ERCC19 Abstracts

May 1 & 2, 2023

The Bethesda North Marriott Hotel & Conference Center

Talks in Order of Appearance

Poster Numbers Randomly Assigned

Virtual Meeting Link & Password will be sent to the email address provided at registration on Wednesday April 26th

NIH Extracellular RNA Communication Consortium
ERCC Program Closeout Meeting
May 1-2, 2023

Bethesda North Marriott Conference Center, Main Ballroom, Salons A & B
5701 Marinelli Road, Rockville MD 20852

Mon, May 1st

Note: All times are ET

Welcome & ERCC Overview	
8:15 a.m.	Badge pickup
8:30 a.m.	Danilo Tagle, Director, Office of Special Initiatives, National Center for Advancing Translational Sciences, NIH: Opening remarks & ERCC overview
8:45 a.m.	ERCC2 goals & accomplishments - Louise Laurent & Saumya Das (ERCC2)
Session I: Carrier Sorting Technologies & Applications I	Session Chairs: Angela Zivkovic & Meenu Srinivasan
9:05 a.m.	Hsueh-Chia Chang (ERCC2), College of Engineering, University of Notre Dame: High-throughput purification, fractionation and characterization of extracellular vesiclesand nanoparticles for diagnostic and therapeutic applications
9:25 a.m.	Robert Coffey (ERCC2), Dept. of Cell & Developmental Biology, Vanderbilt University: Extracellular vesicles and nanoparticles: Emerging complexities
9:45 a.m.	Louise Laurent (ERCC2), Dept. of Obstetrics, Gynecology & Reproductive Sciences, UC San Diego: Developing an immunomagnetic separation strategy for mapping extracellular vesicle heterogeneity
10:05 a.m.	*Break & Networking (30 min)*
10:35 a.m.	Bogdan Mateescu (ERCC2), Brain Research Institute, Universit of Zurich: PRISM: Purification of exRNA by Immuno-capture and Sorting using Microfluidics
10:55 a.m.	Ken Witwer (ERCC2), School of Medicine, Johns Hopkins University: Asymmetric flow field-flow fractionation for separation of exRNA carriers: Blood plasma lipoproteins and extracellular vesicles
11:15 a.m.	Daniel Chiu (ERCC2), Depts. of Chemistry & Bioengineering, Univsersity of Washington: Digital flow cytometry for the analysis of single extracellular vesicles and particles
11:35 a.m.	Shannon Stott, Center for Engineering in Medicine & Surgery, Massachusetts General Hospital: Microfluidics for cell-specific EV isolation
11:55 a.m.	Justus Ndukaife, Dept. of Electrical Engineering, Vanderbilt University: Next generation optical nanotweezers for unraveling the heterogeneity of extracellular vesicles and particles (EVPs)
12:05 p.m.	*Lunch, Networking, Posters (unattended) (90 min)*
12:45 - 1:15 p.m.	Ontology Discussion
Sesson II: ERCC Resource & Technology Showcase	Session Chairs: Jeff Franklin & Olesia Gololobova
1:35 p.m.	Aleks Milosavljevic (ERCC2), Dept. of Molecular & Human Genetics, Baylor College of Medicine: Untangling the complexity of EVs and their cargo using the exRNA Atlas
1:55 p.m.	Joel Rozowsky (ERCC2), Dept. of Molecular Biophysics & Biochemistry, Yale University: Integrative analysis of extracellular RNA profiles and associated tools for analyzing exRNA sequencing data
2:15 p.m.	Sharon Stack, Harper Cancer Research Institute, University of Notre Dame: Application of Asymmetric Nanopore Membrane (ANM) technology to evaluate extracellular vesicle-mediated tumor-host communication
2:35 p.m.	Roger Alexander (ERCC2), Extracellular RNA Communication Consortium: Overview of ERCC2 technology development
2:55 p.m.	Justin Chang (ERCC2), Dept. of Molecular Biophysics & Biochemistry, Yale University: Visualizing dimensionally-reduced Atlas data: the exRNA Explorer tool
3:05 p.m.	Jessie Arce (ERCC2), Dept. of Molecular & Human Genetics, Baylor College of Medicine: The NanoFlow Repository: a resource for sharing standards-compliant metadata and data for flow cytometry experiments involving extracellular vesicles and other particles
3:15 p.m.	*Break & Networking (30 min)*
Plenary Speaker	Introduction: Saumya Das
4:15 p.m.	**Plenary Speaker: Eduardo Marbán**, Smidt Heart Institute, Cedars-Sinai Medical Center: Novel ncRNA drugs bioinspired by EV contents
4:45 p.m.	Day 1 Summary
Session III: Poster session	
5:00 p.m.	Poster Sesssion, Main Ballroom, Salon C
6:30 p.m.	Day 1 adjourns

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Tue, May 2nd

Note: All times are ET

Welcome		**NIH: Opening Remarks & ERCC Overview**
	8:30 a.m.	Patricia Labosky, Office of Strategic Coordination – Common Fund Division of Program Coordination, Planning, and Strategic Initiatives, Office of the Director, NIH: Opening Remarks
Session IV Carrier Sorting Technologies & Applications II		Session Chairs: Bogdan Mateescu & Marsalas Whitaker
	8:45 a.m.	Eduardo Reategui (ERCC2), Dept. of Chemical & Biomolecular Engineering, Ohio State University: RNA characterization in single extracellular vesicles and particles from complex biofluids for cancer diagnostics
	9:05 a.m.	David Routenberg (ERCC2), Meso Scale Diagnostics: Identification and isolation of EVs with multi-marker signatures
	9:25 a.m.	Jeff Franklin (ERCC2), Dept. of Cell & Developmental Biology, Vanderbilt University: Overview of ERCC2 benchmarking studies: Complementary technologies to analyze a colorectal cancer cell secretome
REMOTE	9:45 a.m.	An Hendrix, Laboratory of Experimental Cancer Research, University of Ghent: A versatile toolbox for a comprehensive view on extracellular vesicles
	10:05 a.m.	Steven A. Soper, Center of BioModular Multi-scale Systems for Precision Medicine, University of Kansas Mixed-scale fluidic systems for the high efficiency selection of disease-associated EVs and their subsequent analysis for disease management
	10:25 a.m.	*Break & Networking 30 min*
	10:55 a.m.	Tony Jun Huang (ERCC2), Pratt School of Engineering, Duke University: Acoustofluidic technologies for the manipulation of cells and extracellular vesicles
	11:15 a.m.	Ionita Ghiran (ERCC2), Beth Israel Deaconess Medical Center: Identification of post-transcriptional modifications in nucleic acid sequences using purpose-designed molecular beacons
	11:35 a.m.	Gijung Kwak, Center for Nanomedicine, Johns Hopkins University: Extracellular vesicle-associated adeno-associated virus for inhaled gene delivery
REMOTE	11:55 a.m.	Giovanni Camussi, Dept. of Medical Sciences, University of Turin: Edible plant-derived extracellular vesicles as a carrier for an oral SARS-COV-2 vaccine
	12:15 p.m.	Priyanka Gokulnath (ERCC2), Cardiovascular Research Center, Massachusetts General Hospital: Extracellular vesicle microRNA cargo drives ventricular arrhythmia in heart failure patients by recapitulating developmental genes
	12:35 p.m.	*Lunch, Networking, Posters (unattended) (90 min)*
Session V: ExRNAs as Biomarkers		Session Chairs: Jennifer Jones & Jack Zheng
	2:05 p.m.	Tijana Jovanovic-Talisman (ERCC2), Dept. of Cancer Biology and Molecular Medicine, Beckman Research Institute, City of Hope: Integrated computational, "omics," and imaging approaches to high resolution identification of tissue-specific EVs
	2:25 p.m.	Desmond Brown, Neurosurgical Oncology Unit, Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke: Primary cilia: Exploitable glioblastoma signaling hubs
	2:45 p.m.	Dennis Jeppesen (ERCC2), Dept. of Cell & Developmental Biology, Vanderbilt University: Ubiquitination of extracellular proteins is specific for tetraspanin-enriched small extracellular vesicles
	3:05 p.m.	Julie Saugstad, School of Medicine, Oregon Health & Science University: miRNAs as biomarkers for and mediators of Alzheimer's Disease
	3:25 p.m.	*Break 10 min*
Session VI: Remaining Challenges and Future of the Field		Moderators: Matt Roth & Ken Witwer
	3:35 p.m.	Panel discussion. Panelists: Eduardo Marban, Shannon Stott, Justus Ndukaife & Sharon Stack
	4:30 p.m.	Christine Happel, Office of Special Initiatives, National Center for Advancing Translational Sciences (NCATS), NIH: Closing Remarks
	4:45 p.m.	Meeting adjourns
	6:30 p.m.	Close-out ERCC dinner – All invited; self-pay

NIH Extracellular RNA Communication Program Overview

The NIH Common Fund Extracellular (exRNA) RNA Communication program (https://commonfund.nih.gov/exrna) was established in 2012 to uncover fundamental principles of exRNA generation, secretion, and transport; identify and develop a catalog of exRNA found in normal human body fluids; and investigate the potential for using exRNAs in the clinic as therapeutic molecules and/or biomarkers of disease. The ERCC is funded via the Cooperative Agreement mechanism, which allows NIH program staff to assist, guide, coordinate, and participate in project activities.

ExRNAs can act as endocrine signaling molecules, both locally and systemically, representing a novel paradigm in intercellular communication. ExRNAs are transported in body fluids in association with an array of carrier vehicles of varying complexity including extracellular vesicles (EVs), ribonucleoproteins (RNPs), and lipoproteins (LPPs). While these carriers (EVs, RBPs, and LPPs) share the functional attribute of protecting exRNAs from degradation by ubiquitous extracellular RNAses, their respective contributions to biodistribution, uptake, and/or function of their respective exRNA cargo in target cells is less clear. In humans, exRNAs have been found in virtually all body fluids examined, including blood, saliva, urine, breast milk, cerebral spinal fluid (CSF), amniotic fluid, semen, ascites, and pleural effusions. Reports in the literature suggest that exRNAs can have both protective and pathogenic roles in a variety of human diseases, and concomitantly serve as biomarkers for disease states. Taken together, the above findings highlight the important role secreted exRNAs have in regulating human health and disease. A brief overview of exRNA communication is presented in the video "Unlocking the Mysteries of Extracellular RNA Communication".

Stage 1 of the program defined many fundamental principles of exRNA biogenesis, distribution, uptake, and function. The ERCC developed the tools to enable these studies, and generated a reference catalog of exRNAs found in a variety of body fluids. They also developed a community resource, the exRNA Atlas, to provide access to exRNA data, standardized exRNA protocols, and other useful tools and technologies generated by the exRNA consortium.

Since the program was founded, awardees have published over 740 peer-reviewed publications, including numerous publications describing refinement of protocols. They have generated over 7,500 samples in 29 datasets totaling more than 39.94 billion RNA-seq reads. They have published 8 software packages and 49 protocols, all of which have been made available through the exRNA Portal (exRNA.org) and exRNA Atlas (exRNA-Atlas.org). They also published a landmark collection of papers released April 4, 2019 on the biology and possible clinical applications of exRNA in the Cell family of journals, available at https://www.cell.com/consortium/exRNA.

exRNA

NIH Extracellular RNA Communication Stage 2

While significant strides have been made in the field of exRNA biology, specific technological challenges remain. For example, RNA concentrations in biofluids are significantly lower than in tissues, necessitating the development of improved methods for their isolation and analysis. Currently available separation approaches are time-consuming and do not adequately discriminate exRNAs encapsulated in EVs from exRNAs associated with non-vesicular RNPs or LPPs. Ultimately, this can lead to unintended biases in exRNA population profiling based upon the specific isolation method used. Further, little is known about the normal physiology of exRNAs, the functional consequences of exRNA uptake, or deficiency of uptake, by recipient cells, and the level of variation existing at the subpopulation level in both normal and disease states. These technological impediments limit the utility and impact of current efforts to catalog exRNAs and assess their contribution(s) to human health and disease.

These technology challenges will be addressed in the second stage of the NIH Common Fund exRNA Communication Program. Development of these tools, technologies, and resources will complement Stage 1 deliverables and fill remaining knowledge and technology gaps. As in Stage 1, awardees from all 3 initiatives have formed a consortium, with the overarching goal of determining fundamental principles associated with exRNAs. Comparisons across studies are essential to establish these cross-cutting principles so investigators must be willing to act as part of the consortium. Meeting the goals of the Stage 2 Initiatives will have a transformative effect on the field of exRNA biology as this will enable a greater understanding of the fundamental role of exRNAs in intercellular signaling and the translational potential to diagnose and treat diseases.

The exRNA Stage 2 Initiatives include:
a) The Data Management and Resource Repository (DMRR), which will continue to develop the exRNA Atlas, curate and disseminate information regarding critical reagents and resources, as well as facilitate cross-project collaborations (RFA-RM-18-026)
b) Developing improved separation technologies to rapidly and reproducibly sort exRNA carrier vehicles (EVs, RBPs, and LPPs) and identify their respective exRNAomes and associated molecular cargo. (RFA-RM-18-027)
c) Developing single EV isolation and analysis tools to assess exRNA heterogeneity and enable mapping exRNAs to their cell or tissue of origin (RFA-RM-18-028)

This initiative is funded through the NIH Common Fund, which supports cross-cutting programs that are expected to have exceptionally high impact. All Common Fund initiatives invite investigators to develop bold, innovative, and often risky approaches to address problems that may seem intractable or to seize new opportunities that offer the potential for rapid progress.

exRNA

Programmatic Goals

Data Management and Resource Repository (DMRR) on Extracellular RNA (U54)

Program Director - John Satterlee, Ph.D., NIDA

The DMRR will serve as a community-wide resource for data, protocols, and standards. It will coordinate across the CF Consortium of investigators to ensure data are comparable and to facilitate collaborations. It will develop an outward facing community resource website for easy access to CF-generated data, tools and ultimately, for data generated by the community at large. It will develop rigorous quality control measures of the data it stores. It will provide computational tools to enable the average investigator to query the data. It will also serve the CF consortium by organizing steering committee and grantees meetings. It will organize workshops and community outreach activities as necessary to develop a "field" of exRNA researchers and to disseminate new tools, technologies, and data. This Limited Competition Funding Opportunity Announcement (FOA) invited an application from the currently funded NIH Common Fund-supported Extracellular RNA Communication Consortium (ERCC) Data Management and Resource Repository (DMRR) to support Stage 2 efforts of this program. The overall programmatic goal of the DMRR is to integrate the efforts of all funded components of the ERCC and serve as a community-wide resource for extracellular RNA (exRNA) standards, protocols, and data. In 2020, the DMRR was awarded additional funds for data integration efforts from Stage 1 and 2 of the ERCC.

Advancing Extracellular RNA (exRNA) Communication Research: Improved Isolation and Analysis of exRNA-Carrier Subclasses (UG3/UH3 Clinical Trial Not Allowed)

Program Director - T. Kevin Howcroft, Ph.D., NCI

Circulating extracellular RNAs (exRNAs) can act as endocrine signaling molecules, both locally and systemically, representing a novel paradigm in intercellular communication. ExRNAs are transported in body fluids in association with a variety of carrier vehicles of varying complexity including extracellular vesicles (EVs), ribonucleoproteins (RNPs), and lipoproteins (LPPs). These distinct carriers protect exRNAs from degradation and are thought to contribute to the biodistribution, uptake, and functional impact of exRNAs in target cells. The overarching goal of this Funding Opportunity Announcement (FOA) is to develop and evaluate innovative separation tools, technologies, and approaches that will enable the scientific community to rapidly and reproducibly sort complex biofluids into homogenous carrier populations of EVs, (including EV subsets), RNPs, and LPPs, and that also support high-throughput isolation and analysis of their extracellular RNA content and associated molecular cargo.

Advancing Extracellular RNA (exRNA) Communication Research: Towards Single Extracellular Vesicle (EV) Sorting, Isolation, and Analysis of Cargo (UG3/UH3 Clinical Trial Not Allowed)

Program Director - Danilo Tagle, Ph.D., NCATS

The ability to isolate and analyze single EVs and their cargoes from human biofluids would provide a unique opportunity to understand the cell or tissue from which their respective exRNAs originate (heterogeneity) and, importantly, add significant depth to our understanding of exRNA communication. The overarching goal of this Funding Opportunity Announcement (FOA) is to develop and demonstrate innovative technologies and reagents towards isolating single EVs and to characterize the exRNA cargos associated with specific EV subpopulations based on cell of origin and their intended target cell. Shedding light on the diversity of exRNAs carried by EVs will allow for a better understanding of the precise role of exRNAs as signaling molecules for both physiological and pathophysiological processes, ultimately accelerating development of exRNAs as therapeutics and diagnostics.

External Program Consultants

Xandra Breakefield, Ph.D.
Professor of Neurology
Harvard Medical School
Geneticist, Neurology and Radiology
Massachusetts General Hospital

Dr. Breakefield focuses on researching the genetic mechanisms that underlie diseases of the nervous system, as well as treatments and biomarkers for these diseases. Her work is primarily on movement disorders such as early onset torsion dystonia (DYT1) and X-linked dystonia parkinsonism (XDP). She also has extensive experience working with brain tumors (especially glioma/glioblastoma) and with tumor suppressor syndromes (neurofibromatosis type 1 & 2 and tuberous sclerosis type 1 & 2). Dr. Breakefield was an investigator with Stage 1 of the Extracelllular RNA Communication Consortium, where she investigated how exosomes and microvesicles released by brain tumor cells modify their microenvironment to promote tumor growth. Her lab continues to explore how these extracellular vesicles can be used as biomarkers of disease, and how they can be modified to treat disease by delivering RNA or other treatments to the nervous system.

Michael Davis, Ph.D.
Professor/Associate Chair for Graduate Studies
Director, Children's Heart Research and Outcomes (HeRO) Center
Georgia Institute of Technology

Dr. Davis' work focuses on cardiac regeneration and preservation after injury, using molecular-based and biomaterials-based approaches. His lab has engineered 3D bioprinted cardiac components (such as aortic valves) used induced pluripotent donor stem cells. Additionally, he utilizes systems biology and bioinformatics to identify and study therapeutic RNA clusters in cardiac progenitor cells. He also has extensive experience with exosomes and bioactive nanoparticles, engineering exosome-like vesicles and investigating the effects of exosomes and their miRNA cargo on cardiac function, and developing a biocompatible nanoparticle capable of drug delivery to cardiomyocytes.

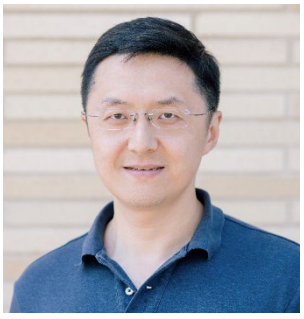
Scott Fraser, Ph.D.
Provost Professor
Director of Science Initiatives
Elizabeth Garrett Chair in Convergent Bioscience
University of Southern California

Dr. Fraser has extensive experience working in the field of quantitative biology, combining approaches from chemistry, engineering, and physics to study questions in biology and medicine. His lab specializes in the imaging and molecular analysis of intact biological systems, and works on developing new technologies for innovative multiplex and multimodal assays. He is especially interested in early development, organogenesis, and medical diagnostics.

Hakho Lee, Ph.D.
Associate Professor in Radiology
Massachusetts General Hospital/Harvard Medical School
Dr. Lee has extensive experience in extracellular vesicle (EV) analyses. His research focuses on developing sensitive, cost-effective, and user-friendly tools for EV characterization. For instance, by integrating principles of nanomaterials, biophysics, and engineering, his group advanced new analytical technologies, including nPLEX (nanoplasmonic exosome), iMEX (integrated magneto-electrochemical exosome), iMER (integrated magnetic exosomal RNA), and SEA (single EV analyses). Some of these platforms are now being translated for clinical uses. Dr. Lee currently directs the Biomedical Engineering Program at the Center for Systems Biology, Massachusetts General Hospital (MGH), and is also a Hostetter MGH Research Scholar.

Tushar Patel, M.B., Ch.B.
Professor of Medicine, and Cancer Biology, Dean for Research
Mayo Clinic
Dr. Patel is an expert in understanding the role and regulation of noncoding RNA in the development of liver and biliary tract cancers. The focus of his lab is to deploy the power of RNA for next generation diagnostics and therapies for liver cancer. He is particularly interested in the application of emerging knowledge on exRNA for diagnosis and therapies, with an emphasis on liver diseases and cancer, and has worked with Stage 1 of the Extracellular RNA Communication Consortium to explore exRNAs as biomarkers of hepatobiliary cancers.

Marina Sirota, Ph.D.
Associate Professor, Pediatrics
University of California, San Francisco
Dr. Sirota is currently an Assistant Professor at the Bakar Computational Health Sciences Institute at UCSF. Prior to that she was the Lead Research Scientist in the Division of Systems Medicine at Stanford University and has worked as a Senior Research Scientist at Pfizer where she focused on developing Precision Medicine strategies in drug discovery. Her research interests lie in developing computational integrative methods and applying these approaches in the context of disease diagnostics and therapeutics. Her primary focus is on leveraging and integrating different types of omics and clinical data to better understand the role of the immune system in disease.



Siyang Zheng, Ph.D.
Professor of Biomedical Engineering and Electrical & Computer Engineering
Carnegie Mellon University
Dr. Zheng is the principal investigator of the Micro & Nano Integrated Biosystem (MINIBio) Laboratory at CMU. Dr. Zheng's main research theme is to develop micro/nano technologies for precision healthcare, at the interface of material, device and biomedicine. His recent work includes developing liquid biopsy technologies for non-invasive cancer diagnosis using nanomaterials and microdevices, innovative nanomaterials and biomaterials for cancer therapeutics, nanomaterial-integrated microdevices for virus discovery and diagnosis, new methods for protein/peptide enrichment and proteomics, DNA analysis, volatile odor detection, implantable pressure sensor, in vivo gene transfer materials and devices, on-chip physiological or disease models, and fabrication of biodegradable and biocompatible polymers.

exRNA

TALK ABSTRACTS

Talk #	Presenter last name	Title of Abstract
1	Chang	High-Throughput Purification, Fractionation and Characterization of Extracellular Vesicles and Nanoparticles for Diagnostics and Therapeutics Applications
2	Coffey	Extracellular vesicles and nanoparticles: Emerging complexities
3	Laurent	Developing an immunomagnetic separation strategy for mapping extracellular vesicle heterogeneity
4	Mateescu	P.R.I.S.M: Purification of exRNA by Immuno-capture and Sorting using Microfluidics
5	Witwer	Asymmetric flow field-flow fractionation for separation of exRNA carriers: blood plasma lipoproteins and extracellular vesicles
6	Chiu	Digital flow cytometry for the analysis of single extracellular vesicles and particles
7	Stott	Microfluidics for cell-specific EV isolation
8	Ndukaife	Next generation optical nanotweezers for unraveling the heterogeneity of extracellular vesicles and particles (EVPs)
9	Milosavljevic	exRNA Atlas: a gateway into the world of exRNAs and their carriers in human biofluids.
10	Rozowsky	Integrative Analysis of Extracellular RNA Profiles and Associated Tools for Analyzing ExRNA Sequencing Data
11	Stack	Application of Asymmetric Nanopore Membrane (ANM) Technology to Evaluate Extracellular Vesicle-Mediated Tumor-Host Communication In Ovarian Cancer
12	Alexander	An overview of ERCC2 technology development
13	Chang	Visualizing dimensionally reduced Atlas data: the exRNA Explorer Tool
14	Arce	The NanoFlow Repository: A Resource for Sharing Standards-Compliant Metadata and Data for Flow Cytometry Experiments involving Extracellular Vesicles and other Particles
15	Marban	Novel ncRNA drugs bioinspired by EV contents
16	Reategui	RNA characterization in single extracellular vesicles and particles from complex biofluids for cancer diagnostics
17	Routenberg	Identification and isolation of EVs with multi-marker signatures
18	Franklin	Overview of ERCC2 benchmarking studies: Complementary technologies to analyze a colorectal cancer cell secretome
19	Hendrix	A versatile toolbox for a comprehensive view on extracellular vesicles
20	Soper	Mixed-Scale Fluidic Systems for the High Efficiency Selection of Disease-Associated EVs And Their Subsequent Analysis for Disease Management
21	Huang	Acoustofluidic sEV Isolation Devices with High Yield, High Biocompatibility, and High Repeatability
22	Ghiran	Identification of posttranscriptional modifications in nucleic acid sequences using propose-designed molecular beacons
23	Kwak	Extracellular vesicle-associated adeno-associated virus for inhaled gene delivery.
24	Camussi	Edible plant-derived extracellular vesicles as a carrier for an oral SARS-COV-2 vaccine
25	Gokulnath	Extracellular vesicle microRNA cargo drives ventricular arrhythmia in heart failure patients by recapitulating developmental genes
26	Jovanovic-Talisman	Integrated computational, ‘omics’, and imaging approaches to high resolution identification of tissue-specific EVs
27	Brown	Primary Cilia: Exploitable Glioblastoma Signaling Hubs
28	Jeppesen	Ubiquitination of extracellular proteins is specific for tetraspanin-enriched small extracellular vesicles
29	Saugstad	MiRNAs as Biomarkers for and Mediators of Alzheimer’s Disease

POSTER ABSTRACTS

Poster #	Last Name	Abstract title
1	Kumar	Quantifying Cancer-associated Exosomes, Supermere and Lipoprotein Markers with Novel Janus Nanoparticle Diffusometry Assay and Charge-based Electrokinetic Biosensors
2	Lai	From Labeling, Dosage, and Size to Biodistribution and Function of Extracellular Vesicles
3	McCarthy	A microfluidic platform for the detection of Alzheimer's Disease from co-localized surface proteins on neuron-derived exosomes
4	Ng	Refining Methods for Isolating & Detecting exRNA Carriers
5	Sharma	A scalable, high-throughput isoelectric fractionation platform for exRNA nanocarriers: Comprehensive and bias-free isolation of EVs, lipoproteins (LLPs), and Ribonucleoproteins (RNPs) from blood plasma, urine, and saliva
6	Tosar	RNA as its own carrier: nicked tRNAs are stable reservoirs of tRNA halves in biofluids
7	Zheng	Measurement of high-density lipoprotein particle size in clinical samples using transmission electron microscope provides better resolution for particle size and subgroup analysis
8	Arora	SalivaDB - A comprehensive database for salivary biomarkers in humans
9	Dayarathna	Multiparametric characterization of polarized microglial-derived extracellular vesicle (EV) mRNA cargo: shedding new light on neuroimmune crosstalk
10	Denniss	Extracellular vesicle-derived HIV RNA in CSF and serum reservoirs and its role in mediating neurocognitive dysfunction
11	Muse	Relationships Between Human Milk EVP miRNA Content and Attained Infant Anthropometry at One Year: A Pilot Study
12	Nguyen	Predicting Responses to Immunotherapy of Non-Small Cell Lung Cancer Patients via Single Extracellular Vesicular mRNA and Protein Detection
13	Rima	Sorting Single Extracellular Vesicles and Bioparticles and Simultaneously Detecting RNA and Protein In Situ on a Microdomain-Array Assay
14	Spanos	Tissue-Specific RNA Cargo of Plasma Extracellular Vesicles in COVID-19 Pathogenesis: A Dissection of EV RNA Origins and Pathways
15	Sun	β-cell-specific extracellular vesicles as an early diagnostic biomarker of Type 1 Diabetes
16	Yang	Aged and Young Breast Matrix Bound Vesicles in Breast Cancer
17	Ronan	Age and Sex Influence the Behavior of Exosomal miRNA in Tissue-Bound Exosome Populations to Affect Outcomes of Cardiac Fibrosis through Synergistic miRNA Effects
18	Sheikh	The Outer Membrane Vesicles of Bacteroides fragilis Contain Unique Small RNA Species and Trigger Immune Responses in Host Cells
19	Whitaker	Capture of Fluoro-labelled EGFR+ Extracellular Vesicles using Bio-functionalized Magnetic Particles
20	Carnel-Amar	Identification of Macrophage-specific Markers on Extracellular Vesicles in a Transgenic Mouse Model & Humans
21	Chatterjee	An In Vivo Model to Profile Cardiac Specific Extracellular Vesicles During Ischemic Reperfusion Condition
22	Chen	Extracellular RNA (exRNA) Analysis of Human Biofluids Derived Extracellular Particles Using Different Computational Tools.
23	Ciullo	Small noncoding RNA, yREX3, mined from therapeutic cell extracellular vesicles, reprograms macrophages through epigenetic silencing of Pick1 to limit ischemic heart injury
24	Liao	Long non-coding RNA BCYRN1 and its short synthetic derivatives promote regulatory T Cell proliferation, migration and activation
25	Sanada	The function of TRBP as a pro-inflammatoryExtracellular-RNAs sensor in obesity
26	Byappanahalli	Extracellular vesicles and their associated cargo as biomarkers in midlife frailty
27	Park	Extracellular vesicles from septic plasma of mice induce brain inflammation via MIRNA cargo
28	Srivastava	Utilizing SERS of immunocaptured exosomes to track exRNA

29	Wang	A Next-Generation High-Efficiency Extracellular Vesicle (EV) Isolation Platform, NanoEX, for Diagnostics and Therapeutics
30	Cheung	Characterizing RNA Packaging into Extracellular Vesicles (EV) by Comparing RNA in EV and other fractions from Conditioned Culture Media
31	Hakim	Developing an Automated High-Throughput RNA Isolation Pipeline for Extracellular RNAs from Various Biofluids for Small-RNA Sequencing
32	Srinivasan	k-TSP Algorithm Selected Serum miRNA Predictors of Gestational Age in Pregnancy
33	Reddy	Small extracellular vesicle loaded bevacizumab reduces the frequency of intravitreal injection required for diabetic retinopathy
34	Gao	exRNA carried by M2-like Macrophage Exosomes Suppress Cardiac Inflammation & Prevent Chronic Heart Failure after Myocardial Infarction
35	Arce	The NanoFlow Repository: A Resource for Sharing Standards-Compliant Metadata and Data for Flow Cytometry Experiments involving Extracellular Vesicles and other Par□cles
36	Pantham	Transcriptomic Profiling of Urinary Extracellular RNA in Pregnancy and Severe Preeclampsia
37	Tiwary	Efficacy of exRP250- a biologic molecule against Imiquimod-induced Psoriasis in BALB/c Mice

TALK ABSTRACTS

Hsueh-Chia Chang
University of Notre Dame

High-Throughput Purification, Fractionation and Characterization of Extracellular Vesicles and Nanoparticles for Diagnostics and Therapeutics Applications

Hsueh-Chia Chang1, Satyajyoti Senapati1, David Go2, Crislyn D'Souza-Schorey3
1. Department of Chemical and Biomolecular Engineering, University of Notre Dame, Notre Dame IN. 2. Department of Aerospace and Mechanical Engineering, University of Notre Dame, Notre Dame IN. 3. Department of Biosciences, University of Notre Dame, Notre Dame IN.

We review the technologies we have developed in the UG3UH3 project. Our approach is to create new technologies that can isolate, purify and characterize the biomarker nanocarriers at high throughput, yield and sensitivity but with very low-cost instrumentation. Due to these specifications, our technologies are based on new principles in ultrafiltration, electrokinetics and superparamagnetics. The nanocarriers include sEVs, HDLs, LDLs and supermeres. The biomarkers include proteins and nucleic acids.

These technologies include:

1. A high-throughput sEV isolation and purification technology that can process 800 ml of cell culture medium or 20 ml 40x diluted plasma in 2 hours. The key invention is an Asymmetric Nanopore Membrane (ANM) fabricated from ion-track polymer membranes with straight conic pores that minimize hydrodynamic resistance and protein fouling. The sEV purity and throughput are significantly better than current commercial products. It is particularly suitable for purifying therapeutic EVs. A company, Aopia Biosciences, has been founded to commercialize ANM. It is now taking service orders and will be placing the instrument on the market this year.

2. A high throughput/yield magnetic trap for immuno-nanobeads based on a Magnetic Nanoporous Membrane (MNM) version of the ANM. We developed an electroplating technique to plate a crystalline magnetic film around the pores of the MNM. A wedge geometry of the film at the pore produces a high magnetic field that can rapidly pull down nanomagnetic beads, with antibody-captured EVs, from a flowing solution with nearly 100% yield. The enriched beads near the pores of the MNM enables high-sensitivity and rapid affinity and activity assays of the proteins on the captured EVs. The captured EVs can then be analysed for downstream assay of the interior EV cargo. [Zhang et al, Comm Bio, 5:1358(2022)].

3. A high-throughput continuous-flow isoelectric fractionation (CIF) technology that can fractionate sEV, HDL, LDL and RNP by their isoelectric points from plasma, saliva and urine with >80% yield and >90% purity. It outperforms affinity-based and highly biased gold standards. It has a resolution of 0.3 ΔpI, sufficient to separate many nanocarriers and even subclasses of nanocarriers. Separation of HDL from LDL and mitochondrial sEVs from the main sEV population will be demonstrated.

4. An ion-exchange membrane (IEM) sensor to characterize nanocarriers with specific colocalized membrane proteins. The depletion action of the IEM amplifies the electrokinetic effects of the nanocarriers or their reporters to produce a large voltage signal from the membrane ion current. Sequential addition of different antibody functionalized charge reporters then allows large-dynamic range (4 logs) and sensitive (fM) characterization of multiple colocalized proteins on the nanocarriers. PON1-HDL, aEGFR-CD63 sEV and CEA-Supermere characterization will be reported for GBM, CRC and CVD clinical samples with >0.99 AUC scores, significantly higher than ELISA, fluorescence or SPR based sensor technologies. [Kumar S et al, Nature Comm, 14:557 (2023); McCarthy K et al, LabChip, 23:285(2022)].

Abstract # 2

Robert Coffey

Vanderbilt University Medical Center

Extracellular vesicles and nanoparticles: Emerging complexities

Robert J Coffey1, Qin Zhang1, Dennis K Jeppesen1, James N Higginbotham1, Oleg Tutanov1, Sarah E Glass1, Clark Massick1, Tuoye Xu2, Kasey Vickers1, Jeffrey L Franklin1, Al Charest2

1. Vanderbilt University Medical Center, Vanderbilt University, Nashville TN
2. Harvard BIDMC, Boston MA

The diversity of vesicular and nanoparticle carriers of extracellular protein, nucleic acids, and lipids in various biofluids is emerging through the use of multiple complementary technologies that can separate and analyze these components and their cargo. In this work, we developed new methods to parse and analyze both EV and nanoparticle constituents. Using these methods, we characterized a novel small non-vesicular extracellular nanoparticle (NVEP), termed supermere, and further refined the composition and functions of exomere NVEPs. Furthermore, we purified and analyzed subclasses of EVs and reevaluated various constituents and subclasses including extracellular RNAs (exRNAs). We uncovered various functional activities of NVEPs and EVs, showing their ability to signal, affect drug resistance, and alter organoid tumor growth amongst others. Some of this work has been published in a series of papers[1-4]. To broaden these results, we analyzed functional cargos of EVs and NVEPs from glioblastoma (GBM) and colorectal cancer (CRC) to find common and unique protein, lipid and exRNA cargos. We demonstrated that exRNA is critical for the biodistribution of supermeres, particularly their ability to cross the blood brain barrier. Results also suggest that NVEPs are highly inflammatory towards immune cells, e.g. microglial cells, potentially through bioactive lipid cargo. We compared genes implicated in regulating the trajectory of precancer cell states to cancer [5] to our existing compendium of EV and NVEP markers to determine important descriptors of precancer cell states that are represented in the biofluid of cancer patients. Such work has led us to focus on several genes. Two are the EV-associated dipeptidase 1 (DPEP1) and the supermere-associated TGFBI. The GPI-linked membrane protein DPEP1 can regulate the local tumor microenvironment through its enzymatic activity and the immune environment through its interaction with neutrophils. TGFBI, one of the most abundant proteins in supermeres, interacts with the collagen matrix and cellular integrins. TGFBI-containing supermeres have been affinity isolated and found to interact with multiple other identified supermere proteins and exRNAs. TGFBI-containing supermeres can also be found in the plasma from CRC patients. Our work demonstrates the value of orthogonal biochemical purification strategies to parse constituents from both cell culture and endogenous biofluids to determine potential biomarkers and functional players in the tumor microenvironment.

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Abstract # 3

Louise Laurent

University of California San Diego

Developing an immunomagnetic separation strategy for mapping extracellular vesicle heterogeneity

Willi Cheung1,3,4, Abbas Hakim1,4, Peter De Hoff1,4, Amber Morey1,4, Anelizze Castro-Martinez1,4, Isabella Saldana1,2,4, Anil Sood 5, Tao Liu6, Kendall Jensen 7, Robert Raffai8, John Nolan9, Saumya Das10, Louise C. Laurent1,4

1. Department of Obstetrics, Gynecology & Reproductive Sciences, University of California San Diego, La Jolla, CA, USA
2. Department of Biological Sciences, California Polytechnic State University, San Luis Obispo, San Luis Obispo, CA, USA
3. Department of Biology, San Diego State University, San Diego, CA, USA
4. Sanford Consortium for Regenerative Medicine, La Jolla, CA, USA
5. MD Anderson Cancer Center, University of Texas, Houston TX
6. Pacific Northwest National Laboratory
7. TGEN
8. University of California San Francisco
9. Scintillon Institute
10. Massachusetts General Hospital

Non-invasive assessment of tissue function is a major motivation for the study of extracellular vesicles (EVs). EV's represent a subset of the heterogenous particles secreted by cells into biofluids and are themselves diverse in both structure and in cargo. EV surface protein markers, derived from and specific to their cells of origin, are attractive targets for immunomagnetic separation (IMS) techniques used to isolate and purify these lipid bilayer-bound particles from the other components of their biofluid milieux. We have optimized a bead- based IMS protocol that enables selective enrichment of EVs carrying each of the canonical exosome markers (CD9, CD63, CD81) and the placental trophoblast-specific marker placental alkaline phosphatase (PLAP), and used it to isolate EV subpopulations from conditioned media from a variety of culture cell types, as well as human plasma. The resulting material was analyzed using NGS techniques (Small/Long RNA Seq) and mass spectrometry-based (proteomics/lipidomics). We found, as expected, distinct cargo profiles for EVs from different cell type and biofluid sources. Interestingly, the canonical EV markers enriched for the same subpopulation of EVs for some sources (e.g. DiFi cell culture conditioned media), but different subpopulations for others (e.g., placental explant culture conditioned media, human plasma). These results provide evidence for the presence of different biogenesis pathways with active content sorting systems rather than strictly a passive random assortment model for EV cargo loading.

Bogdan Mateescu

University of Zurich

P.R.I.S.M: Purification of exRNA by Immuno-capture and Sorting using Microfluidic

Stürchler S1,2, Meng Y1, Amaral G2, Barragan-borrero V1, Ng M8, Meechoovet B12, Joanna Palade12, Alsop E12, LaPlante EL3, Fullem R3, Chen D3, Starner AC3, Esquivel E3, Jackson AR3, Ghiran I4, Pereira G4, Rozowsky J5, Chang J5, Gerstein M5, Alexander R6, Roth ME3, Laurent L9, Wang YT10, Tsai CF10, Liu T12, Jones J11, Franklin J7, Coffey R7, Van Nostrand E3, Mansuy I1,2, McManus M8, Milosavljevic A3, Stavrakis S1, Van Keuren-Jensen K12, Raffai RL8, deMello A1, Mateescu B1,2.
1. ETH Zürich, Zürich, Switzerland
2. University of Zürich, Zürich, Switzerland
3. Baylor College of Medicine, Houston, TX, USA
4. Harvard Medical School, Boston, MA, USA
5. Yale University, New Haven, CT, USA
6. Pacific Northwest Research Institute, Seattle, WA, USA
7. Vanderbilt University School of Medicine, Nashville, TN, USA
8. University of California, San Francisco, CA, USA
9. University of California, San Diego, La Jolla, CA, USA
10. Northwest National Laboratory, Richland, WA, USA
11. Laboratory of Pathology Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA
12. TGEN, Phoenix, AZ, USA

Extracellular RNAs (exRNAs) have emerged as promising biomarkers for various diseases, however, the identification of their specific carriers remains a challenge (Mateescu B*, Jones J*, et al, iScience, 2022). RNA-binding proteins (RBPs) play a crucial role in the release and transport of exRNAs by sorting and loading them into different extracellular carriers, such as extracellular vesicles (EVs), lipoprotein particles (LPPs), and ribonucleoprotein (RNP) particles. Despite their importance, the current understanding of RBPs as carriers of exRNA fragments in human biofluids is limited. The PRISM project aims to address this gap in knowledge by identifying new carriers of exRNAs and developing microfluidic and affinity-capture methods to fractionate biofluids into their most relevant exRNA carrier components.

In collaboration with Milosavljevic lab, we used eCLIP/exRNA intersection analysis to identify 34 putative exRBPs with detectable footprints in both cell supernatant and healthy human plasma, and predicted specific exRBPs associated with EVs and non-EVs carrier classes across biofluids. Using Western-bloting, we demonstrated differential release of 15 RBPs in conditioned media of 293T, human mesenchymal stem cells and DiFi cancer cells (LaPlante E*, Stürchler A*, et al, Cell Genomics, in press). To further explore the role of RBPs as exRNA carriers, we generated a comprehensive exRBP atlas by creating 293T KO lines for 35 exRBP candidates and sequencing their cellular and conditioned medium small RNA. Our atlas revealed that specific RBPs are crucial for the biogenesis and extracellular stability/export of some exRNA biotypes and sequences (Stürchler et al, in preparation).

Building on these findings, we developed new exRBP pull-down assays that can enrich specific exRNA biotypes (up to 80-90% recovery and 95% purity) and from low-volume input (50-200 µl) of saliva, plasma, and milk samples in a 96-well format prior to RNA sequencing (Amaral G*, Barragan-Borrero V*, et al, in preparation). Additionally, we successfully generated a series of llama nanobodies reactive against DiFi EVs, which will represent an important recombinant binder resource for EV pulldown and detection by nanoflow cytometry. In parallel, we developed microfluidic platforms based on viscoelastic focusing that allow us to isolate cellular compartments, as well as large (>500-1000 nm), medium (200-500 nm), and small (80-200 nm) EVs at high purity from whole human blood (Meng et al, in preparation). All these purification approaches are being benchmarked using highly purified exRNA carrier fractions (e.g., fluorescent EVs,

HDL/LDL/chylomicrons) isolated from established methods (e.g., cushion gradient, size-exclusion chromatography) (Ng M et al, in preparation).

The P.R.I.S.M microfluidic and affinity-capture platform and resources (e.g., fluorescent EVs, nanobodies) for fractionating RBPs, LPPs, and EVs from biofluids will improve the sensitivity and specificity of exRNA-based biomarker assays. Moreover, our exRBP atlas constitutes the first systematic analysis of the impact of RBPs on extracellular small exRNA profiles in human cells.

Ken Witwer
Johns Hopkins Univercity

Asymmetric flow field-flow fractionation for separation of exRNA carriers: blood plasma lipoproteins and extracellular vesicles

Olesia Gololobova1, Wyatt N. Vreeland2, Angela M. Zivkovic3, Juan Pablo Tosar4, Michael E. Paulaitis1, Zhaohao Liao1, Kenneth W. Witwer1
1. Johns Hopkins University School of Medicine
2. National Institute of Standards and Technology
3. University of California Davis
4. Institut Pasteur de Montevideo

Cells can transform their gene expression and functions through secretion and uptake of RNA, thereby involving RNA as mediators of intercellular communication. The study of extracellular RNA (exRNA) as a signaling molecule can lend insights into the regulatory roles of RNA in cell biology and may lead to translational potential. exRNA carriers include lipoproteins (LP) and extracellular vesicles (EVs), the diversity of which remains incompletely appreciated. To date, the separation of exRNA carrier subclasses has focused mostly on EVs, and characterization has often been limited to verifying the presence of the target particles but not the absence of co-isolating particles. Here, we present two methods to isolate LP and EVs from human plasma. The first is a four-step process to separate highly pure plasma EV, low-density lipoproteins LDL, and high-density lipoproteins HDL particles using combinations of size- and density-based techniques. For this method, we also performed full “process controls” of passing buffer through all steps of the separation techniques. The second method is an application of asymmetric flow field-flow fractionation, which permits rapid separation and characterization of exRNA carriers. For the first time, we use this technology to separate particles from small volumes (20 µL) of plasma. Throughout, subclasses of exRNA carriers were defined by depletion or enrichment of lipoproteins ApoB100 (VLDL, LDL), ApoA1 (HDL), EV-borne tetraspanins CD9 and CD81, and albumin, among others. Single-particle analyses were performed with nano-flow cytometry (nanoFCM) and interferometric reflectance imaging sensing (SP-IRIS). The size of lipoproteins was determined using dynamic light scattering. The detected diameter range of particles was from about 45 to 200 nm (EVs), 5-12 nm (HDL), and 15-40 nm (LDL, VLDL). Finally, we assessed the presence of specific RNAs in the various particle fractions. We conclude that both methods provide reproducible yields of EVs and lipoproteins that are substantially separated from free proteins and other common plasma co-isolates.

Daniel T Chiu
University of Washington

Digital flow cytometry for the analysis of single extracellular vesicles and particles

Daniel T Chiu
University of Washington, Department of Chemistry and Bioengineering, Seattle, WA

We have developed a multi-parametric high-throughput flow-based method for the analysis of individual extracelluar vesicles and particles (EVPs), which are highly heterogeneous and comprise a diverse set of surface protein markers as well as RNA cargoes. Yet, current approaches to the study of EVPs lack the necessary sensitivity and precision to fully characterize and understand the make-up and the distribution of various EVP subpopulations that may be present. We termed our platform digital flow cytometry, which provides single-fluorophore sensitivity to enable phenotyping single EVPs with unprecedented precision and sensitivity.

Shannon Stott
Center for Engineering in Medicine & Surgery, Massachusetts General Hospital

Microfluidics for cell-specific EV isolation

Shannon Stott
Center for Engineering in Medicine & Surgery, Massachusetts General Hospital

Advances in microfluidic technologies and molecular profiling of tumors have propelled the rapid growth in blood-based tests for cancer. Through a collaborative effort between bioengineers, biologists, and clinicians, my laboratory has developed microfluidic devices that can isolate and characterize cell-specific EVs from blood. Data from these devices will be presented with a focus on our recent effort to characterize extracellular vesicles from the blood of patients with highly aggressive brain tumors. Transcriptomic and protein based signatures have been identified that suggest potential utility in the clinic to guide patient care. I will also share our most recent work repurposing these technologies to isolate SARS-CoV-2 virus particles and extracellular vesicles related to COVID19 infection. We are actively pursuing the testing of blood, stool and saliva to better predict which patients might have more severe outcomes. Through the microfluidic isolation of circulating biomarkers in cancer and COVID, our goal is to obtain complementary data to the current standard of care to help better guide treatment and identify new biomarkers and putative therapeutic targets.

Justus Ndukaife
Vanderbilt University

Next generation optical nanotweezers for unraveling the heterogeneity of extracellular vesicles and particles (EVPs)

Justus Ndukaife
Vanderbilt University

The heterogeneity of extracellular vesicles and particles (EVPs) presents a significant challenge to comprehending the biological roles of EVPs1. Existing EVP analysis methods, such as mass spectrometry and western blotting, rely on ensemble assays and cannot identify the composition of individual EVPs. Recently, Raman spectroscopy combined with laser trapping has emerged as a potent technique to characterize EVs and achieve global biomolecular composition analysis of EVs2–4. This approach involves using a focused laser beam to trap EVs at the laser focus, excite them, and collect Raman signals resulting from the inelastic scattering of incident photons to obtain molecular composition information of trapped EVs. However, the current Raman tweezer technologies have certain limitations, including the inability to focus light to the nanoscale required to trap single nanoscale EVPs and long wait times of several minutes to tens of minutes before trapping of EVs can occur. Furthermore, the spontaneous Raman signals are inherently weak, requiring long integration times and trapping of multiple EVs to collect sufficient data.

To address these limitations, we present an innovative approach for high-throughput nanoscale scale optical trapping and enhanced Raman spectroscopy of single EVPs which can rapidly trap thousands of single EVPs in parallel within seconds. This approach combines nanoplasmonic aperture cavities with microfluidics to transport and trap thousands of single EVPs in parallel near the nanoplasmonic cavities within seconds. The nanoplasmonic cavities generate substantial optical force upon illumination to achieve stable trapping of single nanocale EVPs and high local light field intensity enhancements for boosting Raman signals. Our results show that this innovative platform can stably trap nanoscale EVPs and precisely place individual EVPs to the regions of highest local light field intensity enhancement where the Raman signals can be boosted from the trapped EVPs.

Future studies will focus on conducting experiments to characterize individual EVPs using the plasmon-enhanced Raman scattering, paving the way to unravel the heterogeneity of EVPs and improve our understanding of their biological roles.

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Aleksandar Milosavljevic
Baylor College of Medicine

exRNA Atlas: a gateway into the world of exRNAs and their carriers in human biofluids.

Aleksandar Milosavljevic
Baylor College of Medicine

Construction of the exRNA Atlas resource (https://exrna-atlas.org) was an early goal of the ERCC project. Analysis of the original Atlas (version 4P1, 2018) via computational deconvolution revealed high complexity of exRNA carriers, with at least six vesicular and non-vesicular carriers of distinct exRNA cargo observable across human biofluids (Murillo et al. Cell 2019). A more recent exRNA-eCLIP intersection analysis (In Press) further revealed a map of extracellular RNA binding proteins (exRBPs) and associated RNAs. The two maps provide foundational knowledge for the studies of extracellular RNA communication in humans.

Atlas resource hosts tools for analyzing individual datasets and subsequent optional sharing of the uniformly processed data. Atlas-hosted exceRpt RNA-seq processing pipeline (Rozowsky et al Cell Syst 2019) has seen major uptake well beyond ERCC. Recent additions include tools for interactively exploring and visualizing Atlas datasets and custom analysis (Abstract #10). Future expansions of the resource include integration with NanoFlow Repository (Abstract #14) and support for multi-omic methods for comprehensive characterization of both the exRNAs and their carriers.

Taken together, our results demonstrate the power of sharing exRNA data and tools through the exRNA Atlas resource. We anticipate that the foundational maps enabled by data sharing and the tools readily accessible through the Atlas resource will continue to stimulate community involvement with the resource and catalyze discovery.

Joel Rozowsky
Yale University

Integrative Analysis of Extracellular RNA Profiles and Associated Tools for Analyzing ExRNA Sequencing Data

Joel Rozowsky
Yale University

We present an integrative analysis of extracellular RNA (exRNA) expression profiles spanning 6,907 samples from various human biofluids. Each profile originates from the NIH-funded Extracellular RNA Communication Consortium (ERCC), which sequenced the samples and uniformly processed the results to quantify the expression of detected RNAs (e.g., miRNAs, piRNAs, tRNAs, longer RNAs) as well as potential exogenous RNAs. We build machine learning models that effectively distinguish between biofluids and identify the exRNA features, frequently biofluid-specific, that are most informative for this classification. We also show how to construct machine learning models that differentiate between biological conditions, such as disease state, using human plasma exRNAs. Furthermore, we show how the small exRNA profiles of human biofluids can be related to those of a compendium of human tissues to infer the tissue-of-origin for exRNAs present in cell-free biofluids. Lastly, we performed dimensionality reduction on this dataset through a pipeline incorporating PCA, tSNE, VAE, UMAP, and PHATE. We also provide a tool, amenable to the submission of custom user analyses, for interactively exploring and visualizing low-dimensional representations of the dataset. We also present the other tools and pipelines for the analysis of extracellular RNA sequencing data. In particular we present the extracellular RNA-seq processing pipeline of the ERCC.

Abstract # 11

Sharon Stack
University of Notre Dame

Application of Asymmetric Nanopore Membrane (ANM) Technology to Evaluate Extracellular Vesicle-Mediated Tumor-Host Communication In Ovarian Cancer

M. Sharon Stack1,2, Jeff Johnson1,2, Reihaneh Safavi-Sohi1,2, Ceming Wang3,
Rebecca Whelan4, and H.C. Chang2,3

1. Department of Chemistry & Biochemistry, University of Notre Dame
2. Harper Cancer Research Institute, University of Notre Dame
3. Department of Chemical and Biological Engineering, University of Notre Dame
4. Department of Chemistry, University of Kansas

Ovarian cancer (OvCa) is the deadliest gynecologic malignancy, with a five year survival rate of 30%. Age is the most significant risk factor for OvCa, with a median age of 63 at diagnosis and median age of 70 for cancer-related deaths. Epidemiological studies performed more than three decades ago documented a disparity in cancer-related deaths between young and aged patients independent of treatment pathway. Pre-clinical studies from our group using murine models of aging support these data, showing increased peritoneal tumor burden in aged mice relative to young in both C57Bl/6 and FVB backgrounds; however, the molecular mechanisms by which aged individuals exhibit enhanced metastatic growth remains unclear. As one potential mechanism is via altered extracellular vesicle (EV)-mediated host:tumor communication with aging, the objective of this study is to evaluate EVs obtained from young (Y) vs aged (A) hosts and assess their role in a variety of functional studies. In collaboration with the Chang lab, we have used asymmetric nanopore membrane (ANM) technology for rapid purification of high-quality EVs from the peritoneal lavage of tumor-naïve Y vs A female mice and from ascites of tumor bearing Y vs A animals. Our results show that EVs from A hosts induce pro-metastatic behaviors in a variety of in vitro assays. Moreover, relative to EVs from Y mice, EVs from A hosts significantly enhance tumor cell adhesion to the peritoneal mesothelium in vivo, a key early event in OvCa metastatic dissemination. In collaboration with the Whelan laboratory, studies are underway to undertake a detailed comparison of the EV proteome in vesicles purified from Y vs A hosts. Future studies will examine how EVs regulate the peritoneal immune landscape and will evaluate EV priming of the pre-metastatic niche in ovarian cancer.

Roger Alexander
Extracellular RNA Communication Consortium

An overview of ERCC2 technology development

Roger P. Alexander1, Eduardo Reategui2, John Nolan3, Louise Laurent4, for the Extracellular RNA Communication Consortium
1. Extracellular RNA Communication Consortium, Phoenix, AZ 85254
2. Department of Chemical & Biomolecular Engineering, The Ohio State University, Columbus, OH 43210
3. The Scintillon Institute, San Diego, CA 92121
4. Department of Obstetrics, Gynecology, and Reproductive Medicine, University of California San Diego, La Jolla, CA 92093

The two main goals of the second stage of the Extracellular RNA Communication Consortium (ERCC2) are to develop technologies 1) to characterize single extracellular vesicles and their cargo, and 2) to better characterize the molecular carriers of extracellular RNA. New techniques developed by ERCC2 researchers join an already diverse array of methods and instruments that have been brought to bear on the problem of studying EVs and exRNAs.

We have developed an ”ERCC2 Technology Matrix” to try to bring order to this complex landscape. We grouped the different methods for exRNA / EV research by their main aim — Fractionation, Bulk Analysis, or Single Particle Analysis — and on a different axis by a set of metrics like speed, throughput, scale, limits of detection, and instrument cost. The resulting overview table shows instruments from both commercial vendors and ERCC2 research groups. In the overview table, metrics are binned into the rough categories of High, Medium, and Low, but further information about each technology is available in a more in-depth table that includes numerical estimates of the metrics and details about things like preparation of input materials. Each lab was responsible for categorizing its own instruments, and we together are responsible for the grouping of commercial instruments. In some areas we do not have precise numbers, or there is no consensus in the field about the most important metrics, but what we present in the table is the best we think is currently possible based on our collective effort.

We hope that this ERCC2 Technology Matrix will provide useful guidance to researchers new to the field and will inform the existing exRNA- and EV research communities about how ERCC2 technology development efforts fit in to the broader landscape of research tools.

Justin Chang
Yale University

Visualizing dimensionally reduced Atlas data: the exRNA Explorer Tool

Justin Chang, Joel Rozowsky
Molecular Biophysics and Biochemistry, Yale University, New Haven CT

Background

Extracellular RNAs (exRNAs) and their associated RNA binding proteins (RBPs) comprise an influential mechanism of cellular communication. Although these roles are yet to be fully elucidated, the packaging of gene-encoding exRNAs and the regulatory roles of exRNA-associated RBPs in transcription are areas of active investigation. The Extracellular RNA Communication Consortium (ERCC) has generated an exRNA Atlas for the broader scientific community, which records the exRNA expression profiles of over 6000 samples and the presence of over 1,400,000 distinct RBP sites in detected exRNAs. Due to improved filtering, standardization, and robustness in exRNA sequencing, the volume of complex data available to researchers will only increase in coming years.

Aims

Therefore, we aim to apply dimensionality reduction - encoding high-dimensional tabular data into a lower number of dimensions - in order to identify and visualize patterns in exRNA / RBP data. The exRNA Explorer Tool, available through the exRNA Atlas, stores precomputed analyses on AWS S3 and retrieves them for interactive visualization or export for subsequent downstream processing.

Methodology

Current datasets include miRNAs, piRNAs, tRNAs, circular RNAs, exogenous miRNAs, RNAs from microbes in the human body, and RBP site availability counts. The data were aggregated from the exRNA Atlas V2 and assembled into tables, with samples as rows and sequence counts as columns. Based on the parameters for each requested analysis, corresponding scaling and normalization methods are applied. We then apply a variety of dimensionality reduction methods, each with unique strengths and weaknesses. Specifically, we use principal component analysis (PCA), trained variational autoencoders (VAE), uniform manifold approximation and projection (UMAP), Potential of Heat diffusion for Affinity-based Transition Embedding (PHATE), and t-Distributed Stochastic Neighbor Embedding (tSNE). Users are also able to set expression thresholds and count sequences across tissues through UpSetR plots, which generalize the functionality of Venn diagrams.

Results

Currently, we have precomputed over 60,000 analyses and have functionality for fulfilling custom requests. Dimensionally reduced data can be interactively visualized in one of several forms: two-dimensional scatterplots, three-dimensional scatterplots, box plots, UpSetR plots, or heatmaps. In these visualizations, colors, shapes, and labels are used to differentiate samples across available metadata characteristics, such as disease state, biofluid, or tissue. Subsetting is supported before and after dimensionality reduction, allowing for reduction based on certain disease states or biofluids, as well as identifying tissue-specific or disease-specific patterns in broader reductions. To view the reduced data directly, interactive tables are also supported.

Conclusions

The exRNA Explorer Tool enables the interactive exploration of dimensionally reduced exRNA / RBP profiles at scale. We welcome discussion on the tool and whether it may be applicable to research in the broader ERCC community.

Jessie Arce
Baylor College of Medicine

The NanoFlow Repository: A Resource for Sharing Standards-Compliant Metadata and Data for Flow Cytometry Experiments involving Extracellular Vesicles and other Particles

Jessie E. Arce1*, Joshua A. Welsh2*, Sean Cook2, John Tigges3, Ionita Ghiran3, Jennifer C. Jones2, Andrew Jackson1, Matthew Roth1, Aleksandar Milosavljevic1
1. Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA.
2. Translational Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA.
3. Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA.

Background: Extracellular Particles (EPs), including extracellular vesicles (EVs), have been identified as important carriers of exRNA and mediators of intercellular communication and implicated in various biological and pathological processes. Flow cytometry is a widely used method for analyzing EPs, but the need for standardized reporting of experimental metadata limits the reproducibility and comparability of EV flow cytometry studies.

Aims: To develop a resource for sharing standard-compliant metadata and data for EP-related flow cytometry experiments and to promote rigor and reproducibility in EP and exRNA research.

Methodology: We developed The NanoFlow Repository as an online platform to facilitate sharing of EP characterization data and standards-compliant metadata. This initially focused on single EV flow cytometer (EV-FC) and bead-based assays and has been expanded to include Rresistive pulse sensing (RPS) analyses. The metadata includes information about the experimental design, samples, instrument configuration, and analysis parameters, following the MIFlowCyt-EV framework. The platform allows for easy dataset discovery, exploration, and download, with the ability to filter datasets based on various parameters, including the type of flow cytometer used to generate the data. For enhancing reproducibility and standardization, the repository supports the deposition of FCMPASS, MPAPASS, and RPSPASS calibrated datasets, enabling the scientific community to share, compare, and validate EP-FC experiments more comprehensively. Researchers can create an account to upload EP-FC data files and link them to publications. In addition, the platform provides access to MIFlowCyt and MIFlowCyt-EV reports, samples, and related data files for each dataset.

Results: The NanoFlow Repository currently hosts 48 public datasets with 546 FCS files generated by various flow cytometers, providing a valuable resource for the EP research community. The repository enables users to access standards-compliant metadata and reproduce published results, facilitating validation or refutation of previous findings and promoting collaboration and data reuse for meta-analyses.

Conclusions: The NanoFlow Repository represents a valuable resource for the EP and exRNA research communities, enabling the sharing of standards-compliant metadata and data for EP-related flow cytometry experiments. Its use will improve the rigor and reproducibility of research and promote collaboration and data reuse. We invite the scientific community to explore and contribute to the NanoFlow Repository: https://genboree.org/nano-ui/ld/datasets.

Eduardo Marban

Smidt Heart Institute, Cedars-Sinai Medical Center

Novel ncRNA drugs bioinspired by EV contents

Eduardo Marban, MD, PhD1
Smidt Heart Institute, Cedars-Sinai Medical Center, Los Angeles, CA

The development of regenerative therapy has benefited greatly from serendipity. Cardiac stromal cell therapy has shown promise in phase 1 and 2 trials of Duchenne muscular dystrophy1,2, and is now in phase 3 testing. But cell products vary in potency and can be difficult to manufacture. In the process of mechanistic investigations into cell therapies for heart disease, we discovered a central role for extracellular vesicles (EVs) as paracrine mediators of therapeutic bioactivity3. EVs reproduce the benefits of cells, and they are easy to isolate, store and deliver, making them viable cell-free therapeutic candidates. But EVs also have value as a discovery platform for biologically-active molecules. EVs work by delivering RNA and protein payloads to target cells, so it is logical to mine EV contents in a search for defined factors. In so doing we have found several noncoding RNA (ncRNA) species, including short Y RNAs, which themselves have intriguing biological actions4,5. This talk will trace the arc of discovery from cells, to EVs, to mining EV contents for novel bioactive ncRNAs, and finally using natural ncRNAs as bioinspiration for novel compounds that follow the structural conventions for approved ncRNA drugs. Lead compound TY1, a 24-nucleotide new chemical entity derived from a short Y RNA plentiful in EVs, has remarkable disease-modifying bioactivity in scleroderma with systemic sclerosis, a lethal treatment-refractory rare disease6. The next generations of cell-free biologics (EVs and ncRNAs) may provide the benefits of cell therapy without the intrinsic limitations.

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Eduardo Reategui

The Ohio State University

RNA characterization in single extracellular vesicles and particles from complex biofluids for cancer diagnostics

Eduardo Reategui
Department of Chemical & Biomolecular Engineering, The Ohio State University

Liquid biopsies are minimally invasive technologies that isolate and characterize circulating tumor biomarkers in bodily fluids. Thus, extracellular vesicles (EVs) have become attractive diagnostic and prognostic alternatives to tissue biopsies in oncology due to their abundance and easy accessibility. We will present a single-EV and particle (siEVP) assay to simultaneously detect mRNAs, miRNAs, and proteins in EVs and lipoproteins (LPs) subpopulations. The technology immobilizes and sorts particles via positive immunoselection on distinct microdomains, focusing biomolecular signals in situ. By detecting EVPs at a single-particle resolution, our technology outperformed the sensitivities of bulk-analysis benchmark assays for RNA and proteins. We could also perform multiplexed detection of protein/RNA and RNA/RNA colocalization within single EVs and LPs. Thus, our technology effectively examines intravesicular, intervesicular, and interparticle heterogeneity with diagnostic promise. The translational potential of our technology is shown through proof-of-concept experiments performed with serum, plasma, saliva, and tissue samples from cancer patients.

David Routenberg
Meso Scale Diagnostics, LLC.

Identification and isolation of EVs with multi-marker signatures

Evan Gizzie, Collin Nelson, Henri Butler, Talis Spriggs, Deepak Nanjappa, Sigal Shachar, Jacob Wohlstadter, Alex Tucker-Schwartz, David A. Routenberg
Meso Scale Diagnostics, LLC, Rockville MD

Extracellular vesicles hold great promise as a source of diagnostics and prognostics biomarkers due to their rich and multifaceted information content; however, identifying a population of interest in a biofluid and separating it from the background of non-targeted EVs remains a considerable challenge. Identifying, enriching, and characterizing vesicles from specific cells, tissues or organs has long been a goal of the EV research community and this consortium, but the use of single markers for EV immunoprecipitation has generally produced insufficient specificity and has led us to conclude that combinations of more than one marker may be necessary to define specific EV populations.

Here we present a highly multiplexed digital immunoassay for identifying combinations of surface markers present on EVs in a biofluid sample and quantifying the EVs with these multi-marker signatures. To demonstrate the technique, we developed a library of antibody conjugates targeting >60 surface proteins, mostly putative markers of EVs from T-cells, monocytes, macrophages, granulocytes, endothelial cells, epithelial cells, and platelets. These conjugates are used in a combinatorial assay, which enables simultaneous surveying of >200,000 combinations of surface markers in each sample and up to 96 samples at a time. After verifying the performance of the antibodies and the assay platform using EVs from numerous cell lines, we applied the system to human plasma from healthy subjects and colorectal cancer patients. We identified specific two- and three-marker combinations for each target cell type as well as specific populations that were elevated in late-stage colorectal cancer but not detected in healthy controls.

In order to enable further study of highly specific populations of EVs defined by two- or three-marker signatures, we developed a multi-marker immunoaffinity isolation technique. This method relies on multiple antibody conjugates to isolate a population of EVs with the logical AND of two or three markers using a single solid-phase pull-down step. We demonstrate this technique on several populations identified using the combinatorial assay, enabling further studies of the contents and the heterogeneity of these EVs populations.

Jeff Franklin
Vanderbilt University Medical Center

Overview of ERCC2 benchmarking studies: Complementary technologies to analyze a colorectal cancer cell secretome

Jeffrey L. Franklin1, James N. Higginbotham1, Oleg Tutanov1, Qin Zhang1, Dennis K. Jeppesen1, Zheng Cao1, Mark Castleberry1, Clark Massick1, Marsalas Whitaker1, Sarah E Glass1, Tuoye Xu2, Willi Cheung4, Abbas Hakim, 4 Anelizze Castro4, Martin Ng5, Chenguang Zhang8, Ceming Wang8, James Kim3, Seung Ryoung Jung3, Alessandra Stürchler12, 13, Stephanie Chidester10, Kendall Van Keuren-Jensen11, Michael Gertz10, Jubin George10, Joshua Welsh10, Elizabeth Hutchins10, Jennifer Jones10, Bogdan Mateescu12, 13, Peter De Hoff4 , Daniel Chiu3, Satyajyoti Senapati8, John P. Nolan9, Hsueh-Chia Chang8, Robert L. Raffai5, 6,7 , Louise Laurent4, Al Charest2, Todd Giorgio1, Kasey Vickers1 and Robert J Coffey1
1. Vanderbilt University Medical Center, Vanderbilt University, Nashville TN.
2. Harvard BIDMC, Boston MA.
3. University of Washington, Seattle WA.
4.UCSD, San Diego, CA
5. Northern California Institute for Research and Education, San Francisco, CA.
6. Department of Surgery, Division of Vascular and Endovascular Surgery, University of California San Francisco, San Francisco, CA.
7. Department of Veterans Affairs, Surgical Service (112G), San Francisco VA Medical Center, San Francisco, CA.
8. University of Notre Dame, Notre Dame, IN.
9. The Scintillon Institute, San Diego, CA.
10. NIH/NCI, Bethesda MD.
11. TGen Phoenix, AZ.
12. Institute for Chemical and Bioengineering, ETH Zürich, 8093 Zürich, Switzerland.
13. Brain Research Institute, University of Zürich, 8057 Zürich, Switzerland.

Biofluids contain a complex mixture of secreted proteins, extracellular vesicles (EVs) and non-vesicular extracellular nanoparticles (NVEPs), that carry various RNAs (exRNAs), DNAs and proteins, which can be used as analytes. A standardized reagent was developed to assess the strengths, weaknesses and commonalities of various techniques and technologies used to purify and analyze these biofluid constituents. The nucleotide and protein cargo of the EVs and NVEPS (exomeres and supermeres) from a human colorectal cancer (CRC) cell line, DiFi, has been molecularly defined. EVs from DiFi cells contain high levels of EGFR, which is an analyte of interest amongst multiple groups within ERCC2. Hollow fiber bioreactors (HFB) were used to produce consistently large amounts of this complex biofluid used in several different assays. A large pool of this material was frozen as either a crude preparation or as highly purified EVs that have been sub-fractionated using cushioned density gradient ultracentrifugation (DGUC) and size exclusion chromatography (SEC). This material was shared among multiple groups to facilitate comparison of technological and methodological analysis, as well as determining sources of variability that can impact the overall protein and RNA profile of readouts. DiFi cells provided to the NCI intramural Center for Cancer Research Translational Nanobiology Section are being used as a producer cell line for EV and NVEP reference material for cross institutional surface marker and RNA cargo studies that are ongoing and include multiple ERCC groups. The following studies have been implemented to facilitate cross platform and group comparisons:

1) DiFi EVs and NVEPs have been used as a common sample to benchmark the inter-lab reproducibility and reported EVP characteristics using different commercial flow-cytometry instruments as well as new technologies developed under the ERCC projects. The DiFi HFB CM pool was used for inter-laboratory validation of single EV flow cytometry measurements according to established best practices using a “top-down” approach. The Difi pool was also used to evaluate the performance of assays using new flow cytometers with higher sensitivity that enable calibration based on single

molecule detection (a “bottom-up” approach). As a result of these single EV FC measurements, the DiFi pool now represents a well-characterized reference preparation that can be used to evaluate the performance of other single EV measurement platforms including those based on EV capture from solution.

2) It was found that affinity selection using antibodies raised against any one of the commonly used tetraspanins (CD63, CD9, or CD81) followed by small RNA sequencing produces similar miRNA expression profiles, suggesting that these three markers identify the same population of EVs.

3) A high throughput bead capturing magnetic nanoporous membrane (MNM) technique developed by the Chia Chang group at the University of Notre Dame suggests that the MNM captured DiFi EGFR sEVs contain half of the amount of CD63 and CD9 than the total DiFi sEVs before MNM capture, which is also consistent with miR-21 qRT-PCR results, showing partitioning of these miRs in different EV populations.

4) DiFi EVs purified by DGUC and SEC were used to generate nanobodies.

An Hendrix

Ghent University

A versatile toolbox for a comprehensive view on extracellular vesicles

An Hendrix
Laboratory of Experimental Cancer Research, Department of Human Structure and Repair, Ghent University, Ghent, Belgium; Cancer Research Institute Ghent, Ghent, Belgium

Analyzing extracellular vesicles (EV) from biofluids (blood plasma, urine and feces) is challenging and complicates their biological understanding and biomarker development (PMID: 33568799). We fractionate biofluids by combining size-exclusion chromatography (SEC) and OptiPrep density gradient centrifugation to study clinical context-dependent and time-dependent variations in the biomolecular landscape of EVs (PMID: 31776460; 34429857; 32284825) . Using biofluids from cancer patients and reference materials to ensure standardized EV measurements (PMID: 31337761; 33452501), we demonstrate the technical repeatability of biofluid fractionation. Using serial samples from cancer patients we show that EV carry a clinical context-dependent and/or time-dependent protein and small RNA composition. In addition, differential analysis of biofluid fractions provides 1) a catalogue of putative EV corona proteins, 2) a list of proteins not associated with EV, and 3) reveals the presence of EV from diverse origins (PMID: 30518529; 35033427).

In conclusion, the implementation of biofluid fractionation allows to advance the biological understanding and biomarker development of EV.

Abstract # 20

Steven Soper

University of Kansas, Lawrence

Mixed-Scale Fluidic Systems for the High Efficiency Selection of Disease-Associated EVs And Their Subsequent Analysis for Disease Management

Steven A. Soper and Andrew K. Godwi
University of Kansas, Lawrence & University of Kansas Medical Center

We have been developing tools for the diagnosis of a variety of diseases. The commonality in these tools is that they consist of microfluidic devices made from plastics via injection molding. Thus, our tools can be mass produced at low-cost to facilitate bench-to-bed side transition and point-of-care testing (PoCT). We have also been generating novel assays focused on using liquid biopsy samples that are enabled using microfluidics. In this presentation I will talk about the evolution of our fabrication efforts of plastic-based microfluidic and nanofluidic devices as well their surface modification to make the devices biocompatible for in vitro diagnostics. One tool that we have generated is a plastic device (38 × 42 mm) that consists of 1.5M pillars, which are surface decorated with affinity agents targeting certain disease-associated extracellular vesicles (EVs). The affinity agents are covalently attached to the surface of the microfluidic device using a hetero-bifunctional linker, which consists of a coumarin moiety to allow for the photolytic release of the captured EVs using a blue-light LED to minimize photodamage to the EVs' molecular cargo. In this presentation, I will discuss the utility of these microfluidic devices using EVs as a source of mRNAs for molecular sub-typing of breast cancer patients. EVs were affinity selected from breast-cancer patients' plasma by searching for both epithelial and mesenchymal expressing EVs to allow for highly efficient sub-typing using the PAM50 gene panel and the nCounter.

Abstract # 21

Tony Jun Huang

Duke University

Acoustofluidic sEV Isolation Devices with High Yield, High Biocompatibility, and High Repeatability

Chaoran Chang,1 Arindam Ghosh, 1 Jianping Xia,2 Tony Jun Huang,2,* Lin Wang1,*

1. Ascent Bio-Nano Technologies, Inc., Morrisville, NC 27560

2. Thomas Lord Department of Mechanical Engineering and Materials Science, Duke University, Durham, NC 27708

In recent years, small extracellular vesicles (sEVs) have gained popularity as potential biomarkers for liquid biopsies due to the plethora of molecular cargo they carry, such as nucleic acids and proteins. However, the clinical application of sEVs is severely hindered by the lack of high-yield, high-biocompatibility, and high-repeatability techniques for sEV isolation. We have developed and validated acoustofluidic based sEV isolation devices that achieve significantly higher yield, biocompatibility, and repeatability than conventional approaches. We performed a comparison study of acoustofluidics devices to conventional approaches such as ultracentrifugation, size exclusion column, Exoquick, and ExoEasy. The isolated EV fractions were characterized by four techniques: NTA, Western Blotting, Electron Microscopy, and total RNA quantification. Our results showed that samples isolated using the acoustofluidic device have a much higher sEV yield and higher integrity than the conventional approach. We also verified that our device work consistently with different operators over multiple samples on different days for different sample types: plasma, saliva, tissue, etc. With its advantages in yield, biocompatibility, speed, and repeatability, the acoustofluidic technology has the potential to greatly simplify and expedite workflows in sEV-related biomedical research and aid in the discovery of new sEV biomarkers.

Ionita Ghiran

Beth Israel Deaconess Medical Center

Identification of posttranscriptional modifications in nucleic acid sequences using propose-designed molecular beacons

Parul Sahu1, Getulio Pereira1,2, Jian Wang, Kamil Szeliski1, Nikolay Dokholyan, Vivian Tran3, Miroslaw Korneck5, John Tigges3, Jennifer Jones4, Ionita Ghiran1

1. Department of Anesthesia, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States;
2. Department of Chemistry and Chemical Biology, Barnett Institute of Chemical & Biological Analysis, Northeastern University, Boston, MA, USA
3. Nano Flow Core Facility, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States;
4. Laboratory of Pathology Center for Cancer Research, National Cancer Institute, Bethesda, MD, United States;
5. Department of Internal Medicine I, University Hospital Bonn of the Rheinische Friedrich-Wilhelms-University, Bonn, Germany

Background: Post-transcriptional RNA modifications (PTxMs) present in small RNA species, specifically circulating extracellular RNAs, were recently identified as clinically relevant readouts, often more indicative of disease severity than the classical “up and down” changes in their copy number alone. While identification of PTxMs requires multiple and complex sample preparation steps, microgram-range amounts of RNA, followed by expensive and protracted bioinformatics analyses, the clinically relevant information is usually a yes/no for a particular genetic variant(s), and an up/down answer for relevant biomarkers. We have previously shown that molecular beacons (MBs) can identify specific nucleic acid sequences with picomolar sensitivity and single nucleotide specificity by exploiting the target-dependent change in their electrophoretic mobility profile.

Results: We now show a direct, “mix and read” detection of specific RNAs molecules in cells and extracellular vesicles using gel electrophoresis. The detection is based on discreet changes in the hydrodynamic surface profile, the overall charge and charge distribution of the MB-target hybrid. Furthermore, using an RNA tertiary structure prediction algorithm (iFoldRNA) and molecular dynamics simulation (DMD) developed by us, we designed MBs containing targeted nucleobase modification coupled to a unique fluorochrome-quencher pair, which identified the presence of m6A-modified nucleotides in target RNA sequences.

Conclusion: The sample preparation method coupled to the software package affords the design of specific MBs and sensitive, multiplex-type detection of targets in a wide variety of biofluids and cells.

Abstract # 23

Gijung Kwak

Johns Hopkins University

Extracellular vesicle-associated adeno-associated virus for inhaled gene delivery

Gijung Kwak1,2, Olesia Gololobova3, Neeraj Sharma4, Colin Caine5, Marina Mazur6, Kathleen Mulka3, Natalie E. West7, George M. Solomon6, Garry R. Cutting4, Kenneth W. Witwer3, Steven M. Rowe6, Michael Paulaitis1, George Aslanidi5,8,9, Jung Soo Suk1,2,10,*

1. Center for Nanomedicine at Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore MD.
2. Department of Ophthalmology, Johns Hopkins University School of Medicine, Baltimore MD.
3. Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine, Baltimore MD.
4. Department of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore MD.
5. Hormel Institute, University of Minnesota, Austin MN.
6. Gregory Fleming James Cystic Fibrosis Research Center, Heersink School of Medicine, University of Alabama at Birmingham, Birmingham AL.
7. Department of Pulmonary and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore MD.
8. Masonic Cancer Center, University of Minnesota, Minneapolis MN.
9. Institute Molecular Virology, University of Minnesota, Minneapolis MN.
10. Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore MD.

Background: Airway mucus is universally appreciated as one of the major hurdles that inhaled gene vectors must overcome to achieve clinically relevant gene delivery efficacy in the lung. Extracellular vesicles (EVs), having an intrinsic ability to shuttle various biological cargoes through extracellular milieus and between cells, are found in human airway mucus and mediate crosstalk between lung-resident parenchymal cells and/or immune cells.

Aims: We thus hypothesized that association of adeno-associated virus (AAV) on EVs would facilitate the penetration of AAV vectors through the airway mucus. We explored EV-associated AAV serotype 6 (EVAAV6), leveraging AAV6's superior ability to resist adhesive interactions with airway mucus compared to other AAV serotypes. Here we introduce EVAAV6, by leveraging on the delivery-promoting properties of individual components, provides highly efficient inhaled gene transfer efficacy as validated by a series of studies conducted in complementary experimental models.

Methodology: AAV6 and EVAAV6 were produced by transfecting HEK 293T cells with AAV-producing plasmids and harvested from the cell lysate and the conditioned media, respectively. Purified AAV6, EV, EVAAV6, and physical mixture of EVs and AAV6 (EV+AAV6) were analyzed by western blotting, single-particle interferometric reflectance imaging, dynamic light scattering, and transmission electron microscopy. Multiple particle tracking (MPT) analysis was used to measure the diffusion rates of EV and EVAAV6 in cystic fibrosis (CF) patient sputum samples. Transduction efficacy of EVAAV6 was assessed with mucus-free human bronchial epithelial (HBE) cell line and mucus-covered CF patient-derived HBE air-liquid interface (ALI) cultures in vitro and C57BL/6 mice following intratracheal administration in vivo. Safety assessment was pursued with histological analysis of treated lungs.

Results: EVAAV6 exhibited stable EV-AAV6 association while retaining the inherent properties of EVs and AAV6. MPT analysis revealed comparably high mobility of EV and EVAAV6 in CF sputum samples. EVAAV6, but not EV+AAV6, exhibited markedly and significantly greater transduction efficacy compared to AAV6 in mucus-free HBE cells in vitro. EVAAV6, but not EV+AAV6, showed significantly greater reporter transgene expression compared to AAV6 in mouse lungs with an excellent safety profile. EVAAV6, but EV+AAV6, exhibited superior transduction efficacy in mucus-covered ALI cultures of HBE cells derived from a healthy donor and a CF patient compared to AAV6.

Conclusions: EVAAV6 overcomes key biological barriers in the lung to provide widespread and robust pulmonary transgene transduc on following local administra on and could serve as a broadly applicable delivery pla orm for inhaled lung gene therapy.

Giovanni Camussi
University of Turin

Edible plant-derived extracellular vesicles as a carrier for an oral SARS-COV-2 vaccine

Margherita AC Pomatto1; Chiara Gai1; Giovanni Camussi2
1. EvoBiotech s.r.l., Turin, Italy
2. Department of Medical Science, University of Turin, Turin, Italy

Edible plant-derived extracellular vesicles (EVs) may represent a suitable carrier for delivery of RNA-based vaccines as they are not cytotoxic and may protect nucleic acids from degradation and environmental stress. The aim of the present study was to evaluate whether EVs extracted from orange (Citrus sinensis) juice engineered with mRNA coding for protein S subunit 1 (S1) could be used as a carrier for development of an oral vaccine.

The mRNA was efficiently loaded into EVs (Patent Application No. WO2022152771A) and protected from degrading enzymes including RNase enzymes and simulated gastric juice as detected by qRT PCR. After incubation with monocytes, engineered EVs were incorporated, and mRNA was translated into protein. S1 mRNA-EVs were stable after lyophilization and storage at room temperature and after reconstitution were resistant to enzyme degradation.

The immunogenic potential of loaded EVs in suspension was first evaluated in mice immunized via intramuscular and oral routes. The intramuscular administration elicited a humoral immune response with production of specific IgM and IgG blocking antibodies and a T cell immune response, as suggested by IFN-gamma production from purified spleen lymphocytes. Oral administration by gavage induced a specific lymphocyte activation and the production not only of specific blocking IgM and IgG but also IgA, that are the first mucosal barrier in the adaptive immune response. Since biodistribution studies showed a dispersion of S1 mRNA-EVs in soluble form within all gastrointestinal tract, we performed experiments in rats immunized by gavage administration of lyophilized S1 mRNA-EVs encapsulated in edible capsules with specific dissolution into the first intestinal tract. The oral administration of capsules also triggered a humoral immune response with production of specific IgM, IgG and IgA and generation of blocking antibodies and specific lymphocyte activation.

In conclusion, EVs extracted from edible plants confer stability to mRNA and may represent an efficient platform for oral vaccines delivery for their optimal mucosal absorption and ability to trigger an immune response. Oral administration in this context was superior to the intramuscular route also conferring IgA mucosal protection.

Abstract # 25

Priyanka Gokulnath

Massachusetts General Hospital - Harvard Medical School

Extracellular vesicle microRNA cargo drives ventricular arrhythmia in heart failure patients by recapitulating developmental genes

Priyanka Gokulnath PhD1,*, Michail Spanos MD1,*, H. Immo Lehmann MD, PhD1, Quanhu Sheng PhD2, Ling Xiao PhD1, Nedyalka Valkov PhD1, Avash Das MD, PhD1, Emeli Chatterjee PhD1, Guoping Li PhD1, Eric Aslop PhD3, Ashley Mingyi Wang BA1, Kendall Van Keuren-Jensen PhD3, Ravi Shah MD4, and Saumya Das MD, PhD1.

1. Cardiovascular Research Center, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA.
2. Department of Biostatistics, Vanderbilt University Medical Center, Nashville, TN, USA.
3. Division of Neurogenomics, The Translational Genomics Research Institute, Phoenix, AZ, USA.
4. Vanderbilt Translational and Clinical Research Center, Vanderbilt University Medical Center, Nashville, TN, USA.

Background: Heart Failure (HF) is the leading cause of death worldwide, with arrhythmias accounting for about 50% of its mortality. In fact, the relationship between Heart Failure and arrhythmias is closely intertwined. For instance, structural changes in the heart caused by fibrosis occurring after myocardial infarction create the substrate for arrhythmogenesis. On the other hand, any intrinsic changes in the electrical properties of cardiomyocytes brought about by changes in ion channel expression and function underlie the triggers for ventricular arrhythmias and, therefore, sudden cardiac death (SCD). Novel biomarkers associated with HF that lead to these arrhythmogenic events are an area of unmet need, and pathways that provide mechanistic insight into the process could be crucial for therapeutic intervention. Extracellular vesicles (EVs) and their RNA cargo in heart failure have been reported to be involved in disease pathogenesis and could play a role in promoting arrhythmias.

Aim: We aim to identify the specific plasma EV-driven microRNA cargo and pathways that could predispose to arrhythmia through electrical remodeling in ventricular cardiomyocytes during heart failure and further target these specific pathways to rescue the arrhythmic phenotype.

Methodology: We treated human pluripotent stem cell (hPSC) derived ventricular cardiomyocytes with plasma-derived EVs from 10 heart failure and control samples each to determine the changes in the action potential duration using optical voltage mapping. These plasma-derived EVs were sequenced to identify their small RNA cargo and then deconvoluted to examine their tissue of origin. We also sequenced the long-RNA transcriptome of the recipient cardiomyocytes to understand the pathways involved in electrical remodeling. Further, we validated the mRNA targets of the EV-miRNA cargo in cardiomyocytes treated with another 10 samples of control and heart failure EVs using qRT-PCR.

Results: The action potential duration in HF-treated hPSC derived ventricular cardiomyocytes was significantly prolonged by 94.41 16.13 msec as compared to the control-treated. We identified 26 downregulated and 70 upregulated miRNAs among the differentially expressed circulating small RNA transcripts between HF and control (FDR<1%). Many of these plasma miRNA transcripts were found to be enriched in the brain, RBCs, monocytes, adrenal glands and other tissues. We prioritized 33 targets for validation based on predicted protein-coding targets of miRNA (from DIANE and mirWalk), and 10 of these mRNAs were different between HF and control EV-treated cardiomyocytes. Interestingly, some of these mRNAs (EIF4E, NEDD4, ID3, ID1, CDKN1A, HSPD1, RBL1, SERPINE1, NRAS) are known to be expressed during development. Further, pathway enrichment analysis of the validated targets as well as the topmost regulated miRNAs converged in the TGF beta signaling pathway and Hippo pathway, which has been implicated in cardiac developmental arrhythmogenic abnormalities.

Conclusions: Circulating EV small RNA cargo of heart failure patients can alter the electrophysiological properties of cardiomyocytes. A large number of these EV-microRNAs in heart failure patients are derived from various tissues, predominantly the brain. More importantly, the arrhythmic phenotype is triggered when there is a recapitulation of developmental genes induced by the EV-derived miRNAs. While these miRNAs could be identified as putative biomarkers for SCD, targeting these developmental genes may be a fruitful therapeutic approach to rescue electrical remodeling in HF patients.

Tijana Jovanovic-Talisman
Beckman Research Institute, City of Hope

Integrated computational, 'omics', and imaging approaches to high resolution identification of tissue-specific EVs

Michail Spanos1, Bessie Meechoovet2, Andras Saftics3, Emeli Chatterjee1, Natacha Carnel1, Priyanka Gokulnath1, Guoping Li1, Sarah Abuelreich3, Ima Ghaeli3, Dorothy Palomares2, Francesca Galasso2, Elizabeth Hutchins2, Patrick Pirrotte4, Kendall Van Keuren-Jensen2, Tijana Jovanovic-Talisman3, Saumya Das1
1. Cardiology Division and Corrigan Minehan Heart Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA
2. Neurogenomics Division, Translational Genomics Research Institute, Phoenix, AZ, USA
3. Department of Cancer Biology and Molecular Medicine, Beckman Research Institute, City of Hope Comprehensive Cancer Center, Duarte, CA, USA
4. Collaborative Center for Translational Mass Spectrometry, Translational Genomics Research Institute, Phoenix, AZ

Background: Extracellular vesicles (EVs) are released from every known cell type in the human body and contain a variety of cargoes, including RNAs and proteins, that are derived from their source cell. The content of EVs can change with cellular stress; therefore we hypothesized that changes in the EV transcriptome in diseases may accurately reflect the 'state' of the cell or tissue of origin, providing an accurate highly precise biomarker reflecting disease trajectory. However, to date, tools and reagents to identify tissue (or cell) specific EVs have been lacking. Here we describe our integrated methodology to identify specific markers from cardiomyocytes, brain (neuronal and glial), and immune (macrophage) cells that allow for identification of cell-specific transcripts in the plasma.

Methods: Using a computational approach combined with bottom up proteomics, we identified putative membrane proteins with specific or highly enriched expression in cardiomyocytes, neurons, glial cells, and macrophages. Tissue-specificity and presence on EVs was validated with western blotting and dot blotting. Quantitative single molecule localization microscopy (qSMLM) was advanced to confirm the presence of these markers on individual EVs, and we have begun characterization for some of the markers. The ExoMap mouse model for tissue-specific EV reporting was secondarily used to confirm the presence of these markers on EVs derived from specific organs of interest in the plasma (or CSF). Finally, immune-pulldown was optimized to allow for isolation of tissue specific EVs in human plasma for downstream RNAseq.

Results: We identified 2 promising markers for cardiomyocyte-derived EVs (POPDC2 and CHRNE) that were specific for (or highly enriched on) cardiomyocytes and their secreted EVs. Notably, ExoMap-MHC-Cre mice were used to isolate cardiac-specific EVs, which demonstrated the presence of these markers in both heart tissue and plasma EVs. qSMLM demonstrated that heart-enrich EVs from ExoMap-MHC-Cre mice are larger in size, in line with our observation for cardiac-derived EVs from plasma. Immuno-pulldown of cardiac EVs from the plasma of healthy patients showed enrichment of cardiac transcripts. Notably, cardiac EVs were enriched in RNA transcripts that differentiated heart failure from controls. Similarly, we also identified CD68 and CD169 as markers for macrophage specific EVs. Using a similar parallel strategy, we validated their presence on macrophage EVs that could be immune-isolated from the plasma. Finally, we were able to identify MOG and GLAST as markers for oligodendrocytes and astrocytes respectively. We have optimized pulldowns of EVs containing these markers, characterized MOG-enriched EVs with qSMLM, and demonstrated enrichment of transcripts from these cell types.

Conclusions: Our integrated methodology using computational, omics, and imaging approaches, was successfully able to identify tissue-enriched EVs. Optimization of our methods has led to efficient isolation of these EVs from complex biofluids. We are currently assessing the changes in the RNA cargo in tissue-specific diseases in the context of several cardiovascular, immune-related, and neurological diseases.

Desmond Brown
SNB/NINDS/NIH

Primary Cilia: Exploitable Glioblastoma Signaling Hubs

Mi-Yeon Jung, PhD1; Joshua Welsh, PhD2; Josie Trichka, BS2; Stanley Adoro, PhD,2; Jennifer C. Jones, MD, PhD2; Desmond A. Brown, MD, PhD1
1. Neurosurgical Oncology Unit, Surgical Neurology Branch, National Institutes of Neurological Disorders and Stroke, NIH
2. Center for Cancer Research, National Cancer Institute, NIH

Glioblastoma (GBM) is the most common primary malignant brain tumor. It is uniformly fatal with a median overall survival of approximately 15 months and 5-year survival of approximately 6-8%. While immunotherapy has revolutionized cancer therapy, GBM-mediated immunosuppression limits utility in GBM. PD-L1 on the surface of GBM-derived extracellular vesicles (EVs) are implicated in immunosuppression through the induction of myeloid-derived suppressor cells (MDSCs).

Primary cilia are microtubule-based organelles that project from the cell surface in a dynamic fashion and receive and process molecular and mechanical signaling cues. They are ubiquitous having been found on essentially all human cell types. We show that GBM-mediated immunosuppression– defined as MDSC induction following exposure of normal donor monocytes to GBM-derived EVs–requires functional glioblastoma cell primary cilia in vitro. Importantly, primary cilia do not appear to significantly alter EV release or other biophysical properties and our data suggests that the presence of primary cilia alters content of the EVs to modulate their effect on immunosuppression. These findings are novel and have implication for potentially mitigating EV-mediated immunosuppression in GBM and other cancers.

Abstract # 28

Dennis Jeppesen

Vanderbilt University Medical Center

Ubiquitination of extracellular proteins is specific for tetraspanin-enriched small extracellular vesicles

Dennis K. Jeppesen, Qin Zhang, James N. Higginbotham, Robert J. Coffey
Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA

Background: Extracellular vesicles (EVs) are a heterogeneous group of lipid bilayer membrane vesicles released by cells to the extracellular space. They range in size from 50-10,000 nm but are often categorized as small EVs (sEVs) and large EVs (lEVs) based on diameters smaller or larger than 200 nm, respectively. Exosomes are a type of sEV that form as intraluminal vesicles (ILVs) in multivesicular endosomes (MVEs) and are highly enriched in specific tetraspanin proteins, including CD63 and CD81. In contrast, microvesicles are generated by direct outward budding of the plasma membrane and span the sEV and lEV size ranges. Non-vesicular extracellular nanoparticles (NVEPs) are amembraneous particles that include the newly discovered exomeres and supermeres, and they also appear to be released by many cell types. The post-translational modification of proteins by ubiquitin regulates many processes in eukaryotic cells.

Aims, Methodology and Results: We have investigated the presence of ubiquitin-modified proteins in the extracellular space and find that they are specifically enriched in sEVs. Strikingly, the enrichment of Ub in sEVs was as dramatic as the established exosome marker tetraspanin, CD63. In contrast, proteins in microvesicles and exomeres are not associated with ubiquitin modification. Isolation of sEVs by direct immunoaffinity capture of CD63/CD81/CD9-enriched vesicles indicated that tetraspanin-enriched sEVs account for most of the ubiquitinated proteins in the extracellular space. Likewise, CD63/CD81 double-positive exosomes, sorted by fluorescence-activated vesicle sorting (FAVS), account for the majority of the extracellular ubiquitin signal. Neddylation, post-translational modification by NEDD8, has been linked to negative regulation of exosome secretion, and, in contrast to ubiquitination, we find that post-translational modification of proteins with NEDD8 is more associated with exomeres than with sEVs.

Conclusion: Modification of extracellular proteins with ubiquitin is enriched and specific for tetraspanin-enriched sEVs and not other types of EVs. In contrast, neddylation of extracellular proteins is a predominant feature of exomeres and not sEVs.

Julie Saugstad
Oregon Health & Science University

MiRNAs as Biomarkers for and Mediators of Alzheimer's Disease

Ursula Sandau1, Trevor McFarland1, Jack Wiedrick2, Joseph Quinn3, Julie Saugstad1
1. Department of Anesthesiology & Perioperative Medicine, Oregon Health & Science University, Portland OR
2. Biostatistics & Design Program, Oregon Health & Science University, Portland OR
3. Department of Neurology, Oregon Health & Science University; Parkinson Center and Movement Disorders Program; Portland VAMC Parkinson's Disease Research, Education, and Clinical Center, Portland, OR

Background: Alzheimer's disease (AD) is the most common cause of dementia, and brain changes in AD begin 10-20 years before symptoms appear. During ERCC1, we discovered and validated miRNAs in human cerebrospinal fluid (CSF) that differentiate AD patients from controls, and then identified a subset of the CSF AD miRNAs that trend to decrease in expression from Control to Mild Cognitive Impairment to AD. As blood is a preferred biofluid for rapid noninvasive biomarker profiling, we first evaluated the intraindividual longitudinal stability of human miRNAs in plasma, then evaluated the CSF AD miRNAs in plasma. We examined whether the AD miRNAs in CSF are in extracellular vesicles (EVs), and evaluated the effect of APOE4 genotype and sex, two biological factors that increase the predisposition to AD, on miRNA expression in CSF EVs.

Aims: Our aims are 1) to establish the utility of miRNAs in blood as biomarkers for AD, and 2) to establish miRNAs in CSF EVs as mediators of AD pathology.

Methodology: All procedures were approved by the OHSU Institutional Review Board. For the longitudinal stability study, blood samples were collected by venipuncture biweekly over a 3-month period from 22 donors who had fasted overnight. Total RNA was isolated from 200 µL of platelet-free plasma using the miRNeasy Serum/Plasma Advanced Kit (217204, Qiagen), and miRNAs analyzed using TaqMan Advanced miRNA Human A Cards (A34714, Thermo Fisher (TF)). For the CSF:Plasma study, 320 donor- and date- matched CSF and plasma samples from AD and Controls were obtained from the AD Neuroimaging Initiative. Total RNA was isolated from 250 µL of both CSF and plasma using the Urine miRNA Purification kit (Norgen, Thorold, ON), and miRNAs analyzed using a custom TaqMan Advanced Human miRNA Card (TF). For the CSF EV study, 5.0 mL of CSF was concentrated by ultrafiltration, fractionated by size exclusion chromatography (IZON Science, Christchurch, New Zealand), and miRNAs analyzed using TaqMan Advanced miRNA Human A Cards (A34714, TF).

Results: For the longitudinal stability study, 134 miRNAs amplified in plasma, 74 were stable in an individual over the 3-month time period, and relevant to AD, 13 of 17 miRNA biomarkers were stable in plasma over the 3-month time period. For the CSF:Plasma study, preliminary analysis showed that 24 of 25 validated CSF miRNAs differentiate AD from Controls. For the CSF EV study, most AD miRNAs are in EVs, and both APOE4 genotype and sex, two biological factors for AD, alter the expression of CSF AD miRNAs in EVs.

Conclusions: Our studies i) identified miRNAs that show intraindividual longitudinal stability in human plasma over a 3-month time period, including many candidate biomarkers for AD; ii) validated AD CSF miRNA biomarkers differentiate AD from Controls in plasma, an easily accessible biofluid; iii) 4 of 5 CSF miRNAs that decrease in expression from Controls to Mild Cognitive Impairment to AD are located in EVs. Thus, miRNAs may serve as biomarkers for AD, and miRNAs in EVs may serve as mediators of AD pathology.

POSTER ABSTRACTS

Poster Abstract # 1

Sonu Kumar

University of Notre Dame

Quantifying Cancer-associated Exosomes, Supermere and Lipoprotein Markers with Novel Janus Nanoparticle Diffusometry Assay and Charge-based Electrokinetic Biosensors

Sonu Kumar, John Alex Sinclair, Satyajyoti Senapati and Hsueh-Chia Chang

Department of Chemical and Biomolecular Engineering, University of Notre Dame, Notre Dame, IN-46556

Background: Several cancer markers, including EGFR, GPC1, Enolases, PON1, and TGFBI, have been found on the surface of exosomes, supermeres, and lipoproteins. These markers are often colocalized and reflect their parent cell's proteomic signature, providing necessary information for early non-invasive cancer diagnosis. These proteins are frequently only active on cell membranes and are colocalized with specific other proteins due to their role in intracellular cancer pathogenesis. Hence, the exclusion of their free-floating counterparts significantly improves the AUCs of diagnosis. Classical assays such as ELISA, Western Blot, and proteomic mass spectrometry suffer from low sensitivity and cannot study the colocalization of proteins on these particles in any reasonable timeframe, which often require vesicle isolation. Flow cytometry for these vesicles also gives inaccurate measurements due to the small size of these particles.

Aims and Methodology: Here, we propose two novel methods to measure the concentration of exosomes, supermeres, and HDL without any sample isolation. The first method uses a charge-based sensor that utilizes voltage-gating phenomena for ion exchange membranes to measure the concentration of these particles. This method achieves a limit of detection of ~1-10 fM for exosomes and supermeres and ~100 fM for lipoproteins. The second method uses the change in rotational blinking of Janus particles when an exosome binds to it, reaching a limit of detection of ~1-10 fM. Both methods have a 3-4 log10 dynamic range and can measure concentrations directly from human plasma in 30-60 minutes.

Results and Conclusion: The platform has high enough sensitivity to measure tumor-specific surface markers from the entire EV/Supermere/Lipoprotein population in plasma, representing less than 0.01% of the ensemble. Preliminary results and ongoing preclinical studies with human patients include aEGFR and EGFR-positive exosomes from Glioblastoma patients, PON1-positive high-density lipoprotein for coronary artery disease patients, GPC1-positive exosomes for pancreatic cancer, CEA-positive supermeres for colorectal cancer, and TGFBI-positive supermeres for pan-cancer detection. Current AUC for PON1-HDL ~ 0.99 for coronary artery disease and aEGFR-exosomes ~ 1.0 for glioblastoma. A comparison between the diagnostic potential of supermeres and exosomes for cancer is done for the same surface protein (GPC1 and CEA on exosomes vs supermeres).

Poster Abstract # 2

Charles Lai

Academia Sinica, Taiwan

From Labeling, Dosage, and Size to Biodistribution and Function of Extracellular Vesicles

Bryan John Abel Magoling1,2,3, Yan-Tang Wu1,3, Yen-Ju Chen1, Wan-Ting Wong1, Ting-Yu Chuo1, Hsi-Chien Huang5,6, Yun-Chieh Sung5,6, Hsin Tzu Hsieh5, Poya Huang1, Kang-Zhang Lee1, Kuan-Wei Huang5, Ruey-Hwa Chen2,3,4, Yunching Chen5, Charles Pin-Kuang Lai1,3

1. Institute of Atomic and Molecular Sciences, Academia Sinica, Taipei, Taiwan.
2. Graduate Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan.
3. Chemical Biology and Molecular Biophysics Program, TIGP, Academia Sinica, Taipei, Taiwan.
4. Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan.
5. Institute of Biomedical Engineering and Frontier Research Center on Fundamental and Applied Sciences of Matters, National Tsing Hua University, Hsinchu, Taiwan.
6. Department of Chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan.

Extracellular vesicles (EVs) are released by cells to mediate intercellular communication under pathological and physiological conditions. While small EVs (sEVs; <100–200 nm, exosomes) are intensely investigated, the properties and functions of medium and large EVs (big EVs [bEVs]; >200 nm, microvesicles) are less well explored. Here, we identify bEVs and sEVs as distinct EV populations, and determine that bEVs are released in a greater bEV:sEV ratio in the aggressive human triple-negative breast cancer (TNBC) subtype. PalmGRET, bioluminescence resonance energy transfer (BRET)-based EV reporter, reveals dose-dependent EV biodistribution at non-lethal and physiological EV dosages, as compared to lipophilic fluorescent dyes. Remarkably, the bEVs and sEVs exhibit unique biodistribution profiles, yet individually promote in vivo tumor growth in a syngeneic immunocompetent TNBC breast tumor murine model. The bEVs and sEVs share mass spectrometry (MS)-identified tumor progression-associated EV surface membrane proteins (tpEVSurfMEMs), which include SLC29A1, CD9 and CD44. tpEVSurfMEM depletion attenuates EV lung organotropism, alters biodistribution, and reduces protumorigenic potential. This study identifies distinct in vivo property and function of bEVs and sEVs in breast cancer, which suggest the significant role of bEVs in diseases, diagnostic and therapeutic applications.

Kyle McCarthy
University of Notre Dame

A microfluidic platform for the detection of Alzheimer's Disease from co-localized surface proteins on neuron-derived exosomes

Kyle McCarthy1, Sonu Kumar1, Satajyoti Senapati1, Hsueh-Chia Chang1
1. Department of Chemical and Bio-molecular Engineering, University of Notre Dame, Notre Dame, IN.

Background - Alzheimer's disease (AD) is the most prevalent type of dementia and a debilitating neurodegenerative disease. AD is famously marked by extracellular amyloid-β plaques and intracellular accumulations of phosphorylated tau in different regions of the brain that can be identified post-mortem by positron emission tomography (PET). Which allows an effective but invasive method of postmortem confirmation of AD progression. The need for early onset detection and a manageable method of monitoring progression is growing as quickly as the number of people who suffer from AD each year. Monitoring patients' cognitive strength with screening tests like the mini-mental state examination (MMSE) and the Montreal cognitive assessment (MoCA), which are non-invasive and are relatively easily administered, can give a general picture of cognitive decline. Examinations like these are useful for monitoring decline in mental state, but test numbers can be skewed by individual performance and on their own are poor at determining if a patient has AD or a different disease. Biomarkers in cerebral spinal fluid (CSF) were discovered to hold strong correlations and potential diagnostic relevance for confirming AD diagnosis, but require invasive and potentially dangerous collection of CSF from patients making it a less preferred, and less easily repeatable, method. Recently, the discovery of neuron-specific proteins on neuron-derived exosomes (NDE) has led to the study of these markers in plasma. With hallmark biomarkers discovered in plasma exosomes, and more specifically in plasma NDE, there is great promise in the detection of AD through blood tests over the more invasive CSF tests.

Many of these proteins found in NDEs have shown viability as potential AD biomarkers, especially those that are involved in pathways for amyloid fiber plaques and intracellular neurofibrillary entanglement. These include proteins like glial fibrillary acidic protein (GFAP), which increased levels serve as markers of astrogliosis that is observed around Aβ plaques, neurofilament light (NfL), an axon cytoskeletal protein is released into CSF and blood following neurodegeneration or axonal damage to neurons, phosphorylated tau, specific sites on tau causes aggregation of neurofibrillary tangles (NFTs), and amyloid beta (Aβ), the β peptide being prone to aggregation and formation of plaques in neurons. Increased plasma concentrations of each of these proteins, or subunits of these proteins for different phosphorylated tau proteins, ex. Thr181 and Thr231, or, in the case of Aβ, the decrease in relative ratio of Aβ42/40, have great potential in the clinical diagnosis of AD. While many of these show strong potential as biomarkers on their own, identifying strong co-expressed markers should increase the robustness of a diagnostic test and the specificity of that test for AD diagnosis over other neurodegenerative diseases.

Aims - Develop a microfluidic platform capable to detecting colocalized proteins on the surface of exosomes for use in AD diagnostics.

Methodology - Plasma from 5 Alzheimer's patients and 5 healthy control patients of similar age and ethnicities was collected for this study and exosomes were purified by centrifugation. Measurements of relative colocalized surface concentration were made using ion-exchange membrane sensors developed and reported previously by the Chang lab. Measurements were conducted with a sandwich assay to capture a specific surface marker with an antibody anchored to the sensor membrane surface and a secondary reporter antibody functionalized to the surface of a silica reporter particle. Antibodies for NfL, GFAP, phosphorylated tau(p-Tau)181, p-Tau217, p-Tau231, amyloid beta and CD63 were used for capture and antibodies for L1 cell adhesion molecule (L1CAM) and CD63 were used as reporters.
Results - NfL, GFAP and different phosphorylated tau levels on the surface of NDEs were significantly increased compared to healthy patients. P-Tau181, p-Tau217 and p-Tau231 on the surface of neuro-derived exosomes were found to have the strongest independent markers of neurodegeneration. Amyloid beta on the surface of neuro-derived exosomes was found to be a poor independent measure of AD in patients.

Conclusions - This work shows the potential for diagnosis of AD and other neurological diseases using colocalized exosomal surface proteins. Very few studies have looked at the relative concentrations of exosomal surface proteins as potential biomarkers in AD and other dementia patients, nor have the relative surface concentrations been paneled as they have here. The use of these anion exchange-based sensors allows for a rapid test that can screen multiple NDE targets simultaneously with the sensitivity to detect their concentrations in plasma.

Martin Ng
University of California, San Francisco, San Francisco VA Medical Center, NCIRE

Refining Methods for Isolating & Detecting exRNA Carriers

Martin Ng1,2, Alex Gao1,2, Robert L. Raffai1,2,3
1. Department of Veterans Affairs, Surgical Service (112G), San Francisco VA Medical Center, San Francisco, CA 94121, USA.
2. Northern California Institute for Research and Education, San Francisco, CA 94121, USA.
3. Department of Surgery, Division of Endovascular and Vascular Surgery, University of California, San 16 Francisco, CA 94143, USA.

Background - Extracellular RNAs (exRNA) are increasingly investigated as biomarkers of human disease onset and resolution, as well as effectors of intercellular signaling in complex biological networks. Despite mounting enthusiasm in the study of exRNA in molecular medicine, the field is hampered by technical limitations that stem from their isolation and detection. Complexities associated with the study of exRNA arise from the fact that they do not exist as free molecular structures, but rather are associated with carriers that protect them from hydrolysis and ensure their delivery to target tissues and cells.

Aims - As participants of the NIH ERCC, our laboratory has led the way in refining techniques to isolate and detect exRNA carriers based on their biophysical and biological properties, with the goal of advancing the study of exRNA biology in human personalized medicine.

Methodology - To address the challenge of resolving the diversity of exRNA carrier species present in biofluids and conditioned cell culture media, such as extracellular vesicles and lipoproteins, we combined the use of cushioned-density gradient ultracentrifugation (C-DGUC), sequential density ultracentrifugation (SD-UC), size-exclusion chromatography (SEC), and immunoaffinity capture-based bead systems in collaboration with the Laurent lab. Furthermore, to address the challenge of accurately detecting extracellular vesicles (EVs) in biofluids, we have been collaborating with Dr. John Nolan to establish the use of vesicle flow cytometry (vFC). The sources of exRNA carriers used in our studies consisted of conditioned cell culture media and plasma. They included primary mouse and human THP1 macrophages, cancer cells including HEK293, DiFi, Kuramochi, and iPSC cells, as well as mouse and human plasma.

Results - Our first series of studies sought to improve the resolution of EVs by combining C-DGUC fractionation to Izon SEC. We did so by first resolving conditioned cell culture media using C-DGUC, followed by pooling EV-rich CD81-positive fractions 6-8 that were subsequently applied onto a first generation, 35nm qEV original column. This two-step approach achieved an improvement in EV purity and size resolution of a defined density. Outcomes of resolving EVs by this approach was probed with RNA sequencing in the Laurent lab and mass spectrometry in the Liu lab, while surface-enhanced Raman spectroscopy (SERS) was performed in the Xie lab.

To broaden our exRNA isolation and detection approaches, we implemented the use of immunoaffinity-based bead pulldowns and vesicle flow cytometry. Anti-human tetraspanin magnetic beads from Miltenyi served to isolate human plasma EVs, while anti-human APOB/E/AI IgG, coupled to Spherotech magnetic beads, will serve to isolate lipoprotein subclasses. ExRNAs present in these carriers will next be examined using qRT-PCR and RNA-sequencing in collaboration with the Jensen and Laurent labs. Finally, ongoing collaborations with the Nolan lab has established the use of vesicle flow cytometry as a robust tool to identify and distinguish EVs in cell conditioned media and biofluids.

Conclusions - Our efforts have led to new methods to improve the isolation and detection of exRNA carriers from cell conditioned media and biofluids that will help advance their study as biomarkers and effectors of human health and diseases.

Poster Abstract # 5

Himani Sharma

University of Notre Dame

A scalable, high-throughput isoelectric fractionation platform for exRNA nanocarriers: Comprehensive and bias-free isolation of EVs, lipoproteins (LLPs), and Ribonucleoproteins (RNPs) from blood plasma, urine, and saliva

Himani Sharma1, Vivek Yadav1, Crislyn D'Souza-Schorey3,4, David B. Go1,2, Satyajyoti Senapati1, Hsueh-Chia Chang1,2,3

1. Department of Chemical and Biomolecular Engineering, University of Notre Dame
2. Department of Aerospace and Mechanical Engineering, University of Notre Dame
3. Harper Cancer Research Institute, University of Notre Dame
4. Department of Biological Sciences, University of Notre Dame

Background: Extracellular nanocarriers (EVs, lipoproteins, and ribonucleoproteins) of proteins and nucleic acids mediate intercellular communication and are clinically adaptable as circulating biomarkers. Both the nanocarrier identity and their molecular cargo are relevant for detecting and characterizing various diseases. However, the overlapping size and density of the nanocarriers have so far prevented their efficient physical fractionation, thus impeding independent downstream molecular assays. Moreover, affinity-based and liquid extraction technologies tend to exhibit significant bias and contamination, thus corrupting the quantification of expression level. Hence, there is a need for a new technology to provide bias-free, high-throughput, and high-yield nanocarrier fractionation.

Aims: Our aim is to develop a novel free-flow isoelectric focusing (charge-based) technique to fractionate different exRNA-nanocarriers at high throughput, yield, and purity from various biofluids (plasma, urine, and saliva).

Methodology: Our platform, called the Continuous Isoelectric Fractionation (CIF) device, consists of two modules of microfluidic chips: (i) a pH gradient chip and (ii) a separation chip. The pH gradient chip incorporates a pair of bipolar membranes, which split water into H3O+ and OH– ions under optimized reverse bias voltage due to the Wien effect. The separated H3O+ and OH– ions are separated and stabilized by flow (~12 ml/hour), zero-flux Boltzmann distributions of the inert buffer ions, as well as rapid and reversible water dissociation reaction to form a linear pH gradient (pH 3 to 11) without using commercial ampholytes. Depending on the application, the desired portion of the effluent from the pH gradient chip is then injected downstream into a separation chip(s) to produce a high-resolution pH gradient where the nanocarrier mixture is injected (~3 ml/hour) and separated based on their charge (isoelectric point). To facilitate desired pH transfer from the pH gradient chip to the separation chip, a machine learning procedure is developed. For quantitative assessments, the effluents collected from all the outlets of the chip were qualitatively analyzed by various methods such as ELISA, gel electrophoresis, zeta potential, and TEM images.

Results and Conclusion: We optimize the CIF technology by fractionating various combinations of binary mixtures of exRNA nanocarriers (EVs, LLPs, and RNPs) spiked in 1x PBS buffer (yield >80% and purity >90%). Its performance is then evaluated with several biofluids, including plasma, urine, and saliva samples. Comprehensive, high-purity (plasma: >93%, urine: >95%, and saliva: >97%), high-yield (plasma: >78%, urine: >87%, and saliva: >96%), and probe-free isolation of ribonucleoproteins in 0.75 ml samples of various biofluids in 30 minutes is demonstrated, significantly outperforming affinity-based and highly biased gold standards having low yield and day-long protocols. The optimized technique has a resolution of 0.3 ΔpI, sufficient to separate many nanocarriers and even subclasses of nanocarriers. This device represents a significant advancement as it overcomes the various limitations of commonly utilized traditional isolation technologies, including ultracentrifugation, immunocapture, and ultrafiltration techniques.

Juan Pablo Tosar
Institut Pasteur de Montevideo

RNA as its own carrier: nicked tRNAs are stable reservoirs of tRNA halves in biofluids

Bruno Costa 1 2, Marco Li Calzi 1, Mauricio Castellano 1 3, Valentina Blanco 1 3, Ernesto Cuevasanta 2 4 5, Irene Litvan 6, Pavel Ivanov 7, Kenneth Witwer 8, Alfonso Cayota 1 9, Juan Pablo Tosar 1 2
1. Functional Genomics Laboratory, Institut Pasteur de Montevideo, Montevideo 11400, Uruguay.
2. Analytical Biochemistry Unit, Center for Nuclear Research, School of Science, Universidad de la República, Montevideo 11400, Uruguay.
3. Biochemistry Department, School of Science, Universidad de la República, Montevideo 11400, Uruguay.
4. Laboratory of Enzymology, School of Science, Universidad de la República, Montevideo 11400, Uruguay.
5. Centro de Investigaciones Biomédicas, Universidad de la República, Montevideo 11800, Uruguay.
6. Department of Neurosciences, University of California San Diego, La Jolla, CA 92093.
7. Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115.
8. Molecular and Comparative Pathobiology, Neurology, and The Richman Family Precision Medicine Center of Excellence in Alzheimer's Disease, Johns Hopkins University School of Medicine, Baltimore, MD 21205.
9. Hospital de Clínicas, Universidad de la República, Montevideo 11600, Uruguay.

Nonvesicular extracellular RNAs (nv-exRNAs) are abundant in cell-conditioned media and biofluids, but little is known about their stability, function, and potential use as disease biomarkers. Herein, we measured the stability of several naked RNAs when incubated in human serum, urine, and cerebrospinal fluid (CSF). We identified extracellularly produced tRNA-derived small RNAs (tDRs) with half-lives of several hours in CSF. Contrary to widespread assumptions, these intrinsically stable small RNAs are full-length tRNAs containing broken phosphodiester bonds (i.e., nicked tRNAs). Standard molecular biology protocols, including phenol-based RNA extraction and heat, induce the artifactual denaturation of nicked tRNAs and the consequent in vitro production of tDRs. Broken bonds are roadblocks for reverse transcriptases, preventing amplification and/or sequencing of nicked tRNAs in their native state. To solve this, we performed enzymatic repair of nicked tRNAs purified under native conditions, harnessing the intrinsic activity of phage and bacterial tRNA repair systems. Enzymatic repair regenerated an RNase R-resistant tRNA-sized band in northern blot and enabled RT-PCR amplification of full-length tRNAs. We also separated nicked tRNAs from tDRs by chromatographic methods under native conditions, identifying nicked tRNAs inside stressed cells and in vesicle-depleted human biofluids. Dissociation of nicked tRNAs produces single-stranded tDRs that can be spontaneously taken up by human epithelial cells, positioning stable nv-exRNAs as potentially relevant players in intercellular communication pathways.

Jack Jingyuan Zheng
University of California, Davis

Measurement of high-density lipoprotein particle size in clinical samples using transmission electron microscope provides better resolution for particle size and subgroup analysis

Jack Jingyuan Zheng1, Brian Vannak Hong1, Joanne K. Agus1, Xinyu Tang1, Nola Klebaner1, Fei Guo2, Carlito B. Lebrilla3, Izumi Maezawa4, Lee-Way Jing4, Angela M. Zivkovic1
1. University of California, Davis, Department of Nutrition. CA, 95616, USA.
2. University of California, Davis, Department of Molecular and Cellular Biology. CA, 95616, USA.
3. University of California, Davis, Department of Chemistry. CA, 95616, USA.
4. University of California, Davis, Department of Pathology and Laboratory Medicine, School of Medicine, CA 95817, USA

Introduction: Various technologies exist for assessing extracellular vesicle and particle size. Negative-stain transmission electron microscopy (NS-TEM) allows direct visualization and precise size quantification of individual particles, features that are not obtainable by most other sizing methods. Yet it is rarely used in a clinical scale, mainly for its laborious procedures and training requirements.

Methods: In this study, we report the use an NS-TEM protocol to quantify high-density lipoprotein (HDL) particles isolated from human subjects with Alzheimer's disease (AD), with mild cognitive impairment (MCI), age-matched without dementia (age-Control), or young without dementia (Control). With approval from the UCD Institutional Review Board, samples were collected from the University of California, Davis (UCD) Alzheimer's Disease Research Center Biorepository with participant consents, and from the Ragle Human Nutrition Center, UCD. HDL samples were isolated from plasma using density-adjusted ultra-centrifugation followed by size-exclusion chromatography. Samples were negatively-stained using 2% uranyl formate and viewed using a JEOL 1230 model TEM at 40k magnification, 120 kV. Micrographs were obtained using bottom-mounted Ceta camera (4k × 4k pixels) and particle size analyzed using ImageJ software. The size data from NS-TEM to that reported by dynamic light scattering (DLS), a commonly used sizing method, and ultraviolet light absorption were compared.

Results: Particle size results reported by NS-TEM have higher repeatability than DLS (inter-run CV of 0.34% vs. 2.8% for size standards, and 3.503% vs. 10.43% for biological samples, respectively). Particle size distributions of HDL showed that specific subgroups had different abundance between the AD, MCI, age-Control, and the Control groups that were not observable by other sizing techniques.

Conclusion: We reason that NS-TEM provides high resolution data, is less confounded by random and directional effects common in other sizing methods, and should therefore be practiced more frequently in HDL particle size studies.

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Akanksha Arora
Indraprastha Institute of Information Technology (IIIT)

SalivaDB - A comprehensive database for salivary biomarkers in humans

Akanksha Arora, Dashleen Kaur, Sumeet Patiya, Dilraj Kaur, Ritu Tomer, Gajendra P. S. Raghava
Indraprastha Institute of Information Technology, Delhi, India

Background and objective: Saliva as a non-invasive diagnostic fluid has immense potential as a tool for early diagnosis and prognosis of patients. The information about salivary biomarkers is broadly scattered across various resources and research papers. It is important to bring together all the information on salivary biomarkers to a single platform. This will accelerate research and development in non-invasive diagnosis and prognosis of complex diseases.

Methods: We collected widespread information on five types of salivary biomarkers – proteins, metabolites, microbes, miRNA, and genes found in humans. This information was collected from different resources that include PubMed, HMDB and SalivaTecDB.

Results: Our database SalivaDB contains a total of 15821 entries for 201 different diseases and 48 disease categories. These entries can be classified into five categories based on type of biomolecules; 6067, 3987, 2909, 2272, and 586 entries belong to proteins, metabolites, microbes, miRNAs, and genes respectively. The information maintained in this database includes analysis methods, associated diseases, biomarker type, regulation status, exosomal origin, fold change, and sequence. The entries are linked to relevant biological databases to provide users with comprehensive information. We developed a web-based interface that provides a wide-range of options like browse, keyword search, and advanced search. In addition, a similarity search module has been integrated that allows users to perform a similarity search using BLAST and Smith-Waterman against biomarker sequences in SalivaDB.

Conclusions: We created a web-based database – SalivaDB, which provides information about Salivary Biomarkers found in humans. A wide-range of web-based facilities have been integrated to provide services to the scientific community. SalivaDB is freely available at - https://webs.iiitd.edu.in/raghava/salivadb/

Poster Abstract # 9

Thamara Dayarathna

Nationwide Children's Hospital

Multiparametric characterization of polarized microglial-derived extracellular vesicle (EV) mRNA cargo: shedding new light on neuroimmune crosstalk

Thamara Dayarathna1, Paola Loreto Palacio1, Michael Parthun2, Mireia Guerau-de-Arellano2, Bradley Butsch1, Kim Truc Nguyen2, Eduardo Reátegui2, Setty M. Magaña1*

1. Translational Neuroimmunology, Center for Clinical and Translational Research, Nationwide Children's Hospital, Ohio, OH

2. School of Health and Rehabilitation Sciences, The Ohio State University, Ohio, OH

3. Department of Chemical and Biomedical Engineering, The Ohio State University, Ohio, OH

Background: Microglia are the resident macrophages of the central nervous system (CNS) and are key regulators of neuroinflammation. Microglia are capable of releasing EVs—however, the role of microglial EVs in propagating CNS neuroinflammation is not fully characterized. Increasing our understanding of microglial EV and EV-mRNA cargo can shed light on mechanisms of neuroimmune crosstalk and help expand the therapeutic landscape. The objectives of this study are 1) to explore the mRNA repertoire in cellular vs. EVs from polarized microglia and macrophages 2) to determine if EVs secreted from polarized cells (i.e. pro- or anti-inflammatory phenotype) can modulate the polarization of resting microglia or macrophages, 3) to establish EV-pro and anti-inflammatory positive controls for future studies.

Methods: Two macrophage cell lines were utilized in this study (HMC3—microglia; THP1—macrophage). Both cell lines were polarized into M1 and M2 phenotypes using IFNy/LPS and IL-10/IL-4 respectively. Cells and EV RNA were analyzed via RT-qPCR to determine the expression levels of M1 and M2 related genes. Cells were also analyzed via immunoblotting and immunocytochemistry to determine the presence of M1 and M2 markers. EVs were isolated from microglia and macrophage cell culture media using tangential flow filtration (TFF). Microfluidic resistive pulse sensing (MRPS) measured particle size and concentration. EVs markers were characterized via immunoblotting and Exoview. EV morphology was assessed by transmission electron microscopy (TEM).

Results: Microglial and macrophage polarization resulted in increased expression of expected M1 and M2 genes and proteins. The THP1 cells were more consistent and reproducible. EVs from M1 and M2 microglia and macrophage conditioned media exhibited classic EV markers and M1/M2 candidates. TEM demonstrated a cup-shaped morphology characteristic of EVs. Co-cultures of EVs derived from M1 and M2 cells with resting macrophages and microglia will be investigated. Cross-validation of EV characterization via Exoview and MRPS are ongoing. Co-cultures with polarized EVs will follow.

Conclusions: In this study, we performed multiparametric characterization of EVs derived from microglia and macrophage cell lines. Exploration of EV vs. cellular mRNA and protein targets will shed light on the potential role of macrophage derived EVs in propagating neuroinflammation. Co-cultures of polarized EVs with resting macrophages will allow investigation of the immunomodulatory role of M1 and M2 EVs. Lastly, establishment of M1 and M2 phenotype EV positive controls will increase reproducibility of subsequent studies.

Julia Denniss
National Institute of Neurological Disorders and Stroke

Extracellular vesicle-derived HIV RNA in CSF and serum reservoirs and its role in mediating neurocognitive dysfunction

Julia Denniss, B.S.1, Catherine DeMarino, PhD1, Bryan Smith, MD1, Joseph Snow, PhD2, Lisa Henderson, PhD1, Darshan Pandya, MD1, Avindra Nath, MD1
1. Section of Infections of the Nervous System, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD.
2. National Institute of Mental Health, National Institutes of Health, Bethesda, MD

Approximately 37 million people are infected with HIV-1, which causes acquired immunodeficiency syndrome (AIDS) and is among leading global causes of both mortality and burden. While combined antiretroviral therapy (cART) has extended life expectancy by targeting the virus at nearly every stage of its life cycle, there remain no inhibitors of transcription, and the integrated provirus retains its capability to produce viral RNAs, which have been detected in patient serum and cerebrospinal fluid (CSF). HIV-associated neurocognitive disorders (HANDs) still represent a significant long-term burden and affect up to 30-50% of those being treated with cART. The degree of neurocognitive impairment can be assessed with clinical scores of global deficit, attention/working memory, verbal fluency, and other categories.

Extracellular vesicles (EVs) are a varied class of membrane-bound cell components released from most cell types which have been shown to carry lipids, proteins, mRNAs, non-coding RNAs, and DNA. It has been shown that exosomes, a subtype of EVs, can transport HIV-1 proteins and RNA from infected cells to uninfected cells even in the presence of cART. Additionally, it was found that exosomes from uninfected cells can stimulate viral transcription in infected cells.

Having previously demonstrated the presence of HIV-1 RNA in EVs isolated from patient CSF and serum in a cohort of HIV-infected individuals with well-controlled disease on cART, we used seven Digital Droplet PCR (ddPCR) assays to evaluate the transcriptional status of the reservoir at various sequence regions, including (i) readthrough (affected by transcriptional interference) (ii) TAR (required for transcriptional initiation) (iii) R-U5/pre-Gag (long LTR, suggests elongation proximal to the 5’ end) (iv) Pol (indicating elongation past the region of the initial Gag viral protein) (v) Nef (distal protein) (vi) U3-polyA (indicating complete transcription of the HIV protein) and (vii) Tat-Rev (the multiply-spliced complete protein, indicating productive infection).

We found that levels of the RNA transcripts differed significantly depending on the reservoir from which they were derived. The transcripts were found at higher levels in the CSF, suggesting poorer viral control in the CNS reservoir. Additionally, the percent of patients with detectable levels of viral transcripts (defined as a concentration > 0) differed by reservoir. We did not find a correlation between matched serum and CSF RNA concentrations. These findings provide support to our hypothesis of a separate CNS reservoir with a unique transcriptional profile.

Our results indicate a significant positive correlation between R-U5/pre-Gag (Long LTR) and global deficit score (which increases with worsening deficit) and significant negative correlations between Long LTR and attention/working memory, information processing, and verbal fluency, as well as a summative T-score of the seven categories (all of which decrease with worsening deficit). The Long LTR transcript serves as a scaffold which recruits suppressive transcription remodelers, contributing to viral latency via epigenetic silencing. Our findings suggest that there is basal viral transcription associated with neurological complications of long-term disease. This novel mechanism, which contributes to neurological dysfunction, could be targeted to improve quality of life and achieve a functional cure for HIV-1 infection.

Meghan Muse
Dartmouth College Geisel School of Medicine

Relationships Between Human Milk EVP miRNA Content and Attained Infant Anthropometry at One Year: A Pilot Study

Meghan E Muse1, David A Armstrong2,3, Anne G Hoen1,4, Diane Gilbert-Diamond1, Jiang Gui4, Thomas J Palys1, Brock C Christensen1, Margaret R Karagas1, Caitlin G Howe1
1. Department of Epidemiology, Geisel School of Medicine at Dartmouth, 1 Medical Center Dr, Lebanon, NH, USA
2. Department of Dermatology, Dartmouth-Hitchcock Medical Center, 1 Medical Center Dr, Lebanon, NH, USA
3. Research Service, VA Medical Center, 15 N Main St, White River Junction, VT, USA
4. Department of Biomedical Data Science, Geisel School of Medicine at Dartmouth, 1 Medical Center Dr, Lebanon, NH, USA

Background: Extracellular vesicle and particle microRNAs (EVP miRNAs) in human milk are hypothesized to facilitate communication between mother and child during the postnatal period. While an emerging body of literature has begun to characterize the composition of miRNAs in human milk EVPs, little is known about their potential roles in infant growth.

Aims: Our objective was to assess potential associations between the composition of EVP miRNAs in human milk and attained anthropometry at 12 months of age in a pilot study of breastfed infants in New Hampshire.

Methodology: EVP miRNAs were extracted from the supernatant fraction of human milk samples (n = 48) collected approximately 6 weeks postpartum from participants in the New Hampshire Birth Cohort Study. The Nanostring nCounter platform was used to profile levels of 798 miRNAs. Three measures of milk EVP miRNA content were evaluated: 1) the total quantity of EVP miRNA transcripts (log2-transformed), 2) the number of unique miRNA transcripts present (sample richness), and 3) sample evenness, calculated as the Shannon Diversity Index divided by the natural log of the sample richness score, bound between 0 and 1. We assessed relationships between each measure of EVP miRNA composition with age and sex-standardized WHO Z scores for infant weight, length, weight-for-length, and head circumference measured at approximately 12 months of age, using robust linear regression, adjusting for gestational age at delivery, anthropometry at birth, and duration of breast feeding.

Results: Infant weight (Beta = 0.304, P = 0.011) and length (Beta = 0.421, P = 0.005) z-scores at 12 months were positively associated with total EVP miRNA content. EVP miRNA evenness was inversely associated with infant weight (Beta = -5.763, P = 0.022), length (Beta = -6.789, P = 0.039), and head circumference (Beta = -6.959, P = 0.017) z-scores at 12 months.

Conclusions: In a pilot study of 48 breastfed infants in New Hampshire, the EVP miRNA composition of human milk was associated with infant attained weight, length, and head circumference at one year. Given the absence of associations with weight-for-length, and the consistent directionality of the associations for weight and length, our findings additionally suggest that milk EVP miRNA composition influences growth, but not adiposity, of infants. Observed inverse associations with sample evenness may suggest that increased presence of specific EVP miRNAs are associated with higher measures of infant anthropometry. However, larger studies are needed to confirm these findings and to investigate whether specific miRNAs in human milk EVPs influence growth and/or adiposity in infants.

Truc Nguyen
The Ohio State University

Predicting Responses to Immunotherapy of Non-Small Cell Lung Cancer Patients via Single Extracellular Vesicular mRNA and Protein Detection

Luong T. H. Nguyen1†, Jingjing Zhang1†, Xilal Y. Rima1†, Xinyu Wang1, Kim Truc Nguyen1, Kwang Joo Kwak2, Tamio Okimoto3, Joseph Amann3, Min Jin Yoon1, Takehito Shukuya3,4, Chi-Ling Chiang1, Nicole Walters1, Yifan Ma1, Donald Belcher1, Hong Li1, Andre F. P
1. William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University; Columbus, OH 43210, USA.
2. Spot Biosystems Ltd.; Palo Alto, CA 94301, USA.
3. Comprehensive Cancer Center, The Ohio State University; Columbus, OH 43210, USA.
4. Department of Respiratory Medicine, Juntendo University; Tokyo, Japan.
†The authors contributed equally to this work
*Corresponding author. reategui.8@osu.edu

Background: NSCLC cells enhance their ability to evade immune responses by increasing the expression of immune checkpoint proteins, which can be targeted to improve survival rates. Although immunohistochemical (IHC) staining of tissue samples for PD-L1 protein can identify suitable candidates for immune checkpoint inhibition (ICI), this method has limitations such as invasiveness, poor spatiotemporal resolution, and an accuracy rate of 20-40%.

Aim: A non-invasive assay called AuSERP (immunogold single extravesicular RNA and protein assay) was developed to identify candidates suitable for immune checkpoint inhibition (ICI) by measuring PD-1/PD-L1 proteins and RNA on individual non-lysed extracellular vesicles (EVs).

Methods: The AuSERP assay employed a gold-coated coverslip that was modified with gold nanoparticles coated with antibodies to separate individual extracellular vesicles (EVs) based on their membrane antigens. These isolated EVs were then analyzed for proteins and mRNA with low abundance using tyramide signal amplification and molecular beacons contained within cationic lipoplex nanoparticles.

Results: Fluorescent signal was observed with total internal reflection fluorescence microscopy to detect both protein and mRNA of PD-1/PD-L1 in individual extracellular vesicles (EVs). PD-L1 protein and mRNA expression were measured in EVs obtained from IFN-γ-stimulated H1568 cells mixed with healthy donor serum, using ELISA/qRT-PCR and AuSERP assays. Notably, the AuSERP assay was found to be 1000 times more sensitive than ELISA/qRT-PCR. A small cohort of 54 NSCLC patients was recruited before undergoing immunotherapy, out of which 27 patients exhibited positive responses while 27 patients did not. These four biomarkers were evaluated in combination, and the results demonstrated that incorporating both PD-1/PD-L1 mRNA increased the assay sensitivity, leading to an NSCLC diagnosis accuracy of 93.2% and a prediction accuracy of ICI response of 72.2%.

Conclusions: A novel assay was created to detect low-abundance protein and mRNA molecules simultaneously in single non-lysed extracellular vesicles (EVs) with high sensitivity. The use of single-EV detection instead of bulk-analysis methods led to a notable improvement in sensitivity, making it a promising alternative to immunohistochemistry (IHC) and a significant advancement for immune checkpoint inhibitor (ICI) therapy.

Xilal Rima

The Ohio State University

Sorting Single Extracellular Vesicles and Bioparticles and Simultaneously Detecting RNA and Protein In Situ on a Microdomain-Array Assay

Xilal Y. Rima,1† Jingjing Zhang,1† Xinyu Wang,1† Luong T. H. Nguyen,1 Kristin Huntoon,2,3 Yifan Ma,1 Paola Loreto Palacio,4 Kim Truc Nguyen,1 Karunya Albert,1 Minh-Dao Duong-Thi,1 Nicole Walters,1 Kwang Joo Kwak,5 Min Jin Yoon,1 Thamara Dayarathna,4 Hong

1. William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, OH 43210

2. Department of Neurosurgery, The University of Texas MD Anderson Cancer Center,
Houston, TX 77030

3. The Brain Tumor Center, The University of Texas MD Anderson Cancer Center, Houston, TX 77030

4. Department of Pediatrics, Division of Neurology, Nationwide Children's Hospital, Columbus, OH 43205

5. Spot Biosystems Ltd., Palo Alto, CA 94301

6. Department of Radiation Oncology, The University of Texas Southwestern Medical Center, Dallas, TX 75235

7. Institute for Systems Biology, Seattle, WA 98109

8. Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210

†JZ, XYR, and XW contributed equally to this work

*corresponding author

Background: Extracellular vesicles (EVs) are heterogeneous physically and molecularly and the co-isolation of similar bioparticles confounds their characterization. In situ detection of single EVs has augmented sensitivities and aided in qualitatively demonstrating intravesicular heterogeneity. Herein, we demonstrate single-particle detection of EVs and lipoproteins (LPPs) on controllable microdomains and highlight the importance of single-EV detection in analyzing intervesicular, intravesicular, and interparticle heterogeneity.

Aims: Develop a customizable single-EV in situ method for the facile colocalization of biomolecular species, including protein, miRNA, and miRNA in non-lysed single EVs and other bioparticles.

Methodology: Microdomains for particulate capture were generated via photoetching of a non-biofouling polymer monolayer on a glass coverslip, allowing for the adsorption of avidin and subsequently biotinylated antibodies to immobilize single bioparticles on the coverslip surface. Intravesicular heterogeneity investigations were realized with the use of fluorescently-labeled antibodies and molecular beacons to target protein and RNA biomarkers in single non-lysed bioparticles. Intervesicular and interparticle investigations were performed by customizing the antibodies utilized to immobilize the bioparticles.

Results: Being at the forefront of single-EV RNA in situ detection, an in-depth characterization of exogeneous RNA transfected in human EVs was carried, whereby three miRNA species were co-detected in single non-lysed EVs. Colocalization analyses were performed for different regions of an mRNA strand, tetraspanin analyses, and protein-RNA interspecies detection, demonstrating the breadth of the technique. Intervesicular heterogeneity investigations were performed by immobilizing EVs into ARF6, annexin A1, CD63, CD9, EGFR, and IgG subpopulations and detecting nine biomarkers spanning across protein, mRNA, and miRNA. Clustering of the biomolecular species and linear discriminant analysis demonstrated similarities between CD63+ and CD9+ subpopulations, while ARF6+ and annexin A1+ subpopulations demonstrated similarities, with the former providing enhanced biomolecular profiling. Furthermore, due to the co-isolation of LPPs and EVs, interparticle heterogeneity was investigated by decorating the microdomains with anti-CD63/CD9 and anti-apolipoprotein A1/B cocktails. Herein, we demonstrated the presence of CD63+/CD9+ LPPs that are obscured in bulk-analysis methods. Lastly, to illustrate the clinical utility of the technique for diagnosis, six glioma cell lines were subjected to small RNA sequencing, whereby four RNA species were found to be the most upregulated. Serum from a small cohort of 10 glioblastoma patients and 10 healthy donors was collected whereby the EVs were isolated and screened for the RNA species. Despite the possible interaction of CD63+/CD9+ LPPs glioblastoma patients demonstrated significantly higher levels of the vesicular RNA.

Conclusions: The success of this work opens avenues in the investigations of intravesicular, intervesicular, and interparticle heterogeneity for biomarker discovery, diagnostics, biogenesis investigations, and deconvolution of circulatory bioparticle heterogeneity.

Michail Spanos

Massachusetts General Hospital / Harvard Medical School

Tissue-Specific RNA Cargo of Plasma Extracellular Vesicles in COVID-19 Pathogenesis: A Dissection of EV RNA Origins and Pathways

Michail Spanos1*, Priyanka Gokulnath1*, Elizabeth Hutchins3, Bessie Meechoovet3, Sophia LY Root1, Emeli Chatterjee1, Guoping Li1, Ana Chiaretti1,Elvira-Markela Antonogiannaki2, Vassiliki Apollonatou2, Eirini Maratou4, Effrosyni D. Manali2, Jasmin Shehade

1. Cardiovascular Research Center, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA.
2. 2nd Pulmonary Medicine Department, General University Hospital Attikon, Medical School, National and Kapodistrian University of Athens
3. Division of Neurogenomics, The Translational Genomics Research Institute, Phoenix, AZ, USA
4. Second Department of Internal Medicine and Research Institute, General University Hospital "Attikon", School of Medicine, National and Kapodistrian University of Athens, Athens, Greece

Background - COVID-19 is an infectious disease caused by SARS-CoV-2, which has rapidly spread across the globe, causing significant morbidity and mortality. Although substantial progress has been made in understanding the clinical manifestations of COVID-19, the precise mechanisms that drive disease severity remain poorly understood. Extracellular vesicles (EVs) have emerged as key players in intercellular communication, and their RNA cargo has been shown to be involved in the pathogenesis of various diseases. However, the specific tissue or cell type origin of EVs that drive COVID-19 severity is currently unknown. By characterizing the RNA content of severe COVID-19 patients EVs we sought to identify the specific tissue signatures contributing to the EV RNA pool that drives COVID-19 pathogenesis.

Aim - We aimed to define the tissue or cell origin of plasma EV RNA signatures in COVID-19, focusing on the identification of tissue-specific RNAs within EVs that could help to determine the tissues and pathways that are affected by or contribute to severe disease.

Methodology - To identify EV RNA cargo implicated in severe disease, plasma from hospitalized (severe) and non-hospitalized (mild/moderate) COVID-19 patients was collected. Plasma EV RNA was sequenced and differential expression (DE) analysis between groups was performed using DESeq2 v1.26.0. To determine the tissue origin of the differentially expressed EV RNAs Tissue-Specific Enrichment Analysis (TSEA) using the TissueEnrich R package was used. Each gene was assigned a Tau tissue specificity score using the tispec package with CPM normalized count data from GTeX v8 as input. Integrative Genomics Viewer (IGV) was used to overlay EV RNA fragments for primer design. Tissue specific EV RNAs were validated by qPCR in a cohort of patients with moderate (non-ICU, n=12), severe (ICU, n=12) COVID-19 and healthy controls (n=12).

Results - Patients with severe COVID-19 had 64 upregulated EV RNAs originating from adipose tissue, salivary glands, and thyroid glands as well as the heart, spleen, skeletal muscle, and brain. Pathway analysis showed that these genes were part of muscle contraction, developmental, and ion channel pathways. The downregulated EV RNAs in those patients were 677 and derived mostly from immune-related tissues such as bone marrow, spleen, lymph nodes, tonsils, and appendix. Downregulated EV transcripts were mostly associated with translation-related pathways, cytokine signaling, and vesicle-mediated transport. Tissue specific EV RNAs AL133373.2, PRSS16 (pancreas), ANK1, MB (skeletal muscle), SYT3, KCNJ6 (brain), AHNAK (peripheral nerve), BCL2A, HSPA8, EZR (lung), HLA-A (immune) were significantly deregulated in severe COVID-19 patients of the validation cohort.

Conclusions - Our findings revealed that plasma EVs RNA from COVID-19 patients were derived from a range of tissues, including bone marrow, heart, lung, liver, and kidney, indicating the multi-organ involvement in COVID-19 pathogenesis. Furthermore, we identified specific RNA signatures associated with severe disease, suggesting potential biomarkers of disease severity. Overall, our results highlight the importance of EV RNA cargo in COVID-19 pathogenesis and offer valuable insights into the underlying mechanisms driving disease severity. Our approach could have important implications for the development of targeted therapies aimed at specific cell or tissue types and ultimately, the improvement of patient outcomes.

Lingfei Sun
Massachusetts General Hospital

β-cell-specific extracellular vesicles as an early diagnostic biomarker of Type 1 Diabetes

Lingfei Sun[1], Michail Spanos[1], Ritin Sharma[3], Khadija EI Jellas[2], Patrick Pirrotte[3], Rohit Kulkarni[2], Guoping Li[1], and Saumya Das[1]
1. Cardiovascular Research Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA.
2. Islet Cell and Regenerative Biology, Joslin Diabetes Center; Department of Medicine, BIDMC, Harvard Medical School, Boston, MA, USA.
3. Translational Genomics Research Institute, City of Hope Medical Center, Phoenix, AZ, USA

Background: Type 1 diabetes (T1D), a chronic disease characterized by a gradual loss of insulin-producing β-cell function over time due to the autoimmune death of β-cells, affects nearly 2 million people in the U.S. However, the clinical diagnosis of T1D is usually made in the presence of fasting hyperglycemia, by which time the majority of functional β cell mass has already been lost. Therefore, there is an urgent need to identify a novel circulating biomarker for the earlier diagnosis of T1D. Extracellular vesicles (EVs) are phospholipid bilayer-enclosed nanosized particles that can be detected and assayed in virtually all human biofluids. Their cargo, including small and long RNA transcripts may serve both as novel mediators of intercellular signaling and contain potential biomarkers of cellular stress.

Aims: The work aims to characterize β-cell-specific EVs in circulation and identify the signatures of β-cell-derived EVs during early T1D development, which may be translated into novel diagnostics for identifying patients with early T1D.

Methodology: EndoC-βH1 cells are used to interrogate the β-cell specificity and model T1D development. In detail, EndoC-βH1 cells are treated with inflammatory cytokines (IL-1β and IFN-γ) for 48 hours to recreate the T1D environment. Cellular RNAs are extracted and analyzed to confirm the stress response of cells under cytokine challenges. EVs are enriched using size-exclusion chromatography and characterized using Spectradyne and Dot/Western blotting. Subsequently, the expression of miRNAs, tRNAs, and tRNA-derived small RNAs (tDRs) in these EVs are examined by using digital droplet PCR and northern blotting.

Results: Cellular RNA analysis shows a 20-fold increase in PD-L1, a 3.5-fold increase in ERAP1, and an 11-fold increase in ERAP2 in EndoC-βH1 cells treated with inflammatory cytokines, indicating the successful modeling of T1D in vitro. Spectradyne detected approximately 10E9 EV particles/mL conditional cell culture medium, and Dot/Western blotting confirmed their EV identity. Digital droplet PCR demonstrated the abundance of miR-216a-5p, a β-cell-specific miRNA, but not hepatocyte-specific miR-122, in β-cell-derived EVs. The EV amount appears to be higher in the inflammatory cytokine challenge group. Consistently, miR-216a-5p expression in EVs is significantly increased. Interestingly, lower expression of whole-length tRNA-Asp-GTC and higher levels of their fragments are detected in the EVs derived from EndoC-βH1 cells after cytokine treatment.

Conclusions: Our preliminary data indicate that inflammatory cytokines-treated EndoC-βH1 cells, an in vitro cell culture system modeling the early T1D development, appear to secrete more EVs, whose RNA cargo is clearly changed, including a higher level of miR-216a-5p and higher tRNA cleavage events when compared with untreated cells. Further transcriptomic analysis of these EVs will facilitate the development of β-cell-specific EVs or their RNA cargo as novel diagnostics to identify patients with early T1D.

Jun Yang
University of Notre Dame

Aged and Young Breast Matrix Bound Vesicles in Breast Cancer

Jun Yang1, Gokhan Bahcecioglu2,4, George Ronan2,3, and Pinar Zorlutuna1,2,3,4
1. Department of Chemical and Biomolecular Engineering, University of Notre Dame, Notre Dame IN
2. Department of Aerospace and Mechanical Engineering, University of Notre Dame, Notre Dame IN
3. Bioengineering Graduate Program, University of Notre Dame, Notre Dame IN
4. Harper Cancer Research Institute, University of Notre Dame, Notre Dame IN

While most extracellular vesicle (EV) studies in cancer research focus on cancer cell secreted EVs, recently an unconventional, bioactive subgroup of EVs has been identified within the extracellular matrix (ECM) of tissues and have shown to provide tissue specific activities in multiple different tissues including urinary bladder, small intestine and heart. Given the recently recognized importance of tumor microenvironment, especially the ECM surrounding the tumor, matrix-bound EVs (MBVs) might have important roles in cancer initiation and progression. We have previously investigated the breast ECM alterations with older age, one of the biggest risk factors for breast cancer, but little is known on EVs in the aging breast microenvironment in general, especially the ECM-bound EVs. Here, we showed for the first time the presence of MBVs in the breast tissue, visualized their uptake from both native decellularized tissue sections and 3D hydrogels, investigated and compared the effects of MBVs from aged and young tissues on breast cancer progression and metastasis.

EVs were isolated from the fourth mammary glands of young (2-6 months) and aged (20-23 months) mice and characterized with nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). Exosomal markers were visualized with immunogold EM. Normal (KTB21) and cancerous (MDA-MB-231) mammary epithelial cells were used in this study. Cellular uptake of the MBVs were visualized with fluorescence labeling. Cell motility and invasion were assessed by tracking cells microscopically or quantifying invaded cells in transwell assay, respectively. Cytokine and miRNA contents of MBVs were profiled and specific targeted factors were selected for epithelial cell invasion assays.

NTA and TEM revealed the presence of round shaped, CD9 and CD63 positive vesicles in breast ECM with ~140 nm diameter. Interestingly, treatment with MBVs from aged mice (MBV-aged) significantly increased cell motility in both KTB21 and MDA-MB-231 cells compared to untreated control, while treatment with MBVs from young mice (MBV-young) posed weaker influence on cell motility. Moreover, both MBV-aged and MBV-young treatment significantly increased the invasion of KTB21 and MDA-MB-231 cells, with higher impact of MBV-aged on the MDA-MB-231 cells than MBV-young.

Cytokine profiling and NanoString sequencing revealed a system of cytokines and miRNAs encapsulated in the MBVs, including significantly higher levels of adiponectin and selected oncomiRs in the MBV-aged than MBV-young. Adiponectin treatment significantly increased cell invasion compared to non-treatment groups in both cell types. Quantitative RT-PCR confirmed higher levels of the oncomiRs in the MBV-aged than MBV-young. Transfection of epithelial cells with a cocktail of selected oncomiR mimics led to increased invasion, indicating that these miRNAs are involved in MBV-aged-driven increase in cell invasion.

In conclusion, we showed that as an integral component of the breast ECM that could interact with and be taken up by cells, these previous-unknown MBVs could play a pivotal role in how the breast tumor microenvironment alters epithelial cells toward cancer and metastasis as well as how the aging microenvironment further enhances these influences. Our results also show that the cargo upregulated in MBV-aged could exert crucial influences in promoting the MBV-driven invasive cell behavior and be potential targets for future treatment development.

George Ronan
University of Notre Dame

Age and Sex Influence the Behavior of Exosomal miRNA in Tissue-Bound Exosome Populations to Affect Outcomes of Cardiac Fibrosis through Synergistic miRNA Effects

George Ronan1,2; Gokhan Bahcecioglu2; Jun Yang3; Pinar Zorlutuna1,2,3,4
1. Bioengineering Graduate Program, University of Notre Dame, Notre Dame, IN.
2. Department of Aerospace and Mechanical Engineering, University of Notre Dame, Notre Dame, IN.
3. Department of Chemical and Biomolecular Engineering, University of Notre Dame, Notre Dame, IN.
4. Harper Cancer Research Institute, University of Notre Dame, Notre Dame IN

Background: Extracellular vesicles (EVs) have been increasingly recognized as essential agents in microenvironment signaling in recent years. Novel extracellular matrix (ECM)-bound EVs (MBVs), as opposed to biofluid EVs, were implicated in onset of chronic diseases on the tissue level. MBVs may contribute to tissue aging and subsequent increase in disease risk and mortality. Aging is a major risk factor for cardiovascular disease (CVD), the leading cause of death worldwide, and correlates with clinical outcomes via poorly understood mechanisms. These differences result from fibrotic, inflammatory, and other chronic changes in the microenvironment. The onset rate of these changes also differs significantly between men and women. Intervention in these changes is limited by a lack of understanding of complex cardiac microenvironment interactions. Recent data suggest that age and sex-dependencies in clinical outcomes are mediated by long-term changes to the cardiac microenvironment, but the utility of MBVs in progressing understanding of field has not been well described. Greater understanding of chronic changes in MBVs in the microenvironment is essential for advancing understanding of CVD and developing therapeutic strategies.

Aims: We show that previously unreported EVs in human cardiac ECM modulate fibrotic phenotype dependently on the age and sex of the donor, consistent with clinical data, and that anti-fibrotic effects can be recapitulated using synergistic identified miRNAs.

Methods: MBVs were isolated from left ventricle (LV) ECM from aged male (AM), aged female (AF), young male (YM) or young female (YF) donors. LV vesicles (LVVs) were characterized by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and western blot. LVV content was assessed by cytokine blot, miRNA profiling, and PCR. LVV effects on cardiac fibroblast (CF) fibrotic activity were assessed by wound healing and gel contraction assays, and α-SMA expression. Treatment with individual or a cocktail of identified miRNAs were also assessed, as was cell survival and proliferation under infarct-like conditions.

Results: LVVs had exosome-like characteristics. LVVs showed differences in cytokine and miRNA profiles between AM and other groups, with AM showing higher cytokine and significantly lower miRNA content. Increased cytokines were related to oxidative stress and aging. Identified target miRNA were previously implicated in beneficial CVD patient outcomes. Treatment of CFs with LVVs showed differential effects based on age and sex of donor. AM significantly decreased wound healing and increased transdifferentiation and contractility compared to untreated control, while AF, YF, and YM showed opposite effects. Treatment with a cocktail of identified miRNA could recapitulate these effects, but not individual miRNAs. Treatment also enhanced cell survival under infarct-like conditions.

Conclusions: MBVs contribute to age and sex-dependent differences in cardiac microenvironment consistent with clinical outcomes. LVVs of young or female donors promoted anti-fibrotic effects, while AM were pro-fibrotic. The anti-fibrotic effects were partially recapitulated by identified miRNAs together, but not individually. Thus, the beneficial effects of LVV treatment may be mediated by a combination of miRNAs, not just one. Analysis of these synergistic effects will enhance understanding of endogenous anti-fibrotic pathways and advance therapeutic intervention.

Poster Abstract # 18

Aadil Sheikh

Baylor University

The Outer Membrane Vesicles of Bacteroides fragilis Contain Unique Small RNA Species and Trigger Immune Responses in Host Cells

Aadil Sheikh1, Alysia Martinez1, Michelle Pujol1, Bernd Zechmann2, Joseph Taube1, Leigh Greathouse1,3

1. Department of Biology, Baylor University, Waco TX.
2. Center for Imaging and Microscopy, Baylor University, Waco TX.
3. Department of Nutrition, Baylor University, Waco TX

Alterations in the community structure of the gut microbiome are associated with changes in the host physiology, including inflammation. A critical component of the inflammatory response system are receptors capable of sensing foreign nucleic acids (e.g. small RNAs) that are carried as cargo in bacterial outer membrane vesicles (OMVs). The mechanism by which human extracellular RNAs immune responses have been well established, the contribution of bacterial sRNA to the host immune system is unclear. We hypothesize that pathogenic and commensal microbes use OMV-associated small RNA species to differentially affect host inflammatory responses. First, we profiled the small RNA species of purified OMVs from a commensal strain (NTBF) and a pathogenic strain (ETBF) of Bacteroides fragilis. To distinguish the differences in the sRNA profiles of both strains and their OMVs, we conducted small RNA-seq and identified enrichment of specific sRNA species in OMVs that were differentially expressed between strains. This evidence led us to investigate the differential effects of these OMVs in intestinal epithelial cells. Using Caco-2 and HT29 cells exposed to OMVs from each strain, we ran qPCR to test several pro- and anti-inflammatory cytokines. We observed that both strains upregulate the expression of IL-1β and TGFβ, but NTBF stimulates a greater response in IL-8 compared to ETBF. These results indicate that bacteria may preferentially load small RNAs into vesicles that target host cells, which differentially affect host immune responses through RNA-sensing pathways. Overall, our data suggest a key function of bacterial small RNAs and their OMV vehicles in controlling host immune system.

Marsalas Whitaker
Vanderbilt University

Capture of Fluoro-labelled EGFR+ Extracellular Vesicles using Bio-functionalized Magnetic Particles

Marsalas Whitaker 1, Chris Haycook PhD 1, Fan Xue 1, Zheng Cao PhD 2, Jim Higginbotham PhD 2, Jeff Franklin PhD 2, Bob Coffey MD 2 and Todd Giorgio PhD 1
1. Department of Biomedical Engineering, Vanderbilt University.
2. Department of Cellular and Developmental Biology, Vanderbilt University Medical Center.

The level of epidermal growth factor receptor (EGFR) present in subpopulations of extracellular vesicles (EVs) is dependent on the level and activation of EGFR within the tumors from which EVs are derived. Currently, there are several techniques used to enrich for EVs. Some examples are differential and gradient ultracentrifugation, surface acoustic wave (SAW) technology and Immuno- capture. There are a couple of pitfalls of these current EV purification techniques. One is relying on size and density-based purification of small-EVs that are labor intensive. Another challenge for purification of EVs from biofluids like plasma and conditioned media is copurification of contaminants from other secreted nanoparticles that are not sufficiently separated from EVs. Co-purification of some such nanoparticles termed supermeres, some of which contain the secreted protein TGFBI, can confound analysis of purified EVs. Here, we developed antibody-conjugated superparamagnetic microparticles with cleavable DNA linkers to enable the capture and non-destructive release of EGFR+ EVs. We utilized DNA linkers using conventional copper-free “click” chemistry synthesis methods to generate cetuximab-DNA (CTX-DNA) conjugates. Linkers contain two unique restriction sites to provide selective labeling and liberation of EVs mediated by BamHI and ClaI restriction enzyme cleavage. Superparamagnetic microparticles were decorated with different concentrations of CTX-DNA to test conditions leading to efficient purification and release of EGFR+ EVs from complex biological samples. To do this, we add our CTX-DNA Magnetoparticles into a conditioned colorectal cancer derived DiFi cell media with fluorescent-labelled EVs. During the incubation period, EGFR+ EVs will bind to CTX then after the reaction is stopped, we perform magnetic separation and a series of washes to purify the “captured” EVs. Afterwards, we quantify our capture analysis using two methods: Spectrophotoflourometer Emission and Western Immuno-blots. Quality control parameters (volume yields, degree of labelling DBCO/Antibody and Nanodrop IgG concentrations) were evaluated and has helped to optimize the capture analysis performed to show signal of EGFR+ EVs. In conclusion, we will determine optimal conditions for improved efficient EV capture from biofluids with intact EV release thus allowing our group to efficiently capture and label EV subsets for proteomic and flow analysis. This will provide an efficient alternative to ultracentrifugation and other techniques that allows specific interrogation of EV subsets containing unique analytes for which we have affinity reagents, such as EGFR antibodies.

Natacha Carnel-Amar
Massachusetts General Hospital

Identification of Macrophage-specific Markers on Extracellular Vesicles in a Transgenic Mouse Model & Humans

Natacha Carnel-Amar1, Sara Abuelreich2, Andras Saftics2, Michail Spanos1, Emeli Chatterjee1, Worawan Limpitikul1, Ionita Ghiran3, Stephen Gould4, Robert L Raffai5, Kendall Van Keuren-Jensen6, Tijana Jovanovic-Talisman2, Saumya Das1
1. Cardiovascular Research Center, Massachusetts General Hospital, Boston, MA, USA.
2. Department of Cancer Biology and Molecular Medicine, Beckman Research Institute, City of Hope Comprehensive Cancer Center, Duarte, CA, USA.
3. Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA
4. Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205, USA
5. Department of Surgery, Division of Vascular and Endovascular Surgery, University of California, San Francisco, San Francisco, CA 94143, USA
Department of Veterans Affairs, Surgical Service (112G), San Francisco VA Medical Center, San Francisco, CA
6. Neurogenomics Division, TGen, Phoenix, AZ 85004, USA

Introduction: Extracellular Vesicles (EVs) are key mediators of intercellular communication and can transfer various types of molecules, including RNA, proteins, and lipids, between cells.
Macrophages, a prominent type of immune cells, are known to release EVs with distinct contents and functions. However, specific surface markers of macrophage-derived EVs are still poorly defined. The ExoMap mice model provides a platform to investigate tissue-specific EV subpopulations and the complexity of EV heterogeneity. We have found that CD169 and CD68 markers are specific to macrophages and have been associated with immune diseases, including Sarcoidosis. In this study we used this novel transgenic mouse model (ExoMap) and a cohort of sarcoidosis patients to probe specific EV populations arising specifically from immune cells and characterize EV heterogeneity at the single EV level.

Method: Double Transgenic vav-cre/ExoMap mice and appropriate littermate wild-type controls were used to assay for immune (vav-expressing) cell derived EVs. In this mouse model, expression of cre in the vav-expressing cells (hematopoietic cells and mostly immune cells in the post-natal mouse), leads to expression of humanized CD81 (hsCD81) and mNeonGreen in the immune cells and in their EVs. Size Exclusion Column (SEC) and immuno-pulldown with either anti-hsCD81 or anti-CD169/CD68 antibodies (from the mouse or human plasma, respectively) were used to isolate macrophage-specific EVs. 3 miRNAs (mir223, mir374c, mir412) specific to macrophages were tested using Droplet Digital PCR (ddPCR). To design our human pilot study, we chose patients with active sarcoidosis (n=10), latent sarcoidosis (n=10), healthy controls (n=10) to compare the level of expression of specific immune markers in their blood.
Results: Bone marrow cells prepared from vav-cre/ExoMap mice but not wild-type litter mate controls were found to express mNeon Green and hsCD81. Immuno-pulldown combined with vesicle flow cytometry defined the presence of hsCD81/mNeonGreen EVs in the plasma of vav-cre/ExoMap but not wild-type mice, demonstrating the specificity of the model for immune cells. Expression of CD68 and CD169 was robust on the EVs isolated using immuno-pulldown with hsCD81 specific antibodies from the Double Transgenic vav-cre/ExoMap mice which is consistent with the immune specificity of the vav-cre expression. Ongoing studies examine the expression of these markers in circulating EVs of mice injected with LPS to stimulate the release of macrophage-EVs. Using human pooled plasma, we detected expression of CD68 on the EVs isolated using immuno-pulldown with CD169 specific antibody, suggesting the presence of both markers on macrophage specific EVs. An examination of the RNA contents carried by EVs derived from control or patients with active sarcoidosis is on-going.

Conclusion: Our findings support these markers as conserved between mouse and human samples, indicating their potential as universal markers of macrophage-derived EVs. Our findings could improve diagnostic specificity in diseases where macrophages play a key role. Ongoing experiments aim to comprehensively characterize individual macrophage-derived EVs using the quantitative single molecule localization microscopy (qSMLM). Together, our findings provide a valuable resource for future studies of macrophage-EV communication and could serve in the development of EV-based diagnostic and therapeutic strategies.

Emeli Chatterjee
Massachusetts General Hospital

An In Vivo Model to Profile Cardiac Specific Extracellular Vesicles During Ischemic Reperfusion Condition

Emeli Chatterjee1*, Sarah Abuelreich2*, Andras Saftics2, Natacha Carnel-Amar1, Ana Chiaretti1, Priyanka Gokulnath1, Michail Spanos1, Guoping Li1, Jochen D. Muehlschlegel3, Tijana Jovanovic-Talisman2, and Saumya Das1
1. Cardiovascular Research Center, Massachusetts General Hospital, Boston, MA, USA.
2. Department of Cancer Biology and Molecular Medicine, Beckman Research Institute, City of Hope Comprehensive Cancer Center, Duarte, CA, USA.
3. Department of Anesthesiology, Perioperative and Pain Medicine, Department of Anesthesiology, Brigham and Women's Hospital , MA, USA.

Background: Ischemic heart disease is the leading cause of mortality worldwide. Extracellular vesicles (EVs) and their contents play particularly significant roles in the biology of cardiac ischemic injury and subsequent remodeling, including mediating cross talk with the immune system and other cell types. Thus cardiac specific EVs and their contents may serve as specific biomarkers in cardiovascular diseases..

Aim: We wanted to focus our analyses on the dynamic regulation of cardiomyocyte-derived EVs in cardiac ischemia by leveraging our EV reporter transgenic mouse model (ExoMap mouse; hsCD81-mNeonGreen, C57BL6 background)to study acute and dynamic changes in cardiac EVs in a murine model of ischemic heart disease. This will provide the framework for future studies to identify cardiomyocyte-specific markers of heart remodeling after ischemia-reperfusion injury.

Method: The ExoMap mice were crossed with the alpha-MHC-cre (C57BL6) mice to create cardiac specific cre expression, resulting in mNeonGreen positive cardiomyocytes. EVs were isolated from the heart tissues of the double transgenic mice followed by standard characterization of EVs. To characterize the double transgenic mice, cardiac tissue derived EVs were an immunocaptured with hsCD81 and probed with cardiac specific EV markers like Troponin T (TnT), CHRNE along with canonical EV markers like CD63 and Alix. To assess whether there is any significant increase of cardiac EVs in the circulation following ischemia-reperfusion injury, we generated an IR model (n=2) with 30 minutes of left anterior descending coronary artery ligation (LAD) followed by reperfusion for 24hrs. Sham operated mouse (n=1) was used as control group. For single EV analysis, plasma derived vesicles from double transgenic IR and sham control mice were affinity isolated using either hsCD81 (heart-enriched EVs) or mCD81 (total-EVs) and labeled with fluorescent antibodies against tetraspanins (mCD9/mCD63/mCD81/hsCD81). EVs were imaged using quantitative single molecule localization microscopy (qSMLM) to detect tetraspanins and total internal reflection fluorescence (TIRF) microscopy to detect mNeonGreen; n=3 (15ROIs).

Result: We were successfully able to perform immune-magnetic pulldown using a human anti-CD81 antibody to specifically isolate cardiac derived EVs from mouse heart tissue. CD63, Alix, TnT and CHRNE were highly/only expressed in the hsCD81 EV pull down fraction of the double transgenic mice demonstrating the markers characteristic for cardiomyocyte EVs. Plasma from IR mouse 1 and IR mouse 2, yielded 5% and 7% HsCD81-positive EVs, respectively while plasma from sham control mouse yielded ~1% HsCD81-positive EVs. For all three mice, HsCD81-positive EVs showed a larger EV size (on average 95-97 nm) compared to "total" EVs (enriched in MmCD81, on average 82-87nm) in line with our previous observations on subpopulations of cardiac-derived EVs.

Conclusion: From our preliminary data, it can be concluded that this transgenic mouse model can be used for cardiac specific EV studies. In addition, we saw a significant increase in cardiac EVs in the circulation following ischemia-reperfusion injury. Assessment of EVs from this mouse model paves the way to test heart-specific markers and to measure molecular changes in EVs in IR conditions.

Tzu-Yi Chen
Icahn School of Medicine at Mount Sinai

Extracellular RNA (exRNA) Analysis of Human Biofluids Derived Extracellular Particles Using Different Computational Tools.

Tzu-Yi (Tina) Chen1#, Edgar Gonzalez-Kozlova3#, Taliah Solemani1, John Fullard2,5, Susmita Sahoo4, Natasha Kyprianou6, Alex Charney2,5, Ash Tewari6, Panos Roussos5, Carlos Cordon-Cardo1, Gustavo Stolovitzki7, Navneet Dogra1,2,7
1. Department of Pathology, Icahn School of Medicine at Mount Sinai, NY.
2. Department of Genetics and Genomics, Icahn School of Medicine at Mount Sinai, NY.
3. Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, NY.
4. Department of Cardiology, Icahn School of Medicine at Mount Sinai, NY.
5. Department of Psychiatry, Icahn School of Medicine at Mount Sinai, NY.
6. Department of Urology, Icahn School of Medicine at Mount Sinai, NY. 7. International Business Machine
# Equal contributions

Extracellular RNA (exRNA), identified as part of the extracellular canopy comprised of microvesicles, exosomes, and lipoproteins, has shown to play a role in cellular crosstalk and pathogenesis. Nevertheless, the differences in short-read aligners and computational tools used in analyses have shown to be correlated with large outcome variability despite similar exRNA experimental setup. In this study, we benchmarked the performances of the most common short read aligners (Bowtie, Bowtie2, BWA, STAR, exceRpt) using ~100 in-house exRNA samples derived from four subtypes (urine, serum, tissue, and cell culture medium) of various disease types (prostate and liver cancer, Alzheimer's, and Parkinson's disease) via three different combinations of isolation methods (UC, UC-DG, SEC, nanoDLD). The alignment performances of each aligner are evaluated based on the percentage of reads aligned relative to the total number of reads under similar alignment parameters (mismatch, etc.). The heterogeneity observed across the five aligners is then reflected in the distinct raw count distributions, exRNA biotype compositions, and top differentially expressed signatures. Interestingly, the technical variance from the aligners surpasses both the batch effects across datasets and the biological variance in sample subtypes, contributing to around 25% of the total variance, and is too large to be corrected via normalization. To investigate the effect of aligner choice on downstream analysis, we also performed differential expression analysis on the prostate cancer cohort, comparing serum derived EVs before and after prostatectomy. Aside from the distinct differential expression profiles across aligners, the corresponding pathway enrichments for the pre-prostatectomy samples are not all associated with cancer or immune responses. In summary, the main findings of our research confirm exRNA specificity due to aligner choice. Ultimately, we aim to highlight the existing bias and ambiguity seen in these computational tools while presenting the key considerations for exRNA analyses of various biofluids and subtypes, as no single tool meets all requirements.

Alessandra Ciullo
Smidt Heart Institute-Cedars-Sinai Medical Center

Small noncoding RNA, yREX3, mined from therapeutic cell extracellular vesicles, reprograms macrophages through epigenetic silencing of Pick1 to limit ischemic heart injury

Alessandra Ciullo1, Liang Li1, Chang Li1, Collin Farrell2, Matteo Pelligrini2, Eduardo Marbán1, Ahmed Ibrahim1
1. Smidt Heart Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA
2. Department of Molecular, Cell, and Developmental Biology, University of California Los Angeles, Los Angeles, CA USA

Background

Small non-coding RNAs (snRNAs) comprise the majority of extracellular vesicle (EV) cargo. Therapeutic cells secrete EVs replete with snRNAs that repair damaged tissue through gene regulation in target cells.

Aims

Here we investigate the role of yREX3, a small RNA homologous to Y RNA4 and enriched in extracellular vesicles in cardioprotection.

Methodology

In vivo, we used ischemia/reperfusion (I/R) injury in rats to evaluate the cardioprotective effect of yREX3. To identify the main target cell of YREX3, we exposed bone-marrow-derived macrophages (BMDMs), cardiac fibroblasts, and cardiomyocytes to yREX3 to assess transcriptomic changes. To identify the gene target of yREX3, we performed whole-genome bisulfite sequencing (WGBS) and methylation-specific sequencing. Finally, to evaluate the role of macrophages in yREX3 cardioprotection, we adoptively transferred BMDMs exposed to yREX3 in I/R injury.

Results

In vivo, yREX3 reduced infarct size (3.45±0.43g) compared to vehicle or scramble control (11.41±1.32g and 11.88±2.44g, respectively) and circulating cardiac troponin I (5.79±0.83ng/ml vs 19.16±2.81ng/ml and 17.42±2.07ng/ml, respectively). BMDMs respond robustly to yREX3 compared to cardiac fibroblasts or cardiomyocytes (5435 genes vs 4114 and 2653, respectively). Methylation analysis identified pick1 as a target of yREX3 (56% methylated compared to control). Pick1 downregulation enhanced Smad3 phosphorylation and phagocytic capacity in BMDMs. Adoptive transfer of BMDMs exposed to yREX3 or pick1 silencing recapitulated the cardioprotective effects of yREX3 in I/R rats as shown by infarct size (6.55± 0.85g) compared to vehicle or scramble control (16.73±2.13 and 16.15±3.43g, respectively) and cardiac troponin I (7.62 ±0.83ng/ml vs 13.27 ±3.09ng/ml and 15.24 ±4.04ng/ml, respectively).

Conclusion

YREX3 is a small Y RNA identified in the EVs of therapeutic cells and limits tissue injury via a novel mechanism.

Ke Liao
Cedars-Sinai Medical Center

Long non-coding RNA BCYRN1 and its short synthetic derivatives promote regulatory T Cell proliferation, migration and activation

Ke Liao, Zahra Mohammadigoldar, Liang Li, Weixin Liu, Natasha Anders, Akbarshakh Akhmerov, Ahmed Ibrahim, Eduardo Marbán
Smidt Heart Institute, Cedars-Sinai Medical Center, Los Angeles, CA.

Background: Regulatory T (Treg) cells are increasingly implicated as modulators of myocardial ischemia-reperfusion injury. Previously, we found that extracellular vesicles isolated from human cardiosphere-derived cells (CDC-EVs) can increase Treg proliferation and anti-inflammatory cytokine (IL-10) production. Here we investigate how a long noncoding RNA, BCYRN1 (Brain Cytoplasmic RNA 1) enriched in CDC-EVs, affects human Treg function. Using mechanistic insights, we seek to create and test BCYRN1-derived binding small sequences (BDSSs) which retain in vitro bioactivity and may be more viable as a potential therapeutic candidates.

Aims: To develop a novel RNA-based approach targeting regulatory T cells as a potential therapy for myocardial infarction (MI).

Methodology: BCYRN1 was identified as a LncRNA species in CDC-EVs using RNA sequencing and validated by qPCR. RNA pulldown and luciferase assays were used to determine whether BCYRN1 works as a miRNA sponge and find out the binding sequences within BCYRN1 for miRNAs. We assessed BCYRN1 and BDSS effects on Treg by measuring cell proliferation (Cell Counting Kit-8 assay), cell migration (trans-well migration assay), and IL-10 (qPCR and ELISA assay). In vivo, MI was induced in 8-10-week-old C57BL/6 mouse by left coronary artery ligation for 45 minutes followed by reperfusion. Fifteen minutes later, the mice (N=5, each group) received (via tail vein) CDC-EV BCYRN1 (CDC-EV overexpressing BCYRN1), BDSSs, mut-BDSSs (scrambled inactive versions of BDSS) or vehicle (PBS). MI size (by TTC staining) and infiltrating Treg (by flow cytometry) were assessed 72 hrs post-MI.

Results: BCYRN1 is one of the most highly enriched LncRNAs in CDC-EVs. RNA pulldown and luciferase assays show BCYRN1 contains binding sites (BDSS-138, -150 and -98) for miR-138, miR-150 and miR-98 that target ATG7, CCR6 and IL-10 mRNAs, respectively. Thus, BCYRN1 serves as a miRNA sponge which, in turn, leads to Treg cell proliferation (3.00± 0.11 fold increase, N=5, via induction of ATG-7 dependent autophagy), CCR-6 dependent migration (1.77 ± 0.10 fold increase, N=5) and increased production of IL-10 (qPCR: 2.60 ± 0.35 fold increase, N=6 and ELISA: 6.12 ± 0.78 fold increase, N=5). Lipid nanoparticles containing 3BDSSs induce Treg cell proliferation (1.89± 0.61 fold increase, N=6), migration (1.32± 0.23 fold increase, N=6), and IL-10 induction (6.75± 0.52 fold increase, N=6). Among the three BDSS species, BDSS-98 dose-dependently and uniquely upregulated IL-10 production. In the MI model (N=5, each group), CDC-EV-BCYRN1 (but not vehicle) and BDSS cocktail (but not mut-BDSS) induced Treg infiltration, proliferation and IL10 production in the heart with a concomitant decrease of infarct size compared to vehicle-treated MI mice.

Conclusions: We have pinpointed BCYRN1 as a bioactive constituent of CDC-EVs, promoting Treg cell-mediated cardioprotection. BCYRN1 serves as a miRNA sponge to mediate beneficial effects on Treg function in the heart. Thus, a single LncRNA and shorter synthetic derivatives mimic the effects of CDC-EV on Treg in vitro and in vivo. Our study provides a novel approach, targeting Treg by a defined LncRNA mined from CDC-EV, to treat MI. The same approach could reasonably be predicted to be useful in other diseases where increased Treg activity is a goal (e.g., autoimmune disorders).

Yohei Sanada
Cincinnati Children`s Hospital Medical Center

The function of TRBP as a pro-inflammatoryExtracellular-RNAs sensor in obesity

Yohei Sanada, Yoshinori Ichihara, Rupinder Gill, Takahisa Nakamura
Division of Endocrinology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH

【Background and aims】

Chronic inflammation has been shown to play a vital role in the pathogenesis of many obesity-related metabolic disorders, such as Type 2 Diabetes (T2DM), non-alcoholic fatty liver disease (NAFLD), and insulin resistance. Extracellular vesicles (EVs) carry biological cargos such as RNA and protein to distant organs and cells. However, while several studies of RNA Cargos (Extracellular-RNAs; ExRNAs) have linked inflammation to the onset of obesity-related diseases, the mechanisms of how ExRNAs exert the regulatory function in recipient cells are still unclear. Our preliminary data indicate that Liver-derived ExRNAs are a primary mediator in inducing obese-related immune cell activation and obese-related inflammation. This study aims to elucidate the mechanisms of how ExRNAs are transferred into recipient macrophage cells and recognized by cellular protein. 【Methodology】 We developed a method to capture interactions between ExRNAs and cellular proteins, as these events are might crucial to exert the function of ExRANs in recipient cells. First, Huh7 were cultured with or without 1 mM 4-thiourigine (4-SU) for 48hr; second, Magcapture exosome isolation kit was used to isolate the EVs containing 4-SU-labeled RNA (4-SU EVs); and third, 4SU-EVs were added to immortalized macrophage cells followed by ultraviolet (UV) irradiation with 365 nm light; and fourth, crosslinked ExRNAs-protein complexes were purified by a modified solid-phase reversible immobilization method (EVer-CLASP). To identify the ExRNAs-protein complexes, we performed silver staining and immunoblot analysis. RNA-binding proteins such as the RNA silencing machinery proteins (Argonaute 2 (Ago2) and TAR RNA-binding protein (TRBP)) that play a major role in intracellular RNA and virus-derived exogenous RNA trafficking have been suggested to be involved in recognizing ExRNAs. We generated macrophage-specific Ago2 and TRBP (Ago2-M, TRBP-M) KO mice to evaluate the function of Ago2 and TRBP. We isolated peritoneal cavity cells from the wild, Ago2, and TRBP M-KO mice and treated them with palmitate-treated Huh7-derived EVs (PA-EVs). Flow cytometry and qPCR analyses were performed to monitor the inflammatory status. We analyzed the inflammatory parameters in serum, pancreas, adipose tissue, and macrophage population of adipose in TRBP M-KO within HFD-induced obesity model. Eventually, we performed the measuring fasting insulin levels, ITT, and GSIS within HFD-induced obesity. 【Results】 After 8hr treatment of 4-SU EVs, Ago2 and TRBP signals were significantly detected by immunoblot analysis, but these signals were decreased after 20hr treatment and couldn't detect in cont EVs treatment group. And also, silver staining analysis indicated a higher signal in 4-SU treatment group than the control. The functional assay by flow cytometry and qPCR analysis showed that the Pro-inflammatory responses by PA-EVs were blunted in Ago2 and TRBP-M KO peritoneal cavity cells. Moreover, inflammatory genes such as TNF-a, IL-6, and Cd38 expression levels in the pancreas, adipose and isolated macrophage from adipose tissue in TRBP M-KO were significantly lower than in WT mice. The fasting insulin levels and ITT showed that M-TRBP KO mice ameliorated the insulin resistance phenotype. Finally, glucose-induced insulin secretary analysis indicated that insulin secretion levels in TRBP M-KO mice were significantly higher than WT mice. 【Conclusions】 EVer-CLASP might be able to identify the interaction of ExRNAs and cellular proteins. Also, TRBP might be one of the ExRNAs sensor to recognize and regulate the function of ExRNAs.

Anjali Byappanahalli
National Institute on Aging (NIA)

Extracellular vesicles and their associated cargo as biomarkers in midlife frailty

Anjali M. Byappanahalli1, Nicole Noren Hooten1, Mya Vannoy1,2, Nicolle A. Mode1, Ngozi Ezike1, Alan B. Zonderman1, Michele K. Evans1
1. Laboratory of Epidemiology and Population Science, National Institute on Aging, National Institutes of Health, 251 Bayview Boulevard, Baltimore, MD 21224
2. Current address: Perelman School of Medicine, University of Pennsylvania, 3400 Civic Center Boulevard Philadelphia, PA 19104

Background: Frailty is an aging-associated clinical syndrome characterized by a low-grade sterile inflammatory state that can commence at midlife with similar risk for morbidity and mortality. Little is known about the factors contributing to this chronic inflammatory state. Damage-associated molecular pattern (DAMP) molecules have been shown to drive sterile inflammation. We recently reported that extracellular vesicles (EVs) contain circulating cell-free mitochondrial DNA (ccf-mtDNA), which can act as a DAMP molecule.

Aims: Our aim was to investigate whether EV cargo, specifically ccf-mtDNA and inflammatory proteins, may act as DAMP molecules in early frailty in a diverse cohort of African American and White participants.

Methodology: EVs were isolated from the plasma of non-frail (n=90) and frail (n=87) middle-aged (45-55 years) participants from the Healthy Aging in Neighborhoods of Diversity across the Life Span study using size exclusion chromatography (SEC). EV-enriched fractions were characterized using immunoblotting, transmission electron microscopy, and nanoparticle tracking analysis. To examine EV mtDNA levels, DNA was isolated from EVs and analyzed by quantitative real-time PCR using four different mtDNA primers. Ninety-two inflammatory proteins were quantified from EVs using proximity extension assays. To investigate the functional effect of EVs from frail and non-frail individuals, we measured mitochondrial energetics in vitro. Levels of EV concentration, mtDNA, and inflammatory proteins were analyzed using linear regression, modeled to the study design of frailty, race, sex, and poverty status.

Results: EV markers were present in our EV-enriched fractions and the EVs were a typical size and morphology of plasma EVs. EV concentration was highest in frail White participants. Levels of EV mtDNA were significantly higher in frail individuals compared to non-frail individuals. Six inflammatory proteins (FGF-21, HGF, IL-12B, PD-L1, PRDX3, STAMBP) were significantly associated with frailty. EV inflammatory proteins were significantly altered by frailty status, race, sex, and poverty status. For example, the levels of EV-associated CD5, CD8A, CD244, CXCL1, CXCL6, CXCL11, LAP-TGF-beta-1, and MCP-4 were higher in frail White participants compared to non-frail White and frail African American participants. EV-associated urokinase-type plasminogen activator (uPA) was highest in frail White participants living below poverty. EV-associated CCL28 levels were highest in non-frail women and CXCL1 were highest in non-frail men. Levels of CD5, CD8A, CXCL1, LAP-TGF-beta-1, and uPA were higher in men living below poverty. CXCL6 levels were significantly higher in individuals living above poverty. EV-associated levels of mtDNA and inflammatory proteins were significantly correlated. Additionally, treatment of THP-1 cells with EVs from frail individuals resulted in increased basal respiration compared to treatment of EVs from non-frail individuals.

Conclusions: These data suggest that DAMP molecules, such as mtDNA, and inflammatory proteins are higher in EVs from frail individuals. EVs from frail individuals can affect mitochondrial energetics. The association of mtDNA with chemokines and other inflammatory EV cargo proteins may contribute to the frailty phenotype. In addition, the social determinant of health, poverty, influences the inflammatory cargo of EVs in midlife frailty.

Poster Abstract # 27

Chanhee Park

University of Maryland, Baltimore

Extracellular vesicles from septic plasma of mice induce brain inflammation via MIRNA cargo

Chanhee Park1, Yun Li1, Zhuofan Lei1, Melian Jones1, Junyun He1, Fengqian Chen1, Hui Li1, Kavitha Brunner1, Jing Zhu1, Steve M. Jay3, Wei Chao1, Junfang Wu1,2, Lin Zou1

1. Department of Anesthesiology & Center for Shock, Trauma and Anesthesiology Research, University of Maryland School of Medicine, Baltimore, MD

2.University of Maryland Center to Advanced Chronic Pain Research, University of Maryland, Baltimore, MD

3. Fischell Department of Bioengineering, University of Maryland College Park

Background: Sepsis-associated encephalopathy (SAE) occurs in sepsis survivors, where neuronal inflammation plays a critical role in its pathogenesis. Our recent studies demonstrated that plasma extracellular (ex) miRNAs, including miR-146a, miR-122, miR-34a, miR-145, increased in sepsis. These ex-miRNAs cause neuronal inflammation both in microglial cultures and in the intact brain. Extracellular vehicles (EVs) have been proposed as a vehicle for ex-miRNA-mediated intercellular communication. However, the biological function of plasma EVs and their miRNA cargo in neuronal inflammation is unclear.

Aim: We hypothesize that septic plasma EVs carrying miRNAs contribute to neuroinflammation.

Methods: EVs from HEK293 cell cultures (293EVs) were labeled with fluorescent dye PKH26 and used to test EV internalization. Plasma EVs were isolated using ultracentrifugation and quantified by Viewsizer 3000 (Horiba). Microglia and RAW cells were treated with PKH26-labeld 293EVs (293EVs-PKH26) for overnight. The uptake of 293EVs-PKH26 by the cells was observed under a confocal microscope (Nikon). Sepsis was created by cecal ligation and puncture (CLP) in mice. Sham mice had laparotomy only. Brain immune cells and cytokine gene expression were measure by flow cytometry and qRT-PCR. Microglia were isolated from neonatal mouse brain. The function of EV-associated miRNAs was tested by preincubating EVs with control oligos or anti-miRNAs combo prior to the cell treatment. The mouse neuronal cells were treated with the CLP EVs or microglial conditioned media (CM), which were collected from the microglial cultures treated with CLP or sham EVs. Neuronal apoptosis was measured by cleaved caspase-3 in Western blot.

Results: Microglia and RAW264.7 cells internalized 293EVs-PKH26 after 24 hours of incubation as confocal fluorescent images. ICV delivery of CLP-EVs, but not Sham-EVs, led to a marked brain infiltration of immune cells, e.g., neutrophils and monocytes. Brain cytokine/chemokine gene expressions, such as CXCL2, TNFα, IL-6, IL-1 β, were also significantly upregulated in the cerebral cortex following ICV injection of CLP-EVs. In microglial cultures, CLP-EVs induced a cytokine production including CXCL2 and IL-6 in a dose-dependent manner, while Sham-EVs did not. Pretreatment of EVs with anti-miRNAs combo (anti-miR-146a, -122, -34a, -145) led to a 49.3% inhibition in CLP-EV-mediated CXCL2 production as compared to that of control oligos. Importantly, neuronal cells exhibited an increase in cleaved caspase-3, an active enzyme for cell apoptosis, 24 h after microglial CM treatment, but not by direct CLP-EV treatment.

Conclusion: Plasma EVs of septic mice cause brain inflammation both in the intact animal and in the microglial cultures. Media from activated microglia treated with the septic EVs induce neuronal apoptosis. The proinflammatory property of the EVs in part attributes to their cargo miRNAs.

Siddharth Srivastava
University of California, Los Angeles

Utilizing SERS of immunocaptured exosomes to track exRNA

Siddharth Srivastava1, Jun Liu1, Tieyi Li1, Yong Kim2, David Wong2, Ya-Hong Xie1
1. Materials Science and Engineering, University of California - Los Angeles
2. School of Dentistry, University of California - Los Angeles

Surface Enhanced Raman Spectroscopy (SERS) is a technique that provides biomolecular “fingerprints” of their entire biochemical content (nucleic acids, amino acids, lipids, etc) via the composition information of the collective Raman-active bonds inside EVs. We describe a platform that is based on SERS in combination with machine learning and multivariate analysis to demonstrate the utility of analyzing vesicles (including RNA content) derived from different biological sources. We demonstrate single vesicle sensitivity and high specificity in distinguishing between individual exosomes. In this study, we present SERS analysis of exosomes isolated from saliva samples for the purpose of exRNA tracking and salivary liquid biopsy for diagnosing gastric cancer (work in progress).

A key challenge faced during SERS of biofluids is the “training data purity” due to a complex analyte environment consisting of a variety of bioparticles. Specifically, EVs from patient body fluids be it saliva, blood or tissue consist of those derived from both healthy cells as well as cancerous cells. Machine learning programs are confused by, e.g. the EVs from healthy cells in cancer patients to treat them as characteristic of cancer patients. To tackle this issue, we utilize surface functionalization of the SERS substrate for immunocapture of exosomes from the sample, by targeting specific EV membrane markers unique to the organ of concern so as to reduce the fraction of the “mistaken identity”. In addition, signatures such tetraspanin on the sEV membrane are used to ensure that SERS spectra are collected to sEVs only avoiding the other possibilities such as HDL, LDL, cell debris protein aggregates, etc.

Data are presented demonstrating the improved specificity before and after surface functionalization. Subsequently, this data is used to obtain exRNA signatures from saliva samples of healthy control and gastric cancer patients. Downregulation of the exRNA is demonstrated, and furthermore, the data is used to demonstrate successful blind tests to diagnose gastric cancer from saliva.

Ceming Wang
Aopia Biosciences, Inc.

A Next-Generation High-Efficiency Extracellular Vesicle (EV) Isolation Platform, NanoEX, for Diagnostics and Therapeutics

Ceming Wang2, Chenguang Zhang1, Sonu Kumar1, Satyajyoti Senapati1, Nan Su2, and Hsueh-Chia Chang1
1. Department of Chemical and Biomolecular Engineering, University of Notre Dame, Notre Dame, IN
2. Aopia Biosciences, Inc. Pleasanton, CA

Background: Filtration-based EV isolation technologies hold great potential in providing advantages in yield, purity, and processing throughput over other methodologies, such as ultracentrifugation, precipitation, affinity purification, and size exclusion chromatography, etc. However, conventional ultrafiltration methods still suffer from issues such as membrane clogging and EV loss due to trapping, damaging, or fusion. Here, we demonstrated the performance of a next-generation EV isolation platform, NanoEX, based on an innovative Asymmetric Nanopore Membrane (ANM) nanofiltration technology to address major bottlenecks in EV-based research, diagnostics, and therapeutics.

Aims: Our goal is to develop an integrated and automated instrument, NanoEX, for EV isolation and concentration, based on our Asymmetric Nanopore Membrane (ANM) nanofiltration technology, to address all the current challenges in EV isolation and to provide solutions to large-scale EV production.

Methodology: The high performance of the ANM is achieved through an innovative nano-fabrication technology to produce filtration membranes with unique pore structures. The low-resistance ANM nanopores have highly uniform pore tip size and an asymmetrical channel geometry, which allows efficient filtration at extremely low pressure (up to 10 times lower than using traditional ultrafiltration membranes) to eliminate EV trapping, minimize EV damage or fusion, and prevent membrane clogging and fouling. The resulting EVs are of high yield and purity, while maintaining their integrity and bio-functionalities.

Results and Conclusions: By isolating EVs from both cell culture medium and human plasma samples, we demonstrated that the NanoEX platform could achieve 1) Simultaneous EV purification and enrichment with high yield (> 80%) and high purity (>99.5% free-protein removal); 2) Rapid and automated processing of a wide range of research and diagnostic samples up to 50ml volume; 3) Gentle filtration process for preserving EV integrity; 4) Enclosed processing that avoids sample cross-contamination, and 5) Scalable processing capabilities (up to liters of input materials) for therapeutic EV production. The highly pure EV isolated by the NanoEX platform allows accurate analysis of EV biomarkers without the interference of contaminants. The ANM technology based NanoEX system represents a next-generation EV isolation platform that provides unparalleled purification efficiency over all existing technologies and will greatly accelerate EV research, and enable wide adoption of EVs for diagnostic and therapeutic applications.

Willi Cheung
University of California, San Diego

Characterizing RNA Packaging into Extracellular Vesicles (EV) by Comparing RNA in EV and other fractions from Conditioned Culture Media

Willi Cheung1,3,4, Abbas Hakim1,4, Peter De Hoff1,4, Amber Morey1,4, Anelizze Castro-Martinez1,4, Isabella Saldana1,2,4, Louise C. Laurent1,4
1. Department of Obstetrics, Gynecology & Reproductive Sciences, University of California San Diego, La Jolla, CA, USA.
2. Department of Biological Sciences, California Polytechnic State University, San Luis Obispo, San Luis Obispo, CA, USA.
3. Department of Biology, San Diego State University, San Diego, CA, USA.
4. Sanford Consortium for Regenerative Medicine, La Jolla, CA, USA.

Background: Extracellular vesicles (EVs) are heterogeneous populations of molecules intensively studied for their potential role as diagnostic biomarkers. EVs participate in intercellular communication through transfer of molecular cargos including microRNAs (miRNAs). Despite their roles in cell-to-cell communication, understanding of the different populations of EVs and their secretion mechanisms remains far from comprehensive. While CD9, CD63, and CD81 are members of the tetraspanin family commonly used as EV markers, it became clear that tetraspanins were not equally expressed in all EVs. However, reliable isolation of EVs represents a considerable challenge. Aim: The aim of this study is to use an optimized immunomagnetic separation (IMS) workflow to selectively enrich EVs from DiFi cells and placental explant supernatant, in order to characterize the various subtypes of EVs produced. In addition, this study aimed to compare the specificity and capture, efficiency of IMS with an alternative biophysical separation method, DGUC. Methodology: CD9, CD63, CD81, and placental specific marker placental alkaline phosphatase (PLAP) EVs were enriched through positive selection from the cell supernatant of each cell line using immunomagnetic separation (Miltenyi Biotec). RNA was extracted in triplicate from the IMS bound and flowthrough fractions, DGUC fractions, DGUC+SEC fractions as well as from the whole supernatant (Norgen Biotek). Small RNA libraries were prepared using a miniaturized NEBNext Small RNA Library Preparation Kit (Illumina). Expression levels and distributions of tetraspanins and PLAP on EVs were analyzed by small RNA sequencing. Results: This study showed that immunomagnetic separation seemed to preferentially isolate specific subtypes of miRNA-rich EVs with high efficiencies. DGUC was able to separate EV with decreased yield and second-step purification by SEC did not improve EV separation. Moreover, our data demonstrated that tetraspanin distribution is unique for different sources of EVs. DiFi and placental explant supernatant (PES) exhibited different patterns of miRNA expression in CD9/CD63/CD81 derived EVs. All three tetraspanins were clustered together in DiFi EVs, while CD63 displayed significantly higher expression than CD9 and CD81 in PES EVs. Conclusions: The use of this immunomagnetic separation method allowed us to identify heterogeneous distributions of tetraspanins enriched EVs and highlighted the potential for isolation of distinct EV subpopulations produced by various cell types and biofluids. Overall, this work provides evidence for understanding of EV heterogeneity and opens up for further evaluation of EVs as promising biomarker and therapeutic targets for various diseases.

Abbas Hakim
University of California, San Diego

Developing an Automated High-Throughput RNA Isolation Pipeline for Extracellular RNAs from Various Biofluids for Small-RNA Sequencing

Abbas Hakim1,2, Peter De Hoff1,2, Cassandra Wauer1,2, Basant ElGhayati1,2, Tyler Ostrander1,2, Anelizze Castro-Martinez1,2, Priyadarshini Pantham1,2, Louise C. Laurent1,2
1. Department of Obstetrics, Gynecology & Reproductive Sciences, University of California San Diego, La Jolla, CA, USA
2. Sanford Consortium for Regenerative Medicine, La Jolla, CA, USA

Background: Extracellular RNAs (exRNAs) play important roles in cell-cell communication and are becoming common targets for development of disease biomarkers. Clinical applications of exRNA disease biomarkers may apply quantitative PCR or sequencing to verify the expression of these biomarkers. However, reproducibility and efficiency of upstream nucleic acid isolation remains a concern in utilizing these diagnostic assays. The development of robotic platforms offers automated and efficient high-throughput RNA isolations which aid the development of exRNA diagnostic assays. While manual RNA extractions allow for easier scaling of reaction volumes, automated extractions are restricted by instrument physical limitations. These limitations present challenges to efficiently obtain viable concentrations of exRNAs. While techniques for manipulating RNA concentrations such as RNA desiccation and RNA bead clean-ups are common, the effect on the resulting small RNAs is not well documented.

Aim: We aim to use miRNA expression profiles to assess differences between isolation methods, sample input volumes, and RNA concentration techniques to develop an automated high-throughput RNA isolation pipeline for small RNA library sequencing.

Methodology: Plasma, serum, and urine were collected from pregnant and non-pregnant patients and pooled for RNA isolation. Samples were processed in triplicate using the automated magnetic bead-based MagMAX kit on the Kingfisher Flex platform (plasma, serum, urine), the manual column-based Norgen kit (plasma, serum) and the manual phenol/chloroform-based miRCURY kit (urine). Serum/plasma input volumes of 100, 166, and 200 uL were tested with the MagMAX kit and 500 uL with the Norgen kit. Urine input volumes include 250 uL and 3.5 mL for the MagMAX and miRCURY kits respectively. Duplicate lysates for a single elution were tested to increase the yield of the automated extractions. MagMAX extracted RNA was used to compare desiccation and bead clean-up as methods for increasing concentration. Small RNA libraries (total n=372) were prepared using an automated and miniaturized version of the NEBNext Small RNA Library Preparation Kit.

Results: The manual kits had greater RNA yield than the MagMAX kit, however, upon desiccation and resuspension, the MagMAX samples reached similar concentrations across all biofluids. The bead cleanup resulted in a loss of smaller species of RNA and no increase in concentration. The MagMAX kit yielded higher percentages of miRNA than the Norgen kit (serum/plasma) but lower than the miRCURY kit (urine). Pregnancy status was the main driver of variation, followed by extraction kit, and sex. Examination of C19MC placental miRNAs showed comparable expression between the two extraction methods in serum/plasma.

Conclusions: Different miRNA populations are enriched between manual and automated kits, however, both methods successfully distinguish female pregnant, non-pregnant, and male serum/plasma samples. Desiccation of RNA produces better libraries but does not increase the overlap of miRNAs expressed between extraction methods. Bead cleanup treated RNA showed the poorest performance and may not be suitable for small RNA-seq. We have demonstrated that an automated high-throughput RNA extraction pipeline is viable for use in small RNA-seq and suited for large scale miRNA profiling studies. Sequencing data from samples that were extracted using duplicate lysates will be included in the final dataset.

Srimeenakshi Srinivasan

University of California, San Digo

k-TSP Algorithm Selected Serum miRNA Predictors of Gestational Age in Pregnancy

Srimeenakshi Srinivasan1,2, Priyadarshini Pantham1,2, Peter De Hoff1,2, Chanond Nasamran, Abbas Hakim1,2, Anelizze Castro1,2, Gabriela Cortes, Arundhati Calambur, Kajal Verma1, Adanna Anyikam1, Antoinette M Mason1, Vy Tran1, Morgan Meads1, Mariko Horii1,2

1. Department of Obstetrics, Gynecology & Reproductive Sciences, University of California San Diego, La Jolla, CA, USA
2. Sanford Consortium for Regenerative Medicine, La Jolla, CA, USA
3. Center for Computational Biology & Bioinformatics
4. Center for Excellence in Translational Immunogenomics

Introduction: Determination of gestational age (GA) using minimally invasive methods is essential for the management of pregnancy and its complications. MicroRNAs (miRNAs) have the potential to be used as biomarkers for prediction of GA. In this longitudinal study, we aimed to identify robust miRNA predictors of GA in maternal biofluids from healthy individuals.

Methods: Serum was collected from 6 healthy pregnant subjects longitudinally throughout pregnancy (n=40). Small RNA was extracted using the Norgen Plasma/Serum Circulating and Exosomal RNA Purification Kits (Slurry Format). Small RNA libraries were prepared using the NEBNext Small RNA Library Prep Kit, sequenced on the Illumina HiSeq 4000, and processed using the Genboree exceRpt small RNA pipeline. The samples were split into pairs according to the clinical trimester into First and Second, First and Third and Second and Third trimesters. Raw miRNA counts were filtered and subject to the k-TSP algorithm using the switchBox package to identify Top Scoring miRNA Pairs (TSP) within each pair of trimesters. These TSPs were validated on an independent cohort (Diurnal) of 132 samples.

Results: The k-TSP algorithm identified 9 miRNA pairs differentiating first and second trimesters, 8 pairs for first and third trimesters and 6 pairs for second and third trimesters from the training data set. These predicted the trimesters in the Diurnal cohort with 57.6%, 84.2% and 73.5% respectively.

Conclusion: The k-TSP algorithm identified several pairs of miRNAs in serum that are predictive of the trimester and have been further validated in in an independent cohort. These miRNA pairs will be further evaluated for their ability to predict the gestational age at a more granular level during pregnancy using other machine learning models.

Shivakumar Reddy
Centre for Molecular Neurosciences, Manipal Academy of Higher Education

Small extracellular vesicle loaded bevacizumab reduces the frequency of intravitreal injection required for diabetic retinopathy

"Shivakumar Reddy K1, Abhijna Ballal R1, Shailaja S2, Raghu Chandrashekar H3, Dinesh Upadhya1"
1. Centre for Molecular Neurosciences, KMC, MAHE, Manipal- 576104, Karnataka, India.
2. Department of Ophthalmology KMC, MAHE, Manipal- 576104, Karnataka, India.
3. Manipal College of Pharmaceutical Science, MAHE, Manipal- 576104, Karnataka, India.

Background: Diabetic Retinopathy (DR) is one of the most common microvascular complications of both types of diabetes. It remains a leading cause of severe vision loss and blindness in working-age people. DR leads to vision loss through abnormal new blood vessel formation through neovascularization in the retina, exudate formation and associated inflammation, oxidative stress, and enhanced retinal cell death. Anti - VEGF therapy through intravitreal injections is an established treatment for reducing VEGF levels in the retina to prevent neovascularization and hard exudates in diabetic macular edema associated with vision loss in DR [9 - 14]. Although clinically anti-VEGF therapy has several advantages, it is initially required monthly for controlling VEGF levels. Repeated intravitreal injections potentially cause devastating ocular complications, including trauma, intraocular haemorrhage, retinal detachment, and endophthalmitis. Several novel drug delivery strategies are currently adopted to reduce the burden of monthly intravitreal injections. Extracellular vesicles are considered next-generation drug delivery platforms. Mesenchymal stem cell-derived small extracellular vesicles (MSC - sEVs) with a size below 200 nm possess tremendous potential to carry drugs for treating many diseases. This ability is due to their biocompatibility, inherent immunomodulatory functions, and low immuno-stimulatory surface markers. Previous studies have shown that intravitreally injected MSC-EVs reached different retinal layers, and their presence was detected for several weeks. This suggests the possibility of MSC - EVs as one of the finest delivery systems to carry drugs for treating diabetic retinopathy. In the present study, we have loaded therapeutic bevacizumab into human bone marrow-derived MSC-sEVs and evaluated their therapeutic efficacy in reducing the frequency of intravitreal injections in a streptozotocin-induced model of diabetic retinopathy in rats.

Methodology: Small EVs were isolated from bone marrow-derived mesenchymal stromal cells (BM-MSC) and characterized. Bevacizumab was loaded into sEVs by freeze-thaw, co-incubation 0.2% saponin, and sonication methods. Bevacizumab loaded in EVs was checked for its antiangiogenic properties by chorioallantoic Membrane (CAM) assay in the egg chick embryo. To track the bevacizumab-loaded EVs in the retina, FITC conjugated bevacizumab was loaded into PKH26 labeled sEVs. Further, DR induced in Wistar Albino rats through the administration of streptozotocin. Blood sugar levels were monitored multiple times over months after the STZ injection, and the presence of exudates in the retina was confirmed through Evans blue leakage and FITC-dextran perfusion. The effect of anti-VEGF-loaded EVs on neovascularization was evaluated at different time intervals using VEGF immunostaining. The anti-apoptotic effect of EV-BZ was measured by quantifying tunnel-positive cells and caspase-3 expression in the retina. ELISA strips method was used to measure the anti-inflammatory effect for the following cytokines TNF-α, IL-1β, IL-1α, IL-6, interferon-γ, MCP-1, MIP-1α and RANTEs.

Results: Characterization of EVs using NanoSight tracking demonstrated a mean size of 167nm, TEM analysis revealed cup-shaped morphology, and western blot analysis revealed positive for TSG101 and CD 63 while negative for GM130. A significant amount of bevacizumab was loaded by all the tested methods. The bevacizumab-loaded EVs significantly inhibited the angiogenesis in the CAM assay. We have tracked bevacizumab-loaded EVs in different retina layers in naive rats. In STZ-induced DR, bevacizumab-loaded EVs significantly decreased the number of exudates in the retina, VEGF expression, and DR-induced cell death in the bevacizumab-loaded EV group compared to bevacizumab alone group over a period of two months. EV-BZ and BZ alone failed to reduce chronic retinal inflammation at two months.

Conclusion: The study provides a clinically important finding that MSC-sEV-loaded bevacizumab reduces the frequency of intravitreal injection required for treating diabetic retinopathy. This study provided the first evidence of prolonged benefits associated with MSC-sEV as a drug delivery system.

Alex Gao

University of California San Francisco, San Francisco VA Medical Center

exRNA carried by M2-like Macrophage Exosomes Suppress Cardiac Inflammation & Prevent Chronic Heart Failure after Myocardial Infarction

Alex Gao1,2, Martin Ng1,2, Anh Phu1,2, Ngan Vu1,2, Robert L. Raffai1,2,3

1. Department of Veterans Affairs, Surgical Service (112G), San Francisco VA Medical Center, San Francisco, CA 94121, USA.

2. Northern California Institute for Research and Education, San Francisco, CA 94121, USA.

3. Department of Surgery, Division of Endovascular and Vascular Surgery, University of California, San
16 Francisco, CA 94143, USA.

Title

exRNA carried by M2-like Macrophage Exosomes Suppress Cardiac Inflammation & Prevent Chronic Heart Failure after Myocardial Infarction

Introduction

Coronary artery disease often results in cardiac ischemia, which in turn can lead to chronic inflammation and heart failure (CHF). We previously reported that the anti-inflammatory compound FTY720 is effective in controlling CHF by suppressing cardiac inflammation following myocardial infarction (MI) in a mouse model of diet-induced occlusive coronary atherosclerosis. Unfortunately, FTY720 is contraindicated for patients with CHF as it causes bradycardia. To this end, we tested whether exosomes produced by M2-polarized human THP-1 macrophages (THP1-IL4-exo) could serve as an alternative therapeutic option. We recently demonstrated that miRNA cargo in THP1-IL4-exo are effective in controlling systemic inflammation and atherosclerosis in mice with hyperlipidemia. In this study, we sought to test whether THP1-IL4-exo could control CHF in mice that experience MI by reducing cardiac inflammation via the delivery of anti-inflammatory microRNA cargo.

Methods/Preliminary results:

We used Hypomorphic ApoE mice deficient in scavenger receptor Type-B1 expression (HypoE/SR-B1−/−) to model CHF that develops in response to MI caused by diet-induced coronary atherosclerosis. Our findings show that tri-weekly intraperitoneal injections of 1010 THP1-IL4-exo into HypoE/SRB1-/- mice fed a high-fat diet (HFD) extended their lifespan. Our data show that THP1-IL4-exo achieved this partially by attenuating inflammation. More specifically, inflammation was decreased by reducing the number of neutrophils in the circulation as well as the expression levels of M1 macrophage-related inflammatory genes (Tnfa, Il6) and by increasing the expression levels of M2 macrophage-related anti-inflammatory genes (Il10, Chil3) in cardiac tissue. THP1-IL4-exo also attenuated the expression of matrix metalloproteinases, a family of proteins upregulated in response to cardiac injury that drive ventricular remodeling, resulting in adverse cardiac dysfunction. Additionally, miRNA associated with cardiovascular health and inflammation were modulated in cardiac tissue of mice treated with THP1-IL4-exo. Such control of systemic and cardiac inflammation by THP1-IL4-exo led to functional improvements in cardiac function as detected by echocardiography that included an increase in fractional shortening and left ventricular ejection fraction.

Conclusion

Our findings support the use of THP1-IL4-exo and their miRNA cargo as therapeutic agents to control CHF in response to coronary artery disease.

Jessie Arce
Baylor College of Medicine

The NanoFlow Repository: A Resource for Sharing Standards-Compliant Metadata and Data for Flow Cytometry Experiments involving Extracellular Vesicles and other Particles

Jessie E. Arce1*, Joshua A. Welsh2*, Sean Cook2, John Tigges3, Ionita Ghiran3, Jennifer C. Jones2, Andrew Jackson1, Matthew Roth1, Aleksandar Milosavljevic1
1. Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA.
2. Translational Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA.
3. Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA.

Background: Extracellular Particles (EPs), including extracellular vesicles (EVs), have been identified as important carriers of exRNA and mediators of intercellular communication and implicated in various biological and pathological processes. Flow cytometry is a widely used method for analyzing EPs, but the need for standardized reporting of experimental metadata limits the reproducibility and comparability of EV flow cytometry studies.

Aims: To develop a resource for sharing standard-compliant metadata and data for EP-related flow cytometry experiments and to promote rigor and reproducibility in EP and exRNA research.

Methodology: We developed The NanoFlow Repository as an online platform to facilitate sharing of EP characterization data and standards-compliant metadata. This initially focused on single EV flow cytometer (EV-FC) and bead-based assays and has been expanded to include Rresistive pulse sensing (RPS) analyses. The metadata includes information about the experimental design, samples, instrument configuration, and analysis parameters, following the MIFlowCyt-EV framework. The platform allows for easy dataset discovery, exploration, and download, with the ability to filter datasets based on various parameters, including the type of flow cytometer used to generate the data. For enhancing reproducibility and standardization, the repository supports the deposition of FCMPASS, MPAPASS, and RPSPASS calibrated datasets, enabling the scientific community to share, compare, and validate EP-FC experiments more comprehensively. Researchers can create an account to upload EP-FC data files and link them to publications. In addition, the platform provides access to MIFlowCyt and MIFlowCyt-EV reports, samples, and related data files for each dataset.

Results: The NanoFlow Repository currently hosts 48 public datasets with 546 FCS files generated by various flow cytometers, providing a valuable resource for the EP research community. The repository enables users to access standards-compliant metadata and reproduce published results, facilitating validation or refutation of previous findings and promoting collaboration and data reuse for meta-analyses.

Conclusions: The NanoFlow Repository represents a valuable resource for the EP and exRNA research communities, enabling the sharing of standards-compliant metadata and data for EP-related flow cytometry experiments. Its use will improve the rigor and reproducibility of research and promote collaboration and data reuse. We invite the scientific community to explore and contribute to the NanoFlow Repository: https://genboree.org/nano-ui/ld/datasets.

Priyadarshini (Priya) Pantham
University of California San Diego

Transcriptomic Profiling of Urinary Extracellular RNA in Pregnancy and Severe Preeclampsia

Priyadarshini Pantham1,2, Marina Mochizuki1,2, Cassandra Wauer1,2, Srimeenakshi Srinivasan1,2, Peter De Hoff1,2, Amber L. Morey1,2, Aishwarya Vuppala1,2, Abbas Hakim1,2, Tyler Ostrander1,2, Basant ElGhayati1,2, Gisselle Santiago1, Dora J. Melber1, Marni B
1. Department of Obstetrics, Gynecology & Reproductive Sciences, University of California San Diego, La Jolla, CA, USA
2. Sanford Consortium for Regenerative Medicine, La Jolla, CA, USA

Background: Preeclampsia is a serious and common disease of pregnancy, which is characterized by maternal hypertension, as well as renal, hepatic, and placental dysfunction. Urine contains extracellular RNAs (exRNAs), which are associated with extracellular vesicles (uEVs) and other carriers. Urine is enriched for kidney-derived exRNAs, but also contains exRNAs from other tissues, including the placenta during pregnancy. Thus, maternal urine is a potential source of exRNA biomarkers for preeclampsia and associated organ disfunction.

Aim: We aimed to identify optimal methods for transcriptomic profiling of maternal urinary exRNAs in pregnancy by testing different exRNA isolation and library preparation methods. The ultimate goal is to apply these methods to identify biomarkers for non-invasive prediction and diagnosis of preeclampsia.

Methodology: RNA was extracted from pooled and/or individual samples of urine obtained from normal non-pregnant and pregnant females as well as males using input volumes of either 0.6 mL, 1 mL, or 4 mL. Samples were extracted in duplicate or triplicate using either vesicular (miRCURY, ExoRNeasy, SeraMir kits), or total (miRNeasy advanced, Norgen kits) exRNA isolation methods. Library preparation was automated and miniaturized to 1/5th of the manufacturers' recommended reaction volumes using a small volume liquid handler (SPT Labtech). Small RNA libraries (total n=208) were prepared using the NEBNext Small RNA library preparation kit, and long RNA libraries (total n=97) were prepared using the Takara SMART-Seq v4 Ultra Low Input RNA or the SMARTer Stranded Total RNA-Seq Kit v2 Pico Input kits.

Results: The greatest sources of variance were exRNA isolation method in small RNA libraries, and library preparation method in long RNA libraries. The miRCURY kit yielded small RNA libraries of significantly higher complexity compared to other methods, and long RNA libraries prepared using the SMART-Seq v4 kit yielded significantly more uniquely mapped reads compared to the Pico v2 kit ($p<0.05$). We have conducted a preliminary study using the miRCURY method to isolate exRNAs from gestational age-matched urine samples from normal (n=24) and severe preeclamptic (n=9) pregnancies, and have discovered 4 miRNAs that are differentially expressed ($p<0.05$).

Conclusions: We have established scalable pipelines for extraction and small and long RNA-Seq profiling of urinary exRNA in urine in a robust and reproducible manner, and have used these methods to identify miRNAs that may be differentially expressed between normal pregnancy and severe preeclampsia. More research is required to identify optimal methods of normalizing urinary vesicular and exRNA content due to the intrinsic variability of this biofluid in normal and diseased states.

Vinay Tiwary
exRNA Therapeutics Limited

Efficacy of exRP250- a biologic molecule against Imiquimod-induced Psoriasis in BALB/c Mice

Vinay Kumar Tiwary, Satyam Tiwary

Psoriasis, a chronic inflammatory skin disease, affects approximately 2-3% of the global population. It is characterized by abnormal keratinocyte differentiation, hyperproliferation of the epidermis, excess leukocyte infiltration, and pro-inflammatory cytokine production. The imiquimod (IMQ)-induced mouse model is widely used for testing new psoriasis drugs. exRP250 is an extracellular vesicle-based drug isolated from bacteria, containing tRNA fragments with antioxidant, anti-inflammatory, and immunomodulatory properties. This study aims to investigate the efficacy of exRP250 drug in the IMQ-induced psoriasis model in BALB/c mice.

Methods:

42 male BALB/c mice were divided into the control group (Group I) and six experimental psoriasis groups (Groups II to VII). Psoriasis was induced in the animals of Groups II to VII by topical application of IMQ cream for 14 days. After the confirmation of psoriasis model, exRP250 drug along with the reference standard drug Clobetasol Propionate was applied topically to the mice in Groups III to VI for 14 days. Group VII was treated with high-dose exRP250 drug plus IMQ for 14 days. PASI score and ear thickness were measured during the treatment phase. At the end of the experiment, cytokine levels were measured in serum, ear and skin tissue, and histopathological examination was performed on ear and skin tissue.

Results:

Spleen weight and ear thickness were significantly reduced in a dose-dependent manner in Groups III-V treated with exRP250 drug compared to Group II ($P<0.0001$; $P<0.001$). The PASI score was significantly decreased in Group V ($P<0.0001$) and partially decreased in Groups III and IV compared to the reference drug and Group VI ($P<0.05$). ExRP250 drug significantly reduced cytokine levels (IL-17, IL-23, and TNF-α) in serum ($P<0.0001$; $P<0.001$) and skin tissue (NF-kB and IL-23) ($P<0.001$; $P<0.05$) in all dose levels compared to Group II and VI. Gene expression levels of IL-17A, IL-10, TLR-9, NF-kB, and INF-G were also significantly reduced in the exRP250-treated groups compared to Group II, VI, and VII. Histopathological analysis showed an improvement in all doses of exRP250-treated groups compared to Group II and VI.

Conclusions:

The results suggest that exRP250 is a promising anti-psoriatic drug with antioxidant, anti-inflammatory, and immunomodulatory properties. It significantly improved the PASI score and reduced cytokine levels in the serum, ear and skin tissue, and gene expression in BALB/c mice with IMQ-induced psoriasis. These findings may have significant implications for the development of new therapeutic strategies in psoriasis treatment.

Keywords: exRP250 drug, Imiquimod, Psoriasis, Cytokines, PASI