Reshke, Tsuneya Ikezu, Hameeda Sultana, Killian P. O'Brien, Chukwumaobim D. Nwokwu, Inge S. Zuhorn, Simon Carding, Dennis A. Steindler, Frederik J. Verweij, Pieter Vader, Martin Olivier, Crislyn D'Souza-Schorey, Moran Amit

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***The Abstract of the American Society for Exosomes and Microvesicles 2020 Annual Meeting, November 16-19, 2020 [Table 1]**

Table 1. Table of Content

1. Argonautes in extracellular vesicles: artifact or selected cargo?

Author: Alissa M. Weaver

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Abstracts: Argonaute 2 (Ago2) is the essential component of the RNA-Induced Silencing Complex (RISC) that binds miRNAs and promotes mRNA degradation. Extracellular vesicle (EV)-carried miRNAs have been shown to influence gene expression and functional phenotypes in recipient cells. Many investigators have found Ago2 in EVs and it is postulated that Ago2 is a major transporter of miRNAs into small EVs (SEVs), such as exosomes. Others have reported that extracellular Ago2 is non- vesicular. I will discuss issues that may lead to diverging results and show data on the role of culture conditions in the detection of Ago2 in SEVs.

2. Super-resolution microscopy-based resolving of membrane-associated proteins on extracellular vesicles

Authors: Ryan P. McNamara[1,2,*], Yijun Zhou[1,2], Meredith G. Chambers[1,2], Anthony. B. Eason[1,2], Justin T. Landis[1,2], Brian Yang[1,2], Blossom A. Damania[1,2], Dirk P. Dittmer[1,2]
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Abstracts: Extracellular vesicles (EV) are secreted from most cell types and are intimately involved in homeostasis. Given that their size can drop well beneath the limit of diffraction of typical light microscopy [< 250 nanometers (nm)], direct visualizations and characterizations of single EV have proven daunting. Conventional approaches to their visualization include light scattering methods such as nanoparticle tracking analysis, transmission and scanning electron microscopy (EM), and highly sensitive nanoscale flow cytometry. Super-resolution microscopy, such as direct stochastic optical reconstruction microscopy (dSTORM) has also been recently employed to view EV. Here, we show that dSTORM can be employed in multiple channels to resolve membrane-associated proteins to nanometer precision. Our dSTORM- based characterizations of EV typically had resolutions of ± 20 nm on the XY axis using excitation lasers of 473 and 640 nm. Size distributions of EV were consistent with other methods of measurement such as light scattering and resistive pulse sensing and were independent of the excitation laser. Moreover, we were able to resolve tetraspanins on the membrane of an individual EV, both by conjugation to- mCherry or through indirect antibody-mediated detection. In conclusion, we have rendered individual EV to nanometer detail through super-resolution microscopy and confirmed the presence of tetraspanin molecules on their surface. We propose that a highly sensitive analysis like this could be adapted to rapidly scan for rare- event biomarkers of disease through fluorescence-based machine learning.

3. Exosomal carboxypeptidase econfers and CPE-shRNA loaded exosomes inhibit tumorigenesis

Authors: Sangeetha Hareendran[1], Bassam Albraidy[1], Xuyu Yang[1], Aiyi Liu[2], Anne Breggia[3], Clark C Chen[4], Y Peng Loh[1]
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Abstracts: Carboxypeptidase E (CPE) and its splice variant CPE- N has been shown to promote cancer growth and metastasis in various cancer types. Exosomes carry biomolecules (proteins, DNA, mRNA and

miRNA) unique to their cell of origin and deliver them to recipient target cells thereby mediating cell-cell communication. We have investigated whether exosomes from high metastatic HCCH (liver cancer) cells can confer growth and metastatic properties to HCCL (low metastatic). Exosomes were isolated from the supernatant culture media of cancer cells and a correlation was found between elevated CPE mRNA levels in exosomes from high *vs*. low metastatic cell lines across various cancer types. Content analysis of exosomes derived from HCC97H cells revealed CPE-WT mRNA and protein. We showed that exosomes released from HCC97H cells were able to enhance invasion of HCC cells with poor metastatic ability (HCC97L) in Matrigel invasion assay and proliferation in MTT assay. However, when CPE expression was suppressed in the HCC97H cells before exosome isolation, the exosomes had no effect on proliferation and invasion. These data demonstrate the ability of exosomes to confer metastasis in cancer cells and the role of exosomal CPE in driving the process. We then utilized the inherent property of exosomes to act as efficient delivery tools to carry a therapeutic agent such as shRNA. Previously it was shown that down-regulation of CPE expression by shRNA can reverse tumor growth and metastasis in an HCC mouse model. We therefore loaded CPE-shRNA into exosomes by infecting HEK293 (Human Embryonic Kidney) cells with adenovirus carrying CPE-shRNA-GFP. These modified exosomes were harvested from the cell medium, purified and then used to transfer CPE-shRNA to HCC97H cells. The exosomes taken up by the recipient cells resulted in significant reduction of CPE mRNA levels and decrease in colony formation of these cells. Thus, these studies demonstrate the ability of exosomal CPE to enhance invasion in low metastatic HCC cells and the potential to use shRNA loaded exosomes to target CPE as a therapeutic strategy to treat liver and other cancers. Finally, in a pilot study we measured CPE mRNA copy numbers in serum exosomes of patients with cancer (glioma, breast, ovarian, colon, cervical, pancreatic or prostate cancer) and healthy controls. Significantly elevated levels of CPE mRNA copy numbers were found in serum exosomes of cancer patients versus healthy controls, suggesting that exosomal CPE mRNA could potentially be used as a biomarker in an initial screen for diagnosing cancer.

4. Bacterial vesicles: vehicles for Inter-Kingdom communication and modulators of plant immune response

Authors: Hannah M. McMillan[1], Sophia G. Zebell[2], Jean B. Ristaino[3], Xinnian Dong[2], Meta J. Kuehn[4]
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[4]Department of Biochemistry, Duke University, Durham, NC, USA.

Abstracts: Bacterial outer membrane vesicles perform a variety of functions in bacterial survival and virulence. In mammalian systems, vesicles activate immune responses and have been exploited for use in vaccines. However, little work has focused on the role vesicles play in dry-land environments or in the context of plant-microbe interactions, and research has only just begun on their role as natural nanoscale vehicles. We show that vesicles from the plant pathogen Pseudomonas syringae pv tomato DC3000 and the plant beneficial Pseudomonas fluorescens activate plant immune responses that protect against future bacterial and oomycete challenge. Interestingly, our results also expose differences in vesicle packaging between pathogens and beneficials and in vesicles isolated from various environmental conditions that lead to different sensitivities to biochemical stressors. Importantly, these studies reveal unique differences in pathogen- versus beneficial-induced immune activation and support the use of vesicles as a tool to

probe type III secretion system-independent immune responses. Uncovering the mechanisms through which pathogen-and beneficial-derived vesicles mediate plant immune responses and exploring their role as natural nanoparticle vehicles will reveal new targets for agricultural disease management and highlight novel roles for vesicles in overall ecosystem function.

5. Staphylococcus aureus secretes immunomodulatory RNA via extracellular membrane vesicles

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Abstracts: Bacterial-derived RNA can function as ligands for intracellular receptor activation and induce downstream signaling to modulate the host response to bacterial infection. The mechanisms underlying the secretion of immunomodulatory RNA by human pathogens, such as Staphylococcus aureus, and their delivery to intracellular host cell receptors are not well understood. Recently, extracellular membrane vesicle (MV) production has been proposed as a general secretion mechanism that could facilitate the delivery of functional bacterial nucleic acids into host cells. S. aureus produce membrane-bound, spherical, nano-sized, MVs packaged with a select array of bherefore, in the context of neurotrophic vioactive macromolecules and they have been shown to play important roles in bacterial virulence and in immune modulation through the transmission of biologic signals to host cells. The present study sought to examine the nature of the association between RNA and MVs produced by S. aureus. We also sought to analyze the immunostimulatory potential of MV- associated RNA and to evaluate receptor-mediated recognition of MV-associated RNA and DNA molecules by innate immune cells. Here we report that S. aureus produces MV-associated RNA molecules that are protected from nuclease degradation. MV-associated nucleic acids were transferred to cultured murine macrophages and induced significant Interferon-β mRNA expression largely through endosomal Toll-like receptor (TLR) signaling. Upon exposure to nuclease-treated MVs, TLR3-/- and TLR7-/- macrophages produced very little IFN-β mRNA. TLR3 recognizes dsRNA, which points to the possibility that S. aureus MVs are packaged with immunostimulatory dsRNA molecules. TLR7 has previously been found to recognize S. aureus tRNA, as well as ssRNA molecules. Altogether, these data indicate that endosomal nucleic acid receptors are activated in cultured mouse macrophages upon MV exposure, likely due to immunostimulatory properties of MV-associated nucleic acids. Our findings show for the first time an MV-mediated pathway by which *S. aureus*-derived immunomodulatory RNA molecules are delivered to host cells. How MV-associated nucleic acids are trafficked intracellularly and recognized by endosomal TLRs will be examined in future experiments.

6. Separation of EVs from virions in coronavirus infections

Authors: James Erickson[1], Maria Cowen[1], Yuriy Kim[1], Anoop Pal[2], Heather Branscome[1,3], Archana Gupta[4], Fatah Kashanchi[1]

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Abstracts: Since the severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) was declared a pandemic in mid-March of 2020 by the World Health Origination (WHO), laboratories around the world started research into diagnostics, therapeutics, and treatments[1]. In recent years, the importance of extracellular vesicles (EVs) in the pathogenesis of viral infections have been found in the cases of many viral pathogens including few DNA and RNA viruses including human T-cell leukemia virus-1 (HTLV-1) and human immunodeficiency virus-1 (HIV-1)[2,3]. EVs from HIV-1 infected cells on uninfected macrophages induces an increase in the proinflammatory cytokines[3]. While EVs from HTLV-1 infected cells on uninfected recipient cells promoted the localization and cellular contact by cells, this directly influences the pathogenesis of HTLV-1 as the virus mainly infects other cells by cell to cell contact[2]. Similar to retroviruses, coronaviruses are also positive strand RNA viruses, except they replicate in the cytoplasm and may regulate chromosomal DNA depending on the strain of virus. We have recently began working on beta- coronaviruses, including OC43 (BSL2 strain) and SARS-CoV-2 (BSL3 strain). Our initial experiments focus on isolation of EVs away from virions using either an iodixanol gradients or Izon sizing columns. We have successfully separated the two from one another mainly due to their density and potentially size differences. We found that EVs from multiple coronaviruses are not infectious and viral particles treated with UV irradiation are also not infectious. We also have found that coronavirus EVs caused T-cell death, which may corelate with lymphopenia observed in COVID patients. Along these lines coronavirus EVs can activate other viral genes (i.e., HIV-1 or HTLV-1) when these genes are integrated into the genome, further implying that these EVS regulate chromosomal gene expression. Finally, the mechanism(s) of how these EVs may cause such diverse effects on T-cells and other viral gene expression will be discussed.

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7. Use of stem cell extracellular vesicles as a holistic approach towards CNS repair

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Abstracts: Neurological diseases and disorders are leading causes of death and disability worldwide. Many of these pathologies are associated with high levels of neuroinflammation and irreparable tissue damage. We have previously shown that extracellular vesicles (EVs) from infected cells contain viral by products (non-coding RNAs and proteins) and that these EVs can exert deleterious effects on recipient cells[1-3]. Therefore, in the context of neurotrophic viruses EVs may contribute to or perpetuate processes relating to neuroinflammation and neurodegeneration. Due to their multipotent properties, stem cells have broad applications for tissue repair; additionally, stem cells have been shown to possess both immunomodulatory and neuroprotective properties. In recent years it has been well-established that stem cell EVs play a critical role in the functionality associated with stem cells. The diverse biological cargo contained within these

vesicles are proposed to mediate their effects and, to date, the reparative and regenerative effects of stem cell EVs have been demonstrated in a wide range of cell types. While a high potential for their therapeutic use exists, there is a gap of knowledge surrounding their characterization, mechanisms of action, and how they may regulate cells of the central nervous system (CNS). We have isolated and recovered high yields of EVs from large scale cultures of both induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs) using tangential flow filtration. Our EV characterization includes both phenotypic (size, tetraspanin expression) and biochemical assays. EV functionality has also been assessed *in vitro* utilizing several cell-based assays related to cellular viability, migration, angiogenesis, and immunomodulation in both healthy and damaged recipient cells with relevance to the CNS. Our data suggests that EVs from different sources of stem cells display unique phenotypes, exhibit differential association with various cytokines, proteins, and long non-coding RNAs, and have the ability to significantly enhance processes that are critical for cellular repair[4]. Lastly, utilizing an iPSC-derived neurosphere model, we have observed a robust uptake of stem cell EVs and have found that these EVs are able to effectively penetrate these 3D structures. Collectively, these results highlight the "holistic" properties of stem cell EVs by demonstrating their ability to partially reverse or reduce damage in various cell types.

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8. Extracellular vesicles from HTLV-1 infected cells regulate viral spread and pathogenesis

Authors: Sarah Al Sharif[1], Daniel O. Pinto[1], Gifty Mensah[1], Maria Cowen[1], Pooja Khatkar[1], James Erickson[1], Heather Branscome[1], Catherine DeMarino[1], Weidong Zhou[2], Lance A. Liotta[2], Fatah Kashanchi[1]
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Abstracts: Human T-cell lymphotropic virus type 1 (HTLV-1) is the causative agent of adult T- cell leukemia/lymphoma (ATLL) and HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP)[1,2]. Approximately 10 million worldwide are infected with HTLV-1, however, it is most likely that the true figure is greater than this number as many cases are not reported[2]. It is endemic in many areas such as southern Japan, the Caribbean, and parts of Africa[2]. In recent publications, we showed that HTLV-1 infected cells release Extracellular Vesicles (EVs) containing viral RNA and proteins (gp61/Tax/HBZ) and are not infectious[3,4]. However, they can enhance cell-to-cell contact of uninfected cells, eventually aid in spread of virus[4]. We separated distinct EV subpopulations from HTLV-1 infected cell supernatants by differential ultracentrifugation (DUC) into 2k, 10k, and 100k EVs based upon centrifugation speed. Proteome profiling of various HTLV-1 EV subpopulations showed that viral/host protein was highly abundant in 2k subpopulation, compared to other subpopulations. Western Blots revealed that p19 and Tax (i.e., viral proteins), as well as LC3 and p62 (i.e., autophagy proteins) were mainly present in the 2k and 10k subpopulation. Using an *in vitro* angiogenesis assay, 2k HTLV-1 EVs were primarily responsible for tubular deterioration. Different EV subpopulations were incubated with cells involved in neuroinflammation (i.e., astrocytes, macrophages, and neurons) where the highest level of IL-8 expression

(involved in cell migration) was found in astrocytes and monocyte-derived macrophages. IL-6 (involved in inflammation) was only present in neurons treated with these EVs (i.e., 100k > 10k > 2k). HTLV-1 EVs were able to facilitate HTLV-1 viral spread in monocytic cell-derived dendritic cells via cell-cell contact. Finally, an increase in proviral DNA and RNA levels in humanized mice tissue (i.e., Blood, Lymph Node, and Spleen) were noticed following treatment with 2k and 10k HTLV-1 EVs, indicating the importance of these EVs in HTLV-1 spread. These findings indicate that different HTLV-1 EV subpopulations induce cytokine expression, tissue damage, and viral spread. These EV subpopulations could potentially contribute to the development of ATLL or HAM/TSP. Further mechanistic understanding of these EVs in HTLV-1 pathogenesis will be discussed for the development of preventative measures and treatment options for this devastating disease.

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9. Proteomics of cerebellar exosomal proteins: therapeutic and biomarker implications in spinocerebellar ataxia-1

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Abstracts: Exosomes are observed to carry proteins, RNA, and also some chromosomal DNA released by cells. These biomolecules are found to travel from one cell to another and thus acts as a messenger to communicate signals between different cells. In the nervous system, they are found to be critical for neuron-neuron, neuron-glia communications. The emerging roles of exosomes in different neurodegenerative pathologies like Alzheimer's, Parkinson, and Prion suggests that exosomes can help in the spread of these toxic proteinaceous inclusions and hence they are a potential therapeutic target in such diseases. Exosomal biology in the diseases of Spinocerebellar ataxia-1 (SCA1) is not understood with no report present to define the role of exosomes in the SCA1 pathology. Since cerebellar cells are the primary cells affected in the SCA1, hence, we have characterized the exosomes isolated from the primary mixed culture of cerebellar cells using a variety of biochemical methods and biophysical techniques. Furthermore, we have identified the proteomic content of the exosomal lysate using the LC-MS/Mass Spectrometry and have found proteins that are enriched for the biological functions in the extracellular matrix, myelin sheath formation, axonal growth cone development. We will use this data to identify proteins that are necessary for the development and functions of the cerebellum and can derive a conclusion on the role of exosomes in SCA1. Exosomes also carry molecules that are relevant to disease-specific diagnosis and so they are also proposed as a biomarker tool in neurodegenerative pathologies of Alzheimer's, Parkinson. However, their role as a biomarker candidate in SCA1 pathology is not defined and their use of a biomarker strategy is tested for the very first time by us. To validate exosomes as a biomarker in SCA1 pathology, we plan to isolate and characterize exosomes from the body fluids (blood and CSF) of $WT^{2Q/2Q}$ and $SCA1^{154Q/2Q}$ knock-in mouse models in different age groups of pre-symptomatic, early symptomatic, and late symptomatic. The exosomal proteins will be identified using the various proteomics-based approach and then compared between $WT^{2Q/2Q}$ and $SCA1^{154Q/2Q}$ knock-in mice and any alterations in the level of proteins will be studied further for use as a biomarker in SCA1 pathology. The results of the above work will also be tested as a biomarker candidate in SCA1 patients. The overall findings of our work will provide clues on the role of exosomes in the pathology of SCA1 and may well establish them as a biomarker in the pathology of SCA1.

10. Advancing extracellular vesicle characterization with quantitative single molecule localization microscopy

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Abstracts: Quantitative Single Molecule Localization Microscopy (qSMLM) can quantify individual biomolecules in cells with nanoscale precision. However, because of several technical hurdles, a similarly robust quantification is often difficult to achieve when the methodology is applied to extracellular vesicles (EVs). One significant challenge has been to rigorously isolate and characterize disease- specific and tissue-specific subpopulations of EVs. Our approach to probe EV subpopulations entails three main advancements: 1) more effective methods to label the EVs for SMLM imaging; 2) an optimized protocol for affinity isolation and imaging of EVs; and 3) advanced algorithms to robustly detect size and count the number of biomolecules within individual EVs. Briefly, size exclusion chromatography was first used to isolate EVs from either cultured cell media or patient plasma. Then, EV membranes were labeled with fluorescent reagents: dyes, lectins, or antibodies. The excess fluorescent molecules were removed by size separation using concentrating filters, core beads, or size exclusion columns. Finally, EVs enriched in specific receptor(s) were affinity isolated onto coverslips, imaged, and analyzed. Using this comprehensive approach, we have quantified EVs isolated from the plasma of patients who have pancreatic cancer. Specifically, we have determined the number of isolated EVs, their size, and the abundance of several biomarkers. Compared to healthy controls, patients with pancreatic cancer exhibited a distinct population of larger EVs enriched in epidermal growth factor receptor and carbohydrate antigen 19-9. Ultimately, when this approach is paired with traditional methods, it may shift the paradigm for comprehensively prehensively characterizing cancer-specific and organ-specific EVs.

11. Hypocalcemic-induced exosome secretion drives differential metastatic progression of epithelial ovarian cancer

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Abstracts:

Introduction: Serous epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy and peritoneal fluid (ascites) build-up is common in late stage EOC. This ascites is an abundant source of cell-secreted exosomes. Gynecological conditions are often associated to decreased blood serum calcium concentrations and elevated exosome production. Exosomes are continuously secreted through the endosomal pathway; however, their release can be triggered by various physiologic stimuli, leading to exosome heterogeneity. We have demonstrated that mimicking hypocalcemia conditions by chelating extracellular calcium releases a unique population of exosomes from an EOC cancer cell line. We

hypothesize that naturally secreted exosome (SEC-exosomes) *vs*. chelation-induced exosome (CI-exosomes) treatment will lead to differential biophysical and molecular cellular responses to mediate more invasive tumor microenvironment (TME).

Methods: OVCAR-3 EOC cells were cultured in exosome-free media for 48 hours. SEC-exosomes were collected from media and harvested using standard ultracentrifugation methods. OVCAR-3 were washed and treated with EDTA to harvest CI-exosomes. CI-exosomes were isolated using ultracentrifugation. Exosome diameters and ubiquitous surface protein markers for CI- and SEC- exosomes were validated using dynamic light scattering and immunoblots, respectively. Patient-derived EOC fibroblasts were treated with either exosome population. High-throughput physical and molecular assays - single-cell migration, immunocytochemistry, adhesion assays, and miRNA microarrays - were used to examine unique differences between populations and their interactions with fibroblasts.

Results: Microarray data showed unique miRNA profiles in SEC- *vs*. CI- exosomes, suggesting heterogeneity in cell-secreted exosomes. Specifically, there were 1,019 differentially expressed miRNAs between either exosome population. Highly regulated miRNAs were linked to mechanosensitive pathways that impact cell motility and cytoskeletal organization. Both populations altered actin fiber and focal adhesion protein organization to affect fibroblast morphologies. Exosome populations further increased random and directional fibroblast migration; however, fibroblast adhesion strength was only increased with CI-exosome treatment. For co-culture OVCAR-3 and fibroblast studies, CI-exosomes promoted adhesion and spreading of OVCAR-3 cells, while SEC-exosomes led to dramatic elongation in a small percentage of OVCAR-3 cells.

Discussion/Conclusions: We showed that decreased extracellular calcium levels, which mirror various gynecological conditions, led to the release of a unique exosome population (CI-exosomes) from a single cancer cell line. This CI-exosome population contained unique miRNA content and led to different molecular/physical fibroblast phenotypes compared to SEC-exosomes. This highlights that tumor cells can secrete multiple exosome populations to mediate cancer progression.

12. Orchestration of human macrophage NLRP3 inflammasome activation by Staphylococcus aureus extracellular vesicles

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Abstracts:

Staphylococcus aureus (*S. aureus*) is an opportunistic pathogen that causes a variety of diseases, including bacteremia, endocarditis, skin and soft tissue infections, and food poisoning. The bacterium produces a myriad of virulence factors, including toxins, enzymes, surface proteins, and multiple glycopolymers, many of which are components of staphylococcal extracellular vesicles (EVs). We purified EVs from a community-associated methicillin-resistant *S. aureus* strain and showed that EV-associated pore-forming toxins, particularly alpha hemolysin and members of the leukocidin family, lysed a variety of cell types. Staphylococcal alpha-type phenol-soluble modulins promoted EV biogenesis by disrupting the cytoplasmic membrane, whereas peptidoglycan crosslinking and autolysin activity modulated EV production by altering the permeability of the cell wall. Purified *S. aureus* EVs were internalized into human macrophages in vitro, and this process was blocked by inhibition of the dynamin-dependent endocytic pathway. EVs triggered NLRP3 inflammasome activation, resulting in the cellular release of IL-1β and IL-18 and induction of

pyroptosis. Consistent with this result, a dose-dependent cytokine response was detected in the extracellular fluids of mice challenged intraperitoneally with *S. aureus* EVs. Pore-forming toxins associated with EVs were critical for NLRP3-dependent caspase-1 activation of human macrophages, but not for TLR2 signaling. In contrast, EV-associated lipoproteins not only mediated TLR2 signaling to initiate the priming step of NLRP3 activation but also modulated the toxin content and the biogenesis of EVs, resulting in alterations in IL-1β, IL-18, and caspase-1 activity. Our study describes mechanisms by which *S. aureus* EVs induces inflammasome activation and reveals an unexpected role of staphylococcal lipoproteins in EV biogenesis. S. aureus EVs may serve as a novel secretory pathway to transport protected cargo to host cells during infection to modulate cellular functions.

13. Beta-glucan stimulated neutrophils secrete IL-1α through exosomes

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Abstracts: Interleukin-1α (IL-1α) and IL-1β are leaderless pro-inflammatory members of the IL-1 family of cytokines that are secreted via unconventional, ER and Golgi-independent pathways. Secretion of processed, bioactive IL-1β by macrophages is mediated by Gasdermin D (GSDMD); in contrast, little is known about the mechanism of IL-1α release by myeloid cells. Here, we demonstrate that IL-1α production by macrophages is completely dependent on Gasdermin D. We found that neutrophils produce IL-1α following inflammation induced by Aspergillus fumigatus spores that express cell surface β -glucan, and show that in contrast to macrophages, IL-1α secretion by β-glucan stimulated neutrophils occurs independently of GSDMD. Instead, we found intracellular IL-1α co-localized with extracellular vesicle (EV) markers CD63, CD9, and CD81, and that IL-1α is encapsulated in EVs isolated from β-glucan stimulated neutrophils. Preincubation of neutrophils with GW4869 inhibited exosome release and significantly reduced IL-1α levels in cell-free supernatant. Further, we found that exosomal IL-1α can signal through its receptor, IL-1R1, and EVs isolated from neutrophils, and can activate macrophages as measured by proinflammatory cytokines IL-6 and TNFα release. Collectively, these findings demonstrate that the mechanism for IL-1α secretion in neutrophils is distinct from macrophages and identify a role for neutrophil extracellular vesicles, specifically exosomes, in this process.

14. Hypoxia-responsive bovine milk exosomes as targeted carrier for chemotherapeutics

Authors: **Jessica Pullan[1], Li Feng[1], Kaitlin Dailey[1], Sangeeta Bhallamudi[1], James Froberg[2], Lina Alhalhooly[2], Amanda Brooks[3], Sathish Venkatachalem[1], Yongki Choi[2], Sanku Mallik[1]**

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Abstracts: Exosomes are secreted nanosized extracellular vesicles with innate transportation ability making them ideal candidates for drug delivery. The inherent cellular cargo of an exosome can negatively impact its ability to carry and deliver cargo safely. Bovine milk exosomes have not shown the same effect as other sources in throughout the literature and therefore are a safe and effective source of exosomes. In order to increase targeting and predictable control drug release of bovine exosomes, a neuropilin receptor agonist peptide (iRGD) and a hypoxia-responsive lipid have been incorporated into the lipid bilayer of bovine

milk exosomes. Hence, peptide targeted, hypoxia-responsive bovine milk exosomes (iHRX) encapsulating the anticancer drug doxorubicin will decrease cell survival of triple-negative breast cancer cell population. Doxorubicin has been encapsulated using electroporation and shows encapsulation efficiencies of above 50%. Transmission electron microscopy, atomic force microscopy, and dynamic light scattering have demonstrated the stability of modified exosomes in normoxic conditions and exosome fragmentation under concentrations greater than 5 mM glutathione, which directly corresponds to tumor oxygen levels. Additional studies to confirm the presence of iRGD has been performed indicating strong adhesion to the avb3 integrin most abundantly found in tumors. Future studies will include testing of iHRX's toxicity and cellular internalization in monolayer and three-dimensional cultures prior to *in vivo* studies.

15. Liver tumor-derived exosomes (small extracellular vesicles) induce macrophage polarity

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Abstracts:

Our recent data demonstrate that nanoscale tumor-derived small extracellular vesicles (sEVs), otherwise known as exosomes, induce macrophage (Mφ) functions. Tumor-associated M1 (proinflammatory) or M2 (immunosuppressive) Mφs are pivotal in the progression of inflammation-related cancers such as hepatocellular carcinoma. However, the ability of liver tumor-derived sEVs to influence Mφ- related pro-tumor processes is largely unexplored. Using human HepG2 liver tumor and THP-1 Mφ models, we investigated the hypothesis that HepG2-sEVs induce Mφ polarity, indicative of pro-tumor inflammatory processes. Preliminary ELISA results revealed a dose dependent increase in production of the proinflammatory M1 cytokine TNF-α and immunosuppressive M2 cytokine IL-10 at a treatment concentration of 0.05 μg sEV protein/μL. Subsequent ELISA experiments demonstrated batch to batch variations in the induction of IL-10 by HepG2-sEVs at the 0.05 μg sEV protein/μL dose. In contrast, normal plasma (np) control sEVs did not increase IL-10 production. TNF-α production was significantly induced by all np-SEV and HepG2-sEV lots and batches tested. Use of qRT-PCR showed that both np control and HepG2 sEVs favored induction of M1 polarization factors with HepG2's also trending toward induction of a few mixed M1/M2 and M2 markers. Overall, HepG2-sEVs induced Mφ polarity, indicative of pro-tumor inflammatory processes. Future investigations will explore these processes and derivative therapeutic strategies.

Grant Support: University of Louisville Cancer Education Program (R25-CA134283), The Elsa U. Pardee Foundation (PI J.L. Hood), University of Louisville Department of Pharmacology and Toxicology Faculty Start-up funds to J.L. Hood, and UofL Hepatobiology and Toxicology COBRE NIGMS NIH P20GM113226.

16. Small RNA-binding molecules inhibit HIV-1 transcription in CNS latent reservoirs

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Abstracts: Despite the emergence of combinational antiretroviral therapy (cART), 50% of all HIV-1 infected patients demonstrate some degree of HIV-1 associated neurocognitive disorder ranging from asymptomatic neurocognitive impairment to mild neurocognitive disorder to severe HIV-associated dementia. Although cART is highly effective in suppressing viremia, the lack of an FDA approved viral transcription inhibitor allows for persistent non-processive transcription which results in the production of short non-coding RNAs (TAR and TAR-gag)[1,2]. Our lab and others have previously shown that these RNAs are released from the cell in extracellular vesicles (EVs), specifically exosomes, and can induce production of proinflammatory cytokines in recipient myeloid cells[3,4]; a factor that may contribute to the neuroinflammation observed in HIV-1 infected individuals with HIV associated neurocognitive disorder (HAND). Here, we have screened a panel of small molecules that have been previously found to noncanonically bind HIV-1 Trans Activation Response (TAR) element, a 5' RNA element that is required for activated viral transcription[5]. We have identified 5 candidates that effectively inhibit viral transcription in myeloid and T-cells without toxicity. The mechanism of inhibition may be directly linked to Tat-activated transcription and epigenetics modification. These data also suggest a direct link and communication between Nuclear Transcription and EV cargo biogenesis. Collectively, our results suggest that the use of small RNA-binding molecules to inhibit viral transcription could potentially be used to complement existing cART drugs to address the current therapeutic gap in current regimens.

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17. Extracellular vesicles and microRNAs in maternal milk are important for growth and gut health during weaning in murine pups

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Abstracts:
Background: Human milk contains approximately 2.2×10(exp)11 exosome-size EVs per mL, which harbor more than 200 microRNAs. Mammals absorb EVs and microRNAs from milk, and microRNAs regulate approximately 60% of human genes. Studies in transgenic mice suggest that EVs in maternal milk accumulate primarily in the liver, brain and gastrointestinal (GI) mucosa in neonate pups.
Objective: Assess whether maternal EVs and microRNAs promote postnatal growth and GI health in neonate mice.
Methods: WT pups were fostered to homozygous Tsg101 KO dams (impaired exosome biogenesis), heterozygous Dicer KO dams (loss of microRNA biogenesis) or WT dams (control) from synchronized

pregnancies (4 pups/dam). We assessed milk EVs and microRNAs, gut development, barrier function, mRNA expression profile in the jejunum, postnatal weight gain, and milk quality and intake. Statistics: unpaired *t*-test (Tsg101/Dicer *vs*. control); $P < 0.05$.

Results: KO of TSG101 and Dicer caused an 80% and 60% decrease of EVs and microRNAs, respectively, in milk. The loss of milk EVs and microRNAs led to an up to 20% shorter length of the gut, 20% decrease of villi height and 15% crypt depth, 50% increase in leakiness of the gut (appearance of FITC-dextran in blood), and a 50% loss of postnatal weight gain in pups. Approximately 400 mRNAs were differentially expressed in the jejunums of pups fostered to TSG101 KO dams or WT dams. Nutritional quality of milk and milk intake were not study confounders.

Conclusions: Mothers communicate with their offspring through EVs and microRNAs in milk, and the maternal message plays a role in optimal growth and gut health in neonate mice.

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18. High-capacity membranes for simple, rapid extracellular vesicle isolation with high yield and purity

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Abstracts:

Introduction: Despite their small size, extracellular vesicles (EVs), such as exosomes, play important roles in normal physiological processes and diseases. A critical bottleneck in EV research is the isolation of the vesicles, which has historically been accomplished via ultracentrifugation (UC). However, UC is time consuming, is not scalable, requires specialized equipment, may damage vesicles during the high-speed spins, can pull down non-EV proteins and nucleic acids, and suffers from low yield. More recently, precipitation solutions have been utilized to simplify EV isolation protocols, but these techniques are often inconsistent, provide low yields, and reduce purity. Thus, there is a strong need for a rapid EV isolation method that does not compromise purity or yield.

In order to overcome these shortcomings, we developed a new method of purifying EVs using a membrane-column-based approach. This method comprises a novel membrane conjugated to a lectin-based compound that selectively binds EVs. The membrane is chemically modified to have increased surface area, allowing higher binding capacity and yield, while also providing a highly pure and concentrated EV preparation. Additionally, the membrane is assembled into benchtop-centrifuge- compatible spin columns, which can be used to isolate EVs in under 30 minutes, improving on lengthy UC protocols. These kits provide a new and reliable method to rapidly enrich EVs from biological fluids for downstream analyses.

Results: Capturem-isolated EVs exhibited a more uniform and smaller particle size distribution, with an average particle size of 81 nm and a D90 value of 110 nm. Conversely, UC-isolated EVs were larger, with an average particle size of 135 nm and a D90 value of 203 nm. Additionally, the particle sizes isolated from UC were more variable than those from Capturem isolation. EVs isolated from plasma using the Capturem kit or UC were labeled with fluorescent dye and subjected to fNTA analysis. The results showed that UC-isolated EVs were highly contaminated with other particles and only 20% exhibited EV-specific labeling. In contrast, Capturem-isolated EVs demonstrated more than 4X this enrichment, with over 84% EV-specific labeling. Thus, Capturem columns consistently provide a pure, intact, and concentrated EV population in less than 30 minutes.

Conclusions: Research and clinical studies could greatly benefit from technology that provides rapid and simple isolation of EVs from various biofluids-especially as it concerns methods that generate pure, concentrated EV samples with enough yield for subsequent proteomic, genomic, and transcriptomic analyses. We have designed the Capturem™ Extracellular Vesicle Isolation Kit to meet these high standards, enabling researchers to consistently obtain pure EVs. These tools have the potential to enable faster drug discovery and better diagnostics for a variety of diseases.

19. Isolation and characterization of extracellular vesicles in saliva of children with asthma

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Abstracts:
Asthma is the most common chronic disorder in children and is a heterogeneous disease, making identification of objective biomarkers of its subtypes and their underlying pathology a research priority. Identifying biomarkers that can profile clinical subtypes early in the course of asthma is critical in applying tailored therapy, yet the clinical utility of current biomarkers is very limited because they are either invasive or non-specific and unable to identify clinical subtypes. Non- invasive methods to study and monitor childhood asthma severity and distinguish pathophysiological subgroups is a critical research gap. Extracellular vesicles (EVs) and their bioactive cargo are attractive candidates for biomarkers of asthma endotypes.
Saliva, regarded as the "mirror of the body", harbors constituents that provide sources for monitoring of health and disease states. Thus, we hypothesize that saliva EVs and their cargo may serve as a novel biomarker for asthma subtypes. Here, we first confirm the presence of EVs in saliva of children with asthma and characterize the EV population isolated from saliva supernatant using a high- throughput EV isolation method that can be scaled to large epidemiological studies of childhood asthma such as the School Inner City Asthma Intervention Study (SICAS-2).
Objective: To characterize the population of EVs isolated from saliva supernatant of children with asthma. Methods: EVs were isolated from saliva supernatant of 209 SICAS-2 study participants using ExoQuick-TC (System Biosciences) following a modified protocol. Pelleted EVs were re-suspended in filtered 1X dPBS. 5 μL of re-suspended EVs were aliquoted from each sample and combined to create a pooled sample for downstream analyses. EVs were visualized by morphological analyses (TEM). Capillary immunoassays confirmed the presence of EV-associated proteins. Microfluidic Resistive Pulse Sensing (MRPS) and Nanoparticle Tracking Analysis (NTA) were performed to determine EV concentration and size distribution. The concentration and size distribution of specific tetraspanin subpopulations (CD9, CD63, CD81) was determined using the ExoView R100 (NanoView Biosciences).
Results: Rounded, membrane-bound structures (25-350 nm) identified by TEM were positive for CD9, CD63, CD81, ANXA5, and ICAM-1 with limited cellular contamination (negative for CANX). MRPS detected a broad distribution of particle sizes with a significant population at 800 nm (concentration of peak population: 5.9×10^6 particles/mL, 0.01× stock). Further analysis of small (< 200 nm) EVs with fluorescent NTA revealed a bi-modal distribution of EVs at 90 nm and 150 nm. 28% of EVs were positive for CD9, 35% were double positive for CD9 and CD63, 25% expressed CD9 and CD81, and 12% expressed all three tetraspanins.
Conclusions: Our findings characterize saliva EVs from children with asthma and show that saliva EVs can be isolated in a high-throughput method, opening a new avenue for asthma epidemiology studies.

20. Immunoregulatory properties of cancer stem cell derived extracellular vesicles

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Abstracts: Head and neck squamous cell carcinoma (HNSCC) is the 6th most common malignancy globally, and the five year survival rate for stage III and IV patients is only 50%. HNSCC patients who relapse have a mean survival rate of less than one year. As such, understanding the mechanism of local recurrences and their immunoevasive properties is imperative to improving disease outcomes for stage III and IV HNSCC patients. Cancer stem cells (CSCs) are a subpopulation of cells within a tumor that possess self-renewing properties that have been linked to tumor initiation, neoplastic maintenance, and recurrence post treatment. Residual CSCs not eliminated by surgical resection, or chemoradiation, are thought to induce recurrence in many patients. A key feature of CSCs is their immunoregulatory properties that allow them to evade immunosurveillance and elimination. Several studies have established that CSCs secrete canonical secretory proteins with immunoregulatory properties, however, it remains unclear what role, if any, extracellular vesicles play in CSCs immunomodulatory properties. Using a co-culture system and multichromatic flow cytometry analysis we have evaluated CSCs and their derived EVs ability to regulate the polarization of the monocyte cell line, THP-1 macrophages towards an M2-like immunosuppressive phenotype. We have also analyzed the expression patterns of inhibitory immune checkpoint receptors and glycan factors in CSCs and CSC-EVs. Our preliminary data suggests that CSCs and CSC-EVs express numerous immune checkpoint proteins and associated glycans such as sialic acid residues. Our data also suggests that CSC-EVs induce M2-like polarization of macrophages.

21. Extracellular vesicles in diabetes mellitus carry inflammatory cargo that affects cellular behavior

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Abstracts: Type 2 diabetes mellitus is a global health problem evidenced by its rising prevalence and incidence worldwide. This chronic metabolic disease causes multiple end organ complications including inflammation-related atherosclerotic peripheral vascular disease, which results in devastating morbidity and mortality. Previously, we reported that individuals with diabetes mellitus have higher levels of circulating extracellular vesicles (EVs). EVs from diabetic individuals are more readily internalized by monocytes and induce inflammatory signals in these cells. To further elucidate the molecular cargo that may elicit these responses in cells, we quantified inflammatory protein levels in plasma-derived EVs from

a longitudinal cohort of euglycemic and diabetic individuals. We report many significant associations between EV inflammatory protein levels and diabetes status. Most importantly, we found that vascular endothelial growth factor A (VEGF-A) was significantly associated with diabetes status in our longitudinal cohort. The plasma levels of this angiogenic factor were higher in EVs from individuals with diabetes compared to euglycemic individuals. Furthermore, EV levels of VEGF-A were significantly associated with homeostatic model assessments of insulin resistance (HOMA-IR) and β-cell function (HOMA-B), which are mathematical models that measure diabetes severity. To examine whether EVs with different inflammatory cargo can affect target cell behavior, we performed *in vitro* cell biological assays to assess the functional effects of these EVs on endothelial cells. We found that EVs from diabetic individuals induced actin cytoskeletal rearrangements including cell lamellipodia formation and increased cellular migration when compared to EVs from euglycemic individuals. Here we demonstrate that EV inflammatory protein profiles differ by diabetes status. Hence, our data suggest that EVs may play important roles in the end organ complications of diabetes mellitus, such as peripheral vascular disease.

22. IMP1 modulates extracellular vesicle production in the GI tract through regulation of endosome and autophagy pathways

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Abstracts:

Background: RNA binding proteins regulate protein expression through coordinated binding to a series of related transcripts referred to as an RNA operon. Previous work demonstrates context and tissue specific roles for the RNA binding protein insulin-like growth factor 2 mRNA binding protein 1 (IMP1) in the context of cancer, especially colorectal cancer (CRC). Recent studies have linked IMP1 to extracellular vesicles and demonstrated that Imp1 is a potential regulator of autophagy during intestinal repair in response to damage. The endosomal, extracellular vesicle, and autophagy pathways are interrelated; however, a direct role for IMP1 in coordinating these pathways in the GI tract has not been explored. Hypothesis: IMP1 alters vesicle production and secretion by altering expression of vesicle pathway proteins in colon cancer and the non-transformed intestinal epithelium.

Methods: Ribosomal profiling and RNA sequencing were used to compare transcript and translational efficiency profiles between IMP1 null and IMP1- overexpressing CRC cell lines. Extracellular vesicles were assessed by nanoparticle tracking analysis, western blot, and electron microscopy in CRC cells and non-transformed, mouse intestinal enteroids. Effects of IMP1 on endosome, vesicle, and autophagy pathways were assessed by electron microscopy, immunofluorescence, and western blot in cell lines and enteroids.

Results: A number of extracellular vesicle and exosome-related pathways were the most significant differentially regulated pathways by gene ontogeny analysis of RNA sequencing data in IMP1 null *vs.* IMP1-overpressing CRC cells ($P = 1.78\times10^{-10}$, 2.43×10^{-10}). Over 42% of the changed transcripts are associated with a

vesicle pathway and differentially regulated by IMP1 expression. We found that IMP1 increases extracellular vesicle secretion in HT-29 (4483 +/- 403 *vs.* 1934+/- 414, $P = 0.006$) and SW480 (3604+/- 399 *vs.* 2293+/- 464, $P = 0.049$) CRC cells *vs.* null controls. IMP1 modulates translational efficiency or protein levels of early endosome proteins, including: EEA1, SNX15, PLA2G4B, and F8A2. IMP1 decreases expression of autophagy proteins LAMP1 and LC3-II. By contrast, Imp1 overexpression in non-transformed enteroids does not enhance vesicle secretion, but may alter expression of endocytic proteins.

Conclusions: Our novel findings suggest that IMP1 plays an important role in vesicle production and secretion in the context of colon cancer through increased early endosomal pathway activity and reduced autophagy-mediated degradation. Importantly, the effects of IMP1 expression differ between non-transformed and cancer cells. Our findings have implications for the development of novel early detection and therapeutic approaches in CRC where IMP1 is overexpressed.

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23. Simple workflow for isolation and Western blot detection of MISEV-recommended EV protein-markers

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Abstracts:

Introduction: Characterization of isolated extracellular vesicles (EV) is often challenging, due to the limited amount of material and the variety in approaches for experimental EV preparations. The "Minimal Information for Studies of Extracellular Vesicles" (MISEV) guidelines advice to characterize isolated EVs by their protein composition. Nevertheless, following these recommendations can result in a significant effort due to work-intensive and variable EV isolation procedures and time-consuming standard western blot protocols. Here we present a complete workflow for isolating intact EVs via membrane-affinity columns and detection of MISEV-recommended protein markers using Simple Western Blotting.

Methods: Intact EVs were isolated using a bind-wash elute procedure from 0.25-8 mL pre-filtered plasma or 10 mL of pre-filtered urine (exoEasy Kit, QIAGEN). EVs were eluted in 250 µL elution buffer for analysis and compared to the EV-depleted fraction in the column flow-through and to the neat biofluid sample. Multiple EV and non-EV protein markers were analyzed by subjecting 4 µL sample directly to Simple Western system, which entails automated capillary electrophoresis-based protein separation and chemiluminescence-immunodetection (Jess platform, ProteinSimple, Bio-Techne).

Results: A total of ten primary antibodies were identified and optimized for the workflow, including multiple targets in each of the three categories suggested by the MISEV-guidelines. Input volume titrations were used to define the dynamic range for the complete workflow and replicate isolations were used to demonstrate its repeatability. EV markers are generally low abundant in urine and plasma but were readily detected after enrichment of EVs by isolation. The EV fractions showed a strong enrichment of EV-proteins, whereas non-EV proteins were significantly reduced - and increasing the number of wash steps in the EV isolation lead to a substantially improved signal-to-noise ratio.

Conclusions: We developed a simple, robust and quantitative workflow for isolating and analyzing EV proteins. EVs obtained by membrane-affinity spin columns were enriched in the relevant EV proteins and depleted for non-EV proteins, establishing a method for easy compliance with official MISEV guidelines.

24. Nanomechanical fingerprinting of single extracellular vesicles

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Abstracts: As potential a class of novel diagnostics and therapeutics, the physio-chemical characterization as well as the biomolecular composition of EVs are widely investigated. However, there is emerging evidence suggesting that biomechanical analysis of lipid-bilayer membrane-bound single EVs may provide key insights into their biological structure, biomarker functions, and potential therapeutic functions. Using multi-parametric AFM imaging and force spectroscopy we compared the structure-mechanical properties (including Young's modulus, stiffness, deformability, and adhesion maps) of invasive and noninvasive breast cancer EVs at nanoscale resolution. Our findings reveal that secreted EVs reflect the biomechanical signatures of parent cancer cells that vary in invasive potential. Irrespective of the EV isolation method employed, single EVs derived from non- invasive (biomechanically stiffer) cancer cells were also significantly biomechanically distinct compared to EVs derived from invasive (biomechanically soft) breast cancer cells. In particular, we propose multi-parametric AFM structure- mechanical analysis augmented with machine learning capabilities to further advance label-free, orthogonal biophysical understanding of EVs beyond biomolecular or particle size characterization and analysis, with significant implications for research and clinical use of EVs.

25. Development of non-invasive clinically applicable *in vivo* tracking of extracellular vesicles using magnetic resonance imaging

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Abstracts: As researchers continue to explore the therapeutic potentials of extracellular vesicles (EVs) for the treatment of many diseases, there is a growing unmet need for real-time *in vivo* monitoring of these therapeutic EVs after they are injected into a subject to understand their safety, targeting, and effectiveness. While current optical imaging solutions like bioluminescence and fluorescence are useful for EV tracking studies in animal models, there is limited utility in clinical applications. Here, we present a novel EV labeling technology that enable real time, non-invasive tracking and quantitative assessment of EVs *in vitro* and *in vivo* utilizing magnetic resonance imaging (MRI). Leveraging clinically applicable magnetic agents, mesenchymal stem cells-, neural stem cells-, and amniotic fluid stem cells (AFSCs)- derived EVs were labeled directly or indirectly by labeling the secreting cell first prior to vesicle collection. The magnetic

labeling did not affect the physiological characteristics of the cells, and the MR detectability of labeled-EVs were confirmed by magnetometer and *in vitro* MRI phantoms. To demonstrate the utility of MRI- assisted EV tracking, a proof of concept *in vivo* biodistribution study was conducted by injecting labeled AFSC EVs into WT and Alport mice (a model of chronic kidney disease) via different routes of administration, and tracking them via MRI at 10 min and 3 h post injection. MRI studies showed that homing of AFSC EVs to the kidney injected intra-cardiacally into Alport mice were more efficient versus the retro-orbital route, and Prussian blue staining of kidney sections confirmed the MR findings. In summary, we have developed a clinically applicable novel magnetic nanoparticle agent that can be used to label and track the biodistribution of EVs in living subjects using non- invasive, safe, and effective MRI technology that's widely available. This technology is highly adaptable and can be deployed in both preclinical and clinical settings.

26. Glomerular heterogeneity and modulation of miR-93-5p: the role of extracellular vesicles

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Abstracts: miRNA play important roles in the pathogenesis of renal diseases. Modulation of miRNA in podocytes and glomerular endothelial cells (GEC) has been associated with cellular damage and development of renal diseases. miR-93-5 is a potent regulator of various genes and pathways responsible of glomerular damage during pathological conditions, like VEGF, TGF and Msk2. We have evidence that miR- 93-5p is altered in the glomeruli of mice affected with X-linked Alport syndrome (AS), as well as in human AS glomeruli. In this study, we investigated the role of miR- 93-5p in glomerular cells from healthy and AS mice. We also used extracellular vesicles (EVs) derived from amniotic fluid stem cells (AFSC), which are naturally enriched with mir-93-5p to asses their therapeutic potential to rescue glomerular damage *in vitro* and *in vivo* by regulating miR-93-5p target genes. Mesangial cells, podocytes and GEC were sorted from glomeruli of male and female WT and AS mice at different stages of disease and miR-93-5p expression was evaluated by qRT-PCR. We assessed renal cortices from patients affected by AS and disease-modifying activity of human AFSC EVs by 1) applying EVs to damaged GEC and podocytes *in vitro* followed by analysis of mir-93-5p targets and 2) injecting EVs into AS mice followed by RNAseq analysis of isolated glomeruli and survival. Glomerular miR-93-5p expression differed between male and female mice and in glomerular cells throughout the progression of disease *vs*. WT. In glomerular cells from AS male mice, miR-93-5p levels were significantly lower in GEC, but not in podocytes or mesangial cells, relative to WT. Consistently, decreased miR-93-5p expression was detected in human samples from AS patients. Expression of WT1 and miR-93 in puromycin aminonucleoside damaged podocytes and expression of fibronectin and miR-93 in VEGF damaged GEC was restored to basal level in the presence of hEVs. *In vivo*, single injection of hEVs showed therapeutic effect by ameliorating the level of proteinuria and increasing life span, as shown for mouse EVs (Sedrakyan, 2017). Differential gene expression and pathway enrichment analysis showed stark differences between male and female glomeruli in WT, involving respiratory and metabolic pathways, extracellular matrix and cell adhesion molecules. In AS males, genes with functional role in lipid metabolism and angiogenic pathways were most highly regulated; AS males injected with EVs showed improved gene modulations in metabolic function and genes with functional role in the development of vasculature and angiogenesis were most highly enriched. Gender specific variation in miR-93-5p expression in glomerular cells might indicate important differences in their biology and potential response to injury during development of kidney diseases. EVs from AFSC demonstrate great potential to restore lost miR-93-5p expression in glomeruli of AS and therefore can present powerful therapeutic approach for treatment of CKD.

27. Use of high-capacity membranes for simple, rapid exosome isolations with high yield and purity

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Abstracts:

Introduction: Despite their small size, exosomes play important roles in normal physiological processes (e.g., immune response, neuronal function, and stem cell maintenance) and diseases (e.g., cancer and liver disease). The isolation of the vesicles has historically been accomplished via ultracentrifugation. However, ultracentrifugation is time-consuming, is not scalable, requires specialized equipment, may damage vesicles during the high-speed spins, and suffers from low yield. More recently, precipitation solutions have been utilized to simplify exosome isolation protocols, but precipitation-based techniques are often inconsistent, with low yield and reduced purity. Thus, there is a significant need for a method to isolate exosomes without compromising purity or yield rapidly.

Methods: Here we describe the use of novel membranes conjugated to a proprietary, non- antibody based exosome-binding compound to isolate exosomes selectively. The membranes, which we have named Capturem™ membranes, have been chemically modified to have increased surface area, which allows higher binding capacity while providing highly pure and concentrated samples. Additionally, the membranes have been assembled into benchtop centrifuge-compatible spin columns, which can be used to isolate exosomes from plasma in under 30 minutes.

Results and Conclusions: Isolations performed with the Capturem exosome isolation spin columns produced exosomes of sizes comparable to experimental values reported in the literature; containing the key exosome protein markers CD63, CD9, and Alix; and with little or no expression of the exosome-negative markers Calnexin and Albumin. As a whole, the Capturem columns enable researchers to study exosomes to accelerate the pace of their research by obtaining high yields of noncontaminated exosomes simply and rapidly.

28. Detection of EGFR mutations in extracellular vesicle RNA and protein corresponds to disease status in metastatic lung cancer patients

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Abstracts:

Introduction: Current cancer detection and characterization methods require a lung tissue biopsy, an invasive procedure, performed only when the patient is showing symptoms that often only arise in late-stage cancers. Extracellular vesicles offer a stable, abundant biomarker in the blood to serially profile molecular characteristics of patient tumors through a non-invasive liquid biopsy. In non-small cell lung cancer (NSCLC), identifying the presence of sensitizing and resistance epidermal growth factor receptor (EGFR) mutations informs sensitivity to tyrosine kinase inhibitors (TKIs) and dictates treatment regimes.

Methods: This pilot study reports the longitudinal study of EGFR mutations carried in extracellular vesicle protein (evProtein) and RNA (evRNA) to monitor metastatic non-small cell lung cancer patients. We enrolled 10 NSCLC patients: 8 with exon 19 deletion mutations and 2 with co-harbored L858R sensitizing and T790M resistance mutations. We used ultracentrifugation to isolate EVs from dilute plasma before testing the EVs for mutations based on the patient's original tumor biopsy. In our work, we used droplet digital PCR (ddPCR) to test for mutations in the EV-RNA and western blots to test for sensitizing mutations in EV-protein.

Results: In our cohort of 10 NSCLC patients, mutant EV-RNA was detected in 9/10 patients. The overall detection rate for all EV-RNA mutations was 60%. Exon 19 del was detected in 7/8 patients, and in 78% of samples tested. While the detection rate of L858R remained moderate at 60%, the detection of the notoriously challenging T790M mutation was low, at 25% Sensitizing mutations were detected in 12/21 (57%) EV-Protein samples. EV-RNA and EV-Protein were then compared to clinical data and showed that increasing EV-RNA mutation burden mirrored disease progression. For 6/7 patients who were longitudinally monitored, EV-RNA mutation burden mirrored clinical trajectory. Increasing exon 19 del exoRNA burden mirrored disease progression in two patients, while decreasing burden mirrored stable disease in three patients. The three patients who saw a decrease in exon 19 del burden have remained clinically stable for an average of 192 ± 9 days after the final blood draw, while those who had progressive disease are now deceased. Of the two patients with L858R/T790M mutations, one patient's EV-RNA burden mirrored disease trajectory, while the other did not. Conversely, EV-Protein did not appear to mirror clinical progression and was largely patient dependent.

Conclusions: In this novel proof of concept study, extracellular vesicles were screened for previously identified EGFR mutations carried by each patient. Changes in EV-RNA were found to correlate with disease trajectory; however, the clinical implications of EV-Protein remain unclear. This study demonstrates the potential utility of characterizing both extracellular vesicle RNA and protein cargo, allowing for multi-faceted analyses of a patient's disease.

29. The role of extracellular vesicles in cancer-related fatigue

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Abstracts:

Purpose/Background: Cancer Related Fatigue (CRF) is the most prevalent and distressing symptom associated with cancer and its treatments. The etiology of CRF is shown to be multifactorial, yet the exact mechanisms of CRF are poorly understood. The purpose of this study was to investigate the role of extracellular vesicles (EVs) in the inflammation mechanism of CRF. EVs are ubiquitously present in the body, exchange material between cells and can serve as biomarkers for diagnosis and treatment. No prior studies have investigated EV markers as a measure of inflammation in CRF.

Methods: Plasma was collected from men with non-metastatic prostate cancer receiving external beam radiation therapy (EBRT) (n = 40) at two time points: at the start of (T1) and 3 months after EBRT (T2). Fatigue was assessed using the Functional Assessment of Cancer Therapy - Fatigue (FACT-F). Characterization of the EVs included: size and concentrations using Nanoparticle Tracking Analysis (NTA), morphology using electron microscopy, and EV markers (CD9, CD81, TSG101) using western blot. Cytokines were measured in EV-associated and soluble (EV free) fractions using a multiplexed immunoassay system.

Findings: Log fold change of EV-associated analytes showed increase in Eotaxin, hsp27, IP10, MIP3α from T1 to T2 in fatigued patients compared to non-fatigued. Whereas, in soluble fraction, only survivin had a

log fold increase at T2 for the fatigued cohort.

Implications: There is an association between EV markers and severity of fatigue in men treated with EBRT for prostate cancer. EV-associated and soluble analytes should be used for early detection of persistent CRF that can continue for months after treatment completion.

30. Mechanistic analysis of protein transport to extracellular membrane vesicles of a hypervesiculating bacterial strain, Shewanella vesiculosa HM13

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Abstracts: Shewanella vesiculosa HM13 is a hypervesiculating Gram-negative bacterium isolated from the intestine of a horse mackerel. The strain produces extracellular membrane vesicles (EMVs) that contain a single major cargo protein, P49, of unknown function. P49 is expected to be useful as a carrier to deliver foreign proteins to EMVs. To develop an EMV-based heterologous protein production system by using S. vesiculosa HM13 as the host, we analyzed the mechanism of transport of P49 to EMVs. Whole genome sequencing of S. vesiculosa HM13 revealed that the P49 gene is located downstream of a gene cluster coding for homologs of components of a type II protein secretion system (T2SS). Disruption of the genes coding for these proteins caused disappearance of P49 from EMVs. Thus, it is very likely that the T2SS-like machinery functions as a protein translocon to transport P49 to EMVs. In the vicinity of the P49 gene in the genome, we also found the genes coding for proteins that are presumably involved in the synthesis of surface glycolipids/polysaccharides. When these genes were disrupted, P49 was secreted to the extracellular space without being loaded to EMVs. The results raised the possibility that P49 is loaded onto EMVs through interaction with surface glycolipids/polysaccharides of EMVs. We further studied the transport mechanism *in vitro* by incubating purified P49 with P49-free EMVs prepared from various gene-disrupted mutants. We found that P49 was loaded onto EMVs prepared from the mutants that lack the T2SS-like machinery, whereas the loading efficiency was markedly decreased for EMVs prepared from the mutants that lack the putative surface glycolipid/polysaccharide biosynthesis genes in the P49 gene cluster. These results suggest that P49 is translocated across the outer membrane through the T2SS-like machinery and loaded onto EMVs through interaction with surface glycolipids/polysaccharides of EMVs.

31. Mycobacterial dynamin like proteins are necessary for extracellular vesicle release in M. tuberculosis

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Abstracts: Mycobacterium tuberculosis (Mtb) secretes pathogenicity factors and immunologically active molecules via extracellular vesicles. However, little is known regarding the mechanisms and molecules involved in mycobacterial vesicle biogenesis. This study investigates molecular determinants of vesicle

production in Mtb by analyzing Mtb cells under conditions of high vesicle production, such as iron restriction and cells lacking VirR. Transcriptomic analysis showed common upregulation of the iniBAC operon in association with high vesicle production in Mtb. Genetic and vesicle production analysis demonstrated that the dynamin-like proteins (DLPs) encoded by this operon, IniA and IniC, are necessary for release of extracellular vesicles by Mtb in culture and in infected macrophages. The first line antibiotic, isoniazid was found to stimulate vesicle release in a DLP-dependent manner. Extracellular vesicles purified from WT Mtb cultures or concentrated from the extracellular medium of infected macrophages stimulated release of pro- inflammatory cytokines in uninfected bone marrow-derived macrophages, whereas an iniAC mutant showed poor immunostimulatory activity. Our results provide a new understanding of the function of mycobacterial DLPs and mechanistic insights into vesicle biogenesis and enable studies that address the role of extracellular vesicles in TB pathogenesis.

32. Intracellular delivery methods using biofunctional peptide-modified extracellular vesicles

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Abstracts: Extracellular vesicles (exosomes, EVs) with encapsulation of biofunctional molecules (e.g., enzymes and genes) are highly expected to be next-generation therapeutic carriers because of their pharmaceutical advantages including effective usage of cell-to-cell communication routes, controlled immunogenicity, absence of cytotoxicity, brain targeting through BBB. However, methods for increasing the cellular EVs uptake efficacy must be developed to achieve effective intracellular delivery of EV contents. In this presentation, I will introduce novel techniques to enhance cellular EV uptake by modification of functional peptides on EV membranes[1-3] especially using an antimicrobial protein, CAP18 derived cell-penetrating peptides[4] for macropinocytosis induction and effective cellular uptake. We previously found that macropinocytosis (accompanied by actin reorganization, ruffling of the plasma membrane, and engulfment of large volumes of extracellular fluid) is important route for enhanced cellular EV uptake[5]. CAP18- derived sC18 peptide has high abilities for cellular uptake, and dimer-type structure of sC18 peptides, (sC18)2, shows further cell membrane penetration[4]. In this study, we found that (sC18)2 peptides induced macropinocytosis via glycosaminoglycan on plasma membranes of targeted cells. Furthermore, modification of the sC18 peptides or (sC18)2 peptides with stearyl-moiety on EV membranes significantly enhanced cellular EV uptake via macropinocytosis induction. In addition, ribosome-inactivating protein, saporin-artificially encapsulated EVs with modification of the (sC18)2 peptides showed glycosaminoglycan-dependent cell-killing activity. Our experimental techniques and findings are considered to contribute to the development for EV-based intracellular delivery system via macropinocytosis.

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33. Effects of marijuana on viral transcription in HIV-1 infected cells and resulting extracellular vesicle release

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Abstracts: As of 2016, roughly 18.2 million of the approximately 36.9 million people living with HIV globally were receiving combination antiretroviral therapy (cART). Despite decades of research and development of this complex drug regimen, which is effective in the prevention of new infections, cells with an integrated HIV-1 genome have leaky transcription which can produce viral RNAs and proteins. These viral products can then be packaged into extracellular vesicles (EVs) and released from the infected cell. EVs, specifically exosomes, produced from HIV-1 infected cells contain viral mRNAs and incubation of these exosomes with cells caused a significant increase in the production of the proinflammatory cytokines, implicating EVs as a possible mechanism for the chronic inflammation observed in the CNS of people living with HIV-1 on antiretroviral therapy[1-4]. Previous studies have shown that marijuana use in people living with HIV is associated with a lower viral load and high CD4+ T-cell count, suggesting a potential therapeutic application. Here, we investigated the effects of cannabinoids, CBD and THC, on viral transcription in HIV-1 infected cells and resulting changes in EV release. Our data suggests CBD and THC can act as viral transcription inhibitors, potentially through two independent mechanisms. Here we show that treatment of CBD and THC on virally infected myeloids results in lowered production of intracellular and extracellular viral RNAs, such as short non-coding (TAR) and genomic (env) transcripts, as well as lowered downstream viral proteins, such as capsid protein (Pr55), cleaved capsid protein (p24) and accessory protein (Nef). Additionally, the results show a significant reduction in EVs released from infected cells. These studies are significant in that marijuana may provide a protective effect by alleviating the pathogenic effects of EVs in HIV-1 and CNS-related infections.

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34. Extracellular vesicles release from infected cells prior to virion release

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Abstracts: Recently, it has become evident that Extracellular Vesicles (EVs) play a major role in the viral pathogenesis. Our lab has been able to elucidate the role of EVs in the pathogenesis of several different viruses including HIV-1, HTLV-1, Rift Valley Fever Virus and Ebolavirus[1-7]. However, timing difference between EV and virions release from infected cells has not been previously reported. We have attempted to address the spatiotemporal dynamics of EV and virions release from HIV-1 and HTLV-1 infected cells. The infected cells were synchronized in G0 quiescent stage using serum starvation. Viral latency was reversed by increasing gene expression with the addition of serum rich media and inducers (20% FBS + PMA/PHA). Supernatants and cell pellets were collected post induction at 0, 3, 6, 12, and 24 h and assayed for the presence of markers of EVs, autophagy and for the viral proteins and RNA transcripts. Results from supernatants of uninfected cells showed a peak of tetraspanin proteins (CD63, CD81, and CD9) at 6 h and a gradual decrease of all EV associated proteins by 24 h. However, the EV from HIV-1 infected cells showed all three tetraspanins present at 3 h and expression gradually increased up to 24 h. When compared to HTLV-1 infected cells, the three tetraspanin proteins peaked at 6 h and expression continued to decrease up to 24 h. HTLV-1 infected cells also showed a unique pattern of CD81 expression. Autophagy associated proteins (LC3A, LC3B and p62) from uninfected cells and HTLV-1 infected cells plateaued at 6 h, whereas in HIV-1 infected cells their expression continued to increase and peaked at 24 h. HIV-1 viral proteins (p24, gp120, Nef) expression was present at 6 h and continued to increase and peaked at 24 h. HTLV-1 proteins (p19 and gp46/61) peaked at 6 h and decreased overtime. HIV-1 and HTLV-1 gene expression was quantified, and data correlated with viral protein expression. EV release was analyzed by nanoparticle tracking analysis and significant increase of EV concentration overtime in both uninfected and infected samples was observed. Finally, virus rescue assay with the use of naïve cells was performed on 6- and 24-h supernatants. HIV-1 supernatant from 6-h sample was found not to be infectious, however the virus from 24-h sample was rescued. Our data indicates that EV release may occur prior to virion release from infected cells. The released EVs can be implicated in the facilitation of virus spread and deleterious effect on the naive recipient cells.

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35. Separation of EVs from virions in coronavirus infections

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Abstracts: Since the severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) was declared a pandemic in mid-March of 2020 by the World Health Origination (WHO), laboratories around the world started research into diagnostics, therapeutics, and treatments[1]. In recent years, the importance of extracellular vesicles (EVs) in the pathogenesis of viral infections have been found in the cases of many viral pathogens including few DNA and RNA viruses including human T-cell leukemia virus-1 (HTLV-1) and human immunodeficiency virus-1 (HIV-1)[2,3]. EVs from HIV-1 infected cells on uninfected macrophages induces an increase in the proinflammatory cytokines[3]. While EVs from HTLV-1 infected cells on uninfected recipient cells promoted the localization and cellular contact by cells, this directly influences the pathogenesis of HTLV-1 as the virus mainly infects other cells by cell to cell contact[2]. Similar to retroviruses, coronaviruses are also positive strand RNA viruses, except they replicate in the cytoplasm and may regulate chromosomal DNA depending on the strain of virus. We have recently began working on beta- coronaviruses, including OC43 (BSL2 strain) and SARS-CoV-2 (BSL3 strain). Our initial experiments focus on isolation of EVs away from virions using either an iodixanol gradients or Izon sizing columns. We have successfully separated the two from one another mainly due to their density and potentially size differences. We found that EVs from multiple coronaviruses are not infectious and viral particles treated with UV irradiation are also not infectious. We also have found that coronavirus EVs caused T-cell death, which may corelate with lymphopenia observed in COVID patients. Along these lines coronavirus EVs can activate other viral genes (i.e., HIV-1 or HTLV-1) when these genes are integrated into the genome, further implying that these EVS regulate chromosomal gene expression. Finally, the mechanism(s) of how these EVs may cause such diverse effects on T-cells and other viral gene expression will be discussed.

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36. Reduction of the therapeutic dose of silencing RNA by packaging it in extracellular vesicles via a pre-microRNA backbone

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Abstracts: Current delivery vehicles enable less than 1% of silencing RNA (siRNA) cargoes to escape into the cytoplasm. This necessitates high doses in patients that have demonstrated toxicity and constrained use of siRNA therapeutics to targets expressed in liver. Small extracellular vesicles (sEVs) are naturally released

from virtually all cell types and can traffic RNA between cells, and are touted as a bio- inspired delivery vehicle. However, doses of siRNA used to suppress targets in many published studies using sEVs far exceed those of other delivery vehicles. This suggests that sEVs are quantitatively poor at delivering cargoes into target cells and questions the model in which the principal role of sEVs is intercellular delivery of cargoes. We demonstrate that sEVs naturally contain thirty copies or less of specific miRNA per sEV. Nonetheless, pre-miR-451 derivatives are enriched by 1,000-fold in sEVs produced by many cell types. Reprogramming the unique Dicer- independent pre-miR-451 secondary structure with new siRNA sequences enables robust siRNA enrichment in sEVs and these sEVs reduce siRNA target expression in mouse liver, intestine and kidney podocytes with doses of siRNA that are at least 10-fold lower than lipid nanoparticles. The capacity of sEVs to deliver siRNA is abrogated when their membranes are disrupted by electroporation. This demonstrates that intact sEVs can be highly efficient at RNA delivery and provides an RNA stem-loop to harness this by enabling robust, scalable packaging of siRNA into sEVs.

37. Cell type-specific and disease-associated protein networks in extracellular vesicles isolated from human iPSC-derived neural cells and Alzheimer's disease brain tissues

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Abstracts: Extracellular vesicles (EVs) have gathered great interest in studying neurodegenerative diseases with the capability of transferring pathogenic molecules and the source of liquid biopsies. Here we performed combined label-free and tandem mass tag-labeling based quantitative mass-spectrometry of EVs isolated from human induced pluripotent stem cells (hiPSCs) and Alzheimer's disease (AD) brain tissues to conduct a comprehensive EV proteomics study on AD. Cell type-specific EV protein signatures were identified from hiPSC-derived excitatory neurons, astrocytes, microglia-like cells and oligodendrocytes. Furthermore, a whole protein co-expression network analysis identified a module most significantly associated with AD pathology and cognitive function and enriched in astrocytic markers, particularly reactive astrocytes. Proteins within this module were regulated by pro-inflammatory molecules for the inflammatory processes. Our study presents unique human neural cell type-specific EV markers and their application for liquid biopsy-based EV AD biomarkers and disease monitoring.

38. Exosomes mediate Zika virus transmission through SMPD3 neutral Sphingomyelinase in cortical neurons

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Abstracts: The transmission dynamics of ZIKA virus (ZIKV) in or between neurons, or within the developing brains of the fetuses are not fully understood. Using primary cultures of murine cortical neurons, we show that ZIKV uses exosomes as mediators of viral transmission between neurons. Increased production of exosomes from neuronal cells was noted upon ZIKV infection. Neuronal exosomes contained both ZIKV viral RNA and protein(s) that were highly infectious to naïve cells. RNaseA and neutralizing antibodies treatment studies suggested presence of viral RNA/proteins inside exosomes. Exosomes derived from time- and dose-dependent incubations showed increasing viral loads suggesting higher packaging and delivery of ZIKV RNA and proteins. Furthermore, we noted that ZIKV induced both activity and gene expression levels of neutral Sphingomyelinase (nSMase)-2/SMPD3, an important molecule that regulates production and release of exosomes. Silencing of SMPD3 in neurons resulted in reduced viral burden and transmission through exosomes. Treatment with SMPD3 specific inhibitor GW4869, significantly reduced ZIKV loads in both cortical neurons and in exosomes derived form these neuronal cells. Taken together, our results suggests that ZIKV modulates SMPD3 activity in cortical neurons for its infection and transmission through exosomes perhaps leading to severe neuronal death that may result in neurological manifestations such as microcephaly in the neonatal developing embryonic brains and other complications associated with Guillain-Barré syndrome in adults.

39. Understanding Intracellular Fate of EV-delivered Content

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Abstracts: Despite much work performed on evaluating the potential effects of extracellular vesicles (EVs), the functional uptake of their cargo is still controversial. This project aimed to demonstrate that EV content (protein and mRNA) is protected and can be subsequently transferred with functional activity into recipient cells, while also developing a tool to assess and quantify functional EV uptake.
Methods: Fusion proteins used were mitochondrial localized coxVIII-CFP-nanoluc(Cox) and nuclear localized H2B-RFP-nanoluc(H2B).
Results: HEK293T cell-derived EVs protected Cox proteins from proteinase K digestion while demonstrating significantly improved efficiency of uptake when compared to free protein, as measured by bioluminescence that was still detectable in recipient cells 96 hrs post EV-exposure. To confirm functional uptake, recipient cells exposed to EVs containing H2B for 72 hrs were imaged and some recipient cells manifested fluorescent red nuclei. To demonstrate the presence of functional mRNA within EVs, producer cells were transfected for such a duration as not to have detectable levels of protein in the EVs while still containing detectable levels of mRNA (qPCR) even after RNaseA treatment. Transfer of these EVs to HeLa cells showed an increase in expression of H2B which was blocked by cyclohexamide, confirming translation of the mRNA (2.2 kb). To determine if recycling of EV delivered proteins occurs, recipient HeLa cells were exposed to EVs containing Cox for 72 hrs. All extracellular EVs were removed and cells were trypsinized (0.25% for 30 min) to remove any non-internalized Cox protein. 48 hrs later, EVs (CD63+ and CD9+)

released from cells contained Cox suggesting recycling of protein or possibly recycling of entire EVs. Lastly, an assay was developed to measure functional EV uptake. Nanoluc protein was split in two and fused to mTurquoise2(N65) or mScarlet-I(66C). Expression of each fragment alone exhibited non-detectable levels of luminescence while expressing both together had a significantly increased signal. Delivery of either fragment within an EV to a cell expressing the corresponding fragment worked as confirmation and quantification of EV uptake (HEK293, U87, HeLa cells).

Summary/Discussion: This study robustly demonstrates EV delivery of functional mRNA and protein to cells, while also establishing a simple assay to quantify and validate functional EV uptake.

40. Smart Microprobes Imbued with Recognition Element as a Sensitive Bioanalysis Platform for Exosomes

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Abstracts: Circulating exosomes have become useful biomarkers for precise and noninvasive diagnosis and disease monitoring. However, sample purity is a drawback for current liquid-phase methods for exosome isolation. We report a selective solid-phase technology for isolation of pure exosome populations. Microneedles (300 μm × 30 mm) were functionalized with exosome-specific anti-CD63 antibodies and their capture efficiency assessed via Fluorocet assay post-incubation in astrocyte-derived exosome suspension (EXO) enriched by a standard kit, as well as direct incubation in conditioned astrocyte medium (CAM), while blocking non-specific binding with 0.1% BSA in PBS. Our results indicated a 6-fold increase in exosomes captured by microprobes incubated overnight on ice in EXO (23 × 106 exosomes/probe) vis-à-vis 2 h incubation (3.0 × 106 exosomes/probe), and 2 folds more than the overnight probes at room temperature (12.9 × 106 exosomes/probe). The microprobe's exosome loading capacity decreased when incubated in conditioned astrocyte medium, indicating that longer incubation at lower temperatures in enriched exosome suspension favors more efficient exosome capture. Our designed probe was also amenable to exosomal protein and miRNA extraction, in amounts sufficient for downstream analyses. Future works will focus on its integration into a lab-on-a-chip platform.

41. Extracellular vesicles for drug delivery to the brain

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Abstracts:

Extracellular vesicles have advantageous properties for drug delivery applications, including non-immunogenicity and homing capacity. Important parameters for efficient drug delivery to the brain using (natural) nanocarriers are efficient crossing of the blood-brain barrier (BBB) and spatiotemporal control of cargo release.

Here we show that EVs derived from neural stem cells (NSCs) are taken up by brain microvascular endothelial cells following binding to heparan sulfate proteoglycans, and efficiently cross the BBB[1].

In addition, we developed an analytical methodology, combining state-of-the-art molecular tools and correlative light and electron microscopy to demonstrate that EV cargo release occurs from endosomes/lysosomes[2].

Finally, we have explored the potential of neural stem cell derived EVs enriched with DNAJB6 as a therapeutic intervention for Huntington's disease (HD). HD is a neurodegenerative disorder characterized by aggregation of the huntingtin (HTT) protein containing expanded polyglutamine (polyQ) tracts. DNAJB6, a DNAJ chaperone, has been reported to efficiently inhibit polyQ aggregation *in vitro* in cell models, and *in vivo* in HD animal models[3]. Administration of DNAJB6-containing EVs to cells expressing expanded polyQ tracts suppressed HTT aggregation. Furthermore, intrathecal injection of DNAJB6-enriched EVs into R6/2 transgenic HD mice significantly reduced mutant HTT aggregation in the brain[4]. Taken together, our data suggest that EV-mediated molecular chaperone delivery may be an effective way to reduce polyQ aggregation and potentially treat polyQ diseases, including HD.

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42. The uptake, trafficking, and biodistribution of Bacteroides thetaiotaomicron (Bt) generated outer membrane vesicles

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Abstracts: The human gastrointestinal tract is home to hundreds of trillions of microorganisms (the microbiome) that perform a vital role in food digestion and providing essential nutrients and vitamins. They also play an important role in metabolizing medicines and drugs, and in resisting infection by pathogens. Gut microbes are however susceptible to change with alterations in their makeup and activity occurring as a result of exposure to various environmental factors such as diet, drugs, pathogens and behaviour. Such changes have been associated with more than 90% of human diseases affecting the gut,

liver, joints, heart and brain. A central question to discriminating between association and causality is, how do gut microbes communicate with their host to affect physiological changes at the cellular and organ level within the gut and beyond? We have uncovered roles for microbiota-derived metabolites and highly stable, nanosized microvesicles (bacterial extracellular vesicles; BEVs) naturally produced in the gut by prominent members of the intestinal microbiota in cross-kingdom communication. We have shown that specific bacteria and their metabolites can affect various sensory cells of the immune, endocrine and nervous systems within the intestinal mucosa and that BEVs can bring about changes in host cell physiology by delivering various cargo including metabolic enzymes and mediators of intracellular signaling. Via their ability to cross the intestinal and respiratory epithelium and access the lymphatic and vascular system they can activate innate and adaptive immune cells locally and throughout the body to promote local and systemic regulatory immune responses. Furthermore, we have exploited this BEV-mediated cross-kingdom communication pathway to develop a biologics delivery technology platform using BEVs to deliver therapeutic proteins and vaccine antigens directly to mucosal tissues. Pre-clinical studies highlight the utility of this technology in both boosting natural immunity and in preventing and treating infection and autoimmune mediated pathologies that affect the gut and lungs, and other tissues.

43. Infectious Exosomes/Microvesicles in Degenerative and Neoplastic Stem Cell Pathologies

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Abstracts: The role of exosomes/microvesicles ("EMVs") in cell-cell communication is important in understanding infectious-like transmission of disease in everything from COVID-19 to Parkinson's and cancer. Our previous studies have linked infectious disease to neurodegenerative diseases and cancer[1], where stem cells have been shown to have a vulnerability to EMV conveyance of disease-associated nucleic acids and proteins[2,3]. We also showed[4] that lectins, toxins and viruses have the ability to bind to particular glycoconjugates on the surfaces of stem and differentiated neural cells, and transcellularly transport to distant central nervous system ("CNS") sites. This hijacking of the neural connectome leads to stem cell pathologies where not enough differentiated progeny are generated and potentially contribute to neurodegenerative disease, or, on the contrary, lead to too many progeny being generated as in the case of glioblastoma[5,6], where the transcellular transport of such potentially infectious EMV molecular cargoes may underlie gliomagenesis and spread[7]. We have been studying such an infectious nature of Parkinson's disease by way of characterizing stem cell EMVs from normal, idiopathic, gene-identified (e.g., LRRK2 G2019S mutant) and gene-corrected Parkinson's Disease patients using Nanosight technology, immunocytochemistry and gene expression profiling to identify at-risk networks involved in the initiation and propagation of disease. We have identified EMV-associated genes including SOD1, SOD2, HIF1a, APP, JAK2 and GSK3B involved in both stem cell behavior and neurodegeneration, and furthermore showed that LRRK2 gene correction altered the molecular profile of EMVs to a near normal expression pattern as seen in control iPSC-derived dopamine neuron EMVs. Knowing the cell and molecular bases for route of entry and system to system transmission of pathogenic elements will help us to better design precision therapies that can target particular virus-cell, cell-cell and multisystem interactions underlying standard brain versus disease-associated neural functions. For example, the highly pathogenic H5N1 virus has been shown to enter the CNS[8], potentially from peripheral, e.g., vagus nerve innervation of primary

tissue infection sites including the lungs and GI system, and can lead to a CNS Parkinsonian phenotype. With evidence growing for neurotropism of the SARS-CoV-2 coronavirus and potentially for many other emerging viruses, it is important to determine the precise mechanisms by which infectious agents and EMVs may contribute to latent disease, including neurodegenerative diseases and cancers that may arise later in life[9].

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44. Tracking exosomes *in vitro* and *in vivo*

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Abstracts: Exosomes are a subclass of Extracellular Vesicles able to elicit a phenotypic response in target cells, and correspond to the intraluminal vesicles (ILVs) of multivesicular endosomes (MVEs) formed during endosome maturation. Before these MVEs fuse with the plasma membrane - the final step of exosome biogenesis - they undergo dynamic interactions with other endosome-subtypes and organelles that will impact the dynamics of exosome secretion. After secretion, they face the complex 3D environment of the body, before being taken up by target cells. Due to a lack of appropriate model systems, it remains challenging to understand how factors impact their physiology *in vivo*. We developed *in vitro* and *in vivo*

model systems to study exosome dynamics and function by expressing CD63-pHluorin in cells and zebrafish embryos[1-3]. This work represents a comprehensive and complimentary approach to study endogenous EV regulation and function *in vitro* and *in vivo* at high spatiotemporal accuracy.

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45. A CRISPR-Cas9-based reporter system for single-cell detection of extracellular vesicle-mediated functional transfer of RNA

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Abstracts: Extracellular vesicles (EVs) play an important role in communication between cells through functional transfer of bioactive cargo, including RNA. Despite increasing interest in EV-mediated RNA transfer, in-depth knowledge on mechanisms underlying EV-mediated RNA delivery and processing is limited due to a lack of suitable readout systems. Here, we report a novel CRISPR/Cas9-based reporter system that allows for studying EV-mediated RNA transfer at a single-cell level. After validation of this system by studying the role of known targets involved in EV uptake and intracellular membrane trafficking, we have employed this system to uncover various novel genes that play a regulatory role in functional RNA transfer. Thus, this novel system may be used for the study of specific genetic targets and pathways underlying EV-mediated functional RNA delivery.

46. Exploitation of the Leishmania Exosomal Pathway by Leishmania RNA virus 1

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Abstracts:

Leishmaniasis, a complex pattern of diseases caused by sand fly-transmitted Leishmania sp. causes over 2 million new infections and 30,000 deaths each year. In mammals, Leishmania parasites establish a persistent infection by inducing MØ dysfunction through direct manipulation of MØ signaling. We have deciphered the mechanisms whereby Leishmania exploits MØ signaling pathways to block microbicidal functions and innate inflammatory responses during infection. Work from my lab discovered that Leishmania major GP63 was enriched in Leishmania exosomes and to play a pivotal role in those deactivation process of MØ responses.

We reported that Leishmania exosomes are released in the gut of its sand fly vector and co-inoculated with Leishmania promastigotes during blood meals. Co-egested Leishmania exosomes were found to exacerbate cutaneous leishmaniasis skin lesions by overproducing inflammatory cytokines fueling Th17 immune response.

Recently, Leishmania RNA virus 1 (LRV1) infecting certain Leishmania species was found to be associated with aggressive mucocutaneous disease triggered in response to this dsRNA virus. However, it was unclear how LRV1 is exposed to the mammalian host cells. In higher eukaryotes, some viruses are known to utilize the host exosome pathway for their formation and cell-to-cell spread. As a result, exosomes derived from infected cells contain viral material or particles. Recently, we found that LRV1 exploits the Leishmania exosome pathway to reach the extracellular environment. Biochemical and electron microscopy analyses of exosomes derived from LRV1-infected Leishmania revealed that most dsRNA LRV1 co-fractionated with exosomes, and that a portion of viral particles was surrounded by these vesicles. Transfer assays of LRV1-containing exosome preparations showed that a significant number of parasites were rapidly and transiently infected by LRV1. Remarkably, these freshly infected parasites generated more severe lesions in mice than non-infected ones. Moreover, mice co-infected with parasites and LRV1-containing exosomes also developed a more severe disease. Overall, jpeg, with resolution of 300-this work provided evidence that Leishmania exosom as viral envelopes, thereby facilitating LRV1 transmission and increasing infectivity in the mammalian host.

47. Delivery of pre-miRNA Cargo to Tumor Microvesicles

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Abstracts: The regulated shedding of extracellular vesicles (EVs) is now understood to serve as an important means of intercellular communication. Tumor-derived microvesicles (TMVs) comprise a class of extracellular vesicles released from tumor cells that are now understood to facilitate communication between the tumor and the surrounding microenvironment. Despite their significance, the regulation and mechanisms governing the trafficking of bioactive cargos, including microRNAs (miRNAs), to TMVs at the cell surface remain poorly defined. While miRNA recruitment ito exosomes has been reported, including the sequence specific mechanisms for targeting miRNA, current understanding of miRNA loading into TMVs has only recently begun to be elucidated. We have described a molecular pathway for the delivery of miRNA cargo to nascent TMVs involving the dissociation of a pre-miRNA/Exportin-5 complex from Ran-GTP following nuclear export and its subsequent transfer to a cytoplasmic shuttle comprised of ARF6-GTP and GRP1. As such, ARF6 activation increases the pre-miRNA cargo contained within TMVs via a process that requires casein kinase 2-mediated phosphorylation of Ran-GAP1. Further, TMVs were found to contain pre-miRNA processing machinery including Dicer and Argonaute 2, which allow for cell-free

pre-miRNA processing within shed vesicles. These findings offer cellular targets to block the loading and processing of pre-miRNAs within TMVs. As with the exosomes, it is possible that as we continue to clarify the heterogeneity of microvesicle populations we will similarly decode additional mechanisms of miRNA sorting to microvesicles.

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48. Head and Neck Cancer Exosomes Drive MicroRNA-mediated Reprogramming of Local Neurons

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Abstracts: Solid tumors are complex collections of cells surrounded by benign tissues that both influence and are influenced by the tumor. These surrounding cells include vasculature, immune cells, neurons, and other cell types that are collectively known as the tumor microenvironment. Tumors manipulate their microenvironment for the benefit of the tumor. Autonomic neurons innervate and drive malignant growth in a variety of solid tumors. However, the mechanisms by which these neuron-tumor relationships are formed have not been well understood. Recently, Amit et al. described that trophic relationships between oral cavity squamous cell carcinomas (OCSCCs) and nearby autonomic neurons arise through direct signaling between tumors and local neurons. An inducible tumor model in which 4NQO was introduced into the drinking water of *Trp53* knockout mice was used to model OCSCC-microenvironment interactions. Using this model, this group discovered that loss of p53 expression in OCSCC tumors resulted in increased nerve density within these tumors. This neuritogenesis was controlled by tumor-derived microRNA-laden extracellular vesicles (EVs). Specifically, EV-delivered miR-34a inhibited neuritogenesis, whereas miR-21 and miR-324 increased neuritogenesis. The neurons innervating p53-deficient OCSCC tumors were predominantly adrenergic and arose through the transdifferentiation of trigeminal sensory nerve fibers to adrenergic nerve fibers. This transdifferentiation corresponded with increased expression of neuron-reprogramming transcription factors, including POU5F1, KLF4, and ASCL1, which were overexpressed in the p53-deficient samples, and are proposed targets of miR-34a-mediated regulation. Human OCSCC samples enriched in adrenergic neuron markers are associated strongly with poor outcomes, thus demonstrating the relevance of these findings in cancer patients.

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Extracellular Vesicles and Circulating Nucleic Acids

Commentary

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Head and neck cancer exosomes drive microRNA-mediated reprogramming of local neurons

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Abstract

Solid tumors are complex collections of cells surrounded by benign tissues that influence and are influenced by the tumor. These surrounding cells include vasculature, immune cells, neurons, and other cell types, and are collectively known as the tumor microenvironment. Tumors manipulate their microenvironment for the benefit of the tumor. Autonomic neurons innervate and drive malignant growth in a variety of solid tumors. However, the mechanisms underlying neuron-tumor relationships are not well understood. Recently, Amit *et al.* described that trophic relationships between oral cavity squamous cell carcinomas (OCSCCs) and nearby autonomic neurons arise through direct signaling between tumors and local neurons. An inducible tumor model in which 4NQO was introduced into the drinking water of *Trp53* knockout mice was used to model OCSCC-microenvironment interactions. Using this model, this group discovered that loss of p53 expression in OCSCC tumors resulted in increased nerve density within these tumors. This neuritogenesis was controlled by tumor-derived microRNA-laden extracellular vesicles (EVs). Specifically, EV-delivered miR-34a inhibited neuritogenesis, whereas EV-delivered miR-21 and miR-324 increased neuritogenesis. The neurons innervating p53-deficient OCSCC tumors were predominantly adrenergic and arose through the transdifferentiation of trigeminal sensory nerve fibers to adrenergic nerve fibers. This transdifferentiation corresponded with increased expression of neuron-reprogramming transcription factors, including POU5F1, KLF4, and ASCL1, which were overexpressed in the p53-deficient samples, and are proposed targets of miR-34a-mediated regulation. Human OCSCC samples enriched in adrenergic neuron markers are associated strongly with poor outcomes, thus demonstrating the relevance of these findings to cancer patients.

Keywords: MicroRNA, microenvironment, adrenergic neurons, solid tumors, neurotrophic growth, neuron-tumor crosstalk

Cancers are predominantly characterized by their loss of proliferative control. Genetic changes drive this increased proliferation, resulting in the formation, growth and spread of tumor cells throughout the body. Mutations that commonly drive tumor growth, however, also drive molecular changes within the tumor and the surrounding tissues. Encircling the solid tumors are collections of healthy cells that typically act to support cell types and tissue functions in the local region. These cells include structural fibroblasts, immune cells, neurons, blood vessels, and other cell types that in combination are known as the tumor microenvironment. In the context of cancer, these supporting cells can be manipulated to support the growth and spread of the tumor. These manipulative relationships become essential for the survival of the cancer and are found in prostate[1,2], gastric[3-5], pancreatic[6], skin[7,8], glioma[9-11], and a variety of other tumor types[12–15]. Subsequently, efforts to interrupt the relationships between solid tumors and immune cells[16], as well as between tumors and blood vessels[17] have shown efficacy in stymying tumor growth, demonstrating the widespread therapeutic potential borne from understanding these tumor-microenvironment relationships. To date, the relationships between tumors and other members of the tumor microenvironment are not well understood. Additionally, the mechanisms by which these relationships are formed and sustained are not well documented. Recent work from Amit *et al.*[18] has uncovered that tumors use extracellular vesicular signaling to drive nearby neuron survival, growth, spread, and subtype switching, which, in turn, drives tumor growth.

This recent work focused on oral cavity squamous cell carcinoma (OCSCC), an aggressive tumor arising from mouth epithelial cells. To study these tumors in greater depth, the group leveraged laboratory mouse models of OCSCC. These include mice in which the tumor suppressor p53 was conditionally knocked out of epithelial cells ($Krt5^{cre}$; $Tp53^{flox/flox}$). The control group for these mice lacked the $Krt5^{cre}$ allele, leaving *Tp53* intact in all cells. In these models, tumor initiation was induced via introduction of the carcinogen, 4-nitroquinoline 1-oxide (4NQO) into the drinking water. Additionally, the group used patient-derived xenograft models in which $p53^{WT}$ or $p53^{null}$ OCSCC cells were injected into the tongues of mice and allowed to grow. Finally, in cell culture dishes, dorsal root ganglia (DRG) were co-cultured with oral keratinocytes and $p53^{WT}$ or $p53^{null}$ OCSCC cells to model neural interactions with OCSCC tumors *in vitro*.

Initially, the group found that in tumor samples derived from both their conditional *p53* knockout mice, as well as their patient-derived xenograft models, loss of *p53* coincided with increased adrenergic nerve density within the tumor. These findings were recapitulated in human OSCSS samples, indicating that tumor cells lacking *p53* were driving increased adrenergic neuritogenesis. When testing this same hypothesis using *in vitro* OCSCC-DRG co-cultures, Amit *et al.*[18] concordantly found increased DRG innervation of $p53^{null}$ OCSCC cells. When $p53^{WT}$ OCSCC-DRG co-cultures were incubated with conditioned medium containing extracellular vesicles (EVs) from $p53^{null}$ OCSCC cells, the same neuritogenic effect was observed. Additionally, when the $p53^{null}$ OCSCC cells co-cultured with DRGs were inhibited from releasing EVs, the effect was lost, thus demonstrating that the $p53^{null}$-derived EVs and their contents were the driving force behind the described neuritogenic effect.

Extracellular vesicles serve as important vehicles of small regulatory RNA species, which are known to be essential for proper neuronal development and function. By comparing the small RNAs found within EVs derived from $p53^{WT}$ and $p53^{null}$ cell lines, Amit *et al.*[18] found that 17 microRNAs (miRNAs) were down-regulated in $p53^{null}$ cells. After narrowing this collection of miRNAs to the most down-regulated species, they found that several of these miRNAs, including miR-34a, were also down-regulated in the $p53^{null}$

tumors found within *in vivo* mouse models. Thus, these data suggest that miR-34a, and other similarly acting miRNAs, prevent neuritogenesis and that, when lost in $p53^{null}$ EVs, the surrounding neurons exhibit increased neuritogenesis. In concert with this hypothesis, the group next inhibited miR-34a by transfecting cultured neurons with an antagomiR that specifically inhibits miR-34a. This experiment resulted in increased neuritogenesis in the antagomiR-34a transfected neurons when compared to neurons transfected with scrambled antagomiRs, thus confirming the hypothesis that miR-34a from $p53^{WT}$-derived EVs acts to prevent neuritogenesis.

To complement these findings, they next identified candidate miRNAs that drive neuritogenesis. Towards this effort, they uncovered that antagomiR-mediated inhibition of miR-21 and miR-324 resulted in decreased neuritogenesis in transfected neurons when compared to those transfected with scramble antagomiRs. Conversely, transfection of neurons directly with a combination of miR-21 and miR-324 resulted in a robust increase in neuritogenesis when compared to neurons transfected with scramble miRNA molecules. Moreover, adding miR-34a to this combination of miR-21 and miR-324 decreased neuritogenesis, demonstrating that cancer-driven neuritogenic processes lie in a delicate balance, dictated, at least in part, by these miRNAs.

The researchers noticed that these newly formed neurites stained positively for the adrenergic marker tyrosine hydroxylase (TH), demonstrating thereby that the responding neurons were adrenergic in nature. However, upon exposure to EVs derived from $p53^{null}$ OCSCC tumors, they found that the number of adrenergic neurons increased dramatically in both the *in vitro* and *in vivo* models, suggesting that $p53^{null}$-derived EVs were promoting an adrenergic state. Conversely, exposure to $p53^{WT}$-derived EVs decreased the number of intratumoral TH-positive adrenergic neurites, suggesting that these wildtype EVs inhibit cancer-associated adrenergic neuritogenesis. At this point, the researchers were uncertain about the origin of these adrenergic neurites. Amit *et al*.[18] wanted to know whether these adrenergic neurites arose from previously existing adrenergic neurons or whether these newly-formed adrenergic neurites arose from another neuron type. They subsequently found that transfection of trigeminal sensory neuronal cultures with miR-21 and miR-324 resulted in increased adrenergic staining, suggestive of neurotype switching from a sensory to a sympathetic nature. However, when miR-34a was added to the combination of miR-21 and miR-324, the effect was lost, demonstrating that along with inhibiting neuritogenesis, miR-34a activity also inhibits neo-adrenergic neurotype switching. These findings were subsequently bolstered by transcriptomic analysis, which demonstrated that neurons incubated with EVs derived from $p53^{null}$ OCSCC tumors expressed increased levels of catecholamine biosynthesis-related genes and decreased levels of sensory neuron signaling genes. Specifically, the transcription factors POU5F1 and KLF4 were found to be upregulated in trigeminal neurons following incubation with EVs derived from $p53^{null}$ OCSCC tumors. These transcription factors are sufficient to drive neuronal differentiation of adult neural stem cells. Additionally, these two factors are directly regulated by miR-34a activity. NEUROG2 and ASCL1 are two additional factors that were upregulated in trigeminal neurons following incubation with $p53^{null}$ OCSCC EVs. These factors are also candidate targets of miR-34a regulation, and their activity drives neuronal differentiation, specifically to an adrenergic fate. These expression changes illustrate a neurotype switching event in sensory neurons adjacent to $p53^{null}$ OCSCC tumors. Moreover, the combination of the findings described thus far suggest that depletion of miR-34a in EVs released by $p53^{null}$ OCSCC tumors is the mechanism by which these tumors drive increased neo-adrenergic innervation of the tumor body.

Knowing that the tumor-innervating neurons that drive neurotropic tumor growth are adrenergic in nature opens up avenues for the use of readily available therapies to treat patients with OCSCCs. Beta adrenergic receptor blocking medications, for example, are already approved for the treatment of hypertension, heart arrhythmias, angina, migraines and other illnesses, and are widely available[19]. Additional data from published clinical trials support the use of anti-adrenergic approaches to treating several types of cancers,

including breast cancer and hepatocellular carcinoma, among others[20-25]. Additionally, several ongoing clinical trials are testing anti-adrenergic medications as an adjuvant or stand-alone therapy in prostate and pancreatic cancers (NCT03152786, NCT02944201, NCT03838029, NCT04245644). Within this study, Amit *et al.*[18] found that Carvedilol, which non-selectively blocks α1, β1, and β2 adrenergic receptors, dramatically decreased tumor growth and proliferation rates when compared to tumors within vehicle-treated mice harboring patient-derived xenografted $p53^{null}$ OCSCC tumors. Supporting the use of anti-adrenergic therapies in humans, this group found that the level of TH staining in human OCSCC samples was an independent predictor of increased tumor recurrence and decreased patient survival. Thus, this work argues for an increased focus on anti-adrenergic approaches for treating OCSCC as well as other cancers.

Similar to the findings by Amit *et al*[18], Magnon *et al.*[1] found an increased density of TH-positive adrenergic neurons within the tumor microenvironments of patients with high-risk prostate adenocarcinomas[1]. This group showed that the number of adrenergic neurons within the tumor microenvironment was an independent predictor of tumor recurrence. They also found a similar relationship between tumor aggression and the amount of parasympathetic vesicular acetylcholine transporter (VAChT)-positive staining within the tumor body. However, they were not able to determine whether this increase in autonomic innervation was due to neurogenesis, neuritogenesis, neuron-type switching, or some other mechanism. Additionally, they were not able to determine if the tumor was driving this increase in autonomic neurite number, and by what means these aggressive tumors were communicating with local neurons. In contrast, the findings presented by Amit *et al.*[18] shed light on possible mechanisms of communication between prostate tumors and local innervating autonomic neurons. These experiments add to the already rich literature describing the role that EVs play in regulating the tumor microenvironment and cancer metastasis. Future work in prostate adenocarcinoma should examine the EVs released by these tumors, and the miRNA contents that may be manipulating local neurons. A recent report systematically demonstrated the importance of EV contents in the development and growth of a variety of cancers[26]. Though small RNA messengers and regulators were found to be important in mediating tumor growth in this study as well, this group provided ample evidence supporting damage-associated molecular patterns (DAMPs) and other cancer-associated proteins as essential mediators of neuron-tumor trophic interactions. These findings point to the possibility that protein messengers are important in establishing and maintaining the trophic relationships between prostate adenocarcinomas and local autonomic fibers, as well as $p53^{null}$ OCSCC tumors and local sensory/adrenergic neurons.

Though the mechanisms governing neuron-tumor relationships described by Amit *et al.*[18] were worked out between OCSCC tumors and sensory/adrenergic neurons of the oral cavity, many of these principles might be generalizable to other tumor types and neuronal subtypes. Already, increased neuritogenesis and sensory-autonomic neurotype switching has been reported in pancreatic nerves in the context of pancreatic cancer[27]. However, the findings presented here also differ from those published in previous work. In contrast to the findings of Magnon *et al.*[1], Amit *et al.*[18] found no increase in parasympathetic VAChT-positive neurites in $p53^{null}$ OCSCC tumors. Thus, it is possible that for each tumor type and each region of local autonomic fibers, a balance of sympathetic and parasympathetic neurons guides tumor growth and survival. Future work will need to examine these balancing forces within each tumor type and the corresponding microenvironment.

This work describes novel mechanisms of EV-mediated regulation of tumor activity, which highlights new hypotheses regarding the biology underlying these mechanisms. Differential delivery of miR-34a *vs*. miR-21 and miR-324 to local neurons could be the result of differential loading of miRNAs into EVs. Alternatively, the differing contents of these cancer-derived EVs could be dictated strictly by expression changes within $p53^{null}$ and $p53^{WT}$ cancer cells. Specific analysis of transcriptional changes in $p53^{null}$ and $p53^{WT}$ cancer cells, combined with detailed profiling of EV contents will allow for a deeper understanding of the mechanisms governing this described specificity of EV contents. In tandem with this, specific subpopulations of $p53^{null}$

and $p53^{WT}$ tumor cells likely contribute more to the specific release of different EV-delivered miRNAs than other subpopulations of $p53^{null}$ and $p53^{WT}$ tumor cells. Cellular heterogeneity within tumors is a well-described aspect of tumor biology that affects tumor activity, and the different mutations and transcriptional profiles found within different subpopulations may be an important driver of differential miRNA expression and release through EVs. Modern sequencing techniques, including single-cell RNA sequencing of tumor samples will be valuable in unraveling the link between specific transcriptional profiles and EV-mediated miRNA delivery. Additionally, this work argues for the increased use of EV sampling in the diagnosis, surveillance and treatment of various cancer types. Sampling EV contents from blood, plasma, and other bodily fluids is a non-invasive and cost-effective method of providing researchers and care teams with myriad signals and transporters shared between the tumor and the microenvironment[28–31]. Moreover, while these contents serve as potent biomarkers of cancer presence, diagnosis, and progression, they also provide clues as to which treatment approaches may be most effective in treating specific patients. Stemming from this work, analysis of EV contents will also identify novel proteins and RNA messengers, thereby identifying alternative signaling pathways, and furthering our understanding of how neuron-tumor trophic relationships are established and sustained.

In this work, Amit *et al.*[18] uncovered a novel and potent method by which OCSCC tumors manipulate nearby sensory neurons to drive a neo-adrenergic neurotype switching event, thereby driving tumor growth and spread. These findings constitute a paradigm shift in our understanding of how tumors and neurons within the microenvironment interact with each other. Moreover, this work illustrates the importance of EV contents, and more specifically, miRNA signaling in mediating the trophic relationships between tumors and neurons that drive tumor growth. Future work will focus on further deciphering EV-mediated signaling, with a focus on developing novel approaches of using EV analysis to better understand tumor biology, and to better treat human cancer patients.

DECLARATIONS

Author's contribution

PJH and MA Conceptualized the manuscript:.
PJH wrote the first draft of the manuscript. MA supplied technical knowledge to support the manuscript throughout the revision process. PJH created the graphical abstract for the manuscript. PJH and MA contributed to the manuscript revision and approved the submitted version.

Availability of data and materials

Not applicable.

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Conflicts of interest

The authors declare no conflict of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm. [Last accessed on 30 Oct 2017]

Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. Proc Natl Acad Sci U S A. Forthcoming 2002.

For other types of references, please refer to U.S. National Library of Medicine.
The journal also recommends that authors prepare references with a bibliography software package, such as EndNote to avoid typing mistakes and duplicated references.

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