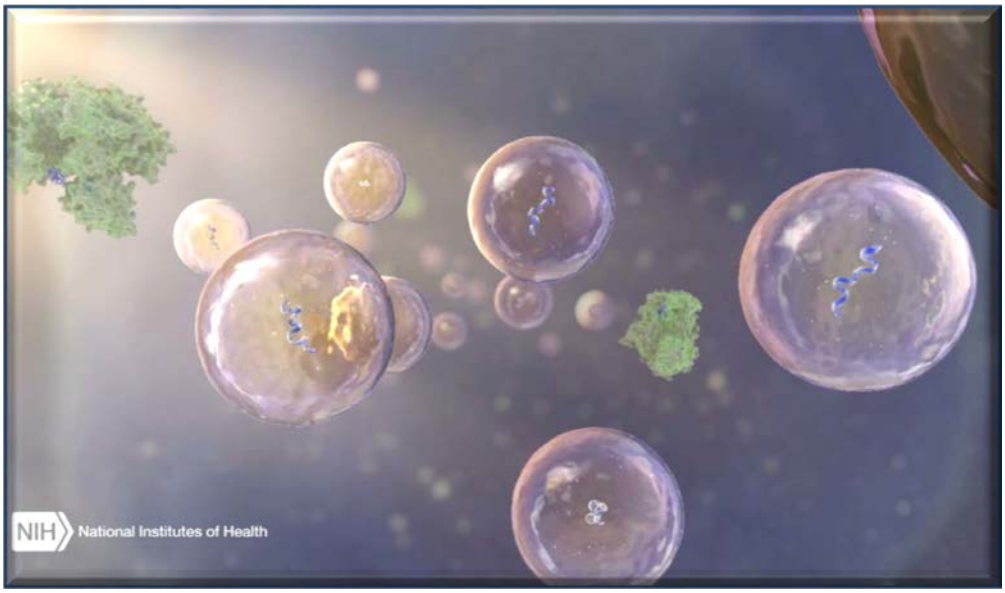
exRNA

COMMUNICATION PROGRAM

November 3rd and 4th, 2016

NIH Extracellular RNA Communication Consortium
7th Investigators' Meeting
November 3rd and 4th, 2016
Bethesda North Marriott Hotel, Rockville, MD



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MEETING AGENDA

Thursday November 3rd		
7:30 AM	Registration and Poster Set Up	
8:15 AM	Announcements and Housekeeping	Matt Roth/Tania
8:20 AM	Common Fund Opening Remarks	Patricia (Trish) Labosky
8:30 AM	Key note presentation: Bioengineering studies of exosomes using tissue chip models of human tumors	Gordana Vunjak-Novakovic
Session I: ERCC Studies of Extracellular RNA for Human Therapeutics. Chairs: Anastasia Khvorova and Xandra Breakefield (Salons A and B)		
9:00AM	Exosomes as Therapeutic RNA Delivery Vehicles	Anastasia Khvorova
9:20AM	Therapeutic Targeting of Prostate Cancer Cell-Derived Exosomes by High Throughput Screening of Potent Biogenesis and Secretion Inhibitors	Amrita Datta
9:40AM	HER2-targeted Extracellular Vesicle Delivery of Therapeutic mRNA for Enzyme Prodrug Therapy	AC Matin
10:00AM	Lack of toxicity and immunogenicity in mice following 3-week dosing of engineered exosomes derived from HEK293T cells	Mitch Phelps
10:20AM	Development of a Cross Consortium Standardized Platform for Creation of Specific Cell Types/Exosomes from Induced Human Pluripotent Stem Cells	Richard Kraig
10:40 AM	Break (15 Minutes)	
Session II - Genetic Models and Tools to Study exRNA. Chairs: Michael McManus and Saumya Das (Salons A and B)		
10:55AM	Utilizing Hollow Fiber Bioreactors as a Scalable Source for Extracellular Vesicle Production	Irene Yan
11:10AM	Target Mapping of Red Blood Cell Derived Extracellular Vesicles in a Transgenic Murine Model	Avash Das
11:25AM	Semantic Web Enabled Integration of Wikidata and Wikipathways for Exploring Impact of exRNAs in Health and Disease.	Sebastian Burgstaller-Muehlbacher
11:40AM	Panel Discussion, Maximum Time: 20min	
12:00PM	LUNCH ON YOUR OWN	
	Workshop on EV Isolation and Biochemical Characterization (Salon C). 12:10 – 12:50pm George Pavlakis, NCI: Scale-up Production, Purification and Enhanced Tumor Delivery of Engineered Extracellular Vesicles	12:50 – 1:30pm ERCC PIs short talks *Jeff Franklin:* Functional Assays for Evaluating Colorectal Cancer Secreted Vesicle Activity Saumya Das: Assays to Test Functionality of Extracellular Vesicles in Cardiovascular Diseases *Tushar Patel*: Functional Role of Stem Cell Extracellular Vesicles in Reparative Responses to Liver Injury *Peter Quesenberry*: Approaches to Isolation of Functionally Active Vesicles *Anastasia Khvorova*: Scale Up of Exosomes Manufacturing from Mesenchymal Stem Cells
Session III - Heterogeneity of exRNA Carriers and Implications in Function and Disease. Chairs: Anna Krichevsky and Alissa Weaver		
1:45PM	Sources and Composition of Extracellular Vesicles in Inflamed Tissue	Mark Ansel
2:05PM	Influence of Hyperglycemia on Macrophage-derived Exosomes in Diabetic Atherosclerosis Regression	Robert Raffai
2:25PM	Incorporation of Full Length mRNA Messages in Vesicles by Binding to RNPs	Erik Abels
2:35PM	Optimization of exRNA Isolation from Biofluids: Progress Report from the RNA Isolation WG	Leonora Balaj
2:55PM	Break (15 Minutes)	
3:10PM	Round Table Discussions in Salon C	
4:20PM	Poster and Networking Session (Foyer outside Salons A, B and C)	
Adjourn Day 1		

Friday, November 4th			
Session IV: Extracellular RNA and Function. Chairs: Andrei Goga and Bogdan Mateescu (Salons A and B)			
8:00AM	In Vivo Regulated Release and Function of Extracellular Small RNAs		Andrei Goga
8:20AM	exRNA Released by Glioblastoma Alters Brain Microenvironment		Xandra Breakefield
8:40AM	Secreted RNA During CRC Progression Biogenesis Function and Clinical Markers		James Patton
9:00AM	Genetic Models for exRNA Communication		Michael McManus
9:20AM	Definition of Serum Ribonucleoprotein Composition and Its Regulation and Function		Thomas Tuschl
Session V: Comprehensive Reference Profiles of Extracellular RNA in Human Biofluids and Bioinformatics Tools Update. Chairs: Kendall Jensen and Louise Laurent (Salons A and B)			
9:40AM	Small RNA Profiles in Normal Human Biofluids		Paula Godoy
10:00AM	Phylogenetic Analysis of Exogenous Sequences from Extracellular RNA-seq Data Using the exceRpt Pipeline.		Joel Rozowsky
10:20AM	Systematic, Multi-institution and Multi-protocol Assessment of Next Generation Sequencing for Quantitative Small exRNA Profiling		Maria Giraldez
10:40AM	Highlights from round table session : Total time 40min (Tables 1 to 4)		
11:20AM	Break (15 Minutes)		
Selected Working Group Parallel Meetings			
11:35AM	MADS/RNA Seq (Salon A)	RNA Isolation (Salon B)	Resource WGs – U19s, EVB (Salon C)
12:15PM	U01s (Salon A)	Therapy UH3s (Salon B)	Scientific Outreach WG (Salon C)
12:55PM	Lunch on your own (90min) -- NIH/ESA Closed Meeting (Room TBD)		
Session VI - Extracellular RNAs as Biomarkers and Liquid Biopsy in Human Disease. Chairs: Roopali Gandhi and Julie Saugstad (Salons A and B)			
2:30PM	MicroRNAs in Human Cerebrospinal Fluid as Biomarkers for Alzheimer's Disease		Theresa Lusardi
2:50PM	Isolation of exRNA from Clinical Biofluid Specimens and exRNAseq Analysis		Klaas Max
3:10PM	Validation of miRNA as Diagnostic and Prognostic Biomarkers for Multiple Sclerosis		Roopali Gandhi
3:30PM	Panel Discussion, Maximum Time: 20min		
3:50PM	Highlights from Round Table Session: Total Time 40min (Tables 5 to 8)		
4:30PM	Closing Remarks, Announcements. Steering Committee Chairs to Adjourn the Meeting		

Day One Roundtable Topics and Discussion Leaders

3:10pm in Salon C

Table 1: Exosome and exRNA biogenesis (Mark Ansel)

Table 2: Exosome cargo function & cargo sorting (A C Matin)

Table 3: Genetic models and tools to study exRNA function (Ionita Ghiran)

Table 4: Exosome characterization & production (Thomas Schmittgen)

Table 5: Non-human exRNAs and interspecies exosome-mediated communication (Joel Rozowsky)

Table 6: Non-exosome mediated transport of (and signaling by) cell-free nucleic acids (Julie Saugstad)

Table 7: Exosome lipidomic profiles in disease (Anastasia Khvorova)

Table 8: exRNA and RNA-binding proteins, implications in disease (Xandra Breakfield)

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.

Confidentiality Disclosure Agreement

Extracellular RNA Communication Program

I certify that, to the best of my knowledge, I have disclosed all conflicts of interest that I may have with the Extracellular RNA Communication Program Consortium. I fully understand the potential confidential nature of discussions and presentations, and agree to respect and maintain the privileged status of information. I further understand and acknowledge that materials provided and discussions held prior to and during meetings may reveal confidential information. As a presenter, I agree to employ all reasonable efforts to preserve and safeguard my own confidential information.

For speaker presentations, we are asking the first slide for every presentation to be a conflict of interest disclosure slide. This is to protect you and the rest of the Consortium members. A conflict of interest is any situation where a speaker may have interests that may cause a conflict with the ERC Consortium. Conflicts of interest do not preclude the delivery of the talk, but should be explicitly declared. These may include financial interests (e.g. owning stocks of a related company, having received honoraria, consultancy fees), organizational interests (e.g. private advisory board membership) and gifts.

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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 President of the International Society for Extracellular Vesicles (ISEV)

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.

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
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Project Team Coordinators:

T. Kevin Howcroft, Ph.D. Program
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Division of Cancer Biology, Cancer Immunology and Hematology Branch National Cancer Institute (NCI)

Danilo A. Tagle, Ph.D.
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National Center for Advancing Translational Sciences (NCATS)

Members:

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National Cancer Institute (NCI)

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Division of Blood Diseases and Resources
National Heart, Lung, and Blood Institute (NHLBI)

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Genes, Genomes and Genetics Center for
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National Institute on Drug Abuse (NIDA)

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National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

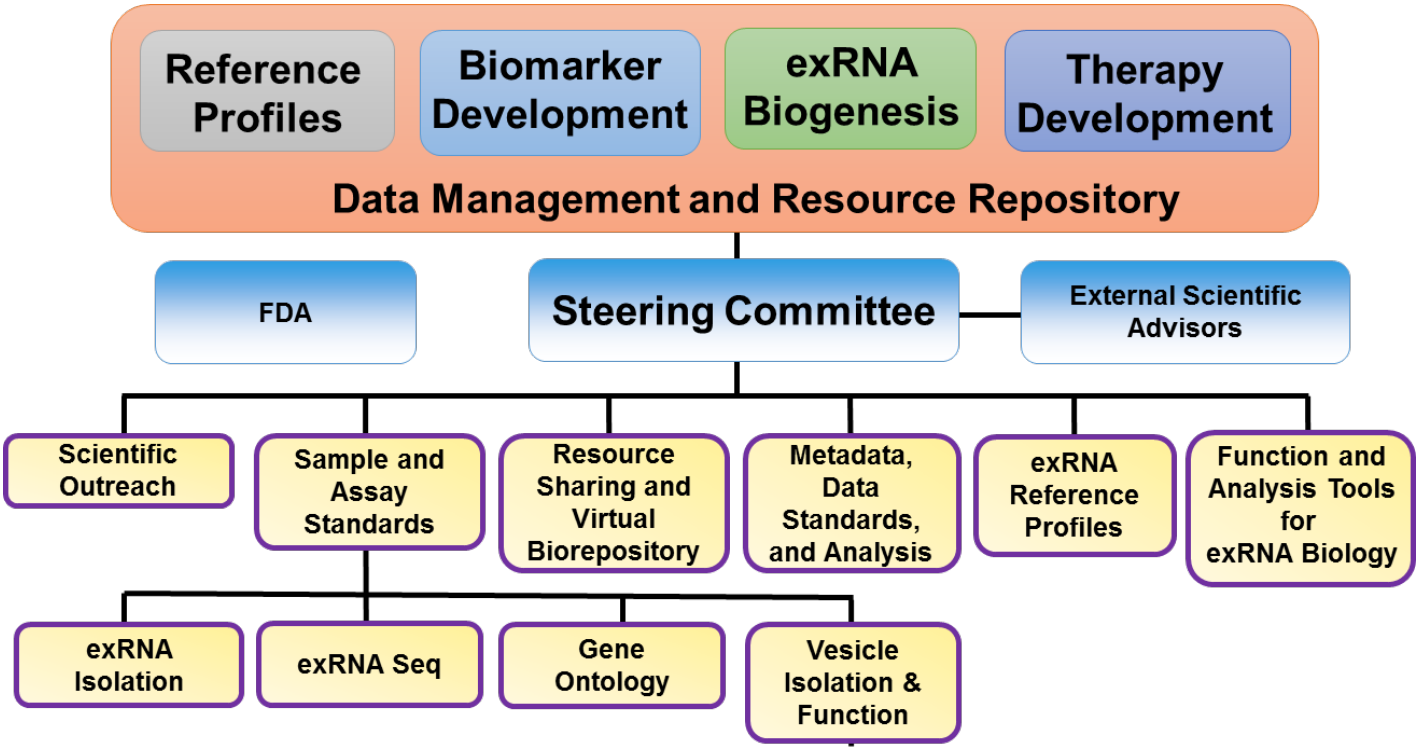
Margaret Sutherland, Ph.D.
Program Director
Neurodegeneration Cluster
National Institute of Neurological Disorders and Stroke (NINDS)

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

Sundar Venkatachalam, Ph.D. Program Director, Integrative
Biology and Infectious Diseases Branch
National Institute of Dental and Craniofacial Research (NIDCR)

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.



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)

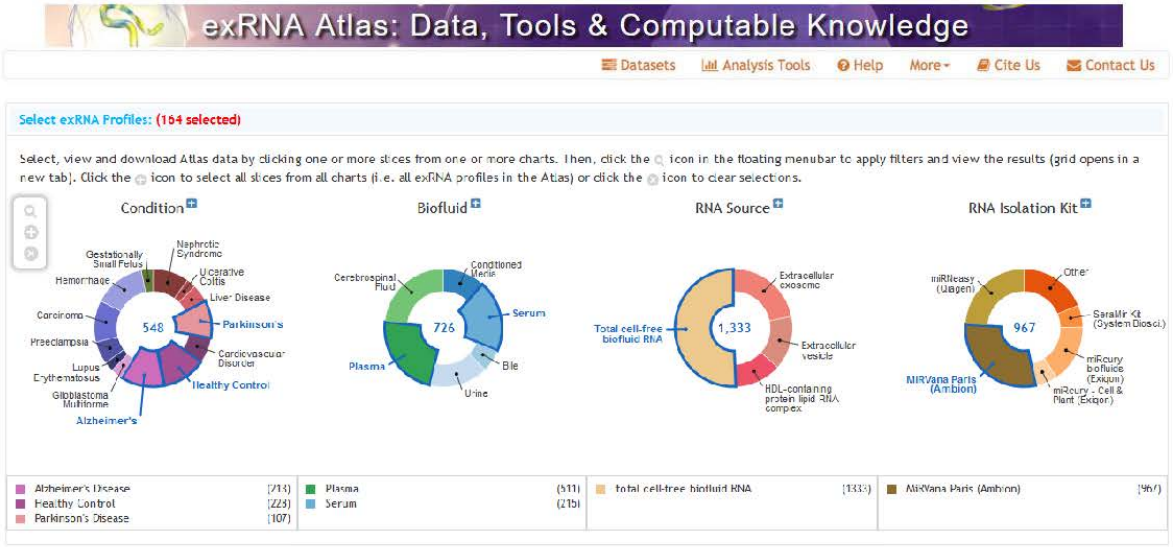
Resource Sharing Working Group - Fred Hochberg and Matt Huentelman

The goals of the Resource Sharing Working Group 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

exRNA Atlas Overview

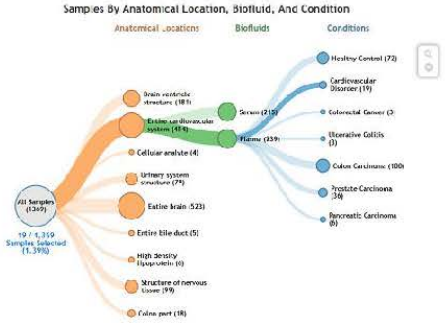


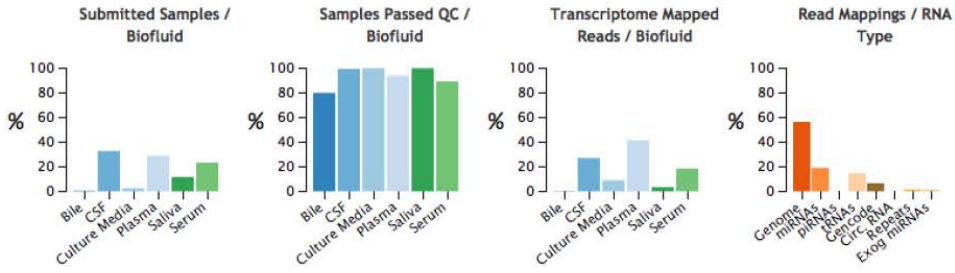
Overview

- The exRNA Atlas is a growing data resource for the scientific community, produced by the NIH Common Fund's Extracellular RNA Communication Consortium (ERCC).
- Currently, the atlas includes **1369 exRNA profiles** from different biofluids and health conditions, profiled using RNA-seq as well as RT-qPCR assays.
- RNA-seq profiles were derived from about **11.3 billion reads** and processed using the *exceRpt small RNA-seq pipeline*. ERCC-developed data quality metrics are uniformly applied to these datasets.

Features

- Faceted filtering and data navigation tools for sub-selecting samples of interest as well as data and metadata downloads are enabled by rich metadata standards developed by the consortium and metadata annotations contributed by the data producers.
- Data analysis tools for comparing datasets are also available.
- The atlas also displays relevant statistics and summaries of the deposited datasets.





Contact

- The Atlas is developed and maintained by the Data Management and Resource Repository (DMRR) of the ERCC. You can access the Atlas by visiting **exrna-atlas.org**.
- For questions, comments and suggestions or for depositing your exRNA data in the Atlas, please contact Sai Lakshmi Subramanian (sailakss@bcm.edu) or William Thistlethwaite (thistlew@bcm.edu).

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Revised August 2016

exceRpt Overview

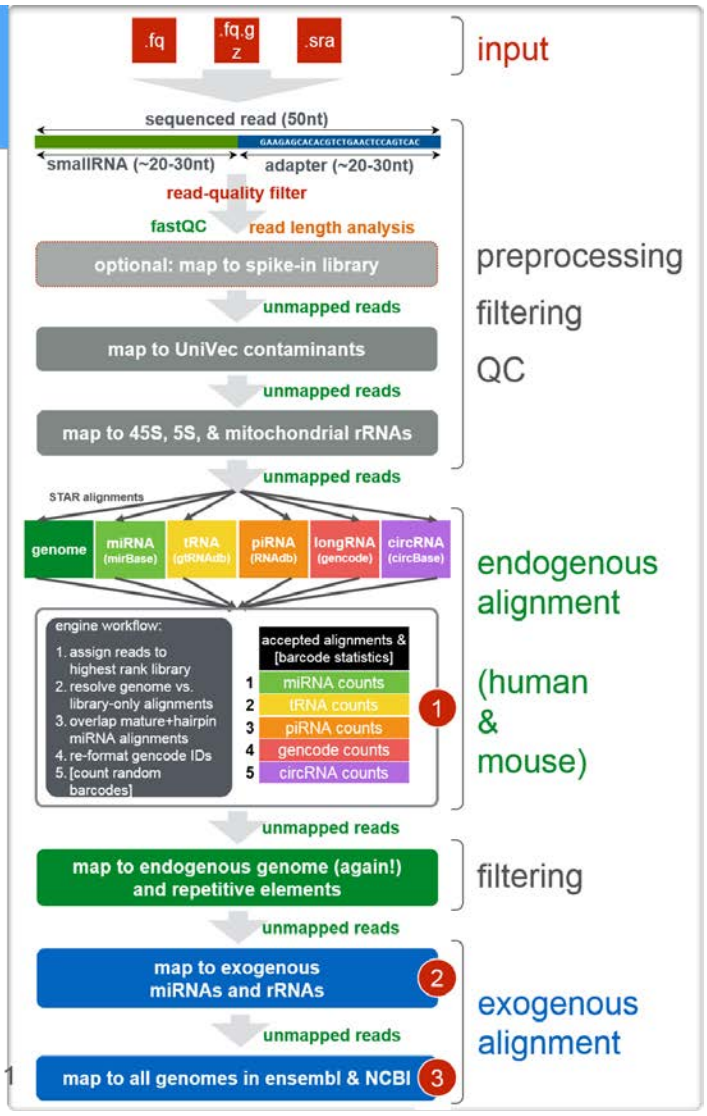
exceRpt

extra-cellular RNA processing toolkit

Key Updates:

1. Revised mapping parameters to support all biofluids including QC evaluation
2. Incorporation of exogenous genome sequence characterization using phylogenic trees.
3. Exogenous ribosomal sequence characterization using phylogenic trees.
4. Upcoming (Nov) release of long version of exceRpt pipeline.

R. Kitchen, J. Rozowsky, M. Gerstein



Oral Presentations

Oral Presentations Abstracts

Session I: ERCC Studies of Extracellular RNA for Human Therapeutics

Exosomes as Therapeutic RNA Delivery Vehicles

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Extracellular vesicles (EVs) have received much interest for clinical use as vehicles for oligonucleotide therapeutic (ONT) delivery as well as endogenous carriers of disease biomarkers for diagnostic purposes. Currently, efficient and non-toxic ONT delivery to the central nervous system represents a significant barrier to their use in the treatment of neurological disorders, such as Huntington's disease. Exosomes have the potential to act as "native" ONT delivery vehicles, but robust and scalable methods for loading therapeutic RNA cargo into exosomes are lacking. We show that hydrophobically modified siRNAs (hsiRNAs) efficiently load into exosomes upon co-incubation, without altering vesicle size, distribution, or integrity, and promote efficient neuronal internalization and Huntingtin mRNA silencing both in vitro and in vivo.
Exosomes are highly efficient in the functional intracellular transfer of small endogenous RNAs. The RNA content of exosomes is under thorough investigation as a fingerprint for disease diagnosis. There is limited information available on the protein and lipid composition of EV membranes, despite the importance of this information for understanding EV biogenesis and biomarker trafficking. We will present recent data from a high resolution proteomics and lipidomics analysis of exosomes from different cell sources and discuss their functional contributing components.

Therapeutic Targeting of Prostate Cancer Cell-Derived Exosomes by High Throughput Screening of Potent Biogenesis and Secretion Inhibitors

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Background: Exosomes are implicated in cell-to-cell communications and in modulating the function of recipient cells. Cancer cell-derived exosomes promote metastasis, primarily via the trafficking of metastasis-promoting genomic and proteomic factors. Our investigations also showed the ability of prostate cancer (PC)-secreted exosomes in enabling neoplastic reprogramming of adipose stem cells (ASC). Thus, persistent cues from tumor-secreted exosomes can educate the metastatic niche to facilitate aggressive tumor growth and therapeutic resistance, underscoring the importance of exosome- targeted therapies. The pathophysiologic roles of exosomes are also recognized in many other diseases, and highlight the potential for therapeutic interventions. Although the biogenesis and secretion of exosomes are known to be tightly controlled by cytosolic mechanisms that include endosomal sorting complexes and the Rab proteins, pharmacologic agents that potently inhibit exosome biogenesis and/or secretion are currently unavailable.

Aim: To optimize a high throughput screen (HTS) assay to identify compounds that potently inhibit exosome biogenesis and/or secretion pathways in aggressive prostate cancer cells.
Methods: An aggressive PC cell line, C4-2B, was stably transfected with CD63-GFP (exosome-marker) and was used to quantify the effect of drugs on exosome production in vitro, by using an Acumen cell imaging scanner. Briefly, cells were seeded on poly-D-Lysine (PDL) coated plates for 48 hours (recovery period). Following which, cells were starved for 16 hours (synchronization) and then exposed to different concentrations of compounds for 96 hours, and the GFP signal was quantified. These optimized assays showed that the amount of CD63-GFP expressed in cells produced a robust fluorescent signal for 1536-well HTS assay of compounds, both in terms of

total fluorescence intensity and the number of exosomes per cell. The LOPAC library (a collection of 1,280 pharmacologically active compounds) and the NPC library (NCGC collection of 3,300 compounds approved for clinical use) were screened for compounds that inhibit exosome biogenesis and/or secretion. The activity of lead compounds was further verified by measuring both the number & size of secreted exosomes, by using the qNano-IZON system; and by documenting the protein markers of exosome biogenesis and secretion, e.g. Alix, TSG101 and Rab27a.

Results: Our optimized HTS-compatible assay identified a number of high potency inhibitors of exosome biogenesis and/or secretion, including Manumycin-A (Antibiotic), Mephensin (NMDA-R antagonist), Triadimenol, Neticonazole and Climbazole (Antifungals) and Ketoprofen (NSAID).

Conclusions: Assays for robust HTS of inhibitors of exosome biogenesis and/or secretion from prostate cancer cells have been established and validated in vitro. Several high potency compounds have been identified. Current efforts (UH3-phase) are to test the pharmacokinetics (PK) and pharmacodynamics (PD) of these lead compounds in both normal and tumor-bearing mice, in vivo.

HER2-targeted extracellular vesicles delivery of therapeutic mRNA for enzyme prodrug therapy

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Background: HER2 is overexpressed in aggressive breast cancers with poor prognosis. We have generated dendritic cell (DC) extracellular vesicles (EVs), the "body's antigen delivery system", decorated with HER2-specific single chain antibodies (scFv) for directed therapeutic delivery to HER2+ cancers. Our prodrug regimen (CNOB/ChrR6) is being used; its cytotoxic product, MCHB, can be visualized noninvasively in living mice, providing facile means for assessing targeted activation. We have loaded the EVs with ChrR6 enzyme-encoding mRNA -- mRNA eliminates the transcriptional step needed for DNA-mediated gene delivery. The strategy can potentially be personalized using EVs of patients' own DCs. Aims: To use HER2-targeting EVs for specific and effective delivery of the prodrug therapy to HER2+ cancer, and characterize pharmacokinetics/pharmacodynamics (PK/PD) of the regimen.

Methodology: Using exosome-display technology employing the p6mLSC1C2 plasmid, we constructed a chimeric gene, consisting of the coding sequences of anti-HER2 scFv (ML39) fused to lactadherin C1C2 domains. EVs were harvested from HEK293 cells transiently transfected with the plasmid, and pure chimeric protein was isolated using His-tag columns. EVs from naïve HEK293 or DC cells were decorated with the externally added chimeric protein via the capability of the C1C2 domains to bind to the EV phosphatidylserine. ELISA quantified the specificity for HER2. Cell binding was determined with CFSE-labeled directed EVs using BT474 (high HER2) or MCF7 cells (low HER2) and visualized by fluorescence microscopy. mRNA transfer to EVs was accomplished using 293FT cells transiently transfected with plasmids containing the EV-loading zipcode. Two plasmids with the CMV promoter (pAdTrack-CMV and pCDH-CMV) were compared for EV mRNA loading efficiency. ChrR6 mRNAs was quantified by qRT-PCR. EVs decorated with anti-HER2 scFv and enriched the ChrR6 mRNA were incubated overnight with BT474 cells. After washing, the cells were treated with CNOB. MCHB production and cell viability were quantified by fluorescence and the MTT assay, respectively. In vivo PK/PD was examined with imaging and LC/MS/MS for tissue drug quantification, followed by modeling and simulation via WinNonlin.

Results: Plasmid-transfected HEK293 cells generated EVs expressing the anti-HER2 scFv/C1C2 chimeric protein. DC EVs, which lack lactadherin, were incubated with the chimeric protein: the resulting directed EVs showed 10-times greater binding capacity than EVs from the transfected HEK293 cells. The directed EVs bound to a much greater extent to BT474 than to the MCF7 cells. Directed and loaded EVs conferred transcription-independent CNOB activation capability specifically on BT474 cells indicating successful delivery of the mRNA; they also generated much greater killing of BT474 cells than the non-directed EVs. Ca. 275 secreted EVs could transfer 1 copy of mRNA. The CMV promoter proved more efficient than the MSCV promoter, and the plasmid pAdTrack better than pCDH,

generating 36 and 87% improvement, respectively. PK/PD studies pointed to a more effective in vivo prodrug dosing regimen for cancer treatment.

Conclusions: We have successfully engineered EVs that specifically bind HER2 cells and can deliver functionally competent therapeutic mRNA to and specific killing of these cells by the prodrug regimen. Tests are now underway to demonstrate the efficacy of these EVs to treat implanted HER2+ breast tumors in mice.

Lack of toxicity and immunogenicity in mice following 3-week dosing of engineered exosomes derived from HEK293T cells

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4. Department of Biostatistics, The Ohio State University, Columbus OH

Background and Aims: Exosomes are under evaluation as delivery agents for therapeutics. Ongoing studies often use mouse disease models for evaluation of drug disposition, activity or efficacy. A comprehensive study of the potential immunogenic and/or toxic effects of exosomes in mice has not been reported. The aim of our study was to thoroughly assess the potential of wild type or engineered HEK293T cell-derived exosomes to induce toxicity and immune responses in mice.
Methods: Exosomes harvested from wild type (WT) or HEK293T cells engineered to express a targeting peptide (Full no 199), a targeting peptide plus therapeutic miR-199 (Full) or empty vector control (Empty) were administered as ten injections of 8.5 μg protein, every other day for 3 weeks to C57BL/6 mice by intravenous and intraperitoneal routes. Mice receiving PBS were included as vehicle controls. All mice were monitored throughout the 3-week study for visual signs of toxicity. Blood samples were collected before the first dose (day 1), one day after the first dose (day 2), and prior to euthanasia (day 22) for complete blood count analysis. Serum was prepared after euthanasia for biochemical profiles and evaluation of immune markers using the RodentMAP test. Single cell suspensions from spleens were prepared for immunophenotyping analysis. Tissue samples were harvested and fixed for histopathological examination.

Results: All 50 mice (10 mice/group) from the 5 treatment groups (PBS, WT, Empty, Full no 199 and Full) were monitored throughout the experimental period, and no signs of abnormality or behavioral changes were observed. The increase in body weight was normal, and no statistical differences were observed between the 5 groups. Gross and histopathological examination did not reveal any abnormalities in the organs examined. Complete blood count and blood chemistry panels indicated no significant difference between the 5 groups for all tested markers. To determine if the injected exosomes derived from human cell lines would induce an immune response in the mice, 42 cytokines and chemokines were measured using the RodentMAP test. Of the 23 markers that were present at quantifiable levels, we observed no significant differences in these markers compared to the PBS control group. Results from splenocyte immunophenotyping showed no significant differences between the 5 groups for all tested immune cell populations, indicating that exosome treatment did not affect the spleen immune cell composition.

Conclusions: Exosomes derived from wild type and engineered HEK293T cells and dosed at 8.5 μg protein per dose over a 3 week period did not induce toxicity or immune response in immune competent C57BL/6 mice.

Development of a Cross Consortium Standardized Platform for Creation of Specific Cell Types/Exosomes from Induced Human 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 producing analogous human-derived exosomes. 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 goal here is to conduct basic research on human-derived SDC-Exos that will form the basis for future clinical trials. To achieve this, we must: 1) produce human SDC-Exos that recapitulate the effects of rat SDC-Exos; 2) produce sufficient quantities of SDC-Exos for treatment; 3) show that SDC-Exos are non-toxic / do not induce negative immune responses; 4) produce SDC-Exos under GMP standards. Accordingly, we are developing human induced pluripotent stem cells (hiPSCs) as a robust and highly scalable means to produce therapeutic SDC-Exos.

Methods: Use of hiPSCs presents us with several advantages. Donor cells obtained from adult patients can 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 SDC-Exos. Our plan is to use 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 SDC-Exos as "biological medicinals". Importantly, hiPSCs have unlimited growth capacity and can be used for generation of exosomes from specific cell types of all three germ layers.

Results: We have determined that DCs derived from human bone marrow can be used to produce effective SDC-Exos. Preliminary studies indicate that these human SDC-Exos significantly increase myelin levels and reduce microgliosis in rat brain slice cultures, and contain high levels of miR-219. We are in the process of performing a full profile of SDC-Exo miRNA contents and testing their effect on human oligodendrocyte precursor cells. Based on this work, we believe that using hiPSCs (vs. bone marrow) will be a viable method to increase the scale of SDC-Exo production by up to 10,000 fold. Updates on the status of hiPSC work will be reported.

Conclusions: Though we have successfully produced SDC-Exos from human bone marrow-derived DCs, obtaining patient bone marrow is a painful, invasive procedure. Thus, we suggest use of hiPSCs derived from skin biopsies and differentiated into immature DCs as an alternative source. The ease of obtaining skin fibroblasts, the ability to generate hiPSCs from these fibroblasts, and the capacity for hiPSCs to differentiate into DCs all suggest that this approach constitutes a minimally invasive, reproducible and scalable method of obtaining patient-specific therapeutic SDC-Exos. Currently, there is little commonality among the methods used to produce therapeutic exosomes in various ERCC projects. It is our hope to create a common platform for the reproducible fabrication of large quantities of exosomes derived from any cell type of interest. Such a program would form the basis for long-lasting collaborative efforts between ERCC members and the scientific community at large.

Session II: Genetic Models and Tools to Study exRNA

Utilizing Hollow Fiber Bioreactors as a Scalable Source for Extracellular Vesicle Production

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Background: Extracellular vesicles (EV) are particles released from various cell types and tissues. Collection of EV at analytic lab scale by approaches such as ultracentrifugation, agglomeration or adsorption chromatography are not amenable to large-scale production. Current approaches for large- scale purification involve culture in multiple large flasks and require centrifugation. Culture in hollow fiber bioreactors could provide high surface-to-volume ratios, support large numbers of cells at high densities, and reduce the need for large volume centrifugation.

Objectives: To evaluate the potential for hollow fiber bioreactor technology to produce a continuous source of highly concentrated EV from cells in culture.

Methods: We evaluated EV production by three different types of cells, human hepatocytes (HH), cholangiocarinoma cells (CCA), and bone marrow derived mesenchymal stem cells (MSC) during culture using the FiberCell system hollow fiber bioreactor (FiberCell Systems, Frederick, MD). Cells were seeded into a hollow fiber cartridge, which adhere to the outside of the fibers, and regular medium was perfused inside the hollow fibers. Serum free media was used in the extracapillary space surrounding the cells. Supernatant was harvested and replaced with fresh serum free media every 24-48 hours. EV were isolated from the extracapillary space using sequential ultracentrifugation, and size and number were analyzed using Nanoparticle Tracking Analysis (Malvern, UK).

Results: Cells were seeded into a C2011 cartridge at 3-7x 107 cells. The intracapillary media was monitored daily with glucose checks and replacement as appropriate. EV were isolated from 20mL of the extracapillary fluid collected every 2 days. The EV isolated from HH, CCA or MSC had mean size of 133-188 nm, 74-215nm and 118-286 nm over sequential collections with a mean CV of 32%, 45% and 45% respectively. The mean % of EV collected with size less than 200nm was 89, 93 and 86, while the mean number of particles / ml from HH, CCA and MSC were 1.03E+11, 3.87E+09 and 2.01E+10. Compared with culture in flasks, there was a 10-fold increase in mean number of particles/ml using the hollow fiber bioreactor.

Conclusion: Hollow fiber bioreactors can be used as a source of scalable EV production from diverse cell types. EV can be continuously isolated over the life of the bioreactor, without passaging of the cells and with yields that are greater than those from culture in flasks. The functional potential of these EV remains to be evaluated.

Target Mapping of red blood cells derived extracellular vesicles in a transgenic murine model

Avash Das[1], Olivia Ziegler[1], Kirsty Danielson[1], Bridget Simonson[1] Shulin Lu[2], Vasilis Toxavidis[2], Tigges John[2], Ravi V Shah[1], Saumya Das[1*], Ionita Ghiran[2*].
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Introduction: There is emerging evidence on the role of extracellular vesicles (EVs) as mediators of intercellular communication during both, normal and pathological conditions. EVs derived from circulating red blood cells (RBCs) have been implicated in the pathogenesis of various diseases such as malaria and atherosclerosis. Here, we describe a novel, fluorescence switch-based, experimental model to study EV- mediated communication between RBCs and various tissues and organs that allowed us to describe for the first time, the cross-talk between RBCs and different cell types at baseline homeostasis.

Methods: Mice with RBC-specific expression of cre (Erythropoietin Receptor (EpoR) Cre) were crossed with reporter mGmT Rosa mice to yield EpoRCre/mGmT offsprings that expressed membrane bound Cre-GFP specifically in RBCs. Plasma EVs from EpoRCre/mGmT mice (n=3) were studied with nano-flow cytometry and fluorescence microscopy, while organs from EpoRCre/mGmT (n=3) and mGmT (n=3) mice were cryo-sectioned and imaged with confocal microscopy. Real-time PCR and western blotting for Cre mRNA were conducted on RBCs, bone marrow and RBC derived EVs of EpoRCre mice and wild-type mice. In vitro, dermal fibroblasts cultured from mGmT mice were treated with RBC-derived EVs from EpoRCre mice and fluorescence microscopy was performed after 96 hours. In vivo, mGmT mice (n=3) were transfused with EVs derived from RBCs isolated from EpoRCre mice and mGmT mice respectively, and their organs were imaged using fluorescence microscopy followed by deconvolution and 3-D reconstruction.

Results:
1. The expression of EVs from RBC-GFP in plasma accounted for about 9% of total fluorescent EVs as detected by nano-flow cytometry and microscopy-based dynamic flow-analysis. Analysis of bone marrow and other organs by microscopy demonstrated m-GFP staining in the background of m-Tomato staining, suggesting a low-level transfer of Cre from RBCs to other cell-types.
2. While Cre mRNA expression was found to be negligible in RBCs, bone marrow and RBC derived EVs of EpoRCre mice, Cre-recombinase protein can be detected in EpoRCre RBCs and bone marrow.
3. In-vitro dermal fibroblasts from mGmT mice showed red to green trans-membrane color switch with EpoRCre EVs derived from RBCs, suggesting transfer of functional cre.
4. Organs harvested from mGmT mice, EpoRCre/mGmT mice and mGmT mice transfused with EpoRCre vesicles showed varying expression of membranous GFP in the latter two experimental conditions with
the bone marrow, heart, lungs, kidney and spleen being the foremost among the organs with considerable m-Tomato to m-GFP membrane bound color switch under confocal microscope.

Conclusion: Our data demonstrate for the first time, the EV-based 'cross-talk' between circulating RBCs and tissues and organs under normal homeostasis. Employing similar transgenic murine model can be used to study the pathogenesis of several diseases where the role of RBC has often been suspected. The model presented here is fully applicable to studying the other cells.

Semantic Web Enabled Integration of Wikidata and Wikipathways for Exploring Impact of exRNAs in Health and Disease.

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Background: Extracellular RNAs (exRNAs) have emerged as an essential intercellular communication factor in health and disease. The effects of exRNAs are multi-faceted and frequently affect gene expression of their target cells and tissues. In order to interpret the effects of exRNAs on gene expression and therefore on downstream signalling pathways, open and easily accessible resources and approaches are required. Wikidata (www.wikidata.org) is an open, community driven, document based database and general graph of knowledge. It contains a large biological dataset involving genes, proteins, drugs, diseases and many interactions between them. In addition, with Wikipathways, a large, community driven resource for biological signaling pathways exists, providing insights into essential genes in signalling pathways. In addition to their completely open and Wiki-type nature, both resources provide SPARQL endpoints, allowing for fast retrieval of integrated data packages of interest for any downstream application, based on federated SPARQL queries.
Aims: Data on human miRNAs should be integrated into a Wikidata data model, representing pre- miRNAs and mature miRNAs as separate Wikidata items. Furthermore, miRNAs should be linked to the genes they are expressed by and the target genes they are regulating. Finally, SPARQL queries should be designed for efficient identification of target pathways.
Methodology: We used the public domain miRNA database miRBase (http://www.mirbase.org/) as an open data source for miRNAs and wrote a Python program (also termed a bot) in order to generate Wikidata items for human pre-miRNAs and mature miRNAs. For miRNA target genes, we used the TargetScan predicted targets data with a score cutoff of >0.79. Mature miRNAs were linked to target genes via the Wikidata 'regulates' property. SPARQL queries were executed on the Wikipathways SPARQL endpoint.
Results: In total, 2,619 human pre-miRNAs, 4,400 mature miRNAs and their corresponding identifiers were imported from miRBase. Furthermore, miRNA families, based on RFAM were imported to allow classification and categorization of the distinct miRNAs. This categorization allows for higher-level queries on miRNA family basis. In addition, we added links to the gene targets of these miRNAs onto the mature miRNA items in Wikidata. Finally, we created SPARQL queries which allow for retrieval of pathways in Wikipathways which might be affected in case a certain miRNA is expressed intracellularly or secreted as part of an extracellular vesicle. The reverse query enables to retrieve all genes involved in a pathway and search for miRNAs involved in its regulation. Gene Ontology terms of the target genes also allow inference of more general effects of miRNAs on cellular functions and biological processes.
We also construct queries which retrieve all miRNAs impacting pathways that are e.g. involved in immune regulation. ExRNAs, co-expressed with expression of certain genes, and so potentially regulating other signaling pathways can also be retrieved with such queries.
Conclusions: We created an open, community driven resource and knowledge base for exRNAs (and more broadly for all miRNAs) which integrates Wikidata and Wikipathways via the semantic web and SPARQL, allowing for users to execute queries, contribute data and share their findings on Wikidata. This resource should help elucidating the effects of exRNAs in health and disease.

Session III: Heterogeneity of exRNA Carries and Implications in Function and Disease

Sources and composition of extracellular vesicles in inflamed tissue

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Background and aims: We have previously shown that T lymphocytes release extracellular miRNAs in response to inflammatory stimuli. We hypothesize that immune cells are important sources of extracellular vesicles and extracellular RNA during tissue inflammation. Moreover, we speculate that this process may be critical for the immune function of these source cells. The major goals of our project are to define the molecular mechanisms of Ago protein and ex-miRNA release by activated T cells, and to determine the immune cell contribution to body fluid ex-miRNAs in homeostatic and inflamed conditions.

Methodology: We established small RNA sequencing with unique molecular identifiers to obtain robust small RNA profiling in the small fluid specimens that we can obtain in mouse models of tissue inflammation. We compare bronchoalveolar lavage supernatant and blood

serum of control mice and mice with induced allergic airway hypersensitivity. In this model of asthma, inflammatory cells including lymphocytes, eosinophils and macrophage are recruited into the lung parenchyma and airways in response to allergen challenge. We investigate particle size, vesicle composition and RNA content using nanoparticle tracking analysis (Nanosight), vesicle flow cytometry and small RNA sequencing, which we analyzed with the Genboree exceRpt Small RNA-seq Data Analysis Pipeline. In addition, we optimized and validated methods for exosome and other microvesicle purification by differential ultracentrifugation followed by flotation in density gradients, and used these together with small RNA qPCR and immunoblotting to characterize released exRNA form and content from in vitro activated T cells. Our ability to perform highly efficient RNAi in primary T cells using next generation electroporation allows us to test the requirements for vesicle and RNA release in this system.

Results: Serum and bronchoalveolar lavage supernatants contain distinct sets of extracellular miRNA and other small RNAs. Both profiles include miRNAs that are highly expressed by lymphocytes and other immune cells, but airway epithelium miRNAs are also abundant in bronchoalveolar lavage fluid.
Genetically modified mice that lack lymphocytes exhibit altered exRNA profiles, consistent with the idea that lymphocytes are important exRNA source cells. T cells activated in vitro change their mode of ex- miRNA secretion, indicating that activating signals alter Rab protein dependent membrane trafficking and vesicle sorting.

Conclusions: Immune cells and epithelial cells make distinct contributions to the exRNA pool in inflamed airway lining fluid and blood serum. Signals that activate T lymphoctyes induce changes in the mechanism of vesicle and extracellular RNA release. These changes likely alter both the exRNA milieu in inflamed tissues and the gene expression program of source T cells.

Incorporation of full length mRNA messages in vesicles by binding to RNPs

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Background/aims: Extracellular vesicles (EVs), including exosomes, have shown to play a role in various biological processes and diseases. EVs are small vesicles (50-1000 nm) that are shed by cells. Their content reflects in large extent that of the donor cell and includes proteins, RNAs, and DNA. Multiple proteins and ribonucleoproteins (RNPs) have been implicated in active RNA sorting into EVs. In this study we evaluated the loading of full-length mRNAs by association with RNPs. We focused on a specific RNP, Staufen 1 (Stau1) which has a number of mRNA partners. This multifunctional RNP has been described to regulate mRNA stability, localization, transport and translation and is known to be present in EVs. In addition, the binding potential to a specific mRNA, Arf1, is well established.

Methodology: HEK293T stable cell lines were stably transduced with a lentiviral vector expressing fusion protein hStau1-GFP [Köhrmann et al., 1999]. EVs from wild type and stable cell lines were isolated using exoRNeasy (Qiagen) and differential ultracentrifugation. Protein expression was analyzed using Western blots. mRNA content was analyzed using RT-(q)PCR with primers in coding region (CDS) and 3'UTR. Full length mRNA was captured using primers spanning 5'UTR, CDS and 3'UTR regions and amplified using high fidelity polymerase.

Results: Initial results show full-length mRNA enrichment of specific message Arf1 that is associated with RNP partner Stau1. When bound to Stau1, most of the mRNA incorporated into EVs is full-length, while low expression levels of 3'UTR fragments are observed. Several known Stau1 targets confirmed similar expression patterns in EVs. Other messages show different expression patterns with high abundance of fragmented RNA, supporting the theory that association with RNPs can promote incorporation of full-length mRNAs.
Conclusions: Our present data suggests RNP-mRNA combinations enrich the levels of full-length mRNA messages present in vesicles. Even though previous studies have shown an increased presence of 3'UTR fragments of mRNAs in vesicles [Batagov et al., 2013], this enrichment appears to be reduced when mRNA is bound to a protective RNP partner. This data emphasizes the potential of horizontal transfer of intact messages by EVs; suggesting RNP-RNA incorporation into vesicles may represent a good candidate for therapeutic nucleic acid delivery.

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Influence of Hyperglycemia on Macrophage-derived Exosomes in Diabetic Atherosclerosis Regression

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Background: Atherosclerosis is dynamic process that has been shown to regress in response to sustained plasma lipid reduction. However, studies have also shown that diabetic individuals do not fully benefit from the cardioprotective effects of existing lipid-lowering therapies. Studies, including from our laboratory, have shown that hyperglycemia impairs atherosclerosis regression in mice by altering myeloid cell activity. What is not known is the impact that hyperglycemia can exert to alter the release of microRNA into exosomes produced by myeloid cells to enhance systemic and vascular inflammation and thereby impair atherosclerosis regression.

Aims: We sought to explore the impact that hyperglycemia exerts to alter the microRNA content and increase the pro-inflammatory properties of exosomes produced by cultured macrophages and those present in the plasma of diabetic mice. We also sought to explore if a control of cellular oxidative stress in macrophages exposed to hyperglycemia can prevent dysregulated microRNA secretion into exosomes to suppress their pro-inflammatory properties.

Methodology: Bone marrow derived macrophages (BMDM) prepared from WT mice and transgenic mice over- expressing the human Catalase gene (Cat-BMDM) were cultured in normal or high glucose medium. Cell- derived exosomes were isolated through the use of our cushioned-density gradient ultracentrifugation (C-DGUC) method followed by a battery of biochemical tests including Nanosight analysis to quantify particle numbers. Circulating exosomes were isolated from the plasma of WT mice and Akita diabetic mice using C-DGUC and similarly assessed for particle numbers. The pro-inflammatory properties of exosomes were tested by exposing them to cultures of J774 macrophage reporter cells that were stimulated with LPS as well as Ehy926 endothelial cells that were stimulated with TNF-α.

Results: Our findings show that hyperglycemia increases levels of microRNA-155 in exosomes secreted by cultured macrophages and well as in exosomes isolated from the circulation of diabetic Akita mice. Our data also show that such exosomes display pronounced pro-inflammatory properties when exposed to cultured macrophages and endothelial cells by increasing the expression of NF-kB responsive genes. Interestingly, our findings show that an enzymatic control of OS in Cat-BMDM cultured in high glucose results in the secretion of exosomes that suppress inflammation in endothelial cells.

Conclusions: Our findings show that hyperglycemia increases pro-inflammatory properties of exosomes secreted by macrophages and those circulating in diabetic mouse plasma, including by increasing levels of miR-155 that could serve as an unsuspected source of inflammation to impair atherosclerosis regression in diabetes. Our findings also point to increased cellular oxidative stress (OS) as a cause for microRNA dysregulation in exosomes derived from macrophages exposed to hyperglycemia, and suggest that exosomes derived from Cat-BMDM could serve to facilitate the regression and stabilization of atherosclerosis in diabetic mice.

Optimization of exRNA isolation from biofluids: Progress report from the RNA Isolation Working Group

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Extracellular RNAs (exRNAs) are found in a variety of biological fluids, but at low levels. Anecdotal evidence and small studies have suggested that the biofluid type and the specific RNA isolation method can markedly impact the yield of exRNA and its compatibility with different downstream assays. This project was designed to be a systematic study of these parameters for five types of biofluids, 10 RNA isolation methods, and 2 downstream analytical methods. Here, we report our progress thus far and future plans.

We focused on 5 biofluid types: plasma, serum, urine, cell culture supernatant, and bile. Plasma, serum and urine were collected from 10 healthy male and 10 healthy female individuals (18 – 40 years of age) and mixed to generate large volume pools of these biofluids. Plasma samples were distributed to 4 labs (Breakefield, Das, Gandhi, and Sood), serum samples to 3 labs (Breakefield, Gandhi, and Sood), and urine samples to 1 lab (Laurent) for RNA isolation. Cell culture supernatants were collected from three different labs (Das, Laurent, and Patel), each using a separate cell line, and distributed for exRNA isolation and analysis. Bile from four patients was collected and analyzed in one lab (Patel).

We tested 10 different RNA isolation methods. Seven of which included an initial extracellular vesicle isolation step (the ExoRNeasy kit [Qiagen], the ME kit [New England Peptide], the Plasma/Serum Circulating and Exosomal RNA Purification Kit [Norgen] with and without an Amicon concentration step, the Exoquick kit [System Biosciences], concentration using small-pore filters [Millipore], and ultracentrifugation). The other three methods isolated the exRNA from the entire unfractionated sample (the miRNeasy Micro kit [Qiagen] using either Qiazol or a "homebrew" lysis buffer, and the miRcury Biofluids kit [Exiqon]). The resulting exRNA samples were quantified using the Nanodrop and RiboGreen assays. The RNA size distributions were determined using Agilent RNA 6000 Pico Chips on the Agilent 2100 BioAnalyzer System. ExRNA samples were also evaluated by qRT-PCR using TaqMan probes for miRNAs (hsa-miR-let7a-5p, hsa-miR-16-5p, hsa-miR-223-3p and cel-miR-39-3p spike-in) and mRNA (GAPDH, using single- exon and exon-exon junction primer sets) along with a cel-miR-39-3p spike-in control.

We used multivariate analysis of data from the serum, plasma, urine, bile, and cell culture samples, which was used to evaluate the technical and biological variability introduced by the use of different RNA isolation kits, lab sites, and biofluid sample types. We found marked differences in the quantities of individual miRNAs detected by qRT-PCR among biofluids. The quantities of individual miRNAs varied among kits for any given biofluid, with the pattern differing between the three different miRNAs tested. For any given miRNA, the relative performance of the difference kits varied between biofluids. Our results also indicated significant variability in the process of obtaining qRT-PCR results among laboratories. These observations led us to conclude that it will be necessary to apply an analysis method that is able to interrogate many more miRNAs at one time, and to perform this method at a single site. We therefore, are in the process of performing small RNA sequencing on the isolated exRNA samples at one site, and will present preliminary results from these experiments.

Session IV: Extracellular RNA and Function

Collective abstracts below are describing each of the projects, sub-projects and core activities from the Biogenesis initiative. Five oral presentations on Session IV will highlight the major accomplishments per project.

In Vivo Regulated Release and Function of Extracellular Small RNAs – Robert Blelloch (Contact PI)

Sources and composition of extracellular vesicles in inflamed tissue

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Background and aims: We have previously shown that T lymphocytes release extracellular miRNAs in response to inflammatory stimuli. We hypothesize that immune cells are important sources of extracellular vesicles and extracellular RNA during tissue inflammation. Moreover, we speculate that this process may be critical for the immune function of these source cells. The major goals of our project are to define the molecular mechanisms of Ago protein and ex-miRNA release by activated T cells, and to determine the immune cell contribution to body fluid ex-miRNAs in homeostatic and inflamed conditions.

Methodology: We established small RNA sequencing with unique molecular identifiers to obtain robust small RNA profiling in the small fluid specimens that we can obtain in mouse models of tissue inflammation. We compare bronchoalveolar lavage supernatant and blood serum of control mice and mice with induced allergic airway hypersensitivity. In this model of asthma, inflammatory cells including lymphocytes, eosinophils and macrophage are recruited into the lung parenchyma and airways in response to allergen challenge. We investigate particle size, vesicle composition and RNA content using nanoparticle tracking analysis (Nanosight), vesicle flow cytometry and small RNA sequencing, which we analyzed with the Genboree exceRpt Small RNA-seq Data Analysis Pipeline. In addition, we optimized and validated methods for exosome and other microvesicle purification by differential ultracentrifugation followed by flotation in density gradients, and used these together with small RNA qPCR and immunoblotting to characterize released exRNA form and content from in vitro activated T cells. Our ability to perform highly efficient RNAi in primary T cells using next generation electroporation allows us to test the requirements for vesicle and RNA release in this system.

Results: Serum and bronchoalveolar lavage supernatants contain distinct sets of extracellular miRNA and other small RNAs. Both profiles include miRNAs that are highly expressed by lymphocytes and other immune cells, but airway epithelium miRNAs are also abundant in bronchoalveolar lavage fluid. Genetically modified mice that lack lymphocytes exhibit altered exRNA profiles, consistent with the idea that lymphocytes are important exRNA source cells. T cells activated in vitro change their mode of ex-miRNA secretion, indicating that activating signals alter Rab protein dependent membrane trafficking and vesicle sorting. Conclusions: Immune cells and epithelial cells make distinct contributions to the exRNA pool in inflamed airway lining fluid and blood serum. Signals that activate T lymphoctyes induce changes in the mechanism of vesicle and extracellular RNA release. These changes likely alter both the exRNA milieu in inflamed tissues and the gene expression program of source T cells.

AKT-driven intracellular sorting of extracellular microRNAs in prostate cancer

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Extracellular miRNAs have been identified as biomarkers and potential effectors of cancer progression, yet little is known about how disease pathways influence miRNA release. We seek to uncover how the PI3K/AKT/mTOR pathway, the most commonly misregulated pathway in prostate cancer, affects the sorting and secretion of miRNAs. Using a normal prostate epithelial cell line, RWPE-1, overexpressing myristoylated-AKT or a control vector, we have found that Akt activation induces changes in the selective secretion of miRNAs of prostate cancer. Furthermore, we have used a cell fractionation protocol to demonstrate that Akt activation induces changes in the subcellular localization of miRNAs, potentially leading to its selective secretion. Of particular interest, the oncomiR miR-106a, was enriched in exosomes relative to cells as well as in intracellular multivesicular bodies (MVBs) relative to P-bodies, in the constitutive

activated Akt cells. These results support a model of Akt driven active sorting of this miRNA, a miRNA implicated in Akt driven prostate cancer progression. However, the constitutive expression of activated Akt makes it difficult to determine primary versus secondary effects of the pathways on these events, so we have developed two new systems. First, we have established a Tet-On inducible system to gain a better understanding of the acute effects of Akt on differential sorting and release of miRNAs. Secondly, we sought to understand how the immediate attenuation of the PI3K/AKT/mTOR pathway affects miRNA sorting and secretion. To this end, we have optimized conditions for treating PC-3, a PTEN deficient prostate cancer cell line, with potent mTOR inhibitors, Rapamycin and Ink128. While preparing these samples, we have had to make significant improvements in our work stream in order to handle the multiple conditions being simultaneously tested. Of particular importance, we discovered that filters dramatically reduced the yield of exosomes. Using a series of differential centrifugation and sucrose gradients without filtration, we have optimized a new exosome isolation protocol that yields purer and high quantities of CD63+ exosomes. We have also improved our cell fractionation protocol for consistent reproducibility and higher MVB yields. For instance, by using an Isobiotec cell homogenizer, we have been able to lyse more than 80% of cells, instead of ~30% with needles or dounce, without disrupting nuclei or organelles. Furthermore, we adjusted the concentrations of our sucrose gradients to yield more material in the vesicular fractions. The resulting fraction is highly enriched for CD63 intracellular vesicles. Interestingly, with this enhanced enrichment of both exosomes and MVBs, we discovered that the RNA content was qualitatively very different between exosomes and MVBs, potentially challenging the dogma of the MVB origin of the extracellular exosome fraction. To tackle this question with robust quantitative methods, we have enhanced our small RNA sequencing protocol to address ligation bias, differential jackpotting, and excess adapter dimers. We have done so by using degenerate ends on adapters, additional internal unique molecular identifiers, addition of modifications to the 5' adapters, and titrated adapter and carrier concentrations. With all these new tools in our work stream, we plan to determine the effects of the Akt pathway on the sorting and origins of resulting extracellular RNA changes.

Regulation of exRNA production by different oncogenes in tumor models

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Extracellular RNAs (exRNAs) released from cells can be found within vesicles, bound to lipoproteins or RISC components. They are crucial for cell-to-cell communication and thought to play important roles in cell development, diseases, and cancer metastasis. Using a panel of isogenic MCF10A cell lines that express most commonly overexpressed oncogenes we aim to identify oncogenic signals that alter extracellular vesicle production. We purified exosomes via ultracentrifugation and confirmed the presence of exosome markers such as Tsg101, Alix, Clathrin and CD9 by western blotting. Nanosight particle-tracking analysis revealed that MYC overexpression induces increased exosome release within a panel of ~12 different oncogenes. Similarly, we confirmed the MYC-dependent increase in exosome production using MYC inducible MTB-TOM cell lines. RNA microarray and metabolomic analysis identified significantly up and down-regulated genes involved in ceramide and sphingolipid metabolism in cells overexpressing MYC. Interestingly, sphingomyelinase inhibitor GW4869 significantly decreased exosome production in MYC compared to other oncogenes suggesting a ceramide-dependent pathway for exosome production in cells overexpressing MYC. We aim to dissect mechanisms involved in exosome biogenesis by altering levels of differentially expressed candidate genes. Moreover, our preliminary miRNA profiling from both intracellular and extracellular miRNAs found significantly up and/or down-regulated miRNAs present in extracellular vesicles. This finding suggests that oncogenes can alter which miRNAs are selectively secreted or retained in the cells. We sought to identify key regulators of these selections and their contribution to cancer progression.

In addition, we aim to elucidate the temporal regulation of exRNAs by the oncogenes MYC and RAS in primary liver tumor formation and regression. We are not only interested in exRNAs within vesicles but also bound to lipoproteins. In collaboration with Dr. Robert Raffai, we analyzed lipoproteins and lipoprotein particles from plasma collected from tumor driven by RAS, MYC, and MYC+RAS and compared to non-tumor bearing mice. The data indicate that the induction of liver
cancer by MYC or RAS oncogenes results in a differential accumulation of lipoprotein classes. The induction of RAS causes mainly an accumulation of HDL and chylomicron cholesterol whereas MYC-driven tumors have increased accumulation of LDL cholesterol. We also showed that different miRNA species were upregulated by RAS and MYC oncogenes in these conditional tumor models (i.e., miR-21 in RAS-driven tumors, miR494 in MYC-driven tumors). We now seek to determine if these oncogenic miRNAs can assemble into exRNAs and if so in which format (i.e., vesicle and or bound to lipoproteins) to regulate gene expression of target genes and whether they play a role in tumor metastasis.

Profiling membrane trafficking for exRNA release by endogenous protein tagging

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In order to elucidate the mechanisms regulating the release of exomes and exRNAs, it is highly desired to visualize these processes in living cells. Traditionally, live imaging of cellular proteins have usually been achieved by overexpressing a fluorescent protein fusion to the target. Nevertheless, overexpression artifacts are widely present (e.g. leading to the overpopulation of certain membrane compartments). To overcome this challenge, we have developed a scalable method to tag endogenous proteins for microscopy analysis by combining CRISPR/Cas9-mediated genome editing with split- fluorescent protein labeling (Kamiyama et al., Nat Comm 2016; Leonetti et al., PNAS 2016). Specifically, a short peptide sequence, GFP11, derived from the 11th beta-strand of an optimized super-folder GFP, is knocked-into endogenous genes. This peptide complements with the separately expressed GFP1-10 fragment to form a full fluorescent GFP. The small size of the GFP11 tag enables cloning-free knock-in with low cost and extremely high efficiency. Moreover, it can also be arranged as tandem repeats to amplify the fluorescence signal, enabling the detection of lower abundance targets. Recently, we have engineered two new split fluorescent protein tags, split mNeonGreen and sfCherry2, that are significantly (8 fold in the case of sfCherry) compared to the previous split GFP and sfCherry labels. With these two tags, we have demonstrated double knock-in for two-color imaging of endogenous proteins. We have also developed a photoactivatable split sfCherry2 for super-resolution microscopy analysis of cellular ultrastructures. Using this endogenous protein tagging method, we are generating human cell line libraries tagging Rab proteins the control intracellular vesicle trafficking (in collaboration with Robert Blelloch in the same U19 consortium), aiming at uncovering the pathways for exome and exRNA release. We are also collaborating Noelle L'Etoile and Andrei Goga to generate NRDE2 knock-in cell lines for the understanding of nuclear RNAi in exRNA target cells.

Genetic approach to discover pathways that produce and utilize exRNA.

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Background: Addiction, learning deficits and cognitive decline are all mounting public health concerns. Each pathology results in a large part from aberrant neuronal plasticity. Thus, understanding the cellular and molecular basis for neuronal plasticity is of great interest. We propose to use the powerful cell biological and genetic model organism *C. elegans* to discover pathways that produce and utilize exRNA. Mounting biochemical evidence has accumulated for exRNAs in mammals. In *C. elegans* the phenomenon of RNAi spreading, which requires exRNAs, is well documented. However, it is still unclear how exRNAs are produced, secreted, trafficked from one tissue type to the next, or how they alter target tissue biology. These gaps result from a paucity of genetic screens for pathways required for the production and function of environmentally induced exRNAs.
We have evidence that *C. elegans* sensory neurons produce small RNAs in response to environmental stimulation. Though these small RNAs arise from the sensory neuron, we find they induce organism-wide increases in small RNA and we can visualize the effects of the exRNA in distant tissues. These effects are dependent upon the double stranded RNA channel, SID-1.

Aim: Our aim is to identify genes required for biogenesis, secretion, trafficking and utilization of stimulus-induced exRNAs.

Methodology: Our first approach is to develop robust exRNA reporter strains based on the published (Juang et al., 2013) work and robust artificial triggers to use in screens for genes required for biogenesis, secretion, trafficking and utilization of stimulus-induced exRNAs. Our second approach is to identify novel mobile RNA species that arise in neurons and are trafficked throughout the organism and to use these in similar screens. Our third approach is to use optogenetic tools to determine what aspects of neuronal activity control release of the exRNA signal.

Results: We have developed a robust reporter for olfactory stimulus-induced spread of RNA silencing and we have begun to examine the role of neuronal activity in producing this signal. Our initial screen has identified a novel autophagy pathway required for spreading of RNA interference. Our sequencing efforts identified new endogenous RNA species that are likely to travel from neurons to other tissues including the germline. We made the unexpected finding that some endogenous interfering RNA species are likely to arise from

splicing intermediates. We also have evidence that the pathway that utilizes mobile RNA species is required in the germ cells for proliferation. The conserved pathway members are being analyzed in collaboration with the Goga lab (Project 3) as possible interpreters of the exRNA signal in cancer cells.

Conclusions: We have developed a robust reporter for exRNA activity and we are in the midst of carrying out candidate screens for genes required for export of this signal from neurons. We are probing the link between neuronal activity and export. We have identified a new biosynthetic pathway for endogenous interfering RNA. We have new evidence that one role for exported RNA signals from olfactory neurons is in the production or function of gametes. This study will provide detailed genetic and cell biological insight into how endogenously triggered 22G RNAs are produced and act at a distance from their source cell to affect organismal biology.

Influence of Hyperglycemia on Macrophage-derived Exosomes in Diabetic Atherosclerosis Regression

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Background: Atherosclerosis is dynamic process that has been shown to regress in response to sustained plasma lipid reduction. However, studies have also shown that diabetic individuals do not fully benefit from the cardioprotective effects of existing lipid-lowering therapies. Studies, including from our laboratory, have shown that hyperglycemia impairs atherosclerosis regression in mice by altering myeloid cell activity. What is not known is the impact that hyperglycemia can exert to alter the release of microRNA into exosomes produced by myeloid cells to enhance systemic and vascular inflammation and thereby impair atherosclerosis regression.

Aims: We sought to explore the impact that hyperglycemia exerts to alter the microRNA content and increase the pro-inflammatory properties of exosomes produced by cultured macrophages and those present in the plasma of diabetic mice. We also sought to explore if a control of cellular oxidative stress in macrophages exposed to hyperglycemia can prevent dysregulated microRNA secretion into exosomes to suppress their pro-inflammatory properties.

Methodology: Bone marrow derived macrophages (BMDM) prepared from WT mice and transgenic mice over-expressing the human Catalase gene (Cat-BMDM) were cultured in normal or high glucose medium. Cell-derived exosomes were isolated through the use of our cushioned-density gradient ultracentrifugation (C-DGUC) method followed by a battery of biochemical tests including Nanosight analysis to quantify particle numbers. Circulating exosomes were isolated from the plasma of WT mice and Akita diabetic mice using C-DGUC and similarly assessed for particle numbers. The pro-inflammatory properties of exosomes were tested by exposing them to cultures of J774 macrophage reporter cells that were stimulated with LPS as well as Ehy926 endothelial cells that were stimulated with TNF-α.

Results: Our findings show that hyperglycemia increases levels of microRNA-155 in exosomes secreted by cultured macrophages and well as in exosomes isolated from the circulation of diabetic Akita mice. Our data also show that such exosomes display pronounced pro-inflammatory properties when exposed to cultured macrophages and endothelial cells by increasing the expression of NF-kB responsive genes. Interestingly, our findings show that an enzymatic control of OS in Cat-BMDM cultured in high glucose results in the secretion of exosomes that suppress inflammation in endothelial cells.

Conclusions: Our findings show that hyperglycemia increases pro-inflammatory properties of exosomes secreted by macrophages and those circulating in diabetic mouse plasma, including by increasing levels of miR-155 that could serve as an unsuspected source of inflammation to impair atherosclerosis regression in diabetes. Our findings also point to increased cellular oxidative stress (OS) as a cause for microRNA dysregulation in exosomes derived from macrophages exposed to hyperglycemia, and suggest that exosomes derived from Cat-BMDM could serve to facilitate the regression and stabilization of atherosclerosis in diabetic mice.

exRNA Released by Glioblastoma Alters Brain Microenvironment - Xandra Breakefield (Contact PI)

Project 1 - Stephen Gould: Exploring & Exploiting Exosome and exRNA Biogenesis and Uptake

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Working in human tissue culture cells (HEK293, 293T, BDEneu (cholangiocarcinoma), and MDA-MB-231 (breast cancer) cell lines, we generated a series of small expression vectors that drive high-level expression of natural and synthetic exosomal marker proteins. In addition, we generated a series of Cas9/CRISPR-expressing plasmids that are designed to disrupt genes involved in exosome biogenesis, and used these vectors to make cell lines lacking these key genes. Using these reagents, we show that loss of 'exosomal' tetraspanins CD9, CD81, or CD63 do not disrupt the biogenesis of exosomes, and furthermore, that loss of HRS, Alix, and several other candidate exosome biogenesis factors have little if any effect on the vesicular secretion of exosomal tetraspanins. These results are mirrored by a parallel genetic screen in *Drosophila* S2 cells, which shows no substantive role for Alix or other ESCRT-associated proteins in vesicular secretion. In the course of these experiments, we observed that the vesicular secretion of CD63 varies widely from cell to cell, whereas the vesicular secretion of CD9 and CD81 are far more stable, indicating that CD63 is a relatively unreliable marker for studying exosome biogenesis. Our studies also explored the ability of exosomes to deliver molecules from cell-to-cell, both in vitro and in vivo. In vitro, we observed that expression of fusogenic membrane proteins in exosome-producing cells enhanced exosome-mediated, intercellular traffic of exRNAs, demonstrating the potential of exosomes as molecular delivery vehicles. In vivo, we observed that fibroblast/stellate cell-derived exosomes delivered cargo molecules selectively to cancer cells, allowing the inhibition of cancer growth and extension of survival in a rat model of cholangiocarcinoma.

Project 2 - Anna Krichevsky: Full-coverage landscape of extracellular RNA secreted by glioblastoma stem cells

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Background: Extracellular RNAs (exRNAs) are packaged in various extracellular complexes, including microvesicles (MVs), exosomes, and non-vesicular ribonucleoprotein complexes (RNPs). Secretion of exRNA is universal for many types of cells and tissues. However, exRNA may have specific functions associated with the development of malignant tumors such as glioblastoma brain tumor (GBM), where the communication between the tumor cells and brain microenvironment alters the parameters of tumor growth and host response. The global repertoire of exRNA released by glioma cells is not fully characterized, and the degree of its functional uptake by the normal cells of the brain is unknown.

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 investigate the spectrum of the GBM-derived exRNA, enrichment of specific RNA classes and individual species in exRNA complexes, and the levels of uptake by diverse cell types of the brain.

Methodology: Low passage patient-derived tumorigenic glioma cell cultures that represent the most therapy-resistant stem-like cell population (GSC) have been utilized. A novel exRNA isolation protocol has been established to recover vesicular and non-vesicular exRNA fractions from the cell conditioned media. This protocol has been shared with and utilized by other ERCC members. RNA composition of MV, exosome, and RNP fractions was profiled using RNAseq with optimized library preparation steps that increased the coverage for all classes of RNA and minimized the sequencing bias. The data was analyzed using different bioinformatics pipelines, and selected transcripts validated by qRT-PCR. The uptake level by neurons, astrocytes, and microglia has been further investigated using the co-cultures of primary cells and RNA markers highly abundant in glioma exRNA.

Results: We have characterized and quantified the RNA composition of MV, exosomes, and RNP fractions secreted by heterogeneous patient-derived GSC cultures. Different GSC exRNA fractions exhibit highly distinct RNA composition. Patient-derived GSC cultures secret variable amounts of RNA. The majority of exRNA is represented by fragmented rRNA species (~90% in EVs; ~ 64-90 % in RNPs). GSC exRNA is relatively enriched in small RNA species and deprived in mRNA fragments. Among three exRNA fractions analyzed, GSC MV transcripts most closely reflect the cellular transcriptome, making MV the promising (and yet unexplored) fraction for the biomarker discovery. In addition to confirmed miRNA enrichment in exosomes, tRNA and Y RNA fragments are the most abundant and enriched in exRNA fractions. We also compared the cellular RNA composition of normal human brain recipient cells with GBM exRNA and predicted the potential impact of miRNA transfer. Our RNAseq data have been shared with the ERCC consortium.

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.

Project 3 - exRNA Released by Glioblastoma Alters Brain Microenvironment

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Background. Our group has focused on developing tools to monitor the functional exchange of exRNA between glioma cells and normal cells in the brain.

Aims. We have been particularly interested in the extracellular exchange of functional miRNAs and other non-conding RNAs (with Dr. Anna Krichevsky) and mRNAs, as well as retrotransposon sequences between tumor and normal cells, and in how these RNAs change the phenotype of normal cells, including their transcriptome and epigenetic state.

Metholology. We have developed a number of new tags to visually monitor: membrane vesicles using palmitoylated fluorescent proteins; RNA using phage MS2 sequences in the 3'UTR of the message and a fluorescent coat protein that binds to them; and vesicle fate using a membrane bound form of luciferase.
Experiments have been carried out in culture and in a syngeneic glioma mouse model with correlative neuropathology, FACS, intravital imaging in brain chambers (with Dr. Thorsten Mempel); RNA sequencing (with Dr. David Ting) and DNA methylation analysis (with Dr. Aleks Milosavljevic).

Results. We have verified the functional transfer of miRNA and mRNA sequences through the extracellular space in cultures of glioma cells and normal cells, including microglia. We have also found that full length mRNAs can be transferred in extracellular vesicles in combination with the appropriate RNA binding protein (see Scheepbouwer et al., abstract this meeting). Retrotransposon sequences are also transferred between cells in retrovirus-like particles generated from human endogenous retrovirus sequences leading to transient expression of RNA carried within them in recipient cells. Non-coding RNAs from glioma cells are also implicated in changes in the DNA methylation status of recipient endothelial cells (see Rocco et al., abstract this meeting). Using cell and vesicle fluorescent labeling we have confirmed the release of vesicles from glioma cells in the brain and their uptake by surrounding normal cells *in vivo*. Uptake of RNA-containing vesicles by microglia exaggerates their transcriptome to the "M2" type which is supportive of tumor growth and immune suppressive, thus supporting tumor progression.

Conclusions. We have generated tools which can be used by the field to monitor the distribution and effects of exRNA and extracellular vesicles in culture and *in vivo*. Our work supports the functional effects of exRNA on the phenotype of recipient cells, which in the case of tumor exRNA is consistent with promoting tumor growth.

Project 4 - Alain Charest: EGFR Signaling Regulates Extracellular Vesicle microRNA Contents

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Background: Extracellular Vesicles (EVs) are rapidly becoming recognized as an important mode of intercellular communication in normalcy and disease. EVs contain complex cargos, the identity of which remains mostly poorly characterized. It is clear that the contents of EV can serve as bio- monitors of the transcriptional, post-translational and metabolic status of their cells of origin but the mechanisms that dictate the type and identity of molecules that populate cargos are not known.

Aims: Our Project aims to establish an extensive catalog of the extracellular RNA species produced by our mouse Glioblastoma tumors and to study the impact of EGFR activity, one of the most common oncogenes in malignant glioma, on EV cargo composition, with a focus on miRNAs.

Methodology: As a source of exRNA, we used mouse glioblastoma (GBM) cell lines that were isolated from an EGFR-driven genetically engineered mouse model that we created. We isolated different exRNA fractions through differential filtration methods and performed RNA-seq from these fractions to obtain quantitative levels of exRNA. We focused on the miRNA contents of EVs

isolated from cells that had been subjected to EGFR inhibition with targeted therapeutic agents (tyrosine kinase inhibitors) compared to the EV miRNA content from control GBM cell lines.

Results/Current Achievements: We have generated RNA-seq data from small RNA and long RNA isolates from our control and treated cells. We demonstrate that specific species of miRNAs are preferentially included and excluded from EV cargo upon inhibition of EGFR kinase activity.

Conclusions: Our results establish a link between the oncogenic activities of EGFR and the composition of exRNA and miRNA molecules within EVs from GBM cells.

Core: Intravital Microscopy Core: Visualizing cell-cell communication in the tumor environment

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Background: It is currently beyond the reach of even the most sophisticated in vitro methodology to simulate the complex interplay of physical, cellular, biochemical, and other unknown factors that determine the cellular behavior in vivo. This is particularly relevant for the tumor microenvironment (TME), where both cancer cells and a multitude of both innate and adaptive immune cells locally communicate with each other both through direct physical interactions as well as through secreted factors, which determines the outcome of the anti-tumor immune response. The Intravital Microscopy Core is designed to assist the U19 Project: “exRNA released by glioblastoma alters brain microenvironment” in using advanced intravital imaging approaches to examine the role of tumor-derived extracellular vesicles (EVs) in exRNA-mediated communication with the immune system.

Aims: To (i) provide expertise in the design of specific experiments utilizing multiphoton intravital microscopy- based analysis of EV biogenesis by glioblastoma, their properties in the extracellular space and uptake by non- malignant brain cells, as well as of functional exRNA- and EV-mediated intercellular communication between tumor cells and tumor stroma, (ii) provide access to the required instrumentation and to aid in the execution of MP-IVM experiments using mouse models of glioblastoma, and (iii) provide computational resources for the storage, processing, in-depth analysis, and interpretation of digital imaging data.

Methodology: Our lab has adopted the use of fluorescent protein-based reporter systems to visualize cancer cells as well as various cells of the innate and adaptive immune system in transplanted tumor models in mice. Implantation of tumor cell lines either into dorsal skinfold chambers or chronic brain window chambers permits longitudinal imaging of their interaction with the immune system through repeated multiphoton intravital microscopy session under general anesthesia.

Results: In collaboration with the Breakefield lab we have continued to use a GL261 glioma cell line expressing membrane-targeted GFP or RFP to monitor the fate of in vivo-produced EVs in the tumor environments and specifically to characterize their uptake by tumor-associated myloid cells. Furthermore, we have been evaluating the intracranial implantation of GL261 tumor cells encapsulated in nanoporous silica shells filled with micorporous hydrogel. These shells are impermeable to cells but permeable to EVs and will allow us to assess the effects of secreted tumor-derived factors, including EVs, in separation from cell contact-dependent effects on the tumor

environment. Initial data demonstrate that both shells and embedded tumor cells can be visualized by MP-IVM.
In collaboration with the Charest lab we have implanted glioma cells that inducibly express membrane-targeted GFP as well as Cre recombinase into reporter mice that express the red fluorescent protein tdTomato upon Cre- induced recombination that deletes an upstream translational STOP signal. Initial experiments suggest a low rate of horizontal transfer of Cre activity from tumor cells to brain cells that by morphological criteria resemble neurons.
Finally, in collaboration with the Krichnesvsky and Breakefield labs, we are adopting the use of a fluorescent miRNA reporter system developed by the McManus lab in order to assess the horizontal transfer of miRNAs from tumor cells to cells of the tumor stroma. Initial studies will address the role of miR21 in this context.

Conclusions: This Core's capabilities have been well integrated into exRNA and EV-related studies conducted by U19-supported and other groups investigating the TME.

Secreted RNA During CRC Progression Biogenesis Function and Clinical Markers – Robert J. Coffey (Contact PI)

Project 1: Functional changes in secreted RNA biogenesis

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The heterogeneity of secreted RNA (exRNA) carriers has become increasingly evident. Determining the nature of different exRNA carriers is critical for understanding the biogenesis and function of such exRNAs. One class of exRNA carriers are vesicular, larger microvesicles (above 130 nm) and smaller exosomes (40–130 nm). Exosomes represent a heterogeneous group of vesicles with multiple distinct cargos with separable functions; purifying these small vesicles into different pure populations is an important technological hurdle to overcome. Another class of distinct exRNA carriers is ribonucleoprotein complexes, unassociated with lipid vesicles. We have developed specific methods for purifying and analyzing these different exRNA carriers. In one method, we use a commercial flow cytometer and directly labeled fluorescent antibodies to analyze and sort individual exosomes first isolated by sequential ultracentrifugation. This method is termed fluorescence-activated vesicle sorting (FAVS). Using antibodies to EGFR and the exosomal marker, CD9, we have flow-sorted subclasses of EGFR/CD9 double-positive and double-negative exosomes from colorectal cancer (CRC) cells; we have assessed EGFR activation status on individual CRC exosomes using a monoclonal antibody (mAb) that recognizes "conformationally active" and mutant VIII EGFR (mAb 806); we have detected human EGFR and CD9 on exosomes isolated from the plasma of athymic nude mice bearing human CRC tumor xenografts; we have performed multicolor FAVS simultaneously identifying CD9, EGFR and an EGFR ligand, amphiregulin (AREG), on normal human plasma-derived exosomes. These studies demonstrate the feasibility of using FAVS to both analyze and sort individual exosomes based on specific cell-surface markers. In collaboration with Xandra Breakefield's and Bob Carter's labs, we are using FAVS to purify and analyze plasma exosomes isolated from patients with glioblastoma multifome (GBM) that often contain amplified and/or mutant VIII EGFR. By FAVS, we are able to detect plasma-derived exosomes that contain activated EGFR from four patients with GBM with documented mutant VIII EGFR-containing tumors; by immunoblotting, we detect an EGFR isoform of the predicted size for the mutant EGFR and this isoform is tyrosine phosphorylated. We have not identified exosomes containing activated EGFR (mAb 806 positive exosomes) when purified from normal patient plasma, although, as mentioned above, normal plasma exosomes contain total non-active EGFR. Another aspect of exRNA heterogeneity concerns its association with non-vesicular components; we have determined that ultracentrifuge "pellet-pure" exosomes represent a mixture of small vesicles and protein complexes, as determined by flotation density gradient fractionation. Preliminary analysis of exosome gradient fractions from a CRC and GBM cell lines indicate distinct fractions with different protein and miRNA content. We are continuing our work to understand differences in regulation of exRNA biogenesis in these various vesicular and non-vesicular exRNA carriers.

Vanderbilt Project 2: Trafficking of exRNAs in KRAS Mutant Colon Cancer

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Mutant KRAS colorectal cancer (CRC) cells release exosomes that can alter the tumor microenvironment. The goal of our project is to test the hypothesis that KRAS signaling selectively controls the sorting of specific RNAs and RNA-binding proteins into exosomes. In recent work funded by this project, we have previously shown that some exosomal miRNAs are differentially sorted into exosomes based on KRAS mutant status and that those miRNAs can contribute to changes in gene expression in recipient cells. Furthermore, we have demonstrated that the RISC machinery component Ago2 is differentially sorted to exosomes based on KRAS-MEK-ERK signaling. To test whether mutant KRAS might regulate the composition of other secreted RNAs (mRNAs and lncRNAs), we compared RNAs from cells and matched exosomes derived from isogenic colorectal cancer cell lines differing only in KRAS status. We show that many RNAs are differentially enriched in exosomes compared to parent cells. From mRNA seq data, we observe strong enrichment of Rab13 in mutant KRAS exosomes. In addition to coding genes, we find enrichment of antisense RNAs and transcripts that map back to pseudogenes in exosomes compared to cellular RNA profiles. To test if extracellular RNAs can be transferred between cells (Aim 1), we have developed a method of tracking RNAs through modification of the CRISPR-Display system, which allows specific guide RNAs from donor cells to enable activation of a luciferase reporter in recipient cells. Additionally, to test if full length mRNAs can be trafficked extracellularly between cells, we have also designed an HA-tagged mRNA transferred from donor to recipient cell, allowing for RT-qPCR of transferred mRNA abundance through amplification of the HA-tag RNA coding sequence found on the 3' end of our RNAs of interest. These new methods aim to test if long extracellular RNAs are potentially trafficked in a functional state and which kinds of vesicles preferentially transfer long versus short RNAs. We are also working on the biogenesis mechanism for Ago2 and RNA-carrying vesicles, testing both ceramide and ESCRT-mediated pathways for their role in Ago2-RNA sorting to exosomes (Aim 3). Thus far, we find that inhibition of ceramide synthesis in DKs-8 colon cancer cells causes a ~50% decrease in exosome release. However, analysis of equal numbers of vesicles by Western blot reveals a slight increase in the Ago2 content of exosomes with no alteration in the levels of the ESCRT component TSG101, and other exosome markers CD63 and HSP70. Furthermore, prion protein, a reported receptor for Ago2 was also not decreased. We are confirming these data in separate cell types and also plan to perform RNA-Seq on control and ceramide-inhibited vesicles to identify any changes in RNA and RNA-binding protein content. These data will test previous reports that the RNA-containing vesicles are sensitive to ceramide pathway inhibition and relate Ago2 and Ago2-dependent RNAs to that pathway. As a future goal, these latter experiments can be combined with the CRISPR-Display and HA-mRNA systems to relate biogenesis mechanisms to RNA transfer and uptake by recipient cells.

Vanderbilt Biotechnology and Bioinformatics Core

Secreted RNA during CRC progression: biogenesis, function and clinical markers

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The biotechnology and bioinformatics core provides transcriptome sequencing and data analysis services to all research groups in the Vanderbilt U19 project. During the grant period, the core has generated long RNA-Seq data for ~60 samples and small RNA-Seq data for ~60 samples. All these data sets have been analyzed by standardized bioinformatics pipelines that include algorithms for quality analysis, reads mapping, transcript quantification, and differential expression analyses. All raw data, intermediate results, and final results are stored and backed up by the Vanderbilt Advanced Computing Center for Research and Education (ACCRE). The ACCRE servers can be directly accessed by all project investigators. Selected data sets have been uploaded to the Genboree and the Gene Expression Omnibus (GEO).

Small RNA sequencing and data analysis for three isogenically-matched human colon cancer cell lines that differ only in the mutation status of the KRAS oncogene showed that more than 85% of the reads from the cellular RNA libraries could be mapped to the genome, whereas only 50–71% from the exosomal libraries were mappable. In cells, the majority of small RNA reads mapped to microRNAs (miRNAs), whereas in exosomes, the majority of small RNA reads mapped to repetitive elements. The size distribution of cellular small RNA matched that expected from miRNA-derived reads (21–23 nucleotides). However, the small RNA read distribution from exosomes was much broader with many reads smaller than 22 nucleotides in length. A comparison of miRNA 3' trimming and tailing between cells and exosomes showed that exosomes had a slight increase in trimmed miRNAs. Our quantitative analysis showed that exosomal miRNA profiles were distinct from cellular miRNA profiles. Importantly, KRAS-dependent differential miRNA expression affected miRNA expression patterns in exosomes more prominently than in the parent cells, indicating that mutant KRAS may alter sorting of specific miRNAs to exosomes.

In addition to providing routine services, the core also develops new bioinformatics methods for data analysis. We developed a new computational pipeline for analyzing circular RNAs (circRNAs) based on RNA-Seq data generated from total RNA preparations and applied the pipeline to the long RNA-Seq data generated from the three human colon cancer cell lines mentioned above. We showed that circRNAs are significantly down-regulated at a global level in mutant KRAS cells compared to wild-type cells, indicating a widespread effect of KRAS mutation on circRNA abundance. In all three cell lines, circRNAs were also found in exosomes. circRNAs were more abundant in exosomes than cells, suggesting that circRNAs may be more stable than their linear transcripts and may serve as promising cancer biomarkers. Interestingly, some circRNAs down-regulated in KRAS mutant cells were also down-regulated in KRAS mutant exosomes, whereas other circRNAs followed different trends in the cells and exosomes, suggesting that there is a complex exosomal trafficking mechanism for circRNAs.

Genetic Models for exRNA Communication - Michael McManus(Contact PI)

Next generation exRNA posi-sensors

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Background: We have spent effort developing and optimizing our posi-sensor systems to provide the most robust and sensitive readouts for exRNAs in cell culture systems with mixed success. Although the TetR exRNA posi-sensor worked wonderfully in cell culture, both in sensitivity and rheostat reporting (ie fluorescence intensity depending on the concentration of the exRNA), its performance lacked in the broad compendium of cell types due to poor CMV promoter activity in most tissues. We spent time reconfiguring the TetR system to use different promoters, with some minor successes, but ultimately we needed to completely overhaul the system. The discovery of dCas9 offered us a special opportunity to re-engineer the posi-sensor circuit design for primary mouse tissues. However additional hurdles abound. Would the dCas9 system work in primary tissues? What level of sensitivity could it achieve? Could it provide rheostat readout, where fluorescence intensity depends on the concentration of the exRNA?

Aims: Our overall aim is to develop a second-generation posi-sensor system that is based on dCas9 mediated silencing of a reporter, where exRNA activity releases silencing and triggers reporter expression. This requires us to develop and characterize dCas9 transgenic mice and develop new approaches that obviate challenges associated with in vivo TetR based systems.

Methodology: To test the dCas9 based posi-sensor concept, we created two basic dCas9 sensor systems, neither of which depends on the CMV or TetR systems. Importantly, our new strategies can be used with any promoter. For our purposes we developed the system with the most robust ubiquitously expressed mouse promoter (CAGGS).

Results: In the first strategy, miRNA binding sites were placed in the sgRNA. We chose to create sgRNA constructs that contained binding sites in the structural loops of the sgRNA, with the hypothesis that sgRNA activity could be retained despite the presence of a larger RNA loop. We tested this system using an sgRNA targeting GFP expression in GFP+ dCas9-KRAB+ 293T cells. We found that placing the miRNA binding sites within one of the loops of the sgRNA totally disrupted sgRNA activity, rendering this position ineffective for posi-sensing. However, another loop position retained much of its native activity. Unfortunately, no miRNA dependent fluorescence change occurred when this construct was tested for posi-sensing. These data suggest that the sgRNAs are not effectively targeted by miRNAs, diminishing our enthusiasm for further exploring strategy one. In a second strategy, miRNA binding sites were engineered into the 3'-UTR of the dCas9 mRNA. This strategy is most similar to the TetR strategy, since it is based on the silencing of the effector protein molecule. To test this strategy GFP+ 293T cells were infected with both an sgRNA targeting GFP and a dCas9-KRAB construct harboring miRNA binding sites for either miR-h155 (not expressed negative control), miR-21 (expressed at a moderate level), or miR-17 (an abundantly expressed miRNA). We observed that the percentage of GFP positive cells increases with miRNA abundance, suggesting this as a viable strategy for a dCas9-based posi-sensor. One notable caveat is that unlike the TetR based system, GFP fluorescence intensity is not proportional to the miRNA level of expression, ie the system may not give a rheostat readout.

Conclusions: One of our tested dCas9 posi-sensor system shows promising results, offering the highest expression and lowest background that we have seen in any of our assays. Considering these favorable results, we are now in the process of conducting in vivo experiments for our exRNA sensors.

The role of *SidT2* in exRNA biogenesis in mouse

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Background: The *C. elegans* SID-1 protein is a transmembrane channel that transports extracellular RNA into cells [1, 2]. The mouse SID-1 homologs (*SidT1* and *SidT2*) also transport ExRNA into cells [3]. *SidT1* and *SidT2* knockout (KO) mice are viable, but *SidT2(KO)* mice are deficient for viral-induced interferon responses and succumb more readily to virus infection than controls [3]. *SidT2(KO)* pups are also significantly smaller than control pups.

Aims: We hypothesize that *SidT1/T2* interacting RNAs are likely to be functional ExRNAs. Our immediate aims are to use *SidT1/T2(KO)* mice and *SidT1/T2* knock-in epitope tagged mice to identify *SidT1/T2*-dependent ExRNAs.

Methodology: We are (1) adapting single-cell RNA-Seq protocols [4] to plasma ExRNA samples. We are also (2) adapting published CLIP-Seq protocols to membrane proteins using heterologous SID-1::FLAG expression in *Drosophila* S2 cells as a proving ground for eventual SidT1/T2 CLIP-Seq analysis of mouse cells and tissues. We have (3) used modified CRISPR-based approaches to efficiently tag both genes in mouse.

Results and conclusions:
(1) We use Trizol, followed by DNAse, phenol::$CHCl_3$ and ethanol precipitation to isolate 50-100ng of total RNA per ml of cell-cleared and filtered (0.45 micron) plasma. We then use the single-cell CelSeq protocol [4] to prepare RNA-Seq libraries from a dilution series of the purified RNA, including samples spiked with lysed whole-blood samples to determine whether RNA from lysed cells is a concern. Because this protocol is selective for mRNA, analysis is limited to annotated mRNAs, which constitute only a minor fraction of all reads. Efforts to annotate and quantify the non-mRNA reads continue. Our ongoing analyses of two-to-four technical replicates of four biological replicates of each genotype indicate: a) that 70-250 μl of plasma (5-25 ng of total RNA) is sufficient for single-cell RNA seq analysis, thus this protocol should enable analysis of ExRNA from less abundant fluids; b) RNA from lysed cells does not contribute to the mRNA counts; and c) compared to controls *SidT2(KO*) plasma has more RNA (2X more reads, 4-5 more unique sequences detected). The increased sequence depth suggests a biological impact of *SidT2KO* and also complicates identification of *SidT2*-dependent ExRNA.
(2) Transfected S2 cells expressing SID-1::FLAG transport RNA and the fusion can be readily immune-precipitated from cell lysates. However, radiolabeled dsRNA, a SID-1 transport substrate, co-immune precipitates with SID-1::FLAG independent of the cross-linking treatment. This indicates that the RNA may be interacting indirectly with SID-1 in the lysates (protein-protein-RNA and/or membrane-protein-RNA aggregates) obscuring detection of signal from any direct interactions. Specificity-control experiments to identify and correct the situation are in progress.
(3) Although genetic complementation experiments indicate that *SidT2-HA_3* is functional, we cannot yet detect the tagged protein by either IF or western blot. Experiments are in progress to determine whether this is an issue of protein abundance or tag stability.
(4) Genetic and cross-fostering analysis indicates that the *SidT2(KO)* small pup phenotype is a maternal effect. We have not detected any maternal care deficiencies, but because *SidT2* is expressed in lactating breast tissue we are exploring whether the *SidT2(KO)* affects the RNA content or abundance of milk and whether this may be responsible for the small pup size.

1 Science. 295:2456-9 (2002).
2 Science. 301:1545-7 (2003).
3 Nguyen T.A. et al., (in review).
4 Cell Rep. 2:666-73 (2012).

Milk-derived RNAs as paradigm to study exRNAs biogenesis and function

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Background and Aims: Many classes of extracellular RNAs (exRNAs) are detected in mammalian body fluids, including breast milk, raising questions about their origins and putative functions. The ease to isolate high amount of milk, and its high exRNAs content (including miRNAs), establish milk as an attractive model to investigate exRNA biogenesis and function. Our objective is to characterize the biogenesis, stability and potential function of milk microRNAs (miRNAs) and their biogenesis/effector proteins (RNA silencing pathway). We notably aim at (1) deciphering if the different milk fractions (e.g. vesicular vs non-vesicular) contain distinct miRNA populations, (2) determining which proteins are associated with milk miRNAs, (3) monitoring their stability and their potential functional transfer in mouse neonate and in *in vitro* cultured cells.

Results and conclusions:
(1) We confirmed that human, bovine, and mouse milk contains high amount of RNA (200-400 ng/ml) mostly concentrated in milk fat globules (MFGs). Interestingly, MFGs exRNAs contain non-degraded ribosomal RNAs, are qualitatively different from milk-associated cells RNAs (e.g. by agarose/acrylamide gel analysis), and carry high level of distinct miRNAs (miR-22, mir-200s, miR-148a, miR-21). Moreover, in MFGs, we could detect high level of several cytoplasmic (but not nuclear) miRNAs biogenesis factor (High: DICER, TRBP, PACT; Low: DROSHA, DGCR8), and also high level of the miRNA-loading effector AGO1 (but low level of AGO2). As AGO2 is generally the most abundant argonaute (AGO1-4) in mammalian tissues and cells, this observation strongly suggests that an Argonaute sorting mechanism leads to preferential incorporation of AGO1 in MFGs. We are currently validating this hypothesis *in vivo* by characterizing Argonaute expression profile, and subcellular localization in lactocytes.

(2) By tracing milk-borne miR-21 or miR-22 in corresponding knockout pups fed by heterozygous mothers, we confirmed their accumulation in their stomach content (at postnatal day 3, 6 and 10). However, milk-borne miR-21 or miR-22 level rapidly drops in the small intestine to reach undetectable level (by qRT-PCR) within the first half of the duodenum. We could also not detect these milk-born miRNAs in neonate peripheral organs, including liver, spleen, kidney and thymus. Surprisingly, level of miR-22 and miR-21 in milk are not impacted by maternal AGO1/3/4 triple knockout, suggesting that these specific argonaute factors are not required for the sorting and/or stabilization of milk miRNAs in this context.

(3) Finally, we developed a mouse model allowing specific expression of a membrane-bound GFP in lactocytes, and showed efficient i*n vivo* fluorescent labeling of milk EVs. By combining this model with high-resolution flow cytometry we demonstrate that, while milk EVs are most abundant in duodenum lumen, they also accumulate at more distal part of the intestine indicating that a significant portion survive the harsh digestive environment. In parallel, we apply a similar approach with a lactocyte-specific expression of GFP-AGO2 to characterize the bio-distribution and possible uptake of this particular miRNA effector protein in neonates. Finally, we are also testing whether uptake and functional transfer of milk EVs can occur *in vitro* using breast or intestine epithelial cell models.

exRNA mouse core

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Background: We have created a bevy of Cas9 mice to offer a faster and less expensive means to interrogate exRNAs. This allowed us to more economically expand our repertoire for producing exRNA deleted alleles in the posi-sensor system and as it saves time in mating our existing microRNA knockouts. We have developed turnkey protocols for ablating exRNAs in mouse zygotes, using sgRNA:wtCas9 complex injection. We have also begun developing novel technologies that employ CRISPR perturbation in mice, including a degron based dCas9 system. Collectively these data give us great enthusiasm for the system in our exRNA studies.
Aims: As described in the original proposal, the Core serves two major roles: to produce reagents to advance the project, namely to produce mice and lentiviral constructs and tittered virus. We have been productive over the last year, continuing to develop and refine new mouse-based systems as described in last years progress report (site-specific targeting of transgenes in mouse models and Cas9 based mouse models). We have focused on systems that help us maximize time and quality of data. New developments will impact investigators using mouse models: optimizing the use of Cas9 in mouse model systems, development of site-specific targeting protocols for the rapid creation of transgenic exRNA sensor mice, and a degron-based dCas9 tool for inducibly altering exRNAs.

Methodology: Most of our site-specific targeting technologies have focused on delievery to the *Hipp11* locus, although we have a ROSA26 system available for use, particularly for our future goal of creating orthogonal multi-component systems. Briefly, in the case of the *Hipp11* locus targeting, constructs are recombined into an attB-site containing site, aided by the Phi31 integrase protein. This yields a single copy heterozygous transgenic mouse containing the specific transgene. Although this system is not foolproof, it avoids

many of the issues related to other technologies such as traditional transgenesis and embryonic stem cell based mouse production. We have also recently begun to characterize an inducible Cas9 system, based on N-terminal rule degron technology. The goal is to advance the current crop of Cas9 systems to allow targeting in the adult animals. We will continue to develop and refine these methods during the remainder of the project period with hopes that it will be of value to investigators.

Results: The above systems can be remarkably fast and efficient; at the same time, they are not perfect. For example, we have encountered situations where sgRNA zygote injections to cause embryo death (we suspect that the RNA concentration is to blame). We have also encountered genetic mosaicism (the nuisance of genotyping the F1 population and determining that a CRISPR edit did not go germline). An additional caveat we encountered related to a limitation where large numbers of edited loci (3 or more) are impossible to achieve within the same zygotic cell and have them go germline. Nonetheless, CRISPR technology is really shining a light on the ability to rapidly and economically make mice and the Core is continuing to serve our needs.
Conclusion: We hope that others in the consortium reach out to us in 2016 and hereafter—as we stand ready to help generate reagents for other labs in the cooperative network.

Definition of Serum Ribonucleoprotein Composition and Its Regulation and Function - Thomas Tuschl (Contact PI)

Definition of serum ribonucleoprotein composition and its regulation and function

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Core: Tuschl lab
Immune responses against RNA binding proteins (RBP) and extracellular RNAs are a hallmark of certain autoimmune diseases including Lupus nephritis. To better understand the pathogenesis and progression of these autoimmune diseases we sought to determine the origin of exRNAs, factors influencing their availability in blood and plasma as well as mechanisms contributing to the activation of RNA-mediated stress pathways by (1) studying fluctuations in exRNA composition in normal controls and (2) identifying RNA binding targets of autoantigen RPBs and (3) characterizing exRNAs in SLE patients carrying distinct classes of autoantibodies

Subproject 1:
exRNA isolation was performed on cell-free of serum (96) and plasma (216) samples from 13 healthy controls collected at twelve different time points throughout the period of two months, using an automated isolation protocol. After performing multiplexed RNA-based cDNA library preparation, exRNA was reverse-transcribed, PCR-amplified and subjected to Illumina HiSeq sequencing. RNAseq data was demultiplexed and mapped against a curated small RNA (sRNA) transcriptome. miRNA analysis of normal controls is currently conducted using unsupervised clustering and differential expression analyses. Other major RNA classes have been mapped and their read-length distributions have been generated. We are currently analyzing variabilities in exRNA compositions amongst normal controls and aim to evaluate the significance in deviating profiles observed in some individuals, while performing sample collection of samples for a larger cohort for reference profiling.

Subproject 2: Putterman lab (Einstein College of Med) and Tuschl lab (Rockefeller University).
Additional 230 subjects were recruited to the Einstein Lupus Cohort with a total of >480 individual subjects currently in the cohort. 10% of the cohort subjects are <18 years old. Immortalized B-Cell lines of lupus nephritis have been generated and sRNA and mRNA analyses have been completed. exRNAs present in sera from patients with defined serological profiles including SSB/La positive, SSB/La negative profiles and combinations will be analyzed and compared to the PAR-CLIP dataset to identify immune-stimulatory

RNAs. Next, we will enrich autoantibodies against SSB/La and TROVE2/SSa2/Ro from patient blood samples by immunoprecipitation using protein G-coupled magnetic beads and isolate captured RNAs. This attempt should enable us to identify highly immunostimulatory RNPs, which are released in circulation and may contribute to autoimmunity.
To study cellular stress responses in a cellular model systems, we established all cell lines (SSB/La, SSA/Ro60, Ro52/TRIM21, and additional 100 cell lines) to catalog the composition of SG triggered by different environmental stresses. CRISPR KO cells were generated for many proteins implicated in cellular stress response, such as TIA1, TIAR, G3BP1, HuR. We could show that TIA1/TIAR proteins are important for cell viability since a double knockout of both proteins is lethal. Upon TIA1/TIAR KO, activation of PKR leads to phosphorylation of EIF2S1and SG formation.
Furthermore, we established assays using cGAMP as a power full reagent for activation of innate immunity in STING-expressing cells, such as THP1 monocytes, serving as a critical reference point in testing RNA-based innate immunity pathways.

Subproject 3 Patel lab (Memorial Sloan Kettering Cancer Center) and Tuschl lab (Rockefeller University).
The RNA targets of SSB/La were identified using an optimized version of the PAR-CLIP protocol in HEK293 cells inducibly producing SSB/La protein. This optimized protocol is currently being used to identify the RNA targets of further Lupus autoantigens RBP (e.g. TROVE2/SSa2/Ro). RNA targets of SSB/La are predominantly RNA polymerase III transcripts, and in particular 3' ends of tRNA precursors, 5S rRNA, and ncRNAs, such as 7SL small cytoplasmic or U6 small nuclear RNA. Efforts are currently underway to generate stable RNA bound complexes of both La and Ro towards efforts at crystallization and structure determination.
Bacterially expression constructs of human Translin and of C3PO and were expressed and recombinant proteins were purified. RNA-binding properties of both Translin and C3PO were studied by electrophoretic mobility shift assay (EMSA) using short RNA oligonucleotides and binding characteristics were determined. We have purified both Translin and C3PO protein-RNA complexes by gel-filtration chromatography and performed extensive crystallization screening of the complexes. We obtained well-diffracted crystals of Translin crystallized in presence of two single stranded RNA sequences. The structures were determined at 2.2 Å and 2.74 Å resolutions, respectively, and revealed minor differences in the arrangement of octameric Translin, but no bound RNA substrate in the expanded hollow interior of the closed-barrel structures. We have also obtained small crystals of the truncated wild-type C3PO in the RNA-free state, which are currently being optimized to attain diffraction quality.

Session V : Comprehensive Reference Profile of Extracellular RNA in Human Biofluids and Bioinformatics Tools Update

Small RNA Profiles in Normal Human Biofluids

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A diverse range of small RNAs can be found in human biofluids. To characterize these, we are performing small RNA sequencing analysis on 11 different biofluids, from 5-10 normal human donors. To date, we have processed plasma, serum, urine, bronchoalveolar lavage fluid (BALF), cerebrospinal fluid (CSF), saliva, sputum, cord plasma, and amniotic fluid. Here, we report the results obtained from a modified small RNA-Seq protocol that uses adaptors with randomized ends to reduce bias and is suitable for small amounts of RNA. For each of the samples, we were able to detect small RNAs, including miRNAs and fragments of tRNAs, and Y-RNAs, long non-coding RNAs and mRNAs. The fraction of mapped small RNA reads annotated as miRNAs ranged from 0.2%-86.8%, with relatively low amounts of miRNA in urine, amniotic fluid, and CSF and higher amounts in plasma, serum, BALF, and plasma from cord blood. tRNA fragments represented <1%-72.8% of reads, with relatively few tRNA fragments in plasma, cord plasma, and BAL, and more tRNA fragments in urine and amniotic fluid. Unsupervised hierarchical clustering based upon highly variable miRNAs showed good separation of samples by the biofluid of origin, except that saliva and sputum samples were similar. Our results provide a useful comparison between small RNA populations in a large set of diverse biofluids utilizing the same massively parallel sequencing method for each sample. Ongoing analyses are addressing the likely cellular origins and mRNA targets of miRNAs found in different biofluids.

Phylogenetic analysis of exogenous sequences from extracellular RNA-seq data using the exceRpt pipeline.

Joel Rozowsky[2], Robert Kitchen[1,2,3], Timur Galeev[1,2], Sai Subramanian[4], William Thistlethwaite[4], Matthew Roth[4], Aleksandar Milosavljevic[4], Mark Gerstein[1,2,5]

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The exceRpt pipeline (github.gersteinlab.org/exceRpt) of the Extracellular RNA Communication Consortium (ERCC) has for several years allowed users to analyze small-RNA-seq data from extracellular preparations. A major feature of the pipeline is a comprehensive series of quality filters designed to reduce wet-lab contamination and the variable quality of small-RNA annotation. An additional advantage of these filters is that they provide greater confidence when attempting to detect exogenous nucleic acids using reads not mapped to the endogenous (human or mouse) genome or transcriptome.

We present updates to the exceRpt pipeline focusing on these exogenous analysis components. In addition to the current phylogenetic tree based analysis of exogenous reads mapping of reads to non- host genome species, we have extended the pipeline to separately perform a similar phylogenetic analysis using sequences mapping to non-host ribosomal sequences. Existing approaches to studying the microbiome tend to rely either on alignments to 16S bacterial/archeal (or 28S fungal) rDNA sequences or to a pre-processed set of reference genomes in which highly degenerate sequences (i.e. those that co-occur across multiple species) have been removed. This, however, results in an extraordinary loss of potentially valuable data as the occurrence of reads aligning to non-ribosomal or to multiple species/strains is very frequent (given the similarity of many of these genomes). To resolve this we have developed a novel algorithm for characterizing alignments to all available exogenous rDNA sequences and genomes in terms of the NCBI taxonomy tree. By traversing downwards through the taxonomy tree in search of the optimal level at which each read should be allocated, we can provide the most specific description possible for each read while still allowing it to multi-map to a large number of individual species.

The advantage of aligning to both rDNA and full genomes is that we can capture as many reads as possible. The ~3 million bacterial, archeal, and fungal rDNA sequences in the RDP enable an extremely broad survey of the microbiome. However we typically capture 10x more reads from only the ~12,000 sequenced genomes, suggesting that many of the exogenous exRNA reads are not of ribosomal origin. We show that the alignments to the rDNA and genomes are largely consistent, but we also show how taxonomy-resolved

alignments to the rDNA sequences can reveal gaps missing species/families/etc in the exogenous genomes. We will also compare the resulting exogenous ribosomal based trees against those from the full exogenous genome based trees for a variety of different biofluids using extracellular RNA-Seq datasets from the exRNA Atlas (exRNA.org).

Systematic, multi-institution and multi-protocol assessment of next generation sequencing for quantitative small exRNA profiling

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Background: Extracellular RNAs (exRNAs) are of increasing interest both because of their potential utility as non-invasive biomarkers and their potential functional roles as mediators of cell-cell communication. The advent of next generation sequencing offers unprecedented opportunities for characterization and study of exRNAs. Whereas performance characteristics of NGS for long intracellular RNAs are rather well-understood, NGS for exRNA commonly requires small RNA profiling from low-input samples, which involves fundamentally different library preparation methods with distinct protocols and technical biases. The accuracy, precision and quantitative capabilities of small RNA profiling across varying protocols, platforms and laboratories is not well known, even though such reference data is critical for enabling reproducible research.
Aims and Methodology: With the aim of evaluating the accuracy, reproducibility and technical bias of current exRNASeq protocols, we conducted a Cross-U01 exRNASeq study using a highly standardized protocol and standard reference samples. We provided a detailed set of instructions for library preparation and sequencing as well as common reference samples. The samples, (i) a synthetic equimolar pool containing 1,152 synthetic RNA oligos including microRNAs and non-microRNA sequences of varied sequence and length from 15 nt to 90 nt, (ii) two ratiometric pools A and B containing 330 sequences in 15 different ratios, and (iii) a human RNA pool isolated from a pool of plasma samples, were distributed to all the participants. Nine participating groups (Laurent lab, UCSD; Erle lab, UCSF, Ghiran lab, BIDMC; Jensen lab, TGen; Nolte lab, UUTR-Netherlands; Freedman lab, UMass; Wang lab, ISB; Galas lab, PNRI and Tewari lab, U. of Michigan) prepared and sequenced quadruplicate libraries from each of the common samples using the Ilumina TruSeq small RNA kit and an alternative library preparation protocol(s) of their choice. As a result we accessed a range of protocols and labs with a common set of samples. Sequencing data were centrally analyzed using the Genboree exceRpt Small RNA-seq pipeline.
Results: Using the equimolar reference sample, we confirmed that commonly used exRNASeq protocols are affected by sequence-dependent bias as a consequence of library prep procedures, with protocol- specific differences in the observed bias. Interestingly, "home-brewed" protocols using adaptors with degenerate bases at the end were able to partially ameliorate this bias. Using the ratiometric pools, SynthA and SynthB, we found that small RNA seq is remarkably accurate and reproducible even across multiple laboratories using different methods and platforms. For example, the method was able to distinguish samples with as little as 0.5-fold difference in relative abundance of exRNA species. In addition, the accuracy of differential expression was remarkably reproducible between labs (e.g., %CV was generally of <20% for the majority of RNA sequences).
Conclusions: In conclusion, our results suggest that although small RNA library preparation protocols have sequence-dependent technical biases, exRNA-Seq is a powerful and fairly reproducible approach for relative quantification of exRNAs across diverse samples.

Session VI: Extracellular RNAs as Biomarkers and Liquid Biopsy in Human Disease

MicroRNAs in Human Cerebrospinal Fluid as Biomarkers for Alzheimer's Disease

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Background: Currently available biomarkers of Alzheimer's disease (AD) include cerebrospinal fluid (CSF) protein analysis and amyloid PET imaging, each of which has limitations. The discovery of extracellular microRNAs (miRNAs) in CSF raises the possibility that miRNA may serve as novel biomarkers of AD.

Objective: Investigate miRNAs in CSF obtained from living donors as biomarkers for AD. Methods: We profiled miRNAs in CSF from 50 AD patients and 49 controls using TaqMan® arrays.
Replicate studies performed on a subset of 32 of the original CSF samples verified 20 high confidence miRNAs. Stringent data analysis using a four-step statistical selection process including log-rank and receiver operating characteristic (ROC) tests, followed by random forest tests, identified 16 additional miRNAs that discriminate AD from controls. Multimarker modeling evaluated linear combinations of these miRNAs via best-subsets logistic regression, and computed area under the ROC (AUC) curve ascertained classification performance. The influence of ApoE genotype on miRNA biomarker performance was also evaluated.

Results: We discovered 36 miRNAs that discriminate AD from control CSF. 20 of these retested in replicate studies verified differential expression between AD and controls. Stringent statistical analysis also identified these 20 miRNAs, and 16 additional miRNA candidates. Top-performing linear combinations of 3 and 4 miRNAs have AUC of 0.80–0.82. Addition of ApoE genotype to the model improved performance, i.e. AUC of 3 miRNA plus ApoE4 improves to 0.84.

Conclusions: CSF miRNAs can discriminate AD from controls. Combining miRNAs improves sensitivity and specificity of biomarker performance, and adding ApoE genotype improves classification.

Isolation of exRNA from clinical biofluid specimens and exRNAseq analysis

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Background: Very few biomarkers or indicators exist that allow determining diagnosis and prognosis of kidney disease or allow predicting rejection of kidney transplants.

Aims: Characterization of urine RNA composition and identification of novel urine-derived RNA-based biomarkers for kidney diseases.

Methodology: We developed protocols for isolation of extracellular RNA (exRNA) from urine and plasma/serum for parallel processing of hundreds of clinical samples to obtain healthy reference and disease signature RNA profiles. Reproducible recovery of exRNA requires highly denaturing RNA isolation conditions to inactivate abundant extracellular ribonucleases (RNases) present in these bodily fluids. By adjusting organic extraction conditions, it is possible to sequentially extract exRNA and exDNA. This will allow performing DNA profiling approaches, without requiring additional biofluid samples. exRNAs were characterized using barcoded small RNA cDNA library preparation, followed by 50-nt 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 abundance of fragments of other RNA classes, such as tRNAs, snRNAs, scRNAs, rRNAs, mRNAs and lncRNAs. High content of non-HG19-mapped reads, which frequently exceeds 50% of the total reads in urine samples, required the development of approaches to identify non-human RNA sources.
Results: At present we have isolated exRNA from more than 1200 urine samples of patients with chronic kidney disease, lupus nephritis and kidney transplant patients as well as several hundred plasma/serum samples. Automated contig assembly techniques combined with BLASTN-based sequence queries against the non-redundant sequence database allowed to identify sources of foreign RNA, which originate from the normal microbial flora, potential asymptomatic colonization and infections as well as secondary colonization from other microbial reservoirs (e.g. skin).

Conclusions: The majority of urine miRNA composition mirrors the composition in plasma. Links between miRNA composition and kidney-derived biometric parameters such as estimated glomerular filtration rates were identified. Microbial composition, which can vary considerably between samples, may influence the development and outcome of kidney disease.

Validation of miRNA as diagnostic and prognostic biomarkers for multiple sclerosis

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Background: Multiple sclerosis (MS) is an autoimmune disease characterized by progressive neuronal demyelination, resulting in varying degrees of disability over time. MS is usually diagnosed based on the clinical symptoms and MRI analysis. There is an urgent need to find blood-based biomarker for MS disease diagnosis, disease stage, and prognosis and to study treatment response.

Aims: Following a two-year discovery and validation phase, we performed a multi-center international validation study aiming to investigate the potential use of miRNA expression as non-invasive biomarkers for MS disease diagnosis, disease stage, and association with MS clinical parameters.

Methodology: Serum samples from MS patients and healthy controls (HC) were collected across the following four centers; 1) CLIMB (Comprehensive Longitudinal Investigation of MS at BWH, (n=74), 2) AUBMC Multiple Sclerosis Interdisciplinary Research (AMIR), Lebanon (n=87), 3) The STOMPII- Stockholm Prospective Assessment of Multiple Sclerosis, Sweden (n=120), and 4) The Multiple Sclerosis Genetic Susceptibility Study (MSGSS), UCSF (n=67). RNA was extracted, and miRNA expression levels were detected by LNA-based qPCR (Exiqon). miRNA expression was then correlated to disease state (MS patients vs. HC groups) as well as to disability score assessed using the Kurtzke Expanded Disability Status Score (EDSS).

Results: We identified several previously validated miRNAs that correlate with disease state and disability status that were corroborated by data obtained from several independent centers. One miRNA was consistently up-regulated in MS patients in comparison to the HC groups among two independent centers. Of note, this molecule has been demonstrated to enhance NF-κB activity by targeting NF-κB regulators. This is intriguing, as NF-κB has emerged to be a major player in MS pathophysiology.
Differential expression of four previously validated miRNAs was established across two of the four centers in patients with secondary progressive MS (SPMS) in comparison to the HC groups. Excitingly, the molecule of interest that regulates NF-κB activity was also validated in this SPMS vs. HC comparison. In addition, we discovered that the expression of three previously validated miRNAs significantly correlated with patient disability score in at least two independent centers. Notably, one of these miRNAs has been shown to regulate COX2, which is overexpressed in MS lesions, and has been implicated in MS pathophysiology. A very exciting finding was that the expression level of one previously validated miRNA that negatively correlated with disability score was validated across all four independent centers. These findings suggest that this miRNA is a strong candidate biomarker that may be useful in predicting or assessing patient disability, and perhaps response to treatment.

Conclusions: We found several miRNAs of interest that exhibit clear biological significance in MS pathophysiology and are promising candidates for their potential use in developing predictive diagnostic, prognostic, or therapeutic tools for MS.

Note: The name of miRNAs were not mentioned in the abstract due to their consideration for patent filing.

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Poster Presentations Abstracts

Session I: ERCC Studies of Extracellular RNA for Human Therapeutics

1. Novel extra cellular RNA-based combinatorial RNA inhibition therapy

Pinar Kanlikilicer[1], Mohammed Helmy Rashed[1], Justyna Filant[2], Maria Ines Almedia[1], Rahul Mitra[2], Theresa J. O'Halloran[3], George Calin[1], Anil Sood[2], Gabriel Lopez Berestein[1]

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RNA molecules are secreted into extracellular space (exRNAs) and play an important role in cell-cell communication. The innovative therapeutic concept in our UH2 program is the depletion of oncogenic microRNAs (miRNAs) from malignant cells by enhancing their secretion as exRNAs or blocking suppressor microRNAs delivery into extracellular space by blocking secretion mechanisms. We aimed to maximize therapeutic benefit by the combination of siRNA and miRNAs to target exRNA production. We have identified some molecules of interest for targeting exosome release as well as candidate long non-coding RNAs and miRNAs; miR-6126, miR-940, miR-29b and miR-1246 as the significantly differentially over expressed in exosomes compared to the originating cells in an ovarian cancer panel. We showed that miR-6126, miR-940 andmiR-29b act as a tumor suppressor miRNAs by inhibiting oncogenic pathways. We also inhibited the exosome release using both siRNA and small molecule inhibitors. The purpose is to increase the tumor suppressor miRNAs in the target cancer cell by blocking their secretion. Our project on novel extra cellular RNA-based combinatorial RNA inhibition therapy, using exosome-derived RNA as a target is novel and has a great impact on the creation of improved cancer therapy strategies.

2. Lack of toxicity and immunogenicity in mice following 3-week dosing of engineered exosomes derived from HEK293T cells

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Background and Aims: Exosomes are under evaluation as delivery agents for therapeutics. Ongoing studies often use mouse disease models for evaluation of drug disposition, activity or efficacy. A comprehensive study of the potential immunogenic and/or toxic effects of exosomes in mice has not been reported. The aim of our study was to thoroughly assess the potential of wild type or engineered HEK293T cell-derived exosomes to induce toxicity and immune responses in mice.

Methods: Exosomes harvested from wild type (WT) or HEK293T cells engineered to express a targeting peptide (Full no 199), a targeting peptide plus therapeutic miR-199 (Full) or empty vector control (Empty) were administered as ten injections of 8.5 μg protein, every other day for 3 weeks to C57BL/6 mice by intravenous and intraperitoneal routes. Mice receiving PBS were included as vehicle controls. All mice were monitored throughout the 3-week study for visual signs of toxicity. Blood samples were collected before the first dose (day 1), one day after the first dose (day 2), and prior to euthanasia (day 22) for complete blood count analysis. Serum was prepared after euthanasia for biochemical profiles and evaluation of immune markers using the RodentMAP test. Single cell suspensions from spleens were prepared for immunophenotyping analysis. Tissue samples were harvested and fixed for histopathological examination.

Results: All 50 mice (10 mice/group) from the 5 treatment groups (PBS, WT, Empty, Full no 199 and Full) were monitored throughout the experimental period, and no signs of abnormality or behavioral changes were observed. The increase in body weight was normal, and

no statistical differences were observed between the 5 groups. Gross and histopathological examination did not reveal any abnormalities in the organs examined. Complete blood count and blood chemistry panels indicated no significant difference between the 5 groups for all tested markers. To determine if the injected exosomes derived from human cell lines would induce an immune response in the mice, 42 cytokines and chemokines were measured using the RodentMAP test. Of the 23 markers that were present at quantifiable levels, we observed no significant differences in these markers compared to the PBS control group. Results from splenocyte immunophenotyping showed no significant differences between the 5 groups for all tested immune cell populations, indicating that exosome treatment did not affect the spleen immune cell composition.
Conclusions: Exosomes derived from wild type and engineered HEK293T cells and dosed at 8.5 μg protein per dose over a 3 week period did not induce toxicity or immune response in immune competent C57BL/6 mice.

3. Large-Scale Isolation of High Purity Ginger Derived Exosome-Like Nanovesicle For Gene Delivery Therapy

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Exosomes is an emerging option for non-viral gene delivery therapy. One of the biggest blockage for application is the high cost and low yield of exosomes derived from human cell line. Ginger derived exosome-like nanovesicles(GDENs) had been proved to have the similar characteristics as human derived exosomes, which have potential application as economic substitute of human derived exosomes. Here we introduce a large-scale purification method combined cushion ultracentrifuge and equilibrium ultracentrifuge to isolate concentrated GDENs in large scale (7.5*1011 particles/mL). We determine the quality and quantity of GDENs by characterize size distribution (92.74 +/- 46.64 nm), zeta potential (-13.8 +/- 9.9 mV) and Electronic Microscopic morphology. We also prove the concept of utilize GDENs as the substitute of human derived exosomes for gene delivery by determine RNA loading efficiency as well as in vitro therapeutic siRNA delivery assay.

Session II: Genetic Models and Tools to Study exRNA

4. Semantic web enabled integration of Wikidata and Wikipathways for exploring impact of exRNAs in health and disease.

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Background: Extracellular RNAs (exRNAs) have emerged as an essential intercellular communication factor in health and disease. The effects of exRNAs are multi-faceted and frequently affect gene expression of their target cells and tissues. In order to interpret the effects of exRNAs on gene expression and therefore on downstream signalling pathways, open and easily accessible resources and approaches are required. Wikidata (www.wikidata.org) is an open, community driven, document based database and general graph of knowledge. It contains a large biological dataset involving genes, proteins, drugs, diseases and many interactions between them. In addition, with Wikipathways, a large, community driven resource for biological signaling pathways exists, providing insights into essential genes in signalling pathways. In addition to their completely open and Wiki-type nature, both resources provide SPARQL endpoints, allowing for fast retrieval of integrated data packages of interest for any downstream application, based on federated SPARQL queries.

Aims: Data on human miRNAs should be integrated into a Wikidata data model, representing pre- miRNAs and mature miRNAs as separate Wikidata items. Furthermore, miRNAs should be linked to the genes they are expressed by and the target genes they are regulating. Finally, SPARQL queries should be designed for efficient identification of target pathways.

Methodology: We used the public domain miRNA database miRBase (http://www.mirbase.org/) as an open data source for miRNAs and wrote a Python program (also termed a bot) in order to generate Wikidata items for human pre-miRNAs and mature miRNAs. For miRNA target genes, we used the TargetScan predicted targets data with a score cutoff of >0.79. Mature miRNAs were linked to target genes via the Wikidata 'regulates' property. SPARQL queries were executed on the Wikipathways SPARQL endpoint.

Results: In total, 2,619 human pre-miRNAs, 4,400 mature miRNAs and their corresponding identifiers were imported from miRBase. Furthermore, miRNA families, based on RFAM were imported to allow classification and categorization of the distinct miRNAs. This categorization allows for higher-level queries on miRNA family basis. In addition, we added links to the gene targets of these miRNAs onto the mature miRNA items in Wikidata. Finally, we created SPARQL queries which allow for retrieval of pathways in Wikipathways which might be affected in case a certain miRNA is expressed intracellularly or secreted as part of an extracellular vesicle. The reverse query enables to retrieve all genes involved in a pathway and search for miRNAs involved in its regulation. Gene Ontology terms of the target genes also allow inference of more general effects of miRNAs on cellular functions and biological processes.
We also construct queries which retrieve all miRNAs impacting pathways that are e.g. involved in immune regulation. ExRNAs, co-expressed with expression of certain genes, and so potentially regulating other signaling pathways can also be retrieved with such queries.

Conclusions: We created an open, community driven resource and knowledge base for exRNAs (and more broadly for all miRNAs) which integrates Wikidata and Wikipathways via the semantic web and SPARQL, allowing for users to execute queries, contribute data and share their findings on Wikidata. This resource should help elucidating the effects of exRNAs in health and disease.

5. Milk-derived RNAs as paradigm to study exRNAs biogenesis and function

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Background and Aims: Many classes of extracellular RNAs (exRNAs) are detected in mammalian body fluids, including breast milk, raising questions about their origins and putative functions. The ease to isolate high amount of milk, and its high exRNAs content (including miRNAs), establish milk as an attractive model to investigate exRNA biogenesis and function. Our objective is to characterize the biogenesis, stability and potential function of milk microRNAs (miRNAs) and their biogenesis/effector proteins (RNA silencing pathway). We notably aim at (1) deciphering if the different milk fractions (e.g. vesicular vs non-vesicular) contain distinct miRNA populations, (2) determining which proteins are associated with milk miRNAs, (3) monitoring their stability and their potential functional transfer in mouse neonate and in in vitro cultured cells.

Results and conclusions:
(1) We confirmed that human, bovine, and mouse milk contains high amount of RNA (200-400 ng/ml) mostly concentrated in milk fat globules (MFGs). Interestingly, MFGs exRNAs contain non-degraded ribosomal RNAs, are qualitatively different from milk-associated cells RNAs (e.g. by agarose/acrylamide gel analysis), and carry high level of distinct miRNAs (miR-22, mir-200s, miR-148a, miR-21). Moreover, in MFGs, we could detect high level of several cytoplasmic (but not nuclear) miRNAs biogenesis factor (High: DICER, TRBP, PACT; Low: DROSHA, DGCR8), and also high level of the miRNA-loading effector AGO1 (but low level of AGO2). As AGO2 is generally the most abundant argonaute (AGO1-4) in mammalian tissues and cells, this observation strongly suggests that an Argonaute sorting mechanism leads to preferential incorporation of AGO1 in MFGs. We are currently validating this hypothesis in vivo by characterizing Argonaute expression profile, and subcellular localization in lactocytes.

(2) By tracing milk-borne miR-21 or miR-22 in corresponding knockout pups fed by heterozygous mothers, we confirmed their accumulation in their stomach content (at postnatal day 3, 6 and 10). However, milk-borne miR-21 or miR-22 level rapidly drops in the small intestine to reach undetectable level (by qRT-PCR) within the first half of the duodenum. We could also not detect these milk-born miRNAs in neonate peripheral organs, including liver, spleen, kidney and thymus. Surprisingly, level of miR-22 and miR-21 in milk are not impacted by maternal AGO1/3/4 triple knockout, suggesting that these specific argonaute factors are not required for the sorting and/or stabilization of milk miRNAs in this context.
(3) Finally, we developed a mouse model allowing specific expression of a membrane-bound GFP in lactocytes, and showed efficient in vivo fluorescent labeling of milk EVs. By combining this model with high-resolution flow cytometry we demonstrate that, while milk EVs are most abundant in duodenum lumen, they also accumulate at more distal part of the intestine indicating that a significant portion survive the harsh digestive environment. In parallel, we apply a similar approach with a lactocyte-specific expression of GFP-AGO2 to characterize the bio-distribution and possible uptake of this particular miRNA effector protein in neonates. Finally, we are also testing whether uptake and functional transfer of milk EVs can occur in vitro using breast or intestine epithelial cell models.

6. Characterization of the extracellular content released from glioblastoma cells.

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Tumor cells release a variety of contents in the extracellular milieu including RNA contained in membrane-bound vesicles, ribonucleoprotein (RNP) complexes and high and low density lipoproteins. Extracellular vesicles (EVs) range in size from 50 nm to 1μm and above, the latter being more common in cancer cells. miRNAs are also found bound to Argonaute(Ago) complexes and are released from cells. mRNA, miRNA, ncRNA, DNA and proteins have all been found in the extracellular environment, but it is currently unknown the extent to which each subpopulation is present at any given time. There is also evidence to suggest that each subgroup can vary its content in response to the cellular status. Previous work has also shown that the miRNA content is distinct between EVs and RNPs (Arroyo et al., 2011).
Here we have optimized a one-step digital PCR protocol to directly count individual RNA and DNA molecules from a small amount of conditioned media. Glioblastoma cells were cultured serum –free in 48-well plates at concentrations varying from 7 to 15,000 cell/well. Representative miRNAs, mRNAs and genomic DNA were quantified using the one-step digital PCR irrespective of their carrier status. The number of particles (EVs) in the media was also counted using two different particle tracking systems (Malvern and ParticleMetrix), and levels of miRNAs, mRNAs and DNA molecules were determined per particle and per cell. Let-7a was used to infer the presence of EVs in the conditioned media, while miR- 16 was used to evaluate the levels of Ago2 ribonucleoproteins. Primers specific for GAPDH mRNA and genomic DNA were also used to determine the levels of mRNA and genomic DNA. Cells were grown under normal and heat shock conditions (42°C for 30 minutes) to determine differences in the release of these molecules. Preliminary data indicates that most extracellular miRNA is bound to RNPs and the EV/RNP ratio was approximately 1:100. mRNA copies were the lowest while gDNA was the most abundant molecule but it did not vary with different conditions. Data will be reported on counting of these molecules from two primary human glioblastoma cell lines under normal and stress conditions.

Session III: Heterogeneity of exRNA Carries and Implications in Function and Disease

7. Incorporation of full length mRNA messages in vesicles by binding to RNPs

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Background/aims:
Extracellular vesicles (EVs), including exosomes, have shown to play a role in various biological processes and diseases. EVs are small vesicles (50-1000 nm) that are shed by cells. Their content reflects in large extent that of the donor cell and includes proteins, RNAs, and DNA. Multiple proteins and ribonucleoproteins (RNPs) have been implicated in active RNA sorting into EVs. In this study we evaluated the loading of full-length mRNAs by association with RNPs. We focused on a specific RNP, Staufen 1 (Stau1) which has a number of mRNA partners. This multifunctional RNP has been described to regulate mRNA stability, localization, transport and translation and is known to be present in EVs. In addition, the binding potential to a specific mRNA, Arf1, is well established.
Methodology:
HEK293T stable cell lines were stably transduced with a lentiviral vector expressing fusion protein hStau1-GFP [Köhrmann et al., 1999]. EVs from wild type and stable cell lines were isolated using exoRNeasy (Qiagen) and differential ultracentrifugation. Protein expression was analyzed using Western blots. mRNA content was analyzed using RT-(q)PCR with primers in coding region (CDS) and 3'UTR. Full length mRNA was captured using primers spanning 5'UTR, CDS and 3'UTR regions and amplified using high fidelity polymerase.
Results:
Initial results show full-length mRNA enrichment of specific message Arf1 that is associated with RNP partner Stau1. When bound to Stau1, most of the mRNA incorporated into EVs is full-length, while low expression levels of 3'UTR fragments are observed. Several known Stau1 targets confirmed similar expression patterns in EVs. Other messages show different expression patterns with high abundance of fragmented RNA, supporting the theory that association with RNPs can promote incorporation of full-length mRNAs.
Conclusions:
Our present data suggests RNP-mRNA combinations enrich the levels of full-length mRNA messages present in vesicles. Even though previous studies have shown an increased presence of 3'UTR fragments of mRNAs in vesicles [Batagov et al., 2013], this enrichment appears to be reduced when mRNA is bound to a protective RNP partner. This data emphasizes the potential of horizontal transfer of intact messages by EVs; suggesting RNP-RNA incorporation into vesicles may represent a good candidate for therapeutic nucleic acid delivery.

8. Isolation, Identification, and Characterization of Novel Nanovesicles

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A main class of EVs is thought to be exosomes. Exosomes carry various proteins, bioactive lipids and genetic information to alter the phenotype and function of recipient cells. Thus, exosomes have been implicated in numerous biological and pathological processes. Like

other EVs, exosomes are heterogeneous in size and in function, and are released from many types of cells. The heterogeneity of EVs makes it very challenging to determine which subpopulation of EVs plays a dominate role in the phenotypes observed. Current strategies for charactering EVs are limited to multiple in vitro manipulations for isolation and purification, followed by analytic approaches that generate data which may not represent what takes place in vivo. Therefore, the ability to identify, isolate, and molecularly characterize EVs with minimal in vitro manipulation is urgently needed. We hypothesize that a predominant EV population is released from cells and plays a key role in intercellular communications. In this study, we demonstrated that unlike other identified EVs including exosomes which cannot be detected using a nanosizer without concentration in vitro, HG-nanovesicles (HG-NVs) can be readily detected from blood and cell cultured supernatants without in vitro manipulations. Five lines of evidence support that HG-NVs are a previously unrecognized and predominate nanovesicle. First, unlike exosomes, HG-NVs are much smaller in size (8-12 nm in diameter versus 50-150 nm in diameter of exosomes), much more homogenous in size and less negatively charged (-10±5mV) than exosomes (- 40±10 mV) released from the same cell types. Second, after depletion of exosomes using a standard protocol, HG-NVs are still present in samples. Third, based on composition analysis, we identified a number of unique proteins and RNAs being present/absent in the HG-NVs compared with exosomes released from both human and murine breast tumor cells. Fourth, in order to characterize exosomes, they must be concentrated using different technologies that could cause an alteration in their properties. Determining whether the properties of exosomes have actually been altered after in vitro concentration is a challenging problem. This is not the case with HG-NVs. In contrast, without concentration or other forms of laboratory manipulation, HG-NVs from blood or cell supernatants can be detected with a Nanosizer. Finally, from a biological effects perspective, our data indicate that HG-
NVs are different from exosomes (1) in their RNA profile from tumor bearing mice and LPS challenged mice; (2) in their cytokine profile from macrophages, dendritic cells and immature myeloid cells; and (3) in promoting tumor growth based on two different mouse tumor models used in this study. This discovery may have broad implications in advancing both cell biology research and clinical management.

Session IV: Extracellular RNA and Function

9. Profiling membrane trafficking for exRNA release by endogenous protein tagging

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In order to elucidate the mechanisms regulating the release of exomes and exRNAs, it is highly desired to visualize these processes in living cells. Traditionally, live imaging of cellular proteins have usually been achieved by overexpressing a fluorescent protein fusion to the target. Nevertheless, overexpression artifacts are widely present (e.g. leading to the overpopulation of certain membrane compartments). To overcome this challenge, we have developed a scalable method to tag endogenous proteins for microscopy analysis by combining CRISPR/Cas9-mediated genome editing with split- fluorescent protein labeling (Kamiyama et al., Nat Comm 2016; Leonetti et al., PNAS 2016). Specifically, a short peptide sequence, GFP11, derived from the 11th beta-strand of an optimized super-folder GFP, is knocked-into endogenous genes. This peptide complements with the separately expressed GFP1-10 fragment to form a full fluorescent GFP. The small size of the GFP11 tag enables cloning-free knock-in with low cost and extremely high efficiency. Moreover, it can also be arranged as tandem repeats to amplify the fluorescence signal, enabling the detection of lower abundance targets. Recently, we have engineered two new split fluorescent protein tags, split mNeonGreen and sfCherry2, that are significantly (8 fold in the case of sfCherry) compared to the previous split GFP and sfCherry labels. With these two tags, we have demonstrated double knock-in for two-color imaging of endogenous proteins. We have also developed a photoactivatable split sfCherry2 for super-resolution microscopy analysis of cellular ultrastructures. Using this endogenous protein tagging method, we are generating human cell line libraries tagging Rab proteins the control intracellular vesicle trafficking (in collaboration with Robert Blelloch in the same U19 consortium), aiming at uncovering the pathways for exome and exRNA release. We are also collaborating Noelle L'Etoile and Andrei Goga to generate NRDE2 knock-in cell lines for the understanding of nuclear RNAi in exRNA target cells.

10. Regulation of exRNA production by different oncogenes in tumor models

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Extracellular RNAs (exRNAs) released from cells can be found within vesicles, bound to lipoproteins or RISC components. They are crucial for cell-to-cell communication and thought to play important roles in cell development, diseases, and cancer metastasis. Using a panel of isogenic MCF10A cell lines that express most commonly overexpressed oncogenes we aim to identify oncogenic signals that alter extracellular vesicle production. We purified exosomes via ultracentrifugation and confirmed the presence of exosome markers such as Tsg101, Alix, Clathrin and CD9 by western blotting. Nanosight particle-tracking analysis revealed that MYC overexpression induces increased exosome release within a panel of ~12 different oncogenes. Similarly, we confirmed the MYC-dependent increase in exosome production using MYC inducible MTB-TOM cell lines. RNA microarray and metabolomic analysis identified significantly up and down-regulated genes involved in ceramide and sphingolipid metabolism in cells overexpressing MYC. Interestingly, sphingomyelinase inhibitor GW4869 significantly decreased exosome production in MYC compared to other oncogenes suggesting a ceramide-dependent pathway for exosome production in cells overexpressing MYC. We aim to dissect mechanisms involved in exosome biogenesis by altering levels of differentially expressed candidate genes. Moreover, our preliminary miRNA profiling from both intracellular and extracellular miRNAs found significantly up and/or down-regulated miRNAs present in extracellular vesicles. This finding suggests that oncogenes can alter which miRNAs are selectively secreted or retained in the cells. We sought to identify key regulators of these selections and their contribution to cancer progression.

In addition, we aim to elucidate the temporal regulation of exRNAs by the oncogenes MYC and RAS in primary liver tumor formation and regression. We are not only interested in exRNAs within vesicles but also bound to lipoproteins. In collaboration with Dr. Robert Raffai, we analyzed lipoproteins and lipoprotein particles from plasma collected from tumor driven by RAS, MYC, and MYC+RAS and compared to non-tumor bearing mice. The data indicate that the induction of liver

cancer by MYC or RAS oncogenes results in a differential accumulation of lipoprotein classes. The induction of RAS causes mainly an accumulation of HDL and chylomicron cholesterol whereas MYC- driven tumors have increased accumulation of LDL cholesterol. We also showed that different miRNA species were upregulated by RAS and MYC oncogenes in these conditional tumor models (i.e., miR-21 in RAS-driven tumors, miR494 in MYC-driven tumors). We now seek to determine if these oncogenic miRNAs can assemble into exRNAs and if so in which format (i.e., vesicle and or bound to lipoproteins) to regulate gene expression of target genes and whether they play a role in tumor metastasis.

11. Non-coding RNAs in exosomes secreted by cancer cell lines

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Exosomes are extracellular vesicles of 50–150 nm in size, consisting of nucleic acids, proteins and lipids. The spectra of these molecules depend on their cell origin. Because exosomes are released from cells into body fluids, they may provide a rich source for non-invasive diagnostics. For the RNA in exosome, it has been reported that the exosomes contain mRNA, microRNA (miRNA), and long non-coding RNA (lncRNA). The miRNAs and lncRNAs are known modulator for transcriptome, but their function in exosome is still unclear. In this study, we utilized high-throughput expression profiling techniques, microarray and RNA-Seq to analyze RNAs in eight different cancer cell lines and corresponding exosomes. We observed that The exosomes contain higher percentage of lncRNA compared to the corresponding cell lines. We also identified sets of miRNAs and lncRNAs that are either in common or specific in different exosomes. This suggests some lncRNAs and miRNAs may serve as novel targets for cancer treatment. We are in the process of validating some of the findings and also conduct function studies on selected lncRNAs.

Session V : Comprehensive Reference Profile of Extracellular RNA in Human Biofluids and Bioinformatics Tools Update

12. sRNAnalyzer - A flexible and customizable small RNA sequencing analysis pipeline

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The function and application of small non-coding RNAs (ncRNA), especially microRNA (miRNA) has gained significant interest in recent years. As is well known, miRNA is a group of 17 to 23 nucleotide-long evolutionary conserved regulatory RNA in the cells. Numerous reports have shown the existence of RNA molecules, including miRNA, in different bodily fluids. These extracellular RNAs are either encapsulated in lipid vesicles or complexed with proteins to prevent RNase degradation. The spectrum of extracellular miRNA in various body fluids shows strong correlation with physiopathological conditions and has been introduced as biomarker for disease diagnosis. Therefore, the ability to quantify specific miRNA levels accurately in biological samples is critical for further development of miRNA-based biomarkers. Although many experimental and computational tools have been developed, it is still a challenge to accurately and comprehensively analyze the small RNA population. The short sequence length, high sequence similarity among family members, and sequence length variations (isomiR) all contribute to the difficulty of miRNA quantification. Besides the endogenous RNA molecules, recent studies have revealed that a significant fraction of the RNA in circulation is derived from exogenous species including bacteria and fungi. Thanks to the wealth of information provided and decrease of cost, various next generation sequencing (NGS) platforms have gradually become the common tool for studying small RNAs. The NGS data allow the assessment of global small RNA profiles in a sample, since it can resolve closely related sequences, is not affected by sequence length variation, does not require prior knowledge of the sequence, may provide information to identify new miRNA sequences and allows the profiling of exogenous RNAs in the sample. However, the current tools for small RNA sequencing (sRNA-Seq) data analysis are still deficient, including poor specificity in both endogenous and exogenous species derived RNA. To address some of the small RNA analysis problems, especially for miRNA and exogenous RNA, we built a comprehensive and customizable small RNA sequencing (sRNA-Seq) data analysis pipeline – sRNAnalyzer. Our pipeline enables 1) Additional adapter trimming process to generate cleaner data, 2) Comprehensive microRNA profiling strategies to better handle isomiR issues, 3) Summarization for each nucleotide to detect potential SNPs on miRNAs, 4) Multiple assignment to simulate miRNA array and qRT-PCR platforms, 5) A local probabilistic model to map reads to the most-likely entry IDs, 6) Comprehensive ribosomal RNA filtering for more accurate mapping results, 7) More specific species assignment on exogenous RNAs in circulation, and 8) Taxonomy annotation/summarization at major ranks for exogenous species. These features should increase the flexibility for users to interrogate the results and gain a global view of the cell free small RNA in circulation. To evaluate the performance of sRNAnalyzer, we tested the pipeline with both artificial (synthetic miRNA panel) and biological samples (bacterial culture samples, human tissue and plasma samples). The results showed that sRNAnalyzer not only achieve better miRNA analysis results but also able to identify the origin of exogenous RNA with improved confidence. sRNAnalyzer is implemented in Perl and is available at: http://srnanalyzer.systemsbiology.net/

13. The exRNA Atlas: Cloud-hosted repository, tools and infrastructure for processing, storage and analysis of extracellular RNA profiles

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The Data Management and Resource Repository (DMRR), as part of the Extracellular RNA Communication Consortium (ERCC), has created a comprehensive pipeline to process and store extracellular RNA profiles in the exRNA Atlas, a public data repository accessible via web browser and FTP client. Datasets stored in the exRNA Atlas are profiled using either RNA-seq or RT-qPCR assays, and all RNA-seq datasets are uniformly processed using the extracellular RNA processing toolkit (exceRpt). The exceRpt pipeline generates sample-level quality control metrics, produces abundance estimates for small RNA species, and provides detailed alignment information for visualization and validation. The Atlas also stores metadata for all samples using the GenboreeKB exRNA Metadata Tracker, a MongoDB-backed database service.

The public exRNA Atlas currently stores 850 exRNA profiles from 12 studies, with nearly nine billion small RNA-seq reads processed through exceRpt. A faceted search feature on the Atlas webpage allows users to easily select samples of interest on various criteria such as condition, biofluid, exRNA source, and RNA isolation kit. Users can also select samples via a linear tree drill-down diagram and via grid views that display biosamples by condition or assay type. Once users select samples of interest, they can download data and metadata as well as submit samples for further downstream analysis via the Analyze Selected Samples feature.

The DMRR has recently made several important updates to the Atlas. First, all small RNA-seq samples in the public Atlas have been reprocessed using the most recent (4th) version of exceRpt. Consequently, exRNA profiles in the Atlas are expanded and now include read mappings to the genomes of all sequenced species in Ensembl/NCBI. Second, Atlas users no longer have to visit the Genboree Workbench to use the Atlas' integrated analysis tools like DESeq2. Instead, tool jobs can be launched directly from the Atlas via the Analyze Selected Samples feature, with result files available via email.

Third, the Atlas is now capable of storing and presenting RT-qPCR data, with 99 RT-qPCR profiles currently available in the public Atlas. The DMRR is in the process of developing an automated FTP data submission pipeline for RT-qPCR data, as opposed to manual curation of data by DMRR administrators.

The exRNA Atlas can be accessed either directly (www.exrna-atlas.org) or via the Quick Links section in the exRNA Portal (www.exrna.org). Tutorials on using the Atlas and exceRpt can be found in the Data section under the Resources tab on the exRNA Portal (www.exrna.org/resources/data/).

14. Infrastructure and Development of the exRNA Virtual Biorepository

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The Data Management and Resource Repository (DMRR) and Resource Sharing Working Group, as part of the Extracellular RNA Communication Consortium (ERCC), are currently developing an exRNA virtual biorepository (EVB) for convenient biosample tracking

and sharing within the scientific community. The EVB will follow a hub-node model, where the hub will function as a central entity that communicates with each participating institution (or node) and returns information to the researcher about available biosamples. Each node will store its own set of metadata documents for samples associated with that node and will have its own set of node management tools, allowing for self-maintenance and flexibility for node administrators. The Genboree REST API infrastructure will allow the hub to seamlessly gather sample metadata from nodes using Linked Data technologies.

Researchers will request samples of interest by using the EVB Hub. The EVB Hub's portal page will contain a Sample Request UI where users can search and filter available samples by various metadata properties like biofluid type, condition, and donor demographics. The page will then display a results grid showing all matching samples, and each listing will have a link to a full page report where users can read more about a given sample, including information about its associated study and contact information for the sample submitter. As part of the sample request process, users are prompted to provide information about why they are making the request. Certain restrictions, set by node administrators, may also apply to requests, such as minimum and maximum quantities that can be requested for a given sample. After the request has been placed, users will use the sample request status tracking page to check whether their requests have been approved or denied.

All metadata about samples will be stored in the nodes. Node members will add, edit, and view documents using the node management tools. Members will add new documents by either using the node's web-based Data Entry UI or by uploading documents in bulk (in tabbed format).

The first test case for the EVB will involve the evaluation of cerebrospinal fluid (CSF) for the diagnosis of various brain-related conditions. The list of participating institutions includes UCSD, TGen, OHSU, and BNI, each institution having its own node within the hub-node infrastructure described above.

15. Small RNA Profiles in Normal Human Biofluids

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A diverse range of small RNAs can be found in human biofluids. To characterize these, we are performing small RNA sequencing analysis on 11 different biofluids, from 5-10 normal human donors. To date, we have processed plasma, serum, urine, bronchoalveolar lavage fluid (BALF), cerebrospinal fluid (CSF), saliva, sputum, cord plasma, and amniotic fluid. Here, we report the results obtained from a modified small RNA-Seq protocol that uses adaptors with randomized ends to reduce bias and is suitable for small amounts of RNA. For each of the samples, we were able to detect small RNAs, including miRNAs and fragments of tRNAs, and Y-RNAs, long non-coding RNAs and mRNAs. The fraction of mapped small RNA reads annotated as miRNAs ranged from 0.2%-86.8%, with relatively low amounts of miRNA in urine, amniotic fluid, and CSF and higher amounts in plasma, serum, BALF, and plasma from cord blood. tRNA fragments represented <1%-72.8% of reads, with relatively few tRNA fragments in plasma, cord plasma, and BAL, and more tRNA fragments in urine and amniotic fluid. Unsupervised hierarchical clustering based upon highly variable miRNAs showed good separation of samples by the biofluid of origin, except that saliva and sputum samples were similar. Our results provide a useful comparison between small RNA populations in a large set of diverse biofluids utilizing the same massively parallel sequencing method for each sample. Ongoing analyses are addressing the likely cellular origins and mRNA targets of miRNAs found in different biofluids.

16. Epigenomic Profiling Provides Information about the Mechanism of Action and Cell Type of Origin of Tumor-Derived Exosomal Cargo

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The following three questions are central to the study of tumor-derived exosomal exRNAs: (1) Which pathways mediate any phenotypic changes observed in target cells? (2) What is the cell type of origin of the exosomes found within tumor tissue or body fluids of cancer patients? (3) What is the connection between pathological processes within tumor tissue and the predictive power of exRNA biomarkers found in body fluids? Using glioblastoma multiforme and breast cancer as case studies, we present results suggesting that epigenomic profiling –in conjunction with the more commonly used experimental and profiling data – may help address these questions.
Using cell-line models, we explored epigenomic reprogramming of pathways involved in tube formation and hyper-vascularity induced in brain endothelium cells upon the uptake of EVs secreted from Glioblastoma Multiforme (GBM) stem-like cells. Our assay demonstrates that EV exposure recapitulates the angiogenic effect of a growth factor cocktail stimulus, consistent with hyper-vascularity observed in GBM. Differentially methylated regions (DMRs) in endothelial cells that are specifically caused by the EV exposure point to the specific reprogramming of the glomerulogenesis pathway, consistent with the glomerular patterns of hyper-vascularization observed in glioblastoma. Further, GO term enrichment analysis based on these DMRs suggests epigenomic reprogramming of biological processes consistent with other pathological features of GBM. To investigate upstream mediators, GBM EV content was characterized by RNA-seq. Results from subsequent pathway analysis of EV-miRNA target transcripts are also consistent with the role of EV-miRNAs in GBM pathology.
The exosomes found in the body fluids of cancer patients may originate from any of a number of different cell types present within heterogeneous tumor tissue. However, large scale assessment of cell type composition, of molecular profiles of individual cell types within tumors, and of exosome-mediated heterotypic interactions remains challenging. To address those issues, we developed Epigenomic Deconvolution (EDec), an in silico method that infers cell type composition of complex tissues as well as DNA methylation and transcription profiles of constituent cell types [In Press, Cell Reports]. By applying EDec to GBM and breast tumors from TCGA we detect changes in immune cell infiltration and other variation in heterotypic interactions that may be exosome-mediated and that predict patient survival. To determine cell type of origin of exRNAs found in body fluids from cancer patients we are currently comparing cancer patient profiles from the exRNA Atlas against the deconvoluted RNA profiles from corresponding tumor types.
In summary, our preliminary results suggest that epigenomic footprints of exosomes in the form of DMRs and epigenomic deconvolution of complex tumor tissues may help address key questions in exosome biology.

17. Diagnostic and prognostic potential of smallRNA biomarkers in fetal development

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Background: Circulating and cellular miRNAs have potential value as diagnostic and prognostic biomarkers for a variety of conditions, including pregnancy, cancer and organ dysfunction. Placental Dysfunction (PD), including preeclampsia and fetal growth restriction, is a significant cause of maternal and fetal morbidity and mortality. The current methods for prediction of PD have sensitivities between 55-95% and require both sophisticated equipment and highly trained technicians on site. A cost-effective, non-invasive, highly sensitive, and specific strategy for identification of patients at high risk of PD, would enable screening for the general obstetric population with the goal of targeting resources to appropriate cases, while sparing low-risk patients unnecessary anxiety. Ideally, such a method would not only identify those at risk for placental dysfunction, but also provide prognostic information regarding the likely severity and timing of onset of the clinical manifestations of placental dysfunction, which would allow personalized schedules for clinical monitoring and timing of delivery.
In previous work, we have identified candidate miRNA biomarkers in the maternal serum that are present at different levels in those patients who will develop PD compared to uncomplicated pregnancies. We have also observed that placental and maternal serum miRNA profiles are different in early and late pregnancy. In order to develop a test that can be used at any time during pregnancy, we need to

validate these candidate biomarkers in an independent cohort and also generate a reference miRNA profile at high temporal resolution throughout pregnancy.
Small RNAs account for a very small percentage of the total RNA that can be isolated from tissues or cell free compartments. Thus, we have developed protocols with increased sensitivity, reliability and robustness that will enable us to extract meaningful information from such rare molecules.
Aims:
1. Generate a week-by-week reference placental miRNA profile
2. Validate candidate maternal serum miRNA biomarkers to predict PD
Methods: RNA was isolated from placental tissues at various gestational ages and from normal and preeclamptic maternal serum. Reduced volume small RNA libraries, using the NEBNext smallRNA kit were constructed using a Mosquito HTS nanoliter scale liquid handling robot. In order to increase the miRNA fraction, the libraries were size selected on a PippinHT. Library characterization and quantification was performed using a Bioanalyzer DNA High Sensitivity microfluidic chip and Quant-iT Picogreen or AccuBlue NextGen dsDNA assays. Libraries were sequenced on a HiSeq 4000.
Results: Small RNAseq libraries were prepared from 0.3-1ng of maternal serum exRNA or 100ng of total placental RNA at reduced (1/5th) volumetric scales. Preliminary analysis of the placental miRNA data suggests that miRNA profiling can determine the gestational age of placentas at high temporal resolution.
Conclusion: We have shown that we can reduce the cost and labor required for the preparation of small RNAseq libraries from exRNA and tissue RNA using automation at reduced volumes, while maintaining technical reproducibility. This workflow is now being used to validate candidate biomarkers and generate high resolution reference miRNA profiles.

18. Evaluation of Normalization Strategy for Extracellular small RNA Sequencing Data

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Extracellular vesicles have been shown to regulate intercellular signaling by transmitting RNA materials such as mRNA, microRNA and snRNA that may partially reflect cellular content within the human body and may show disease specific variation: for example RNA material secreted by cancer cells may be distinct. As such, extracellular small RNA profiles offer great potential as clinical biomarker. Recent advances in high throughput sequencing technology allow for rapid generation of extracellular small RNA sequence data (sRNASeq) derived from clinical liquid biopsy. However, extracellular sRNASeq data have vastly different characteristics that require specific consideration to analyze and make direct application of methods developed for mRNASeq potentially problematic. First, the extracellular sRNASeq data features are often rare and scattered in contrast to mRNASeq data where majority of the data features is accounted for. Second, extracellular sRNA sequencing are highly susceptible to under-sampling due the difficulty of obtaining sufficient RNA quantity.
To gather information on selecting appropriate normalization methods to remove noise in extracellular sRNASeq data, we have assessed the performance of five different normalization methods, namely Cumulative Sum Scaling normalization (CSS), geometric mean (DESeq), total-sum scaling (TSS), trimmed median (TMM), and upper quantile (UP), on both simulated and published extracellular data sets. We used Gini index to evaluate extracellular sRNASeq data sparsity and use it to guide our data simulation. We first assessed the tightness, stability, and correctness of clusters formed after applying each normalization method on simulated data across a range of sparsity. We then evaluated the same set of characteristics on extracellular sRNASeq data derived from clinical samples. We showed that commonly applied normalization methods vary with different degree of data sparsity and that method that accounts for under-sampling variation, such as CSS, are generally more stable. This work provides data on selecting more appropriate normalization method and hopefully will improve our ability in detecting differential sRNA abundance from extracellular sRNASeq data.

Session VI: Extracellular RNAs as Biomarkers and Liquid Biopsy in Human Disease

19. Genome-wide Profiling of Urinary Exsosomal microRNAs Associated with Diabetic Nephropathy in Type 1 Diabetes

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Diabetic Nephropathy (DN) is a form of progressive kidney disease, initiated by microvascular complications due to diabetes that often leads to end-stage renal disease. While microalbuminuria (MA) provides the earliest clinical marker of DN among patients with Type 1 diabetes (T1DM), it lacks the sensitivity and specificity to detect early onset of DN. Recently, microRNAs (miRNAs) have emerged as critical regulators of diabetes as well as various forms of kidney disease, such as renal fibrosis, acute kidney injury, and progressive kidney disease. Additionally, circulating extracellular miRNAs (both associated with microvesicles/exosomes and those circulating freely in various biofluids) have garnered significant attention as potential non-invasive diagnostic biomarkers for various diseases and health conditions. As part of the University of Pittsburgh Epidemiology of Diabetes Complications (EDC) study, urine was collected from T1DM individuals with various grades of MA (normal, overt, intermittent, and persistent) over several disease cycles. We isolated exosomes from urine, and analyzed the small-RNA with sequencing and found a number of miRNA showing concentration changes not only associated with MA status, but also with gender, and HbA1c levels. Additionally, many of the miRNAs associated with MA status and DN occurrence directly target pathways (including TGF-β and PTEN) associated with renal fibrosis, which is one of the major contributors to pathology of DN. We are currently validating these miRNAs and trying to develop a potential diagnostic signature for DN.

20. Comprehensive analysis of circulating RNA in patients with PTSD

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Post-traumatic stress disorder (PTSD) is a systemic and multifactorial disorder that involves dysfunction of multiple biological systems, such as brain circuitry, endocrine, and immune functions. The mechanism and pathophysiology of PTSD have been studied in epigenetic, genetic, metabolism and endocrine aspects. In recent years, dysregulation of miRNAs have emerged as potential factors in psychiatric diseases like schizophrenia, bipolar disorder, major depressive disorder, and autism spectrum disorders. However, studies on the involvement of miRNAs in PTSD are few. In this study, we conducted the first comprehensive profiling of small RNAs of plasma, exosome and exosome depleted plasma samples in PTSD by utilizing next-generation sequencing (NGS) platform. The study subjects consisted of 24 male patients with (n=12) and without (n=12) PTSD of Iraq and Afghanistan combat veterans. Based on the distribution of miRNA in three types of samples, we identified miRNAs showing concentration changes associated with PTSD. In addition, we also observed a set of miRNAs are enriched in exosome. To identify the possible source of these PTSD-associated miRNA in circulation, we conducted miRNA profiling analysis on different human brain subregions. By searching miRNAs enriched in specific brain subregions, we can identify possible involvement of brain subregions in the disease. In addition, by integrating brain subregion enriched miRNAs and differentially expressed circulating miRNAs in PTSD, we propose potential biological processes associated with miRNAs affected by PTSD.

21. Stroke and Circulating Extracellular RNAs

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Background and purpose: There is increasing interest in extracellular-RNAs (ex-RNAs) with numerous reports of associations between selected microRNAs (miRNAs) and a variety of cardiovascular disease (CVD) phenotypes. Previous studies of ex-RNAs in relation to risk for CVD have investigated small numbers of patients and assayed only candidate miRNAs. None has investigated links between novel ex- RNAs and stroke.

Methods: We conducted unbiased next-generation sequencing using plasma from 40 participants from the Framingham Heart Study (FHS, Offspring Cohort Exam 8) followed by high-throughput PCR of 471 ex- RNAs. The RT-qPCR included 331 of the most abundant miRNAs, 43 small nucleolar RNAs (snoRNAs), and 97 piwi-interacting RNAs (piRNAs) in 2763 additional FHS participants and explored the relations of ex-RNAs and prevalent and incident stroke and coronary heart disease.

Results: After adjustment for multiple CVD risk factors, seven ex-RNAs were associated with stroke prevalence or incidence; there were no ex-RNA associated with prevalent or incident CHD. Statistically significant ex-RNA associations with stroke were specific with no overlap between prevalent and incident events.

22. Salivary EBV exRNAs as biomarkers for gastric cancer detection

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BACKGROUND: Gastric cancer is one of the most common cancer worldwide. It is responsible for nearly one million new cases and over 700,000 deaths annually. The 5 year survival rate in United States is under 15%. The diagnosis is often delayed due to lack of early symptoms. Ebstein-barr virus (EBV), also called human herpesvirus 4 (HHV-4) is one of the most common viruses in humans (95% of the population) with oncogenic activity. It is associated with Hodgkin's lymphoma, Burkitt's lymphoma, nasopharyngeal carcinoma, gastric cancer (GC), etc. Infection with EBV occurs by the oral transfer of saliva. Studies have demonstrated positive correlation with EBV infection and gastric cancers in EBV- associated gastric carcinomas. Therefore, the presence of viral genomes in EBV-positive GC-tissues strongly suggests that EBV can be the causative agent in gastric cancer (GC). We hypothesized that EBV- specific RNAs are present in salivary exRNA and could serve as biomarkers for gastric cancer detection. AIM: To test the hypothesis that salivary exRNA in gastric cancer patients contain EBV RNA that can discriminate gastric cancer from non-gastric cancer patients.

METHODOLOGY: The study cohort included 99 gastric cancer (GC) and 100 non-gastric cancer control Korean patients. The GC cases included 54 with early stage (EGC) and 45 with advanced stage (AGC) diseases. RNA sequencing was performed using the Illumina sequencing platform. Bioinformatic analysis of RNA-Seq data included mapping the reads to Korean-specific EBV-associated GC strain (SNU-719 EBV) (obtained from the NCBI GenBank) using HISAT2. After quality control, DESeq2 was used for differential expression

analysis between three groups: GC vs. control, EGC vs. control, AGC vs. control. The most differentially expressed EBV genes will be advanced for validation by means of quantitative polymerase chain reaction.

RESULTS: DESeq analysis revealed 8 significantly differentially expressed genes between GC and control ($p<0.006$), 4 genes between EGC and control ($p<0.001$), and 13 genes between AGC and control ($p<0.02$). Among them, 8 genes were identified with most significantly increased differentially expressed profile (BTRF1, LMP2A, BPLF1, BOLF1, BMRF2, BXRF1, BDLF4, BVRF1) and 8 genes with most differentially decreased expressed profile (BXLF2, BRRF2, BFLF2, BFRF3, BGLF2, BPLF1, BcLF1, BXLF2).
Specifically, five genes: BTRF1 ($p=0.0001$), LMP2A ($p=0.00009$), BPLF1 ($p=0.0003$), BOLF1 ($p=0.0005$),
BMRF2 ($p=0.002$) were significantly differentially increased, while two other genes: BOLF1 ($p=0.0005$) and BMRF2 ($p=0.002$) decreased between GC and non-GC controls.

CONCLUSIONS: Eight salivary EBV exRNAs are significantly associated with GC. Seven most differentially expressed Korean-specific EBV-associated genes in saliva will be verified in the UH3 validation phase of the GC biomarker development project. In addition, further analysis of Korean EBV strain appeared to be crucial since their genome is not included in the most common NCBI viral genome database.

23. Robust Statistical Analysis Pipeline for Selecting Promising Biomarkers from RT-qPCR Experiments

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Background: The potential for extracellular microRNA (miRNA) to serve as disease biomarkers is enhanced by the ability to reliably measure them in human body fluids via technologies such as RT- qPCR. However, commonly employed statistical methods for selecting promising biomarker candidates from multi-dimensional RT-qPCR data (e.g. cutoffs based on p-values or standardized distance in differential expression) are not necessarily appropriate, as "statistically significant" findings may not possess adequate validity, robustness to process noise, or even summarize biomarker potential. Better candidate selection from RT-qPCR experiments requires tight process control, proper normalization, correct handling of censored values, and application of rigourous statistical consensus methodology.
Objective: Describe a robust set of statistical methods for selecting potential biomarkers from RT-qPCR expression experiments.
Methods: We designed a biomarker selection pipeline for RT-qPCR expression data that addresses salient weaknesses in typical analytical approaches, and tested its use in a study comparing miRNA from cerebral spinal fluid from Alzheimer's disease (AD) cases versus controls. First, we use data visualization techniques to identify technical process heterogeneities and measurement failures; filters are applied to remove candidates with poor assay performance. Normalization is based on modeling sources of variation in the behavior of internal reference standards under the gamut of measurement conditions, and cycle time (Ct) censoring thresholds are determined. Next, we perform a battery of statistical assessments targeting different aspects of biomarker potential: strong expression-disease associations, large group separation based on expression and/or censoring, consistent classification ability across alternative metrics, and complementarity with existing clinical markers. Candidates are ranked with respect to consensus performance across the battery of tests, and the diagnostic performance of the top selection is internally validated using resampling methods.

Results: Our statistical pipeline for biomarker selection appears to reduce the rate of false positive and false negative selections compared to typical methods. In the AD study, we were able to reduce a set of hundreds of candidate miRNA from CSF to those that demonstrated top classification performance, which were then verified by results of a follow-up experiment.
Conclusions: Our analytic pipeline will result in more efficient and reliable selection of top performing biomarkers based on RT-qPCR data.

24. Small RNA Profiles of Patients Post Bariatric Surgery

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Extracellular RNAs (ex-RNAs) are ubiquitous in the circulation, and may function to regulate gene expression. The dynamic expression profile of ex-RNAs in response to metabolic stress can be quantified via next generation sequencing, allowing for the detection of thousands of RNA species simultaneously. We are currently investigating the use of ex-RNA expression in plasma to identify small RNA profile changes before and after significant surgical weight loss with bariatric surgery. The small RNA profile in plasma at baseline (before surgery) and 12 months post-surgery yield interesting differences. Further investigation of these differences before and after surgery may elucidate the biology of weight loss and diabetes remission associated with bariatric surgery. The poster will cover the technologies used in the sequencing of these RNA biomarkers and the analysis methodology.

25. Analysis of Extracellular RNA in Cerebrospinal Fluid

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Background: CSF RNA offers a 'liquid biopsy' revealing diagnostic markers of neurologic disease. We evaluated cerebrospinal fluid (CSF) RNA content from six major neurologic diagnoses to develop a template protocol for optimization of collaborative, multi-site studies.
Objective: Provide a template for analysis of CSF pooled from human subjects for application to individual CSF studies. We considered 1) CSF EV size and quantity, 2) RNA yields from four isolation kits,
3) RNA expression from three analytic platforms.
Methods: We created CSF pools from patients with five neurologic diagnoses: Alzheimer's disease, Parkinson's disease, Low Grade Glioma, Glioblastoma, and Subarachnoid Hemorrhage, and neurologically normal controls. We analyzed concentration and distribution of EVs and RNAs. We then measured RNA expression by RT-qPCR and RNA sequencing.

Results: We show overall agreement in the intra- and inter-institutional replication of results for EV characterization, RNA yield and quality. For expression outcomes from RT-qPCR and RNAseq, in general more RNA species were detected in total RNA vs the EV fraction. The number of detectable RNAs correlated with CSF collection site: less RNAs in lumbar than cisternal CSF. For miRNAs detected by PCR, the control pool had ~50% detected in the EV fraction, while the neurologic pools had ~75% in the EV fraction. For miRNAs detected by small RNAseq, there were less miRNAs detected overall relative to PCR, and the GBM and LGG had ~50% miRNA in the EV fractions. 93 specific CSF mRNAs were chosen for assay by RT-qPCR, and long RNAseq was used to detect all mRNAs from neurologic diagnostic CSF pools.
For LGG and SAH, >75% of mRNAs detected by PCR were in the EV fraction compared to ~50% of control and degenerative disease. For long RNAseq, ~25% of mRNA were detected in the EV fraction. We also identified RNAs unique to the EV fraction for each diagnosis, relative to control, and for the miRNAs the pattern of detection was consistent across PCR and RNAseq.
Conclusions: Based on outcome from this first replicate comprehensive study of exRNAs in CSF across neurologic diagnoses, we provide a template protocol for biofluid EV studies that are critical for ensuring the consistency of results in large replicate studies among many sites, and for reporting results from such studies. We have now implemented this template for evaluation of miRNAs and mRNAs in individual CSF samples to identify signatures for each distinct neurological diagnosis.

26. exRNA biomarkers for mechanical and electrical remodeling in post-myocardial infarction patients

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Adverse mechanical and electrical remodeling of the heart following myocardial infarction (MI) contributes to 550,000 cases of heart failure (HF) and 300,000 cases of sudden cardiac arrest (SCA) annually. However, the current clinical parameters for prognosticating cardiac remodeling lack adequate sensitivity and specificity, necessitating the development of complementary biomarkers. We have performed RNAseq on plasma RNA from patients with or without SCA (n=9 each) and with beneficial or adverse left ventricular remodeling (n=11 each) and identified 34 differentially expressed miRNA candidates for validation in a larger cohort (331 patients) by qPCR. Principal components analysis identified 4 principal components (PCs) that accounted for 61% of the total variance in RNA abundance in plasma. PC1 consisted of miR-21-5p, miR-30a-5p, miR-30d-5p, miR-1, miR-423-5p, miR-100-5p, miR- 146a-5p, miR-146b-5p, miR-223-3p, miR-744-5p, and miR-98-5p. PCs describing RNA variation were associated with diffuse myocardial fibrosis, LV end-systolic volume, LV ejection fraction, and LV mass at baseline. Changes in LV mass over time was associated with PC1 (RNAs measured at baseline). In addition, we found that miRNA candidates are expressed differentially across murine cardiac cell types, with miR-30a-5p and miR-30d-5p expressing highest in cardiomyoctes and miR-21-5p, miR-1, miR-423- 5p, miR-100-5p, miR-146a-5p, miR-146b-5p, miR-223-3p, miR-744-5p, and miR-98-5p being expressed at higher levels in non-cardiomyocyte cells. Mouse and pig models of MI showed temporal regulation of miRNA candidates in plasma and tissue. For example, miR-146a was up-regulated in left ventricular tissue and down-regulated in plasma at 24 hours post infarction, and returned to baseline levels in the left ventricle at 1 and 4 weeks post-infarction, with a 2-fold increase in plasma levels at 4 weeks. In conclusion, this study has identified miRNA candidates that are differentially expressed in patients with beneficial or poor remodeling following MI and are temporally regulated in both plasma and tissue in animal models of MI.

27. Exosomal RNA: A More Informative Biomarker for Spontaneous Preterm Birth

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Preterm birth complications are estimated to be responsible for 35% of the world's 3.1 million annual neonatal deaths. Preterm birth has lifelong effects on neurodevelopmental functioning such as increased risk of cerebral palsy, impaired learning and visual disorders, and an increased risk of chronic disease in adulthood. The economic and social cost of preterm birth is high: lengthy stays in neonatal intensive care, long term medical and educational support, and stresses to families experiencing loss of a preterm baby or lengthy stays in the hospital. Current methods for predicting spontaneous preterm birth in women who have been admitted for threatened preterm labor involve clinical risk factor assessment, measuring cervical length (at risk <15mm) and presence of fetal fibronectin in the cervicovaginal fluid. However, these tools have low sensitivities (<50%). Our goal is to explore the possibility of using circulating miRNA as a biomarker for preterm birth by comparing the miRNA profiles in maternal plasma, exosome and exosome depleted plasma between individuals who had a spontaneous preterm birth and those with uncomplicated pregnancies. Plasma samples were collected from women who had spontaneous preterm births between 24-34 weeks (n=20) and matched with respect to gestational age and other variables to women not in preterm labor (n=50). Exosome and exosome depleted fractions were obtained from 11 plasma samples per group and used for RNAseq analysis. The human chromosome 19 miRNA (C19MC) gene cluster is expressed almost exclusively in the placenta. Placental microRNAs (miRNA) may be packaged and released into maternal circulation which may be used as an indicator for placenta health. We found 27 miRNAs from the C19MC cluster with significant concentration differences in the exosome between controls and patients with preterm labor. Interestingly, all C19MC cluster miRNA showed a concentration decrease in exosome compared to controls. The decreased concentrations of these miRNAs in exosome may indicate decreased normal placenta function which may reflect pathological conditions for preterm labor. We did not see similar changes in either the whole plasma or exosome depleted plasma. Our findings suggest exosomal miRNA may be more informative for identifying biomarkers related to preterm labor.

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