More Than Tiny Sacks
Stem Cell Exosomes as Cell-Free Modality for Cardiac Repair

Raj Kishore, Mohsin Khan

Abstract: Stem cell therapy provides immense hope for regenerating the pathological heart, yet has been marred by issues surrounding the effectiveness, unclear mechanisms, and survival of the donated cell population in the ischemic myocardial milieu. Poor survival and engraftment coupled to inadequate cardiac commitment of the adoptively transferred stem cells compromises the improvement in cardiac function. Various alternative approaches to enhance the efficacy of stem cell therapies and to overcome issues with cell therapy have been used with varied success. Cell-free components, such as exosomes enriched in proteins, messenger RNAs, and miRs characteristic of parental stem cells, represent a potential approach for treating cardiovascular diseases. Recently, exosomes from different kinds of stem cells have been effectively used to promote cardiac function in the pathological heart. The aim of this review is to summarize current research efforts on stem cell exosomes, including their potential benefits and limitations to develop a potentially viable therapy for cardiovascular problems. (Circ Res. 2016;118:330-343. DOI: 10.1161/CIRCRESAHA.115.307654.)

Key Words: cardiac regeneration ■ cardiovascular diseases ■ exosomes ■ miRNAs ■ stem cells

Great things are done by a series of small things brought together

—Vincent van Gogh

Challenges for Cell-Based Therapies: Where Do We Stand?

Cell-based treatment regimens were hailed as the wonder cure for treatment of cardiovascular diseases. However, early optimism has gradually transformed into pragmatism and an overall sense of precaution. Number of different stem cell types has been used to date with the solitary goal to replace cardiomyocytes lost to cardiac pathological injury. Yet, cell therapy is marred by recurring themes because not a single cell type to date has been able to orchestrate the balance between cardiomyocyte regeneration, neovascularization, and modulation of inflammatory processes. A glance across literature is imperative to summarize current efforts in cell-mediated repair of the heart, present and future challenges, and some of the new directions, including cell-free cardiac therapies.

One of the first cell types to be verified in clinics for the treatment of heart failure was skeletal myoblasts. Subsequent studies have provided disparate results ranging from no change in left ventricular (LV) ejection fraction in heart failure patients to modest improvement in remodeling and clinical parameters after long-term follow up. Essentially, a large number of negative results coupled with issues of arrhythmias have prevented further studies with skeletal myoblasts. In contrast, bone marrow has provided an interesting source for cell-based therapies because it harbors multiple stem cell types, is easy to harvest, and propagate ex vivo. Administration of unfractionated bone marrow, that is, bone marrow mononuclear cells enriched in multiple progenitor cells types, has been used in the largest number of clinical trials to date. Investigators have conducted various double-blinded multicenter trials, such as Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI), Transplantation Of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI), and Transplantation Of Progenitor Cells and Regeneration Enhancement for patients with Chronic ischemic Heart Disease (TOPCARE-CHD), all based on the use of unfractionated bone marrow cells administered to heart failure patients. Meta-analysis of these studies demonstrated improved cardiac function along with reduction in remodeling and the occurrence of adverse effects in patients. Because bone marrow is host to a variety of stem cell subpopulations, many subsequent studies have focused on evaluating the regenerative potential of individual cell types. For instance, the TOPCARE-CHD trial evaluated the potential of endothelial progenitor cells (EPCs) for treatment of patients with LV dysfunction. Similarly, several clinical trials (Transendocardial Autologous mesenchymal stem Cells and mononuclear bone marrow cells in ischemic Heart Failure Trial [TAC-HFT], Percutaneous stem cell injection delivery...
kit + CPCs, and their delivery to heart failure patients resulted in infarct size was coupled to a remarkable 13.5% improvement in ejection fraction at 1-year follow-up. Collectively, these results may encourage large-scale clinical trials but it is critical to assess the fate of the transplanted cells in heart failure patients, and it is largely hypothesized that the adoptively transferred cells transfer their contents to the damaged myocardium before being lost to the harsh ischemic cardiac milieu. Remnants of the transplanted cells that do make it past the first couple of weeks undergo transformation possibly into vasculature and new myocytes as shown by Vrtovec et al. Without doubt, stem cells are beneficial, but their salutary effects may not be restricted to merely cardiomyocyte generation.

Over the years, a large number of reports have shown that the transplanted stem cells mediate their benefits via several indirect mechanisms, such as recruitment of endogenous progenitors, induction of angiogenesis, protection of existing cardiomyocytes, and reduction in fibrosis and inflammation. These processes are regulated by a variety of small molecules, proteins, messenger RNAs/microRNAs (mRNAs/miRNAs), and paracrine factors produced by the adoptively transferred stem cells in the damaged myocardial milieu. Initial evidence highlighted the importance of extracellular factors in cardiac regeneration and implicated released cytokines from damaged cardiac tissue to be involved in recruitment of stem/progenitor cells to the damage region. Similar findings have been observed whereby the donated stem cells, including mesenchymal stem cells, CPCs, and so on, are able to secrete a broad spectrum of cytokines, chemokines, and growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), monocyte chemoattractant protein-1 (MCP-1), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), stromal cell-derived factor-1 (SDF-1), and thrombopoietin-stimulating regenerative processes.

Recently, microvesicles or exosomes have emerged as important regulators of molecular processes and have been included in the definition of paracrine effectors capable of instigating cell autonomous repair response. What Are Exosomes?

Exosomes are tiny microvesicles released by cells in response to different physiological states. Exosomes were first described as early as 1950s in sheep reticulocytes involved in removal of cell surface molecules. Initial findings characterized exosomes as means to shuttle cellular garbage out of the cells. Nevertheless, intense research efforts recently have provided a much broader role for exosomes in regulating various cellular and molecular processes, including cell–cell communication. Typically, exosomes are 30–100 nm in size and are produced by several cell types, such as T-cells, dendritic cells, mast cells, tumor cells, and sperm. In contrast, vesicles greater than 100 nm are referred as microvesicles or microparticles. Several studies recently have shown that various types of stem cells are also able to release exosomes that exert functional effects that mimic the effect of their parent cell of origin.

Exosome Biogenesis Multiple mechanisms have been proposed for exosome generation. Cellular release of proteins through the plasma

**Nonstandard Abbreviations and Acronyms**

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<th>Abbreviation</th>
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<tr>
<td>CPCs</td>
<td>cardiac progenitor cells</td>
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<td>EPCs</td>
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<td>ESC</td>
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membrane is governed by different processes that are collectively called as exocytosis. Exosomes originate in the intraluminal vesicles of multivesicular bodies as part of the late endosome by inward folding of multivesicular body membrane into its lumen.40 It leads to formation of multiple intraluminal vesicles inside the endosome that are called the multivesicular body. Fusion of the multivesicular body to the plasma membrane releases the intraluminal vesicles into the extracellular matrix at which point they are called as exosomes. Alternatively, direct budding of the plasma membrane encompassing cytoplasmic contents can also occur, and in this instance, the vesicles are categorized as microvesicles.42,43

Exosomal Content

Exosomes are generally identified on the basis of their unique content that is reminiscent of the parent cell of origin. Many proteins specific to the plasma membrane and cytoplasm are present in the exosomes. Because of their endosomal origin, exosome contain proteins involved in membrane transport and fusion, such as Rab GTPases, annexins, and flotillins, as well as integrins and tetraspanins.44 However, the protein content is reflective of the physiological state of the parent cell and varies in response to stress and changes in the microenvironment. Exosomes also contain some conserved proteins considered as markers of exosomes, such as heat shock cognate 70 and tetraspanin CD63 irrespective of their cellular origin.45 Many cell-signaling proteins like β-catenin, Wnt5, Notch ligand, δ-like 4, tumor necrosis factor (TNF)-α, tumor growth factor (TGF)-β are also known to shuttle within exosomes, whereas in some cases, exosomes can carry major histocompatibility complex (MHC) class I and II molecules. Many proteins for cytoskeleton and metabolism (GAPDH) are also found in the exosomes.46

In contrast to the proteins expressed in the exosomes, their lipid content is far more unpredictable. Exosomes contain lipid molecules involved in exosome biogenesis, such as lysobisphosphatidic acid.47 Characteristically, exosomes contain many lipid raft molecules like cholesterol, sphingolipids ceramide, and glycerophospholipids.48 Lipid molecules involved in mediating cellular signaling, such as prostanglandins, are also present in exosomes.

One of the most fascinating characteristic of exosomes is their ability to carry cell type–specific mRNA and miRNAs.49,50 Because miRs have been implicated in the regulation of multiple biological processes, their presence within exosomes potentially opens up host of opportunities for studying downstream effects in cell–cell communication.51 Not only, mRNA and miRNA cargoes of the exosomes have been noted to be functional in the recipient cells but importantly have provided a mechanism for intercellular communication. Proportion of miRNAs within exosomes is considered to be much higher than the parent cells,51 and studies recently have found preferential enrichment of certain miRNAs in exosomes.52–54 Current research efforts propose different mechanism for miRNA sorting, such as neural sphingomyelinase 2–dependent pathway,55 miRNA motif, and sumoylated heterogenous nuclear ribonucleoproteins–dependent pathway.56 3′-end of the miRNA sequence-dependent pathway,57 and miRNA-induced silencing complex–dependent pathway.58 These studies indicate that miRNAs have a higher affinity for exosome packaging but still little is known about how specific underlying mechanism for mRNAs/miRs packaging within the exosome or whether all exosomes from one cell type have similar distribution of the particular mRNA/miR.

Role of Exosomes

Intercellular Communication

Identification of exosomes within bodily fluids, such as blood, urine, plasma, semen, bronchoalveolar lavage has provided evidence for a role of exosomes in intercellular communication.45,49 Furthermore, many physiological conditions lead to changes in the exosomes produced and can serve as biomarkers for identification of disease progression. A large number of studies have implicated exosomes in cancer progression. Exosomes from malignant cells have been known to display angiogenic properties,59 carrying signaling ligands that can activate oncogenic pathways,60 and shuttle tumor-promoting mRNAs. Similarly, few studies indicate exosomes in the spread of infectious agents and viral particles, such as HIV-1.61 In the nervous system, exosomes are produced by several different cell types and can affect other cells of the neuronal system. Released exosomes can modulate synaptic transmission and plasticity dependent on synaptic activity of the electrocally stimulated neurons.62

Immune-Modulation

Extensive research efforts have focused on the ability of exosomes to modulate immune responses. Various components of the immune system have been known to produce exosomes that are involved in multiple processes, such as antigen presentation and initiation of adaptive immune responses. Initial interest in immune modulatory properties of exosomes came from the finding that B-lymphocytes-derived exosomes harbor MHC class II molecules and present them to T-lymphocytes.62 Later studies revealed that exosomes from dendritic cells bear MHC I complexes and play a substantial role in antigen presentation to T-cells and subsequent initiation of immune responses. In similar context, stressed or infected dendritic cells are able to disseminate proinflammatory and immunosuppressive signals via exosome secretion. Numerous reports have shown that immunosuppressive nature of tumor exosomes is responsible for inhibition of antitumor immune responses, resulting in the spreading of malignant cells, whereas its manipulation has been proposed as an anticancer treatment.33,63,64

Modulation of Cell Signaling

Transfer of cell type–specific proteins represents a unique manner in which exosomes can manipulate various cell signaling pathways. As mentioned earlier, some of the exosomal proteins are conserved across species, whereas others are selectively packaged, thereby providing clues to the physiological cell state. Because exosomes are bound by a lipid layer along with the presence of protective proteins, they may be resistant to changes in the extracellular matrix. Recent studies in Drosophila have provided evidence that signaling proteins, such as Wnts, shuttle within exosome and convey signals that lead to development and tissue regeneration.65 Similarly, export of β-catenin out of the cell has been proposed to take place via exosomes, resulting in reduction of cytosolic levels
of the protein. Many oncogenic proteins have been found to be expressed in exosomes, for example, HER2 in breast cancer cell lines. Soderberg et al showed that exosomes from melanoma cells carry TNF, as well as TNF receptors 1 and 2, that are effectively delivered to other cell types.

**Stem Cell Exosomes: A Paradigm Shift in Regenerative Biology**

As reviewed earlier, a large number of cell types release exosomes in response to both cell intrinsic and extrinsic changes. Cell therapy has tremendous promise for tissue regeneration but has been marred by issues prompting the idea whether exosomes from stem cells hold regenerative power and, at the same circumvent, limitations associated with direct transfer of stem cells. Some of the recent findings and concepts in the area of stem cell exosomes are summarized in the sections later.

**Pluripotent Stem Cell Exosomes**

Over the years, pluripotent stem cells, including both embryonic stem cells (ESC) and the more recently discovered induced pluripotent stem cells (iPSCs), have been proclaimed to be cells with the highest regenerative potential. Nevertheless, pluripotent stem cell research carries various ethical concerns compounded with reports of tumor formation after in vivo administration in various disease models. Exosomes coming from such cells may exhibit similar power of regeneration but whether adverse effects related to their parent stem cells are inherited by the exosomes must be carefully ascertained.

**Embryonic Stem Cell Exosomes**

ESCs represent one of the most regenerative stem cell types available; yet, their use remains controversial because of lack of donors, ethical concerns, and tumor formation. ESCs efficiently convert into cardiomyocytes; however, the differentiated cardiomyocytes represent more a neonatal phenotype than functional adult cardiomyocytes. Cell-free components, including microvesicles and exosomes, derived from ESCs may provide an interesting alternative to harness the salutary effects of ESCs and at the same circumvent concerns associated with ESC research. Early evidence suggesting that ESC beneficial effects extend beyond directed differentiation came from studies showing that mature somatic cell undergo epigenetic changes, promoting cellular reprogramming when cocultured with ESCs or their extracts. More recently, ESC-derived microvesicles were used to enhance survival and expansion of hematopoietic progenitor cells with consequent elevation in expression of pluripotent (Oct-4, Nanog, Rex-1), stem cell markers (Scl, HoxB4, GATA-2), and phosphorylation of MAPKp42/44 and AKT. Proposed mechanism for the observed results was governed by delivery of mRNAs specific to pluripotent transcription factors, and the effect was abrogated by heat inactivation of the ESC-derived microvesicles. Similarly, skin fibroblasts undergo rapid proliferation when treated with ESC-derived microvesicles as evidenced by increased bromodeoxyuridine (BrDU) levels and protein levels of proliferating cell nuclear antigen (PCNA) and Ki67. Induction of pluripotency and dedifferentiation in Muller cells of retina treated with ESC microvesicles has been established via transfer of mRNA transcripts (Oct-4, Sox-2) and miR-290 cluster, an ESC-specific miRNA. Detailed proteomic profiling of human ESC microvesicles revealed accumulation of proteins characteristic of invasive cancers but involved in cellular activation, metastasis, and inhibition of apoptosis, as well as proteins displaying immunogenic properties. Interestingly, ESC-specific miRNAs packed within ESC microvesicles are efficiently delivered to target cells opening up a host of possibilities to study downstream molecular signaling processes. Because ESC possess a powerful potential for organ repair coupled with reports showing that ESC-derived exosome recapitulate effect of the cell themselves, we think that ESC exosome represent an interesting therapeutic modality for organ repair (Figure 1), circumventing several issues related to pluripotent cell research.

**Induced Pluripotent Cell–Derived Exosomes**

The discovery of iPSCs by Yamanaka et al has revolutionized regenerative medicine by providing means to understand disease progression and develop targeted therapies. Although iPSCs represent a valuable cell type to study disease progression, their use for organ regeneration suffers mainly because of incomplete transformation of the reprogrammed cells into mature adult cell phenotypes. Furthermore, adoptive transfer of iPSCs bears similar burden of compromised survival, proliferation, and cardiac commitment as observed with other stem cell types. In this respect, cell-free components derived from iPSCs may provide an interesting alternative for cardiac regenerative medicine and may extend their benefits via similar mechanism as discussed earlier for ESC exosomes. Few studies have been conducted to date using iPSC-derived exosome; however, in the ischemic kidney model, therapeutic value of exosomes derived from renal pluripotent stem cells was assessed. The authors observed significant protection and improved survival against nephrotoxicity, leading to enhanced kidney function in animals administered with renal pluripotent stem cells exosomes.

**Adult Stem and Progenitor Cell Exosomes**

Accumulating evidence over the years has suggested a critical role for stem cell secretome in extending the beneficial...
effect of various cell therapy treatments for repair of injured heart tissue. Stem cell clinical trials recently show continued improvement in cardiac function, yet follow up studies hinted toward early loss of adoptively transferred cells. This apparent ambiguity suggests an additional mechanism at play, and a developing consensus points to the ability of the transplanted cells to secrete beneficial factors at the site of injury that galvanize and protect existing cardiac cells, thereby resulting in sustained improvement in cardiac function.

**Mesenchymal Stromal Cell Exosomes**

Over the years, MSCs have proved to be an attractive source for cell-based therapeutic modalities because of their relative ease in procurement and multilineage differentiation potential. Earlier studies suggested MSCs to be highly plastic giving rise to a variety of cell types specific to different organs in the body; however, the idea was challenged shortly thereafter with contradictory reports regarding MSC transdifferentiation capacity. Alternatively, the regenerative potential of the cells was proposed to be largely based on the ability to secrete beneficial factors at the site of injury. Characterization of the MSC secretome has revealed a complex myriad of different cyto- kines, growth factors, inflammatory molecules, components of the extracellular matrix, and proteases. Recently, extracellular vesicles have been identified in the conditioned MSC medium, and initial evidence regarding their therapeutic value came from a study in myocardial ischemic model that laid the foundation for application in several other disease models. MSC exosomes demonstrate typical exosomal characteristic and express cell surface markers, such as CD9, CD81, CD29, CD44, and CD73. Analysis of protein content revealed 379, 432, and 420 unique proteins pointing toward variability in protein packaging, possibly reflecting the physiological cell state. Many signaling molecules related to MSC self-renewal, differentiation, and signaling pathways, such as Wnt (ras-related C3 botulinum toxin substrate 1 [RAC1]), protein kinase C beta [PRKCB], serine/threonine protein phosphatase 2A [PPP2R1A], TGF-β (collagen type 1, alpha 2 [COL1A2], cluster of differentiation 105 [CD105], endoglin [ENG]), and MAPK (familin A [FLNA], heat shock protein-8 [HSPA8], epidermal growth factor receptor [EGFR]) were found to be enriched in MSC exosomes, potentially affecting a diverse range of cellular processes, including cell cycle, proliferation, cell adhesion, cell migration, and cell morphogenesis. Similarly, miRNAs are known to shuttle within MSC exosomes but mostly in precursor form, driving downstream signaling pathways. Additionally, MSC exosomes possess immunologic properties, including secretion of anti-inflammatory cytokines, such as interleukin-10, TGF-β, and promoting inhibition of lymphocyte proliferation. Moreover, MSC-derived exosomes have been tested in graft versus host disease and induce M2-like phenotype in monocytes. In tumor biology, MSC exosomes enriched in tumor-potentiating factors have been shown to mediate pro- and antitumorigenic effects, promoting incidence and growth of malignant cells lines and thereby influencing tumor progression. In contrast, miR-16 present in MSC exosomes blocked VEGF suppressing angiogenesis and tumor development.

MSC exosomes have been extensively used for the treatment of various neurological diseases. In a rat model of cerebral artery occlusion, MSC exosomes were shown to communicate with brain parenchymal cells and transfer miR-133b, leading to changes in gene expression supporting neurite outgrowth and functional tissue recovery. In another study, the authors showed that delivery of MSC-derived exosomes led to improved neurological outcome and neurovascular remodeling. Recently, it has been found that adipose-derived MSC secrete nephrilysin, an important enzyme involved in degradation of β-amyloid peptides that are characteristic of Alzheimer’s disease.

**Endothelial Progenitor Cell Exosomes**

EPCs reside primarily in the bone marrow and possess ability to instigate proangiogenic responses. In clinical settings, a subset of EPCs expressing CD34 has been used for treatment of various ischemic disorders. Although the overall physiological impact of EPC administration has been periodically validated, there is still not much evidence connecting structural changes observed in the heart after EPC delivery as the reason behind functional gains. As is the case with other stem cell types discussed earlier, a large part of the benefits associated with EPC administration rest in their ability to secrete various paracrine mediators. This observation has led to the belief that paracrine signaling may have a much broader role to play in extending the benefits of EPCs therapy. In this regard, studies performed on the CD34+ cells from the bone marrow secrete exosomes that possess angiogenic characteristics, enhance tube formation of endothelial cells, and increase neovascularization in vivo. Further analysis revealed enrichment of several proangiogenic miRs (miR-126 and 130a) in CD34+ cell–derived exosomes. Microvesicles derived from human EPCs carry several markers similar to receptors expressed on EPC membrane, such as intracellular adhesion molecule-1, c4-integrin, CD44, and CD29 (β1-integrin), and induce proliferation, survival, angiogenesis as a consequence of horizontal mRNA transfer of genes, including B-cell lymphoma-extra large (BCL- XL), coflin1 (CFL1), catenin beta 1 (CTNNB1), endothelial differentiation-related factor 1 (EDF1), mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), protein tyrosin phosphatase receptor type T (PTPRT), endothelial nitric oxide synthase (eNOS).

In various disease models, EPC microvesicles were shown to enhance expression of angiogenic miRs (miR-126 and 296) and promote neovascularization of pancreatic islet cells and in the ischemic hindlimb. Hypoxia/reoxygenation injury in brain microvascular endothelial cells alters exosomal levels of RNAs associated with ROS and PI3K/eNOS/NO pathway, leading to corresponding effects on cell survival and death, thereby suggesting the impact of physiological cell state on packaging of exosomal content. Similarly, endothelial cell exosomes carrying miR-214 stimulated angiogenesis and prevented senescence in a disease model for ataxia telangiectasia.

**Exosomes for Cardiac Repair**

The heart is generally considered to be a nonsecretory organ but has the ability to release cytokines, growth factors, and proinflammatory molecules under various stress conditions. Many studies conducted recently provide evidence that
cardiomyocytes, fibroblasts, and endothelial cells are all capable of communicating with their neighboring cells via release of extracellular factors. Understanding the role played by these factors, including small microvesicles and exosomes, may provide important clues to regulation of pathological stimuli within the cardiac tissue. This section reviews current research efforts devoted toward characterizing the role of cardiac-derived stem cell on cardiac repair and the effects of exosome release by cardiomyocytes, fibroblasts, and endothelial cells on intracardiac communication, including ability to affect various cellular processes.

Cardiac Progenitor Cell Exosomes

Discovery of CPCs within the heart regulating cardiac homeostasis and repair has brought a paradigm shift in cardiac regenerative medicine. CPCs possess ability to form all 3 cardiac lineages, and their adoptive transfer leads to significant augmentation of cardiac function in animal models of heart failure. Two recently concluded clinical trials using CPCs demonstrated efficacy of the cells in clinical settings and for treatment of patients with heart failure. CPC transdifferentiation into cardiac cells represents the most likely explanation for their beneficial effect, and additional mechanisms, such as secretion of paracrine factors cannot be ruled out. This notion is further strengthened by findings that show CPC-conditioned medium protects cardiomyocytes under stress parallel with induction of tubule formation in endothelial cells. Subsequently, rat c-kit+ CSCs demonstrated improved cardiac function with reduction in scar size, yet no cells were found in the heart. In contrast, there was increase in the number of cardiomyocytes, blood vessels, and endothelial cells, pointing toward the paracrine ability of the transplanted cells. Similar data has emerged from the recently conducted CADUCEUS clinical trial that showed persistent increase in cardiac function after CPC transfer, yet the donated cells have been hard to detect, pointing toward indirect mechanisms as most probable cause for the observed functional increment. Keeping this in view, the next set of studies focused on the analysis of CPC secretome. High levels of numerous cytokines, including chemokines (TCA-3, SDF-1, 6Ckine), vascular growth factors (VEGF, erythropoietin, bFGF, osteopontin, SCF), and cardiac differentiation factors (Activin A, Dkk homolog-1, TGF-β) that could potentially be involved in mediating CPC salutary effects were all found to be enriched within CPC-derived conditioned medium and microvesicles. CSC-derived paracrine factors, such as angiotensin-1, basic fibroblast growth factor (bFGF), HGF, IGF-1, platelet-derived growth factor (PDGF), stem cell factor (SCF), SDF-1, and VEGF are known to possess cardioprotective properties and are able to promote neovascularization and recruitment. Growing evidence indicates CPCs possess a distinct ability to secrete paracrine factors but contrary findings support transdifferentiation ability of CPCs. Therefore, to delineate the specific contribution of direct regeneration versus paracrine effects, a detailed comparative analysis of transplanted human CDCs was done by Chimenti et al. CDCs produce several cytokines and growth factors, including high levels of VEGF, HGF, and IGF-1. Based on the findings, authors concluded that beneficial effect of CDCs on cardiac function was in large because of their ability to release growth factors and cytokines at the site of injury that possibly activates endogenous cardiac repair mechanisms.

Extracellular vesicles including exosomes have emerged recently as the key component within the stem cell secretome. CPC’s ability to secrete exosomes was validated in the study by Chen et al in a mouse model of myocardial ischemia/reperfusion (I/R) injury. Authors showed that CPC-derived exosomes enriched in miR-451/144 exerted significant cardioprotective effects by promoting H9c2 survival in vitro and cardiomyocyte in vivo, whereas cardiomyocyte progenitor cell-derived exosomes possess ability to enhance endothelial cell migration via extracellular matrix metalloproteinase inducer (EMMPRIN), a membrane-bound matrix metalloproteinase activator. In another study, Barile et al reported that extracellular vesicles from human cardiac progenitors exhibit cardioprotective properties, miRNA analysis of extracellular vesicles derived from CPCs indicated high levels of miR-210, miR-132, and miR-146a-3p mediating antiapoptotic and proangiogenic properties via activation of their downstream targets, such as ephrin A3, PTP1b, and RasGAP-p120, ultimately leading to augmented cardiac function after delivery in a myocardial infarction model. Physiological stem cell state can modulate secretion of paracrine factors, and in particular, normoxic conditions have been associated with reduced stem cell repair capability. Because hypoxia preconditioning can enhance CPC therapy, whether there is a similar effect on exosome secretion was the focus of a separate study that aimed to characterize the effect of hypoxia on CPC exosomes. Authors show that CPC exosomes released under normoxic conditions possess diminished reparative potential. In contrast, hypoxia primes CPCs to produce exosomes laden with several miRs, including miR-17, 199a, 210, and 292, and augments exosome ability to repair the injured heart. Recently, exosomes were proposed to be responsible for mediating the cardioprotective effects of gene and cell therapy combinatorial approach. Co-delivery of CPCs with minicircle plasmid carrying HIF1 led to increase in survival of the transplanted CPCs and improvement of cardiac function. Mechanistic studies revealed targeting of minicircle plasmid carrying HIF1 to cardiac endothelial cells, promoting exosome release that are subsequently up-taken by CPCs. Moreover, these exosome are packed with miR-126 and miR-210, and their accumulation in CPC promotes subsequent changes in CPC biological properties.

Modulation of Cardiac Repair Response by Exosomes

Survival and Neovascularization

Cardiomyocytes are typically not considered to be secretory cells but have been known to release cytokines and growth factors, such as atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), TGF-β, TNF-α, and microvesicles that are sometimes referred as cardiosomes. Characterization of cardiac myocyte exosomes revealed enrichment of heat shock protein (hsp60), that when released into the extracellular space induces cardiomyocyte apoptosis via activation of Toll-like receptors. Yu et al showed that hypoxic cardiomyocytes release exosomes loaded with TNF-α that could trigger cell death in other cardiomyocytes. Nevertheless,
Exosomal content of cardiomyocytes is highly dependent on the physical environment and the type of stimulus. Giricz et al and other researchers have shown that ischemic preconditioned hearts promote exosome release and help spread cardioprotective signals within the myocardium. On the contrary, fibroblast secretome carries many adverse effects on cardiomyocytes. Co-culture of fibroblasts with cardiomyocytes or just treatment with fibroblast-conditioned medium led to development of cardiomyocyte hypertrophy. In another study, changes in cardiomyocyte cell size were attributed to exosomal transfer of miR-21*, a passenger strand star miRNA that normally undergoes intracellular degradation, released by cardiac fibroblast in response to hypertrophic insult.

Exosomes from adoptively transferred stem cells have also been implicated to extend prosurvival effects onto the heart. Wang et al demonstrated a cardioprotective role for iPS-derived exosome facilitated via transfer of miR-21 and miR-210, promoting cardiomyocyte survival in response to injury. Authors showed that iPS-exo protect H9C2 cells against H2O2-induced oxidative stress by inhibiting caspase 3/7 activation. Furthermore, iPS-exo treatment in vivo resulted in significant reduction of TUNEL+/cardiac troponin I + apoptotic cardiomyocyte apoptosis 24 hours after I/R injury compared with the control group. In one study, human ESC-derived mesenchymal stem cells were shown to release exosomes, and their delivery into ischemic heart resulted in significant reduction of infarct size. Arslan et al demonstrated the therapeutic efficacy of MSC exosomes in a myocardial I/R model with infarct size reduced by 45% and significant improvement in cardiomyocyte survival. Similarly, CPC-derived exosomes promoted endothelial cell migration parallel with 53% reduction in cardiomyocyte apoptosis in a mouse model for acute I/R. Recently, it was shown that ischemic preconditioning of MSCs enhanced levels of miR-21, miR-22, miR-199a-3p, miR-210, and miR-24 in exosomes released by the cells, and administration of MSC–ischemic preconditioning exosomes resulted in reduction of cardiac fibrosis and apoptosis compared with the hearts treated with control exosomes. Importantly, the antiapoptotic effect of MSC exosomes was tied to miR-21–mediated targeting of methyl CpG-binding protein 2. Genetic modification of MSC with GATA-4 proved to be a novel strategy to increase exosome efficacy for cardiac repair and was associated with increased cardiomyocyte survival, reduced apoptosis, and enhanced cardiac contractile function in mice subjected to myocardial infarction. This antiapoptotic effect of GATA-4 MSC exosomes was attributed to enrichment of miR-19a that targets phosphatase and tensin homologue (PTEN), leading to activation and AKT and ERK signaling pathways. In contrast, extracellular vesicles comprising of both microvesicles and exosomes derived from MSC were shown to promote neangiogenesis and preserve cardiac performance in a mouse model for myocardial infarction. Other stem cell–derived exosomes, such as EPC exosomes, possess the ability to modulate cardiomyocyte survival and confer protection against angiotensin II-induced hypertrophy by activating PI3K/Akt/eNOS pathways via RNA enriched within the exosomes. Similarly, genetic manipulation of CD34+ cells with sonic hedgehog not only led to increased ability of the cells to enhance cardiac function after myocardial infarction but interestingly, sonic hedgehog overexpressing CD34 cells were able to produce exosomes loaded with sonic hedgehog protein and was delivered to various cardiac cells providing mechanistic basis for the increased cardiac function. Recently, the cardioprotective ability of exosomes was assessed in samples from rats and human health volunteers. Authors demonstrated that exosomes can deliver endogenous cardioprotective signals to the heart activating toll-like receptor–mediated Hsp70–dependent mechanism in cardiomyocytes promoting their survival against I/R injury.

Proliferation and Cell Cycle Progression

The renowned cardiomyocyte incapacity to replicate has prompted researchers over the years to devise therapies that not only enhance other features of the cardiac regenerative machinery but also have additional effects on reactivating cell division in spared cardiomyocyte after injury. Recently, exosomes in the heart have been proposed as one of the mechanisms responsible for regulating cell cycle and proliferation in the target cells. In a model for diabetic cardiomyopathy, Wang et al showed that coculture of diabetic cardiomyocytes with endothelial cells (EC) significantly inhibited EC proliferation and migration compared with coculture with normal cardiomyocytes. Further analysis revealed that diabetic cardiomyocytes release exosomes packaged with miR-320 as well as low levels of miR-216 and Hsp20 proteins. In particular, transfer of miR-320 to endothelial cells leads to downregulation of IGF-1, Hsp20, and Ets-2 signaling subsequently blunting proliferation. A similar role has been suggested for stem cell–derived exosomes in proliferating response of the injured heart tissue. Lee et al showed that MSC-derived exosomes have the ability to inhibit hyper-proliferative signals by suppression of STAT-3 phosphorylation in a murine model for pulmonary hypertension. Recently, CDC exosome administration in the heart after myocardial infarction resulted in reduction of cardiomyocyte apoptosis in conjunction with cardiomyocyte proliferation and increased angiogenesis. The salutary effects of CDC exosomes were linked with miR-146a that was enriched within the exosomes and recapitulated the effects observed with exosomes delivery in the heart.

Our work with ESC-derived exosomes has revealed a unique ability of the exosomes to instigate a cardiac proliferative response. ESCs have the ability to produce exosomes ≈40 nm in size, display exosomal protein flotillin-1, and are enriched in pluripotent mRNA transcripts (Oct-4, Sox-2, Nanog) and interestingly the miRNA-290 cluster. ESC exosomes were able to promote survival in response to oxidative stress and enhance tube formation ability in different cell types via transfer of the exosomal contents. Intramyocardial delivery of ESC exosomes to the heart after myocardial infarction resulted in significant augmentation of cardiac function because of enhanced angiogenesis and activation of the cardiac proliferative response. Furthermore, ESC exosome–administered hearts showed elevated cardiomyocyte proliferation and increase in the number of endogenous c-kit+ CPCs in the heart after myocardial infarction. Ex vivo manipulation of CPCs with ESC exosomes led to a substantial increase in cellular function in vitro and the CPC cardiac repair potential after injury in vivo. This proproliferative effect was mediated by...
transfer of miRs specific to ESCs commonly referred as ESC cycle miRNAs. In particular, we demonstrated that miR-294 present exclusively in the ESC exosomes enhanced CPC cell cycle progression and cardiomyocyte histone-3 phosphorylation in the pathological heart.

**Epigenetic Modulation and Cardiac Commitment**

Exosomes possess ability to epigenetically modulate target cells as shown by the study conducted by Waldenstrom et al. Recently, it was shown that exosomes from immature different cell types, including those that make up the immune system, promote macrophage switch toward M2 anti-inflammatory processes part of the cardiac immune response mediated via release of extracellular vesicles.128

Inflammation and Immune Regulation

Cardiac adaptation to pathological stress is largely dependent on remodeling of the cardiac tissue and subsequent activation of fibrotic processes. Accumulating evidence suggests an important role for exosomes in intracardiac communication mediating inflammation, angiogenesis, and cardiomyocyte survival. The inflammatory process is a complicated symphony of many different cell types, including those that make up the immune response. Interestingly, almost all cells of the immune system release exosomes that mediate downstream cellular responses.129,30 Recently, it was shown that exosomes from immature dendritic cells and regulatory T cells can reduce the risk of cardiac allograft rejection, suggesting a possible immunosuppressive role for exosomes.129 Similarly, exosomes produced by macrophages, T cells, and dendritic cells possess the ability to modulate inflammatory processes part of the cardiac immune response after myocardial infarction.126 Microvesicles are known to increase in peripheral blood during inflammation and thrombotic complications that occur during various cardiovascular pathologies.127 Under such conditions, these microvesicles are able to modify inflammatory and immune responses by carrying and delivering receptors, adhesion molecules, and other mediators of inflammation that can ultimately shift the balance of inflammatory process in the heart. Several reports indicate that microvesicles are a source of transcription factors that initiate coagulation, alter endothelial redox balance, and provide phosphatidylinerine, thereby promoting thrombosis and affecting patients with cardiovascular disease.127

In contrast, several studies provide evidence that paracrine factors released by adaptively transferred stem cells at the site of injury can influence inflammatory and immune cell responses.129,129 MSCs have been the most well characterized stem cell type for their role in modulating various components of cardiac immune response mediated via release of extracellular factors. MSC paracrine factors, including microvesicles, increase neutrophil life span via interleukin-6-dependent mechanism,129 promote macrophage switch toward M2 anti-inflammatory phenotype,130 and strongly affect T-cell response (Table).128

**Limitations and Future Perspectives**

Exosome therapy may represent a multifaceted strategy for promoting regeneration and repair in the heart after pathological damage (Figure 2). However, recent excitement over these powerful cell-free components of stem cells must be channeled into meaningful research efforts toward fully understanding the role of these microvesicles in cardiac biology. Recent studies indicate that benefits of cell therapy are largely dependent on transfer of paracrine effectors at the site of injury.39,129 Because exosomes form a significant part of the secreted stem cell fraction, they represent a potential source for cardiac regenerative therapies. Nevertheless, cellular uptake of exosome is quick, resulting in rapid dissemination of the vesicular contents to the target cells. Therefore, an important area for consideration is how long the beneficial effects of exosome therapy last in the heart after delivery? Because of their short half-life, most of the studies using stem cell exosomes implicate exosome administration with activation of the endogenous cardiac repair response as the main reason behind augmented cardiac function after injury.122,123 Delivery of miRNAs, proteins, and miRNAs to cardiac cells, including cardiomyocytes and resident cardiac stem cells, may promote their survival, proliferation, and generation, yet long-term revival of cardiac repair processes may require multiple injections of exosome in the heart. Commonly used routes for exosome delivery include direct intramyocardial injection, increasing cardiac localization and possible a cell type–dependent response of cells that receive the exosomes. In contrast, systemic administration via intravenous infusion affords the provision for repeated exosome dosage but may carry the risk of off-target effects in other organs besides the heart. Similarly, it has been shown that intracoronary transfer of stem cells results in migration of the cells across the vessel barrier and into the neighboring myocardium,132 providing the rationale for a similar delivery method for exosomes in the future. Optimal exosome-based treatment would warrant tailored exosomes designed for specific cell types within an organ to minimize adverse effects, but this approach has not been tested yet and certainly represents a limitation. Recently, it has been shown that systematically delivered exosomes can be directed to deliver their content exclusively to the brain, laying down foundation for the development of a similar strategy for the heart.

Another area that needs a careful investigation stems from the realization that all exosomes, even when derived from a defined cellular source, are not created equal, and the constitution of their contents is largely dependent on physiological state of the parental cell of origin. It is well-established that stem cells obtained from animals and patients with physiological stresses, such as age, diabetes mellitus, and systemic inflammation have vastly reduced reparative activity. Because a large number of patients with cardiac diseases present these symptoms, it is reasonable to predict that stem cell exosomes from these patients may similarly have reduced activity or may potentially aggravate the negative response by delivering altered protein/miR contents to already compromised ischemic tissue. Indeed our ongoing studies (unpublished results) point toward this possibility. Therefore, careful investigation of exosomal biology and function when stem cells are exposed to different stress conditions would be required before their clinical applications. Another related question that requires attention is to determine whether exosome contents are evenly distributed among every exosome produced by the parent...
### Table. Summary of Stem Cell–Derived Exosomes, Their Contents, and Downstream Effects

<table>
<thead>
<tr>
<th>Type</th>
<th>Content</th>
<th>Model</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mesenchymal stromal cells exosomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD81</td>
<td>Human MSCs</td>
<td>Detailed proteomic characterization(^{33,34})</td>
<td></td>
</tr>
<tr>
<td>CD29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD105</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAC1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRKCB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP2R1A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wnt (RAC1, PRKCB, PP2R1A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-B (COL1A2, ENG)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK (FLNA, HSPA8, EGFR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10, TGF-B</td>
<td>Lymphocytes</td>
<td>Anti-inflammatory(^{40})</td>
<td></td>
</tr>
<tr>
<td>miR-16</td>
<td>Breast cancer cells</td>
<td>Tumor progression(^{90})</td>
<td></td>
</tr>
</tbody>
</table>
| miR-133b, Neprilepsin                   | Brain            | Neurite outgrowth,\(^{90}\) 
|                                        |                  | \(\beta\)-amyloid peptides\(^{91}\) |
| miR-21, miR-22, miR-199a-3p, miR-210, Heart |                  | Cardiac fibrosis\(^{92}\) ↓ |
| miR-24                                  | Heart            | Cardiomyocyte survival\(^{102}\) ↑ |
| STAT-3 phosphorylation                  | Pulmonary hypertension | Hyper-proliferation\(^{103}\) ↓ |
| RNA PI3K/AKT/eNOS                       | Heart            | Neovascularization\(^{110}\) ↑ |
| miR-126, miR-130a                       | Heart            | Survival\(^{112}\) ↑ |
| Sonic hedgehog                          | Heart            | Neovascularization ↑ |
| ICAM-1                                  | Endothelial cells | Angiogenesis\(^{115}\) ↑ |
| \(\alpha4\)-integrin                    |                  |                              |                                        |
| CD29 (\(\beta1\)-integrin)              |                  |                              |                                        |
| mRNA (BCL-XL, CFL1, CTNNB1, EDF1, MAPKAPK2, PTPRT, eNOS) |                  |                              |                                        |
| miR-126, miR-296                        | Pancreas         | Neovascularization\(^{114,115}\) ↑ |
| mRNA (PI3K/eNOS/N0)                     | Brain microvascular endothelial cells | Survival\(^{116}\) ↑ |
| miR-214                                 | Ataxia telangiectasia | Angiogenesis\(^{117}\) ↑ |
| **Endothelial Progenitor cells exosomes** |                  |                              |                                        |
| RNA PI3K/AKT/eNOS                       | Heart            | Hypertrophy\(^{118}\) ↓ |
| miR-126, miR-130a                       | Heart            | Neovascularization\(^{119}\) ↑ |
| Sonic hedgehog                          | Heart            | Survival\(^{120}\) ↑ |
| ICAM-1                                  | Endothelial cells | Angiogenesis ↑ |
| \(\alpha4\)-integrin                    |                  |                              |                                        |
| CD29 (\(\beta1\)-integrin)              |                  |                              |                                        |
| mRNA (BCL-XL, CFL1, CTNNB1, EDF1, MAPKAPK2, PTPRT, eNOS) |                  |                              |                                        |
| miR-126, miR-296                        | Pancreas         | Neovascularization\(^{114,115}\) ↑ |
| mRNA (PI3K/eNOS/N0)                     | Brain microvascular endothelial cells | Survival\(^{116}\) ↑ |
| miR-214                                 | Ataxia telangiectasia | Angiogenesis\(^{117}\) ↑ |
| **Cardiac derived stem cells exosomes** |                  |                              |                                        |
| Chemokines (TCA-3, SDF-1, 6Ckine)       | Hypoxic preconditioning | Recruitment\(^{124}\) ↑ |
| Vascular growth factors (VEGF, Erythropoietin, bFGF, osteopontin, SCF) |                  |                              |                                        |
| Cardiac differentiation factors (Activin A, Dkk homolog-1, TGF-\(\beta\)) |                  |                              |                                        |
| Ang-1, bFGF, HGF, IGF-1, PDGF, SCF, SDF- Heart 1, VEGF |                  |                              |                                        |
| miR-451/144                             | Heart            | Survival\(^{125}\) ↑ |
| EMMPRIN                                 | Endothelial cells | Migration\(^{126}\) ↑ |
| miR-146a                                | Heart            | Breakdown of ECM ↑ |
| miR-210, miR-132                        | Heart            | Cardiomyocyte survival\(^{127}\) ↑ |
| miR-17, 199a, 210, 292                   | Hypoxic stimulation | Apoptosis ↓ Angiogenesis\(^{128}\) ↑ |
| miR-17, 199a, 210, 292                   | Hypoxic stimulation | Antifibrotic, Angiogenesis\(^{129}\) ↑ |

ICAM-1 indicates intracellular adhesion molecular; IL, interleukin; and MSC, mesenchymal stromal cells.
The content packaging may largely be dependent on the physiological state of the cell, leading to differential enrichment of various proteins, mRNAs, and miRNAs in the exosomes. Moreover, the consequences of having a variable payload on cardiac signaling pathways must be thoroughly assessed because inconsistency in exosome content may lead to off-target effects because of aberrant packaging of proteins that the cell deems unnecessary or garbage. Similarly, a large number of studies implicate miRNAs delivery as the primary mode of action for exosomes. This association was established by treating the parent cell with a specific antagoniR, consequently, knocking the miR out in the exosome but the treatment may alter cellular characteristics. Alternatively, cells treated with inhibitors blunting exosome production can also lead to reciprocal decrease in the exosome miRs after administration.122 In this respect, studies conducted recently have proposed methodologies and procedures for efficient in vivo imaging and monitoring of extracellular vesicles trafficking.135,136 Therefore, a careful validation of exosomal content transfer is required to correlate the observed molecular effects to the exosomal cargo.

Because exosomes are distributed and enriched in bodily fluids, including blood, plasma, serum, semen, they potentially provide a window of opportunity to serve as biomarkers of disease existence or progression, including their distribution and content before and after stem cell therapy. Indeed, it is reported that routine examination of serum from patients with different malignant disorders reveals high expression of extracellular vesicles that display malignancy-related disorders.133 Similarly, cardiac hypertrophy has been associated with release of exosome from the heart that carry functional angiotensin II type I receptor.137

Figure 2. Stem cell–derived exosomes for cardiac repair. Exosome derived from different types of stem cells, including embryonic stem cells (ESC), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), cardiac stem cells (CSCs), and endothelial progenitor cells (EPCs) carry and deliver message RNAs (mRNAs), microRNAs (miRNAs), and proteins to the damaged heart tissue consequently augmenting resident cardiac stem cell activation/expansion, cardiomyocyte proliferation, neovascularization, and modulation of cardiac inflammatory response (Illustration credit: Ben Smith).


26. Haider HKb, Jiang S, Idris NM, Ashraf M. IGF-I-overexpressing mesenchymal stem cells accelerate bone marrow stem cell...
mobilization via paracrine activation of SDF-1 alpha/CXCR4 signaling to promote myocardial repair. Circ Res. 2008;103:1300–1308. doi: 10.1161/
CIRCRESAHA.108.186742.

dow DW. Exosomes from human CD34(+) stem cells mediate their pro-
CIRCRESAHA.111.253286.

28. Pan BT, Johnston RM. Fate of the transferrin receptor during maturation of shear reticulocytes in vitro: selective externalization of the recep-

nr11567.

MZ. Embryonic stem cell-derived microvesicles reprogram hematopoietic
genitors: evidence for horizontal transfer of mRNA and protein deliver-


lished murine tumors using a novel cell-free vaccine: dendritic cell-de-


37. Stein JM, Luzio JP. Exocytosis caused by sublytic autologous comple-
ment attack on human neutrophils. The sorting of endogenous plas-


42. Squadrito ML, Baer C, Arsenault F, Mardenza C, Giffilin GD, Lyle R, Ihlebahn M, De Palma M. Endogenous RNAs modulate microRNA sort-


44. Vila-Ruiz Beltrá C, Gutiérrez-Vázquez C, Sánchez-Cabero F, Pérez-Hernández D, Vázquez J, Martín-Cofreces N, Martín-Herrera DJ, Pascual-


ing to the dendritic cell-T-cell infectious synapse uses a pathway of tet-

50. Von Barthel CD, Atlick AL. Multivesicular bodies in neurons: dist-

ficient induction of HLA-A*0201-restricted and carcinoembryonic an-


A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse.


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