

Stem cell-derived exosomes as a therapeutic tool for cardiovascular disease

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Abstract

Mesenchymal stem cells (MSCs) have been used to treat patients suffering from acute myocardial infarction (AMI) and subsequent heart failure. Although it was originally assumed that MSCs differentiated into heart cells such as cardiomyocytes, recent evidence suggests that the differentiation capacity of MSCs is minimal and that injected MSCs restore cardiac function *via* the secretion of paracrine factors. MSCs secrete paracrine factors in not only naked forms but also membrane vesicles including exosomes containing bioactive substances such as proteins, messenger RNAs, and microRNAs. Although the details remain unclear, these bioactive molecules are selectively sorted in exosomes that are then released from donor cells in a regulated manner. Furthermore, exosomes are specifically internalized by recipient cells *via* ligand-receptor interactions. Thus, exosomes are promising natural vehicles that stably and specifically transport bioactive molecules to recipient cells. Indeed, stem cell-derived exosomes have been successfully used to treat cardiovascular disease (CVD), such as AMI, stroke, and pulmonary hypertension, in animal models, and their efficacy has been demonstrated. Therefore, exosome administration may be a promising strategy for the treatment of CVD. Furthermore, modifications of exosomal contents may enhance their therapeutic effects. Future clinical studies are required to confirm the efficacy of exosome treatment for CVD.

Key words: Exosomes; Messenger RNA; Cardiovascular disease; Mesenchymal stem cells; Stem cells; MicroRNA

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Core tip: Exosomes are membrane vesicles that contain and transport specific bioactive molecules, such as proteins, messenger RNAs, and microRNAs, to recipient cells. In this review, we describe the mechanisms of

exosome biogenesis, selective sorting of bioactive molecules into exosomes, and exosome secretion. We also discuss preclinical studies in which stem cell-derived exosomes were successfully used to treat cardiovascular disease (CVD). Finally, we discuss the future possibility of exosome-based clinical treatment of CVD.

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INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide. Owing to recent advances in the treatment of acute myocardial infarction (AMI) using percutaneous coronary intervention or bypass surgery, the survival of patients with AMI has substantially improved. However, many of these survivors develop heart failure (HF) as a result of the death of cardiomyocytes and subsequent tissue remodeling. As the induction of the proliferation and differentiation of the remaining cardiac tissue to regenerate heart structure remains challenging, heart transplantation is still the only treatment option for fatal HF. The development of new therapies for AMI and HF is thus required to improve the outcome in these patients.

Recently, many attempts have been made to improve the outcome of AMI and ischemic HF (IHF) using stem cells in preclinical^[1-4] and clinical^[5-10] studies. Among of the various stem cells, mesenchymal stem cells (MSCs), particularly bone marrow-derived MSCs, have been used to treat patients with AMI and IHF in clinical trials, with their safety and efficacy demonstrated in some studies^[5-10]. The earliest preclinical studies suggested that MSCs have the potential to differentiate into multiple cardiac cell types including cardiomyocytes, vascular endothelial cells, and vascular smooth muscle cells^[1-3]. However, subsequent studies did not demonstrate this remarkable differentiation capacity of MSCs. Rather, it was reported that most intravenously injected cells are trapped in the lung rather than engrafted in the heart^[11,12]. Even when MSCs are administered to the swine heart *via* the coronary artery following AMI induction, only 6% of the injected cells remained in the infarct zones 14 d after AMI induction^[11]. Furthermore, the supernatant of MSC cultures reportedly improves cardiac function^[13-15]. These results suggest that MSCs improve cardiac function *via* the secretion of paracrine factors rather than *via* the direct differentiation of MSCs into cardiac cell types. Furthermore, MSC transplantation has several problems such as low survival rate and stem cell tumorigenesis^[16]. However, if MSC-secreted paracrine factors can efficiently repair and regenerate cardiac tissues, cell-free therapy is possibly a safer alternative in

the future.

Recently, a variety of cell types, including stem cells, have been shown to secrete paracrine factors in not only naked forms but also membrane vesicles, such as exosomes, microvesicles, ectosomes, membrane particles, exosome-like vesicles, and apoptotic bodies^[17]. Exosomes are one of the secreted vesicles (also referred to as extracellular vesicles or EVs) that are 30-100 nm in diameter and contain a variety of biologically active molecules, such as proteins, messenger RNAs (mRNAs), and microRNAs (miRNAs)^[18]. In this manuscript, we review the characteristics of exosomes and their possible applications in CVD treatment.

EXOSOME ISOLATION AND IDENTIFICATION

Several strategies have been used to isolate exosomes from tissues. These strategies utilize ultracentrifugation, size-based purification, precipitation using polymers, and immunoaffinity purification as reviewed in some reports^[19-21]. Ultracentrifugation is the most established method of exosome isolation which employs sequential centrifugation combined with sucrose density gradient ultracentrifugation. Size-based purification includes ultrafiltration and gel filtration methods. Alternatively, polymers such as polyethylene glycol, widely used to precipitate proteins and viruses, can also be used to precipitate exosomes. As exosomes express specific proteins and lipids on their surface, antibodies recognizing these molecules (frequently conjugated with magnetic beads) are also used in their isolation.

Identification of exosomes is usually achieved by evaluating their morphology and size, their motion in a solution, and the specific molecules they express, as previously reviewed^[22,23]. Electron microscopy is commonly employed to measure the size and assess the morphology of exosomes. The number of particles corresponding to exosome size can be counted by nanoparticle tracking analysis. This method utilizes the phenomenon of Brownian motion in a liquid suspension to measure particle size. Because exosomes are derived from endosomes and are finally released from cells as described in the following section, molecules involved in exosome formation, such as tetraspanins (CD81, CD9, and CD63), are expressed in exosomes. These markers can be used to identify exosomes.

EXOSOME BIOGENESIS, SECRETION, AND UPTAKE BY RECIPIENT CELLS

Exosomes are derived from endosomes that are formed by the inward budding of the plasma membrane (Figure 1)^[18]. The subsequent inward budding of the endosomal membrane results in the formation of intraluminal vesicles (ILVs) into which cytoplasmic molecules, such as proteins, mRNAs, and miRNAs are sorted^[24,25]. These endosomes containing ILVs, or multivesicular bodies

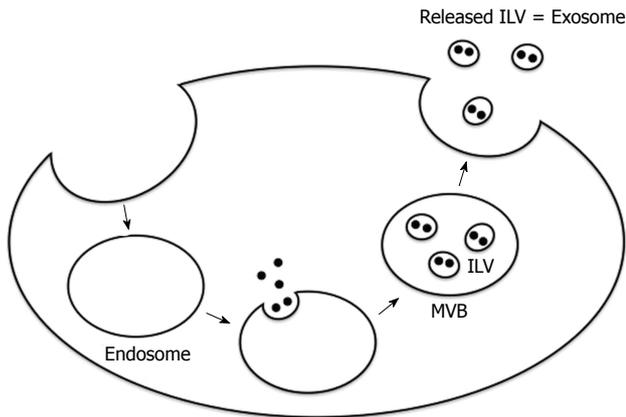


Figure 1 Schematic diagram showing exosome biogenesis and release. ILV: Intraluminal vesicle; MVB: Multivesicular body.

(MVBs)^[18], fuse with the plasma membrane and release ILVs into the extracellular environment by exocytosis. These secreted ILVs containing biologically active molecules are referred to as exosomes.

The mechanisms of exosome formation and processing are just starting to be revealed. The formation of MVBs is reportedly mediated by the endosomal sorting complexes required for transport (ESCRT) system or by systems independent of the ESCRT machinery as summarized in some reviews^[26-28]. The ESCRT machinery comprises four protein complexes, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, together with accessory proteins. ESCRT-0 recognizes ubiquitinated proteins and is recruited to the endosomal membrane, where it initiates processes leading to the uptake of ubiquitinated proteins into ILVs. ESCRT-0 subsequently recruits ESCRT-I to the endosomal membrane, which in turn recruits ESCRT-II and ESCRT-III. ESCRT-III induces the inward budding of the endosomal membrane and formation of ILVs, while accessory proteins (particularly the vacuole protein sorting gene 4 ATPase or VPS4) are implicated in the dissociation and recycling of the ESCRT machinery. In addition, other molecular pathways mediate ESCRT-independent MVB formation including tetraspanins^[29] such as CD81, CD9, and CD63, and proteolipid proteins such as ceramide^[30].

The docking and fusion of MVBs to the plasma membrane appear to be mediated by soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins such as vesicle-associated membrane protein 7 (VAMP7)^[31]. The release of ILVs (exosomes) from cells following the fusion of MVBs to the plasma membrane is mediated by several mechanisms. The small GTPases of the Rab family (Rab27a/b, Rab11, and Rab35) are the most studied molecules involved in exosome release^[32-34]. Other pathways include WNT5A, glycosphingolipids, flotillins, and stress-induced stimuli such as the increase in intracellular calcium concentration, DNA damage, heat shock, and hypoxia^[35-39]. In addition, an acidic environment has been shown to trigger the secretion of exosomes from cells^[40].

Once released from cells, exosomes bind to target cells *via* ligand-receptor interactions. Molecules, such as integrins, intercellular adhesion molecules, and tetraspanins seem to be implicated in the binding of exosomes to recipient cells^[41-43]. After binding, exosomal contents are reportedly internalized by recipient cells *via* two major mechanisms as summarized in some reviews^[23,44]: (1) exosome fusion with the plasma membrane of recipient cells and direct release of contents into the cytoplasm; or (2) internalization by endocytosis into recipient cells. It has been demonstrated that bioactive molecules in exosomes are not only transferred to recipient cells but also exert functional effects^[45-47].

Although the precise mechanism remains unknown, a specific set of proteins, mRNAs, and miRs are selectively accumulated within exosomes^[48]. It has also been demonstrated that exosomes contain a distinct set of mRNAs compared to the donor cells^[49]. Ubiquitination appears to be required for the uptake of some proteins into exosomes^[50], although ubiquitination-independent accumulation of proteins has also been reported^[51]. The accumulation of miRs into the exosomes of T cells appears to require the recognition of a GGAG sequence located in miRs by the heterogeneous nuclear ribonucleoprotein hnRNP A2B1^[49].

Taken together, accumulating evidence indicates that exosomes are a natural vehicle for the efficient and specific transport of biologically active cargo into recipient cells. These properties may be exploited for the delivery of bioactive molecule such as miRs and chemical compounds such as drugs. For instance, stem cell-derived exosomes may be useful for CVD treatment. We review the potential utility of stem cell-derived exosomes for CVD treatment in the following section.

THERAPEUTIC EFFECTS OF STEM CELL-DERIVED EXOSOMES ON CVD

MSC-derived exosomes

Several preclinical studies have demonstrated the efficacy of MSC-derived exosomes for CVD treatment (Table 1). Lai *et al.*^[52] found that the supernatant of human embryonic stem cell (ESC)-derived MSCs contained small particles (50-100 nm in diameter) corresponding to exosomes. When administered to a mouse model of myocardial ischemia/reperfusion injury, these exosomes remarkably reduced infarct size. The same group also administered exosomes secreted from human ESC-derived MSCs to a mouse model of AMI and demonstrated improved cardiac function^[53]. In addition, they found that the tissue levels of ATP and nicotinamide adenine dinucleotide were significantly increased, while those of reactive oxygen species were significantly decreased after exosome administration. Furthermore, they demonstrated that the phosphorylation of Akt and glycogen synthase kinase 3 (that has anti-apoptotic effects) significantly increased and that of c-jun N-terminal kinase (that has proapoptotic

Table 1 Effects of exosome administration on cardiovascular disease models

Origin of exosomes	Experimental model	Findings	Ref.
Human ESC-derived MSCs	AMI	Reduction in infarct size Recovery of cardiac function Decreased oxidative stress Activation of Akt and GSK3 Inhibition of c-JNK	Lai <i>et al</i> ^[52,53]
Human MSCs	AMI	Reduction in infarct size Recovery of cardiac function Increased angiogenesis	Bian <i>et al</i> ^[54]
Mouse MSCs	AMI	Exosomes were enriched in miR-22 miR22 was implicated in the anti-apoptotic effect of exosomes	Feng <i>et al</i> ^[55]
Rat MSCs overexpressing GATA-4	AMI	Reduction in infarct size Recovery of cardiac function Exosomes were enriched in miR-19a	Yu <i>et al</i> ^[56]
Rat MSCs	Stroke	Recovery of neurological function Stimulation of neurogenesis and angiogenesis	Xin <i>et al</i> ^[57]
Rat MSCs overexpressing miR-133b and those whose expression of miR-133b was knocked down	Stroke	Recovery of neurological function was mediated by miR-133b expressed in exosomes	Xin <i>et al</i> ^[58]
Mouse MSCs	Pulmonary hypertension	Reduction in the progression of pulmonary hypertension and right ventricular hypertrophy	Lee <i>et al</i> ^[59]
Mouse CPCs	AMI	Suppression of apoptosis	Chen <i>et al</i> ^[60]
Human CPCs	AMI	Recovery of cardiac function Suppression of apoptosis Stimulation of angiogenesis	Barile <i>et al</i> ^[61]
Human CPCs	AMI	Recovery of cardiac function Suppression of apoptosis Stimulation of angiogenesis miR-146a was enriched in exosomes and partially mediated their function	Ibrahim <i>et al</i> ^[62]
Mouse ESCs	AMI	Recovery of cardiac function Stimulation of angiogenesis and cardiomyocyte survival Stimulation of the survival and proliferation of CPCs miR-294 was enriched in exosomes and miR-294 promoted the survival and proliferation of CPCs	Khan <i>et al</i> ^[63]
Human CD34+ cells	Matrigel plug assay Corneal angiogenesis assay	Promotion of angiogenesis	Sahoo <i>et al</i> ^[64]
Human CD34+ cells expressing SHH	AMI	Recovery of cardiac function SHH was enriched in exosomes and transferred to recipient cells	Mackie <i>et al</i> ^[66]

ESC: Embryonic stem cell; MSCs: Mesenchymal stem cells; CPCs: Cardiac progenitor cells; SHH: Sonic hedgehog; AMI: Acute myocardial infarction; GSK3: Glycogen synthase kinase 3; c-JNK: c-jun N-terminal kinase.

effects) significantly decreased in cardiac tissue following exosome administration. Bian *et al*^[54] demonstrated the proliferation and migration of human umbilical vein endothelial cells in response to EVs (100 nm in diameter) collected from human MSCs. They also administered MSC-derived EVs to a rat model of AMI and showed that MSC-derived EV administration significantly reduced infarct size, restored cardiac function, and stimulated angiogenesis in the ischemic zone. Feng *et al*^[55] demonstrated that exosomes secreted from mouse MSCs following ischemic preconditioning contained a large amount of miR-22. When administered to mice with AMI, these miR-22-enriched exosomes exerted an anti-apoptotic effect on cardiomyocytes *via* the downregulation of methyl-CpG-binding protein 2. Yu *et al*^[56] used MSCs overexpressing the transcription factor GATA-4 (MSC_GATA-4) and demonstrated that the administration of MSC_GATA-4-derived exosomes restored cardiac function and reduced infarct size in a rat model of AMI. The authors also

showed that MSC_GATA-4-derived exosomes expressed a greater amount of miRs, particularly miR-19a, than control MSCs and that miR-19a appeared to be involved in the cardioprotective effect of MSC_GATA-4-derived exosomes *via* the downregulation of phosphatase and tensin homolog (PTEN) and subsequent activation of anti-apoptotic Akt and extracellular signal-regulated kinase.

Preclinical studies have also reported favorable effects of exosome administration on neurological recovery following stroke induction. Xin *et al*^[57] found that the systemic administration of rat MSC-derived exosomes following the induction of stroke by the ligation of the middle cerebral artery significantly accelerated neurological recovery and stimulated neurogenesis and angiogenesis at the border zone between normal and ischemic tissues. The same group also demonstrated that the administration of MSCs overexpressing miR-133b (MSCs_miR-133b+) enhanced the recovery of neurological function in a rat stroke model whereas MSCs

with miR-133b knockdown (MSCs_miR-133b-) did not^[58]. Furthermore, they showed that the level of miR-133b in exosomes isolated from cerebrospinal fluid was higher in the group that received MSCs_miR-133b+. They also demonstrated that MSC-derived exosomes could be transferred to neighboring cells. Finally, they showed that the expression of connective tissue growth factor (CTGF), a target for miR-133b, was significantly reduced in the ischemic boundary zone following MSCs_miR-133b+ administration, while CTGF expression remained unchanged after MSCs_miR-133b- administration. They concluded that miR-133b derived from exosomes was implicated in MSC-mediated recovery of neurological function in this model.

The beneficial effects of MSC-derived exosome administration have also been reported in a mouse model of hypoxic pulmonary hypertension. Lee *et al.*^[59] demonstrated that the administration of MSC-derived exosomes significantly ameliorated the progression of pulmonary hypertension and right ventricular hypertrophy, possibly *via* the suppression of signal transducer and activator of transcription 3 (STAT3).

Cardiac progenitor cell-derived exosomes

Chen *et al.*^[60] demonstrated that the injection of exosomes isolated from murine cardiac progenitor cells (CPCs) into the murine heart following ischemia/reperfusion injury significantly suppressed apoptosis. Barile *et al.*^[61] demonstrated that the administration of EVs (most of which were exosomes) isolated from human CPCs significantly suppressed apoptosis, stimulated angiogenesis, and improved cardiac function in a rat model of AMI. They also showed that specific miRNAs, such as miR-210, miR-132, and miR-146a-3p, were enriched in CPC-derived exosomes. Ibrahim *et al.*^[62] reported that the administration of human CPC-derived exosomes in a mouse model of AMI significantly suppressed apoptosis, stimulated angiogenesis, and restored cardiac function. They also demonstrated that miR-146a was enriched in CPC-derived exosomes and that miR-146a administration partially mimicked the beneficial effects of CPC-derived exosomes on cardiac function.

ESC-derived exosomes

Khan *et al.*^[63] reported that ESC-derived exosomes from mouse stimulated neovascularization, enhanced cardiomyocyte survival, and restored cardiac function in a mouse model of AMI. Furthermore, ESC-derived exosomes augmented the survival and proliferation of CPCs. miR-294 was enriched in ESC-derived exosomes and the treatment of CPCs with miR-294 promoted the progression of the cell cycle to the S phase, suggesting that ESC-derived exosomes transferred miRNAs, such as miR-294, to CPCs, which promoted the proliferation and survival of CPCs.

CD34+ stem cell-derived exosomes

Sahoo *et al.*^[64] isolated exosomes from human CD34+

stem cells (which include endothelial progenitor cells^[65]) and examined their proangiogenic activity. CD34+ stem cell-derived exosomes stimulated tube formation from cultured endothelial cells in Matrigel (*in vitro* assay), and promoted angiogenesis *in vivo*, as assessed by the Matrigel plug assay and the corneal angiogenesis assay. Mackie *et al.*^[66] demonstrated that CD34+ stem cells expressing the pro-angiogenic factor sonic hedgehog (SHH) restored cardiac function in a mouse model of AMI. They also showed that SHH was enriched in exosomes secreted from stem cells and that it was transferred to and expressed functionally in recipient cells, suggesting that exosome-mediated transfer of SHH to recipient cells accounts for the beneficial effects of stem cell administration in this model of AMI.

Collectively, these studies provide compelling evidence that exosomes derived from a variety of stem cells exert beneficial effects on animal models of CVD.

FUTURE DIRECTIONS

Clinical trials

Although clinical trials using exosomes for CVD treatment have not yet started, exosome administration in humans has been tested, particularly for cancer immunotherapy^[67-69]. Phase I and phase II studies have been performed and the safety of the treatment has been confirmed. Future clinical studies will be required to test the safety and efficacy of exosome treatment for CVD.

Modification of exosomes

Given the low toxicity, high stability in the circulation, and high efficiency of transport to donor cells demonstrated by exosomes, several studies have attempted to augment the therapeutic efficacy by modifying exosomal content. For instance, small RNAs such as small interfering RNAs and miRNAs have been loaded into exosomes during exosome formation using lipofection or following exosome formation using electroporation^[70-74]. These modified exosomes reportedly exerted biological effects in recipient cells^[70-74]. Exosomes have also been used as vehicles to transport exogenous chemical compounds to recipient cells stably and efficiently, because some drugs are condensed in the exosomes of donor cells and transferred to recipient cells. Exosomes enriched in curcumin, an anti-inflammatory agent, or chemotherapeutic agents, such as paclitaxel and doxorubicin, have been used to transport these compounds to recipient cells, with their beneficial biological effects confirmed^[75-78]. Another strategy that has been examined is the modification of exosomal membrane proteins to improve the efficiency of uptake by recipient cells. Alvarez-Erviti *et al.*^[70] prepared dendritic cells that expressed Lamp2b, an exosomal membrane protein, fused to a peptide fragment of neuron-specific rabies viral glycoprotein so that exosomes would be accumulated specifically in the brain. The authors demonstrated that these modified exosomes were specifically taken up by

brain tissues when intravenously administered. Therefore, the modification of exosome structure will enhance the specificity and efficiency of transport and the modification of exosome content (for example, by inclusion of specific miRs) will enhance the therapeutic effect in the future.

Exosome-induced tumorigenesis

It has been reported that MSC-derived exosomes promote tumor growth *in vivo* via the stimulation of vascular endothelial growth factor expression in tumor cells^[79]. In most cases, the stimulation of angiogenesis appears to be favorable for the regeneration of cardiomyocytes after AMI. However, angiogenesis may stimulate tumor growth in other tissues. Therefore, it is desirable to explore a strategy to specifically deliver exosomes to target tissues.

CONCLUSION

Exosomes are one of the secreted vesicles that contain bioactive molecules, such as proteins, mRNAs, and miRs. Exosomes transfer these bioactive molecules to recipient cells, thus exerting biological effects. Preclinical studies have suggested that exosomes can be used for the treatment of CVD such as AMI and stroke. Future clinical studies are warranted to confirm the efficacy of exosome administration for CVD treatment. Furthermore, modifications of exosomal structure and content will enhance the efficacy of exosome administration for such treatments in the future.

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