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The Extracellular RNA Communication Consortium: Establishing Foundational Knowledge and Technologies for Extracellular RNA Research

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The Extracellular RNA Communication Consortium (ERCC) was launched to accelerate progress in the new field of extracellular RNA (exRNA) biology and to establish whether exRNAs and their carriers, including extracellular vesicles (EVs), can mediate intercellular communication and be utilized for clinical applications. Phase 1 of the ERCC focused on exRNA/EV biogenesis and function, discovery of exRNA biomarkers, development of exRNA/EV-based therapeutics, and construction of a robust set of reference exRNA profiles for a variety of biofluids. Here, we present progress by ERCC investigators in these areas, and we discuss collaborative projects directed at development of robust methods for EV/exRNA isolation and analysis and tools for sharing and computational analysis of exRNA profiling data.

The Origin of the ERCC1 Program

The discovery that extracellular vesicles (EVs) can transport RNAs between cells (Skog et al., 2008; Valadi et al., 2007) suggested that RNAs carried by EVs may play a previously unrecognized role in intercellular communication and launched the field of extracellular RNA (exRNA) biology. It was quickly recognized that exRNAs might also have utility as biomarkers of disease and as therapeutic agents. There were, however, many gaps in knowledge and technical challenges to overcome. The mechanisms of EV biogenesis and uptake, exRNA cargo selection, and exRNA function were largely unknown. Moreover, efficient and reproducible methods for isolation and analysis of exRNAs were not available, further complicated by early findings that suggesting that exRNAs can associate with multiple subtypes

Table 1. Resources Developed by the ERCC1 Program

Resource	Application	References
Plasmids	Membrane/vesicle labeling with fluorescent proteins	(Chen et al., 2016†; Higginbotham et al., 2016†; Kamiyama et al., 2016†; Lai et al., 2015†; Lai et al., 2014a†, 2014b†; Leonetti et al., 2016†; Ong et al., 2017†)
	CRISPR/Cas9 mediated KO of EV markers and biogenesis components (e.g., Rab27a, nSMase2)	(Poggio et al., 2019††)
Cells	Sensor cells that report on exRNA activity	(Kamiyama et al., 2016†)
Mouse Models	Models of cancer with secretion of fluorescently tagged tumor-derived EVs	(Bronevetsky et al., 2013†; Farmer et al., 2017†; Lim et al., 2014†; van der Vos et al., 2016†)
	Conditional (cre-Lox) fluorescent exosomal reporter strains	
	Strains to study miRNA function	
Protocols	EV and exRNA isolation protocols	Nature Protocol Exchange: 2015.003,004,006, 097-100,107-120; 2016.057; 2017.071-079,084; 2018.049,050,052,081, (Hinger et al., 2018††; Lee et al., 2018†; Li et al., 2018c†; Li et al., 2018d†; Li et al., 2015†; Majem et al., 2017†; Max et al., 2018†; Patel, 2018†; Reátegui et al., 2018†; Srinivasan et al., 2019††)
	exRNA detection and quantification protocols	(Akat et al., 2014†; Belair et al., 2019†; Ben-Dov et al., 2016†; Giraldez et al., 2018†; Gogakos et al., 2017†; Hafner et al., 2012†; Majem et al., 2017†; Patel, 2018†; Yeri et al., 2018†)
Datasets and Computational Tools & Resources	exRNA Portal, a centralized access point for information, data, and resources about exRNAs.	http://exRNA.org
	exceRpt, a comprehensive analytic platform for extracellular RNA profiling.	(Rozowsky et al., 2019††)
	Virtual BioRepository (VBR), a distributed web-based system for biosample search and exchange between collaborating groups.	https://genboree.org/vbr-hub/
	exRNA Atlas, an on-line resource for exRNA data analysis and sharing.	(Ben-Dov et al., 2016†; Freedman et al., 2016†; Godoy et al., 2018††; Murillo et al., 2019††; Saugstad et al., 2017†; Shah et al., 2017a†, 2017b†)
	Small RNaseq data from biofluids and cell culture conditioned media	
	qPCR data from biofluids	

of EVs, as well as with ribonucleoproteins (RNPs) (Arroyo et al., 2011) and lipoprotein (LPP) complexes (Vickers et al., 2011), indicating that heterogeneity of exRNA carriers would be an important challenge.

The first phase of the NIH Common Fund-supported Extracellular RNA Communication Consortium (ERCC1), launched in 2013, was designed to jump-start progress in this nascent field by addressing five major scientific challenges identified by the exRNA research community (Ainsztein et al., 2015), which were adopted as the major goals of the program. Labs participating in the 30 funded ERCC1 projects have worked individually and collaboratively to work toward these goals, resulting in 480 manuscripts to date, including 18 manuscripts now presented by Cell Press, with more to come, and producing a variety of shared resources (Table 1). The exRNA Portal (http://exRNA.org/) provides descriptions of ERCC projects, a continuously updated list of ERCC publications, and links to these shared resources, as well as a calendar of exRNA-related events and an exRNA-focused blog. Throughout this Perspective, citations for ERCC1 manuscripts are designated with a "†", and "††" indicates a paper now presented by Cell Press.

The first goal of ERCC1 was to address the critical need to develop a better understanding of the mechanisms underlying exRNA biogenesis and export, mechanisms of secretion from source cells, uptake into recipient cells, and functions inside recipient cells. Research efforts by ERCC1 investigators have considerably improved our understanding of these processes. Many of these studies also resulted in the development of molecular and informatics tools, technologies, model systems, and imaging modalities, which are available to the broader scientific community, and will enable investigators to more readily approach related studies (Table 1).

The second goal was to provide a reliable and reproducible catalog of the exRNA species present in healthy human biofluids.

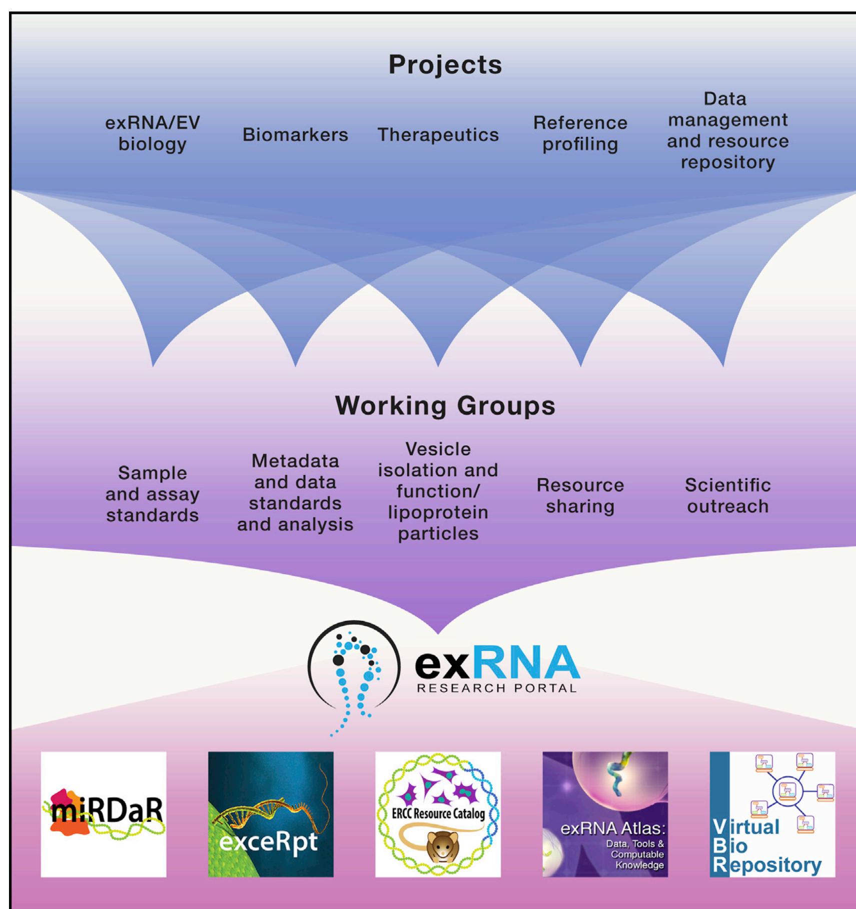

Figure 1. Organizational Structure of Phase 1 of the Extracellular RNA Communication Consortium

This diagram illustrates the major collaborative efforts among the investigators from the 30 individual ERCC1 projects, which were focused on five major domains (exRNA/EV biology, biomarkers, therapeutics, reference profiles, and data management and analysis). ERCC1 investigators interacted with each other in the context of the Working Groups to produce a broad range of shared resources, which can be accessed through the exRNA Research Portal (http://exRNA.org/).

Thus far, exRNA reference profiles for a variety of body fluids, in some cases collected at rest and after physical exercise, have been generated (Ben-Dov et al., 2016†; Freedman et al., 2016†; Godoy et al., 2018††; Saugstad et al., 2017†; Shah et al., 2017b†; Yeri et al., 2017†). These datasets, along with all other exRNA profiling datasets generated by ERCC1, are accessible at the exRNA Atlas (https://exRNA-Atlas.org).

The third goal was to develop computational technologies and tools to enable effective distribution of knowledge and utilization of exRNA profiling data. To accomplish this, the Data Management Resource Repository (DMRR), with input from ERCC1 members, established the exRNA Portal (http://exRNA.org) to serve as a central access point for exRNA resources.

The fourth and fifth goals were to rigorously establish the clinical utility of exRNAs as disease biomarkers and therapeutic agents. Several ERCC1 groups worked to establish the utility of exRNAs in diverse biofluids as biomarkers for a broad range of diseases (Table S1, tabs 1–4). Efforts have also been directed at development of exRNA- and EV-based therapeutic agents (Table S1, tab 5), devising mechanisms for the delivery of therapeutic exRNAs (Table S1, tab 6), and screening of drug libraries for exosome biogenesis inhibitors that can be repurposed for cancer treatment (Datta et al., 2017†, 2018†).

Building and Sharing Resources for exRNA/EV Analysis

Interactions among ERCC1 investigators were promoted by twice yearly meetings and monthly conference calls, resulting in the establishment of several collaborative Working Groups to address fundamental gaps in knowledge and technology, and to promote broad dissemination of methods, samples, and data (Figure 1).

The sample and assay standards working group focused on standardization of exRNA isolation and profiling methods. The low concentrations of exRNAs in biofluids, their vulnerability to contamination (Wei et al., 2016†), the presence of exogenous RNA, and heterogeneity in composition among samples of the same biofluid due to variable contributions from different cell types and exRNA carrier subclasses (Laurent et al., 2015†) all pose challenges to accurate and reproducible measurement. Recognizing that standardization could not be done in a principled manner without understanding the comparative performance of exRNA isolation and measurement methods, this working group embarked upon highly replicated collaborative projects to systematically compare the robustness, inter- and intra-lab reproducibility, and performance of exRNA isolation and measurement methods.

An exRNA isolation project involved six ERCC1 labs and consisted of systematic evaluation of the reproducibility and performance of multiple exRNA isolation methods across standardized samples of diverse biofluids, using quantitative reverse transcription PCR (qPCR) and small RNA-seq as the readouts (Srinivasan et al., 2019††). A key finding of this study was that the reproducibility within methods and concordance among methods varied widely, within and among both biofluids (with the exception of plasma and serum, which were extremely similar) and RNA biotypes. Using computational deconvolution, this study showed that exRNA isolation methods differ substantially in the efficiency and reproducibility with which they access the extracellular exRNAs associated with various carrier subclasses (EVs, RNPs, and LPPs). These results help to explain the low reproducibility observed among published studies and

lead to the conclusion that results obtained from a given combination of exRNA isolation method, biofluid, and RNA biotype cannot be assumed to hold true for other combinations. To enable customized selection of the optimal exRNA isolation method for a specific set of miRNAs in a given biofluid, an interactive web-based application, miRDaR (miRNA detection- and reproducibility-based selection of exRNA isolation methods, https://exrna.org/resources/software/mirdar), was developed, which extracts, analyzes, and displays the relevant data from this dataset based on users' selections.

Two other collaborative projects focused on small RNA measurement. The first project compared three small RNA-seq library preparation methods (NEBNext [NEB], NEXTFlex [Bioo], TruSeq [Illumina]), and three targeted miRNA profiling platforms—hybridization-based Fireplex (Abcam), next-generation sequencing-based EdgeSeq (HTG), and qPCR-based miRNome (QIAGEN)—to examine varying input amounts of standardized tissue RNA (from brain, liver, and placenta) and plasma exRNA (Yeri et al., 2018†). Biological differences among the three tissue miRNA profiles were preserved across all input amounts and profiling methods, particularly for highly expressed miRNAs. For plasma exRNA, the variability attributable to differences among small RNA measurement methods was stronger than that associated with different RNA input amounts. The second project compared four small RNAseq library preparation methods, including methods with fixed and degenerate adapters, using equimolar and ratiometric pools of synthesized small RNAs, to evaluate the absolute and relative bias of each method, and a standardized plasma exRNA sample, to assess inter- and intralab reproducibility on a biologically relevant sample type (Giraldez et al., 2018†). Degenerate adapters markedly decreased sequence-dependent bias, thereby improving library complexity. Despite systematic differences among all methods, relative quantification of any given miRNA with a $\geq$ 1.5-fold difference in concentration between samples was accurately and reproducibly measured by all methods. The results of these two studies indicate that although there were clear systematic differences among protocols, the overall reproducibility of all of the tested methods was excellent, and relative quantification was preserved among methods. Thus, while a single measurement method should be used for a given study, relevant differences between biological groups should generally be reproducible among studies, even if they used different small RNA measurement methods.

The metadata and data standards and analysis working group included computational and data scientists at the DMRR and Data Coordination Center (DCC), as well as other ERCC1 investigators, who focused on development and implementation of computational tools, data quality and metadata standards, workflows for data deposition, and sharing and integrative analysis of data from multiple studies. To address the critical need for a standardized workflow optimized for exRNA data processing, mapping, and normalization, members of this working group developed the exceRpt (extracellular RNA processing toolkit) pipeline (Kaczor-Urbanowicz et al., 2018†; Rozowsky and Gerstein, 2019††). exceRpt includes a modular cascade of alignments/quantifications against multiple RNA biotypes from diverse species. The default order of the annotations used is based on the expected abundance in commonly profiled biofluids, but can be easily customized by the user. exceRpt has been used to uniformly process all the datasets that are available in the exRNA Atlas and is available at exRNA-Atlas.org (website) and github.gersteinlab.org/exceRpt (source code). The exRNA Atlas and an accompanying suite of tools were created to facilitate exRNA data deposition, coordination, processing (using exceRpt), analysis, visualization, and sharing (Murillo et al., 2019††, this issue of *Cell*). Guided by detailed online and tutorials, users can select existing exRNA profiles of interest based on a rich set of clinical and technical metadata using an intuitive Graphical User Interface and/or upload and analyze their own exRNAseq or qPCR data using the available tools.

The first cross-Atlas integrative analysis of 5,309 exRNAseq profiles from 19 studies encompassing 23 health conditions and 5 biofluids (Murillo et al, 2019††) revealed that despite uniform processing using the exceRpt pipeline, substantial sample-to-sample and cross-study variability remained, posing an obstacle to cross-study comparisons. This variability was addressed by unsupervised computational deconvolution, which revealed six exRNA cargo types (CT1, CT2, CT3A, CT3B, CT3C, CT4). Sample-to-sample and cross-study variation in the relative abundance of cargo types explained 50%–90% of the observed variability. Four of the cargo types were associated with known exRNA carriers (low- and high-density EVs, RNPs, and LPPs) and largely corresponded to the carrier subclasses identified in the exRNA isolation study (Srinivasan et al., 2019††). Deconvolution was then used to estimate cargo type proportions in a published dataset of plasma samples collected pre- and post-exercise (Shah et al., 2017b†), which enabled identification of exercise-responsive pathways that were not detected without deconvolution, thus demonstrating the utility of this approach. To facilitate application to more datasets, deconvolution analysis has been made accessible along with the exceRpt pipeline (Rozowsky and Gerstein, 2019††) through the exRNA Atlas site (exRNA-Atlas.org).

The resource sharing working group facilitated the exchange of biofluid samples and fostered collaborations via the development of the Virtual BioRepository (VBR), a web-based system for biosample search and exchange between collaborating groups (https://genboree.org/vbr-hub/). The VBR currently provides access to over 56,000 biofluid samples from six institutions (Table 2) using common IRB and uMTA protocols. The VBR captures metadata, including deidentified donor demographics, sample preparation and storage, residual volume, and chain of custody, and includes an intuitive search interface consisting of a dashboard and query tools for keyword/keyphrase and faceted searching based on rich sample metadata. The VBR also includes features that facilitate open, auditable, and traceable communication between the interested parties through a detailed sample exchange process.

The vesicle isolation and function/lipoprotein particle working group was comprised of ERCC1 members interested in refining methods for the scalable and efficient production and purification of EVs for downstream functional studies. Ongoing efforts focus on applying and validating emerging methods, including hollow fiber and stirred tank bioreactors and tangential flow filtration to concentrate EVs from large volumes of conditioned medium, often in combination with standard purification

Table 2. VBR Biosamples and Institutions

	Mayo	BNI	OHSU	NXDC	TGen	UCSD	Total
No. Biosamples	48,746	7,503	118	20	5	5	56,397
No. Donors	1,595	730	118	10	5	5	2,463
Biofluid/Disease	Serum,Plasma/ Liver diseases	CSF	CSF	CSF	CSF	CSF	

Mayo: Mayo Clinic, Jacksonville, FL; BNI: Barrow Neurological Institute, Phoenix, AZ; OHSU: Oregon Health & Science University, NXDC: NX Development Corporation; TGen: Translational Genomics Institute Phoenix, AZ; UCSD: University of California, San Diego. CSF: Cerebrospinal Fluid.

methods, to produce EVs with reproducible functional effects in model systems.

The scientific outreach working group established and maintains the exRNA Portal (exRNA.org), a centralized access point for exRNA-centered information, data, and resources, including the exRNA Atlas, the exceRpt pipeline, other exRNA data analysis tools (https://exrna.org/resources/software/), a comprehensive collection of exRNA protocols (https://exrna.org/resources/protocols/), the Virtual BioRepository, and other resources, including archived presentations by ERCC1 investigators (https://exrna.org/resources/presentations/). ERCC1 investigators have also generated biological reagents for tracking, characterization, and functional analysis of EVs and their cargo (Table 1). An itemized list of these highly validated tools, including primary applications, detailed protocols, and originator contact information, can be found at the exRNA Portal.

Additional ERCC1 Contributions and Future Directions in exRNA/EV Research

Biology of exRNAs and exRNA Carriers

A key barrier to progress toward a comprehensive understanding of exRNA biology and function has been the heterogeneity of exRNA carriers, including EVs, RNPs, and LPPs. Due to substantial overlap in their physico-chemical properties, these diverse carrier classes co-purify in many commonly used isolation protocols (Tauro et al., 2012), making it challenging to quantify the exRNAs associated with specific carriers and to delineate their functions. In this section, we first discuss current and emerging approaches for separation and characterization of known and novel exRNA carriers, then present a brief summary of our current understanding of exRNA carrier biogenesis, including exRNA packaging, highlighting areas in which our knowledge is particularly limited.

Multiple methods are used for separation of exRNA carriers, and each has its own limitations. Commonly used approaches that both concentrate and separate carriers by density are based on density-gradient ultracentrifugation (DGUC). While sequential DGUC is widely used to isolate LPP subclasses (Li et al., 2018d†), cushioned ultracentrifugation followed by DGUC (C-DGUC) is useful for isolation of EVs (Li et al., 2018c†). However, some exRNA carrier subclasses, e.g., exosomes and HDL, have similar densities and thus fractionate together on DGUC (Michell et al., 2016†; van der Pol et al., 2012). Size exclusion chromatography (SEC) can separate HDL (10 nm in diameter) and exosomes (50–100 nm in diameter), but co-fractionates exosomes and VLDL (30–80 nm in diameter), and also dilutes the input samples. Ultrafiltration can fractionate EVs by size (Cheruvanky et al., 2007; Quintana et al., 2015; Wei et al., 2017†) but is difficult to scale and may damage vesicles due to the forces used to pass EVs through small porosity filters. To address these issues, some investigators have turned to tangential field flow (TFF) (Heinemann et al., 2014) and asymmetric flow field-flow fractionation (A4F). A4F has been used to isolate EVs (Wagner et al., 2014; Zhang et al., 2018a) and LPPs (Kuklenyik et al., 2018; Rambaldi et al., 2009), as well as a novel class of nanoparticles termed exomeres (Zhang et al., 2018a) that have recently been shown to transfer functional cargo to recipient cells (Zhang and Coffey, 2019††). Other methods for EV and LPP enrichment include immunoaffinity methods (including immunoprecipitation, immunoaffinity chromatography, protein capture microarrays, and microfluidic systems [Reátegui et al., 2018†]), ion exchange sequential chromatography, and acoustofluidic separation (Wu et al., 2017). Fluorescence-activated vesicle sorting (FAVS) has been used as a single-vesicle analysis technology (Higginbotham et al., 2016†), although specific sorting of EVs has been difficult to scale. Some investigators have combined multiple methods to enable more precise isolation of exRNA carriers of interest. Combining C-DGUC and SEC enables both processing of high input volumes and separation of EVs from HDL and other LPPs (Karimi et al., 2018; Michell et al., 2016†). Contrary to conclusions from previous studies, use of a combination of DGUC and direct immuno-affinity capture recently showed that exosomes do not contain cytoskeletal elements, common glycolytic enzymes, DNA, or Ago-associated RNPs (Jeppesen and Coffey, 2019††). In addition to physical separation methods, computational deconvolution techniques have the potential to unravel the signatures of specific exRNA carrier subclasses from profiles of complex mixtures. This strategy has been applied to RNA profiles of bulk tissue samples, including tumors, to infer the RNA profiles of different constituent cell types (Onuchic et al., 2016; Newman et al., 2015) and was utilized in (Murillo et al., 2019††; Srinivasan et al., 2019††).

A variety of exRNA carrier detection methods are available, again, each with their own limitations. The presence of EVs, RNPs, and LPPs in a sample can be assessed by measuring the amount of carrier-specific proteins or lipids. The small size of RNPs and HDL particles make them incompatible with single-particle analysis methods, but EVs and larger LPPs can be evaluated by nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), multi-angle light scattering (MALS), microfluidic resistive pulse sensing (MRPS), FAVS, transmission electron microscopy, scanning electron microscopy, single particle interferometric reflectance imaging (SPIRI) (Daaboul et al., 2016), and super-resolution microscopy approaches, including stochastic

optical reconstruction microscopy (STORM) (Nizamudeen et al., 2018). The utility of these emerging technologies is expected to be greatly enhanced by advances in labeling of specific EV subtypes, such as systems that use expression of cell-type-specific membrane fluorescent proteins (Pua et al., 2019††) to enable tracking of EVs by cell source.

Our understanding of the biogenesis of exRNA carriers (even those that are already known) remains incomplete. EVs are produced by a variety of biogenesis mechanisms and range from 50 nm to over 1 μm in diameter. EVs include exosomes, microvesicles (MVs), apoptotic bodies, microparticles, and other less studied subtypes, with $\sim 10^{9-11}$ EVs/mL blood (Chevillet et al., 2014†). LPPs, including chylomicrons (75–500 nm in diameter), very low-density lipoproteins (VLDL, 30–80 nm in diameter), low-density lipoproteins (LDL, 25 nm in diameter), and high-density lipoproteins (HDLs, 10 nm in diameter) are significantly more abundant than EVs in circulation, with $\sim 10^{17-18}$ LDL and HDL particles per 1 mL of blood. RNPs include complexes containing Nucleophosmin (Wang et al., 2010) and Argonaute proteins (Arroyo et al., 2011; Turchinovich and Burwinkel, 2012; Turchinovich et al., 2011; Wei et al., 2017†).

The biogenesis of exosomes has been the most extensively studied and shown to involve recognition of monoubiquitinated cargo by the ESCRT-0 components Hrs and STAM, followed by sequential recruitment of ESCRT I-IV machinery proteins to promote budding and fission of intralumenal vesicles within multivesicular bodies (MVB) (Henne et al., 2011; Hurley and Hanson, 2010). Mechanisms of exosome biogenesis that are less well characterized include synthesis of the lipid ceramide to induce membrane budding (Trajkovic et al., 2008). Genetic manipulation of the biogenesis pathway provides a powerful tool to study the function of exosomes *in vivo*. For example, by blocking exosome biogenesis or release by either deleting nSMase2 or Rab27a, respectively, Poggio et al. showed that exosomes play a major role in suppressing the anti-tumor immune response across multiple cancer models by presenting the immune checkpoint protein PD-L1 (Poggio et al., 2019††).

The biogenesis of MVs is less well understood but may involve lipid flipping from one leaflet of the plasma membrane to the other to induce membrane curvature and budding (Awojoodu et al., 2014; Bianco et al., 2009; Hoehn et al., 2017; Hugel et al., 2005; Tuck, 2011; Wehman et al., 2011) or budding from the plasma membrane after recruitment of the same ESCRT machinery that induces exosome formation within MVBs (Nabhan et al., 2012).

Relevant to packaging of exRNA cargo into carriers, sequencing of small and long RNAs has shown enrichment of specific RNAs in EVs compared to their parental cells (Cha et al., 2015†; Hinger et al., 2018††; Kosaka et al., 2010; Santangelo et al., 2016; Shurtleff et al., 2016; Skog et al., 2008; Squadrito et al., 2014; Valadi et al., 2007; Wei et al., 2017†). There is evidence that this is a regulated process—for example, by KRAS (Cha et al., 2015†; Dou et al., 2016†; Hinger et al., 2018††), T cell antigen receptor and co-stimulatory signals (Chiou et al., 2018††), and other signaling pathways. Specific RNA sequence motifs can lead to EV packaging in certain contexts (Bolukbasi et al., 2012; Hobor et al., 2018; Koppers-Lalic et al., 2014; Santangelo et al., 2016; Shurtleff et al., 2016; Villarroya-Beltri et al., 2013). This sequence-specific targeting can be mediated by RNA-binding proteins, including hnRNPA2B1 (Villarroya-Beltri et al., 2013), Ago2 (McKenzie et al., 2016; Melo et al., 2014), YB-1 (Kossinova et al., 2017; Shurtleff et al., 2016), SYNCRIP (Santangelo et al., 2016), NSUN2 (Kossinova et al., 2017), and others (Statello et al., 2018). It remains unclear how non-vesicular carriers are generated and pick up RNA cargo. It has been speculated that RNPs are released from dead and dying cells, which may explain their abundance in body fluids. Among LPPs, HDL has been shown to accept miRNAs from macrophages *in vitro* (with inhibition of exosome biogenesis increasing this process) and in the mouse circulation *in vivo* (Vickers et al., 2011).

Taken together, this work suggests that exRNAs are associated with diverse carriers, each with its own biogenesis pathway and characteristic exRNA cargo. Several exRNA carriers have been described, but it is likely that others remain to be discovered. Even for known carriers, a comprehensive catalog of their molecular contents and a detailed delineation of the mechanisms underlying their biogenesis and cargo loading remain to be fully elaborated. Given that differences in the relative representation of each carrier may contribute to sample-to-sample variability, careful consideration of heterogeneity in carrier composition is critical for all exRNA studies. To enable investigators to fully account for this heterogeneity, it will be necessary to comprehensively define the carrier subclasses that exist for each biofluid type and develop strategies for measuring the exRNA profile of each carrier subclass in each sample. These strategies may be based on either physical separation and profiling of each carrier subclass or generation of reference exRNA profiles for each carrier subclass so that deconvolution analysis can be used to determine the contribution of each carrier to the overall exRNA profile for each sample.

Extracellular RNAs as Biomarkers of Human Disease

Ten ERCC1 groups focused on exRNA biomarker discovery (Quinn et al., 2015†) and produced exRNA signatures in diverse biofluids for a range of diseases biomarkers (Table S1, tabs 1–4). While most prior studies focused on circulating miRNAs, the broad adoption of small RNAseq by ERCC1 groups enabled examination of other RNA biotypes (Danielson et al., 2017†; Freedman et al., 2016†; Li et al., 2018a†; Yeri et al., 2017†). An emerging theme is that performance of exRNA signatures comprised of multiple exRNA markers, possibly in combination with established protein biomarkers and/or clinical imaging, may be superior to measurements of single exRNAs (Li et al., 2014; Lusardi et al., 2017†; Shah et al., 2018†; Wiedrick et al., 2019†). Algorithms and computational platforms for developing such integrated signatures require further development and continue to evolve. Another concept that has grown out of this early exRNA biomarker work is the potential role of exRNA biomarkers in disease pathogenesis, which in turn suggests that exRNAs may be therapeutic targets (Melman et al., 2015†; Regev et al., 2017†). It has been demonstrated that "functional biomarkers" identified in humans studies are often dysregulated in similar fashion in animal models, and can serve as probes for pathway characterization and discovery (Danielson et al., 2018††; Shah et al., 2018†).

Selection of appropriate controls for exRNA biomarker studies is critical. Case-control studies are appropriate for diseases with defined separable outcomes, such as cancers with or without metastasis. However, studies requiring healthy controls are plagued by the potential presence of asymptomatic diseases, particularly in older age groups due to the increasing prevalence of many chronic diseases with age. To address this need, one of the major goals of ERCC1 was to create large databases of reference exRNA profiles across multiple biofluids and physiologic states for healthy subjects, resulting in a number of publications to date (Ben-Dov et al., 2016†; Freedman et al., 2016†; Godoy et al., 2018††; Saugstad et al., 2017†; Shah et al., 2017a†, 2017b†), with additional studies on large cohorts in progress.

During the course of these biomarker discovery and reference profiling projects, we have identified important challenges that need to be overcome before the clinical utility of exRNA biomarkers can be fully realized. These were also discussed at an NIH Strategic Workshop focused on determining unmet needs and future directions for the ERCC, which included both ERCC and external investigators and resulted in a white paper that summarized the key future directives to address these needs (Li et al., 2018b†). First, there remain biofluids that have not been adequately explored as sources of exRNA biomarkers. Among these is urine, which is easily and non-invasively collected. However, the association of urinary exRNAs with potential carriers and how they enter the urine are poorly understood, and it is unclear to what extent cells outside the urinary tract contribute to urinary exRNAs. Even in genitourinary cancers and kidney disease research, results have been inconclusive (Fendler et al., 2016; Ledeganck et al., 2019), due in part to small sample sizes and lack of standardization of methods for urine collection and processing, RNA isolation and quantification, and data analysis (Hsu et al., 2015). Second, it has become clear that differences in pre-analytical and analytical processing represent substantial challenges to the discovery and validation of exRNA biomarkers. Several studies have demonstrated marked differences in the yield and reproducibility of different exRNA isolation methods (Filant et al., 2018†; Max et al., 2018†; Saugstad et al., 2017†), which are in part due to their biases for different exRNA carrier subclasses (Srinivasan et al., 2019††). Therefore, the scientific community should work toward adoption of standardized methods for sample collection and processing and exRNA isolation and measurement, which are selected based on the yield and reproducibility of available methods. Third, for the growing number of promising exRNA biomarker discovery studies, validation studies on larger cohorts are necessary, such as in (Wiedrick et al., 2019†). Fourth, the increasing recognition that difference carrier subclasses are associated with distinct sets of cargo (Srinivasan et al., 2019††; Turchinovich and Burwinkel, 2012; Wei et al., 2017†) suggests that studies focusing on a specific carrier subclass may decrease variability and increase the signal to noise ratio (van Eijndhoven et al., 2016).

Many of the issues discussed above are addressed by a recent call by NIH to improve rigor and reproducibility in the field (PAR-16-276), which includes these (and other) priorities:

- Development of standards: creation of internal and external ‘‘known standards’’ that can be used to evaluate the validity of methods and reagents.
- Development of tissue-specific markers: identifying a set of specific markers that can be used to enrich for disease- and/or tissue-specific EVs may improve the signal to noise ratio and decrease the variability of measurements.
- Separation of different exRNA carrier subclasses.
- Rigorous evaluation of the outputs of carrier subclass separation and exRNA isolation technologies: the observed wide variability in efficiency and reproducibility among exRNA isolation methods on different biofluids (Srinivasan et al., 2019††) emphasizes the need for rigorous evaluation of the performance of carrier subclass separation, exRNA isolation, and analysis methods going forward.

Therapeutic Applications of Extracellular RNAs

Therapeutic products that exploit biological properties to provide advantages over existing approaches could lead to new exRNA-based therapies. Potential clinical applications of exRNA-and EV-based therapeutics include immune modulation, regenerative medicine, and drug delivery, and can employ EV-based systems to deliver endogenous or modified exRNA or other therapeutic cargoes. Several ERCC1 groups have reported preclinical studies that have used these diverse approaches in selected disease-relevant models and have demonstrated the feasibility of their use for therapeutic purposes (Table S1, tabs 5 and 6).

Naturally occurring EVs, e.g., those produced by stem cells, have properties that allow them to be directly applied as therapeutic agents. For example, bone marrow mesenchymal stromal cell-derived EVs can accumulate at the site of liver or kidney injury and exert a protective effect and can also ameliorate the effects of radiation injury to bone marrow, while interferon-stimulated dendritic cell-derived EVs can increase neuronal myelination (Table S1, tab 5). Another approach for developing EV-based therapeutics is to engineer natural EVs to serve as carriers for delivery of drugs (Table S1, tab 6) or specific native or modified RNA sequences (Table S1, tab 5). Synthetic EV mimetics, such as liposomes that contain a subset of the components of natural EVs, can also be used to deliver drugs or therapeutic RNAs. Major challenges that need to be overcome for all these approaches include efficient loading of the desired cargo, reproducible large-scale production, and efficient delivery of cargo into target cells.

Many approaches have been used to produce large quantities of EVs. For example, EVs derived from bovine milk have been used as delivery vectors to target liver cancers (George et al., 2018†; Matsuda et al., 2019†). Likewise, EV derived from broccoli, grapefruit, or ginger can also be used to target cancer or deliver therapeutics to sites of injury or inflammation (Deng et al., 2017††; Teng et al., 2016†; Zhuang et al., 2015†, 2016†). Therapeutic EVs could be unmodified, or they may have modifications designed to optimize their uptake, surface markers, or cargo. A recent study used engineered EVs to deliver exogenous functional mRNA into cells. Here, EVs directed to the HER2 receptor were loaded with mRNA encoding the enzyme HChrR6, which was used together with the prodrug 6-chloro-9-nitro-5-

oxo-5H-benzo-(a)-phenoxazine to target HER2+ breast cancer cells and xenografts (Wang et al., 2018†). In order to target EVs specifically to cancer cells, RNA aptamer/cholesterol nanostructures have been attached to the outer EV membrane to deliver siRNA cargo efficiently to prostate, breast, and colorectal cells (Pi et al., 2018†).

Preclinical assessment of EV therapeutics to demonstrate safety, including the absence of significant toxicity or negative immune effects, have been the focus of several ERCC1 studies (Table S1, tab 7), but additional investigation is warranted.

Conclusions

The ERCC Program was launched by the NIH Common Fund to tackle the most challenging topics in the nascent field of exRNA research, as well as to develop and disseminate resources that would accelerate progress in the field. In the first phase of the ERCC Program, individual projects have made significant contributions toward: 1) understanding of the biology of exRNAs and their carriers and the variability of exRNA profiles in a variety of biofluids among healthy individuals; 2) identification of exRNA biomarkers for a broad range of diseases; and 3) optimization of methods for large-scale production of safe and effective exRNA- and EV-based therapeutics. Collaborative projects among multiple ERCC1 groups have enabled systematic comparisons between exRNA isolation and analysis methods, which will allow investigators select the optimal currently available methodology for planning future studies, and will provide benchmarks against which novel methods can be compared. Other ERCC1 efforts have produced a broad range of biological, methodological, data, and computational resources, which are publicly accessible through the exRNA Portal (exRNA.org). Key among these resources are: validated plasmids, mouse strains, and protocols for characterization, tracking, and functional analysis of exRNA carriers and their cargo; and the exRNA Atlas, a searchable repository of exRNA profiling data from ERCC1 and non-ERCC1 investigators. These resources are accompanied by a suite of computational tools, including an integrated workflow for small RNAseq data processing (exceRpt), analysis (including computational deconvolution and case-control differential expression analysis) and visualization tools, and links to functional enrichment and pathway analysis tools (WikiPathways, exRNA BioGPS, VesiclePedia). It is clear to us that many of the accomplishments from ERCC1 were facilitated by interactions among groups with diverse and complementary expertise. While we anticipate that the knowledge and resources acquired and shared by the first phase of the ERCC program will contribute substantially to future progress in this field, we recognize that important challenges remain. Some (but certainly not all) of these challenges, including development of methods for single-vesicle characterization and rigorous and scalable methods for separation and analysis of exRNA carrier subclasses, will be addressed by the second phase of the ERCC program.

SUPPLEMENTAL INFORMATION

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CONSORTIA

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DECLARATION OF INTERESTS

S.D. is a founding member of Dyrnamix, which has a patent on extracellular RNA biomarkers for cardiac remodeling. K.V.K.-J. is on the scientific advisory board for Dyrnamix. M.B. is a Clinical Advisor to Opsidio, LLC.

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