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REVIEW

Advances, challenges, and opportunities in extracellular RNA biology: insights from the NIH exRNA Strategic Workshop

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Extracellular RNA (exRNA) has emerged as an important transducer of intercellular communication. Advancing exRNA research promises to revolutionize biology and transform clinical practice. Recent efforts have led to cutting-edge research and expanded knowledge of this new paradigm in cell-to-cell crosstalk; however, gaps in our understanding of EV heterogeneity and exRNA diversity pose significant challenges for continued development of exRNA diagnostics and therapeutics. To unravel this complexity, the NIH convened expert teams to discuss the current state of the science, define the significant bottlenecks, and brainstorm potential solutions across the entire exRNA research field. The NIH Strategic Workshop on Extracellular RNA Transport helped identify mechanistic and clinical research opportunities for exRNA biology and provided recommendations on high priority areas of research that will advance the exRNA field.

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Introduction

Cells can alter their phenotypes through the dynamic secretion and uptake of RNA, thus implicating RNA molecules as critical mediators of intercellular communication (1, 2). The discovery of extracellular RNA (exRNA) as a signaling molecule represents a fundamental change in our understanding of the regulatory role of RNA in cell biology and has enormous translational potential for human health. The Extracellular RNA Communication Program was launched to address a collective need to develop community standards, best practices, and innovative tools in exRNA research. The overarching goals of the Extracellular RNA Communication Consortium are to increase understanding of exRNA biogenesis, transport, and functional effect on recipient cells; identify exRNA biomarkers that can serve as signatures of health or disease; demonstrate the clinical utility of exRNAs as therapeutics; and develop community resources through the creation of a data and resource repository (3). This cross-cutting program has expanded our understanding of the role of exRNAs in health and disease and generated many tools, technologies, and data for the entire research community, all of which are publicly accessible through the exRNA Portal (https://exrna.org/).

While significant advances have been made, the exRNA field still faces challenges, in part due to the inherent diversity of exRNAs and heterogeneity of their respective transport carriers, i.e., extracellular vesicles (EVs), RNA-binding proteins (RBPs), and lipoproteins (LPPs). Other challenges include technical difficulties in isolating and analyzing exRNAs from biological samples and the lack of robust experimental and conceptual models for mechanistic studies (4–7).

To address such challenges, a strategic workshop focused on assessing the current state of the science in the field of exRNA communication was held in June 2017. Key areas were identified to both increase the rigor and reproducibility of findings as well as address the technological and scientific roadblocks to advancement of the field. In particular, there was emphasis on the need to develop tools and techniques to more reproducibly measure and characterize EVs and exRNAs and use validated model systems to study the biogenesis, transport, and function of exRNAs. To this end, there was consensus to identify synergies between physical and synthetic sciences alongside nanotechnology that could accelerate progress in exRNA research. In addition, pressing scientific questions in the exRNA field and a set of recommendations outlining the knowledge, tools, or community resources needed to accelerate exRNA research were discussed.

Biological significance of exRNA transport

Transmission of viral resistance at the maternal-placental-fetal interface. In eutherian organisms, the placenta protects the embryo from the hematogenous spread of pathogens. The placental trophoblasts at the fetomaternal interface are highly resistant to viral infection (8). This effect is attributed, at least in part, to the expression of the primate-specific chromosome 19 miRNA cluster (C19MC) (9, 10). Yoel Sadovsky revealed a new communication paradigm between the placenta and fetomaternal compartments, which is mediated by exRNAs in EVs. Cultured trophoblasts package these placenta-specific C19MC miRNAs into EVs, which can confer viral resistance to recipient cells (11). C19MC miRNAs direct a robust autophagy response in trophoblasts, as revealed by electron microscopy and LC3B puncta formation using fluorescence microscopy (11). This pathway constitutes a powerful evolutionary adaptation to confer nontrophoblasts with antiviral activity and enhance the protection of the developing fetus against infections. Since many other placenta-derived miRNAs have reported to be delivered to diverse cell types (8, 12–14), this cell-cell crosstalk within the placental microenvironment via exRNAs may be involved in broader aspects of placental physiology, such as the maintenance of immune barrier and fetal tolerance.

Friend or foe in viral pathogenesis. EVs released from virus-infected cells contain a variety of viral and host cellular factors that can regulate recipient host cell responses (15, 16). However, the underlying mechanisms by which this EV content contributes to viral pathogenesis and the development of autoimmune diseases remain incompletely understood. Fatah Kashanchi discussed the interplay between HIV-1 and exosomes in the pathogenesis of AIDS. Release of HIV virions and exosomes share overlapping biogenesis pathways, including the ESCRT pathway (17–19). Exosomes containing the HIV *trans*-activating response (TAR) element RNA, an HIV TAR-gag RNA that can be packaged into exosomes and released from infected cells, enhance susceptibility of undifferentiated naive cells to HIV-1 infection (20–22). Importantly, this single-stranded 5′ or 3′ processed stem RNA was found to bind TLRs in recipient cells (21), activating an NF-κB–dependent inflammatory pathway (23). These data demonstrate that exRNAs can serve as ligands for innate pattern recognition receptors and induce inflammatory responses, which represents a novel mode of action for exRNAs.

Michiel Pegtel demonstrated that innate sensing of latent viral exRNAs contribute to pathogenic inflammation and autoimmunity (24). EBV-encoded small RNA, EBER1, is secreted by the infected B cells via sorting into exosomes. These exosomes are preferentially captured and internalized by human plasmacytoid DCs (pDCs). Transfer of unshielded 5′ppp-EBER1 into the pDCs via endocytosis of the exosomes triggers antiviral immunity (24). Interestingly, using Dynasore, a potent inhibitor of endocytosis (25), his group showed that phosphatidylserine receptors, which are used by enveloped viruses for cell entry (26, 27), are also involved in exosome uptake. Due to the similarity in physical and chemical characteristics, as well as biogenesis, export, and uptake pathways between EVs and viruses, especially retroviruses (16), the existing knowledge of these viruses and their interactions with the host may provide some insight into EV biology.

EV-associated exRNAs shape the hematopoietic stem cell niche. Hematopoiesis occurs in a specialized microenvironment, termed the hematopoietic stem cell (HSC) niche, which provides signals that regulate HSC self-renewal, differentiation, and proliferation (28–30). How the HSC niche is remodeled to suppress normal hematopoiesis and promote leukemogenesis in acute myeloid leukemia (AML) is incompletely understood. Peter Kurre demonstrated that AML-derived EVs suppressed residual hematopoietic stem and progenitor cell (HSPC) function indirectly through stromal reprogramming of niche retention factors and directly on HSPC and long-term repopulating HSCs. HSPCs cocultured with the EVs isolated from cultured AML cells, or plasma from mice bearing AML xenografts, exhibited impaired clonogenicity (31). Mechanistically, miR-150 and miR-155 were shown to suppress the hematopoietic transcription factor c-Myb, a known regulator for the erosion of stem cell progenitor function (32, 33). Along with recent studies showing that mesenchymal stem cell–derived EVs regulate HSC function (34), these data suggest a complex model in which EVs and their exRNA cargo coordinately regulate the HSC bone marrow niche.

Biomarkers for cardiovascular diseases. EVs and their contents (including exRNAs) hold great potential as diagnostic and prognostic biomarkers for cardiovascular diseases (CVDs) (35, 36). To search for the miRNA signatures that could be useful biomarkers to distinguish between acute myocarditis and myocardial infarction with nonobstructive coronary arteries syndrome, Francisco Sánchez-Madrid and his group used a transcriptome-based approach in a mouse model of Th17-mediated experimental autoimmune myocarditis (EAM) and identified a set of miRNAs that were overexpressed in antigen-specific Th17 cells and enriched in plasma EVs of EAM mice (our unpublished observations and ref. 37). Interestingly, the human homologs of these miRNAs appeared to be significantly enriched in plasma EVs of myocarditis patients but not expressed in healthy donors or ischemic myocardial infarction patients.

Saumya Das showed that increased levels of plasma extracellular miR-30d were associated with beneficial cardiac remodeling in response to resynchronization therapy in advanced heart failure patients with dys-synchrony (38). Moreover, miR-30d overexpression in cultured cardiomyocytes provided protection against apoptosis by targeting mitogen-associated kinase 4 (MAPK4), a key regulator of TNF signaling. These data underscored that exRNAs could be used as prognostic biomarkers that guide the treatment of patients at risk of heart failure and emphasized the need for understanding CVD pathogenesis by decoding EVs and exRNA signals.

exRNA functional diversity and sorting. There is accumulating evidence that, in addition to microRNAs, EVs contain many other species of noncoding RNAs (39–42). However, the functionality of these RNA species is largely unknown. For example, Y RNAs and tRNAs were substantially enriched in EVs derived from human glioma stem cells, leukemic cells, and breast cancer cells (42–44). Thomas Gingeras presented evidence that, while EVs derived from primary cells contained copies of the full-length (83-nt) human Y5 (hY5) RNA as well as processed 23-nt or 31-nt variants, only the 31-nt hY5 RNA fragments were found in cancer cell-derived EVs (43). Interestingly, only EVs derived from cancer cells or transfection of the 31-nt hY5 RNA fragment could cause apoptotic cell death in human primary fibroblasts (43). A recent study showed tRNA fragments carried by EVs could regulate gene expression in the embryo (45). It will be very interesting to determine the contribution of these poorly studied noncoding RNAs to the function attributed to miRNAs in EVs.

Developing experimental approaches to manipulate the RNA content of EVs is critical for studying the functional role of exRNAs. However, progress has been hampered by a lack of deep understanding of the molecular mechanisms by which exRNAs are processed and packaged in the cell. Alissa Weaver presented evidence that some mature miRNAs were associated argonaute 2 in the cytoplasm and sorted into exosomes in a process regulated by KRAS signaling (46, 47) and by subcellular trafficking between different organelles. There is accumulating evidence that some other RBPs are involved in sorting miRNAs into EVs. Y-box protein 1 (YBX1) has a broad role in sorting a non–Ago2-bound miRNA (miR-223) and other

small noncoding RNAs into exosomes (48, 49). A sumoylated form of hnRNPA2B1 binds and packages miRNAs containing specific motifs into exosomes in T cells (50). In hepatocytes, SYNCRIP (hnRNPQ) has been reported to sort miRNAs with a GGCU sequence at the 3′ end into EVs (51). These findings collectively demonstrated that RBPs play an important role in defining the RNA composition of EVs.

Non-EV exRNA carriers. exRNAs have also been found in extracellular fluids as part of LPP particles, e.g., HDL (52, 53), or in association with RBPs (54, 55). The exRNA species present in these non-EV carriers and their physiological and clinical relevance have not been well defined. Kasey Vickers revealed that the predominant types of small noncoding RNAs found on LPPs were rRNAs, miRNAs, and tRNA fragments, with very low levels of snRNAs and snoRNAs (52, 56). Monocytes and macrophages export specific miRNAs, including miR-223-3p, to HDL (52), which can be transferred to endothelial cells and exert an antiinflammatory effect on these cells (57). HDL miRNAs have been shown to be altered in CVD and diabetes (52, 53, 58, 59). These findings support the functional importance of the LPP exRNA transport pathway and reveal the potential of HDL-associated miRNAs as biomarkers for cardiometabolic diseases.

RBPs remain the least studied exRNA carriers. Although it is known that most of the microRNAs in blood plasma are carried by argonaute complexes independent from EVs (52, 54), it remains unclear whether circulating argonaute/microRNA complexes are passively released from cells upon apoptosis/necrosis or exported from biologically active cells in a regulated fashion (60). Argonaute-bound miRNAs have been found to stand alone (54) as well as be present in EVs (61) and their precursors (46, 62). It will be important to develop new experimental approaches to separate these different forms of RBPs for unambiguous elucidation of their RNA content and biological functions.

Emerging technologies and tools in EV biology

Visualizing and tracking functional exRNA transport. Significant efforts have been made by the EV research community to determine whether transfer of exRNA occurs in vivo and is functional relevant in physiology and disease (6, 7, 63, 64). The use of fluorescent membrane dyes or fluorescently tagged EV proteins with advanced microscopy has allowed direct visualization of EV dynamic localization, confirming their incorporation into target cells. For example, Peter Kurre took advantage of primary murine mesenchymal stem cells generated from transgenic mice that constitutively express membrane-targeted mTomato protein and utilized confocal immunofluorescent microscopy to determine colocalization partners of stably labeled EVs (34). His group also stably transduced an AML cell line to express either mGFP (for xenografts) or mTomato (for purified exosomes), which allowed for visualization of membranous vesicles in vivo (34).

Novel genetic approaches have emerged to track EVs in more physiological relevant settings. Stefan Momma introduced a Cre-*loxP* mouse model system and recently reported that cells derived from these transgenic Cre recombinase–expressing mice release Cre mRNA–containing EVs (65, 66). His group demonstrated that EV uptake was sufficient for translation of the EV-transferred Cre mRNA and an irreversible Cre recombinase–dependent induction of marker gene expression in target cells. Using this system, his group identified the direct transfer of functional mRNA from blood to neurons in the brain as a novel route of communication between the immune system and the brain (65). Saumya Das also used this mouse model to track exRNA intercellular transport in cardiac remodeling (our unpublished observations).

Yoel Sadovsky took advantage of the fact that C19MC is primate-specific microRNA cluster. His group created a humanized transgenic mouse model that expresses the entire 160-kb human C19MC locus or used lentivirus vectors to direct the expression of specific C19MC members selectively in the placenta (67). To determine the pattern of miRNA transport during pregnancy, his group devised a series of breeding and embryo transfer strategies and elegantly demonstrated the trafficking of placental miRNAs to the maternal and fetal compartments and from the maternal circulation to the fetoplacental unit (67). These findings establish a rigorous model system demonstrating exRNA transport between maternal and fetal compartments in vivo.

Optimizing exosome purification. A significant technical hurdle in the rapidly evolving field of EV biology is a lack of techniques to achieve high yields of pure exosomes from cell-culture media and biofluids (68). Ultracentrifugation (UC) (69), ultrafiltration (UF) (70), and polyethylene glycol (PEG) precipitation are the three commonly used methods for exosome isolation (71). Robert Raffai discussed the drawbacks associated with these three popular methods: UC suffers from a significant loss of exosomes; UF retains a substantial amount of protein contaminant; and PEG precipitation creates nanoparticles. To overcomes these limitations, his group recently refined the UC method by concentrating exosomes onto a high-density cushion followed by density gradient UC (cushioned-DGUC) (72). The cushioned-DGUC approach out-

performs the three conventional methods in terms of both yield and purity: it permits a 3-fold improvement in yield relative to UC and an 8-fold reduction in protein contaminates compared with UF and avoids the generation of nonvesicular nanoparticles, as seen using the PEG method. Optimized exosome isolation methods, such as cushioned-DGUC, provide opportunities to enhance rigor and reproducibility in fundamental discoveries and translational applications of exosomes.

Pushing the technological boundaries for EV biomarker research

EVs have the potential to be used to as biomarkers, given their accessibility, abundance, and stability (68, 73, 74). For clinical testing, capture and detection of EVs in small volumes of samples within limited time frames is required. Many innovative EV isolation and profiling platforms, such as microfluidic chips (75), acoustic devices (76, 77), electrochemical sensors (78), and flow cytometry, have recently been developed to improve the specificity, sensitivity and scalable throughput capability.

Bioengineering approaches. Microfluidics-based micro/nanochip approaches provide enabling platforms for quantitative detection and molecular profiling of EVs (79, 80). Yong Zeng developed an ExoSearch microchip that took advantage of continuous micromixing for rapid and efficient exosome capture from blood plasma and multiplexed immunofluorescence exosome phenotyping (81). Using different nanomaterial-inspired approaches, including graphene oxide–induced formation of polydopamine nanofiles (GO/PDA), the analytical sensitivity in detecting low-level, heterogeneous cancer exosomes directly from clinical plasma specimens was substantially improved (82). Hakho Lee focused on developing a translational nanoplasmonic exosome (nPLEX) platform optimized for high-throughput exosome protein profiling based on optical transmission through periodic nanoholes (83). This strategy provides an ideal sensing scheme for EVs, as the sensor probing depth (<200 nm) could be matched to EV size. The nPLEX platform was successfully used to screen for several cancer markers in EVs from both ovarian (83) and pancreatic cancer patients (84).

Flow cytometry. Different cell types contribute to a diverse repertoire of EVs in biofluids. This heterogeneity complicates identification of clinical biomarkers whose signal may be difficult to sort through a high nonspecific background. High-resolution, single-vesicle analysis methods could help answer fundamental questions in EV biology. Recent advances in high-sensitivity flow cytometry have significantly improved our capabilities to analyze EVs at a single particle level (85–87). By combining membrane-specific staining with high-sensitivity flow cytometry, John Nolan refined vesicle flow cytometry (87, 88) that enabled detection and measurement of individual EVs as small as 70 nm. Huiping Liu optimized the detection of surface proteins at a single-exosome level on a microflow cytometer and demonstrated that CD47 expression on circulating exosomes correlated with breast cancer status (89). These data support the feasibility and potential value of using flow cytometry in studying EV biology and developing EV-based biomarkers. However, it will be important to enhance quantitative rigor by using appropriate standards for fluorescence intensity and vesicle size to calibrate flow cytometers to allow results to be compared across laboratories and over time (90–93).

Synthetic biology approaches to improve exRNA therapeutics

The ability of EVs to target specific tissues (94, 95) and transport functional biomolecules, e.g., nucleic acids to recipient cells, offers great therapeutic promise (96–99), which has attracted enormous interest from both academia and private industry (94, 100, 101). However, there are significant challenges associated with the development of EV-based therapeutics, including loading cargoes into EVs, promoting EV stability, tissue targeting, and functional delivery (96, 102, 103). Many novel synthetic and engineering strategies have recently been developed to overcome these hurdles (96, 97). To overcome inefficient RNA loading by conventional methods, Joshua Leonard developed a targeted and modular exosome loading (TAMEL) platform, which provides a strategy for actively loading specific RNAs into exosomes during biogenesis (104). In addition, glycosylation motifs were added adjacent to cell-targeting peptides displayed on EVs to prevent their proteolytic degradation and enhance EV stability (105). Targeting EVs to specific recipient cell types may be mediated by natural EV components (95) or bioengineered moieties on the EV surface (94). Huiping Liu developed a strategy for targeting breast cancer stem cells by engineering cell-targeting peptides into fluorescent protein-tagged exosomes (89). Despite this progress, realizing the therapeutic potential of EVs will require technical advances in large-scale production of high-quality EVs and the development of best-practice models (96, 97).

Outstanding questions and future perspectives

Despite the tremendous translational potential, the exRNA field continues to face many scientific and technical hurdles. For the field to advance, the consensus at this workshop was that the fundamental mechanisms that generate EV heterogeneity and pathways for exRNA loading in EVs need to be better understood. Further, the field must develop novel imaging, sorting, and high-throughput functional profiling methods to successfully understand the role of exRNAs in human pathophysiology. The following recommendations were prioritized as areas of emphasis for future study (Figure 1).

EV formation and secretion and RNA cargo loading. There is limited knowledge of the molecular details regarding how different EV subtypes, e.g., exosomes and microvesicles, are formed and secreted. Multiple intracellular pathways have been reported to effect EV biogenesis (19, 106–108) and to drive the mobilization of EV precursors and their fusion with the plasma membrane, in particular the Rab family of small GTPases (4, 109) and soluble NSF-attachment protein receptor (SNARE) proteins (110, 111). However, how these pathways contribute to the diversity of EVs observed in the extracellular space remains unclear. Identification of the molecular machineries specific to individual EV biogenesis and secretion pathways will be crucial for developing tools and model systems for selective deletion, inhibition, or augmentation of EV subtypes to elucidate the physiological relevance of their RNA cargo in vivo.

Furthermore, the intracellular sorting machineries that direct RNAs to specific export pathways are not well understood. Although some RNAs may passively diffuse into EVs, accumulating evidence suggests that active sorting of RNAs to specific export pathways likely depends on specific RBPs (46, 48, 50) and specific RNA sorting motifs or modifications (48, 50, 51, 112). The generation of a library of RBPs involved in exRNA sorting in source cells and the exRNA transcriptomes associated with each extracellular RBP would be highly beneficial to the field. This will require further elucidation of the biophysical characteristics of each RBP that could define its specificity for interacting with RNA classes and require complementary engineering or synthetic approaches to determinate the RNA structural features for effective packaging in EVs and other exRNA carriers.

Improved separation technologies. The abundance and accessibility of EVs provides an opportunity for the functional interrogation of their exRNA cargo. However, EV heterogeneity, nanoscale dimensions, and lack of unambiguous physical properties or unique molecular markers have posed substantial challenges for the isolation of specific EV subtypes. The presence of exRNAs in non-EV entities, e.g., RBPs (54, 55) or LPPs (52, 53), adds another layer of complexity. The available approaches for isolating EV subtypes, including UC, filtration, size exclusion, immunoaffinity, or flow cytometric sorting, do not unambiguously separate EVs from RBPs or LPPs. Thus, there is an urgent need to determine and evaluate the potential biophysical (77, 113) and/or biochemical (114, 115) approaches that would allow the development of improved technologies for the separation of different EV subtypes and other exRNA carriers.

In addition, the copy number of a given miRNA molecule has been revealed to be on average lower than 1 per vesicle/particle in EV samples (116). It is still unknown whether all EVs contain very few miRNA molecules or a restricted subtype of EVs contains significant amounts of miRNA molecules. There is a pressing need for methodologies enabling single EV isolation from complex biofluids and computational tools permitting deconvolution of single EV-associated exRNAs to inform their cell of origin.

Significant efforts have been made to standardize the methods for the collection, storage, and processing of EV-containing body fluids (5, 117–119). However, there is a lack of well-characterized, publicly available reference standards for optimizing EV counting, isolation, and exRNA cargo characterization. There was a consensus that the field would benefit from establishing rigorous and reproducible standards using well-validated model systems. These standards and model systems will be crucial to the rigor and reproducibility of exRNA studies. In addition, it will be highly beneficial to establish an atlas of EVs and their associated exRNAs secreted by diverse cell types in different physiopathological settings.

EV uptake by recipient cells. Although exRNAs are well accepted as novel mediators of intercellular communication, the molecular codes by which their carriers are addressed to and taken up by specific recipient cells are ill defined. The specificity of targeting EVs to recipient cells is likely conferred by specific ligand-receptor interactions between the EV and target cell. Recent work highlights the existence of a multiplicity of parallel endocytic processes, including clathrin-coated pits, pinocytosis, caveolae, macropinocytosis, phagocytosis, and extrusion, that may be involved in uptake of exRNAs (120). Multiple complementary experimental approaches, including the use of antibodies to block ligand/receptor interactions, chemical inhibitors, and RNA interference, will be needed to identify the dominant molecular events that permit EV

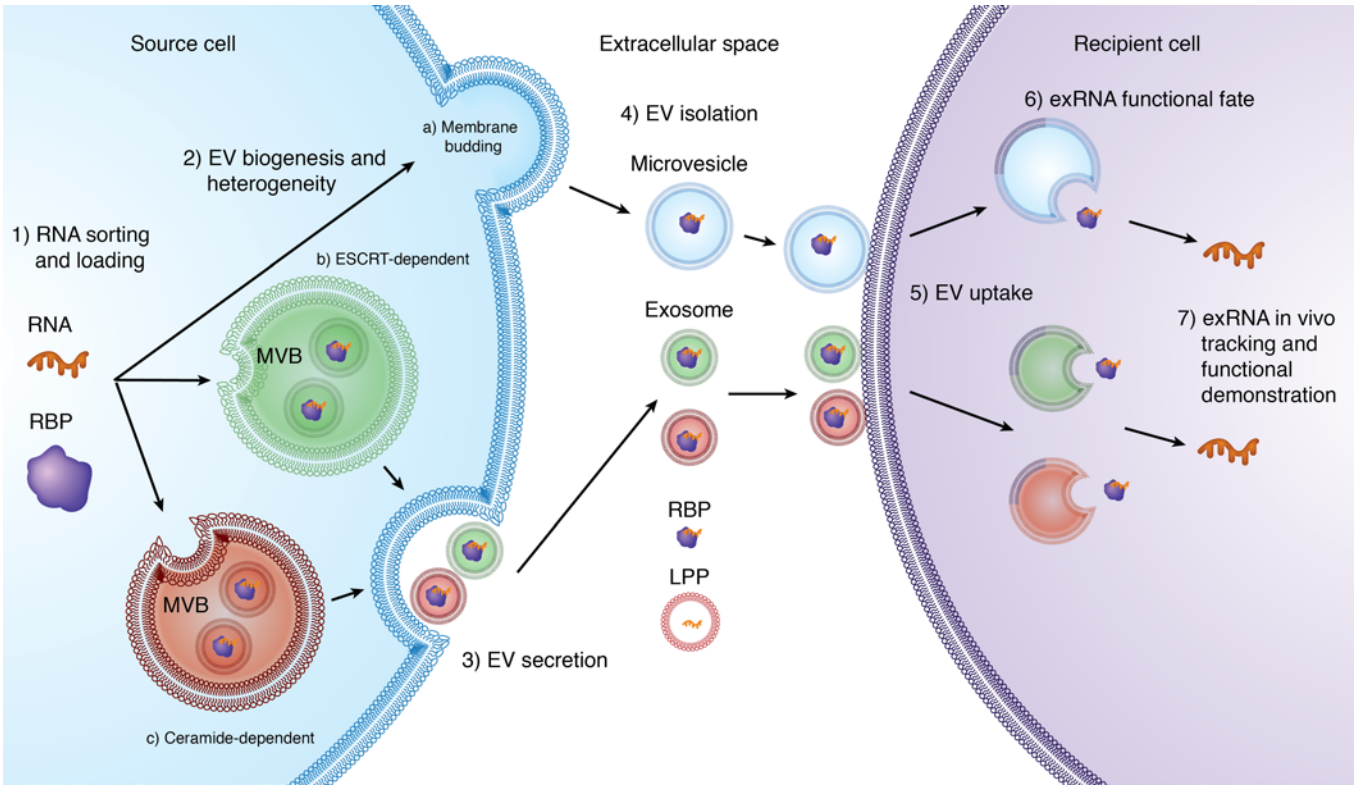

Figure 1. Outstanding questions and research opportunities in extracellular RNA biology. Advancing research on extracellular RNA (exRNA) communication holds great potential for transforming translational science. The NIH Strategic Workshop on Extracellular RNA Transport identified many unanswered fundamental questions and unmet technical challenges and generated recommendations on high priority areas that could catalyze research across the exRNA field. (1) RNA sorting and loading. What are the RNA structural features and RNA-binding proteins (RBPs) that guide RNA to different extracellular vesicle (EV) subtypes, i.e., exosomes and microvesicles? (2) EV biogenesis and heterogeneity. What are the molecules and molecular machineries specific to individual EV biogenesis pathways, i.e., (a) direct membrane budding of microvesicles; (b) endosomal sorting complexes required for transport–dependent (ESCRT-dependent) or (c) ceramide-dependent multivesicular body (MVB) formation? How do different biogenesis pathways contribute to EV heterogeneity? (3) EV secretion. What are the molecular determinants that direct the secretion of EV subtypes along different export pathways? (4) EV isolation. What are the unique biophysical/biochemical characteristics of each EV subtype that could be used for functionally separating EV subtypes from each other and from other exRNA carriers, i.e., RBP, lipoprotein (LPP)? (5) EV uptake. What are molecular interactions between EVs and recipient cells that determine targeted delivery of exRNAs? (6) exRNA functional fate in recipient cells. How are exRNAs processed and through what forms do exRNAs execute their effector functions in recipient cells? (7) exRNA tracking and functional effect in vivo. What are the model systems that allow targeted modification and disruption of EVs to enable monitoring exRNA dynamics in vivo and unambiguous demonstration of their physiological relevance? Illustrated by Rachel Davidowitz.

uptake under different physiological contexts. Importantly, EVs have the capacity to cross biological barriers, such as the blood-brain barrier (94). The identification of specific molecular attributes that allow for efficient fusion and specific egress of EVs through these privileged sites would help enhance delivery of RNA or small-molecule functional cargo through EV engineering for the treatment of neurological disorders.

Functional fate of exRNA in recipient cells. Two modes of action by which exRNAs execute their functions in recipient cells have been described. The first is in line with the canonical role of the RNA in the producer cell. For example, miRNAs can function to regulate cognate mRNA stability in both producer and target cell. A central question with this mode of action is whether sufficient numbers of exRNAs are transferred to have a sustained effect on target cell phenotypes. It will be important to determine whether and how miRNAs delivered by EVs could compete with endogenous miRNAs for the association with the RNA-induced silencing complex in target cells.

The second mode of action relies on the ability of exRNAs to amplify their signal in target cells. In addition to miRNAs, RNA-sequencing analysis has uncovered that EVs contain various fragments derived from mRNAs and ncRNAs, including rRNA, Y RNA, snRNA, snoRNA, lncRNA, and vault RNA (39–42). RNA fragments may be generated by processing inside the EVs as an extracellular maturation process. Some processed tRNAs, Y RNAs, and other RNA fragments have been reported to activate innate RNA sensors in

target cells (20, 121). This category of exRNA solves the "sufficient numbers" problem, as signaling is amplified by the triggering of innate sensing molecules and activation of downstream inflammatory pathways. Another possibility is that signaling amplification could be achieved by transfer of an activated RNA processing machinery between producer and target cell. In this scenario, the exRNAs could serve as the carrier rather than the effector per se. Elucidating the modes of action of exRNAs will be critical for understanding the biological significance of EVs in vivo and for harnessing EVs for therapeutic applications.

Tracking EV distribution and measuring functional effect in vivo. It remains challenging to obtain direct proof of the functional effect of exRNAs on target cells in vivo due to the difficulties in labeling endogenous EVs, tracing their movement, and identifying target cells, without interfering with their function in a physiologically relevant context. A robust model should reflect the physiological properties, concentration, and distribution of EVs in vivo. Novel genetic models in combination with powerful visualization techniques (122, 123) are required to dynamically track individual EVs and understand the functional effect of their exRNA cargos in vivo.

To date, the data regarding the functional role of exRNAs in vivo are mostly derived from animals subjected to injections of various doses of EVs isolated from cell culture systems. There is very limited knowledge regarding in vivo stoichiometry of exRNA carriers and their physiologically relevant concentrations. Assessment of in vivo function of EVs and exRNAs remains challenging and will require robust model systems that allow for targeted disruption of specific EV subtypes, RNAs (124, 125), RBPs, or export pathways. Although efforts have been made to address these questions by targeting key molecules that are involved in EV biogenesis and secretion (108, 126) or interfering with miRNA biogenesis (127), these approaches are often not specific and are thus unable to exclude the effects contributed by EV-independent factors. To achieve unambiguous demonstration of exRNA physiological function in vivo, we need a deeper mechanistic understanding of EV biogenesis and secretion and more advanced molecular tools to manipulate EV subtypes and exRNA loading pathways.

Concluding remarks

Interest in exRNA and EV biology is rapidly growing; yet, there remains a fundamental need for rigorous, hypothesis-driven studies to generate a comprehensive map of exRNA transport pathways. Developing model systems that enable a molecular understanding of EV heterogeneity and exRNA diversity will address knowledge gaps in EV biology and lead to a substantial growth in all facets of exRNA research. The development of these model systems will require complementary skill sets from highly collaborative teams spanning physical, chemical, biological, translational, and clinical sciences. The continuity of the NIH Extracellular RNA Communication Consortium will facilitate these efforts and enhance more rapid progress in realizing the full potential of exRNA in translational and clinical medicine.

Author contributions

TKH conceived this meeting report. KL, RSR, and TKH contributed to writing the manuscript. TKH, LSK, TBL, MJO, and KCV organized the workshop, which was cochaired by SD and RLR. KL, RSR, FK, TG, SJG, LSK, PK, HL, JNL, HL, TBL, SM, JPN, MJO, DMP, YS, FSM, KMV, KCV, AMW, KWW, YZ, SD, RLR, and TKH jointly participated in the workshop and helped edit the text of this manuscript.

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