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REVIEW | *Exosomes and Extracellular Vesicles in Cardiovascular Physiology*

Exosomes in disease and regeneration: biological functions, diagnostics, and beneficial effects

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Lin Y, Anderson JD, Rahnama LM, Gu SV, Knowlton AA. Exosomes in disease and regeneration: biological functions, diagnostics and beneficial effects. *Am J Physiol Heart Circ Physiol* 319: H1162–H1180, 2020. First published September 28, 2020; doi:10.1152/ajpheart.00075.2020.—Exosomes are a subtype of extracellular vesicles. They range from 30 to 150 nm in diameter and originate from intraluminal vesicles. Exosomes were first identified as the mechanism for releasing unnecessary molecules from reticulocytes as they matured to red blood cells. Since then, exosomes have been shown to be secreted by a broad spectrum of cells and play an important role in the cardiovascular system. Different stimuli are associated with increased exosome release and result in different exosome content. The release of harmful DNA and other molecules via exosomes has been proposed as a mechanism to maintain cellular homeostasis. Because exosomes contain parent cell-specific proteins on the membrane and in the cargo that is delivered to recipient cells, exosomes are potential diagnostic biomarkers of various types of diseases, including cardiovascular disease. As exosomes are readily taken up by other cells, stem cell-derived exosomes have been recognized as a potential cell-free regenerative therapy to repair not only the injured heart but other tissues as well. The objective of this review is to provide an overview of the biological functions of exosomes in heart disease and tissue regeneration. Therefore, state-of-the-art methods for exosome isolation and characterization, as well as approaches to assess exosome functional properties, are reviewed. Investigation of exosomes provides a new approach to the study of disease and biological processes. Exosomes provide a potential "liquid biopsy," as they are present in most, if not all, biological fluids that are released by a wide range of cell types.

exosomes; methods; stem cells therapeutics

OVERVIEW OF EXOSOMES

The first observation of extracellular particles and vesicles was in platelet-free serum in 1946 (30). Since then, a substantial number of studies on extracellular vesicles (EVs) have been published (9, 43, 203, 230). Over time, numerous types of EVs have been characterized and assigned various names based on their size, biogenesis, and origin/function such as oncosomes, ectosomes, texosomes, cardiosomes, apoptotic bodies, microvesicles, and exosomes (235). There are overlaps among the different subgroups. However, it is also evident that within these EV populations there are even more subpopulations based on vesicle diameter, density, RNA, DNA, and protein cargo (198).

Currently, EVs are classified into one of three broad categories based on their biogenesis: apoptotic bodies, microvesicles, and exosomes (118). Apoptotic bodies are heterogeneous in size, with an estimated diameter ranging from 200 to 5,000 nm, and are shed from dying cells undergoing programmed cell death (4). Microvesicles, which are also referred to as ectosomes, are shed from the plasma membrane of viable cells and are 100–800 nm in diameter (165, 177). Exosomes are 30–150 nm in size and are released into the extracellular space when multivesicular bodies fuse with the plasma membrane (136). However, it has been demonstrated that vesicles smaller than 100 nm in diameter can bud directly off the plasma membrane (74, 140). EVs can be separated and purified based on their density (194), but the isolated EVs can be heterogeneous, likely due to coisolation of different EV subpopulations during the various purification steps (88). It has been suggested that the vesicles should more properly be called 2 K, 10 K, and 100 K vesicles, or potentially medium/large and small EVs, or high- and low-density EVs, as the biogenesis of each vesicle is indeterminate and might be independent of its size and density (231). Further effort is needed to distinguish among the different types of EVs in a meaningful and useful manner. Size is one parameter, but a greater understanding of the differences among EVs will help focus the field and future studies. Since the exosomal membrane

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retains the characteristics and orientation of the plasma membrane of the cell(s) of origin, investigation of exosomal membrane proteins can provide further insight into the parent cell. Furthermore, known membrane proteins for a given tissue can potentially be used to immunoprecipitate tissue-specific exosomes from plasma and serum samples.

Virtually all cells are capable of secreting types of EVs, and this process is conserved throughout evolution, from bacteria to humans (66, 112). The secretion of EVs was initially reported as the mechanism for eliminating unneeded proteins from maturing reticulocytes (78). However, we now know that EVs have the capacity to exchange components between cells, ranging from nucleic acids to lipids and proteins, and to act as signaling vehicles in homeostatic and pathological cellular processes (169). In the 1990s, EVs were reported to be secreted by B cells and dendritic cells and possessed immunoregulatory properties in antitumoral immune responses (26, 164). Since then, studies of EV cargo and signaling properties have been widely published across a broad range of fields, including cardiovascular disease (22, 32, 54, 65, 171, 181). There is now evidence that cells fine-tune the cargo of EVs, with the rate of secretion depending on their physiological state and exposure to various stimuli (7, 185). Once released into the extracellular space, EVs come into contact with recipient cells and elicit functional responses, promoting phenotypic changes that affect target cells' physiological status (49, 51). EV-mediated intercellular communication can be achieved through various mechanisms, from surface-bound EV ligands binding target cell receptors to initiate signaling cascades to EV internalization either through endocytosis or by fusion with target cells (136).

The first examples of the signaling properties of EVs docking to cells were from B cells and dendritic cells, where EVs were able to present antigens to T cells and induce a specific antigenic response (3, 135, 197). Tumor-derived EVs were shown to transport fibronectin, which bound to integrins on nontransformed fibroblasts and promoted their anchorage-independent growth (one hallmark of tumorigenesis) (13). Similarly, EVs are released along the surface of trophoblasts, thereby promoting embryo implantation (193).

EVs can also transport various lipid species, including sphingomyelin, eicosanoids, fatty acids, and cholesterol, thereby mediating the regulation of cellular bioactive lipid species (166). An example of EV-mediated transfer of pathological material is amyloid proteins (162). Amyloid-β peptide and prion protein (PrP) were found to be present on the surface of EVs, whereas TAR DNA-binding protein and α-synuclein are intraluminal in EVs (56, 58, 145, 155). Consequently, EVs have been found to mediate intercellular spreading of amyloids in neurological disorders (172).

Exosomes have a diabolical role in cancer, as after being released by cancer cells they have been found to promote the spread of cancer by preparing a niche for metastases or premetastases (15, 53, 125). Furthermore, exosomes appear to avoid immune surveillance and have the potential through their cargo to spread drug resistance (53, 125). Although the lipid composition of exosomes is different from that of the parent cells, the function studies of lipids in exosomes is limited (53). Ceramide, a member of the sphingolipid family, is essential for exosome formation, and it is also enriched in EVs Lipids in the exosomal membrane may organize and form "mobile rafts" that convert exosomes into signalosomes, which promote the activation of cell signaling pathways for oncogenesis and metastasis. It is thought that ceramide may modify the function of mobile rafts and their effect on these signaling pathways (53, 125).

An early key study showing a direct link between EVs and tumor invasion of healthy tissues demonstrated that expression of epidermal growth factor receptor (EGFR)vII in glioma cells resulted in markedly enhanced vesiculation, which was detectable in the blood of tumor-bearing mice (6, 70). The resulting EVs transported this oncoprotein to adjacent tumor cells, which led to the production of angiogenic factors such as vascular endothelial growth factor (VEGF) (5). Similar studies by Skog et al. (180) demonstrated that human primary glioblastoma cell-derived EVs transferred not only EGFR but also numerous microRNAs (miRNAs) that stimulated tumor growth and angiogenesis. That group was subsequently able to isolate similar tumor-derived EVs from the blood of patients before treatment (180). Thus, exosomes released by cancer cells can be used to follow the clinical disease state. This foundational work led to the one of the first EV startup companies, Exosome Diagnostics, to develop EV-based biomarkers in the oncology field (175). It is possible that the cancer exosomes could be modified to target metastases and even the original tumor through a cytotoxic cargo. The role of exosomes in cancer is a focus of much research at this time, and new developments may lead to new therapeutic targets as well as new diagnostic and prognostic approaches.

HEART-DERIVED EXOSOMES

Our laboratory was the first to demonstrate that exosomes were produced by adult cardiomyocytes isolated from rat hearts and that these exosomes could be readily purified and characterized. We identified exosomes as the mechanism by which heat shock protein-60 (HSP60) was released from cardiomyocytes (75). The release of exosomes from the cardiomyocytes was augmented by mild hypoxic stress in the absence of necrosis, and the release was inhibited by dimethyl amiloride (DMA; an exosome inhibitor) or methyl-β-cyclodextrin (MBC; an inhibitor of lipid raft formation) with or without hypoxic stress (75). Our later work demonstrated that moderate and heavy consumption of alcoholic beverages, simulated by low and high ethanol concentrations in culture, would increase the release of exosomes from cardiomyocytes (122). Ethanol exposure induced a greater release of exosomes than hypoxia/reoxygenation. Pretreating the cardiomyocytes with a combination of three different antioxidants that inhibit reactive oxygen species (ROS) formation markedly decreased acetylcholine esterase activity, a marker of exosome abundance (122). Exosomal content reflects the cell of origin and cellular stress conditions (8, 86). Although some proteins would commonly be present in the exosomes derived from cardiomyocytes, exposure to these different stimuli resulted in differences in the exosomal protein content, when analyzed by mass spectrometry (122). Some of the proteins, such as HSP27 and HSP90, were found only in hypoxia/reoxygenation-derived exosomes, whereas several other proteins were found only in ethanol-derived exosomes. Unsurprisingly, others have shown that exosomal RNA content also differs depending on the type of stress induced, as shown in endothelial cells (47).

Bodily fluids and cell culture media contain not only exosomes but abundant amounts of protein, which can confound

any analysis of the content of exosomes. Serum and plasma samples have a very large amount of protein, given the high concentrations of albumin and γ-globulins in the blood. It is imperative to separate the exosomes from these other molecules to have a valid analysis of the exosomal contents. We have found that the most efficient way to do this is by size exclusion chromatography (SEC) (Fig. 1), which is quite simple to perform. It separates the large exosomes, which readily pass through the column, from many proteins present in bodily fluids and in culture media. Importantly, the column matrix can be washed, autoclaved for sterility, and reused. This is a more straightforward method to isolate clean exosomes than some other approaches.

The biological content of exosomes is protected by their lipid bilayer. Despite exposure to various physiological and pathological environments, such as ethanol consumption, change in pH, as seen with diabetic ketoacidosis, or fever, exosomes remained very stable and did not release their contents (122). Given the stability of exosomes, they are reliable carriers of the functional content from the releasing cells to recipient cells. For example, both in vitro hypotonic mechanical stretch and in vivo pressure overload induced the cardiac secretion of exosomes containing angiotensin II (ANG II) type 1 receptors (AT1Rs) (159). The AT1R-enriched exosomes were biochemically functional, showing internalization of AT1Rs in the recipient cells after ANG II stimulation. Injection of AT1R-enriched exosomes

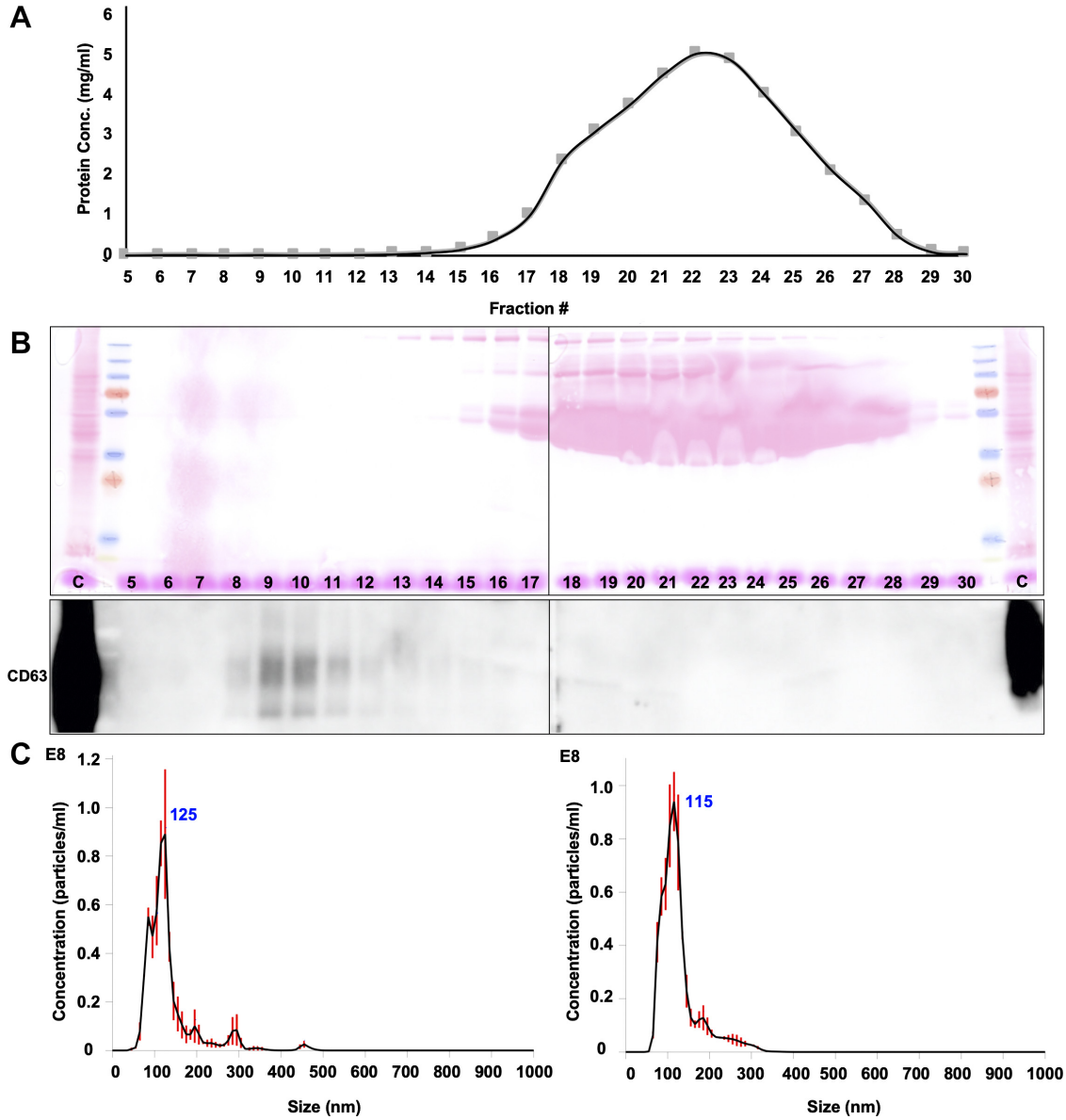

Fig. 1. Exosome isolation using size exclusion chromatography (SEC). Endothelial cells were cultured in medium containing 5% exosome-depleted FBS. The supernatant was collected and ultrafiltered after 24 h and then processed in SEC with 10 mL of bed volume. Each 500 μL of fraction was collected after the sample was added on top of the column. *A*: protein concentration of each fraction was determined using Nanodrop. *B*: Western blot was performed using 2 separate gels to detect the distribution of vast number of proteins and exosomes in fraction 5–30. Ponceau red stain confirmed that the vast number of proteins were eluted in later fractions, whereas anti-CD63 detected exosomes in early fractions. C, parent cell lysates as positive control. *C*: nanoparticle tracking analysis confirmed the exosome sizes in F9 (*left*) and F10 (*right*).

modulated blood pressure in response to ANG II infusion in AT1R knockout mice (159). Valadi et al. (203) demonstrated the presence of mRNA and miRNA in the exosomes derived from different cell types and species and that mouse RNA was transferable via exosomes to human cells, in which the new mouse proteins were then confirmed. Another study identified 1,520 mRNA and 343 chromosomal DNA sequences in the exosomes derived from adult mouse cardiomyocytes in culture (216). The stained DNA and RNA of cardiomyocyte exosomes were detected in the target fibroblasts within 3 h of incubation, and induced changes in the expression of 333 genes, demonstrating that cardiomyocytes can communicate intercellularly via exosomes (216).

Cardiac remodeling is a complex process involving cardiomyocyte hypertrophy and cardiac fibrosis that lead to alterations in ventricular function and structure in response to hypertension, valve disease, ischemic cardiac disease, or cardiac damage. Cardiac fibroblasts are essential in pathological cardiomyocyte hypertrophy in the mouse heart when subjected to transverse aortic constriction in vivo (190). Newborn rat cardiomyocytes cultured in fibroblast-conditioned medium manifested hypertrophy, reduced contractile activity, and increased expression of vimentin (105). This effect of cardiac fibroblasts on cardiomyocyte hypertrophy was likely mediated through paracrine or autocrine pathways, involving cardiac fibroblast-associated cytokines, chemokines, and miRNAs (20, 105, 190). Cardiac fibroblasts have been found to secrete exosomes enriched in star (*) miRNAs, which normally are degraded intracellularly. MiR-21*, one of the fibroblast exosomal derived RNA molecules, was analyzed as a potent paracrine mediator repressing sorbin and SH3 domain-containing protein-2 (SORBS2) and PDZ and LIM domain 5 (PDLIM5) in cardiomyocytes. Silencing either SORBS2 or PDLIM5 in cardiomyocytes resulted in significant cellular hypertrophy (20). In one in vivo study, miR-21* was significantly increased in the pericardial fluid of mice with left ventricular pressure overload–induced hypertrophy, and antagonism of miR-21* in mice was able to attenuate ANG II-induced cardiac hypertrophy (20). ANG II has been shown to enhance exosome release from cardiac fibroblasts that in turn augment ANG II production in cardiomyocytes (120). After administration of exosome inhibitors GW4869 and DMA in ANG-II-treated adult mice, both basal and ANG II-induced exosome release from the isolated cardiac fibroblasts was inhibited, and ANG II-induced cardiac hypertrophy and fibrosis were also prevented (120).

Since cardiomyocytes secrete exosomes, researchers also investigated the role of cardiomyocyte-derived exosomes in the progression of heart failure. Among heart-specific miRNAs, miR-208a levels were shown to be consistently higher in both serum and heart tissue in heart failure induced by either doxorubicin injection or left anterior descending (LAD) coronary artery ligation (236). The increase in miR-208a was confirmed as coming from cardiomyocytes stressed by hypoxia or ANG II in vitro and in the released exosomes, whereas the expression of miR-208a did not change in fibroblasts. The exosomes derived from the stressed cardiomyocytes promoted cardiac fibroblast proliferation, differentiation into myofibroblasts in vitro, and increased cardiac fibrosis in normal rats. Inhibiting miR-208a mitigated these effects in vitro and preserved cardiac function in the infarcted heart (236). Diabetes is a well-known cause of microvascular insufficiency and a risk factor of heart failure. Endothelial dysfunction and angiogenesis impairment are associated with the development of heart failure. Cardiomyocytes isolated from type 2 diabetic rats as well as cardiomyocytes treated with high glucose have been shown to release exosomes enriched in antiangiogenic miR-320 (219). Endothelial cells that incorporated these exosomes exhibited inhibited proliferation, migration, and tube-like formation with the downregulation of IGF-I, HSP20, and Ets2 which are targets of exosomal miR-320 (219).

In contrast to the potentially deleterious effects of the exosomes released in pathological conditions, modified exosomes have a more protective role when released from the heart in the case of diabetes. Cardiac-specific overexpression of HSP20 promoted the production of exosomes, which encapsulated higher levels of antioxidant proteins, protecting cardiomyocytes and endothelial cells against hyperglycemia-induced stress (218). Injection of exosomes derived from cardiomyocytes overexpressing HSP20 attenuated cardiac dysfunction and remodeling in mice whose diabetes was induced by streptozocin (STZ) (218). As discussed, external stimuli can alter the secretion of exosomes, as well as their cargo. Glucose starvation enhanced exosome production by H9C2 cardiomyocytes (64). These exosomes promoted endothelial cell proliferation and tube formation. The enhancement correlated with the upregulated exosomal expressions of miR-17, -19a, -19b, -20a, -30c, and -126, which promote angiogenesis (64). Matrix metalloproteinase-9 (MMP-9), a member of the zinc-metalloproteinase family, is secreted by various cell types, including cardiomyocytes, fibroblasts, and endothelial cells. MMP-9 knockout in diabetic mice has been shown to improve cardiac repair (130). Exercise was later found to stimulate exosome production and downregulate MMP-9 expression in the diabetic heart (31). Altered expression of MMP-9 was associated with markedly upregulated miR-29b and miR-455 in the exosomes secreted after exercise (31).

Circulating exosomes secreted from other cell types can impact the heart or vasculature. Platelets are a major source of plasma exosomes (237). In sepsis, exosome release from platelets was increased secondarily to the increase in nitric oxide (NO) and lipopolysaccharide (LPS). These exosomes subsequently induced caspase-3 activation and apoptosis in endothelial cells, leading to vascular dysfunction, which is a key mediator in sepsis induced shock, which leads to death (62). They also negatively affected myocardial function, as shown in studies of isolated rabbit hearts and rat papillary muscle (14). This may be secondary to the intrinsic production of NO by these septic platelet-derived exosomes (14). Endothelial cells and immune cells also contribute to plasma exosomes. Pretreatment with preconditioned endothelial cell-derived exosomes protected rat cardiomyocytes from cell death secondarily to simulated ischemia/reoxygenation (46). Exosomes can be derived from both activated endothelial cells and apoptotic endothelial cells. These exosomes unsurprisingly, given their different origins, play different roles. The ratio of circulating EVs derived from activated versus apoptotic endothelial cells might be associated with the severity of heart failure (24). As immune cells play sophisticated roles in the progression of various diseases, including cardiovascular disease, interest in the immune response and its modulation by immune cell-derived exosomes on the

injured heart is increasing and was recently addressed in a comprehensive review (232).

CARDIAC REGENERATION VIA EXOSOMES

Nearly 20 years of stem cell research on the treatment of myocardial infarction has generated promising data in animal models as well as in clinical trials (35, 102, 184, 186). This body of work has shown the safety of these therapies, whereas demonstrable efficacy is yet to be fully determined (25, 60, 87). The transplanted stem cells can attenuate expansion of infarct size and preserve the indexes of global cardiac function, thus preventing left ventricular remodeling (40, 199, 246). Some reports suggest that the underlying mechanism of action of transplanted bone marrow-derived stem cells is their ability to adopt a competent cardiomyocyte phenotype, providing de novo myocardial regeneration (45, 241). However, this hypothesis has been challenged by several research groups (127). More conventional studies have established that bone marrow-derived mesenchymal stem cells (MSCs) secrete bioactive molecules such as growth factors and cytokines that potentiate tissue repair and possess immunoregulatory properties (44, 113). Such canonical secretory signaling proteins have been the main research focus of MSCs' therapeutic properties. However, an increasing number of studies support that much of the tissue healing and immunoregulatory properties of MSCs are derived from the exosomes they secrete (108, 109, 173).

Several studies have established that exosomes isolated and purified from medium that has been conditioned by MSCs possess angiogenic and regenerative properties both in vitro and in animal models of myocardial infarction (107, 121, 195, 245). Several studies have demonstrated that the regenerative properties of MSC-derived exosomes can be enhanced through cell culture-based priming conditions, such as hypoxia, serum deprivation, and cytokine stimulation (33, 67, 119). Additional studies have shown that engineering of the miRNA cargo of exosomes is feasible and may enhance MSC exosomes' regenerative properties, including enhanced angiogenic ability of endothelial cells and reduced proliferation rates of activated T cells (Fig. 2) (104, 121, 221, 243). Other groups have used genetic engineering approaches to enrich the protein cargo of MSC exosomes (110, 157, 226). For example, MSCs transduced with GATA4 produced exosomes presenting higher levels of GATA4 (Exo^{GATA4}), which significantly induced expression of antiapoptotic miR-19a and miR-451 (238). GATA4 is a transcription factor highly expressed in cardiomyocytes and important for cardiomyocyte survival and function in the adult heart (156). Exo^{GATA4} enhanced cardioprotection by augmentation of viable cardiomyocytes after exposure to hypoxic stress, improving cardiac function and reducing infarction size in the heart following permanent LAD ligation (238). Exo^{GATA4} were distributed in the border zone of ischemic rat myocardium, where miR-19a was also upregulated (238). Some groups have also used MSC exosomes to precondition cardiac progenitor cells (CPCs.) After being preconditioned with MSC exosomes, CPCs were found to stimulate proliferation, migration, and angiotube formation in a dose-dependent manner (242). CPCs preconditioned with MSC exosomes enhanced differentiation into neovasculature, reduced fibrotic area, and preserved the cardiac function (242).

CPCs alone release exosomes enriched with GATA4-responsive-miR-451 (34). These exosomes inhibited oxidative stress induced caspase-3/7 activation in H9C2 cardiomyocytes and protected the mouse heart by significantly reducing myocardial apoptosis after ischemia/reperfusion (I/R) (34). Preconditioning CPCs with oxidative stress induced higher levels of exosomal secretion and resulted in significantly upregulated miR-21 in these exosomes compared with those derived from untreated CPCs (233). Whereas oxidative stress caused the reduction of miR-21 and increase of cleaved caspase-3 in H9C2 cells, pretreatment with CPC-derived exosomes was protective of the oxidatively stressed H9C2 cells, showing increased miR-21 and resistance to apoptosis. This protective function was amplified in exosomes derived from oxidatively stressed CPCs. The underlying mechanism may involve programmed cell death 4 (PDCD4), a target gene of miR-21 (233). Another group found that 11 miRNAs were upregulated in the exosomes derived from CPCs in response to hypoxia (71). The exosomes from hypoxic CPCs induced endothelial tube formation and reduced profibrotic gene expression in TGFβ-stimulated fibroblasts in vitro and improved cardiac function and reduced fibrosis in the I/R-injured heart (71).

As induced pluripotent stem cells (iPSCs) have a high capacity of cell differentiation that is beneficial to cardiac repair and regeneration, the therapeutic efficacy of exosomes derived from iPSCs or iPSC-derivatives is gaining interest. The iPSC-derived exosomes have been shown to be enriched with cardioprotective miRNAs, miR-21 and miR-210, which are regulated by Nanog and hypoxia-inducible factor-1α. These exosome

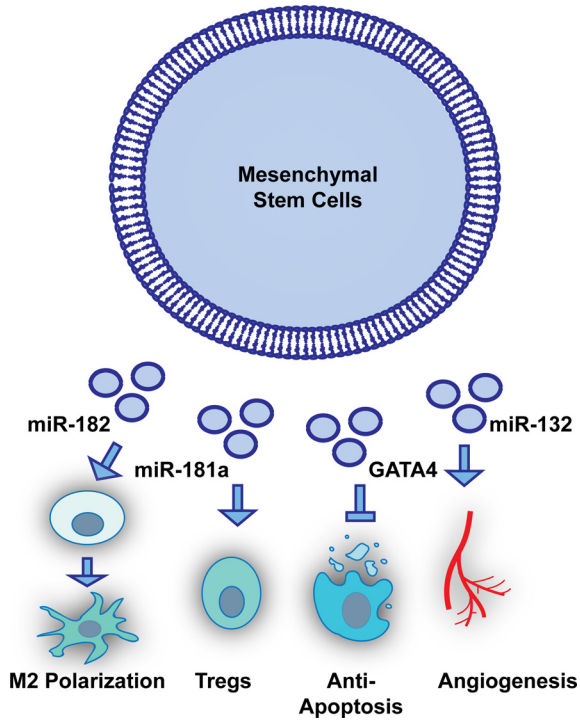

Fig. 2. Engineering of the exosomal cargo is feasible to study mesenchymal stem cell (MSC) exosomes' regenerative properties and may enhance regenerative efficacy in myocardial injury. Figure shows some examples that modulating or overexpressing miRNAs or proteins in MSC exosomes can protect the heart from ischemia injury directly through reducing cardiomyocyte apoptosis (238) and promoting endothelial angiogenesis (121) or indirectly through immunomodulation, such as M_2 macrophage polarization (243) or regulatory T (Treg) cell activation (221).

miRNAs were transferrable to H9C2 cells and protected the cells from oxidative stress-induced apoptosis. Intramyocardial injection of iPSC-derived exosomes immediately after myocardial infarction and before reperfusion was cytoprotective via suppressing apoptosis in the injured heart (220). Another study demonstrated that iPSC-derived EVs, including the fraction of exosomes, were enriched with miRNAs and proteins that are proangiogenic and cytoprotective. The iPSC-EVs enhanced angiogenic capacity, migration, and survival in cardiac endothelial cells. While iPSCs exerted cytoprotective effects on the heart undergoing I/R, iPSC-EVs induced superior cardiac repair, showing attenuated left ventricle dysfunction and hypertrophy and improved neovascularization (2).

CONSIDERATIONS FOR REGENERATIVE EXOSOME SOURCE MATERIAL

There is growing interest in the evaluation of stem cell-derived EVs (Fig. 3) in terms of elucidating their biological role in development and disease and as novel therapeutic approaches (10, 52, 131, 153, 176, 198, 239). The verification of stem cell lines should be given careful consideration due to their inherent sensitivity to the dynamic microenvironmental stimuli experienced during culture (80, 160, 214, 228). For example, MSCs are the most widely studied stem cell source of EV studies to date (52). MSCs possess limited doubling capacity and specific culturing density requirements and have been observed to modulate their morphology and therapeutic properties with continuous culturing (37, 84, 215). Therefore, studies of EVs derived from stem cells necessitate special attention to these factors, which should be reported in detail to allow readers to interpret the state of the parental cell line.

Stem cell lines should be validated for the presence of definitive markers associated with their respective cell type (97, 234). Generally reported markers for verifying pluripotency in embryonic stem cells (ESCs) and iPSCs are OCT4, SOX2, Nanog, SSEA4, TRA-1–60 (PODXL), and TRA-1–81 (PODXL) (29, 76, 244). Some studies also report the presence of additional markers, including SSEA3, LIN28, and SSEA5 (42, 143, 192). For iPSC validation, a verified ESC line like H9 should be used as a positive control for marker expression using either qPCR or immunocytochemistry analysis (114, 117). The karyotypic stability of ESC/iPSC lines should also be established to verify the absence of gross chromosomal abnormalities (188). The surface markers that define MSCs are CD73, CD90, and CD105, which are typically reported as more than 90% positive expression (128). In addition, the trilineage differential potential of MSCs should be demonstrated (adipocyte, chondrocyte, osteoblast) (170).

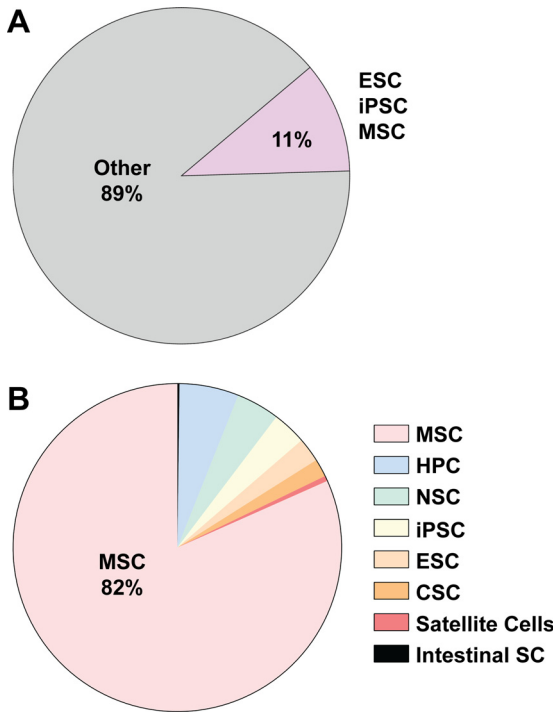

Fig. 3. Distribution of extracellular vesicle (EV) publications across stem cell classes. *A* and *B*: PubMed searches using key terms exosomes, microvesicles, extracellular vesicles, stem cells, MSCs, HPCs, NSCs, iPSCs, ESCs, CSCs, satellite cells, and intestinal stem cells were used to determine the fraction of EV publications associated with different types of stem cells. MSC, mesenchymal stem cell; HPC, hematopoietic progenitor cell; NSC, neural stem cell; iPSC, induced pluripotent stem cell; ESC, embryonic stem cell; CSC, cancer stem cell.

EXOSOME ISOLATION

Ultracentrifugation. Ultracentrifugation has been used as a "gold standard". Samples are cleaned of debris with low-speed centrifugation first (500–10,000 *g*), and then ultracentrifugation is applied to pellet the exosomes, with or without a sucrose cushion, or to separate exosomes in sucrose gradient (1.15–1.19 g/mL) (196). The advantage of ultracentrifugation for exosome isolation is its capacity for large sample volumes (Table 1). The speed and duration are determined depending on rotor dimensions. One of the most commonly used ultracentrifuge rotors, SW28 Ti (Beckman Coulter), takes two 100,000 *g* centrifugations with a minimum of 70 min per run (one for pelleting exosomes and contaminant proteins, followed by one for washing exosomes in PBS) (196). However, the sequential centrifugation requires a significant amount of work and expensive ultracentrifuge equipment. In addition, viscous samples have lower sedimentation efficiency. Increasing ultracentrifugation duration might improve pellet output, but the particle sizes are variable due to protein aggregation or damaged particles. When isolating exosomes from serum samples or cell conditioned media containing serum supplements, these serum proteins might also be pulled down and therefore affect exosome analysis. Serum-free media may be used when isolating exosomes from cell culture. However, serum deprivation will likely affect cell physiology. The duration of serum deprivation needs evaluation depending on the cell type and experimental requirements. It is generally considered that EV isolation via density gradients such as sucrose or OptiPrep yields the most purified exosome populations compared with precipitation and immunoaffinity methods (1, 92, 205). Therefore, the use of density gradient isolation methods can be considered in studies aiming to characterize and investigate the factors specifically packaged into exosomes (230). However, such isolation methods are thought to greatly diminish the functional properties of the resulting vesicle population, thereby limiting the utility of this approach for many studies (27). Alternatively, SEC, which is less expensive because the column matrix can be repeatedly reused after a simple washing, has been shown to be comparable to the density gradient method in terms of exosome purity and yield (116).

Table 1. *Comparison of exosome isolation methods*

Isolation Method	Principle	Pro	Con
Size exclusion chromatography (SEC)	Separate exosomes from other proteins based on size difference	Good exosome purity and reproducibility. Good yield. Good method to remove nonexosomal proteins before proteomics work	May require ultrafiltration prior and post-SEC.
Anion exchange chromatography (AEC)	Separate exosomes from other proteins based on the surface charge	Scalable. Good exosome purity and reproducibility. No pre-AEC concentration of sample required.	Requires ultrafiltration concentration post-AEC.
Affinity purification	Pull down exosomes using antibodies that bind to exosome surface antigens	Specific and high exosome purity. Possible to selectively isolate subset of exosomes.	Low yields and sample capacity. Require prior analysis to select exosome surface markers.
Ultracentrifugation	Sequential centrifugation based on exosome density and size	Large sample capacity and exosome yield. Sucrose gradient can increase exosome purity.	Lengthy process and labor intensive. High equipment cost. Reduced efficiency when sample viscosity increased. Exosomes might be damaged and can clump together due to high speed centrifugation.
Polyethylene glycol-based reagents	Precipitate exosomes based on less solubility in the reagent	Easy procedure. Only basic benchtop centrifuge needed. High yield.	Coprecipitated proteins and reagents can contaminate exosomes and affect downstream applications.
Tangential flow filtration	Generate fluidics flow that is tangential to a filter membrane	Scalable. Moderate exosome purity. No expensive, specialized equipment. High yield.	Yield may be reduced due to substantial contact with plasticware. Some smaller vesicles lost with 100-kDa molecular weight cut-off membranes.

SEC has also been used to remove OptiPrep remnants in isolated EV samples (211).

Commercial polyethylene glycol-based reagents. Since ultracentrifuges are expensive, and using them for isolation is a lengthy process, commercial polyethylene glycol (PEG)-based reagents have been developed to precipitate exosomes with a standard countertop centrifuge. However, the exosome-enriched pellet is contaminated with residual PEG polymer and other soluble proteins (154). An alternate method to enrich exosomes using the countertop centrifuge is centrifugal ultrafiltration. Smaller impurities are allowed to pass through the cellulose membrane of the ultrafilter system, with exosomes retained above the filter. The purity of the exosome samples will depend on the choice of filter pore sizes, whereas smaller pores can provide higher exosome yield but with more large protein complexes and aggregates.

Tangential flow filtration. In a similar fashion to many of the other EV isolation techniques, tangential flow filtration (TFF) has been widely used in the virology field for some time (142). TFF systems generally use a peristaltic pump to generate fluidic flow that is tangential to a filter membrane. Polyethersulfone membranes with a molecular mass cutoff of 100 kDa are most commonly used for EV isolations (77, 81). Although there is no direct conversion from kilodaltons to nanometers, a 100-kDa pore roughly equates to a 10-nm diameter. TFF is fairly efficient at concentrating EVs and allows for a buffer exchange to wash away residual medium contaminants. It should be noted that the more plasticware the source material is exposed to, the more opportunity there is for EV loss due to their charged and sticky surfaces.

Size exclusion chromatography. SEC, as discussed above, facilitates the removal of contaminating extracellular proteins and provides purer exosomes (21). SEC uses porous resin gravity or low-pressure systems. With this method, proteins equilibrate with the resin pores, delaying their passage through the column. On the other hand, exosomes are far too large to equilibrate with the resin pores and therefore pass through the column without delay (137). Sample particles separated in SEC are collected in fractions. The resolution (particle separation) is dependent on the selection of resins and packing dimensions. For example, some of the earliest reports of SEC-based isolation utilized Sepharose CL-2B resin, which has a mean pore size of ~75 nm (111). More recent studies have reported that the use of Sepharose 4B or CL-4B resin, which has a smaller mean pore size of ~42 nm, may be better suited for the separation of smaller EV fractions (23, 111). Generally, exosomes are collected in the earlier fractions, whereas the bulk of serum proteins elute in later fractions. Sample volumes ranging from 0.5 to 4% of packed resin volume can provide good resolution separation, and high resolution can be achieved if the sample volume does not exceed 2% (17). If sample starting volume is large, centrifugal ultrafiltration can be used to concentrate samples before the SEC (23). The sample should be equilibrated in the SEC elution buffer in the final step of ultrafiltration.

Anion exchange chromatography. Anion exchange chromatography (AEC) has been previously used to isolate virus-like particles (VLPs) from cell culture supernatants and separate them away from contaminates (91, 147). AEC takes advantage of the net negative charge observed on the surface of EVs (from glycans/phospho/sulfo groups) to bind EVs to a positively charged chromatographic matrix (79, 101). This allows for minimally processed, precleared conditioned cell culture media to be run on AEC to maximize EV collection under relatively gentle isolation conditions. EVs can then be eluted by using a buffer that possess greater ionic strength than that of the surrounding mobile phase (e.g., higher sodium concentration). Differing glycosylation patterns from parental cell lines and other source material can affect the net charge of the cell and, presumably, the EV surface. Therefore, the optimal ion concentration in the elution buffer needs to be empirically determined for each source EV material. Filtration devices can be used to concentrate the EV fractions and perform a buffer exchange as needed.

Affinity purification. The affinity purification method employs antibodies that capture exosomes by binding to specific surface markers, therefore limiting protein contamination in exosome isolation (18, 141). This can be done with antibodies targeting

CD63 or other established surface markers. A further advantage of affinity purification is to pull exosomes derived from specific cell sources from serum samples by identifying a surface protein marker of interest. This approach is enhanced by the sequential use of two different antibodies to known proteins on the surface of the cell of interest. Although it has high specificity, it requires prior analysis of exosome surface markers and presumable exosome quantities and might result in selection bias from the total population. Furthermore, the yield of exosomes will be much lower with this method but potentially much more specific to the cells of interest.

BIOPHYSICAL AND BIOCHEMICAL CHARACTERIZATION OF EXOSOMES

Electron microscopy. In electron microscopy (EM), a beam of electrons is focused by electromagnetic or electrostatic lenses to illuminate the sample of interest in a vacuum chamber (39). Because the wavelength of electrons is significantly shorter than that of visible light, EM has greater resolution and magnification than light microscopes (38). Ergo, EM has been widely used for particle sizing due to its excellent nanometer resolution (Table 2) (38, 167). Transmission electron microscopy (TEM) is the most common EM method (187). In TEM, the incident electrons pass through the extremely thin sections of sample or are scattered out depending on the sample structure. The transmitted electrons are magnified and projected to create an image on a fluorescent screen or a camera on the bottom of a TEM. Heavy metal stains may be required to enhance the image contrast, where immunolabeling with nanogold particles enables the recognition of samples presenting biomarkers. This approach will not work for regular monitoring of isolated exosomes, as it is very time consuming to prepare samples for EM and then analyze. However, it can be worthwhile to verify by a second method that exosomes are present in a sample using EM and antibodies to exosome markers (122).

Although TEM is able to provide morphological and biochemical information with the use of immunostaining, sample preparation has been reported to cause morphological artifacts, such as cup-shaped appearance of exosomes (90). To avoid the drawback of exosome deformation due to dehydration and chemical fixatives, cryoelectron microscopy (Cryo-EM) can be applied to image cryoembedded exosomes. The very low temperature in this technique can additionally protect the exosomes from radiological damage caused by the incident electron beam (187). Cryoelectron tomography has been reported to verify the spherical structure of exosomes (41) and ultrastructural components within exosomes (89).

Atomic force microcopy. Atomic force microcopy (AFM) drives a cantilever with probe tips at its free end to assess nanoparticle morphology (179). When the probe tips interact with the particles while moving through, the deflection of the cantilever reflects a laser beam (152). The laser reflection is recorded and then analyzed for particle surface structure with resolutions down to $\sim$1 nm. This method has been applied to characterize exosomes derived from human saliva (174) or EVs derived from iPSCs (2). When the AFM tips are functionalized by being coated with antibody, this method can be used to characterize specific individual exosomes. The same group further used anti-CD63 IgG-coated AFM tips to detect exosomal CD63, showing stronger interaction force between the functionalized tips and the exosomes compared with the controls (174).

Dynamic light scattering. The dynamic light scattering (DLS) system detects scattered laser light caused by Brownian motion of the suspended particles (151). It quantifies the average particle sizes ranging from nanometer to micrometer based on monodisperse populations (55). However, the size profile is less accurate if obtained from heterogeneous particle populations, where larger particles scatter more light.

Nanoparticle tracking analysis. Similar to DLS, nanoparticle tracking analysis (NTA) also tracks suspended particles based on Brownian motion (16). This system is equipped with a camera to record the laser light scattered by moving particles at a fixed flow rate driven by a hydraulic pump (187). Each particle is tracked in the recorded video to analyze particle distribution by calculating the particle velocity based on the Stokes–Einstein equation. With this feature, the number and size of heterogeneous vesicle populations can be quantified more accurately with optimal camera and analysis settings (212). The sizing ranges from 30 to 1,000 nm (59). Furthermore, NTA can track fluorescently labeled exosomes, allowing for characterizing exosome phenotypes when labeled with specific antibody-conjugated quantum dots (50, 148). However, the accuracy may be affected by sample handling, and the sample concentration range is limited (10^7–10^9 particles/mL) (59). If the sizes of contaminants are close to the exosome size, the contaminants may be not distinguishable from isolated exosomes and might result in overestimation of the number of exosomes.

Resistive pulse sensing. Resistive pulse sensing (RPS) is capable of accessing particle concentration and size distribution via measuring individual particles (12). When particles pass through the pore filled with conductive fluid in a membrane, the increased electrical resistance produced by each individual is detected by the resistive pulse sensor for analyzing particle size (11). An advanced technique, tunable resistive pulse sensing (TRPS), utilizes a size-tunable pore created in an elastic membrane (183) to improve measurement sensitivity and resolution (168). If the sample consists of heterogeneous particle sizes, multiple membranes of specific pore size ranges may be needed.

Flow cytometry. Flow cytometry is one of the most commonly used methods to qualitatively and quantitatively characterize single cells in a high-throughput and high-sensitivity manner (146). However, limitations in cytometer sensitivity, assay specificity, and the lack of proper reporting of experimental details have produced a body of scientific literature whose rigor is not always clear (132, 204). Recently, a working group of EV flow cytometry researchers from the International Society for Extracellular Vesicles (ISEV), the International Society for the Advancement of Cytometry (ISAC), and the International Society on Thrombosis and Hemostasis (ISTH) developed a framework for the experimental design and minimum amount of information that should be reported with EV flow cytometry studies (MIFlowCyto-EV) (225). Some cytometers are more sensitive than others in detecting EVs (222). Beckman's CytoFLEX and Apogee's MICRO-PLUS are considered two of the more sensitive cytometers currently on the market, with several newer cytometer models that may be even more sensitive and specialized toward EV work making their way to the market (224).

Calibration beads for standardizing EV size and molecules of equivalent soluble fluorophore (MESF) beads are utilized to standardize reporting of EV fluorochrome brightness (68, 223).

Table 2. *Comparison of exosome characterization methods*

Technique	Principle	Resolution	Pro	Con
Electron microscope	Scattered electrons	0.5 nm	Characterize exosome size and morphology. Possible for immunolabeling.	Sample handling may result in morphological artifacts.
Atomic force microcopy	Reflected laser beam based on deflection of the cantilever	1 nm	Characterize exosome size, morphology, and topology. Possible for immunocharacterization.	Probe and sample may be damaged in contact mode.
Dynamic light scattering	Brownian motion of the suspended particles	1 nm–6 μm	Analyze exosome size distribution. Fast and easy procedure.	No absolute quantification. Polydisperse populations results in less accuracy. Sensitive to large particles.
Nanoparticle tracking analysis	Brownian motion of the suspended particles. Analysis of particle distribution based on the Stokes–Einstein equation	30 nm–1 μm	Characterize exosome size, size distribution, and concentration. May use for fluorescence detection, allowing immunocharacterization.	Requires sample exosomes within a limited concentration range.
Resistive pulse sensing	Change in electrical resistance	Depends on the selection of nanopore sizes on the elastic membrane	Characterize exosome size, size distribution, and concentration.	Heterogenous particles might need different nanopore sizes for measurements.
Flow cytometry	Scattered light	*100–200 nm	High throughput. Particle size and count. Possible for fluorescence detection, allowing immunocharacterization.	*Requires high-resolution flow cytometry. High particle concentrations might reduce the accuracy of event count.
Protein detection	Antibody affinity	N/A	Phenotyping. Western blot: semi-quantitation. ELISA: quantitation.	Low sensitivity. No size information.
	Mass spectrometry	N/A	Global characterization of protein content. Quantitative.	Purity of sample influences robustness of data. Requires specialized equipment and data analysis techniques.
RNA detection	qPCR	N/A	Highly sensitive for both mRNA and miRNA. High throughput.	Normalization transcripts need to be empirically derived.
	Transcriptomics	N/A	Highly sensitive and quantitative.	Need large sample size. Low throughput.

N/A, not applicable.

Cellarcus now has EV flow cytometry kits available that contain many of the controls outlined in the new MIFlowCyto-EV standards. In general, groups should perform titration dilutions not only of the antibodies used but also of their EV samples to ensure that concentrated EV preparations are not producing artifacts through the "swarming" effect (48, 68). Researchers should also perform detergent lysis (e.g., 0.3% Triton X) control of EV preparations to verify that the particle population being detected is lipid membrane based (150).

Currently, several EV flow cytometry groups use carboxyfluorescein succinimidyl ester (CFSE)-based dyes as a general stain to discern EVs from other particulate matter in EV preparations as well as cytometer fluidics (48). CFSE is able to passively diffuse into EVs, where its acetate groups are cleaved by esterases, which are then converted to fluorescent esters. CFSE is retained within EVs due to its covalent binding with adjacent molecules via succinimidyl groups (69, 134). This covalent bonding allows CFSE to be retained within EVs for long periods of time and limits its transfer outside of EVs (133). The $CFSE^+$ fraction is then gated on as the EV population for further downstream analysis by fluorochrome-conjugated monoclonal antibodies (124). Given the small surface area of EVs and the lack of robust sensitivity of current cytometers in the submicrometer range, the usage of the brightest fluorochromes available for detecting EV surface protein expression [e.g., R-phycoerythrin (PE) or Brilliant Violet 421 (BV421)] is advantageous (134). Careful application of compensation and fluorescence minus one control are critical for rigorous data acquisition as well as unstained controls in addition to stained buffer controls. Optimization of voltages on cytometers, filtration of buffers, and cytometer fluidics with 20–30-nm filters can greatly reduce background noise due to the presence of non-EV particles. Furthermore, some cytometer companies have specialized filter sets to enhance sensitivity of EV detection (e.g., Fisher's Attune NxT Small Particle Side Scatter Filter).

Protein detection. Immune-based assays, such as Western blot analysis and enzyme-linked immunosorbent assay (ELISA), are widely used to characterize exosomes or exosome subpopulations. Western blot is an antibody-based, semiquantitative or qualitative method to analyze the presence of specific proteins in EV preparations. When doing Western blot analysis, exosomes and the lysates of their producing cells should be loaded side by side in a defined amount for comparing the abundance of detected proteins. Exosome-specific ELISAs provide plates that are precoated with antibodies that can capture exosomes and include lyophilized EV standards for the quantification analysis. Numerous EV-associated proteins (e.g., CD9, CD63, CD81, syntenin-1, flotillin-1) have been assessed systematically (88) and summarized in Minimal Information for Studies of Extracellular Vesicles 2018, including EV-negative control

marker proteins (198). Mass spectrometry-based proteomics approaches have also been widely employed in the characterization of EV preparations (19, 36). Characterization of membrane proteins by use of such approaches requires consideration of the lysis buffer used and the medium in which the peptides are analyzed on the mass spectrometer. Ionic detergents are often required to sufficiently separate out membrane-restricted proteins from lipid components, but such ionic detergents are not compatible with most mass spectrometer methodologies (229). However, EVs may be lysed in strong ionic detergents if it is followed up by a column-based buffer exchange step (229).

RNA detection. EVs are packaged with various subtypes of RNA, including mRNA, miRNA, tRNA, snoRNA, and other classes of noncoding RNA (85). Frequently, quantitative polymerase chain reaction (qPCR) is a common method to detect and quantify RNAs of interest within EV preparations (96). For EVs derived from plasma and other bodily fluids, careful attention should be given to non-EV particles that can coisolate with EVersus Particles such as high- and low-density lipoproteins are abundant in blood and can also be associated with miRNA (213). Therefore, EVs need to be rigorously separated from such contaminants with density gradients to limit the contribution of non-EV-associated RNA (201). To date, there is no consensus on which EV-associated transcripts may serve as effective normalization controls (202). Therefore, effective normalization controls for each class of EV preparation need to be empirically determined and verified for reproducibility. Other methods such as RNA-Seq do not require such normalization genes but are rather normalized to the total number of reads sequenced. To date, several groups have reported on both the detection and EV-based transfer of mRNA transcripts, whereas other groups have had trouble detecting full-length mRNA transcripts in their EV preparations. It is not clear what accounts for this discrepancy, but controls should be used to limit the detection of artifacts, which may be facilitated by longer read lengths and paired end sequencing analyses.

EVALUATION OF EV'S FUNCTIONAL PROPERTIES

Isolation media. The media used for exosome isolations should be given careful consideration. Historically, most published preclinical reports of stem cell-based therapies have utilized serum-containing media (52). However, the use of serum to isolate exosomes is problematic (100). Fetal bovine serum (FBS) is highly enriched for bovine exosomes. Most studies utilizing serum-containing media attempt to preclear the media of bovine EVs via overnight ultracentrifugation (163). However, such depletion strategies are not 100% efficient, resulting in residual FBS-derived exosomes that coisolate with stem cell-derived EVs secreted into the conditioned media (115). Such xenocontaminants interfere with studies investigating the functional properties of exosomes derived from stem cells and limit the future clinical applicability of such EV isolations. There is a growing appreciation that exosomes isolated from serum-free media greatly diminish these issues, yet exposing stem cells to serum deprivation induces cellular stress responses and potentially influences the factors packaged into the resulting exosomes (144, 149). Future studies are needed to investigate the effects of such serum deprivation techniques and whether they greatly influence the composition of the resulting exosomes.

Dosing regimens. Methods of dosing for functional studies should be taken into consideration for studies assessing the functional properties of stem cell-derived exosomes. Some groups determine the administered dose based on the number of EVs present, while other laboratories use total exosome protein content to determine dosing regimens (52). Studies using vesicle concentration methods should verify the accuracy and reproducibility of the methods used to determine vesicle concentration, as previously this has been a limiting factor. The rationale for the use of protein as an appropriate determinant of exosome dosing regimens is based on proteinaceous mass compared with their total RNA content (200). These two classes of macromolecules, protein and RNA, are generally attributed with being the chief mediators of exosomes' functional properties. The preponderance of total protein compared with total RNA among stem cell-derived exosomal populations provides the rationale for using protein as a metric for dosing.

Formulation. Formulation issues associated with exosome cryostorage have garnered increasing attention of late (103). Many published reports utilize PBS as a cryostorage medium for isolated stem cell-derived exosomes for downstream functional assessments. However, PBS is not an ideal cryostorage medium for biologics, as it experiences a significant drop in pH as it approaches freezing temperatures (99, 158). Increasingly, stem cell-derived exosome research groups have started utilizing excipients and carriers to increase their stability. Proteins such as HSA/BSA and sugars such as trehalose are commonly used to stabilize various biologics during product processing and may aid in exosome stability during storage (28). Comprehensive comparison studies of the numerous excipients and carriers available for exosome storage have yet to be published, but such future studies are warranted. Many groups working with stem cell-derived exosomes have also anecdotally reported in personal communications to us that exosomal yield seems to decrease as a function of the amount of plasticware they were exposed to. Limiting exposure to plasticware, using low protein-binding plasticware and preblocking of nonspecific protein binding may potentially aid in minimizing exosomal loss during processing.

In vitro bioassays. Numerous groups have reported multiple functional properties of EV preparations from various source materials, including culture-conditioned media (181, 210). Several have reported the use in vitro immune assays, where generally primary peripheral blood mononuclear cells (PBMCs) or splenocytes, or a subpopulation thereof, are stimulated with an antigen (e.g., LPS) or other stimulant (e.g., phytohemagglutinin) and then treated with a concentrated dose of EVs (72, 98). Outcome measures include cytokine expression analysis, flow cytometry analysis of activation or differentiation markers, and colorimetric activity assays. EVs have also been assessed for their cytoprotective properties in vitro, using a variety of reagents to generate a stress response (e.g., ROS, endoplasmic reticulum stress) in the target cell population, followed by assessments of gene expression or cellular viability rates (73). It is also important to provide evidence that EVs are being taken up by the target cell population, which can be accomplished by fluorescently labeling EVs with various esterase and lipid-incorporating dyes (63). For EV uptake studies using lipid-incorporating dyes, it is important to have appropriate controls demonstrating that the observed EV uptake is not an artifact attributable to dye aggregates forming in the buffers

used, or due to dye leakage out of EVs (which can occur in serum-containing media) (191).

Animal studies. To date, there are over 200 animal studies that have investigated the ability of EVs to modulate tissue physiology in vivo (52). Although these studies have examined a diverse set of diseases and EVs derived from numerous sources, it is clear that EVs have the wide-ranging ability to influence both molecular phenotypes and clinical measures of disease burden in animals. Although most EV animal studies have reported intravenous administration, EV groups should also consider other routes of delivery, including subcutaneous and intraperitoneal, as well as the timing of the EV dosages relative to disease state, both of which may strongly influence outcome measures. When heart disease is treated, the delivery route is an important factor for effective EV homing to the heart. The intravenously delivered exosomes have been shown to be rapidly cleared from the blood circulation, followed by predominant distribution to the liver and then to the lungs (189). To compensate for the unspecific delivery, a larger dose of intravenously delivered exosomes is often used (206, 207). However, a more effective approach to increase the efficacy of intravenously injected exosomes is to modify the exosome surface by either tagging or expressing peptides that target the cells of interest (94, 95, 206, 217). Intramyocardial administration might be an alternative approach, as the intramyocardial injected exosomes that were released by cardiosphere-derived cells were demonstrated to improve heart function in both mouse (83) and porcine (61) models of myocardial infarction. However, the risks of translating the intramyocardial delivery in animal studies to a clinical setting, which will require injection of the exosomes into the heart muscle, are not insignificant (206).

Notably, it is known that bovine exosomes are present in milk, and these exosomes and their miRNAs can be found in milk consumers across species (e.g., humans and mice) (240). Although epidemiological evidence correlates bovine milk intake with the development of chronic diseases of civilization, including obesity, type 2 diabetes mellitus, osteoporosis, common cancers (e.g., prostate, breast, liver, B cells) as well as Parkinson's disease (126), an immune response to the presence of bovine milk exosomes in humans has not been identified despite often daily consumption of milk. In the mouse and porcine studies of milk exosome tracking, the labeled exosomes accumulated in liver, spleen, and brain following suckling, oral gavage, and intravenous administration (123). Repeated challenge of bovine exosomes in mice neither showed any systemic toxicity nor elevated inflammatory cytokines (182). Since bovine milk is a biocompatible and scalable source of exosomes, and the bovine exosomes are protective to the bioactive cargo in a strong acidic environment (e.g., gastric acid), these exosomes are considered effective vehicles to deliver small drug molecules against lung tumor xenografts in vivo via oral or intraperitoneal routes (138).

Although the literature is limited, the bovine milk exosomes open a window of therapeutic exosomes derived from different species, and the lack of evidence of an immune response to exogenous exosomes increases the likelihood of safe repeated dosing with exosomes for therapeutics. Nevertheless, studies employing exosomes to repeatedly deliver therapy should include monitoring for an immune/inflammatory response, given the nascent nature of this approach to therapy. Going forward, it will also be critical for groups to optimize methods, determine dose-response curves, and investigate potential high-dose toxicities as well as potential immunogenicity of EVs derived from different species.

Biodistribution assessment. To date, there have been few published reports on the biodistribution and pharmacokinetics of administered stem cell-derived EVs (57, 93, 106, 129, 227). Such studies often rely on fluorescently labeled lipid-incorporating dyes in conjunction with in vivo optical imaging (e.g., IVIS), assuming that the lipid-incorporating dyes stay embedded in the EV membrane throughout the duration of the experiment (161). Yet several studies investigating commonly used lipid-incorporating dyes have demonstrated that up to 75% of the dye dissociated from the vesicles when incubated in serum (139, 178, 191). Amphiphilic lipid-anchored fluorophores, such as PKH dyes used for poststaining of EVs, also possess the capability to form nanoparticles that are hardly distinguishable from real fluorescently stained EVs (161). Few published reports have investigated the biodistribution of radiolabeled exosomes (57, 82, 209). Another possibility is to label EVs with chemiluminescence-emitting proteins that permit in vivo quantitative evaluation (189). Thus, further studies elucidating the pharmacokinetics of exosome-based therapies are necessary. Nonetheless, current exosome biodistribution methodologies require substantial manipulation of EV populations, which may result in the attenuation of exosome function as a result of degradation or abnormal conformation of proteinaceous factors responsible for cellular uptake. Studies examining the postinsertion function of the resulting exosome fractions are important for validation.

CONCLUSION

Exosomes play a critical role in cell-to-cell communication, as they encapsulate bioactive molecules from the parent cells. Interest in exosomes and their potential physiological and pathological impacts is augmenting in many areas of biological research. Both heart cells and other cell types in different conditions can detrimentally or favorably mediate the status of heart health or diseases through exosomes. Stem cell-derived exosomes have been shown as a regenerative mediator in cardiac repair. Understanding the composition of exosomal cargo enables the investigation of signaling mechanisms and possible genetic modifications in the exosome-releasing cells to improve treatment outcomes. To achieve this, numerous methods of exosome isolation (Table 1) and characterization (Table 2) have been developed. While each method has advantages and disadvantages, adjunct techniques are often employed to enhance the quality and quantity of isolated exosomes. When therapeutic exosomes in preclinical or clinical studies are being applied, several factors should be carefully considered to ensure safety, reproducibility, and efficiency. Overall, it is likely that the investigation and therapeutic use of exosomes has just begun, and we can anticipate more advances in the future.

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AUTHOR CONTRIBUTIONS

Y.L. and J.D.A. conceived and drafted the manuscript. Y.L. and J.D.A. prepared the figures. Y.L., L.M.A.R. and S.V.G. performed the experiments. L.M.A.R. and A.A.K. proofread the manuscript. A.A.K. supervised and revised the article. All authors approved the final manuscript.

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