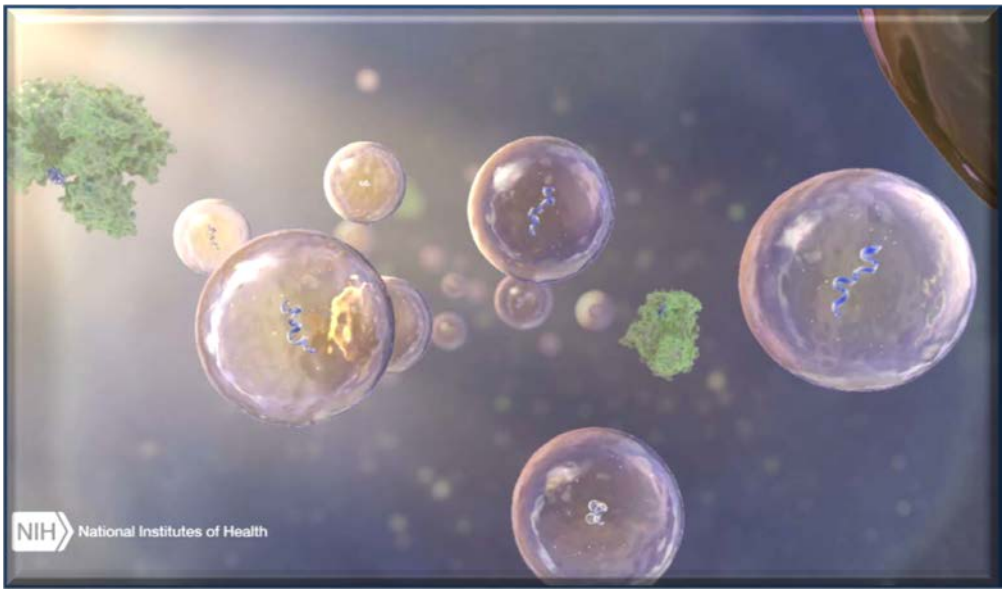
exRNA

COMMUNICATION PROGRAM

November 6 & 7, 2017

NIH Extracellular RNA Communication Consortium
9th Investigators' Meeting
November 6 & 7, 2017
Hilton Rockville Hotel, Rockville, MD



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NIH Extracellular RNA Communication Consortium
9th Investigators' Meeting
November 6-7, 2017

Hilton Washington DC/Rockville Hotel & Executive Meeting Center
1750 Rockville Pike, Rockville, MD 20852

Workshop Descriptions

Vesicle Isolation Workshop: Sunday, November 5th, 2017 6-8 pm Regency Room
Prior Registration Required

The meeting is to discuss vesicle isolation approaches, define vesicle yields, and most importantly, the functionality of vesicles. We have previously discussed many issues around functional studies of vesicles including purity, stability, intrinsic heterogeneity, tissue homing, treatments of originating tissues, and mode of delivery. However, a critical first step preceding these issues is the initial isolation strategy to recover functional vesicles.

There is no limit as to the number of models in which vesicles are used, but there are a finite number of isolation approaches which will be discussed and analyzed. The goal of this workshop will be to discuss isolation techniques and learn from successes and failures from various laboratories. The approaches discussed will be written in detail and then circulated to the participants.

Special Workshop: Translating vesicle biology to clinical therapy.

6:00 p.m.	Ultra-Centrifugation: Pitfalls and benefits	Peter Quesenberry
6:10 p.m.	Tangential flow filtration for scalable GMP-grade purification of EVs	Giovanni Camussi
6:20 p.m.	Refinements to ultra centrifugation for EV isolation	Robert Raffai
6:30 p.m.	Fluorescent activated vesicle sorting (FAVS) and other methods for understanding exosomal heterogeneity	Jeffery Franklin
6:40 p.m.	Making EVs that deliver functionally competent mRNA to the intended target cells/tumors	A.C. Matin
6:50 p.m.	Bacteria-derived sRNAs circulating on lipoproteins	Kasey Vickers
7:00 p.m.	Tangential flow filtration (TFF) as an alternative to ultracentrifugation purification of EVs	Thomas Schmittgen
7:10 p.m. – 8:00 p.m.	**Open Discussion**	

Data Management and Resource Workshop: Monday, November 6th, 2017 11:30am-1pm Plaza Ballroom

Open to all, no prior registration required

The Extracellular RNA Communication Consortium Data Management and Resource Repository (DMRR) workshop will highlight DMRR-developed online resources that are available for the scientific community. The focus will be on a collaborative project of the ERCC, the Virtual Biorepository (VBR) (https://genboree.org/vbr-hub/), which aims to connect researchers in need of biosamples with geographically distributed researchers in possession of shareable biosamples, and to assist in arranging biosample exchange. A brief background on the genesis of the VBR, key goals, and user guidelines will be described, and future plans reviewed. The demo will cover VBR functionality, including content and overview of the 43,724 biosamples from various diseases and conditions, how to execute and save searches, view and store search results, contact biosample providers to request biosamples, and how one may join the VBR as a biosample provider. The VBR demo will be followed by Q & A and discussion. On behalf of the DMRR, the workshop will be hosted by Matt Roth and the VBR demo given by William Thistlethwaite, both from Baylor College of Medicine.

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Monday, November 6th, 2017

Welcome and Registration

8:15 a.m.	Registration opens	
9:15 a.m.	Opening Remarks	

Session I: Extracellular Vesicle Biogenesis and Function

Session Chairs: Drs. Nihal Altan-Bonnet & Xandra Breakefield

9:30 a.m.	HIV-1 Latency: The Relationship between Virus, Exosomes, and TAR-gag, a Novel HIV-1 RNA.	Robert Barclay
9:45 a.m.	Inter-Organismal Vesicular Transmission of Enteric Viruses	Marianita Santiana
10:00 a.m.	CD63, MHC class 1, and CD47 identify subsets of extracellular vesicles containing distinct populations of non-coding and microRNA	Sukhbir Kaur
10:15 a.m.	The RNA binding protein HuR regulates extracellular vesicle secretion in colorectal cancer	Daniel A. Dixon
10:30 a.m.	Oncogene Regulated Production and Release of Vesicles and Extracellular RNAs	Seda Kilinc Avsarglu
10:45 a.m.	***Discussion: Cargo Sorting***	
11:30 a.m.	***Lunch* and DMRR Workshop***	
1:00pm	***Special Presentation:*** Methodologies to Standardize Flow Cytometric Measurement of Extracellular Vesicles and Quantitatively Measure Extracellular RNA	Erica Stein

Session II: exRNA and Extracellular Vesicle Standards and Resources

Session Chairs: Drs. Kendall Jensen & Hameeda Sultana

1:30 p.m.	Definition of serum ribonucleoprotein composition and its regulation and function	Klaas Max
1:45 p.m.	Comparison of different technical platforms for circulating miRNA profiling	Vasily Aushev
2:00 p.m.	Full-coverage landscape of extracellular coding and non-coding RNA released by human glioma stem cells	Anna M. Krichevsky
2:15 p.m.	The exRNA Atlas resource provides insights into exRNA biology through cross-study analysis of exRNA profiles	Aleksandar Milosavljevic
2:30 p.m.	exceRpt: the extracellular RNA-Seq processing pipeline of the ERCC	Joel Rozowsky
2:45 p.m.	***Break***	

Session III: Research, Diagnosis and Biomarkers

Session Chairs: Drs. Leonora Balaj & Tomer Cooks

3:00 p.m.	Extracellular vesicle microRNA signatures reveal tissue heterogeneity in defined glioblastoma subtypes	Agnieszka Bronisz
3:15 p.m.	MicroRNA Biomarkers for Alzheimer's Disease in Human Cerebrospinal Fluid	Julie Saugstad
3:30 p.m.	The bioavailability and distribution of exosomes and their RNA cargos from bovine and porcine milk in mice	Janos Zempleni
3:45 p.m.	Extracellular RNA profiles with human age	Nicole Noren Hooten
3:00 p.m.	Plasma-based detection of the mutant IDH1.R132H in glioma patients	Leonora Balaj
4:15 p.m.	***Break***	
4:30 p.m.-6:30 p.m.	**Poster Session and Networking *(Foyer)***	

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Tuesday, November 7th, 2017

Special Session: Navigating Regulatory Submissions		
9:30 a.m.	Regulatory Considerations for *In Vitro* Diagnostics	Aaron Schetter
10:00 a.m.	Regulatory Considerations for Biologics	Malcolm Moos
Session IV: Therapeutic Uses of exRNA and Extracellular Vesicles	*Session Chairs: Drs. Ana Robles & Tom Schmittgen*	
10:30 a.m.	Anti-HER2 scFv-directed extracellular vesicle-mediated mRNA-based gene delivery arrests growth of HER2-positive human breast tumor xenografts by prodrug activation	A.C. Matin
10:45 a.m.	Modified Extracellular Vesicles Improves Host Immune Response in Colorectal Cancer	Subbaya Subramanian
11:00 a.m.	Optimizing exosome production and intracerebroventricular delivery to brain	Reka A. Haraszti
11:15 a.m.	Tissue Regeneration by Exosome Engineering	James D. Bryers
11:30 a.m.	N6-Methyladenosine (m6A) RNA Modifications Mediate the Beneficial Function of Human CD34+ Stem Cell-Derived Exosomes to Repair the Failing Heart	Susmita Sahoo
11:45 a.m.	***Lunch* and Networking***	
Session V: Cutting-Edge Technologies	*Session Chairs: Drs. Dolores Di Vizio & Jennifer Jones*	
1:15 p.m.	Quantification and characterization of low-abundance exosomes and RNA biomarkers with Interferometric Reflectance Imaging	Derin Sevenler
1:30 p.m.	Role of Red Blood Cell derived extracellular vesicles in cardiac remodeling after myocardial infarction in a transgenic murine model	Saumya Das
1:45 p.m.	Microfluidic Isolation and Molecular Profiling of Tumor-specific Extracellular Vesicles	Berent Aldikacti
2:00 p.m.	Sequencing exosomal mRNA and lncRNA across human biofluids coupled with targeted enrichment of exosomal sub-populations reveals novel biomarkers	Robert Kitchen
2:15 p.m.	Evaluating functional delivery of cargo in extracellular vesicles	Meadhbh Brennan
2:30 p.m.	***Break***	
Session VI: Discussions Not to Miss – Future Directions of Research	*Session Chairs: Drs. Louise Laurent & Aleksandar Milosavljevic*	
2:45 p.m.	Technological Challenges & Opportunities	Jennifer Jones & Mark Ansel
3:10 p.m.	Critical Questions in EV Biology	Alissa Weaver & Mike McManus
3:35 p.m.	The Future of Clinical Applications of EVs/exRNAs	Tushar Patel & Saumya Das
4:00 p.m.	Open Discussion	
5:00 p.m.	**Closing Remarks**	
5:15 p.m.	Meeting Adjourns	

NIH Extracellular RNA Communication Program Introduction

The concept that RNA molecules are secreted in the extracellular spaces and act as endocrine signals to alter the phenotypes of target cells, both locally and at distant sites, represents a novel paradigm in intercellular signaling. Recent advances in RNA sequencing technologies have identified a large and diverse population of extracellular RNA (exRNA) including microRNA and long non-coding RNA (lncRNAs). Given that approximately 60% - 80% of all protein encoding genes is regulated by microRNA and certain lncRNAs have been linked to regulation of the epigenome, extracellular delivery of these RNAs could have profound implications for a wide range of physiologic and pathologic processes.

In humans, exRNAs are found in all body fluids examined, including blood, saliva, urine, breast milk, cerebral spinal fluid (CSF), amniotic fluid, ascites, and pleural effusions. Recent reports in the literature suggest that exRNAs have both protective and pathogenic roles in a variety of human diseases. Further, functional plant- and microbe-derived exRNAs have been identified in human serum and cells, suggesting that trans-kingdom exRNA communication could explain some associations between environmental exposures and health or disease.

Taken together, the above findings highlight the transformative potential that secreted RNAs may have in the regulation of health and disease. However, to realize the potential that exRNAs may have as health/disease indicators and/or as therapeutic molecules, fundamental principles of their biogenesis, distribution, uptake, and function need to be defined. While exRNAs are known to be encapsulated in extracellular vesicles (EVs), recent studies have also demonstrated their presence in nuclease-resistant complexes with RNA-binding carrier proteins, such as HDL and Argonaut, in serum. A better understanding of exRNA sorting to different secretory pathways, regulation of secretion, mechanisms of targeting, and effector function in target cells would generate opportunities to identify novel strategies for prognosis, diagnosis, and intervention of many diseases.

The Common Fund Extracellular RNA Communication Program has been developed to address critical issues in this nascent field. Both fundamental scientific discovery and innovative tools and technologies will be required to advance the field. The key components (and associated FOAs) that need attention include: (a) defining the fundamental principles of exRNA biogenesis, distribution, uptake, and function, developing the molecular tools, technologies, and imaging modalities to enable these studies (RFA-RM-12-012), (b) generating a reference catalog of exRNAs present in the body fluids of normal healthy individuals that would facilitate disease diagnosis and therapeutic outcomes (RFA-RM-13-014), (c) demonstrating the clinical utility of exRNAs as therapeutic agents and/or biomarkers and developing the scalable technologies required for these studies (This FOA and RFA-RM-12-014); and (d) developing 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 (RFA-RM-12-010). Awards funded under these FOAs are anticipated to involve activities conducted by multidisciplinary teams of investigators. Awardees from all 5 initiatives will form a consortium, with the overarching goal of determining fundamental principles associated with exRNAs. Comparisons across studies will be essential to establish these cross-cutting principles so investigators must be willing to act as part of the consortium.

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.

Cooperative Agreement Terms and Conditions of Award

The following special terms of award are in addition to, and not in lieu of, otherwise applicable U.S. Office of Management and Budget (OMB) administrative guidelines, U.S. Department of Health and Human Services (DHHS) grant administration regulations at 45 CFR Parts 74 and 92 (Part 92 is applicable when State and local Governments are eligible to apply), and other HHS, PHS, and NIH grant administration policies.

The administrative and funding instrument used for this program will be the cooperative agreement, an "assistance" mechanism (rather than an "acquisition" mechanism), in which substantial NIH programmatic involvement with the awardees is anticipated during the performance of the activities. Under the cooperative agreement, the NIH purpose is to support and stimulate the recipients' activities by involvement in and otherwise working jointly with the award recipients in a partnership role; it is not to assume direction, prime responsibility, or a dominant role in the activities. Consistent with this concept, the dominant role and prime responsibility resides with the awardees for the project as a whole, although specific tasks and activities may be shared among the awardees and the NIH as defined below.

The PD(s)/PI(s) will have the primary responsibility for:

- Determining experimental approaches, designing protocols, setting project milestones and conducting experiments;
- Adhere to the NIH policies regarding intellectual property, data release and other policies that might be established during the course of this activity;
- Submit quarterly progress reports during the two year pilot phase, in a format as agreed upon by the NIH Common Fund Extracellular RNA Communication Steering Committee. Projects that are selected for continued support through the UH3 mechanism will submit progress reports on a regular basis, at least biannually;
- Accept and implement any other common guidelines and procedures developed for the NIH Common Fund Extracellular RNA Communication Program and approved by the NIH Common Fund Extracellular RNA Communication Steering Committee;
- Accept and participate in the cooperative nature of the NIH Common Fund Extracellular RNA Communication Consortium;
- Attend bi-annual workshops organized by the NIH and the DMRR.

Intellectual Property

The NIH recognizes that intellectual property rights may play a role in achieving the goals of this program. To this end, all awardees shall understand and acknowledge the following:

The awardee is solely responsible for the timely acquisition of all appropriate proprietary rights, including intellectual property rights, and all materials needed for the applicant to perform the project.

Before, during, and subsequent to the award, the U.S. Government is not required to obtain for the awardee any proprietary rights, including intellectual property rights, or any materials needed by the awardee to perform the project.

The awardee is required to report to the U.S. Government all inventions made in the performance of the project, as specified by 35 U.S.C. Sect. 202 (Bayh-Dole Act).

Awardees are expected to make new information and materials known to the research community in a timely manner through publications, web announcements, reports to the NIH Common Fund Extracellular RNA Steering Committee, and other mechanisms.

Citation Language

Each publication, press release, or other document about research supported by an NIH award must include an acknowledgment of NIH

award support and a disclaimer such as "*This [insert specific grant number] is supported by the NIH Common Fund, through the Office of Strategic Coordination/Office of the NIH Director.*" Prior to issuing a press release concerning the outcome of this research, please notify the NIH awarding IC in advance to allow for coordination.
Awardees will retain custody of and have primary rights to the data and resources developed under these awards, subject to Government rights of access consistent with current HHS, PHS, and NIH policies.

Publications

The Program Director(s)/Principal Investigator(s) will be responsible for the timely submission of all abstracts, manuscripts and reviews (co)authored by project investigators and supported in whole or in part under this Cooperative Agreement. The Program Director(s)/Principal Investigator(s) and Project Leaders are requested to submit manuscripts to the NIH Project Scientist within two weeks of acceptance for publication so that an up-to-date summary of program accomplishments can be maintained.
Publications and oral presentations of work conducted under this Cooperative Agreement are the responsibility of the Program Director(s)/Principal Investigator(s) and appropriate Project Leaders and will require appropriate acknowledgement of NIH support. Timely publication of major findings is encouraged.

The successful development of biomarkers and/or therapeutics from exRNA require either substantial investment and support by private sector industries, and/or may involve collaborations with other organizations such as academic, other government agencies, and/or non-profit research institutions not directly involved in the NIH-funded Extracellular RNA Communication Program.

Steering Committee

A Steering Committee will serve as the main governing board of the Extracellular RNA Communications Consortium. The Steering Committee membership will include NIH Project Scientists and the PD/PI of each awarded cooperative agreement. The PD/PI of each award (or designee) will have one vote on the Steering Committee. The Project Scientists may vote, but the total votes will count as a maximum of one-third of the total number of votes. The Steering Committee Chair will not be an NIH staff member. Additional members may be added by action of the Steering Committee. Other government staff may attend the Steering Committee meetings, if their expertise is required for specific discussions. The Steering Committee will:

- Discuss progress in meeting the goals of various exRNA projects;
- Develop recommendations for uniform procedures and policies necessary to meet the goals of the Research Network, for example for data quality measures and assessment, conventions for data deposition, or measuring costs and throughput. Adoption of procedures and policies developed by the Steering Committee will require concurrence by External Scientific Consultants.
- The Steering Committee will also serve as a venue for coordination on the improvement of exRNA scientific methods, for example by disseminating best practices and collectively evaluating new procedures, resources, and technologies.
- The Steering Committee will consider collective goals for the Consortium, will determine how joint publication of results will contribute toward the goals of the exRNA Program, and will coordinate joint publication as needed to demonstrate overarching principles of exRNAs. It will schedule the time for, and prepare concise (3 to 4 pages) summaries of, the Steering Committee meetings, which will be delivered to members of the group within 30 days after each meeting.
- Each full member will have one vote. Awardee members of the Steering Committee will be required to accept and implement policies approved by the Steering Committee.

Dispute Resolution

Any disagreements that may arise in scientific or programmatic matters (within the scope of the award) between award recipients and the NIH may be brought to Dispute Resolution. A Dispute Resolution Panel composed of three members will be convened. It will have three members: a designee of the Steering Committee chosen without NIH staff voting, one NIH designee, and a third designee with expertise in the relevant area who is chosen by the other two; in the case of individual disagreement, the first member may be chosen by the individual awardee. This special dispute resolution procedure does not alter the awardee's right to appeal an adverse action that is otherwise appealable in accordance with PHS regulation 42 CFR Part 50, Subpart D and DHHS regulation 45 CFR Part 16.

The External Scientific Panel

The External Scientific Panel (ESP) will be responsible for reviewing and evaluating the progress of the projects in the Extracellular RNA Communication (ERC) program toward meeting their individual and collective goals. The ESP:

- Will be composed of four to six senior scientists with relevant expertise who are neither PIs nor collaborators of a cooperative agreement involved in the consortia,
- Are appointed by and provide comments and recommendations to the NIH concerning the overall direction of the consortia and the continued support of the components of the consortia,
- May be enlarged permanently, or on an ad hoc basis, as needed,
- Meet at least once a year coinciding with an ERC program investigators meeting to interact with the awardees and NIH staff,
- Meet at least once a year with the ERC program Steering Committee to raise critical topics for consideration by the ERC consortium PD(s)/PI(s),
- Meet at least once a year with NIH Project Team (NIH staff) to offer their individual comments concerning the overall progress of the ERC program in accomplishing its goals and the performance of its various components and recommend any changes that may be necessary by the NIH.
  - At this meeting NIH staff will draft a summary of the comments and recommendations and submit these to the ESP for final approval. The final version will be sent to the consortium PD(s)/PI(s).
  - Prior to the next Steering Committee meeting, the ESP will be provided a written response to their comments and recommendations (usually from the consortia PD(s)/PI(s), with help from NIH program staff as needed). The Steering Committee chair then provides a presentation on the progress the program has made in response to the ESP recommendations and comments.
- Meet with the NIH Project Team (NIH Staff) on an ad hoc basis by teleconference as needed to deal with unforeseen issues
- Will maintain confidentiality of the unpublished and/or proprietary data presented and maintain confidentiality of programmatic discussions with the NIH Project Team (NIH staff)

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Beverly Davidson, Ph.D.
Director of the Center for Cellular and Molecular Therapeutics The Children's Hospital of Philadelphia

Dr. Davidson is an expert in neurodegenerative diseases and novel gene therapy approaches. Her work has utilized RNAi as a tool for gene knockdown to reduce the mutant genes of Spinocerebellar Ataxia and Huntington's Disease. Her research expertise has also extended to understanding how noncoding RNAs participate in neural development and neurodegenerative diseases processes.

Thomas R. Gingeras, Ph.D.
Professor and Head of Functional Genomics
Cold Spring Harbor Laboratory

Dr. Gingeras' research interests are in empirical and computational approaches to study the organization of information found within genomes and the roles non-protein coding RNAs assume both as part of the informational content and regulation of the protein coding content. He is a world leader in studying the genome-wide organization of transcription and the functional roles of non-protein coding RNA. He is also a member of the ENCODE (Encyclopedia of DNA Elements) program.

Jan Lötvall, M.D., Ph.D.
Professor and Chief Physician, University of Gothenburg; Chairman of the Krefting Research Centre, University of Gothenburg
Chief Scientist, Codiak BioSciences
Past President of the International Society for Extracellular Vesicles (ISEV) (2012-2016)

Dr. Lötvall's long-standing scientific interests are in the basic mechanisms of asthma and allergy. He is a clinical specialist in allergy and clinical pharmacology with expertise in basic and clinical research in pharmacological interventions in asthma. More recently, his work in identifying exosome-mediated transfer of mRNAs and miRNAs as a novel mechanism of cellular communication has led the international field of extracellular RNA biology.

Janusz Rak, M.D., Ph.D. Professor of Pediatrics McGill University
Montreal Children's Hospital Research Institute
Dr. Rak's research interests are in understanding the role of the vascular system in the development, progression, and responsiveness to therapy of human cancers. He is an expert the role of extracellular vesicles in oncogenesis. His work has described a glioblastoma vesiculation process where activation of oncogenic pathways increases biogenesis of exosomes.

Gyongyi Szabo, M.D., Ph.D.
Professor of Medicine, Division of Gastroenterology, Department of Medicine Associate Dean for Clinical and Translational Sciences
Director, M.D./Ph.D. Program
University of Massachusetts Medical School
Dr. Szabo is an accomplished physician scientist with research expertise in the regulatory mechanisms of immunity and inflammation in liver diseases. She discovered the molecular and cellular mechanisms of alcohol- and hepatitis C- induced augmentation of inflammation. More recently, her translational sciences and clinical research have focused on therapeutic approaches in non-alcoholic fatty liver disease

Kenneth W. Witwer, Ph.D.
Assistant Professor of Molecular and Comparative Pathobiology. The Johns Hopkins University School of Medicine
Dr. Witwer investigates extracellular vesicles and RNA in the context of HIV infection and inflammatory disease and also actively assessing the effects of diet on extracellular RNA as a potential therapeutic approach.

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Trans-NIH Project Team

Institute/Center Co-Chairs:

Christopher P. Austin, M.D.
Director
National Center for Advancing Translational Sciences (NCATS)

Dinah S. Singer, Ph.D.
Director
Division of Cancer Biology, National Cancer Institute (NCI)

Common Fund Program Leader:

Patricia (Trish) Labosky, Ph.D.
Program Leader
Office of Strategic Coordination,
Division of Program Coordination, Planning, and Strategic Initiatives
Office of the Director, National Institutes of Health (NIH)

Members:

Alexandra Ainsztein, Ph.D.
Program Director
Division of Cell Biology and Biophysics
National Institute of General Medical Sciences (NIGMS)

Philip J. Brooks, Ph.D.
Program Director
Division of Clinical Innovation
National Center for Advancing Translational Sciences (NCATS)

Vivien G. Dugan, Ph.D.
Program Officer in Functional Genomics
Office of Genomics and Advanced Technologies
National Institute of Allergy and Infectious Diseases (NIAID)

Aniruddha Ganguly, Ph.D.
Program Director
Cancer Diagnosis Program, Division of Cancer Treatment and Diagnosis
National Cancer Institute (NCI)

Tina Gatlin, Ph.D.
Program Director
National Human Genome Research Institute (NHGRI)

Max Guo, Ph.D. Chief, Genetics and Cell Biology Branch, Division of Aging Biology National Institute on Aging (NIA)

Christine A. Kelley,Ph.D. Director, Division of Discovery Science and Technology
National Institute of Biomedical Imaging and Bioengineering (NIBIB)

Lillian Kuo, Ph.D.
Program Officer
Division of AIDS
National Institute of Allergy and Infectious Diseases (NIAID)

Tania B. Lombo, Ph.D.
Scientific Program Analyst
National Center for Advancing Translational Science (NCATS)

George A. McKie, D.V.M., Ph.D. Program Director, Ocular Infection, Inflammation, and Immunology
National Eye Institute (NEI)

Project Team Coordinators:

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Program Director
Division of Cancer Biology, Cancer Immunology and Hematology Branch
National Cancer Institute (NCI)

Danilo A. Tagle, Ph.D.
Associate Director for Special Initiatives
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Margaret Ochocinska, Ph.D.
Program Director, Translational Blood Science and Resources Branch
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National Heart, Lung, and Blood Institute (NHLBI)

Richard Panniers, Ph.D.
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Robert Star, M.D.
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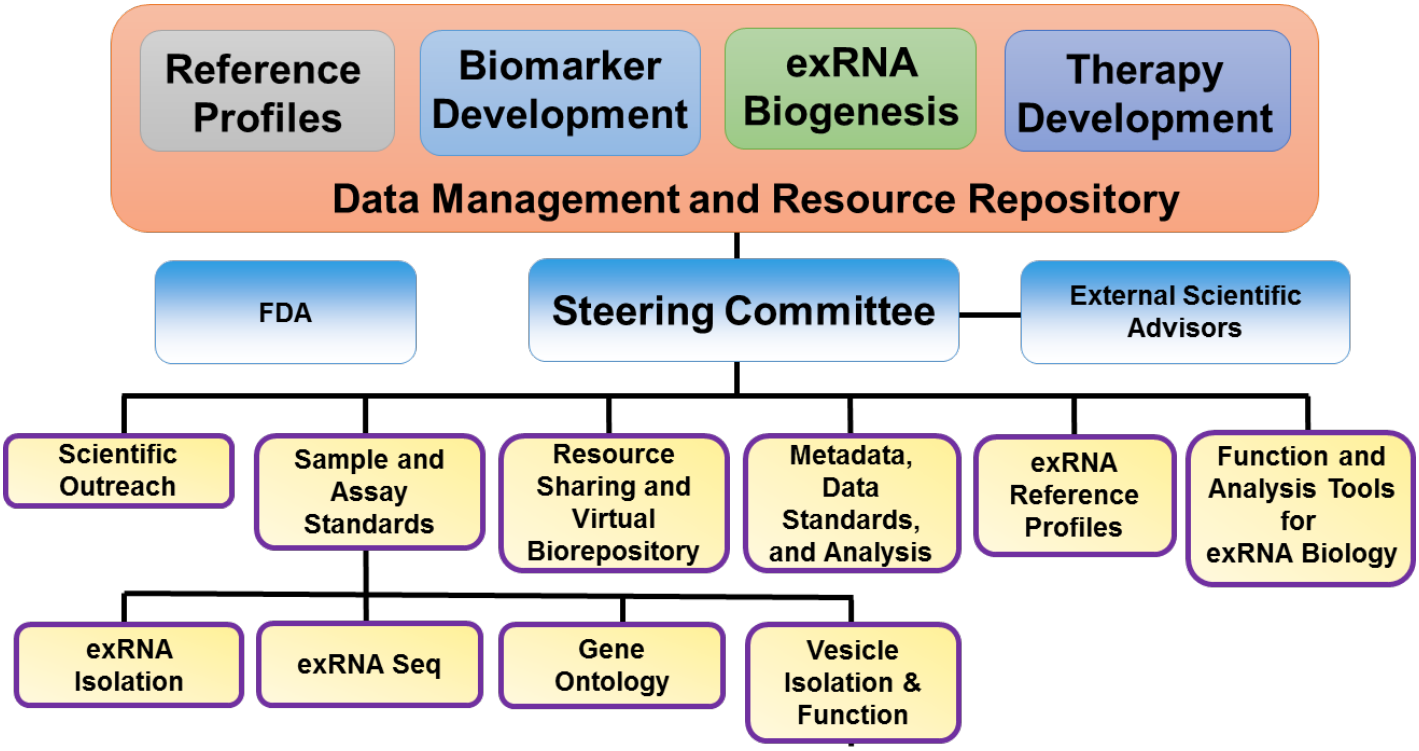
Division of Kidney, Urologic and Hematologic Diseases
National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

Margaret Sutherland,
Ph.D. Program Director
Neurodegeneration
Cluster
National Institute of Neurological Disorders and Stroke (NINDS)

Jessica M. Tucker, Ph.D.
Program Director
National Institute of Biomedical Imaging and Bioengineering

ERCC Structure and Working Groups

The ERCC Steering Committee is the main governing board for the Consortium. Each of the 30 awards from the five research initiatives has one representative on the Steering Committee. The Steering Committee is led by two Consortium investigators. The Steering Committee considers the collective goals for the Consortium, discusses the progress made towards these goals, and coordinates the improvement of exRNA research activities. Currently, there are four Consortium Working Groups: 1) Sample and Assay Standards, 2) Metadata and Data Standards, 3) Resource Sharing and 4) Scientific Outreach. These Consortium Working Groups are designed to address both the collective goals of the Consortium and the needs of individual projects through cross- collaborations. Current Working Groups are phased out when goals are met and new ones formed as needs arise. Accordingly, additional subgroups have formed to address issues with GeneOntology, Non-vesicular or Lipoprotein-associated exRNA, RNA-Seq, RNA Isolation, and Vesicle Isolation and Function.



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Sample and Assay Standards Working Group - Steve Gould and Louise Laurent

The goals of the Sample and Assay Standards Working Group are to survey the current approaches to sample collection and processing, exRNA isolation, and exRNA analysis; to identify high-priority areas that warrant systematic cross-institutional collaborative studies for identification of standardized approaches; and to carry out such studies and distribute the results to the community

Non-vesicular and Lipoprotein-associated exRNAs Subgroup - Robert Raffai and Kasey Vickers

The goals of the Non-vesicular and Lipoprotein-associated exRNA Subgroup are to explore the contribution of lipoprotein-associated RNA as a source of contamination and possible bias in studies of microvesicle isolated using current methods; to explore the impact of hyperlipidemia and storage conditions on the miRNA content plasma lipoproteins; to assist investigators to gain knowledge of methods used to isolate plasma lipoproteins as a potential source of exRNA for future studies

RNA Isolation Subgroup - Louise Laurent

The goals of the RNA Isolation Subgroup are to systematically compare the performance of several RNA isolation methods to determine which methods consistently yield acceptable quantities of high quality exRNA for, qRT-PCR, small RNA sequencing and long RNA sequencing; this will include methods that extract exRNA from unfractionated biofluids, and those that extract exRNA from fractions enriched for extracellular vesicles

Vesicle Isolation and Function Subgroup - Peter Quesenberry and Robert Raffai

The goals of the Vesicle Isolation and Function Subgroup are to focus on the biologic effects of extracellular vesicles/RNA, with particular interest in cell fate changes induced by extracellular RNA, the capacity of restoring injured tissue and the mechanisms underlying such cell fate change

RNA-Seq Subgroup- Saumya Das, Kendall Jensen and Joel Rozowsky

The goals of the RNA-seq Subgroup are to determine a set of library preparation protocols that yield reproducible and accurate RNA-seq data; to compare RNA- seq data across different sites and biofluids from different investigators using the same analysis pipeline to provide a list of commonly expressed exRNAs in biofluids and overcome challenges in comparing data from different data sets; to discuss challenges in RNA-mapping strategies, normalization strategies, analysis pipelines, and platforms for validation of RNA-seq results.

Metadata and Data Standards Working Group - Aleks Milosavljevic and Joel Rozowsky

The goals of the Metadata and Data Standards Working Group are to develop data and metadata standards, develop tools for tracking metadata, define methods to enable streamlined data flow into the DMRR, develop the exRNA-Seq analysis toolset, assist consortium members with their data analysis needs and provide access to exRNA profiling data through a working prototype of the exRNA Atlas

Gene Ontology Subgroup – Kei-hoi Cheung and Louise Laurent

The goals of the Metadata and Data Standards Working Group are to work with domain experts and ontology experts to propose updated terms and relationships to be added to Gene Ontology (GO); this involves liaisons with the international community: Louise Laurent (ERCC), Steve Gould (ASEMV), Suresh Mathivanan (ISEV), and Clotilde Thery (ISEV)

Resource Sharing Working Group - Fred Hochberg and Matt Huentelman

The goals of the Resoure Sharing Working cGroup are to provide a shared resource structure to address issues identified by consortium members, to create a template for IRB filing and consenting that would provide for prospective sharing of materials and biofluids, to create a mechanism for academic and corporate collaboration based upon universal MTA agreement, and to develop a Virtual Biospecimen Repository

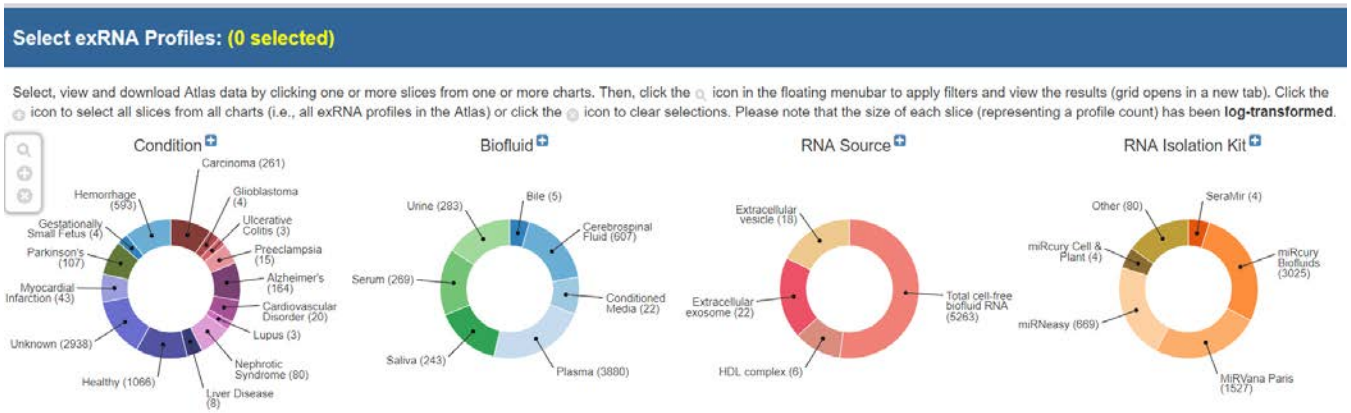
Scientific Outreach Working Group – Roger Alexander, David Galas, and Tushar Patel

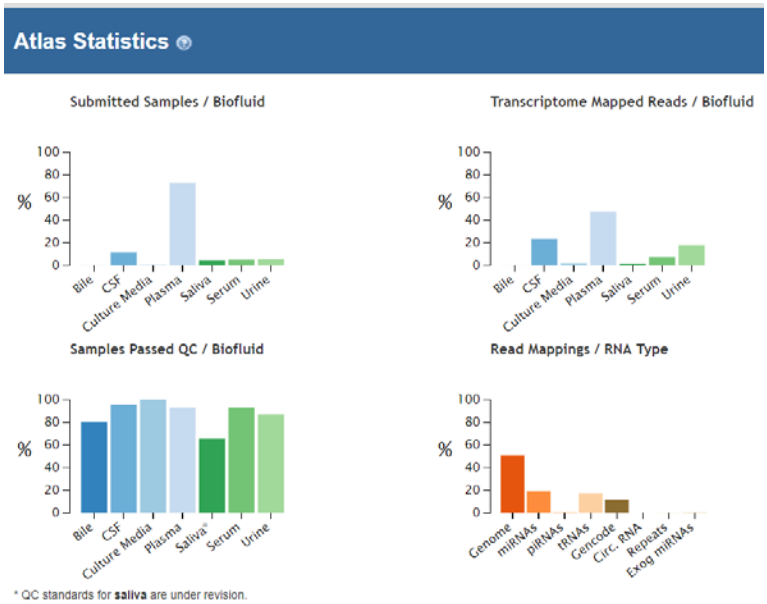
The goals of the Scientific Outreach Working Group are to engage with the scientific community through the web-based exRNA Portal that enables access to research, knowledge, tools, and results of the activities of the consortium, and through development of and participation in workshops and symposia; the four major areas of outreach: (1) establishing the exRNA Portal, (2) policy development for data sharing, (3) education, and (4) literature access, curation and publication

NIH Extracellular RNA Communication Consortium
9th Investigators' Meeting
November 6-7, 2017

Hilton Washington DC/Rockville Hotel & Executive Meeting Center
1750 Rockville Pike, Rockville, MD 20852

exRNA Atlas





New Samples	
Past 3 Months:	3,242
Past 6 Months:	3,740
Past Year:	4,380
Total:	**5,309**

New Datasets	
Past 3 Months:	4
Past 6 Months:	6
Past Year:	7
Total:	**20**

New Reads (Billions)	
Past 3 Months:	2.48
Past 6 Months:	7.75
Past Year:	14.23
Total:	**23.43**

New Conditions	
Past 3 Months:	2
Past 6 Months:	3
Past Year:	10
Total:	**23**

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exRNA VBR

VBR Hub Search More

Virtual Bio Repository

The Virtual Biorepository (VBR) aims to connect researchers in need of biosamples with geographically distributed researchers in possession of shareable biosamples, and to assist in arranging biosample exchange. The VBR Hub search functionality allows scientists to identify biosamples via metadata provided by the participating institutions. Multiple provider institutions may be involved in a single order, and the VBR Hub helps manage the researcher's interaction with each institution.

NOTE: *This is a beta release of the VBR Hub currently under development.* The release focuses primarily on:

- Searching for samples
- Saving queries and search results
- Adding samples to your Cart
  - *(Prior to completion of the inital beta VBR, you can download a tab-delimited copy of your Cart for your records.)*

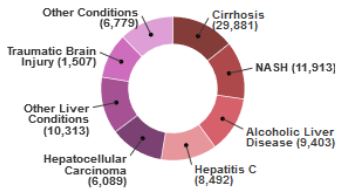
Future releases will complete the initial beta version with support for the full ordering process, help & how-to resources, and an overhaul of this home page.

Alzheimer's Disease X

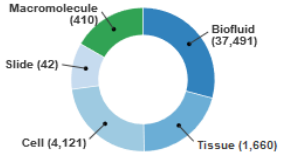
54 samples match this term

Total Biosamples: 43,724

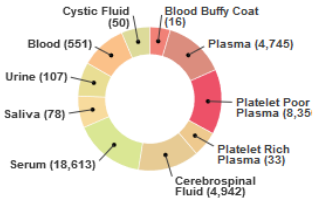
Biosamples by Diagnosis



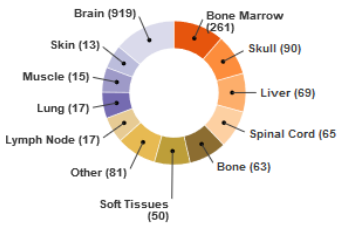
Biosamples by Sample Type



Biosamples by Biofluid



Biosamples by Tissue



Inventory Summary

Contributing Institutions

	TGen	UCSD	OHSU	BNI	NXDC	Mayo
# of Biosamples	5	5	118	7,503	20	36,073
# of Donors	5	5	118	730	10	1,128
# of Studies	1	1	2	1	1	1
# of Available Samples	0	0	114	7,503	16	36,073
Effort (Consortium)	CSF	CSF	CSF	CSF	CSF	Hepatobiliary

Becoming a Contributor: Investigators interested in setting up a VBR node to share their samples may contact the VBR Outreach Coordinator.

Sample Inventory

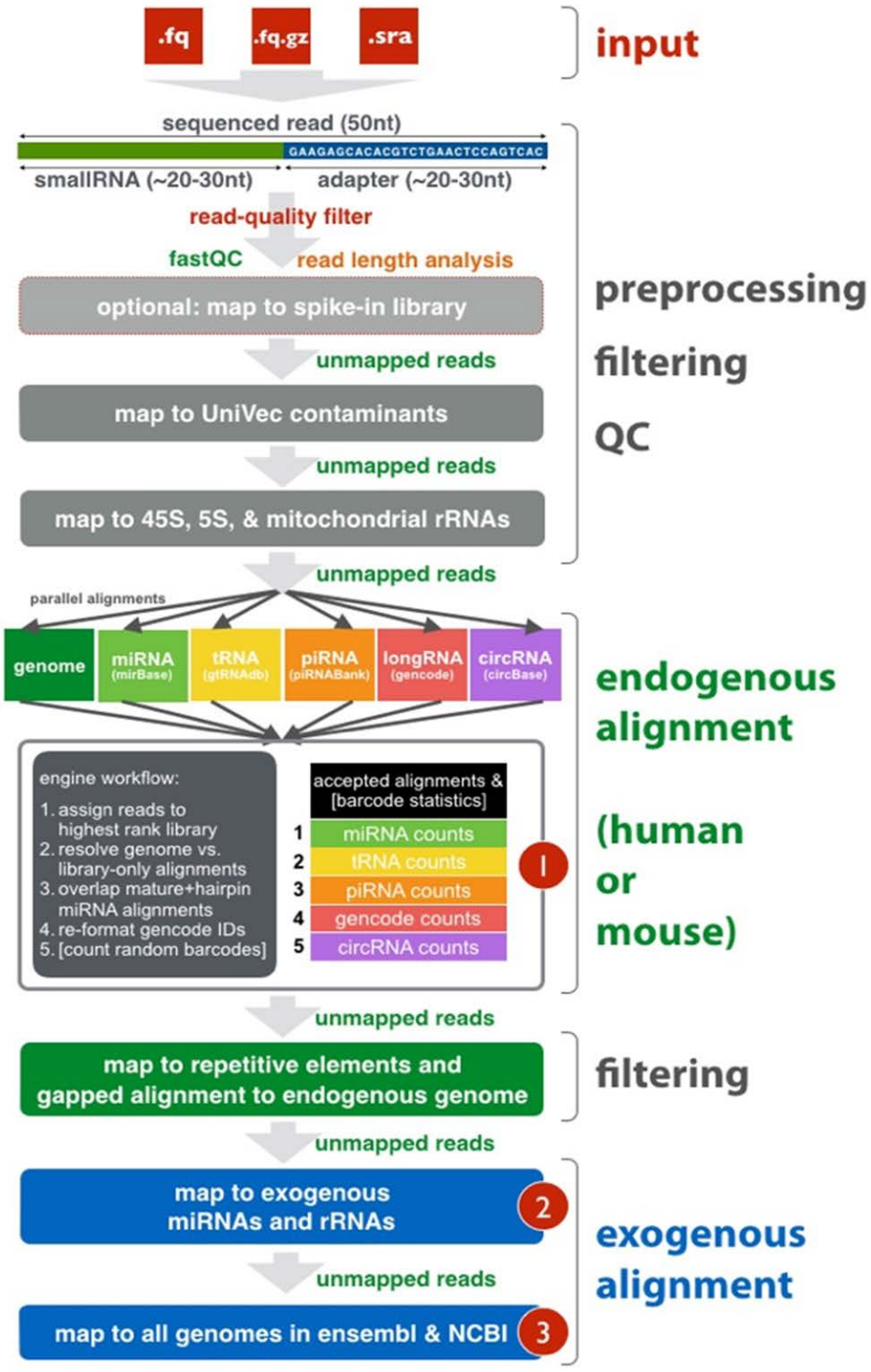
Sample Diversity

Support and Funding

Powered by GENBOREE

Funding Agency:
NIH National Institutes of Health
Office of Strategic Coordination - The Common Fund

exceRpt Pipeline



Oral Presentations

Oral Presentations Abstracts

Session I: Extracellular Vesicle Biogenesis and Function

HIV-1 Latency: The Relationship between Virus, Exosomes, and TAR-gag, a Novel HIV-1 RNA.

Robert Barclay1, Yao A. Akpamagbo1, Catherine DeMarino1, Michelle L. Pleet1, Angela Schwab1, Myosotys Rodriguez2, Gavin Sampey1, Sergey Iordanskiy1, Nazira El-Hage2, and Fatah Kashanchi1.

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2. Department of Immunology, Herbert Wertheim College of Medicine, Miami FL, USA
3. Ceres Nanosciences Inc., Manassas, Virginia, USA.

HIV-1 infection causes AIDS, infecting millions worldwide. Combination antiretroviral therapy (cART) can control the infection but because HIV-1 can establish latent reservoirs in cells, cART is unable to cure the disease. Recently, we have shown that exosomes, a type of extracellular vesicle, from healthy cells can be taken up by HIV-1-infected cells and activate latent HIV-1 even in the presence of cART. This leads to increased transcription of HIV-1 RNA, including TAR (Trans-Activating Response Element) and a novel HIV-1 RNA, TAR-gag. TAR-gag is 615 bases long, containing the TAR element, and terminated within the p17 region of HIV-1 gag. Furthermore, it is a long non-coding RNA and can be packaged into exosomes within infected cells via ESCRT II and exported into the surrounding environment. In the presence of transcription inhibitors F07#13 and CR8#13, TAR-gag is capable of binding to SWI/SNF components, including the mSin3A/HDAC-1 complex, and potentially serves as a scaffolding RNA. Additionally, TAR-gag can recruit suppressive factors and RNA-binding proteins to the HIV-1 promoter, resulting in transcriptional gene silencing (TGS). Finally, F07#13 and CR8#13 suppressed activated virus in a latent humanized mouse model. Collectively, these data indicate that exosomes from uninfected cells activate HIV-1, allowing for increased transcription of viral RNAs, including TAR-gag, which, in the presence of transcription inhibitors, can potentially suppress viral transcription through TGS.

Relevant Manuscripts:

1. Barclay, R., et al. (2017). Exosomes from uninfected cells activate transcription of latent HIV-1. Journal of Biological Chemistry. 292(28): 11682-11701

2. Akpamagbo, Y., et al. (2017). HIV-1 Transcription Inhibitors Increase the Synthesis of Viral Non-Coding RNA that Contribute to Latency. Curr Pharm Des. doi: 10.2174/1381612823666170622101319

Inter-Organismal Vesicular Transmission of Enteric Viruses

Marianita Santiana1, Sourish Ghosh1, Wen-Li Du1, Vignesh Rajesakaran1, Brian Ho1, Yael Mutsafi Ben Halevy1, Dennise De Jesus Diaz2, Stanislav Sosnovtsev2, Eric Levenson2, Gabriel Parra2, Peter Takvorian3, Ann Cali3, Christopher Bleck4, Anastasia Vlasova5, Linda Saif5, John Patton6, Kim Green2, and Nihal Altan-Bonnet1

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Rotavirus and norovirus are highly contagious enteric viruses that are the major causes of mortality and morbidity from enteric pathogenesis(3-5). Their mode of transmission comprises ingestion, replication in the host gut, and shedding into feces for transmission to the next host organism through the fecal-oral route(1,2). Notably, this transmission has always been assumed to occur as free viral particles(1,2). Here we reveal that the optimal infectious form of these viruses in feces is enclosed within membranous vesicles. We show that rotaviruses and noroviruses are selectively packaged as viral populations in phosphatidylserine-enriched vesicles derived from plasma membrane and multivesicular bodies, respectively. We demonstrate that vesicles efficiently transmit infection to other organisms through the fecal-oral route and that this mode of transmission surprisingly is more virulent than free-particle transmission. We find that this is in part due to vesicular transmission suppressing mucosal immune responses. Our

findings reveal for the first time the significance of vesicles in inter-organismal viral transmission and suggest alternative types of therapeutics for disrupting fecal-oral transmission of enteric viruses.

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1.Cortese MM, Parashar UD, Centers for Disease Control and Prevention. 2009. Prevention of rotavirus gastroenteritis among infants and children: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 58:1-25.

2. Hall AJ, Vinje J, Lopman B, Park GW, Yen C, Gregoricus N, Parashar U, Division of Viral Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention. 2011. Updated norovirus outbreak management and disease prevention guidelines. MMWR Recomm Rep 60:1-15.

3. Hall AJ, Wikswo ME, Manikonda K, Roberts VA, Yoder JS, Gould LH. 2013. Acute gastroenteritis surveillance through the National Outbreak Reporting System, United States. Emerg Infect Dis 19:1305-1309.

4. Koo HL, Neill FH, Estes MK, Munoz FM, Cameron A, DuPont HL, Atmar RL. 2013. Noroviruses: The Most Common Pediatric Viral Enteric Pathogen at a Large University Hospital After Introduction of Rotavirus Vaccination. J Pediatric Infect Dis Soc 2:57-60.

Tate JE, Burton AH, Boschi-Pinto C, Parashar UD, World Health Organization-Coordinated Global Rotavirus Surveillance N. 2016. Global, Regional, and National Estimates of Rotavirus Mortality in Children <5 Years of Age, 2000-2013. Clin Infect Dis 62 Suppl 2:S96-S105.

CD63, MHC class 1, and CD47 identify subsets of extracellular vesicles containing distinct populations of non -coding and micro-RNA

Sukhbir Kaur1, Abdel G. Elkahloun2, Arakelyan, Anush3, Lynn Young 4, Tim Myers5, Otaizo-Carrasquero, Francisco5, Weiwei Wu2, Leonid Margolis3, and David D. Roberts1

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3. Section of Intercellular Interactions, Eunice Kennedy-Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda MD 20982
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5. National Institutes of Health Library, Division of Library Services, Office of Research Services , National Institutes of Health, Bethesda, MD 20892, USA

Extracellular vesicles (EVs) are increasingly recognized to have complex functions in mediating cell-cell communication. Some of these functions are mediated by intercellular transfer of mRNA, miRNA and other small RNAs that alter gene expression and function in target cells. The RNA composition of circulating tumor-derived EVs from cancer patients also has potential diagnostic utility. Standardized isolation methods must be developed to understand the physiological functions of RNAs in EVs and their diagnostic utility. However, the heterogeneity in size, density, and composition of EVs has limited progress towards this goal. CD63 and MHC-1 have been used as markers to purify EVs, but it is unclear whether EVs expressing different markers differ functionally. CD47 is also present on EVs, and we have shown that its presence can alter their functional effects on target cells. To further define the functional heterogeneity of EVs, we have captured EVs released from Jurkat T cells using CD47, CD63, and MHC-1 antibodies and evaluated each subset using flow cytometry and RNA expression analysis. CD47, CD63 and MHC class I are present on distinct but overlapping subsets of EVs with similar size distributions. Sequencing of a size-selected RNA library indicates that each subset has similar overall content of tRNAs, small rRNAs, and noncoding RNA, but their miRNA content is highly divergent. The miRNA content of EVs captured by each marker also differs from EVs lacking the respective markers. Marker-specific sorting of miRNAs into EVs identified by RNA sequencing was validated using miRNA microarray analysis and a different EV sorting method. Our results demonstrate that CD63+, MHC-1+, and CD47+ EVs contain distinct but overlapping populations of miRNAs. Therefore, EVs exhibit surface marker-dependent RNA compositional heterogeneity, and specific surface biomarkers may be useful to enrich disease-specific EVs from liquid biopsy.

The RNA binding protein HuR regulates extracellular vesicle secretion in colorectal cancer

Ranjan Preet1, Wei-Ting Hung2, Vikalp Vishwakarma1, Lane K. Christenson2, Jonathan R. Brody3, and Dan A. Dixon1

1. Department of Cancer Biology, University of Kansas Medical Center, Kansas City, KS.
2. Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, KS.
3. Department of Surgery, Division of Surgical Research, Thomas Jefferson University, Philadelphia, PA

Background: Enhanced secretion of extracellular vesicles (EVs) by cancer cells is accepted as a means of transferring specific RNA and protein cargo to recipient cells within the tumor microenvironment, and is being recognized as promising blood-based biomarkers for early cancer detection. In colorectal cancer (CRC), overexpression of the RNA-binding protein HuR (ELAVL1) occurs early during tumorigenesis. When overexpressed, HuR can promote mRNA stabilization of tumor-promoting genes through binding of 3'UTR AU-rich elements (AREs). These same mRNAs are within tumor-derived EVs, suggesting a potential role for HuR.

Aims: To determine how HuR overexpression mediates enhanced CRC cell-derived EV secretion and its functional impact on the RNA cargo present within CRC EVs.

Methodology: Small EV purification was done by ultracentrifugation and characterization utilized nanoparticle tracking analysis (NTA), electron microscopy, and western blotting/ELISA. Starting material was conditioned cell culture/organoid media and in vivo studies used serum/plasma. RNA analysis was done by qPCR.
Results: To identify a role for HuR in EV biology, HuR-inducible HeLa cells were used to demonstrate that HuR overexpression led to a 4-fold increase in EV secretion (50-150nm) and promoted uptake of ARE-containing reporter mRNAs into EVs. Interestingly, HuR was detected as cargo in EVs secreted only from HuR-overexpressing cells. To assess this in CRC cells that endogenously overexpress HuR, EV levels were compared to normal human intestinal epithelial and myofibroblast cells. CRC cells secrete ~3-fold greater EV levels than normal cells, with HuR detected only in CRC-derived EVs. SiRNA knockdown and CRISPER/Cas9 knockout of HuR in CRC cells showed 2- to 3-fold decrease in secreted EVs. The effect of HuR-containing CRC EVs on proliferation and angiogenesis was observed with increased myofibroblast proliferation and HUVEC tubule length as compared to HuR-deficient EVs. These findings were reflected in vivo where GI-tumor bearing APCMin/+ mice produced ~3-fold more serum EVs, with HuR as EV cargo in APCMin/+ mice compared to serum EVs from wild-type mice. Organoids grown from normal intestinal tissue and APCMin/+ adenomas showed similar results to the in vivo findings, indicating that tumor overexpression of HuR drives enhanced EV secretion that can be detected in circulation. A pilot study using purified plasma EVs from late-stage CRC patients and healthy individuals showed HuR was detectable in plasma EVs with ~3-fold increase in CRC patients.
Conclusions: This work has identified a novel connection between HuR-mediated post-transcriptional regulation and CRC tumor-derived EV secretion, along with providing evidence indicating EV HuR as a circulating CRC biomarker.

Oncogene Regulated Production and Release of Vesicles and Extracellular RNAs

Seda Kilinc Avsarglu1, Rebekka Paisner1, Olga Momcilovic1, TJ Hu2, Robert Blelloch2, Dan Nomura3 and Andrei Goga1

1. Department of Cell & Tissue Biology, University of California, San Francisco
2. Department of Pathology, University of California, San Francisco
3. UC Berkeley, Department of Chemistry and Nutritional Sciences

Extracellular vesicles (EVs) have increasingly become a major focus in cell-to-cell communication. Initially thought of as “garbage bags” of cells, they have now been shown to regulate many diverse biological processes such as cell differentiation, development, and tumorigenesis. Despite their importance in tumorigenesis, whether specific oncogenes can regulate vesicle biogenesis and release remains poorly understood. Using a panel of isogenic human mammary epithelial cell lines that have been engineered to express a variety of the most commonly mutated or overexpressed oncogenes, we examined if diverse oncogenic signals differentially regulate extracellular vesicle release. We identified MYC and Aurora kinase B (AURKB) overexpression significantly up-regulate exosome production compared to 12 other oncogenes. MYC-dependent exosome overproduction was confirmed in various inducible mouse cell line models derived from primary transgenic models of MYC-driven breast and liver tumors (i.e., MTB-TOM and EC4 cells). We performed mRNA and metabolomic profiling of these isogenic and inducible cell line models to elucidate altered metabolic pathways that might be involved in exosome biogenesis. Many genes involved in ceramide production are highly dysregulated in MYC and AURKB overexpressing cells and corresponding elevation of ceramide and sphingosine pathway lipids was observed. Inhibiting ceramide production by nSMase inhibitor, GW4869, significantly decreased exosome production in MYC and AURKB cells compared to other oncogenes strengthening the involvement of a ceramide-dependent pathway. In addition to altered amounts of released EVs, oncogenes also resulted in differences in the species and content of extracellular vesicles. While MYC and AURKB released mainly small size vesicles enriched in ALIX, HRAS released large EVs enriched in actinin-4 and apoptotic bodies. Our data provide the first insight into oncogene-specific alterations of extracellular vesicle biogenesis.

Methodologies to Standardize Flow Cytometric Measurement of Extracellular Vesicles and Quantitatively Measure Extracellular RNA

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Biosystems and Biomaterials Division, Materials Measurement Laboratory, National Institute of Standards and Technology, 100 Bureau Drive, Gaithersburg, Maryland, United States of America

Exosomes range in size from 30 nm to 100 nm and carry bioactive molecules, including functional mRNA, microRNA and proteins, with essential roles in intercellular communication and immune system modulation. Exosomes have diagnostic and treatment potential in cancers, and are hypothesized to be the primary mechanism for cancer metastasis. National Institute of Standards and Technology (NIST) has been developing quantitative measurement tools that can be applied to accurately measure different components of exosomes, most notably, extracellular miRNA. Quantitative measurement of external microRNA (miRNA) requires technology that can accurately measure fluorescently labeled nanoparticles in suspension, traceable to a reference measurement. NIST has established basic measurement capabilities using similar biomolecules that can be applied to measure external miRNA.

NIST and ISAC (International Society for Advancement of Cytometry) have developed a two-step methodology to implement flow cytometry quantification. The first step calibrates the fluorescence signal using micro-particles with assigned values of ERF (equivalent number of reference fluorophores). The second step uses a biological cell, with a known number of specific biomarkers, as a reference biomarker to translate the ERF unit to a unit of antibodies bound per cell (ABC). The ABC unit is most relevant and desirable for measurements on biological entities. To apply this scheme to the international EV measurement comparability study, NIST has made equivalent numbers of fluorescein value assignment to flow cytometer calibration beads used in the study for standardizing flow cytometer setting and data reporting.

Additionally, NIST has outlined a method to create a miRNA reference measurement using droplet digital PCR (ddPCR) by applying essential controls, optimization techniques, and an efficacy model to improve the quality of miRNA measurements. Two-step principles are leveraged for miRNA qRT-PCR measurements along with the use of synthetic miRNA targets to evaluate ddPCR following cDNA synthesis with different commercial kits. Applying these criteria to measure miRNA from plasma or serum generates an accurate miRNA copy number reference value. Our measurement technique quantifies specific miRNA copy number in a single sample, without using standard curves for individual experiments.

Due to their extremely small sizes, current exosome purification methods have generated a lot of ambiguity in extracellular miRNA measurements. Flow cytometry has shown the most promise as a quantification method because of multiparameter measurements in nature and feasible incorporation of more advanced excitation and detection technologies to detect nanometer biological entities. By using our quantitative flow cytometry measurement principles on current state-of-art flow cytometers and extrapolating data to miRNA reference measurements using ddPCR, we believe there is an accurate way to quantitatively measure extracellular miRNA.

Session II: exRNA and Extracellular Vesicle Standards and Resources

Definition of serum ribonucleoprotein composition and its regulation and function

Klaas E.A. Max1, Zev Williams1,2, Kemal M. Akat1, Kimberly A. Bogardus1, Michael S. Chang1, Victoria R. Wang1, Nicole Neto2, Xin Li2, Tasos Gogakos1, Iddo Z. Ben-Dov3, Thomas Tuschl1

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2. Department of Obstetrics and Gynecology. Columbia University Medical Center, New York, NY 10032
3. Department of Nephrology and Hypertension, Hadassah Medical Center, Jerusalem, Israel

Aims: Characterization of cell-free blood exRNA composition as a reference in biomarker studies and other profiling approaches and assessment of clinically relevant metadata parameters (individual, gender, female reproductive cycle, nutrition, age, ethnicity) that may contribute to its variability.

Methodology: We developed protocols for isolation of extracellular RNA (exRNA) from urine and plasma/serum to facilitate automated parallel processing of hundreds of clinical samples to obtain reference or disease signature RNA profiles. We recovered nanogram quantities of exRNA available per sample at high purity for direct use in cDNA library preparation. exRNA biofluid composition was characterized using barcoded small RNA cDNA library preparation in batches of 24 RNA samples, followed by single end Illumina HiSeq sequencing yielding up to 150 million reads per batch and lane. Sequence reads were demultiplexed and mapped against our curated human reference transcriptome to obtain miRNA read frequency profiles as well as an abundance of fragments of other RNA

classes, such as tRNAs, snRNAs, scRNAs, rRNAs, mRNAs, and lncRNAs. Sequencing data was used for differential analysis of miRNAs abundance, which allowed identification of indicators discriminating disease subcategories and disease-associated conditions.

Processed samples: To obtain reference profiles and to assess technical, biological and inter-individual variabilities in RNA, we isolated exRNA from cell-free of serum (96) and plasma (216) samples from 12 healthy controls collected at twelve different time points throughout two months. RNA cDNA library sequencing revealed nearly twofold increased epithelial-, muscle-, and neuroendocrine-cell-specific miRNAs in female biofluid, while fasting and hormonal cycle showed little effect. External standardization helped to detect quantitative differences in erythrocyte and platelet-specific miRNA contributions and miRNA concentrations between biofluids. We also discovered a unique exRNA phenotype stable for over a year in one of the volunteers characterized by twofold total miRNA levels and >20-fold elevated endocrine-cell-specific miRNAs in serum and plasma. These results allowed us to conduct our primary study on exRNA composition and variability in cell-free plasma using a larger cohort of 237 healthy individuals with greater diversity in age and ethnic background than previously investigated. We finished sample collection and RNA isolation and obtained sequencing data for 76% of these samples. Sample data analysis is currently in progress.

Comparison of different technical platforms for circulating miRNA profiling

Vasily Aushev, Yula Ma, Jia Chen
Department of Environmental Medicine and Public Health, Icahn School of Medicine at Mount Sinai

Background: There are currently many technical platforms available that allow global miRNA profiling (i.e. measurement of many miRNA species in the same sample). Platform choice is crucial, as each of them has its own advantages and limitations. For example, RNA-seq gives more unbiased snapshot of all transcripts present, not limited by the pre-defined list of targets; at the same time, it is still expensive and makes special demands for the source material. qPCR-based methods, on the other hand, remain the most sensitive for individual transcripts measurement. Blood extracellular miRNAs represent a challenging material due to their low abundance, high variability, and other aspects. We were thus interested to compare different profiling approaches specifically for plasma miRNAs.

Aims: This pilot study pursued two main aims: 1) estimate the global diversity of miRNA species in plasma samples of breast cancer patients and healthy women; 2) compare performance of several technical platforms of miRNA profiling: qPCR-based (Exiqon), hybridization-based (Nanostring), classical RNA-seq, and targeted NGS EdgeSeq (HTG).

Methodology: We created six pooled plasma samples, each contains??? ul plasma from 15-18 individuals with different health status. RNA was extracted using NucleoSpin miRNA Plasma columns (Macherey-Nagel), with spike-in RNAs from ExiSeq NGS QC kit (Exiqon) added prior to isolation. RNA concentration was measured with Quant-It RiboGreen reagent (Invitrogen). Isolated RNA were split into 4 aliquots and profiled in duplicate by 4 platforms (qPCR, Nanostring, RNA-seq, EdgeSeq). Each analysis used an equivalent of 600 ul of plasma.

Results: Profiling of the same set of samples allowed us to compare sensitivity and miRNome coverage of different platforms. As expected, all platforms were able to detect typically highly abundant plasma miRNAs such as miR-451a, -16, -23a etc. Detection of less abundant miRNAs, however, varied across the platforms. Nanostring has shown the lowest sensitivity, with majority of probes giving signal not exceeding the background level. EdgeSeq, on the other hand, displayed extremely high expression levels for such miRNAs as miR-6126, -6131 and other “little-known” miRNAs that are not confirmed by other sources and most probably represent false-positive signal. RNA-seq and qPCR profiling both displayed good reproducibility and sensitivity. For RNA-seq, from 5-10 M raw reads/sample, >1000 miRNA species were detected at least once but only about 250 exceeded an arbitrary threshold of 10 RPM. qPCR, as expected, revealed more miRNA species than NGS for the same samples, giving about 400 miRNAs with reliable qPCR signal. When comparing results of these two platforms, we were able to identify a panel of miRNAs differentially expressed among pooled samples.

Conclusions: Based on the results obtained, we concluded that for our type of samples Nanostring platform is not sensitive enough to detect low-abundant miRNA species, while EdgeSeq platform seems to produce some significant amount of false-positive signal. qPCR and RNA-seq showed good sensitivity and technical reproducibility, but there was certain discordance between them. We believe that our data can be useful for other researchers working with circulating miRNAs and will help to choose optimal analysis approach.

Full-coverage landscape of extracellular coding and non-coding RNA released by human glioma stem cells

Zhiyun Wei1, Arsen O. Batagov2, Sergio Schinelli3, Jintu Wang4, Yang Wang1, Rachid El Fatimy1, Rosalia Rabinovsky1, Leonora Balaj5, Clark C. Chen6, Fred Hochberg7,8, Bob Carter7, Xandra O. Breakefield5, Anna M. Krichevsky1

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5. Department of Neurology and Radiology, Massachusetts General Hospital and Program in Neuroscience, Harvard Medical School, Charlestown, Massachusetts 02129, USA.
6. Neurosurgery Department, University of Minnesota, Minneapolis, Minnesota 55455, USA.
7. Departments of Neurosurgery, University of California, San Diego, La Jolla, California 92093, USA.
8. Scintillon Institute, San Diego, California 92121, USA.

Background: Communication between glioblastoma brain tumor (GBM) and its microenvironment alters the parameters of tumor growth and host responses, and may be mediated in part by tumor-released RNA. While many researchers focus their efforts on miRNA, the global repertoire of extracellular RNAs (exRNAs) released by GBM has not been investigated.

Aims: Our U19 project focuses on the RNA transfer from GBM to the brain microenvironment. In this Sub-Project, as a critical step toward understanding the biological impact of exRNA secretion and transfer, we aimed to characterize the full-coverage landscape of extracellular RNA, coding and non-coding, released by human glioma stem cells, and evaluate the impact of GBM exRNA on the normal cells of the brain.

Methodology: We have developed a protocol enabling quantitative, minimally biased analysis of vesicular and extravesicular exRNA complexes, including microvesicles (MVs) and exosomes (collectively called extracellular vesicles; EVs), as well as ribonucleoproteins (RNPs) and applied it to study exRNA released by patient-derived glioma stem-like cultures (GSC). RNA-Seq was performed to quantify the RNA repertoire of long and small RNA libraries, and qRT-PCR analysis further carried out to validate selected protein-coding and non-coding transcripts in exRNA derived from the cell cultures and human cerebrospinal fluid. We have also examined the endogenous transcriptome of human and mouse brain-derived primary neurons, astrocytes, microglia, and endothelial cells.

Results: Despite the intertumoral heterogeneity, further exacerbated at the exRNA level, the extracellular complexes exhibit distinct RNA composition, with microvesicles most closely reflecting the cellular transcriptome, and exRNPs exhibiting the most discrete repertoire. Up to 90% of exRNA reads represent fragmented rRNA; the remaining content is enriched in small ncRNA species, such as miRNAs in exosomes, and precisely processed tRNA and Y RNA fragments in both EVs and exRNPs. The enrichment patterns were further validated on human cerebrospinal fluid, where commonly mutated mRNAs were also detected. EV-enclosed mRNAs are mostly fragmented, and UTRs are more abundant than ORF regions; nevertheless, some full-length transcripts are present. Relative to the transcriptomes of primary normal brain cells, including neurons, astrocytes, microglia and endothelial cells, GBM exRNA exhibits the higher abundance of several miRNAs, that may exert regulatory functions upon uptake. Overall, there is less than one copy of non-rRNA per EV. Our results suggest that massive EV/exRNA uptake would be required to ensure the functional impact of the transferred RNA information to the normal recipient cells of the brain and predict the most impactful miRNAs in such conditions.

Conclusions: The key outcome of our work is an expansion of a repertoire of potentially transferred small exRNA far beyond the class of miRNA. This conclusion challenges the previously assumed sole role of miRNA in exRNA-mediated intercellular communication and requires in-depth investigation of other classes of exRNA and their impact on the physiology of recipient cells. This study also provides a catalog of diverse vesicular and extravesicular exRNA species useful for biomarker discovery and validates its feasibility on cerebrospinal fluid.
Reference: Wei Z. et al, Nature Communications, 2017 (in press).

The exRNA Atlas resource provides insights into exRNA biology through cross-study analysis of exRNA profiles

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Background: The research into the role of exRNAs in physiological processes and in human diseases requires analyses across independently-produced datasets. Such analyses are hampered by the lack of integration of exRNA profiling data and of associated online resources.

Aims: To help address the open questions in exRNA biology relevant for clinical translation and to catalyze data-intensive exRNA research, NIH Extracellular RNA Communication Consortium (ERCC) created the Extracellular RNA Atlas (exrna-atlas.org).

Methods: exRNA Atlas collects exRNA profiling metadata, supports uniform data processing (RNA-seq, qPCR), standardized data quality assessment, efficient data sharing and analysis. The exceRpt pipeline (v4.6.2) maps RNA-seq data to exogenous rRNAs as well as thousands of exogenous genomes. Metadata validators minimize curation effort, making it possible for the community to contribute on the order of 10,000 profiles per year. Our metadata-driven portal (exrna-atlas.org) displays the data in an accessible way and provides integrated online tools for data browsing, cross-study analyses and knowledge sharing.

Results: The exRNA Atlas contains a total of 2,627 (2,067 currently public) exRNA profiles from nine body fluids (serum, plasma, CSF, bile, urine, saliva, sputum, seminal fluid, and ovarian follicle fluid), with additional 3,000 profiles anticipated by the end of 2017. Over 10,000 Atlas data files have been downloaded by researchers over the last 12 months (5.5-fold increase over last year), and over 21,000 (3-fold increase) have been submitted into Atlas and associated pipelines/tools. The Atlas helps contextualize new data, validate it against expected parameters, and provides opportunities for cross-study analyses.

We complete the first census of ncRNAs in human body fluids and make it available for interactive browsing to inform the design of targeted panels, help focus biofluid-specific studies and contextualize findings on specific exRNAs. We quantitate miRNAs, tRNAs, Y RNAs, and other human exRNAs and identify a core set of over 100 miRNAs that are abundantly present (>10 RPM) in most body fluids. By mapping RNA-seq reads across thousands of non-human genomes we identify taxonomic units of bacterial exRNAs that are recurrently present in saliva and urine.

To examine sources of experimental and biological variation of exRNA profiles, we developed a computational deconvolution algorithm. Deconvolution reveals a striking amount of sample-to-sample variation in relative abundances of exRNA carrier types (vesicular or non-vesicular). Multiple carrier types, each having a characteristic exRNA profile, are detected across most biofluids, the first is a nuclear ncRNA profile matching the exRNA profile of HD exosomes, and multiple cytoplasmic ncRNA profiles, some matching the exRNA profile of LD exosomes. Through deconvolution we assign miRNAs that are differentially expressed in disease conditions to specific carrier types.

Conclusions: By lowering the amount of effort required to contribute exRNA profiling data and associated metadata and by maximizing incentives such as data publication, interoperability and reuse for cross-study analysis, we mobilized the exRNA research community to create the exRNA Atlas, the first compendium of exRNA profiles of human body fluids. Using this integrated resource we addressed some key questions relevant for basic exRNA biology and for clinical utility of exRNA profiling.

exceRpt: the extracellular RNA-Seq processing pipeline of the ERCC

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We will present the tools of the Extracellular RNA Communication Consortium (ERCC) that have been developed for the analysis of extracellular RNA-Seq data and have been used in the construction of a comprehensive atlas of exRNAs in human body fluids. Due

to the process of extracting, purifying, and sequencing of RNA from extracellular biofluids, exRNAs are more vulnerable to contamination than cellular RNA samples. To address this, we have developed the extracellular RNA processing tool (exceRpt), optimized for the analysis of exRNA-seq datasets.

This involves three main steps: 1) Pre-processing: support for random-barcoded libraries, spike-in sequences for calibration or titration, and explicit removal of common laboratory contaminants and ribosomal RNAs. 2) Endogenous processing: Alignment and quantitation of the full set of annotated, potentially spliced, endogenous RNA transcripts including all known miRNAs, tRNAs, piRNAs, snoRNAs, lincRNAs, mRNAs, retrotransposons, and circular RNAs. 3) Exogenous processing: Alignment to annotated exogenous miRNAs in miRBase and exogenous rRNA sequences in the RDP and alignments to the full genomes of all sequenced bacteria, viruses, plants, fungi, protists, metazoa, and selected vertebrates. As part of the exogenous analysis we have developed a novel algorithm for characterizing alignments to all available exogenous genomes in terms of the NCBI taxonomy tree. Existing approaches that remove degenerate sequences (i.e. those that co-occur across multiple species) result in a loss of potentially valuable data as the occurrence of reads aligning to multiple species/strains is very frequent. This is done independently for exogenous reads aligning to exogenous rRNA sequences as well as exogenous genome sequences.

The exceRpt pipeline (available at genboree.org and github.gersteinlab.org/exceRpt) generates a variety of sample-level quality control metrics, produces abundance estimates for various RNA biotypes, and makes available detailed reports of this processing. The exceRpt pipeline (including endogenous and exogenous processing steps) has been used to uniformly process all ~2000 exRNA-Seq datasets that are currently included in the public exRNA atlas which is accessible via the consortium website at exrna-atlas.org. We will also present the quality control metrics as applied to the current available extracellular RNA-Seq datasets of the ERCC in the exRNA Atlas and the special considerations that are needed for specific biofluids such as saliva.

Session III: Research Diagnosis and Biomarkers

Extracellular vesicle microRNA signatures reveal tissue heterogeneity in defined glioblastoma subtypes

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Background: The signatures of protein-coding gene expression and somatic copy-number alterations have revealed the existence of several distinct subtypes among glioblastoma (GBM) patients. The classification is further complicated by the fact that individual tumors contain a spectrum of subtypes and hybrid cellular states and that GBM stem-like cell (GSC) subpopulations retain transcriptome heterogeneity. These findings indicate that tissue-based classification likely shows merely characteristics of the predominant cellular component. Importantly, tissue and GSC subtype classification was also demonstrated by signatures of non-protein-coding genes, such as long non-coding RNAs. Significantly, microRNAs have not been shown to predict GBM classification and prognosis by global signature to date, while being strongly implicated as functionally deregulated in GBM as individual molecules

Aim: Despite the importance of molecular subtype classification of GBM, the extent of extracellular vesicle (EV)-driven molecular and phenotypic re-programming remains poorly understood. We aimed to highlight the magnitude of EV/microRNA-driven propagation of molecular and phenotypic diversity of GSCs.

Methods: Using intracranial xenografts of patient-derived GSCs, we identified subpopulations with distinct transcriptomes, displaying either proliferative/nodular or migratory/invasive modes that were associated with mesenchymal-like or proneural-like subtype, respectively. To reveal complex subpopulation dynamics within the heterogeneous intra-tumoral “ecosystem”, we characterized protein and microRNA expression and secretion in these phenotypically diverse subpopulations of GSC. Bioinformatic analysis followed by functional EV/microRNA transfer between GSCs in vitro and in vivo was used to analyze their molecular and phenotype subtype characteristics.

Results: The highly heterogeneous profile of microRNAs expression in GBMs was distinguishable into two unsupervised classes that partially overlapped with previously determined molecular subtypes, with both subclasses of GSCs displaying differential cellular and EV microRNA profiles. The analysis of microRNA/target networks provided the evidence that EV/microRNAs modified both the molecular landscape and phenotype, acting via cell type-dependent targeting. Importantly, EV proteome retained the subtype specificity and EV protein signatures were associated with worse outcome. The transfer of EVs between subpopulations of GSCs led to increased tumorigenicity in vitro and in vivo facilitating the formation of inter-dependent tumor organization; however it did not alter the phenotypic features.

Conclusions: Our findings demonstrated the existence of previously underappreciated heterogeneity among cancer EVs that contribute to the diverse complexity of the brain tumor "ecosystem", indicating that clinical outcome is influenced by the proportion of tumor cells of varying subtypes which by the exchange of EVs can modify molecular landscape and phenotype, acting in tumor anatomic sites-dependent fashion.

MicroRNA Biomarkers for Alzheimer's Disease in Human Cerebrospinal Fluid

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Background: Currently available biomarkers of Alzheimer's disease (AD) are limited. The discovery of extracellular microRNAs (miRNAs) in cerebrospinal fluid (CSF) raised the possibility that miRNAs may serve as novel biomarkers of AD. Our initial studies discovered a set of miRNAs that that can discriminate AD patients from control subjects.

Aims: Analyze the expression of a set of AD-specific miRNAs in a new and independent cohort of AD and control CSF samples, in order to validate their ability to serve as biomarkers for AD.

Methods: CSF from 47 AD patients and 71 control subjects were obtained from the Shiley Marcos Alzheimer's Disease Research Center at the University of California, San Diego. The expression of 36 candidate miRNA biomarkers identified in the discovery UH2 phase was analyzed using TaqMan® Low Density Custom miRNA Arrays. Stringent data analysis included 7 different methods of classifying methods (LogRank, ROC, CART, CFOREST, CHAID, BOOST, and UH2 assessment) that were each used to independently rank the candidate markers in order (1=best, 26=worst). The total score for each miRNA was added to provide a ranking for each candidate biomarker. Multimarker modeling and covariate analysis were performed on the top ranking miRNAs. Classification performance of miRNA biomarkers were compared to that of ApoE4 genotype. In addition, incremental improvement adding miRNA biomarkers to ApoE4 was assessed.

Results: Analysis of the custom array data validated that the candidate miRNAs discriminate AD from control subjects in a new and independent cohort of CSF samples. Cluster analysis revealed 26 miRNAs in three rank groups. Analysis of the contribution of individual miRNAs to multimarker performance revealed 14 best miRNAs. Top-performing linear combinations of 6 and 7 miRNAs have AUC of 0.775–0.796, relative to ApoE4+ AUC of 0.637 in this sample set. Addition of ApoE4 genotype to the model also improved performance, i.e. AUC of 7 miRNA plus ApoE4 improves to 0.82.

Conclusions: We have validated that CSF miRNAs can discriminate AD patients from controls in a new and independent cohort of CSF samples. Combining the top 14 miRNAs improves sensitivity and specificity of biomarker performance, and adding ApoE4 genotype, and possibly other classifiers such as Abeta:tau ratio, improves classification. The miRNA biomarkers will now be examined in patients diagnosed with mild cognitive impairment (MCI), many of whom progressed to a clinical diagnosis of AD, as well as AD and controls. This study will determine if the AD miRNA biomarker expression changes can be detected earlier in the disease progression.

The bioavailability and distribution of exosomes and their RNA cargos from bovine and porcine milk in mice

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Background: While it is widely accepted that exosomes from bovine milk are bioavailable in humans and mice, the bioavailability of exosomal miRNA cargos remains somewhat controversial. The tissue distribution of milk exosomes and their miRNA cargos is unknown.

Aims: 1) Assess the distribution of bovine and porcine milk exosomes in mice. 2) Assess the bioavailability and tissue distribution of miRNAs, encapsulated in bovine milk exosomes, in humans and mice.

Methodology: 1) Bovine and porcine milk exosomes were labeled exogenously with DiR and endogenously with a ZsGreen1/GnRH fusion protein, respectively, and administered orally and intravenously to mice in imaging studies. 2a) Milk feeding studies were conducted in humans, and bovine miRNAs were analyzed in human plasma by using RNase H2 PCR. 2b) Synthetic, IRDye-labeled miRNAs 34a, 155 and 320a were transfected into bovine milk exosomes, and exosomes were administered orally and intravenously to mice in imaging studies.
Results: 1) Bovine exosomes accumulated in liver and spleen; porcine exosomes (fusion protein) accumulated in liver, spleen and cerebellum. 2a) bta-miR-21-5p and bta-miR-30a-5p were not detectable in human plasma before milk consumption, but were detectable six hours after consumption of 1 L milk [Ct values 27.0 (bta-miR-21-5p) and 28.7 (bta-miR-30a-5p)]. 2b) Distinct miRNAs, delivered orally through bovine milk exosomes, exhibited unique patterns of tissue distribution: miR-34a accumulated in the brain (and heart), miR-155 accumulated in spleen (and brain) and miR-320a accumulated in the liver (and spleen) in mice.

Conclusions: Milk exosomes accumulate primarily in liver and spleen, presumably in resident macrophages. miRNAs in milk are bioavailable in humans and mice and have distribution patterns distinct from that of exosomes in mice. Distinct miRNAs have unique patterns of tissue distribution. We propose that accumulation of miRNAs in the intestinal mucosa or liver is the reason why previous studies failed to detect select miRNAs from milk in plasma and peripheral tissues.
Funding: NIH, NIFA, USDA/Egg Nutrition Center, Gerber Foundation.

Extracellular RNA profiles with human age

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Extracellular RNAs (exRNAs), circulating in the blood, have been identified and used as markers for human development, aging, and disease. However, these studies have been limited in scope, either by profiling one subtype of RNA species (e.g. microRNA [miRNA]) or profiling RNAs of a particular transcript size (e.g. small or large). Here, we have sequenced both large and small RNAs from human serum under one comprehensive protocol within the context of normal aging. We hypothesized that age-related changes in exRNAs are important for normal aging and age-related degenerative processes. Total RNA was isolated from serum from 13 young (30-35 yrs.) and 10 old (80-85 yrs.) African American females chosen from the Healthy Aging in Neighborhoods of Diversity across the Life Span (HANDLS) and the Baltimore Longitudinal Study of Aging (BLSA) studies. Total RNA-seq was performed on the Ion Torrent Proton and sequences were aligned against the human genome version 19 using separate scripts to identify linear RNA, and small RNAs including miRNA and tRNA. There was an average of 8.4 million sequenced reads per individual. We identified mRNA, miRNA, long non-coding RNAs (lncRNA), small nucleolar RNA (snoRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), and Y-RNA transcripts. We found age-dependent changes in transcript biotypes and in specific transcripts. This analysis will provide foundational clues and insight into the diagnostic and functional roles that exRNAs play in human health, and disease, and may uncover important therapeutic targets and/or pathways for future studies and interventions for aging and age-related diseases.

Plasma - based detection of the mutant IDH1.R132H in glioma patients.

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Background: Gliomas are the most common primary cancer of the central nervous system. Recurrent isocitrate dehydrogenase (IDH) gene mutations are found in up to 80% of low grade gliomas and 20% of secondary glioblastomas, identifying tumors with distinct etiology, associated genetic alterations, and overall natural history. As a result, IDH mutant gliomas are now-recognized as separate diagnostic entities within the 2016 World Health Organization Histological Classification. IDH mutant gliomas are typically diagnosed in younger adults ranging from 20-50 years old, initially presenting as lower-grade lesions that can be responsive to standard-of-care treatments such as surgical resection, radiation and chemotherapy. However, these cancers inexorably progress to higher-grade

malignant disease, and prove fatal in most cases. Detection of the mutation prior to surgery would direct the management of care, including the extent of surgical resection and treatment regimen which has been shown to correlate with improved survival.

Aims: Screening plasma samples for the presence of the IDH1.R132H mutant marker.
Methodology: Plasma is a complex biofluid that contains a variety of biomolecules including protein, DNA, RNA and lipids. DNA found free floating or associated with other molecules, while the RNA is encapsulated by lipid vesicles (exosomes, extracellular vesicles; EVs) and therefore protected by degradation. The source from which cell-free DNA (cfDNA) in biofluids is derived is unclear, but it is likely to be released through cell death mechanisms such as necrosis and apoptosis. In contrast, exosomes and other EVs contain both RNA and DNA and are actively released from living cells. As coding mutations such as IDH1.R132H can be found in both cfDNA and exosomal RNA/DNA, combining these sources of nucleic acids (exoRNA+exoDNA=exoNA) have the potential to increase sensitivity. This is of particular benefit to patients with limited copies of circulating IDH1.R132H in the cfDNA fraction, such as those with brain tumors.

Results: In our earlier studies, we showed that the mutant IDH1.R132H is detected in cerebrospinal fluid EVs in patients confirmed to have the mutation. We reported a 70% sensitivity and 100% specificity using EV RNA as a biomarker platform. In a recent pilot study we have tested the possibility of combing the cfDNA, EV-RNA and EV-DNA and have determined the IDH1 mutation in 8/8 samples tested and none of the healthy controls. Correlating the ratios of DNA to RNA mutation may also indicate the disease status where a high level of the mutation at the DNA level indicates tumor cell death, while a high level of mutation at the RNA level, may indicate tumor growth and progression.

Conclusions: We report a novel platform to capture cfDNA and EV-RNA/DNA in circulation in plasma to detect rare copies of a tumor mutations. Combining these two sources of genetic information may lead to improved diagnosis and tracking of the course of the disease.

Session IV: Therapeutic Uses of exRNA and Extracellular Vesicles

Anti-HER2 scFv-directed extracellular vesicle-mediated mRNA-based gene delivery arrests growth of HER2-positive human breast tumor xenografts by prodrug activation

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Background. Extracellular vesicles (EVs), being nature's own antigen delivery entities, may permit gene delivery with minimal immune rejection, and mRNA is likely to be superior to DNA for this purpose. mRNA is translated directly after introduction to cytosol, and eliminates the risk of insertional mutagenesis. However, to be effective, DNA must be transported (for transcription) to the nucleus. This is a highly inefficient process: less than 0.01-0.1% of the DNA delivered into the cytoplasm of mammalian cells enters the nucleus. Moreover, as this transport occurs during nuclear membrane dissolution in mitosis, it is especially inefficient in tumors, where a significant portion of cells is typically quiescent.

Aims. We report a new prodrug, CNOB/HChrR6, treatment for HER2-positive breast cancer that specifically targets HER2 tumors to mitigate off-target toxicity. HChrR6 converts CNOB to the cytotoxic drug MCHB.

Methodology. To confine HChrR6 enzyme generation to the cancer, EVHB protein, with anti-HER2 scFv antibody (ML39) and capable of binding to EV surface, was constructed. Transfection of HEK293 cells with EVHB-encoding plasmid generated EVs displaying this protein. EVHB was eluted from the EV surface and purified using its His tag. A novel plasmid ("pXPort/hChrR6") was constructed, using the 'zipcode' technology and a commercially available plasmid, for loading EVs with HChrR6 mRNA. HER2-overexpressing BT474 human breast cancer xenografts were implanted orthotopically in female nu/nu mice.

Results. pXPort/HChrR6 transfected HEK293 cells generated EVs containing HChrR6 mRNA; incubation with pure EVHB enabled them to target the HER2 receptor, as shown by ELISA, microscopy, and flow cytometry. BT474 cells treated with these "EXO-DEPT"

EVs converted CNOB into MCHB; this effect was actinomycin D-resistant, showing successful HChrR6 mRNA delivery to the cells. EXO-DEPT EV plus CNOB treatment completely arrested implanted HER2-overexpressing tumors in mice, showing for the first time, EV-mediated transfer of exogenous mRNA in functional state to recipient cells, as well as EV-mediated specific sensitization of tumors to a prodrug.

Conclusion. EXO-DEPT/CNOB regimen specifically targets and arrests growth of HER2-overexpressing xenografts in mice. We have evidence that EXO-DEPT EVs can be generated from patient's own dendritic cells, and using mRNA formulations instead of DNA-based plasmids for further immune compatibility. Also, CB1954, which is already in clinical trial, can be substituted for CNOB. These findings raise the prospect of clinical transfer of this treatment scheme. Our EVs can cross the blood brain barrier; thus, the therapy could be useful also in treating brain metastasis of the cancer. ML 39 in EVHB can be replaced with a moiety targeting a different receptor; thus, the approach can be used to treat any disease in which a receptor is overexpressed, by specific targeting of the needed biomolecule(s) and/or drug(s). As many cancers and other disease do overexpress specific receptors, evaluation of this approach in a broad range of clinical indications is warranted.

Modified Extracellular Vesicles Improves Host Immune Response in Colorectal Cancer

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Background: Colorectal cancer (CRC) derived extracellular vesicles (EVs) carry cargo, including nucleotides and proteins, which mediate intercellular communication between cancer cells and the host immune system. We hypothesized that CRC secreted EVs deliver microRNAs that suppress host T-cell function locally and in tumor draining lymph nodes, resulting in a deficient immune response that allows for tumor progression.

Aim: To understand how CRC suppress the immune system, we have studied the interplay between microRNA (miRNA)-containing extracellular vesicles (EVs) secreted by tumor cells and the effect of these EVs on the immune system.

Results: Our data showed that miR-503, its cluster member miR-424, independently downregulate CD28 expression. We found that these miRNAs are present in EVs secreted by CRC cell lines, and are associated with tumor progression in CRC. Connecting those findings, we showed that coculturing T cells with cancer-cell-secreted EVs inhibits the CD28 costimulatory pathway, resulting in suppression of the host immune response. CD28 is an important costimulatory molecule on T cells that is required for T-cell activation, trafficking, proliferation, and function. Strikingly, in orthotopic models of CRC, mice preconditioned or treated with EVs lacking functional miR-503 and/or miR-424 are protected against tumor formation and have a significantly reduced tumor burden, while mice receiving control EVs or control PBS solution all form tumors. Our study shows that EVs without miR-503/-424 are highly immunogenic. These data reveal a new mechanism used by tumors to evade the immune system. Conclusion: Our results provide a strong preclinical evidence that treating patients with EVs lacking immune supressive miRNAs will reduce tumor burden and the chance of recurrence. These results fundamentally advance our knowledge of how cancer cells modulate and suppress the immune response, provide novel targets, and form the basis for a new anticancer therapeutic strategy

Optimizing exosome production and intracerebroventricular delivery to brain

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Introduction: Yield of exosome production is a rate-limiting factor for therapeutic development of exosomes. Here we tested three source cells, two cell culture methods and two exosome production methods for yield and quality of exosomes. We also used the clinically relevant intracerebroventricular delivery for the first time.

Methods: Bone marrow derived (Lonza), adipose tissue derived (ATCC), and umbilical cord Wharton's jelly derived (ATCC) mesenchymal stem cells were cultured in plastic flasks or on microcarriers in stem cell medium (ATCC) as well as in chemically defined stem cell medium (StemPro). Exosomes were purified from mesenchymal stem cells via differential ultracentrifugation or via tangential flow filtration. Purified exosomes were loaded with hydrophobically modified siRNA (hsiRNA) and applied to primary neurons in culture or directly infused into the lateral ventricle of mice. mRNA silencing was quantified QuantiGene® assay.

Results: Umbilical cord derived mesenchymal stem cells yielded 3 times more exosomes in exosome-depleted stem cell medium and 10 times more exosomes in serum-depleted medium than bone marrow derived and adipose tissue derived stem cells. Exosome yield following tangential flow filtration was 10-100 times higher than following differential ultracentrifugation. Exosomes produced by tangential flow filtration had a similar protein profile and hsiRNA delivering efficacy to primary neurons than exosomes produced by differential ultracentrifugation. Infusion of tangential flow filtration – produced, hsiRNA – loaded exosomes into the lateral ventricle of mice resulted in target mRNA silencing in the striatum (up to 44%), motor cortex (up to 36%), thalamus (up to 20%) and in the hippocampus (up to 56%) on both the infused and the non-infused hemispheres.

Conclusions: Microcarrier-based culture of umbilical cord derived mesenchymal stem cells coupled with tangential flow filtration allows the production of exosome amounts capable of powering multiple animal experiments. These exosomes are able spread in the brain to region far from the infusion site, including to the contralateral hemisphere, and efficiently deliver the cargo hsiRNA, resulting in target mRNA silencing.

Tissue Regeneration by Exosome Engineering

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We have previously developed Porous Templated Scaffolds (PTS) that are porous polymer constructs where pore size is uniformly controlled (narrow pore size distribution) throughout the scaffold and the pore interconnects are also uniform in size, with both parameters being adjustable. If pore size is ~40μm, PTS show no chronic inflammation or foreign body response while promoting remarkable healing in numerous soft and hard tissue applications. Using a novel double transgenic mouse strain (LysM-Cre+/0:mT/mG+/0) that uniquely "fingerprints" myeloid cell DNA with an indelible myeloid lineage reporter gene, we provide evidence that only 40μmPTS create an in vivo situation that (a) promotes an early biased polarization of myeloid cells towards a M2-macrophage (MØ) phenotype and (b) also generates extracellular vesicles (exosomes) with unique miRNA content that are capable of re-programming myeloid cells within the PTS. Work presented here suggests myeloid cells, i.e., MØ may be re-programmed into desired non-myeloid lineages through an orchestrated exosomal exchange within a specific pore size PTS.

N6-Methyladenosine (m6A) RNA Modifications Mediate the Beneficial Function of Human CD34+ Stem Cell-Derived Exosomes to Repair the Failing Heart

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Introduction: Previous research from our lab has shown that human peripheral blood-derived CD34+ stem cell-derived exosomes (CD34Exo) have beneficial effects to induce myocardial repair and regeneration in a murine model of myocardial infarction. Emerging evidence suggest that a recently discovered post-transcriptional modifications of RNA are critical regulators of mRNA stability and protein expression. N6-methyladenosine (m6A) is one of the most abundant and functionally relevant modifications of RNA. Analysis of CD34Exo miRNA content revealed selectively enriched miRNAs that target pathways associated with m6A regulations.

Objective: Here we have investigated the therapeutic mechanism of CD34Exosomal miRNAs improving cardiac function via modulating m6A in mRNAs of the treated cells.

Methods and Results: Using clinical human samples, preclinical pig and mouse models and primary cardiomyocyte cell culture, we report decreased expression of an m6A demethylase FTO in failing mammalian hearts associated with increased m6A and decreased contractile function. All of these effects were reversed when the failing murine hearts (post-myocardial infarction surgery) were intramuscularly injected with miR-126-containing CD34Exo. CD34Exo injection mimicked the effects of FTO over expression in reversing ischemia-induced increase in m6A and decrease in calcium dynamics and cardiomyocyte contraction. These benefits were translated in vivo and overall cardiac function ejection fraction and fractional shortening were improved in the failing murine hearts. Moreover, miR-126, one of the most enriched miRNAs in CD34exo, played a critical role in regulating the mRNA modifications by improving FTO expression in cardiomyocytes. In addition, loss of miR-126 in CD34Exo using antimiR-126 reversed the effects of CD34Exo suggesting that miR-126 is a critical regulator of FTO and m6A in mRNA in human cardiomyocytes as well as in the post-MI mouse hearts. Integrating MeRIP-Seq data with SILAC-LC-MS proteomic analysis, we identified that FTO is a key cardiac molecule that demethylates a subset of transcripts encoding cardiac contractile proteins.

Conclusions: Our new data provides first evidence that the human CD34Exo confer beneficial cardiovascular functions at least in part, by regulating the mRNA epitranscriptome in the ischemic myocardium. It also suggests that miR-126 is a critical regulator of FTO and m6A in mRNA in human cardiomyocytes as well as in the post-MI mouse hearts .This is a novel mechanism for CD34Exo in cardiac repair that suggests that post-transcriptional regulation is critical for both cardiomyocyte and cardiac function.

Session V: Cutting-Edge Technologies

Quantification and characterization of low-abundance exosomes and RNA biomarkers with Interferometric Reflectance Imaging

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Background: This study had twofold motivation, based on two related challenges in biomarker analysis:
(a) There remains a need for high-throughput methods to detect and characterize exosomes and extracellular vesicles directly from complex or low-abundance samples. Dynamic Light Scattering and Nanoparticle Tracking Analysis are the most common methods for characterization of exosomes and extracellular vesicles, due to their simplicity and throughput compared to electron microscopy. However, all of these techniques require that vesicles are purified, isolated and concentrated prior to characterization, which complicates their use in diagnostic development or high-throughput studies.
(b) There remains a need for multiplexed and high-throughput methods to rapidly quantify low-abundance extracellular/exosomal RNA biomarkers directly from complex samples. DNA microarrays are an appealing technique for exRNA quantification due to their relatively low cost and high multiplexing capability. However, sensitivity and precision have been this technique's Achilles' Heel compared to approaches that utilize polymerase chain reaction or cDNA sequencing.

Aims: We aimed to develop a platform technology that could address the combined needs for (a) direct detection and characterization of extracellular vesicles without purification and (b) rapid and multiplexed quantification of low-abundance exRNA biomarkers.

Methodology: Interferometric Reflectance Imaging Sensing (IRIS) is a microarray technology that can rapidly enumerate (i.e., count) and characterize biological and metallic nanoparticles that are immobilized onto an IRIS microarray chip. We developed IRIS microarray assays to address the two aims (a-b) described above.

Results: First, we demonstrated that IRIS microarray chips can be used to rapidly count and measure the size of low-abundance exosomes with molecular specificity. Vesicles immobilized on an IRIS antibody microarray chip were individually detected based on their light scattering, in such a manner that enables the optical size of each vesicle to be measured (down to about 50 nm diameter). The IRIS chip is an improvement upon Dynamic Light Scattering or Nanoparticle Tracking Analysis for certain applications since it can be used directly with low-volume/low-abundance samples without isolation or concentration, it individually measures the optical size of tens of thousands of vesicles, and does so with molecular specificity.

Second, we demonstrated that IRIS microarray chips can perform multiplexed RNA expression analysis with a sensitivity and dynamic range that surpasses that of traditional cDNA fluorescence microarrays by about 10,000-fold. In our approach, fluorescent labels are replaced with DNA-conjugated gold nanoparticle labels that are individually detectable by light scattering when immobilized on the IRIS microarray chip. IRIS microarray chips are affordable (about $1 each) and allow quantification of very low-abundance (i.e., sub-femtomolar concentration) biomarkers without amplification. Our high-throughput IRIS RNA-chip scanner can read a typical microarray of 200-500 spots 10 minutes, which is about the same speed as a typical fluorescence reader.

Conclusions: IRIS microarrays are a promising new platform technology for analysis of extracellular vesicles and exRNA biomarkers, particularly in high-throughput applications such as in diagnostics and clinical trials.

Role of Red Blood Cell derived extracellular vesicles in cardiac remodeling after myocardial infarction in a transgenic murine model

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Introduction: Extracellular vesicles (EVs) function as novel mediators of intercellular communication. Here, we describe a novel, fluorescence switch-based, experimental model to study EV-mediated communication between RBCs and other cell types during homeostasis and after myocardial infarction.

Methods: Mice with RBC-specific expression of cre (Erythropoietin Receptor (EpoR) Cre) were crossed with reporter mTmG Rosa26 mice to yield EpoRCre/mTmG off-springs with membrane GFP expression in RBCs and RBC-derived EVs. Cultured dermal fibroblasts from mTmG mice and a mT/floxed/mGFP HEK 293 reporter cell line were used to assess transfer of functional cre in RBC-derived EVs. Gene expression profiling was used to determine functional consequences of EV transfer. To determine targets of RBC-EVs, organs from i)EpoRCre/mTmG ii) mTmG or iii) mTmG mice transfused with RBC-EVs from EpoR-cre mice and targets of RBC-EVs (determined by mGFP expression due to cre-recombination) were assessed by confocal microscopy. Finally, ischemia-reperfusion-infarction (30 min. LAD ligation) was done in EpoRCre/mGmT mice (n=3) and their blood and organs harvested after a span of 4 weeks to analyze changes in quality and quantity of RBC-EV targets following MI.

Results: RBC-EVs (mGFP positive) in plasma accounted for about 9% of total fluorescent EVs as detected by nano-flow cytometry and microscopy. RBC-EVs contained cre protein by EM, and in vitro dermal fibroblasts from mTmG mice or mT/floxed/mGFP HEK 293 reporter cells showed mGFP expression with EpoRCre RBC-EVs, suggesting EV-mediated transfer of functional cre. Recombined HEK cells that were the target of the RBC-EVs have alterations in pathways related to cell proliferation and metabolism. Cre-mediated recombination was noted in diverse organs in EpoRCre/mTmG mice and mTmTG mice transfused with EpoRCre- EVs with the bone marrow, heart, lungs, kidney and spleen showing the largest degree of recombined cells during homeostasis. Target profile of RBC-EVs demonstrated a distinct pattern of EV-mediated communication among the organs following myocardial infarction with a notable increase in recombined targeted cells in the heart particularly in the peri-infarct region, and brain.

Conclusion: We show proof-of-concept for a novel model to study origin and targets of EV-mediated intercellular communication with significant EV-mediated communication between RBCs and cardiomyocytes under homeostatic condition and in a murine model of cardiovascular disease.

Microfluidic Isolation and Molecular Profiling of Tumor-specific Extracellular Vesicles

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Background: Extracellular vesicles (EVs), lipid vesicles present in blood and other biofluids, have sizes that vary from 100 nm to a few microns and carry rich biological cargo that includes proteins, mRNA, microRNA, and DNA. Through their content, EVs preserve the transcriptome information of their parent cells. Thus, they provide unique opportunities for the development of cancer diagnostic tools. However, EVs have not seen widespread clinical testing due to limitations in isolation technologies that suffer from low throughput (ultracentrifugation) or lack of tumor-specificity. To address this, we have developed a microfluidic platform, the EVHB-

Chip, for tumor-specific recovery of EVs using immunoaffinity capture. Using our technology, EVs were isolated from the plasma from glioblastoma multiforme (GBM) patients. The recovered EVs were analyzed for the EGFRvIII mutation, and subsequent RNA sequencing revealed the presence of genes related to resistance, survival and cell signaling.

Materials and Methods: The EVHB-Chip was fabricated using microinjection molding of copolymer olefin (COC. A cocktail of antibodies engineered for EV capture (e.g., anti-EGFR, EGFRvIII, PDGFR) were used to target tumor-specific EVs. After capture, confocal microscopy was used for imaging (e.g., spike EVs experiments), RNA was extracted for ddPCR and RNA sequencing.

Results and Discussion: To mimic conditions present in patient samples, we used engineered exosomes derived from human Gli36 glioblastoma cells that stably express the fluorescent (PalmGFP/tdTomato) and bioluminescent EV reporter (EV-GlucB), and subsequently spiked them in healthy human serum. With confocal microscopy, we identified tumor-specific exosomes at the surface of the EVHB-Chip that were captured using anti-EGFR. Our microfluidic platform, produced a 10 fold increase in tumor-specific EV isolation when compared to traditional methods. Significant gains were seen in the ratio of tumor specific message relative to background (GAPDH or PPBP), often with a 100 fold improvement over ultracentrifugation. These gains were reflected in our ability to detect the presence of the EGFRvIII mutation in GBM patients using digital droplet PCR (5:6 patients tested positive for the EGFRvIII mutation while all healthy controls tested negative). Following, RNA sequencing was performed on a portion of the isolated RNA and the analysis resulted in over 1000 genes significantly upregulated in the patient samples relative to our healthy controls. Our analysis of these genes identified highly specific markers for glioblastoma previously not identified in EVs (OLIG2, BASP) along with genes related to patient survival (MAST3, LRRTM2), chemoresistance (PTPRC, ACTN1), cell signaling (KRAS, PIK3CA, NUCB1), and tumor evolution (CDKN1, ID1).
Conclusions: We have developed the EVHB-Chip for the isolation of tumor-specific EVs. Our results demonstrate the concept of "liquid biopsy" starting from EVs for the detection of somatic mutations (e.g., EGFRvIII) and quantification of up/down-regulated genes by RNA sequencing that may facilitate cancer diagnostics and targeted therapy in a minimally invasive manner.

Sequencing exosomal mRNA and lncRNA across human biofluids coupled with targeted enrichment of exosomal sub-populations reveals novel biomarkers

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Extracellular RNA (exRNA) have recently garnered a high level of interest due to their great potential as sensitive, robust, and easily accessible disease biomarkers. Protected within exosomes and extracellular vesicles, protein complexes, or lipids these RNAs are readily and non-invasively profiled in a wide variety of human biofluids. While the exRNA community remains largely focused on profiling small-RNAs (miRNA, piRNA, etc.), we show that protein coding (mRNA) and long non-coding RNAs (lncRNA) are abundantly represented in exosomes contained within human plasma, serum, cerebrospinal fluid, and urine.
We present our total RNA-sequencing platform, which can reliably and reproducibly quantify transcripts across a wide range of expression levels (from tens to millions of copies). We demonstrate this platform on samples derived from 4 healthy human biofluids, across which we detect more than 15,000 mRNAs and lncRNAs. Many of these genes exhibit significant abundance differences between one or more pairs of biofluids and we offer these data as a community resource. This analysis highlights that the number of potential diagnostic mRNA targets is much larger than that of miRNAs, with >20x more mRNAs detected and >10x more differentially expressed between biofluids.
Paucity of disease-relevant material and high wild-type background are the principal challenges of exosome- or liquid biopsy-based diagnostics. To address this, we have developed the Exosome Dx Enrichment and Depletion (EDDE) platform that can selectively profile sub-populations of exosomes based on surface protein markers. We present this platform as applied to several disease areas using multiple downstream assays including total RNA-seq, qPCR, and ELISA. By enriching for tissue-specific exosomes or depleting non-relevant exosomes from human plasma, we demonstrate superior signal-to-noise characteristics compared to profiling total plasma exosomes.

Evaluating functional delivery of cargo in extracellular vesicles

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Cells release a variety of types of extracellular vesicles (EVs) that contain potentially informational protein, RNA, DNA and lipid cargo. EVs can exert their effects on recipient cells in a number of ways: presentation of ligands that bind to receptors on cells and initiate a

signaling cascade; fusion of the vesicular and plasma membranes such that the contents of the EVs directly enter the cell cytoplasm; and uptake by various forms of endocytosis. In most studies the latter seems to be the dominant mode of uptake, with the consequence that for the EV cargo to be functional it must avoid lysosomal degradation by escaping the endosome into the cytoplasm. A number of studies indicate that some of the protein and RNA cargo is functional in recipient cells. This is especially critical when EVs are used as therapeutic delivery vehicles. We have developed an assay to report on functional activity of EV cargo in the recipient cell cytoplasm. In this assay donor cells express a reporter in which coding sequences for Gluc were fused in-frame at its N-terminus with interleukin-beta (IL1-beta), termed iGLuc (Bartok et al., Methods Mol Biol 2016). Luciferase is inactive in iGLuc, but caspase-1 cleavage of IL1-beta restores luciferase activity in the cytoplasm of recipient cells. We have shown that this EViGluc reporter is intact in EVs released by donor cells and that activity in recipient cells is dependent on caspase-1 activity (which can be endogenous or conferred by a lentivirus expression vector). The assay has been validated by demonstration that treatment of recipient cells with known agents which increase fusion of EVs with recipient cells, and thus delivery the EV cargo directly into the cytoplasm, such as VSVG, lipofectomine, and polybrene increase functional activity of this reporter in recipient cells, as does chloroquine which derails the lysosome degradation. Heparin which blocks uptake of EVs decreases functional activity. This EViGluc reporter assay can be used to screen for drugs which increase or decrease functional EV cargo uptake.

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Poster Presentations Abstracts

Session I: Extracellular Vesicle Biogenesis and Function

1. Exosomes from HIV-1 infected donor cells treated with cART contain TAR and activate the TLR pathway.

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HIV-1 infection results in a chronic illness since long-term HAART can lower viral titers to an undetectable level. However, discontinuation of therapy rapidly increases virus burden. Moreover, patients under HAART frequently develop various metabolic disorders, neurocognitive abnormalities, and cardiovascular diseases. We have previously shown that exosomes containing trans-activating response (TAR) element RNA enhance susceptibility of undifferentiated naïve cells to HIV-1 infection. Up to a million copies of TAR RNA per microliter were also detected in the serum from HIV-1 infected humanized mice suggesting that TAR RNA may be stable in vivo. We recently have found another viral non-coding RNA that we termed TAR-gag which does not code for a protein but is present in exosomes from HIV-1-infected cells. Incubation of exosomes from HIV-1 infected cells with primary macrophages resulted in a dramatic increase of pro-inflammatory cytokines, IL-6 and TNF-β, indicating that exosomes containing TAR RNA could play a direct role in control of cytokine gene expression.

Furthermore, the single stranded 5' or 3' processed stem RNA binding to TLRs activates the NF-κB pathway and regulates cytokine expression. We also found that TAR is capable of controlling the TLR3 pathway by potentially dimerizing TLR3 molecules and phosphorylating the complex. Finally, we found that exosomes from infected cells are increased in numbers when cells are treated with cART. This directly indicates that HIV-1 viral release and exosome release have overlapping biogenesis pathways, including the ESCRT pathway. Collectively, our results imply that exosomes in viral infection control pathogenesis and targeting these particles may be a method to lower overall viral burden in infected hosts.

Relevant manuscripts:

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2. Sampey et., al., J Biol Chem. 2016 Jan 15;291(3):1251-66.
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5. Narayanan A, et al., J Biol Chem. 2013 Jul 5;288(27):20014-33.

2. Neonatal subventricular zone neural stem cells release extracellular vesicles that act as a microglia morphogen

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Subventricular zone (SVZ) neural stem cells (NSCs) are the cornerstone of the perinatal neurogenic niche. Microglia are immune cells of the nervous system that are enriched in the neonatal SVZ. Although microglia regulate NSCs, the extent to which this interaction is bi-directional is unclear. Extracellular vesicles (EVs) are cellular-derived particles that encase miRNA and proteins. It is hypothesized that EVs may transfer information from donor cells to recipient cells. However EVs also carry mis-folded proteins implicated in the pathogenesis of neurodegenerative disorders supportive of EVs encapsulating cellular debris destined for extracellular disposal. Here these hypotheses were tested for SVZ NSC EVs. SVZ NSCs generated EV which could be tracked with the fluorescent fusion EV protein CD9-GFP. CD9-GFP was preferentially expressed within Nestin positive ventro-lateral SVZ NSCs. Extracellular CD9-GFP was detected two days later but subsequently cleared. EVs were preferentially targeted to microglia. Small RNA sequencing identified miRNAs within NSC EVs that regulate microglia physiology and morphology. In support of this finding, EVs induced a transition to a CD11b/Iba1 non-stellate amoeboid morphology. Based on these results, we propose that SVZ NSCs generate EVs that are targeted to and alter microglia within the neonatal brain. These findings implicate a NSC-EV-microglia axis that may provide insight to normal and pathophysiological brain development.

3. Extracellular Vesicles for Viral Transmission: A Rotavirus Perspective in Mouse Model

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Extracellular vesicles have been reported to be used by viruses as “vessels” for transmission in in vitro studies (Chen et al., 2015; etc). However the physiological significance of these exosomal vesicles in viral transmission and host response remains elusive. We have recently found that rotaviruses and norovirus, two enteric viruses that transmit via the oral-fecal route, utilize extracellular vesicles, to exit infected cells (Santiana, Ghosh submitted). We have developed methods to isolate such vesicles from rotavirus infected mouse feces and orally infect them into other mice (Santiana, Ghosh etal., 2017 submitted). Here we interrogate the in vivo role of extracellular vesicles in viral transmission and the immunological response of the host against this mode of infection. In particular we compare the impact of vesicles carrying rotavirus particles relative to free rotavirus particles, in inter-organismal oral-fecal transmission. We qualitatively and quantitatively determine the impact of vesicles on animal physiology, gut morphology, and mucosal immune response. Our results indicate that vesicles are significantly more virulent in rotaviral transmission and lead to suppressed mucosal immune response. We discuss the potential mechanisms by which vesicles can result in enhanced virulence.

4. Ethanol induces enhanced vascularization bioactivity of endothelial cell-derived extracellular vesicles via non-coding RNA regulation under dynamic culturing.

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Background: Although extracellular vesicles (EVs) derived from endothelial cells (ECs), and other cell types, are implicated in promoting vascularization via miRNA transfer, their clinical translation may be hampered by limited potency, as well as lack of standardized production and characterization approaches. Dynamic culture within a perfusion bioreactor can recapitulate the physiological microenvironment of ECs and has been shown to improve their growth kinetics. Additionally, our recent work has shown that exposure of ECs to ethanol at low concentrations in 2D culture results in enhanced vascular bioactivity of their EVs via regulation of miRNA and lncRNA. Thus, in this study, we aim to employ dynamic culturing in combination with ethanol treatment to enhance bioactive EV production for vascularization applications.

Aims: 1) To test the impact of dynamic culturing on EC-derived EV production and 2) to test the effect of ethanol treatment in dynamic culturing on the vascular bioactivity of EC-derived EVs.
Methodology: A 3D printed perfusion bioreactor system containing a scaffold consisting of 5 stacked pillar arrays (total surface area of 72 cm2) was used to culture human dermal microvascular ECs (HDMECs) under flow rate of 4 mL/min of EV-depleted growth media with and without 100mM ethanol. Conventional 2D static culture was used as a control. Conditioned media was collected after 24h and EVs were isolated via differential centrifugation and quantified by NanoTracking Analysis (NTA) using a Nanosight LM10. Vascularization bioactivity of isolated EVs was assessed via a HDMEC gap closure assay. Expression of miRNAs in EVs derived from ECs under different culture conditions was determined using qPCR.

Results: NTA results indicated 20-fold ($n=2$, $p<0.0001$) increase in total EV production when ECs were cultured under flow compared to in 2D static culture in both ethanol treated and untreated groups. No significant increase in EV production was observed between the ethanol treated and untreated groups ($p>0.05$). Interestingly, about 7-fold ($n=2$, $p<0.001$) decrease in total surface protein content per EV was observed in EVs produced under dynamic culture in both ethanol treated and under treated groups. However, ethanol treated EVs under dynamic culture produced EVs with significant vascular bioactivity ($n=2$, $p<0.05$) based on the gap closure assay compared to untreated and negative control. Moreover, our preliminary qPCR results show lower miRNA-106b presence (>4-fold decrease; $n=2$) in ethanol treated EC-derived EVs produced under dynamic culture compared to untreated EC-derived EVs.

Discussion: Our results indicate that higher EV production rates can be achieved by culturing cells under physiological flow environments, however, limited potency remains an issue. Here, we have shown that ethanol conditioning can enhance vascular bioactivity of EVs produced under flow, thus beginning to inform the rational design of a large-scale biomanufacturing approach for therapeutic EV production. The increase in EV vascular bioactivity due to ethanol treatment can be attributed to downregulation of miRNA-106b mediated suppression of CD34, a marker for vascularization phenotype in ECs, as previously shown by our group in 2D culture. Further analysis in EV recipient cells need to be conducted to further establish the mechanism of miRNA and lncRNA mediated increase in EV bioactivity post ethanol treatment in dynamic culturing.

5. Characterization of Exosomes Derived from HIV-1 Infected T cells and Dendritic Cells

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Background: Exosomes are membrane enclosed nano-sized vesicles actively released into the extracellular milieu that can harbor genomic, proteomic and lipid cargos. Functionally, they are shown to regulate cell-cell communication and transmission of pathogens. Though studies have implicated a role for exosomes in HIV-1 pathogenesis, their mechanisms are not well defined.

Aims: We aim to characterize the morphology, molecular composition, biogenesis and functions of exosomes derived from uninfected or HIV-1 infected T cells and dendritic cells (DCs).
Methodology: We isolated exosomes from T cell and DC culture supernatants by ultracentrifugation followed by CD63 or CD81 immuno-affinity purification. Morphology and molecular compositions were analyzed by electron microscopy, mass-spectrometry, miRNA sequencing and Western blotting. Biological functions were studied by HIV-1 infectivity assays and analyzing the pro-inflammatory cytokine expression.

Results: We demonstrate substantial differences in morphological, molecular and biogenesis machinery between exosomes derived from T cells and DCs. Our electron microscopic analysis revealed a significant increase in number of exosomes released from DCs compared to T cells. Further, we observed a significant increase in exosome release from HIV-1 infected T cells and DCs compared to uninfected cells. Next, our analysis of molecules involved in the exosome biogenesis revealed a substantial difference in expression pattern of the endosomal sorting complexes required for transport (ESCRT) complex molecules such as TSG101, Alix, STAM1 and VPS4 between T cells and DCs. In addition, exosomes derived from HIV-1 infected DCs were 4 fold more infective than either cell free HIV-1 or exosomes derived from T-cells. Molecular analysis of exosomes detected the presence of fibronectin and galectin-3 in those derived from DCs, whereas T-cell exosomes lacked these molecules. Addition of anti-fibronectin antibody and β-lactose, a galectin-3 antagonist, significantly blocked DC exosome-mediated HIV-1 infection of T-cells. We also observed increased expression of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-1β and RANTES and activation of p38/Stat pathways in T-cells exposed to exosomes derived from HIV-1 infected DCs. In addition, miRNA sequencing revealed enhanced expression of hsa-miR-374a-3p, hsa-miR-3613-5p, hsa-miR-101-3p, hsa-miR-34c-5p and hsa-miR-32-5p in exosomes derived from HIV-1 infected DCs compared to exosomes derived from uninfected DCs.

Conclusions: Our study provides insight into the role of exosomes in HIV pathogenesis and suggests they can be a target in development of novel therapeutic strategies against viral infection.

6. TNF-**α, exosomes, and microRNAs: Contributions to neuroinflammation**

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Many neurodegenerative diseases are associated with chronic neuroinflammation. Tumor necrosis factor-alpha (TNF-α) is a cytokine that has been observed to be upregulated in the CSF of patients suffering from diseases such as Alzheimer's disease (AD) and stroke. To investigate the potential role of TNF-α in the progression of neuroinflammatory diseases, we investigated its effects on mitochondrial function and miR expression in vitro. Using an immortalized murine hippocampal cell line (HT-22), we exposed cells to TNF-α for 24 hours and assessed mitochondrial function. Additionally, we isolated exosomes from the media of TNF-α exposed cells and exposed naïve cells to these enriched particles. In both cases, we observed significant mitochondrial functional enhancement, characterized by increased respiratory capacity and ATP production, as assessed by the XFe96 analyzer from Seahorse Bioscience. Next, we characterized the exosomes secreted from TNF-α exposed HT-22 cells; using the Malvern NanoSight NS3000, we examined size distributions of isolated particles, namely exosomes. We found that cells exposed to low (0.1 ng/ml) and high (10 ng/ml) doses of TNF-α secreted significantly more exosomes than those in the control or medium (1 ng/ml) dose group. Further, we profiled the expression of three miRs (miR-34a, -146a, and -155) associated with neuro-inflammation. We observed a significant dose-dependent increase in all three miRs contained within isolated exosomes, in contrast the intracellular profiles were vastly different from one another, suggesting differential packaging and secretion, or uptake mechanisms for each of these specific miRs. TNF-α stimulated exosomal secretion may play an important role in the progression of neuro-inflammation within the diseased central nervous system.

7. Inflammation-Stimulated Mesenchymal Stem Cell-Derived Extracellular Vesicles Attenuate Inflammatory Process via Cyclooxygenase Mediated Pathway.

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Extracellular vesicles (EVs) secreted by mesenchymal stem cells (MSCs) have been proposed to be a key mechanistic link in the therapeutic efficacy of cells in response to cellular injuries through paracrine effects. We hypothesize that inflammatory stimulation of MSCs results in the release of EVs that have enhanced anti-inflammatory effects. The present study evaluates the anti-inflammatory properties of EVs derived from inflammation-stimulated and naïve MSCs (MSCEv+ and MSCEv respectively) using a current good manufacturing practice (cGMP)-compliant tangential flow filtration (TFF) system. We extensively characterized the EVs and found a number of differences in protein and cytokine expression and RNA content, despite consistency in size and presentation of common antigens. MSCEv+ further attenuated inflammatory cytokine release compared to MSCEv when co-cultured with activated primary leukocytes, with a distinctly different pattern of cellular uptake. The efficacy of MSCEv+ was attributed to COX2/PGE2 expression, as a COX2 specific inhibitor reduced the potency of MSCEv+ in vitro. The present study demonstrates that EVs can be isolated in a cGMP-compliant manner and characterized using a number of assays that may determine a relevant measure of potency. Inflammatory stimulation of MSCs renders release of EVs that have enhanced anti-inflammatory properties that are due to COX2 expression and PGE2 production.

8. Regulation of macrophage functionality by retinal pigment epithelial cell derived exosomes

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The ocular immune privileged microenvironment programs tolerance in macrophages and microglial cells. Soluble molecules found in the conditioned media of healthy retinal pigment epithelial cell (RPE) eyecups program activated macrophages to be anti-inflammatory, and promote immune tolerance. One of the molecules produced by RPE is the neuropeptide alpha-Melanocyte Stimulating Hormone (alpha-MSH). When the RPE conditioned media is depleted of alpha-MSH, the conditioned media induces apoptosis in the treated macrophages.

We examined for the possibility that membrane FasL and TRAIL are delivered by RPE derived exosomes. From the RPE conditioned media the extracellular vesicles were isolated using Invitrogen dehydration buffer and centrifugation (precipitation method). The isolated vesicles were washed, resuspended in PBS, and added to cultures of macrophages. Caspase 3 activity was assayed at 1, 3 and 18 hours later. In addition, exosomes were collected from the conditioned media of RPE eyecups of mice with eyes suffering from autoimmune uveitis. Also, exosomes were collected from in vitro cultures of ARPE-19 cells, a RPE cell line, grown to a confluent monolayer, or cultures where the monolayer was bisected to stimulate a wound response. Only the exosomes from the RPE eyecups, of healthy and uveitis eyes, activated Caspase-3 activity. Immunoblotting for FasL and TRAIL revealed that the exosomes from the RPE eyecups, and not the ARPE-19 cell line have membrane FasL and TRAIL. This corresponded to the exosome activation of Caspase-3 in macrophages. The exosomes from the RPE eyecups were further assayed for microRNA that can influence apoptotic signaling, and pro-inflammatory activity. The exosomes from RPE eyecups contain the pro-apoptotic miR204, which is also a constitutively expressed miRNA of differentiated RPE cells. In contrast, the anti-apoptotic, pro-inflammatory miR155 was found only in the exosomes from uveitic RPE eyecups, but as pre-miRNA.

These results with our previous findings suggest that as the RPE program tolerance in macrophages through alpha-MSH, the exosomes induce apoptosis in the macrophages not responding to alpha-MSH. This would means that within the healthy immune privileged eye the retina through RPE exosomes select for macrophages and microglial cells programed to suppress inflammation.

9. Identification of molecular signatures of exosomes formed by cancer-cells and their modification in the presence of tumor specific T cells

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This project aims to identify the molecular signature of exosomes that mediate communication between cancer cells and infiltrating T-cells to suppress immune responses to tumor. It will advance scientific knowledge regarding dynamic interactions between tumor and the immune system and identify biomarkers to predict tumor evasion of the immune system as well as tumor response to immunotherapy.

Exosomes are small (30-150 nm) extracellular vesicles that are emerging as major mediators of intercellular communications. These vesicles can shuttle bioactive molecules including proteins, lipids, DNA and RNA species, between cells, leading to the exchange of genetic information and reprogramming of the recipient cells. Recent evidence shows that exosomes transfer oncogenic proteins and nucleic acids to modulate progression and drug resistance of recipient cancer cells. Increasing evidence shows that exosomes generate a microenvironment that promotes tumorigenesis, tumor proliferation and metastasis.

We established a co-culture system for cancer cells (OVA expressing EG.7 cells) and infiltrating T-cells (OT-1 CD8+ T cells) that specifically recognize OVA peptide expressed on tumor cells and have successfully purified exosomes formed in the co-cultured cells as well as in individual cell lines. Exosomes were analyzed using several approaches including RNA-seq, mass spectrometry and transmission electron microscopy (TEM). Novel proteins and RNA molecules have been identified in the isolated exosomes. Exosome content is being examined in the context of reduced affinity of T cells for tumors such as might better reflect spontaneous disease.

By mRNA-Seq analysis of exosomes derived from co-culture cells, we identified upregulated mRNAs related to cell-cell adhesion while downregulated mRNAs are related to immune response. These data suggest that cell-cell interaction and immune responses are key aspects of dynamic crosstalk between tumor and the immune system. The acquired information could facilitate the identification and development of cancer biomarkers.

10. Major vault protein (MVP) dependent sorting tumor suppressor miR-193a into tumor exosomes promotes colon cancer progression

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Tumor exosomes are emerging as key mediators of intercellular communication, however, whether the releasing of exosomes have a biological effect on the exosome donor cells in addition to the recipient cells has not been investigated to any extent. We investigated selective sorting of tumor suppressor miRNAs into exosomes from colon cancer cells and found that this promotes tumor progression. We examined different exosomal miRNA expression profiles in primary colon tumor, liver metastasis of colon cancer, and naïve mouse colon tissue. We found that in more advanced disease, higher levels of tumor suppressor miRNAs are encapsulated in the exosomes. As proof of concept, miR-193a was further tested for the mechanisms underlying it being selectively sorted into tumor exosomes. Affinity purification of miR-193a complex indicated that miR-193a interacts with major vault protein (MVP). Knockout (KO) of MVP led to miR-193a accumulation in the exosomal donor cells instead of exosomes, which resulted in inhibition of tumor progression in mouse colon cancer models. Sorting miR-193a into exosomes by MVP plays a causative role in promoting tumor progression. Furthermore, we demonstrated that miR-193a causes cell cycle G1 arrest and cell proliferation repression through targeting Caprin1 which up-regulates Ccnd2 and c-myc. The role of miR-193a was further demonstrated in colon cancer patients. In human colon cancer patients with more advanced disease, higher level of exosomal miR-193a was circulating in the peripheral blood. Therefore, oncogene protein MVP mediated selective sorting of tumor suppressor miRNA into exosomes

promotes tumor progression. Circulating exosomal miR-193a may be an excellent candidate as a potential biomarker for prognosis of colon cancer progression.

11. Extracellular vesicle-associated miRNAs are pro-inflammatory in sepsis

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Background: We have recently reported that a group of host cellular microRNAs (miR-34a, -122, - 145, -146, -210) are released into the circulation during severe sepsis and their synthetic mimics are capable of inducing complement activation and cytokine production. Extracellular Vesicles (EVs) such as exosomes have been known as vehicles for miRNA-mediated intercellular communication and a source of miRNA biomarkers. However, the exact role of the blood EVs and their loaded miRNAs in sepsis are unclear.

Aims: In the current project, we tested the hypothesis that septic plasma EVs such as exosomes are highly pro-inflammatory and that miRNAs loaded in these exosomes contribute to these effects.
Methodology: A mouse model of sepsis was created by cecal ligation and puncture (CLP). Sham controls were subjected to laparotomy but without CLP. Plasma exosomes were isolated by ultracentrifugation, characterized by electron microscopy and exosome markers, and quantified by a NanoTracking analysis. Exosome-associated RNA was isolated using miRNeasy Micro kit from Qiagen. Exosome-associated miRNAs were profiled by microarray on an immunology-related panel and confirmed by qRT-PCR. Medium cytokines were measured by ELISA.

Results: Electron microscopy identified exosomes isolated from the plasma as “cup-shape” vesicles. Compared with plasma exosomes isolated from sham mice, exosomes isolated from CLP mice were slightly smaller (157.2±2.2 vs. 190.5±6.03 nm, $P<0.0001$) and possessed higher acetylcholinesterase activity and CD-81 expression. However, CLP mice had more abundant exosomes in the plasma compared with sham mice [(3.99±0.34) × 1010 vs. (2.31±0.35) × 1010, $P<0.003$]. qRT-PCR and miRNA array analyses of exosomes revealed that septic mice had significantly higher levels of miRNA expression in their plasma exosomes than sham mice. These include miR-22, -26, -34a, -122, -126, -145, -146a, and -150. Septic, but not sham, plasma exosomes also possessed potent pro-inflammatory properties inducing robust cytokine (IL-6, TNF-α, IL-1β, and MIP-2) production in cultured macrophages. These effects were resistant to polymyxin B (an endotoxin inhibitor) and to RNase treatment, but significantly inhibited by anti-miR inhibitors. Importantly, the septic exosome-induced cytokine production was significantly attenuated in cells deficient of TLR7 or MyD88, but remained the same in cells lacking TLR3 or Trif. In vivo, mice administered i.p. with septic exosomes had an increase in peritoneal neutrophils 22±2.4% vs. 5±1.1%, $p < 0.0001$) and a decrease in macrophages (47±5.5% vs. 73±4.8%, $p < 0.01$) compared to mice treated with sham exosomes. These changes in the peritoneal leukocytes induce by septic exosomes were completely inhibited in mice deficient of MyD88, but surprisingly, unchanged in TLR7-deficient mice.

Conclusions: The plasma exosomes of septic mice prove to be highly pro-inflammatory in macrophage cultures and when injected i.p., and carry significantly higher levels of miR-22, -26, -34a, -122, -126, -145, -146a, and -150. These exosome-associated miRNAs likely contribute to the exosome-induced inflammatory response.

12. Lack of Correlation Between Maternal Serum and Placental miRNA Expression

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OBJECTIVES: Extracellular RNA (exRNAs) in human biofluids have potential utility as predictive, diagnostic, and prognostic biomarkers for a variety of diseases. ExRNAs are carried by extracellular vesicles (EVs), ribonucleoprotein complexes (RNPs), and lipoprotein complexes (LPPs). A potential complicating factor for biomarker discovery is that the exRNAs in any given biofluid originate from many different tissues. Disorders associated with placental dysfunction are of particular interest because the placenta is present only in pregnancy, potentially decreasing the noise associated with biomarkers of placental origin. The goal of this study is to identify miRNAs that display gestational age-specific expression in the human placenta and in maternal serum, and to compare the placental and serum patterns.

METHODS: Small RNAseq was performed on 64 placentas and 72 maternal serum samples collected across gestation. The resulting data were trimmed and mapped using the ExceRpt pipeline. Co-expressed miRNA clusters were identified using Affinity Propagation.

RESULTS: Distinct clusters of miRNAs displaying gestational age-specific patterns of expression were identified in both the placental tissue and maternal serum datasets. However, for many miRNAs, including known placenta-specific miRNAs, the patterns of expression differed markedly between the two datasets.

CONCLUSIONS: miRNAs that display strong gestational age-specific patterns of expression may be useful for development of pregnancy "clocks" that can aid in dating of pregnancies for which standard clinical gestational age dating criteria are unavailable. It is not known whether the alterations in expression across gestational age are due to changes in the cell type composition of the placenta and/or changes in miRNA expression within certain cell types. The lack of concordance between the placental tissue and maternal serum datasets indicates that that complement of placental miRNAs in the maternal serum is not simply a reflection of the placental miRNA-ome, and suggests that release of placental miRNAs into the maternal circulation is impacted by the differential exposure of the different placental cell types to the maternal blood and/or selective secretion of certain miRNAs from the placenta.

13. Placenta-specific miRNAs are carried by a distinct population of EVs in maternal serum

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Extracellular RNAs (exRNAs) are known to be carried by extracellular vesicles (EVs), ribonucleoprotein complexes (RNPs), and lipoprotein complexes (LPPs). However, it is less well understood whether different subpopulations of these carriers are associated with different sets of exRNA cargo. In this study, we used immunoaffinity purification with magnetic bead-conjugated antibodies to enrich for canonical EVs, RNPs, and placenta-specific EVs, and then applied small RNAseq to characterize the miRNA cargo associated with each of these carriers. As expected, our results indicate that there are distinct sets extracellular miRNAs that are predominantly associated with EVs and RNPs. However, there was a separate set of miRNAs that were enriched by an antibody to PLAP (placental alkaline phosphatase), and known placenta-specific membrane protein; this set of miRNAs was enriched for known placenta-specific miRNAs. We also observed a set of miRNAs that was present in the unfractionated serum, but was not immunopurified by antibodies against CD63 or Alix (canonical EV-associated targets), AGO2 (which would enrich for RNPs), or PLAP, suggesting that there is at least one additional as-yet uncategorized exRNA carrier in serum. Overall, our results indicate that placenta-specific extracellular miRNAs are carried in the maternal serum in a PLAP+ EV that is distinct from the canonical EV population, which may have a unique biogenesis mechanism.

14. EXO-Codes for Enhanced Exosome Loading: Nucleic Acids Going POSTAL

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Background: Exosomes are small lipid vesicles 50-100nm that are released by cells and exert phenotypic effects in recipient cells. The molecular cargo of exosomes, including protein and RNAs, are important mediators of this intercellular communication. Although it is appreciated that specific RNAs are actively packaged into exosomes, the molecular mechanisms of this selection are not fully understood.

Aims: Understanding how cells actively package RNA into exosomes would not only provide a richer context of the biology underlying cell-to-cell communication, but would also offer strategies for loading therapeutic RNA cargoes into exosomes.
Methodology: To interrogate the mechanisms of active RNA sorting into exosomes we developed a novel screening method termed POSTAL (Procedure for Organelle-Specific Targeting by Aptamer Library). Exosomes were characterized by flow cytometry, nanoparticle tracking analysis, and transmission electron microscopy. Proteins involved in loading EXO-codes were identified from pull-down assays followed by mass spectrometry. RNA quantification was performed via quantitative PCR and supported with fluorescently labeled RNA analyzed by flow cytometry.

Results: Using the method we have identified de novo 'EXO-codes' which can be utilized to package therapeutic cargo into exosomes of various cell lines. The EXO-codes partition into exosomes up to 400 times more than untargeted control RNA sequences and published RNA sequences. Using a protein pull-down assay and mass spectrometry analysis, diverse sets of RNA-binding proteins have been identified as responsible for sorting the EXO-codes into exosomes of distinct cell lines. The top EXO-code

identified for stem cells was able to load miRNA into exosomes 6-fold more than passively loaded RNA and siRNA 46-fold more than the non-targeted siRNA. The optimized EXO-codes have also been chemically modified to enhance stability. In MDA-MB-231 cells the modified EXO-code loaded siRNA into MDA-MB-231 exosomes more than 100-fold higher than passively loaded siRNA. The exosomes loaded with non-targeted siRNA achieved negligible knockdown of the target gene when plated onto recipient cells while the EXO-Code loaded exosomes knocked down the target gene SNAIL >99% in recipient MDA-MB-231 cells.

Conclusions: These findings not only provide clues into how cells accomplish selective RNA-loading into exosomes, but also offer improvements in therapeutic RNA-loading to pave the way for potent exosome-based therapies.

15. Extracellular miRNA Profiles are Strongly Impacted by RNA Isolation Method

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Background: Extracellular nucleic acid research has generated a lot of interest in the past few years for their potential as intercellular communication molecules, biomarkers and therapeutic agents. However, there are no standardized methodologies to process samples from bedside to sequencing data processing.

Aim: To develop and standardize validated methodologies for sample collection, processing, RNA isolation and next-generation sequencing library construction of exRNA.

Method: ExRNA was isolated from plasma, serum, cell culture supernatant, urine, and bile using 10 different methods. Following quantification and quality controls, qPCR was done for 3 miRNAs using Taqman microRNA kit. Miniaturized small RNA libraries were prepared from selected replicates using the NEBNext small RNA library preparation kit on the nanoliter capacity liquid handler (MosquitoHTS). Fragment analyzer was used to study the size distribution of the library products. Purified small RNA libraries were then quantified by Quantit high sensitivity dsDNA kit and pooled based on both the quantification and fragment analyzer data. Pooled samples were size selected using PippinHT and sequenced on a HiSeq4000.

Results: Many parameters, including intra-lab and inter-lab reproducibility, distribution of small RNA types, and relative abundance of specific miRNAs, vary significantly depending on the RNA isolation method used. Based on qPCR data, differential expression between miRNAs is better correlated among methods and labs than raw Ct values of cell culture supernatant, bile and urine, but not for serum and plasma. For cell culture supernatant, serum and plasma, traditional ultracentrifugation appears to result in the least robust results. ExoRNeasy shows the highest correlation in the Ct values between intra-lab and inter-lab replicates for serum, plasma, cell culture supernatants and bile, followed by miRcury and miRNeasy. Results from small RNA sequencing support the qPCR results the highest reproducibility seen for samples isolated using the ExoRNeasy kit. RNA isolated from urine and bile generated the less reproducible results than the other biofluids. Our results suggest that the primary challenge with the urine samples is the presence of inhibitory factors, while the bile samples appear to suffer mainly from low RNA yield.

Conclusion: In conclusion, based on qPCR and small RNAseq data, ExoRNeasy seems to be the most robust and reproducible commercial kit, which yields high complexity small RNAseq libraries and displays high reproducibility among technical replicates both within and among labs.

Session II: exRNA and Extracellular Vesicle Standards and Resources

16. Integration of normal in vivo biofluid exRNA expression data with Wikidata and the semantic web

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Background: Extracellular RNAs have been determined to be essential players in inter-cell and inter-tissue communication. The expression levels of extracellular RNAs in biofluids are important determinants of normal cell and tissue biology. Deviances in these expression levels might indicate pathologic processes.
Wikidata is a fully open, editable document based graph database which also offers full integration with the semantic web. It currently holds data on human genes, proteins, miRNAs and their targets, tRNAs, lncRNAs and others.

Aims: In order to make normal exRNA expression data in biofluids publicly available and machine readable in a semantic web context, we aimed at annotating RNAs in Wikidata with their in-vivo expression levels from the exRNA Atlas. We also provide example SPARQL (SPARQL Protocol and RDF Query Language) queries which allow for exploration of the data content and also for asking specific scientific questions. Furthermore, all data imported should be linked to appropriate ontologies (e.g. UBERON for anatomy).

Methodology: For the data import, we generated Python scripts which would make use of the Wikidata API (https://www.wikidata.org/w/api.php) and the Wikidata SPARQL endpoint (https://query.wikidata.org). For generating example SPARQL queries, we also made use of the Wikidata SPARQL endpoint.
Results: For the import of exRNA expression data of normal biofluids into Wikidata, we first evaluated which normal biofluid expression data is available in the exRNA Atlas. We found that expression data was available for bile, cerebrospinal fluid, conditioned cell culture media, blood plasma, saliva, serum and urine. The corresponding items in Wikidata were either recovered or created and annotated with the UBERON ontology term matching these anatomical locations. The expression levels were preprocessed and a minimum RPM threshold (>15RPM) was determined in order a certain exRNA would be considered to be expressed in a certain biofluid. If an exRNA would exceed the threshold, a 'part of' (P527) Wikidata statement would be added to the Wikidata exRNA item to indicate expression in a certain biofluid. In addition, each biofluid would receive a reciprocal statement, 'has part' (P361) to list all exRNAs found in this biofluid. If supporting literature existed, a Wikidata reference to the publication was added accordingly. Otherwise, a link to the exRNA Atlas was added. Based on these expression data in Wikidata, SPARQL queries were formulated, which allow for e.g. retrieval of all biofluids an exRNA is expressed or all genes whose expression could be affected by the exRNAs in a certain biofluid.

Conclusions: We created a fully open data store of normal expression levels of exRNAs in biofluids which is computer readable, can be modified by users and can be publicly queried using SPARQL. This should allow biomedical researchers as well as machines to quickly find, retrieve and modify exRNAs expressed in certain biofluids, in a semantic web compatible manner.

17. Comparative analysis of exosome isolation efficiencies between Cushioned–Density Gradient Ultracentrifugation (C–DGUC) and other Commonly Used Methods

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A challenge for the rapidly-evolving field of exosome biology remains the need for approaches that yield pure preparations of exosomes and other extracellular vehicles (EVs) derived from biofluids and cell culture medium.

Aims: In this study, we compared the performance of a method we developed and termed "cushion-density gradient ultracentrifugation" (C-DGUC) with three commonly used methods for exosome isolation from cell culture medium.

Methodology: A hallmark of C-DGUC consists of concentrating exosomes on a high-density iodixanol cushion prior to subjecting them to fractionation by density gradient ultracentrifugation. We tested whether including a cushioned sedimentation step can improve the isolation of murine macrophage-derived exosomes and whether such exosome preparations display higher purity that those isolated by ultrafiltration (UF) and sedimentation with polyethylene glycol (PEG).

Results: At the concentration step, the use of a 60% iodixanol cushion resulted in a recovery of ~90% of all nanoparticles present in the culture medium. This was a threefold improvement of what could be achieved with UC alone. Although UF led to similar nanoparticle recovery rates, it resulted in the retention to ~50% of total protein in the conditioned medium, while the use PEG sedimentation generated a substantial number of artificial nanoparticles. Following density gradient fractionation, non-exosomal protein (97% of total) and nanoparticles (30% of total) in the concentrate produced by c-UC and UC were further eliminated by a second density gradient ultracentrifugation step, while much of the non-exosomal particles produced by PEG were not eliminated. A series of western blotting experiments of the exosome markers TSG101 and Alix confirmed that C-DGUC provides the highest yield of exosomes with minimal protein and nanoparticle contamination.

Conclusions: Taken together, our data show that the use of C-DGUC substantially improves the yields and purity of exosomes from cell culture media to facilitate their study in experimental systems. Ongoing studies explore the benefits of using C-DGUC for the isolation of exosomes and other EVs from complex biofluids including plasma.

18. The exRNA Atlas resource provides insights into exRNA biology through cross-study analysis of exRNA profiles

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Background: The research into the role of exRNAs in physiological processes and in human diseases requires analyses across independently-produced datasets. Such analyses are hampered by the lack of integration of exRNA profiling data and of associated online resources.

Aims: To help address the open questions in exRNA biology relevant for clinical translation and to catalyze data-intensive exRNA research, NIH Extracellular RNA Communication Consortium (ERCC) created the Extracellular RNA Atlas (exrna-atlas.org).

Methods: exRNA Atlas collects exRNA profiling metadata, supports uniform data processing (RNA-seq, qPCR), standardized data quality assessment, efficient data sharing and analysis. The exceRpt pipeline (v4.6.2) maps RNA-seq data to exogenous rRNAs as well as thousands of exogenous genomes. Metadata validators minimize curation effort, making it possible for the community to contribute on the order of 10,000 profiles per year. Our metadata-driven portal (exrna-atlas.org) displays the data in an accessible way and provides integrated online tools for data browsing, cross-study analyses and knowledge sharing.

Results: The exRNA Atlas contains a total of 2,627 (2,067 currently public) exRNA profiles from nine body fluids (serum, plasma, CSF, bile, urine, saliva, sputum, seminal fluid, and ovarian follicle fluid), with additional 3,000 profiles anticipated by the end of 2017. Over 10,000 Atlas data files have been downloaded by researchers over the last 12 months (5.5-fold increase over last year), and over 21,000 (3-fold increase) have been submitted into Atlas and associated pipelines/tools. The Atlas helps contextualize new data, validate it against expected parameters, and provides opportunities for cross-study analyses.

We complete the first census of ncRNAs in human body fluids and make it available for interactive browsing to inform the design of targeted panels, help focus biofluid-specific studies and contextualize findings on specific exRNAs. We quantitate miRNAs, tRNAs, Y RNAs, and other human exRNAs and identify a core set of over 100 miRNAs that are abundantly present (>10 RPM) in most body fluids. By mapping RNA-seq reads across thousands of non-human genomes we identify taxonomic units of bacterial exRNAs that are recurrently present in saliva and urine.

To examine sources of experimental and biological variation of exRNA profiles, we developed a computational deconvolution algorithm. Deconvolution reveals a striking amount of sample-to-sample variation in relative abundances of exRNA carrier types (vesicular or non-vesicular). Multiple carrier types, each having a characteristic exRNA profile, are detected across most biofluids, the

first is a nuclear ncRNA profile matching the exRNA profile of HD exosomes, and multiple cytoplasmic ncRNA profiles, some matching the exRNA profile of LD exosomes. Through deconvolution we assign miRNAs that are differentially expressed in disease conditions to specific carrier types.

Conclusions: By lowering the amount of effort required to contribute exRNA profiling data and associated metadata and by maximizing incentives such as data publication, interoperability and reuse for cross-study analysis, we mobilized the exRNA research community to create the exRNA Atlas, the first compendium of exRNA profiles of human body fluids. Using this integrated resource we addressed some key questions relevant for basic exRNA biology and for clinical utility of exRNA profiling.

19. VBR virtual biorepository: a web-based service for sharing biofluid-, tissue-, cell- and other bio-samples

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Background: VBR Virtual Biorepository originally arose from the needs of investigators within the exRNA Communication Consortium (ERCC) to share biofluid samples across institutions for the purpose of collaborative protocol development and biomarker discovery.

Aims: To enable efficient sample sharing, the ERCC Resource Sharing Working Group, the Data Coordination Center (DCC) and Administrative Core initiated VBR development. The initial goal was to enable the sharing of cerebrospinal fluid (CSF) samples from members of the ERCC-based CSF consortium that includes Translational Genomics Research Institute, Phoenix Children's Hospital, Oregon Health and Science University, and University of California, San Diego.

Method: VBR is designed as a distributed system consisting of a VBR hub and a set of local or cloud-hosted VBR nodes. VBR hub provides an overview of the types and number of biosamples present within the nodes. The hub supports sample queries based on consortia (CSF, hepatobiliary) or institutions and based on publicly shared metadata about anonymized VBR biosamples, including clinical, radiographic, pathologic, and accession metadata. Lists of samples that satisfy search criteria are placed in a shopping cart for ordering from sample providers. Search criteria and results can also be saved for later retrieval and updating via new searches. Upon selecting samples based on the publicly shared metadata, researchers communicate directly with each other to make specific biosample sharing arrangements. Future improvements of the shopping cart feature will allow end-to-end tracking of biosample orders from multiple institutions. VBR nodes are set up independently of the hub under control of sample providers.

Results: The VBR hub (beta, Phase 1) was launched on 9/1/17 (https://genboree.org/vbr-hub/), and is available for use by the global extracellular RNA research community (both ERCC and non-ERCC members). VBR hub currently provides access to metadata for 7,651 CSF and 2,356 hepatobiliary samples from five institutions, with the addition of another 50,000 hepatobiliary samples from the Mayo Clinic planned before the end of 2017. The biofluid samples are suitable for the study of exRNAs and exmiRs from biofluids, and assessment of biomarker sensitivity and specificity.

All the participant institutions agree to common IRB language and uniform MTA to facilitate biosample exchange and maintain separate VBR nodes. The ERCC DCC provides assistance regarding maintenance of data within individual VBR nodes using pre-defined metadata templates. Investigators potentially interested in setting up their VBR node to share metadata about their samples may contact the VBR administrator (thistlew@bcm.edu).

Conclusion: VBR Virtual Biorepository successfully addressed the needs of investigators within the exRNA Communication Consortium (ERCC) to share biofluid samples. The initial goal of 10,000 shared samples is likely to be exceeded by several-fold by the end of this year. The types of shared resources extended from CSF to now also include hepatobiliary samples, tissue, cell,

macromolecular samples and even sample slides. These resources will be particularly useful for catalyzing collaborations for the purpose of collaborative protocol

20. Fractionating human semen for RNA profiling

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Background: Semen is a complex fluid containing both a high concentration of extracellular vesicles (SEV) (average of 2.2 x 10^13 particles per ejaculate), and soluble proteins. We have shown that both fractions contain stable extracellular RNA (exRNA). Semen is exchanged between individuals during intercourse and has effects on recipient cells, though how the exRNA present in semen contributes to this effect remains unknown. A previous study sequencing the small RNA content of SEV from six men revealed small non-coding RNAs with regulatory potential as well as approximately 125 different miRNAs.

Aims: The current study is being undertaken to comprehensively survey the small RNAs present in both SEV and SEV-depleted seminal plasma in twenty different healthy men. RNA yield, protein yield, and vesicle quantity were used to compare methods to optimally purify SEV as well as non-vesicular, protein protected RNAs from individual semen samples.
Methodology: A number of parameters were tested to ensure optimal recovery of RNA from both the vesicular fraction and vesicle depleted seminal supernatant. These included sample handling, filtration of samples, and ultracentrifugation conditions over sucrose or iodixanol density cushions and gradients. Assays used to compare methods included vesicle yield and purity as measured by nanoparticle tracking analysis, ELISAs for the vesicle-associated protein CD63, overall protein concentration in each fraction, RNA yield and quality in each fraction. A preliminary sequencing run using Illumina's TruSeq small RNA protocol on the MiSeq platform was used to validate the purification protocol and to compare RNA biotypes in each fraction.

Results: We found that filtering samples improved the particle to protein ratio for the SEV fraction, but resulted in an unacceptable loss of RNA in the seminal plasma fraction. We concluded that 18 hrs of ultracentrifugation at 100,000 x g of unfiltered samples over an iodixanol cushion, followed by washing of SEV fractions and pooling of protein-containing supernatants yielded the highest amount of RNA without changing the distribution of types of small RNAs found in each fraction. Preliminarily analysis suggests that specific RNAs are very consistently found in human semen. Overall, the small RNAs present in SEV-associated and seminal plasma fractions are similar, though some individual RNAs including tRNA fragments, Y RNA fragments, and some miRNAs are more represented in one fraction.

Conclusion: Biofluids present unique challenges for exRNA purification and profiling. Optimal methodology for each experimental goal should be tested empirically. This study will provide a baseline reference for endogenous RNA present in semen, useful for biomarker development as well as for generating hypotheses about potential functional effects of exRNA exchange between people.

21. Exosomes serve as novel modes of tick-borne flavivirus transmission from arthropod to human cells and facilitate dissemination of viruses to vertebrate neuronal cells

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Molecular determinants and mechanisms of arthropod-borne flavivirus transmission to the vertebrate host are poorly understood. In this study, we show for the first time that a cell line from medically important arthropods, such as ticks, secretes extracellular vesicles (EVs) including exosomes that mediate transmission of flaviviruses RNA and proteins to the vertebrate host cells. Our study shows that tick-borne Langat virus (LGTV), a model pathogen closely related to tick-borne encephalitis virus (TBEV) profusely uses arthropod exosomes for transmission of viral RNA and proteins to the human skin keratinocytes and blood endothelial cells. Cryo-electron microscopy showed the presence of purified arthropod/neuronal exosomes with the size range of 30 to 400 nm in diameter. Both positive and negative strands of LGTV RNA and viral envelope-protein were detected inside exosomes derived from arthropod, murine and human cells. Detection of Nonstructural 1 (NS1) protein in arthropod and neuronal exosomes further confirmed that

exosomes contain viral proteins. Viral RNA and proteins in exosomes derived from tick and mammalian cells were secured, highly infectious and replicative in all tested evaluations. Treatment with GW4869, a selective inhibitor that blocks exosome production affected LGTV loads in both arthropod and mammalian cell-derived exosomes. Transwell migration assays showed that exosomes-derived from infected-brain-microvascular endothelial cells (that constitute the blood-brain barrier) facilitated LGTV RNA and proteins transmission or crossing of the barriers and infection of neuronal cells. Neuronal infection showed abundant loads of both tick-borne LGTV and mosquito-borne West Nile virus RNA in exosomes. Collectively, our results suggest that flaviviruses uses arthropod-derived exosomes as a novel means for viral RNA and protein transmission from the vector, and the vertebrate exosomes for dissemination within the host that may subsequently allow neuroinvasion and neuropathogenesis.

Session III: Research, Diagnosis and Biomarkers

22. Raman Spectroscopy for molecular fingerprinting of liver cancer associated extracellular vesicles

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Introduction: Circulating extracellular vesicles (EV) have emerged as a potential class of biomarkers for the early detection and treatment monitoring in cancer and other diseases. Their use requires the analysis of specific contents within EV, but are challenging because of the lack of single vesicle specificity. Raman spectroscopy can provide a molecular fingerprint of molecular structures within vesicles by providing a spectral plot of energies resulting from vibrational, rotational, and other low frequency modes. Recent studies have shown that circulating EV from cancer patients have higher beta-sheet than alpha-helix content, changes that can result in distinctive Raman spectra. Thus, we evaluated the use of Raman spectroscopy for the detection of molecular changes associated with malignancy in EV from hepatic epithelia.

Methods: Raman spectroscopy was performed on malignant (HepG2) and non-malignant (HH) hepatocytes, and on EV obtained from these cells by differential ultracentrifugation. EV were characterized by determination of their zeta-potential, and assessment of size and concentration using nanoparticle tracking analysis (NTA). For spectroscopic analyses, 1ul samples were placed on a calcium fluoride coverslip and air-dried. Spectra were obtained using a 785nm laser at 95mW power using a 100X objective with a 50.0s exposure. Analysis was performed using a Raman shift library derived from the literature. Concanavalin A and BSA were used as positive controls for beta-sheets and alpha-helices respectively.

Results: EV obtained from HH or HepG2 cells had similar NTA profiles with a peak modal size of 145 and 165 nm respectively. Malignant EVs showed a negative shift in zeta potential compared to non-malignant EVs. Raman spectra were analyzed for Amide I, II, and III regions and their corresponding beta-sheet and alpha helix content. No major differences in spectra were found between malignant and non-malignant cells. However, non-malignant cell EVs had a higher alpha-helical content than beta-sheet content in Amide III region, whereas HepG2 EVs had a higher beta-sheet than alpha-helix content in Amide III and Amide I regions. Amide II region was more prevalent in non-malignant EVs than in cancer-cell EVs, but does not have characteristic peaks for alpha and beta structures.

Conclusions: Raman spectra obtained from EV are different from those obtained from their cells of origin. Although spectra from normal or cancer cells are similar, there are considerable differences in spectra between EV and their cells of origin. Malignant cell EVs have a distinctive spectrum that differs from that obtained from EV obtained from normal cells. Further studies are warranted to evaluate the potential of molecular fingerprinting of EV using Raman spectroscopy as a cancer diagnostic.

23. Differential Expression of Extracellular miRNAs from Plasma following Heart Transplant in Pediatric Patients are Correlative of Rejection.

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Introduction: Heart transplantation is an effective lifesaving treatment for patients suffering from end-stage heart failure. Nevertheless, allograft rejection poses a major obstacle with this treatment option; it can result in the loss of the allograft and mortality. One major challenge currently faced in solid organ transplantation is determining how to screen for heart transplant (HT) rejection. While repeated endomyocardial biopsies (EMBs) have become the accepted standard of practice for monitoring and diagnosing acute heart

rejection, EMB's are invasive and recurring in nature. Thus, there is a need for the discovery of reliable non-invasive biomarkers especially for pediatric patients.
Emerging evidence on the utility of peripheral blood RNAs as biomarkers and their involvement in many biological processes makes them promising candidates as potential biomarkers. These processes include the regulation of immune cell maturation as well as adaptive and innate immune responses. Here we hypothesize that specific miRNAs could serve as reliable non-invasive biomarkers of HT rejection in pediatric patients.

Methods: This study included pediatric HT plasma samples collected post-transplant during their routine rejection surveillance biopsy from Boston Children's Hospital. Using a discovery cohort (n=6) at two time points, baseline (BL) and follow-up (FU) with n=3 acute cellular rejection samples and n=3 non-rejection (NR) samples. Rejection was characterized using histopathology biopsy results on a graded scale from 0R to 3R. Samples were considered NR from 0R- absence of rejection to 1R- mild rejection, while acute cellular rejection (ACR) was considered from 2R- moderate to 3R- severe rejection.
RNA was extracted from 450ul plasma samples using Exiqon's miRCURY biofluids kit and concentrated using Zymogen's RNA Clean and Concentrate kit. Small RNA libraries were prepared using both New England BioLab's "NEBNext small RNA library prep set" and Bioo Scientific's "NEXTflex small RNA sequencing V2 kit". Libraries were then deep sequenced using Illumina's HiSeq single read 50 base pair (SR50) in high output mode producing 100-150 million reads per lane.

Results: One sample was excluded from analysis due to failure to pass quality control post library construction leaving n=5 for data analysis, 3 pairs of ACR samples and 2 pairs of NR. The two analyses were therefore ACR vs BL and NR vs BL. All analyses took into account the paired nature of the samples collected. Although no miRNAs were significantly differentially expressed, 18 miRNAs were significant at a nominal p-value < 0.1 and a fold change ≥ 1.5 in the ACR vs BL analysis, and 13 miRNAs were significant at a nominal p-value < 0.1 and a fold change ≥ 1.5 in the NR vs BL analysis.

Conclusion: Our data did not identify any significantly differentially expressed miRNAs in circulating peripheral plasma from pediatric HT patients. However, our small sample size and limited plasma volume and quality may contribute to this lack of significance. Several novel as well as previously described miRNAs were detected with nominal significance, and our future directions are aimed at creating a panel of miRNAs selected from our results in combination with those previously described in literature to validate in a cohort (n=17; BL and FU) using the FirePlex assay, a method of targeted sequencing. Any miRNAs validated with significance would prove viable targets for peripheral blood biomarker discovery.

24. Exosomes from synovial fluid of patients with osteoarthritis transport miRNAs involved in networks regulating pro-inflammatory response and cell decline

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Osteoarthritis is a common joint disorder and a major cause of disability in the elderly, affecting approximately 30 million people in the United States. Despite the high frequency of osteoarthritis (OA), the cause of the primary form of this disorder is still unknown. Age, genetic and environmental factors contribute significantly to initiation of primary OA. Synovial fluid contains molecules important to maintain mechanical properties of the joint and a wide spectrum of signaling molecules including proteins and nucleic acids, which are freely circulating or are transported inside extracellular vesicles. In the last few years, research studies implicate that exosomes are important components of joint homeostasis and could also be involved in pathogenesis of osteoarthritis. Our project is focused on analysis of exosomes from synovial fluid and to determine whether these vesicles, which are released by synoviocytes into synovial fluid, transport the unique set of miRNAs involved in initiation and progression of osteoarthritis. Current data about the exosomal miRNAs from synovial fluid are limited and a full profile of these molecules is still under investigation.

We isolated exosomes from synovial fluid collected during joint replacement surgery in patients with knee osteoarthritis. Electron microscopy and immuno-gold labeling confirmed the presence of exosomes in an isolated fraction. We identified exosomal markers via Western blot and completed RNA-Seq analysis of miRNAs in nine patients with osteoarthritis. NGS data were generated on the Ion Proton system, which is part of the Ion Torrent platform and were analyzed with small RNA analysis plugin (Ion Torrent Suite software) and R statistical software. NGS results were validated via qPCR in ten new patients. Targets of identified miRNAs and networks were identified with the following databases: DIANA LAB, TargetScan, MIRANDA, miRDB, PICTAR and IPA. We observed that the top ranked miRNAs are part of the networks, which include TP53, IL-6, NFKB complex and ERK1/2, according to IPA.

Additionally, five miRNAs from this list are already linked to osteoarthritis. Target genes of top ranked miRNAs that overlap in four databases include: TP53, MDM4, AFF4, NFIB etc.

Our analysis of the complete miRNA profile of exosomal cargo isolated from synovial fluid of patients with osteoarthritis shows that they are actively involved in the pathogenesis of this disorder. Targets regulated by these miRNAs are in control of cell death, cell cycle and inflammatory responses, which play a significant role in OA pathogenesis. This research was funded by the NIH (NIGMS) grant P20GM103629 (Project 5 to M.C-R.) and by the Irvin Cahen award to V.D. and M.C-R. RNA-Seq was completed in the COBRE Genomics and Biostatistics Core funded by the grant P20GM103629 (to S.M.J.).

25. MicroRNA-30d is a biomarker for heart failure and plays a functional role in cardiac remodeling

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MiRNA-30d is correlated with cardiac remodeling in patients with advanced heart failure (HF) and appears to protect cardiomyocytes from TNF-induced cell death in vitro. However its functional and mechanistic role in cardiac remodeling and HF have not been elucidated. Here, we leverage data from the Framingham Heart Study (FHS) and in vivo data to provide evidence of miR-30d as an important biomarker of cardiac hypertrophy and HF and define its functional role in cardiac remodeling in murine models of cardiovascular disease.

In 2763 patients in the Framingham heart study we determined that the lowest quartile levels of circulating miR-30d are associated with increased LV mass and increased concentricity, both of which are associated with worse outcomes in HF.
In murine models, miR-30d levels were modulated using either LNA to abrogate expression or agomiR to increase expression. In transverse aortic constriction (TAC) and ischemia re-profusion (I/R) induced HF, mice with silencing of miR-30d had significantly worse heart failure and adverse remodeling including fibrosis and inflammation as measured by echocardiography and gravimetric data. Conversely, mice with agomiR-induced increased miR-30d levels had significantly less HF and improved cardiac remodeling compared to control mice. RNA sequencing revealed that miR-30d appeared to regulate key inflammation (MAP4K4) and fibrosis pathways.

Our data provide proof that plasma extracellular miR-30d thus has utility not only as a prognostic biomarker of hypertrophy and heart failure but is implicated in cardiac remodeling in murine models of cardiovascular diseases by regulating MAP kinase and fibrosis pathways. These data support an emerging paradigm that RNA biomarkers of cardiovascular disease may play a functional role in disease pathogenesis.

26. Potential role of circulating extracellular vesicles in the modulation of brain function and behavior

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Background: Peripheral immune alterations have been described in psychiatric disorders such as schizophrenia, depression, and autistic spectrum disorders. In addition, behavioral changes have been observed in various immunodeficient animal models. However, the mechanisms by which peripheral immune system influences brain development and function are not well understood. In this study, we explored the mechanisms by which circulating extracellular vesicles (EVs) mediate immune-brain communication and influence mouse behaviors.

Aims: To understand the role of altered circulating EVs in mice with behavioral deficits

Methodology: Mice deficient for Rag1 or Rag2 gene (Rag KO mice) were used as a model to study the effects of loss of adaptive immune cells (T and B cells) on brain cellular phenotypes and behaviors. Circulating EVs were collected from their sera and analyzed by using electron microscopy, nanoparticle tracking assay, and Western blotting. Brain cellular phenotypes were assessed by immunofluorescent staining and gene expression analysis. Behavioral phenotypes of Rag KO and WT mice were examined in social interaction test. In vivo transfer of EVs was performed to see its effects on behavioral alterations of Rag KO mice.

Results: Rag KO mice displayed social behavioral deficits, accompanying by enhance c-Fos immunoreactivity and altered microglia morphology in the medial prefrontal cortex (mPFC). Circulating EVs were also affected in these mice and lacked the expression of

markers for T cells. Further analysis revealed that a set of microRNAs (miRNAs) in circulating EVs were diminished in Rag KO mice. Our preliminary data also showed that in vivo transfer of circulating EVs rescues the social behavioral deficits of Rag KO mice.

Discussion: Our data showed that circulating EV profiles were altered in mice lacking adaptive immune cells and, accordingly, showing social behavioral deficits. Notably, our in vivo experiments suggest that circulating EVs may contribute to social behaviors. Further study will provide a novel biological insight into the mechanisms underlying peripheral-to-brain immune communication via EVs.

27. Do extracellular vesicles provide a better source for disease biomarkers?

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Circulating RNA has gained significant interest as a biomarker for various diseases in recent years. However the field suffers with inconsistent and irreproducible reports which are caused probably by factors including differences in sample preparation and measurement platform. Extracellular vesicles (EVs) are lipid vesicles released by cells and packed with proteins, RNAs (including miRNA) and other molecules from the cells. We used size-exclusion chromatography and small-RNA sequencing to analyze the miRNA spectra of urine EVs from type 1 diabetes patients with microalbuminuria and plasma EVs from patients with preterm birth, and identified more informative and consistent miRNA signatures associated with their respective disease states. We also identified miRNAs that are enriched in urine and plasma EVs, compared to their respective EV-depleted fractions. In addition, we also observed that the spectrum of miRNA in EVs are less affected by hemolysis, and show little differences between serum and plasma. In summary, we found that the molecular content in EVs better reflect the disease state compared to whole urine or plasma, and is less affected by hemolysis or between serum and plasma.Circulating RNA has gained significant interest as a biomarker for various diseases in recent years. However the field suffers with inconsistent and irreproducible reports which are caused probably by factors including differences in sample preparation and measurement platform. Extracellular vesicles (EVs) are lipid vesicles released by cells and packed with proteins, RNAs (including miRNA) and other molecules from the cells. We used size-exclusion chromatography and small-RNA sequencing to analyze the miRNA spectra of urine EVs from type 1 diabetes patients with microalbuminuria and plasma EVs from patients with preterm birth, and identified more informative and consistent miRNA signatures associated with their respective disease states. We also identified miRNAs that are enriched in urine and plasma EVs, compared to their respective EV-depleted fractions. In addition, we also observed that the spectrum of miRNA in EVs are less affected by hemolysis, and show little differences between serum and plasma. In summary, we found that the molecular content in EVs better reflect the disease state compared to whole urine or plasma, and is less affected by hemolysis or between serum and plasma.

28. Placental Specific Genes are Present in Extracellular Vesicle from Maternal Urine During Pregnancy

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The placenta is a transient organ that is the direct physical interface between the mother and the growing fetus. While studies completed to date confirm the pivotal role of the placenta in the offspring's short- and long-term health, developing new methods to monitor the placenta in real time during pregnancy remains elusive. In order to noninvasively assess placental health both in vivo and in vitro, we are harnessing extracellular vesicles (EVs). EVs are 30-200 nm bilayer cell-derived vesicles that are present in most biological fluids, and they participate in inter-cellular communications. The functional state of the placenta is reflected by changes in the expression of coding and non-coding genes both inside its cells and in its shed EVs. In order to determine what RNA transcripts are packaged in EVs by the placenta and how their profile changes throughout pregnancy, we have obtained 34 urine samples from pregnant women at two gestational time points (16-18 weeks and 34-36 weeks). We isolated EVs and confirmed their presence and size via electron microscopy imaging. Subsequently, urinary EV RNAs were sequenced with Illumina HiSeq4000 for both full length and small libraries. High quality reads were trimmed by trimmomatic, aligned to the human genome using STAR, and quantitated using Cufflinks. We have detected expression of several known placenta specific transcripts including PAPPA2, PSG4, PSG11, LGALS14, MIR3184, and MIR125A. Data from this exploratory research will pave the way for real time assessment of placental health during both normal and abnormal pregnancy with the potential for discovering accurate biomarkers that can be transferred

from bench to bedside. We are also studying the functional role of placental EVs within the placenta and their cross-talk to other organs or systems. Currently, there is a paucity of research exploring the impact of fetal-placental EVs on maternal cells, and few studies convincingly show that this occurs as a physiologic process. EVs are potent modulators of the immune system. To explore the function of placental EVs on the maternal immune system, we isolated EVs from human trophoblast cells and fluorescently labeled RNA inside them. Our live cell time lapse confocal imaging verified trophoblastic EV uptake by human macrophages.

29. The miR-183 cluster: An extracellular signature of aging and bone loss

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The miR-183 cluster including miR-183, miR-182, and miR-96 is highly conserved in vertebrates and plays a role in a number of human diseases. Recently we have found that the miRNAs belonging to this family are elevated in extracellular vesicles (EVs), including exosomes and microvesicles, from the bone marrow interstitial fluid of aged mice. Cellular transfection showed that miR-183 can induce senescence in bone marrow stromal cells, and it has been shown by others to increase osteoclastogenesis by suppressing Hmox1. In vitro studies demonstrate that miR-183 expression is increased with exposure to reactive oxygen species (ROS). These findings point to a role for extracellular miR-183 in age-related bone loss, and others have found that miR-182 can suppress bone formation by targeting Foxo1. We have also observed that EVs isolated from serum of aged mice (22-24 mo) show a 4-fold increase in miR-183 compared to EVs from young, adult mice (4-6 mo). Other studies have shown that miR-183 increases with age in mouse serum but is reduced with calorie restriction, and both miR-183 and miR-96 are downregulated in long-lived centenarians compared to those individuals with shorter lifespans. It is therefore possible that circulating, EV-derived miRNAs including miR-183 may ultimately serve as useful biomarkers for age-associated senescence, stem cell dysfunction, and oxidative stress.

30. MicroRNA changes in blood following blast traumatic brain injury: an exploratory study

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At present, accurate and reliable biomarkers to ascertain the presence, severity, or prognosis of blast traumatic brain injury (bTBI) are lacking. There is an urgent need to establish accurate and reliable biomarkers capable of mbTBI detection. Currently, there are no studies that identify changes in miRNA at varied severities of bTBI. Various biological components such as circulating mRNA and miRNA, could potentially be detected using advanced techniques such as next-generation sequencing. Thus, plasma analysis is an attractive approach with which to diagnose and treat brain injuries. Sub-acute changes in plasma microRNA (miRNA) were evaluated in a murine model of mild-to-moderate bTBI using next-generation and Real Time PCR. Animals were exposed at 17, 17 X 3 and 20psi blast intensities using a calibrated blast simulator. Plasma levels of brain-enriched miRNA, miR-127 were increased in all groups while the let-7a, b and g were reduced in 17 X 3 and 20psi but let 7d was increased in 17psi group. The majority of the miRs and lipids are highly conserved across different species, making them attractive to explore and potentially employ as diagnostic markers. It is tempting to speculate that miR-128 and let-7 family could predict mTBI, while combination of miR-484, miR-122, miR-148a, miR-130a and miR-223 could be used to predict overall status of injury following blast injury.

31. MicroRNA Biomarkers for Alzheimer's Disease in Human Cerebrospinal Fluid

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Background: Currently available biomarkers of Alzheimer's disease (AD) are limited. The discovery of extracellular microRNAs (miRNAs) in cerebrospinal fluid (CSF) raised the possibility that miRNAs may serve as novel biomarkers of AD. Our initial studies discovered a set of miRNAs that that can discriminate AD patients from control subjects.

Aims: Analyze the expression of a set of AD-specific miRNAs in a new and independent cohort of AD and control CSF samples, in order to validate their ability to serve as biomarkers for AD.

Methods: CSF from 47 AD patients and 71 control subjects were obtained from the Shiley Marcos Alzheimer's Disease Research Center at the University of California, San Diego. The expression of 36 candidate miRNA biomarkers identified in the discovery UH2 phase was analyzed using TaqMan® Low Density Custom miRNA Arrays. Stringent data analysis included 7 different methods of classifying methods (LogRank, ROC, CART, CFOREST, CHAID, BOOST, and UH2 assessment) that were each used to independently rank the candidate markers in order (1=best, 26=worst). The total score for each miRNA was added to provide a ranking for each candidate biomarker. Multimarker modeling and covariate analysis were performed on the top ranking miRNAs. Classification performance of miRNA biomarkers were compared to that of ApoE4 genotype. In addition, incremental improvement adding miRNA biomarkers to ApoE4 was assessed.

Results: Analysis of the custom array data validated that the candidate miRNAs discriminate AD from control subjects in a new and independent cohort of CSF samples. Cluster analysis revealed 26 miRNAs in three rank groups. Analysis of the contribution of individual miRNAs to multimarker performance revealed 14 best miRNAs. Top-performing linear combinations of 6 and 7 miRNAs have AUC of 0.775–0.796, relative to ApoE4+ AUC of 0.637 in this sample set. Addition of ApoE4 genotype to the model also improved performance, i.e. AUC of 7 miRNA plus ApoE4 improves to 0.82.

Conclusions: We have validated that CSF miRNAs can discriminate AD patients from controls in a new and independent cohort of CSF samples. Combining the top 14 miRNAs improves sensitivity and specificity of biomarker performance, and adding ApoE4 genotype, and possibly other classifiers such as Abeta:tau ratio, improves classification. The miRNA biomarkers will now be examined in patients diagnosed with mild cognitive impairment (MCI), many of whom progressed to a clinical diagnosis of AD, as well as AD and controls. This study will determine if the AD miRNA biomarker expression changes can be detected earlier in the disease progression.

32. The Potential of Ocular Exosomal Biomarkers as Diagnostic and Prognostic Indicators

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Background: Interest in utilizing 30 -150 nanometer sized exosomes and other extracellular vesicles (EVs) as biomarkers of disease has increased exponentially in recent years. EVs (including exosomes) have several unique features that define ideal biomarkers: (i) a lipid bilayer provides protection for their RNA, DNA, and protein cargo; (ii) they contain tissue-, cell-, or disease-specific proteins and nucleic acids; and (iii) their hardiness enables a wide range of methods for isolation and enrichment from a range of body fluids (e.g. plasma, serum, urine, aqueous humor, tears and vitreous).

Aims and Methodology: To identify biomarkers for retinal disease, we defined the proteome of exosomes from the retinal pigmented epithelium (RPE), which forms the outer blood-retinal barrier in the eye. RPE dysfunction contributes to age-related macular degeneration, the leading cause of blindness in industrialized nations in the elderly. The RPE is a highly polarized barrier, responsible

for the directional secretion of proteins, lipoprotein particles and EVs. Such a division dictates directed interactions of the RPE with the systemic circulation (basolateral side) and the retina (apical side). As a model, we used primary cultures of differentiated porcine RPE monolayers on permeable supports. EVs were isolated from conditioned medium bathing either apical or basolateral RPE surfaces, by differential ultracentrifugation followed by OptiPrep buoyancy density gradient ultracentrifugation. Density fractions containing highly purified exosomes were processed for proteomic profiling. Exosome preparations are inherently heterogeneous mixtures, and traditional mass spectrometry cannot easily identify protein components of low abundance that are nonetheless specific for exosomes. To overcome these technical issues, we performed a mass spectrometry-based proteomic analysis of apically and basolaterally RPE-derived EVs by simultaneously profiling hundreds of proteins in exosome preparations of increasing purity. This approach, termed Protein Correlation Profiling (PCP), permits the analysis of any sub- or extracellular components/complexes that can be enriched by fractionation but not purified to homogeneity. This is the first study to use PCP in determining exosome proteomes. In parallel, EV size distribution and concentration were determined using nanoparticle tracking analysis (ZetaView).

Results: Using PCP mass spectrometry, a total of 631 proteins were identified in exosome preparations, 299 of which were uniquely released apically, and 94 uniquely released from the basolateral side; suggesting distinctly different cargo sorting into EVs released apically versus basolaterally. Exosomes and EVs released basolaterally from RPE cells theoretically enter the systemic circulation (choroid) and thus basolateral RPE-specific exosomal proteins that we identified, represent targets for immunoisolation of RPE-derived exosomes from blood. Preliminary data will be presented which demonstrate the presence of ocular-derived exosomes in systemic circulation.

Conclusions: This study shows that PCP mass spectrometry is a valuable and powerful tool for analyzing extracellular vesicle proteomes. Furthermore, these data serve as a foundation for comparative studies aimed at elucidating the molecular pathophysiology of retinal diseases and to help identify potential therapeutic targets and systemic RNA and protein biomarkers to monitor and stratify such diseases.

33. Optimization of a differential ultracentrifugation protocol for isolation of small extracellular vesicles from human serum

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Background: Ultracentrifugation remains the gold standard for isolation of small extracellular vesicles (sEV), particularly for cancer applications. While immunoisolation offers a relatively clean alternative, such approaches introduce potential bias since the EV-associated tetraspanins that form their basis are frequently downregulated or unexpressed in cancer cells. The gold-standard protocol described by Thery et al (2006) requires 3 lengthy ultracentrifugation cycles, presenting a challenge when using human samples where the total available serum volume is often limited since each additional cycle reduces overall sEV yield, while at the same time substantially increasing the procedure time.

Aims: We sought to determine if an alternative protocol using 2 ultracentrifuge cycles could be employed to increase efficiency.

Methods: We obtained serum from two healthy subjects (40y male and 37y female). One mL aliquots of serum were initially subjected to centrifugation for 30 min at 2,000 × g followed by 45 min at 12,000 × g to remove cells and cellular debris. We subsequently isolated sEV using 3 different ultracentrifugation protocols, each in triplicate: (Protocol 1) three ultracentrifugation cycles at 110,000 × g at 2 hrs, 70 min and 70 min, respectively (Thery et al, 2006); (Protocol 2) two ultracentrifugation steps at 110,000 × g at 2 hrs and 70 min, respectively, with no sucrose cushion; and (Protocol 3) two ultracentrifugation steps with a 30% sucrose cushion, where the sample was first centrifuged at 100,000 × g for 75 min on the sucrose cushion, after which the sucrose gradient was collected and centrifuged for an additional 70 min at 100,000 × g. All centrifugations were performed at at 4ºC. The sEV concentration was quantified for each isolate via nanoparticle tracking analysis (NTA), and sEV were visually confirmed by transmission electron microscopy (TEM) imaging. The presence of sEV was further confirmed by Western blot analysis for the EV-associated tetraspanin CD81 and cytosolic endosomal sorting complex component TSG101. Contamination by potential miRNA-harboring protein was assessed by performing Western blots for ApoA-I, ApoB, and Ago2.

Results: Total high-speed ultracentrifugation time was 260 min for Protocol 1, 190 min for Protocol 2, and 145 min for Protocol 3. sEV concentrations were significantly higher for Protocol 2 ($p < 0.0001$), with an average concentration of 8.63x1010 particles/mL of isolate compared to 9.71x109 particles/mL and 1.57x1010 particles/mL for Protocol 1 and Protocol 3, respectively, although the

difference in concentrations for Protocols 1 and 3 was not significant (p = 0.34). Lipoprotein contamination was noticeably higher for Protocol 2, with some contamination noted for Protocol 1, and very faint or no bands observed for the sucrose cushion protocol (Protocol 3). Ago2 protein was not detected in appreciable quantity in any of the isolates.

Conclusion: Although the 2 ultracentrifuge cycle protocol with no sucrose cushion resulted in a much higher sEV yield, lipoprotein contamination was a substantial issue, drawing into question its utility for scientific and clinical applications. However, 2 ultracentrifugation cycles with a 30% sucrose cushion resulted in slightly higher, albeit non-significant, sEV yields with lower levels of protein contamination compared to the lengthier current gold standard, therefore presenting a more efficient alternative approach for isolation of serum sEVs.

34. Serum Exosomes and miRNAs in Male and Female Current, Former and Non-Smokers

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Significance: Despite recent surge in exosome research, the effect of smoking on serum exosomes remains unclear. Exosomes are present in almost all body fluids including serum. We have evaluated the effect of smoking on serum exosome and its miRNA content in current, former, and non-smokers.

Methods: We isolated exosomes from a total of 24 human serum samples (8 current, 8 former, and 8 non-smokers with 4 males/4 females in each group). Nanoparticle tracking analysis was performed with ZetaView. Zeta potential, an indicator of exosome stability, was measured in 0.05X PBS at 23˚C. Extracted exosomal RNA was quantified and profiled using NanoString Human miRNA assay v3 for 800 miRNAs. miRNA data was exported using the nSolver 3.0 software from NanoString and normalized using the positive controls and the trimmed mean of endogenous probes. After removing miRNAs with background level expression, we used the LIMMA package in R to identify differentially expressed (DE) miRNAs.

Results: The mean age of the study subjects was 55.4, 45.3, and 46.1 years for current, former, and non-smokers, respectively. Exosome diameters were similar across all groups. Significant differences were observed in the size distribution across the spectrum (50-350nm), the amount of the 50-150nm particles, and the zeta potential between the different sex and smoking groups ($p < 0.05$). The transmission electron microscope images indicate that most exosomes were within 50-150nm, consistent with our ZetaView data. We identified 156, 228, and 182 miRNAs in current, former, and non-smokers. With a fold change > 1.5 and $p < 0.05$, we identified 12 DE miRNAs in current vs. non-smokers, 13 miRNAs in former vs. non-smokers, 5 miRNAs in former vs. current smokers, including let-7a-5p, miR-191-5p, miR-19b-3p, miR-1537-3p, miR-125b-5p, miR-146a-5p, and miR-423-5p. Several of them have been associated with cigarette smoking.

Conclusions:
Our study has identified the specific differences in serum exosome and its miRNA contents related with smoking exposure in males and females for the first time. While our findings are exciting, further investigation is needed due to the relatively small sample size.

35. A mouse model for tracking and auditing endothelial cell-derived extracellular vesicles in cancer

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Extracellular vesicles (EVs) play important roles in tumor progression by altering immune surveillance, promoting vascular dysfunction, and priming distant sites for organotropic metastases. The miRNA expression patterns in circulating EVs are important diagnostic tools in cancer. However, multiple cell types in the tumor microenvironment (TME) including cancer cells and stromal cells (e.g. immune cells, fibroblasts, and endothelial cells, ECs) contribute to the pool of circulating EVs. Because EVs of different cellular origins have different functional properties, auditing the cargo within cell type-specific EVs in the TME is essential. Our lab has

previously characterized dysfunctional ECs in tumors and in preliminary studies, we've found that freshly isolated tumor-derived ECs (TECs) shed greater numbers of EVs into culture medium, they harbor unique protein payloads, and they differentially activate immune cells compared to their normal EC counterparts. We've also found that EC lineage-traced mice (Cdh5-CreERT2:ZSGreenloxP/stop/loxP, herein called ECZSGreen mice) stably transport ZSGreen fluorophore into EVs allowing us to chase EC-EVs pulsed into different cellular recipients. We have used these mice to demonstrate that ZSGreen+ EC-EVs purified from plasma are enriched in specific miRNAs; thus, ECZSGreen mice allow labeling and comprehensive auditing of EC-EVs in the cancer setting. We propose that, by shedding EVs, ECs will i) direct the function of immune cells systemically and ii) exchange information with cancer cells and other stromal cells in the local TME.

36. A Novel Approach to Mesothelioma: Exosomal miRNAs

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Malignant mesothelioma (MM) is a devastating cancer arising on the mesothelial cell surface of the pleura, peritoneum, and occasionally the pericardium and tunica vaginalis. The main causal factor of mesothelioma is exposure to asbestos fibers, and the disease has a remarkably long latency period (20-50 years) with very poor prognosis once the disease is diagnosed (6-12 month survival). The diagnosis of this disease is typically in late stages of the cancer, and this is mainly due to a severe lack of useful biomarkers that would equip clinicians for early diagnosis. One of the newest research trends in biomarker discovery is the use of secreted exosomes, 20-140nm membrane bound vesicles disseminated from cells and carrying biological content (proteins, miRNAs, etc.) to target cells with the capacity to alter their phenotype. As molecular cargo packages, exosomes have tremendous potential for uncovering novel biomarkers of disease and also for elucidating biological phenomena. Exosomes are known to play a significant role in normal physiology, also, but their importance in cancer biology is of particular interest to us. Our preliminary microarray data showed that mesothelioma tumor cells secreted exosomes with a unique miRNA composition as compared to non-tumorigenic mesothelial cells, and these findings were validated by qPCR. Interestingly, the miRNAs in highest expression levels from tumor cells were counterintuitively tumor-suppressor miRNAs, notably miR-16-5p. Based on this we hypothesize that mesothelioma tumor cells preferentially secrete high levels of tumor-suppressor miRNAs, such as miR-16-5p, as a sophisticated survival mechanism to avoid such miRNAs from disrupting the tumor's out-of-control growth characteristics. Our findings thus far, suggest that tumor cells do in fact unload their cellular fraction of tumor suppressor miRNAs in a preferential manner heralding the potential for not only biomarker discovery but new mechanistic understandings and therapeutic potentials. We propose that inhibition of exosome secretion may therefore have a beneficial effect on MM cell death and drug resistance. This work is supported by grants from the NIH (ES021110), Department of Defense IDeA Award (W81XWH-14-1-0199) to AS and UVM Pathology Departmental fellowship to PM.

37. Evaluation of pre-analytic factors for the analysis of plasma cell-free RNA in cancer patients

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Background: Analysis of cell-free DNA (cfDNA) is an important source of information for non-invasive molecular diagnostics, and is already implemented in the clinic for prenatal testing and for EGFR screening in non-small cell lung cancer. Despite being less stable than cfDNA, cell-free or extracellular RNA (cfRNA or exRNA) has the potential to provide additional information regarding disease states and may guide treatment options, potentially improving patient care and outcomes. Tumour specific transcripts, such as fusions, not easily identified in cfDNA can theoretically be picked up more readily in cfRNA as they are present in multiple copies. Previous work suggested that cfRNA is released from cells along with cfDNA within exosomes, vesicles, and during normal cell death processes. Much work has been done on cfDNA to optimize the pre-analytic and analytic procedures, but for cfRNA.

Aims: We aim to compare the various methods currently available for the collection, processing, and extraction of plasma cfRNA and establish a standard operation procedure for our facility at Memorial Sloan Kettering Cancer Center (MSKCC), where we anticipate a large number of samples and will need to transport samples from remote affiliated sites.

Methodology: Plasma samples were purchased from a commercial biobank or blood samples were collected from cholangiocarcinoma and lymphoma patients at MSKCC under IRB protocol 12-245 with informed consent by patients. Blood was processed with a double centrifugation protocol (1,400g x 10 min, 14,000g x 10 min) and plasma was frozen at -80C until use. We compared 3 different collection tube types: EDTA, Streck cell-stabilizing cfDNA BCTs and Streck cfRNA BCTs, and we evaluated 5 different extractions protocols: mirVana PARIS kit (Invitrogen), miRNEasy kit (Qiagen) adapted for use with Trizol LS, QIAamp ccfDNA/RNA kit (Qiagen), QIAamp Circulating Nucleic Acid (CNA) Kit (Qiagen) using the standard protocol and the miRNA protocol. We also evaluated the effects of delays in processing of the Streck tubes up to 72 hours. To determine the yields and quality of extracted RNA, bioanalyzer RNA analysis and GAPDH RT-qPCRs were performed.

Results: Extraction kits that presented the highest yields were mirVana PARIS kit and the CNA miRNA protocol, however samples collected in Streck tubes exhibited very low yields using the mirVana PARIS kit. When extracted immediately, tube type did not affect the RNA yields, however increasing delays in processing led to higher levels of total cfRNA when collected in both Streck cfRNA and Streck cfDNA tubes, with Streck cfDNA tubes having significantly greater increase after 72 hour delays.

Conclusions: Our results showed no significant difference in yields from tubes processed immediately and any tube can be used interchangeably at early time points. Additionally, we found that Streck tubes offered the potential to delay processing for several days and still yielded amplifiable cfRNA. As Streck cfRNA tubes showed significantly lower increases in yields at 72 hours, they are preferable to the Streck cfDNA tubes. In terms of extractions, the CNA kit miRNA protocol resulted in the highest yields, whereas the organic extraction reagents in the mirVana PARIS kit demonstrated adverse interactions with the cell-stabilizing agents in Streck BCT tubes. Overall, we report a robust protocol for obtaining cfRNA from plasma, with the option of delaying processing for several days.

38. A Myriad of Novel Tissue-Specific and Disease-Specific Molecules as Candidate Novel Biomarkers

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BACKGROUND: Liquid biopsies are attracting increasing attention from scientists and the popular press. This is due to their tremendous potential as non-invasive, highly-accurate diagnostic tools. Vesicles and extracellular RNAs that are secreted by healthy and diseased cells are of particular interest as they are thought to proxy the source tissue and to reveal disease states. Our work has been focusing on two types of regulatory non-coding RNAs (ncRNAs):
(1) the isoforms of microRNAs (miRNAs), known as “isomiRs;” and,
(2) the fragments of transfer RNAs, known as “tRFs.”
Despite early reports of isomiRs and tRFs in deep sequencing data, there had been no systematic attempt to characterize these ncRNAs for many years.

AIMS: Are isomiRs and tRFs random transcripts? Are they relevant in the context of disease? What processes do they affect? Can they serve as biomarkers? How many loci in the human genome encode miRNAs and tRNAs? Are isomiRs and tRFs found extracellularly?

METHODOLOGY: Generate answers to these questions by mining large collections of transcriptomic data from multiple human tissues.

RESULTS: We analyzed more than 12,000 deep-sequencing short RNA profiles from healthy individuals, patients with different diseases. We discovered that the production of isomiRs and tRFs is highly regimented. Specifically, we showed that miRNA precursors produce constitutively “clouds” of isomiRs with composition and relative abundances that depend on a person’s:

- sex
- population origin, and
- race

as well as on

- tissue
- tissue state, and
- disease type.

In complete analogy to miRNAs, we also showed that tRNA transcripts produce constitutively "clouds" of tRFs with composition and relative abundances that depend a person's:

- sex
- population origin, and
- race

as well as on:

- tissue
- tissue state, and
- disease type.

For isomiRs from the same locus, we additionally showed that they can target non-overlapping collections of genes. This is an important result because it augments considerably our understanding of how many genes are actually controlled by a given miRNA locus. Moreover, for tRFs, we showed that they are loaded on Argonaute in a cell-type dependent manner. Thus, they regulate protein levels though RNA interference.

For triple negative breast cancer and for prostate cancer, we have also linked race-dependent differences in the profiles of isomiRs and tRFs to race-dependent disruptions of regulatory networks between the normal and disease states.
These wide-ranging findings gain additional importance considering that:

a) isomiRs and tRFs are present in vesicles and among extracellular RNAs;
b) we discovered an additional 3,494 human miRNA precursors the vast majority of which are primate-specific and exhibit tissue-specific expression; and,
c) we discovered an additional 351 human genome sequences resembling mitochondrial tRNAs and showed for several dozens of them that they exhibit tissue-specific expression.

CONCLUSION: We showed that many more post-transcriptional regulators exist than originally believed. These regulators (=isomiRs, tRFs) capture tissue state powerfully, can distinguish among diseases, and depend on personal attributes. Moreover, they are found extracellularly. Thus, isomiRs and tRFs could help attenuate the "many-to-one" problem that frequently arises in biomarker research and could prove to be formidable novel biomarkers. This is a possibility that we are exploring currently.

39. MicroRNA-1246 is an exosomal biomarker for aggressive prostate cancer

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Background: Prostate cancer (PCa), a leading cause of male cancer-related mortality, is a remarkably heterogeneous disease with tumors ranging from indolent to very aggressive. A major clinical challenge in PCa clinical management is posed by the inability of current diagnostic tests, to discern between indolent and aggressive disease. Therefore, molecular biomarkers for improving PCa diagnosis and prognosis are highly sought. Also, it is desirable to have an easily accessible, minimally invasive way to determine the molecular imprints of a patient's tumor for risk stratification as molecular characterization based on biopsy sampling is often challenging.

Aims: The primary objective of the current study was to define (i) novel exosomal microRNA (exRNA) biomarkers in aggressive prostate cancer (PCa) to distinguish from benign disease, (ii) exRNA to differentiate between normal and aggressive PCa.

Methodology: Serum samples from PCa patients and clinical information were obtained from the NCI-funded resource Cooperative Human Tissue Network (CHTN). Patient cohort included cases with no prior radiation therapy or chemotherapy and was divided equally into prognostic risk groups defined by American Joint Committee on Cancer as low, intermediate and high categories.

Controls were age and race-matched normal individuals or men with benign prostatic hyperplasia (BPH). Serum derived exosomes (EVs) were isolated using the Total exosome isolation reagent (Life Technologies) and exosomal RNA was prepared using a Plasma/Serum Exosome Purification kit (Norgen Biotek) as per manufacturer's instructions. In a pilot study, we employed a novel, digital amplification-free quantification method using the nCounter technology (Nanostring Technologies) to profile exosomal serum microRNAs (miRNAs) from aggressive PCa cases and BPH/ disease- free controls. For validation of dysregulated miRNAs, mature miRNAs were assayed by quantitative real-time PCR using the TaqMan miRNA Assays (Applied Biosystems).

Results: We identified several dysregulated miRNAs. miR-1246, miR-766-3p and miR-302c-3p were commonly dysregulated in aggressive PCa relative to normal/ BPH. Pathway analyses of dysregulated exRNAs showed dysregulation of key biological signaling and cellular adhesion pathways implicated in metastatic PCa. Of significance, exosomal miR-1246 was identified and validated as the common upregulated exRNA that could distinguish between normal, BPH and aggressive PCa. Ex-miR-1246 expression was correlated with increasing pathological grade, positive lymph node metastasis and poor prognosis pointing to its potential to predict aggressiveness/ localized metastasis. Our data suggests that exosomal miR-1246 is a promising PCa biomarker with diagnostic potential that can predict disease aggressiveness. Further, we found that miR-1246 is a tumor suppressive miRNA that is downregulated in PCa clinical tissues and cell lines and is selectively released into PCa exosomes.

Conclusions: Our findings identify dysregulation of exosomal miRNAs in aggressive PCa leading to alteration of key signaling pathways associated with metastatic prostate cancer. Of importance, we validated miR-1246 as a promising exosomal marker for predicting/diagnosing aggressive prostate cancer.

40. Lead–associated extracellular microRNA in children's urine: a pilot for population-based study

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Background: Urinary extracellular RNAs are potential biomarkers of exposure and disease that may originate from epithelial cells in the renal or urogenital tract. Isolation of these biomarkers in early life could provide a non-invasive method for early detection of nephrotoxic exposure or subclinical renal disease.
Aim: To perform a pilot study to identify lead-associated extracellular microRNA (exmiRNA) in urine samples collected from 10 healthy children 4-6 years of age participating in the Programming Research in Obesity, Growth Environment and Social Stress (PROGRESS) prospective birth cohort study in Mexico City.
Methods: Spot urine samples were collected at the 4-year visit and stored at -80°C until analysis. We performed differential centrifugation on 10 mL aliquots and visualized extracellular vesicles using electron microscopy. ExmiRNA were extracted and expression was assessed using TaqMan OpenArray qPCR technology for 754 miRNAs. Expression data were adjusted for plate-to-plate variability and normalized using the global mean. An exmiRNA was considered 'detectable' if it produced reliable qPCR signal in ≥70% of participant samples. Prenatal blood lead levels (BLLs) were measured by ICP-MS and stratified by the median as high (> 3 µg/dL) or low (≤ 3 µg/dL). ExmiRNA expression was compared using unpaired t-tests with statistical significance defined as $p< 0.05$. Adjustment for covariates and false discovery rate correction will be performed in the larger study.
Results: Over 150 urine exmiRNAs were detected in this pilot study. The top 10 detected exmiRNAs were miR-30c, miR-30b, miR-30a-5p, and miR-30d (from the miR-30 family) as well as miR-204, miR-20a, miR-211, miR-200a, miR-26a, miR-24. Preliminary analysis of all miRNAs identified differentially expressed miRNAs stratified by BLL. miR-551b expression was decreased 9-fold (ΔΔCt: -9.7 (95%CI: -17, -2.5) in the high lead-exposed group ($p=0.01$).
Conclusions: These results suggest that urinary exmiRNA in children are detectable, and associated with prenatal nephrotoxic lead exposure. These results are consistent with a role for lead in the developmental origins of renal disease in children. While this sample size is small, replication is ongoing in a larger sample. ExmiRNA in urine may hold promise as biomarkers of early life renal disease.

41. Elevations in Circulating Extracellular Vesicle miR-21 as a Biomarker of Developing Type 1 Diabetes

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Early detection of developing Type 1 Diabetes (T1D), before widespread destruction of β cell mass, is needed for improved outcomes of T1D prevention strategies. MicroRNAs (miRNAs) released in extracellular vesicles (EVs) have been proposed as ideal biomarkers due to their stability and feasibility of detection. Previous work from our lab demonstrated that β cell miR-21 production is induced by inflammation, and RT-qPCR analysis of diabetic NOD mouse islets revealed a ~4-fold increase in miR-21 expression compared to NOR controls. We hypothesized that the inflammatory milieu of developing T1D may also increase miR-21 in β cell EV cargo. EVs released by β cell lines and human islets exposed to a cytokine mix of IL-1β, INFγ, and TNFα were isolated using ExoQuick reagent.

RT-qPCR revealed an 3-5 fold increase in EV miR-21. Nanoparticle tracking analysis showed no changes in EV quantity or size distribution in response to cytokine exposure, implicating transcript upregulation and changes in EV cargo as responsible for the increase. Serial ultracentifugation was performed to separate β cell EVs by size, and suggested that cytokine-induced increases in β cell EV miR-21 were predominantly determined by exosomes. To assay changes in circulating EV miR-21, we performed longitudinal serum collections on NOD mice and insulitis resistant NOR controls, from 8 wks of age and until diabetes onset. Starting 3 wks prior to diabetes onset, EV miR-21 levels progressively increased in serum of diabetic NODs compared to age-matched NOR controls, peaking at a10-fold increase from baseline levels. To validate relevance to human diabetes, serum EV miR-21 was assayed in samples collected from pediatric T1D patients at the time of diagnosis, as well as healthy pediatric controls (n=16-19/group). Consistent with our NOD data, serum EV miR-21 was significantly increased in diabetic samples compared to controls. We propose that EV miR-21 may be a promising marker of insulitis and developing T1D in susceptible individuals. Ongoing studies will further define relationships between EV miR-21 content and β cell inflammation and death.

42. Predicting And Targeting Protein Ligand-Mediated And Exosome-Mediated Crosstalk Signaling Cascades In Ovarian Tumor Microenvironment With Multi-Cellular Systems Modeling

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Ovarian cancer is the most lethal gynecologic malignancy in the US. While at the fine-grained level, research efforts have been dedicated to identify disease related genes and mutations in cancer cells, the coarse-grained behavior of stroma-tumor or tumor-immune microenvironment in disease progression is relatively unexplored. The identification of cancer-stroma crosstalk networks with prognostic value presents a unique opportunity for developing new treatment strategies to improve patient survival rates. To capture such an opportunity, the Cell-Cell Communication Explorer (CCCExplorer), a Java based modeling tool, has been developed to identify novel bidirectional crosstalk networks among multiple types of cells within tumor microenvironment. CCCExplorer incorporates computational models, matching algorithms, and drug-protein databases to predict, rank and visualize both protein ligand-mediated and exosome-mediated crosstalk signaling cascades and identify rational treatment strategies targeting crosstalk cascades.

For example, with transcriptome profiling data generated from microdissected ovarian cancer cells and cancer associated fibroblasts (CAFs) in high-grade serous ovarian caner tissue (HGSC) as inputs, CCCExplorer was used to identify TGF-β–dependent and TGF-β–independent Smad signaling networks as protein ligand-mediated crosstalk signaling cascades activated in CAFs associated with poor patient survival rates. The microdissection provides valuable spatial information of the sequenced tumor cells within the tumor microenvironment that is lacking in flow cytometry or microfluidics approaches in sorting cells for genomic sequencing. Validation studies by co-culturing ovarian cancer cells with CAFs indicated that activation of Smad signaling in CAFs promoted aggressive phenotypes of ovarian cancer cells while inhibition of Smad signaling in CAFs suppressed ovarian cancer progression in vitro and in vivo.

As an example of a different kind of crosstalk cascades, CCCExplorer used RNAseq data generated from exosomes isolated from CAFs and normal fibroblasts (NFs) as well as from CAF- and NF-derived exosomes treated ovarian cancer cells to discover specific CAF-derived exosomal microRNAs and LncRNAs along with signaling networks associated with chemoresistance in ovarian cancer cells. Note that predicting crosstalk signaling cascades among multiple cell types beyond pairwise comparison results in superlinear scaling of matching complexity and poses non-trivial modeling challenges. After functional validation of predicted crosstalk cascades, CCCExplorer can then be used to query underlying drug information databases in order to identify and rank candidate drug agents that could target cancer progression-associated crosstalk signaling cascades activated in ovarian cancer cells and CAFs.

Our findings demonstrate a powerful tool at multi-cellular level in which a computational framework can be developed for understanding, predicting, and targeting crosstalk signaling cascades and coarse-grained behavior of heterogeneous tumor microenvironment. We are applying CCCExplorer to uncover activated crosstalk networks in ovarian caner cells and neighboring stromal cells and to discover drug compounds targeting these pathways, potentially leading to faster cures for ovarian cancer. Further studies using in vitro and in vivo models to test the efficacy of these drugs in ovarian cancer treatment are warranted.

43. Exosomal microRNA-mediated neuron to astrocyte communication in the CNS

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Neuron to astrocyte signaling is essential for functional tripartite synapses in the mammalian central nervous system (CNS). In particular, neuronal signals are known to up-regulate expression of astroglial glutamate transporter GLT1. We have previously shown that cultured neurons secrete exosomes that contain microRNA miR-124, of which is capable of increasing GLT1 protein expression in cultured astrocytes. In the current study, we investigated the localization and migration of cell-type specifically secreted exosomes in situ in the CNS and mechanisms of exosomal miR-124 mediated up-regulation of GLT1 in astrocytes in vitro and in vivo. We developed a cell-type specific exosome reporter mouse line (CD63 conditional knock-in, CD63-CKI) in which a GFP-fused CD63, an exosome surface marker, can be induced in a particular cell type when bred with the cell-type specific Cre driver mice. By employing this mouse tool, we found that both neuronal and glia-derived exosomes are widely present in the CNS and are routinely taken up among major CNS cell types. Consequently, neuronal miR-124 is transferred into astrocytes through exosomes. In addition, Neuronal exosomes are particularly abundant and migrate extensively in the brain. To understand the dynamics of neuronal exosome release, we measured the quantity of secreted exosomes at different day in vitro (DIV) from cultured neurons using qNano particle analyzer. We found that cultured neurons secrete the most exosomes within 4 days of cultures. The secreted exosomes are positive for typical exosome markers, including CD63, CD81, Tsg101, and GFP/His tag (from the CD63-CKI mice), but are negative for endosome, ER, Golgi, and nucleus markers, respectively. To identify specific miRNA cargo in neuronal exosomes, we further profiled miRNAs from neurons and neuronal exosomes by hybridizing their total RNA with the miRNA chip. We identified 131 and 202 miRNAs that are selectively enriched in neuronal exosomes or neurons, respectively. In contrast, we also found that a subset (21) of miRNAs has a similarly high expression levels (> 8 in microarray scan unit) in both neurons and neuronal exosomes.
We have previously shown that miR-124 is able to significantly up-regulate GLT1 protein expression. Here we further showed that the miR-124 levels in astrocytes are significantly increased following co-culture with neurons, which can be significantly blocked with the treatment of the GW4869, an exosome secretion inhibitor. Both the GW4869 and pre-transfection of miR-124 antisense sufficiently attenuates neuron-dependent GLT1 expression induction. To understand the mechanisms for miR-124-mediated up-regulation of GLT1 expression, we analyzed miRNAs that are predicted to bind to the 3' untranslated region (UTR) of the GLT1 mRNA. By transient transfection of candidate miRNAs and GLT1 immunoblot, we identified several microRNAs (miR-132, miR-218, and miR-128) that significantly reduce GLT1 protein expression in astrocytes. Interestingly, exosomally transferred miR-124 significantly suppress the levels of GLT1-inhibiting miRNAs. In summary, we demonstrated that exosomes are widely present in the CNS and identified miRNAs that are enriched in neuronal exosomes. We also elucidated the mechanisms for exosomal miR-124-induced up-regulation of GLT1 in astrocytes.

44. Mesenchymal stromal cell-derived extracellular vesicles balance the nitrofen-induced alterations of major vasoactive mediators

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Background: Children with congenital diaphragmatic hernia (CDH) continue to have an unacceptably high mortality and those that do survive endure significant morbidity. No definitive treatment for severe CDH and, specifically, CDH-associated pulmonary hypertension (CDH-PH), exists. One of the reasons for this is that the fundamental mechanisms underlying CDH- PH, including the role of vasoactive mediators, remain unclear.Endothelin-1 (ET-1) and endothelial nitric oxide synthase (eNOS) are major vasoactive mediators and alterations of their expression in the pulmonary arteries (PAs) of a rodent model of CDH have not been examined. The nitrofen-induced CDH rat model is a well-established model of CDH, though specific effects of nitrofen on the pulmonary vasculature and vasoactive mediators are unknown. We investigated alterations of the major vasoactive mediators in nitrofen-induced CDH rodent PAs. Extracellular vesicles are small lipid microvesicles, released by cells into the extracellular environment, which facilitate biological responses and may serve as the key mechanistic intermediary between progenitor cells and target cells, tissues, and organs. Mesenchymal stromal cell-derived extracellular vesicles (MSCEv) contain or promote tissue expression of proteins, growth factors, enzymes, and cytokines, and have specifically demonstrated the propensity to attenuate PH. Thus we hypothesized that human MSCEv will balance the nitrofen-induced alterations of major vasoactive mediators. To test this hypothesis, we examined the effects of nitrofen on the expression of major vasoactive mediators in human pulmonary artery endothelial cells (HPAECs). Furthermore, we tested the effect of human MSCEv, on endothelial vasoactive mediators in cell culture.

Methods: Pregnant Sprague–Dawley (SD) rats received either nitrofen (100 mg) or olive oil on gestational day 9.5. The newborn rats (n=20) were sacrificed and PAs were isolated from both CDH and controls. To confirm similar vasoactive mediator expression alterations and assess MSCEv effects, cultured HPAECs were treated with nitrofen (0.1mg/ml), either with or without MSCEv

(1x1010/ml), for 24 hours. For tissues and cells, the expression of endothelial ET-1 and eNOS were measured using western blotting and immunostaining.

Results: Our data showed that the expression of ET-1 was significantly increased in CDH rat PAs (45%, $p<0.05$). However, the expression of e-NOS was significantly decreased (65%, $p<0.01$) in CDH PAs compared with control. In vitro, we demonstrated similar alterations of the major vasoactive mediators (ET-1, 55% increased, $p<0.05$; e-NOS, 60% decreased, $p<0.01$, respectively). Importantly, MSCEv treatment significantly reversed the alterations of the major vasoactive mediators in nitrofen-treated HPAECs. The treatment with MSCEv reduced the expression of ET-1 by 51% ($p<0.05$) and increased the expression of eNOS by 55% ($p<0.05$) compared to nitrofen treated group.
Conclusion: Herein, we elucidate alterations in the expression of endothelial vasoactive mediators specific to nitrofen-induced CDH rodent PAs and confirm similar responses among cultured HPAECs exposed to nitrofen. MSCEv exposure leads to recovery of endothelial cell balance of the vasoactive mediators ET-1 and eNOS. These data may indicate a possible mechanism underlying vascular damage leading to PH in this CDH model and identify MSCEv as a possible therapeutic strategy for CDH-associated PH.

Session IV: Therapeutic Uses of exRNA and Extracellular Vesicles

45. Exosomal transfer of stroma-derived miR-21 confers paclitaxel resistance in ovarian cancer cells through targeting APAF1

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Background and Aims: Ovarian cancer is one of the most lethal gynecologic malignancies. Most ovarian cancers are diagnosed at an advanced stage when the tumor is widely metastatic. The 5-year survival drops to 50% for the cancer cases that spread beyond the pelvis to the omentum, which is a well-vascularized fold of peritoneal tissue and is a major site of adipose tissue accumulation. However, the omental stromal cell-derived molecules that modulate ovarian cancer growth have not been characterized. Recent studies showed that microRNAs (miRNAs) that transfer between living cells that are involved in cell-cell communication are frequently encapsulated in exosomes, which facilitate their targeted exchange, but exosomal miRNA signatures from cancer-associated stromal cells have not be investigated and the functional roles of these exosomal miRNAs in modulating the malignant phenotypes of recipient cancer cells have not been elucidated. Thus, we hypothesize that the transfer of miRNAs and their variants from ovarian cancer-associated adipocytes (CAAs) and fibroblasts (CAFs) to ovarian cancer cells via exosomes may contribute to the malignant phenotypes of ovarian cancer cells.

Methodology and Results: Ion torrent next generation sequencing was performed to identify differential miRNA signatures in exosomes isolated from CAAs, CAFs and ovarian cancer cells. Significantly higher levels of microRNA-21 (miR-21) isomiRs in exosomes and tissue lysates isolated from CAAs and CAFs than in those from ovarian cancer cells were observed. Using a cancer cell/CAA- or cancer cell/CAF-co-culture models with fluorescent FAM-tagged miR-21, it was confirmed that miR-21 is directly transferred from CAAs or CAFs to the cancer cells via exosomes both in vitro and in vivo. Functional studies reveled that exosomal transfer of miR-21 suppressed apoptosis and conferred paclitaxel resistance in the recipient ovarian cancer cells. To delineate the underlying mechanism by which miR-21 exerted its effects on ovarian cancer cells, transcriptome profiling on ovarian cancer cells transfected with pre-miR-21 and pre-miR-negative control was performed. APAF1 was identified as a novel direct target of miR-21. APAF1 was also found to mediate the oncogenic effects of miR-21 on ovarian cancer cells both in vitro and in vivo.

Conclusions: The data suggested that the malignant phenotypes of metastatic ovarian cancer cells can be altered by miR-21 delivered by exosomes derived from neighboring stromal cells in the omental tumor microenvironment, and that inhibiting the transfer of stromal-derived miR-21 is an alternative modality in the treatment of metastatic and recurrent ovarian cancer.

46. Clinical Scale Production and Wound Healing Activity of Human Adipose Derived Mesenchymal Stem Cell Extracellular Vesicles from a Hollow Fiber Bioreactor

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Introduction: Production of extracellular vesicles (EV) such as exosomes at the scale required for clinical applications remains a challenge. Hollow fiber bioreactors are perhaps ideal for producing large quantities of EV at 100X higher concentrations than

conventional protocols. Hollow fiber bioreactors support the culture of large numbers of cells at high densities, 1-2X10e8 cells/ml. Cells are bound to a porous support with a 20kDa molecular weight cut off (MWCO) so cell passaging is not required and EV cannot cross the fiber in either direction.

Methods: 1X109 adipose derived adult MSC were cultured in a FiberCell hollow fiber bioreactor for 8 weeks in DMEM+10% FBS in the circulating medium only. Cells did not expand (monitored by glucose uptake rate) nor did they differentiate (by multiple immunocytochemistry assays) over this time. 20 ml of conditioned medium from the extra-capillary space was harvested weekly.

Results: 8.6X1011 EV particles in a volume of 120mls were harvested from the adult adipose derived MSC culture. Wound healing assays demonstrated significant acceleration of wound healing.

Summary: Hollow fiber bioreactors have demonstrated potential for the manufacturing scale production of EV using cGMP compliant materials and methods. The EV isolated from adult adipose derived MSC cultured in an HFBR show activity that can promote wound healing both in in vitro and in vivo assays. Hollow fiber bioreactors provide a number of significant advantages compared to flask based protocols including higher concentrations, large capacity, time and space efficiency, and perhaps EV quality. Current available technology permits the production of gram quantities of EV, with potential use for clinical applications.

47. Nucleic Acid Delivery to Hematopoietic Stem Cells using Megakaryocytic Microparticles

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Background: Microparticles (MPs) are 0.1 to 1 micron extracellular vesicles (EVs), budding off cellular plasma membranes. MPs play an important role in cell-to-cell communication by transferring cargo (proteins, lipids, or RNAs) from parent to target cells. Megakaryocyte-derived MPs (MkMPs) are the most abundant MPs in circulation (Flaumenhaft et al., 2009). We have shown that, in vitro, MkMPs specifically target and are taken up by human hematopoietic stem & progenitor cells (HSPCs) via fusion or endocytosis following specific receptor recognition (Jiang et al., 2017). MkMPs transfer cargo to HSPCs and induce potent Mk differentiation of HSPCs in the absence of thrombopoietin (Jiang et al., 2014).

Aims: 1) Develop MkMP-based strategies for gene therapies by exploring the capability of human MkMPs to transfer exogenous DNA and siRNA to HSPCs in vitro and in vivo; 2) Investigate the role of exRNA from MkMPs in the induction of Mk differentiation from HSPCs.

Methodology: Human MkMPs were isolated from CD34+-derived Mk cells (Jiang et al., 2014). Electroporation was used for cargo loading to MkMPs. Cy5-labeled plasmid DNA (pGFPns; expressing eGFP) or siRNA (siR-MYB) targeting c-myb were separately loaded into MPs. Silencing of c-myb enhances Mk differentiation of HSPCs (Bianchi et al., 2010). Loading efficiencies were calculated after pDNA purification and quantification, or analyzed by flow cytometry (FC) based on Cy5 fluorescence. Co-culture of cargo-loaded MkMPs with HSPCs was employed to assess the delivery of pGFPns based on pDNA quantification, eGFP RNA levels by qRT-PCR, and eGFP expression by FC. siR-MYB delivery were confirmed with FC analysis on CD41 (an Mk marker) expression at days 3, 5 and 8 of co-culture. Total RNA from Mks, MkMPs or platelet MPs (PMPs; a control MP) were extracted for RNA-seq analysis. The RNA content of these particle/cell populations was compared by differential expression analysis.

Results: We developed a protocol to load ca. 1700 copies of pGFPns into MPs, with over 77% of Cy5+ MPs after electroporation. Delivery of and eGFP expression from pGFPns from MP-HSPC co-culture was confirmed by isolating pGFPns from and measuring eGFP mRNA in HSPCs at days 1 and 3, respectively. At day 3, over 45% of HSPCs were Cy5+ while 20% expressed eGFP, indicating effective and functional MP-mediated pGFPns delivery to HSPCs. We assessed functional RNA delivery by examining the impact of siR-MYB mediated c-myb silencing in enhancing Mk differentiation of CD34+ cells. MkMP delivery of siR-MYB enabled c-myb silencing that resulted in enhanced Mk differentiation: the % of CD41+ and CD34-CD41+ cells increased by 29 % and 25 %, respectively, at day 8 of co-culture versus unloaded MkMPs. Differential expression analysis identified 46 miRNA as highly expressed in MkMPs compared to PMPs, and 58 miRNA enriched from Mks to MkMPs.

Conclusions: Our data demonstrate that HSPCs can be specifically targeted and functional pDNA/siRNA delivered using MPs. This demonstrates the potential of this delivery system for targeting the hematopoietic stem-cell compartment. In separate studies, we have shown that MkMPs target murine HSPCs in vivo, thus demonstrating that MkMPs constitute a potentially useful therapeutic delivery system for gene therapy.

48. Role of miRNAs isolated from synovial fluid extracellular vesicles in pathophysiology of female osteoarthritis

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Osteoarthritis (OA) is a degenerative joint disease affecting the aging population, and the disease is especially prevalent in women. We have previously described a number of unique miRNAs present in extracellular vesicles (EVs) from synovial fluid that characterize the arthritic joint. Furthermore, we also reported novel female-specific, EV-derived miRNAs in the OA population. In this study, we selected several miRNAs that are differentially regulated in female synovial fluid derived-EVs to validate their role in chondrocyte pathophysiology. Female human cartilage and synovial membrane were obtained from the knee joints of tissue donors undergoing total knee arthroplasty procedures. The tissues were transported to the laboratory and then chondrocytes and fibroblast-like synoviocytes were isolated by sequential enzyme digestion. The cells were plated and treated with miRNA mimic of miR-181d-3p, miR-185-5p, miR-7105-5p, miR-16, miR-504, and miR-210 and subsequently analyzed for cell survival assay. We found significant decreases in cell survival with all miRNA mimics except 185-5p when compared to control. We selected miR-181d-3p to further investigate its role in chondrocyte pathophysiology because we previously reported that miR-181d-3p is significantly up-regulated in female synovial fluid EVs. The miR-181d-3p mimic transfection in healthy female chondrocytes showed significant up-regulation of inflammatory genes (IL-6 and TNF) and down-regulation of the anabolic gene aggrecan. Catabolic genes MMP-1 and MMP-13 showed a trend of up-regulation. Furthermore, induction of oxidative stress on fibroblast-like synoviocytes using hydrogen peroxide showed an elevated level of EV-derived miRNA-181d-3p. We hypothesize that the EV miRNA cargo secreted from synovial membranes regulates gene expression of articular chondrocytes in a paracrine fashion. We previously reported that miR-181d-3p regulates estrogen signaling pathway genes such as estrogen receptor-α, estrogen receptor-β and nCOR (Nuclear Receptor Co-repressor) in chondrocytes. Our study suggests that miRNA-181d-3p carried via synovial-derived EVs plays a vital role in articular cartilage degeneration by regulating estrogen, catabolic and inflammatory genes and down-regulating extracellular matrix genes. Further studies are needed to validate our findings in vivo.

49. Exogenous cargo loading can impair endogenous extracellular vesicle bioactivity by displacing RNA

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Background: Extracellular vesicles (EVs) are promising candidates for therapeutic RNA delivery due to their physiological role in cell-cell communication. However, studies have shown that on average, EVs may contain less than one molecule of RNA per vesicle, supporting the need for exogenous loading of genetic cargo to enhance EV bioactivity. While electroporation was initially used for cargo loading, other approaches such as sonoporation have emerged that do not induce aggregation of nucleic acids and EVs. However, in general, it is unclear how exogenous cargo loading impacts the endogenous bioactivity of EVs.

Aims: We hypothesized that sonication will result in a loss of endogenous cargo due to diffusion of RNA after porating the membrane, ultimately leading to reduced bioactivity. We aim to (1) determine the effects of sonication on endogenous EV bioactivity, (2) evaluate the causes of any changes in bioactivity, and (3) ultimately conclude if it is more advantageous to use endogenously bioactive EVs over those with less bioactivity that are more easily produced.
Methodology: Bone marrow derived mesenchymal stem cell (BDMSC) derived EVs were used due to their demonstrated vascularization bioactivity. Endogenous RNA levels were evaluated using a fluorescent membrane-permeable dye, SYTO RNASelect (ThermoFisher) both before and after EVs were subjected to sonication. Exogenous RNA levels were evaluated using fluorescent Cy5-labeled miR-146, a vascularizing miRNA. Human dermal microvascular endothelial cells (HDMECs) were used to asses the vascularization potential of EVs in a gap closure assay.

Results: Sonication of BDMSC EVs led to a decrease in vascularization potential as demonstrated by a gap closure assay. While the unmodified EVs induced 65% closure of the denuded area, sonicated BDMSC EVs only induced 45% closure. This decrease in vascularization bioactivity was mirrored by a decrease in total RNA content of the EV. After sonication, there was a 59% decrease in endogenous RNA cargo. Studies are underway to evaluate the bioactivity of these loaded BDMSC EVs compared to minimally bioactive EVs (HEK293T).

Conclusions: While exogenous loading of RNA can enhance bioactivity of EV therapeutics, it is important to consider the effects of the loading method on the endogenous cargo and bioactivity. Overall, this study demonstrates that sonication-based loading leads to a loss of endogenous miRNA cargo that can in turn decrease EV bioactivity. This study demonstrates the need to thoroughly evaluate the ability of loading methods to maximize the preservation of endogenous EV activity. If the addition of exogenous RNAs is negated by the loss of endogenous cargo, then EV production rate may emerge as the key criteria for selection of source cells for production of EVs to be exogenously loaded.

50. The microRNA regulatory landscape of MSC-derived exosomes: a systems view

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Background: Mesenchymal stem cell (MSC)-derived exosomes mediate tissue regeneration in a variety of diseases including ischemic heart injury, liver fibrosis, and cerebrovascular disease. Despite an increasing number of studies reporting the therapeutic effects of MSC exosomes, the underlying molecular mechanisms and their miRNA complement are poorly characterized.

Aim: Here we microRNA (miRNA)-profiled MSC exosomes and conducted a network analysis to identify the dominant biological processes and pathways modulated by exosomal miRNAs.
Methodology: miRNA-profiling was performed using the Nanostring platform. miRDIP and PANTHER were used to determine significant pathways and biological processes targeted by the most abundant miRNAs.

Results: At a system level, miRNA-targeted genes were enriched for (cardio)vascular and angiogenesis processes in line with observed cardiovascular regenerative effects. Targeted pathways were related to proliferation, apoptosis, Wnt signaling, and pro-fibrotic signaling via TGF-β and PDGF. When tested, MSC exosomes reduced collagen production by cardiac fibroblasts, protected cardiomyocytes from apoptosis, and increased angiogenesis in HUVECs. The intrinsic beneficial effects were further improved by virus-free enrichment of MSC exosomes with network-informed regenerative miRNAs capable of promoting angiogenesis and cardiomyocyte proliferation.

Conclusion: The data presented here help define the miRNA landscape of MSC exosomes, establish their biological functions through network analyses at a system level, and provide a platform for modulating the overall phenotypic effects of exosomes.

51. Generation of Therapeutic Exosomes from Dendritic Cells Derived from Human Induced Pluripotent Stem Cells.

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BACKGROUND: Our project is to develop exosomes from IFNγ-stimulated dendritic cells (SDC-Exos) as a novel therapeutic to promote remyelination and reduce oxidative stress caused by neurodegenerative diseases. We have studied the effects of rat SDC-Exos, and are now producing analogous exosomes from human bone marrow. Our target diseases include multiple sclerosis, migraine, Alzheimer's disease and traumatic brain injury; with Dr. Aya Pusic independently leading development of the latter.

AIMS: Our plan, as previously stated, is to generate human-derived SDC-Exos (hSDC-Exos) for clinical trials. To achieve this, we must: 1) produce hSDC-Exos that recapitulate the effects of rat SDC-Exos; 2) produce sufficient quantities of hSDC-Exos for treatment; 3) show that hSDC-Exos are non-toxic / do not induce negative immune responses; 4) produce hSDC-Exos under GMP standards. Accordingly, we are developing human induced pluripotent stem cells (hiPSCs) from adult fibroblasts as a noninvasive, robust, and highly scalable means to produce therapeutic hiSDC-Exos. Here we will report our progress toward this goal.

METHODS: Donor cells obtained from adult patients provide an autologous source of exosomes which may mitigate potential negative immune responses sometimes seen with allogenic/xenogenic sources. These hiPSCs can be produced from adult fibroblasts obtained via skin biopsies, differentiated into immature DCs, and used to produce hiSDC-Exos. We are using episomal vector-mediated transfection, a non-integrative and non-viral reprogramming method. Since resulting exosomes will not contain trans-gene products, use of this method will likely maintain FDA classification of hiSDC-Exos as a "biological medicinal". Importantly, hiPSCs have unlimited growth capacity and may be used to produce exosomes from specific cell types.

RESULTS: We have nearly completed studies using human bone marrow to derive SDC-Exos, and have found that they recapitulate our prior results using rat SDC-Exos and contain miR-219. Work to develop hiSDC-Exos is progressing: We obtained and verified the

plasmids necessary to reprogram fibroblasts into hiPSCs, and have established efficient transfection protocols for use with adult fibroblasts. Next, we verified pluripotency of human stem cell colonies and their formation into embryoid bodies. Finally, we verified their differentiation into immature DCs. We are now stimulating these cells with IFNγ to test their ability to produce miR-219 containing exosomes. We expect to report miRNA levels and screening at the meeting.

CONCLUSIONS: We estimate that using hiPSCs (vs. bone marrow) will be a noninvasive method to increase the scale of hSDC-Exo production by > 10,000 fold. Results to-date begin to support this conclusion. Once we have established reproducible fabrication protocols, we can estimate hiSDC-Exo amounts that can be produced per run. Since the fabrication procedures will be amenable to bioreactor methods, we anticipate that the high production level necessary for nasal delivery of hiSDC-Exos in clinical trials can be achieved using this method. Conceivably, modifications of the above approach may help provide a common platform for fabrication of large quantities of exosomes derived from any cell type of interest.

52. MiR-34a Targets Cytochrome c and Shapes Stroke Outcomes

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Background: The blood-brain barrier (BBB) is a crucial interface between the central nervous system (CNS) and the circulatory system that maintains cerebral homeostasis by selectively allowing entry of blood solutes into the CNS. BBB dysfunction has been observed in cerebrovascular diseases and neurodegenerative disorders such as stroke. The BBB is opened during stroke, having negative impacts on stroke outcomes. Mitochondria are key players in this abnormal opening of the BBB, and decreased cytochrome c (CYC) levels have been shown in the mitochondrial fraction in stroke. We have recently demonstrated miR-34a regulates the BBB by targeting CYC in vitro.

Aim: To investigate the role of miR-34a in stroke and verify its target CYC in vivo.
Methods and Results: Using a murine transient middle cerebral artery occlusion (tMCAO) model, we demonstrate elevated miR-34a expression in serum and primary cerebral endothelial cells (CECs) from stroke mice. We report that deficiency of miR-34a significantly reduces BBB permeability and improves stroke outcomes. CYC is decreased in the ischemic hemisphere from wild-type (WT) but not miR-34a-/- mice following stroke reperfusion. Uncoupling of electron flow by a pharmacological inhibitor Carbonyl-cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) compromises mitochondrial oxidative phosphorylation (MOP) in cultured CECs and worsens infarction in stroke mice.

Conclusions: Our study provides the first description of miR-34a and electron flow affecting stroke outcomes, which could lead to a revision of current miR-34a targets and may lead to discovery of new mechanisms and treatments for cerebrovascular and neurodegenerative diseases such as stroke and Alzheimer's disease.

53. Pharmacokinetics and tumor distribution of cholesterol-anti-miR-221 loaded EVs in an orthotopic mouse model of hepatocellular carcinoma.

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The potential use of extracellular vesicles (EVs) as drug delivery systems to deliver both small drug molecules as well as nucleic acids and proteins has been increasingly studied in the past few years. The pharmacokinetic properties and biodistribution of EVs and their therapeutic cargo have been evaluated primarily by pseudo-quantitative imaging techniques and have provided a qualitative understanding of the biodistribution of EVs. In this study we have quantitatively evaluated the pharmacokinetics of therapeutic EVs loaded with human cholesterol-labeled antisense miR-221 (chol-ASO-221 EVs) in an orthotopic mouse model of hepatocellular carcinoma (HCC). Surgeries were performed on athymic nude mice to inject luciferase expressing hepatocellular carcinoma SK-Hep1 cells into one lobe of the liver to generate orthotopic tumor xenografts. After tumor growth was confirmed with luminescence imaging, tumor bearing mice received a single intravenous bolus dose of chol-ASO-221 EVs by tail vein injection (52 nmol/kg of chol-ASO-221). Serial blood draws were obtained from each mouse prior to and after dosing at various time points from the submandibular vein. Mice were euthanized predose and at 2, 4, 8, 12, 24 and 48 hours after dosing, and blood and tissue samples were collected.

Absolute quantification of ASO-221 in plasma, liver and tumor samples was achieved by RT-qPCR, and pharmacokinetics parameters in each of these compartments were estimated using non-compartmental and compartmental methods. Plasma levels of ASO-221 followed an IV bolus two compartment model with a half-life of 1.2 hours. Average peak ASO-221 concentrations (485 nM) in the liver was achieved at 12 hours, and therapeutic ASO-221 levels (> 100 nM) were still detected 48 hours post dose. Average peak tumor concentrations were 30 nM within this single dose study, though model simulations indicate the 100 nM target concentration will be achieved with multiple dosing three times weekly. This study provides novel quantitative plasma, liver and tumor pharmacokinetic data for the ASO-221 cargo packaged in therapeutic EVs, which will aid in guiding future efficacy and disposition studies of therapeutic EVs in preclinical mouse models. Current work is ongoing to assess biodistribution of ASO-221 in other mouse tissues as well.

54. Pilot scale and GMP compliant production and purification process of wild-type and engineered extracellular vesicles

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Extracellular vesicles (EVs) are promising therapeutic delivery systems. Production and scale up of EVs is currently limited by a lack of validated, reproducible and good manufacturing practice (GMP) compliant manufacturing processes. Culturing EV producing cells on plastic and EV purification using ultracentrifugation are inefficient processes that are not scalable. To alleviate these short-comings, we propose a tangential flow filtration (TFF) method (100 KDa cut-off cassette membrane) to purify the EVs and a stirred tank bioreactor for culturing. Wild type EVs produced by HEK293T cells were cultured in suspension on Corning enhanced attachment, Cytodex 1 and Cytodex 3 microcarriers and were purified by ultracentrifugation or TFF. Cytodex 3 showed the best cell adhesion, while the Corning one resulted in high total protein content in the sample. We have successfully scaled-up suspension cultures on Cytodex 3 microcarriers to 0.5 l. TFF resulted in EVs with a reduced particle size compared to ultracentrifugation. Purification by TFF produced comparable yields of EVs as ultracentrifugation using significantly reduced time. Future studies include continued development and scale-up of TFF purified and bioreactor culture of both wild-type and genetically engineered EVs.

55. Treating Spinal Cord Injury with Mesenchymal Stem Cell Extracellular Vesicles Improves Locomotor Recovery, Mechanical Sensitivity and Neuroinflammation

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Acute spinal cord injury continues to be a devastating problem worldwide with high morbidity and mortality. Long term morbidities include sensory, motor, and autonomic dysfunction. There is currently no consensus on pharmacological intervention to alter the clinical course of acute spinal cord injury and improve neurological outcome. Bone marrow-derived mesenchymal stem cells (MSC) have been shown to modulate the injury sequelae of SCI via paracrine effects, although the mechanisms are not completely clear. One potential modality is through secretion of extracellular vesicles (EV). EV are heterogeneous particles with lipid bilayer, containing growth factors, lipids, microRNAs, mRNAs, and proteins. We are testing the novel application of MSC-derived extracellular vesicles (MSCEv) to reduce neuroinflammation and improve sensory and motor recovery following SCI.

Animals will be randomly assigned to three groups: sham laminectomy, or T10 contusion + vehicle or T10 contusion + MSCEv. 1,2,3,5,7,10 and 14 days following injury, locomotor recovery will be scored using the Basso, Beattie and Bresnahan (BBB) method. At 14 days post-injury, animals will be tested for mechanical allodynia via the Dixon Up-Down Von Frey method then sacrificed. Spleen will be harvested for neurotransmitter assays, and blood and spinal cord will be harvested for flow cytometry and immunohistochemistry.

Preliminary data indicate significantly higher locomotor recovery scores in SCI + MSCEv animals when compared to SCI + vehicle animals on days 5, 7 and 14 post-injury ($p<0.0001$, $p<0.001$ and $p<0.001$, respectively). Animals treated with MSCEv also demonstrate significantly higher force thresholds in the mechanical sensitivity test compared to vehicle treated animals at 14 days post-injury ($p<0.05$). Flow cytometry analysis of spinal cord reveals increased activation of M1 and M2 microglia in MSCEv treated animals compared to vehicle. Spleen analysis indicates increased myeloid cells and MDSC with decreased NK cells and leukocytes in MSCEv treated animals. These results support our hypothesis that MSCEv are an effective therapeutic after SCI.

56. Arthropod exosomes as novel transmission-blocking vaccine strategies

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The transmission strategies used by flaviviruses to exit arthropods and infect human host were envisioned as best approaches to develop transmission-blocking vaccine(s). Research in our laboratory has shown that both tick and mosquito-borne flaviviruses uses exosomes for transmission from arthropods to human and other vertebrate cells. Our studies have revealed that arthropod-derived exosomes are important means of communication and transmission between the vector and the vertebrate host. We have found that Langat virus (LGTV), a flavivirus member closely related to tick-borne encephalitis virus (TBEV) and mosquito-borne dengue and ZIKA viruses are also transmitted from vector to the vertebrate host through exosomes. The exosomes containing LGTV and dengue/ZIKA viruses RNA and proteins were viable, secured and highly virulent in all tested conditions such as re-infection kinetics, trans-migration assays and viral-plaque-formation assays suggesting exosomes as favorable modes of transmission. In addition, we have identified and characterized novel arthropod exosomal markers that are enriched in exosomes. Using these arthropod exosomal molecules or determinants that facilitates pathogen transmission, we intend to design novel transmission-blocking therapeutics. In summary, arthropod exosomes serve as secured vesicles for transmission of tick/mosquito-borne flaviviruses and blocking their transmission is a novel strategy in the near future.

Session V: Cutting-Edge Technologies

57. Defining the cellular fates of extracellular vesicles using advanced light and super-resolution microscopy

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We previously showed that leukemia derived extracellular vesicles (L-EV) impact the clonogenic behavior of hematopoietic stem and progenitor cells (HSPC) through trafficking of selectively enriched microRNA (Leukemia 2015; Science Signaling 2016). Separately, we reported that exposure to stromal derived EVs prompted HSPC activation and myeloid expansion via TLR4 engagement (J.Bio.Chem, 2016). Here, we set out to resolve the cellular events by which HSPC differentially regulate EV entry and dissect the intracellular fates of internalized vesicles and their cargo. We relied on a combination of both high resolution live cell fluorescence microscopy (LCFM) as well as super-resolution imaging (SRM) strategies in concert with myristoylated fluorophores as an endogenous EV labeling system that can be transitioned to in vivo use. Membrane-derived vesicles released from these cells are brightly labeled and can be resolved using LCFM or SRM without additional staining or manipulation. In initial in vitro studies we investigated the entry of L-EVs into FACS sorted immunophenotypically defined murine HSPCs, as well as mesenchymal stem cells (MSC), and osteoprogenitor cells (OPCs). LCFM studies showed that HSPCs bind to and internalize L-EVs within minutes following exposure and remain intact for hours. Rates of EV internalization were found to be both parent and recipient cell type dependent, and range widely from a single EV to hundreds within 2.5 hours of exposure. For validation, we analyzed FACS sorted HSPCs, MSCs and OPCs from mice xenografted with AML cell lines to determine uptake and position of EVs under in vivo conditions. HSPCs (KSL) sorted at sacrifice had intracellular and/or membrane associated L-EVs in 54%, with an average of 5.7 EVs per cell. Not surprisingly the total L-EVs were fewer than seen in vitro, likely reflecting slower in vivo kinetics. Further studies to understand of intracellular half-life are required to characterize the differences between in vitro and in vivo exposures. During the course of these LCFM studies we further revealed that cells traffic EVs directly into the cytoplasm of bystander HSCPs and MSCs through filopodia-like projections when grown in coculture. In addition to tracking the uptake and fate of EVs at the cellular scale, we have also developed a solid capture approach to stain and image individual EVs. By embedding labeled EVs into hydrogel, we visualized single vesicles at the nanoscale using fluorescence microscopy, and can determine vesicle concentration and relative size distribution across a broad working range. Finally, for increased resolution, we have generated cell lines expressing photoactivable fluorophores that are compatible with SRM. Combining this technique with myristoylation tagging, we have begun to probe EV surface markers and contents in order to learn more about how EVs target recipient cells and package and unload cargo. Using SRM as a screening tool, it may be possible to better categorize EVs and identify markers that will allow for improved isolation, purification and biomarker detection. In aggregate, live-cell, solid capture and super-resolution microscopy techniques will be indispensable tools to resolve the complex biological processes of EV trafficking. Due to their constitutive role in regulating specific cells and tissues, EVs are important intercellular mediators, that, if better understood, could be exploited to advance the detection and treatment of disease.

58. Exogenous nucleic acids in the serum of community-dwelling individuals

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The discovery of circulating cell-free, extracellular DNA (exDNA) and RNA (exRNA) in human body fluids, including serum, has sparked great interest in using these nucleic acids as markers of disease, as diagnostic tools, or as therapeutic molecules. Here, we examined the exDNA/exRNA profiles in community-dwelling individuals. Surprisingly, we found a considerable percentage of sequenced extracellular exDNA/exRNA remains unaligned to the human genome/transcriptome compared to DNA/RNA extracted from cells. In this project, we sought to identify systematically the unaligned reads after filtering out the sequencing adapters and primers. Total RNA was extracted from the serum of >10 young individuals and >10 old individuals and total RNA-seq on the Ion Torrent Proton sequencer was performed using a modified protocol to obtain both small- and large-sized RNAs. The reads were first aligned to the human genome (hg19) using a two-step process with Tophat2 and Bowie2, and unaligned reads were aligned to all known organisms in the Refseq v70 database (54,118 organisms) encompassing prokaryotes (archaea and bacteria) and eukaryotes (fungi, plants, invertebrate animals, and vertebrate animals). Kraken software was used to align against 618.8 billion DNA sequences and 24.2 billion RNA in a sequential manner throughout the different kingdoms. Unexpectedly, different individuals were found to have different percentages of fragments of exDNA/exRNA from numerous organisms. Some of the organisms identified are bacteria (including Clostridium sp. and Pseudomonas sp..), fungi (including Malassezia sp., Saccharomyces), plants (such as corn, tobacco, and poplar), insects (including mites, deer tick, and body lice), and vertebrates (such as turkey and cow). These findings suggest that the species we are identifying in serum may give important information about diet and behavior among the individuals studied. Many of the sequenced reads from the serum of different individuals align to specific regions of the genome of an organism (e.g. Pseudomonas aeruginosa), indicating that perhaps these fragments (some kilobases in size) are particularly stable, which allows them to withstand degradation in body fluids. The identification of these organisms through computational methods awaits experimental validation.

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59. A multiplexed assay for low abundance microRNA biomarkers

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Background: Mature microribonucleic acids (miRNAs) are 18-24 nucleotide long noncoding RNAs. They have important roles in regulation of gene expression in living cells such as by inhibiting translation of mRNAs. Although their detailed regulation mechanisms are not fully understood yet, they have been linked to many diseases. Recent studies have shown that changes in miRNA levels in bodily fluids are in close relation with various diseases. Specifically, miR-451 plays a role in tumorigenesis, self-renewal and chemoresistance in many types of cancer such as breast, gastric and colorectal, and has been implicated as a diagnostic and prognostic biomarker.Expression of miR-223 is deregulated during influenza or hepatitis B infection, inflammatory bowel disease, type 2 diabetes, leukemia and lymphoma. Detection of these small RNAs in biofluids is a growing trend for diagnostics; therefore, easy, accurate and cost effective diagnostic devices/methods are needed.

Aims: The absolute miRNA copy number per serum volume is very small, so techniques used to detect miRNAs must be very sensitive and able to work with small sample volumes to be clinically effective. A device capable of quantitative detection of miRNAs could have significant utility as a bedside platform for diagnosis and prognosis of many diseases.

Methodology: Multiplexed microarrays serve as a key method for the rapid sensing of various biomarkers for diagnostics. Applications of multiplexed biomarker detection require the ultrasensitive detection of nucleic acids at extremely low (femtomolar) concentrations from total sample volumes in the microliter range. Single-particle interferometric reflectance imaging sensor (SP-IRIS) is an optical technique that rapidly counts the absolute number of nanoparticles captured onto a DNA or protein microarray. Here, SP-IRIS has been used for high-throughput and direct detection of miRNA targets on a DNA microarray via DNA-conjugated gold nanoparticle labels in a sandwich assay scheme. This assay has also been integrated with microfluidics to enable dynamic detection of target

biomolecules at attomolar concentrations. Briefly, a DNA microarray of miRNAs-complement ssDNA was printed onto the SP-IRIS chip. Then, the sample solution containing miRNAs was incubated with the chip to allow hybridization. Finally, the chip was incubated with a labeling solution of gold nanorods, and placed in an SP-IRIS reader which automatically counted individual nanorods captured on the surface.

Results: We evaluated the potential application of SP-IRIS in quantitative detection of miR-451 and miR-223. The number of immobilized particles within the target-complement microarray spots is proportional to the concentration of the target solution. To measure the sensitivity and specificity of the assay, we measured dilution curve of two synthetic miRNA sequences from 1pM – 100aM. Preliminary results indicate a limit of detection in the aM range. Moreover, we aim to improve the LOD and sensitivity with real-time measurements of target hybridization through particle tracking modality of SP-IRIS platform through dynamic measurements of individual nanoparticle binding and de-binding.

Conclusions: We demonstrate the clinically effective application of SP-IRIS in miRNA detection.We have developed a sensitive, multiplexed biomarker assay.Based on these preliminary results, this is a promising approach for a variety of miRNA-based diagnostic applications.

60. Noninvasive detection of extracellular RNAs via cationic lipoplex nanoparticle biochip for lung cancer early detection

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Extracellular RNAs carried by exosomes have emerged as promising diagnostic and prognostic cancer biomarkers because of their presence and remarkable stability in body fluids. Exosomal RNAs in blood samples are typically quantified using a workflow that involves isolation of RNA from the blood samples followed by analyte-specific quantitative reverse transcription (qRT)-PCR of RNA. However, this RNA isolation-qRT-PCR workflow cannot distinguish extracellular RNAs secreted by cancer cells from those released physiologically and ubiquitously by all other, non-tumor cells of the body, making it potentially less sensitive or incapable of detecting cancer-specific RNA biomarkers.

We have developed a novel and simple tethered cationic lipoplex nanoparticle (tCLN) biochip with pre-loaded molecular beacons (MBs) in the nanoparticles as probes to capture and detect cancer-specific RNAs in human serum without requiring pre- or post-sample processing. In the tCLN biochip, the fusion of tCLN and exosomes due to electrostatic interaction leads to the mixing of RNAs and MBs within the nanoscale confinement near the biochip interface, which provides the “focusing” effect, allows the discrimination of cancer cell-derived exosomes from their non-tumor counterparts, and thus achieves higher detection sensitivity and specificity than the RNA isolation-qRT-PCR workflow.

The tCLN biochip was first demonstrated using non-small cell lung cancer cells (A549) and normal human bronchial epithelial cells (HBEC). Exosomes secreted by A549 and HBEC cells were collected using ultracentrifugation and applied on the tCLN device to detect exosomal miR-21 and TTF-1 mRNA. A549 exosomes showed higher miR-21 and TTF-1 mRNA expression compared to that of HBEC exosomes, which was consistent with the results obtained by the RNA isolation-qRT-PCR workflow.

Next, we evaluated the tCLN biochip on a training cohort of patients and identified the biomarker signature for lung cancer early detection. The training cohort consists of healthy individuals (n=15), therapy-naive early stage (stage I/II, n=20) and late stage (stage III/IV, n=24) lung adenocarcinoma patients. Based on an informal meta-analysis of existing literature on extracellular RNAs whose levels are dysregulated in lung cancer, five microRNAs (miR-21, miR-25, miR-155, miR-210 and miR-485) and TTF-1 mRNA were selected as biomarker candidates for examination with the tCLN biochip and the RNA isolation-qRT-PCR workflow. We performed a best subset logistic regression analysis where the Bayesian information criteria was used to identify the biomarker signatures. For the tCLN biochip, miR-21 and miR-25 were the biomarker signatures to distinguish no cancer controls from early stage patients (AUC=1.000), and miR-21 and TTF-1 mRNA were the biomarker signatures to distinguish no cancer controls from late stage patients (AUC=0.972). The tCLN biochip has shown much higher detection sensitivity and specificity than the RNA isolation-qRT-PCR workflow (AUC=0.710 and 0.861 respectively).
We are validating the tCLN biochip in a large cohort of patients (n=120) using the discovered biomarker signatures for lung cancer early detection. We will demonstrate the superior sensing performance of the tCLN biochip over the RNA isolation-qRT-PCR workflow. We aim to develop the tCLN biochip into a complementary or companion test for cancer diagnosis.

61. Rapid magnetic isolation of extracellular vesicles via lipid-based nanoprobes

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Background: Extracellular vesicles (EVs) are lipid bilayer-enclosed entities that can mediate intercellular communication by transferring cargo proteins and nucleic acids. However, the existing isolation approaches, including ultracentrifugation, immunoisolation, polymer-based precipitation, and filtration, need to overcome lengthy procedure, vesicle damage, and other difficulties.

Aim: We developed a lipid nanoprobe (LNP) system for rapid isolation of nEVs from both serum-free cell culture supernatant and plasma.1

Methodology: Approximately 1.4 × 109 MDA-MB-231 cells derived nEVs were resuspended in 100 μl of serum free medium, which served as model nEV samples. Blood samples were centrifuged at 300 g for 5 min and then at 16,500 g for 20 min at 4 °C followed by collection of plasma. Lipid nanoprobe (LP), biotin-tagged 1,2-distearoyl-sn-glycero-3-phosphethanolamine-poly(ethylene glycol) (DSPE-PEG-biotin) powder (Avanti) was dissolved in pure anhydrous ethanol at a final concentration of 1 mM. A 100 μl volume of each nEV model sample or blood plasma was added into 1 ml Diluent C. LP (0.001, 0.01, 0.1, 1, 5 or 10 nmol) was added to the other 1 ml of Diluent C before being added to the nEVs. The samples were mixed gently at 4 °C for 5 min and incubated with ~1012 capture probe (CP, avidin-coated magnetic sub-micrometre particles) at room temperature for 30 min. The nEV cargo RNA was extracted using Trizol (Life Technologies). The RNA concentration in the nEVs was measured using a Qubit Fluorometer (Life Technologies).

Results: Each model sample contains on average 348.5 ng of total RNA. Isolation efficiency of nEVs using LP is boosted gradually with increasing amounts of LP. The maximal efficiency of 77.6% is achieved using 10 nmol LP. Extra amount of LP does not further increase isolation efficiency. Next, the effect of incubation time on isolation of nEVs was optimized with 10 nmol LP. Although a gently increase in average isolation efficiency with incubation time is witnessed, prolonged incubation has no statistically significant benefit ($p > 0.05$). For operability and reliability, we incubated LP with nEVs for 5 min. Furthermore, we found the incubation period for fully interaction between LP and CP can be shortened to 10 min with continuous gentle rotation. In brief, approximately 80% of nEVs from the model sample can be labeled and isolated using 10 nmol LP and excessive CP, and the whole isolation procedure takes 15 min. In processing of plasma, given albumin might interfere the insertion of LP into nEVs membrane, we increased its amount, up to 200 nmol, to label and isolate nEVs from 100 μl blood plasma of a healthy donor containing approximately 13.2 ng RNA. Isolation efficiency of 48.3% is achieved using 100 nmol of LP and excessive CP. Doubling LP amount only slightly increase the efficiency to 49.5%.

Conclusions: The LNP system can used for rapid isolation of nEVs. To note, the lipid nanoprobes can be further optimized for nEV isolation.

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