

Invited Mini Review

Multiplexed targeting of microRNA in stem cell-derived extracellular vesicles for regenerative medicine

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Regenerative medicine is a research field that develops methods to restore damaged cell or tissue function by regeneration, repair or replacement. Stem cells are the raw material of the body that is ultimately used from the point of view of regenerative medicine, and stem cell therapy uses cells themselves or their derivatives to promote responses to diseases and dysfunctions, the ultimate goal of regenerative medicine. Stem cell-derived extracellular vesicles (EVs) are recognized as an attractive source because they can enrich exogenous microRNAs (miRNAs) by targeting pathological recipient cells for disease therapy and can overcome the obstacles faced by current cell therapy agents. However, there are some limitations that need to be addressed before using miRNA-enriched EVs derived from stem cells for multiplexed therapeutic targeting in many diseases. Here, we review various roles on miRNA-based stem cell EVs that can induce effective and stable functional improvement of stem cell-derived EVs. In addition, we introduce and review the implications of several miRNA-enriched EV therapies improved by multiplexed targeting in diseases involving the circulatory system and nervous system. This systemic review may offer potential roles for stem cell-derived therapeutics with multiplexed targeting. [BMB Reports 2022; 55(2): 65-71]

INTRODUCTION

Regenerative medicine has the potential to assist the spontaneous regeneration of damaged tissues and organs, as well as restore normal function to birth defects. Thus, regenerative medicine strategies are increasingly being improved using materials and new generative cells to contribute to regeneration, as well

as various combinations of these (1). Cell therapy, a regenerative medicine that meets this need, has become an attractive option to induce in situ tissue regeneration through host cell transplantation and stem cell transplantation. In particular, stem cells derived from fetuses or adults are attracting more attention than general cells in terms of their clinical application ability because they have self-renewal, multipotent or pluripotent properties as undifferentiated cells. Stem cells can be classified into three categories: embryonic stem cells (ESCs), pluripotent stem cells (PSCs), and mesenchymal stem cells (MSCs) (2). Among them, MSCs as tissue-specific stem cells, which do not have ethical problems compared to ESCs, and show less tumorigenicity compared to PSCs, are widely used in tissue damage repair and regenerative medicine.

Although stem cell transplantation has various beneficial effects, abnormal cell differentiation and low engraftment rate at damaged sites are their limitations. Considering that the general consensus of the regenerative mechanism following stem cell transplantation is the paracrine effect through the release of growth factors and cytokines, research to solve the limitations should be continued (3). 1) Nutritional imbalance with the transplant site when culturing stem cells at the laboratory level, 2) the need for hundreds of millions of stem cells, 3) the generation of a large number of stem cells that cannot be delivered to the transplant site, 4) the presence of carcinogenic potential are the problem of stem cell therapeutics that it must be a necessary and sufficient condition for clinical application in regenerative medicine (4-8). In the ischemic heart, it was reported that 1% of cells at 24 hours and less than 0.44% at 4th day after MSC transplantation were survived (9, 10). Furthermore, it has been reported that most of the administered stem cells did not penetrate the blood-brain barrier (BBB) and exert a therapeutic effect due to the limited characteristics of the BBB in the ischemic brain (11).

Stem cell-free-based therapies that can ameliorate the shortcomings of cell therapies are emerging as safe and effective treatment options compared to conventional cell therapies. MSC secretome secreted by paracrine effect, i.e. culture medium, protein, RNA, and lipid, can be a strategy for cell-free therapy, which contributes to physiological or pathological improvement of tissue damage. The MSC secretome effect is manifested by secreting extracellular vesicles (EVs), which are biochemical

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components according to large amounts of activation signals, rather than single growth factors or cytokines. EVs play a role in delivering functional genetic material to other cells, and it transmit various information surrounding it to neighboring cells (microenvironment), thereby activating cell-to-cell interaction as a mediator of cell communication (12). In particular, it has been known that microRNA (miRNA), a short RNA molecule that regulates several cellular processes through post-transcriptional gene silencing, communicates between neighboring cells, however it has reported recently that miRNAs have been commonly or artificially delivered by inducing multiplexed target into the microenvironment via EVs (13, 14).

In this review, we mainly focus on the potential regenerative medicine by which stem cell-free-based therapies attenuate cardiovascular and brain tissue damage. We also discuss specific strategies of miRNA-enriched EV therapeutics for multiplexed targeting.

EXTRACELLULAR VESICLE DERIVED FROM STEM CELLS

EVs are cellular derivatives with a size of 40-1000 nm composed of adhesion molecules and soluble mediators. It has become clear that they exchange cellular biological information and deliver contents using soluble mediators including growth factors, cytokines and small molecules as small extracellular membrane fragments containing various types of cellular information and physiological properties. EVs are mainly classified into exosomes, microvesicles (MVs), microparticles, ectosomes, oncosomes, apoptotic bodies, and many other names according to their size and biogenesis. Proposed through Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines for the field in 2014 in the International Society for Extracellular Vesicles (ISEV), it was promulgated to be collectively referred to as EV (12, 13, 15).

Origin and size

EVs are largely divided into exosomes, MVs and apoptotic bodies according to their biogenesis, release pathway, size, content, and function. Although the specific protein markers that distinguish EVs have not been established, each protein profile is different. Exosomes are released to the outside of the cell in the form of microvesicular endosomes, and are extracellular release materials of 40-150 nm that mainly contain CD63 (CD81/CD9). MV is a plasma membrane formed by the budding of the cell plasma membrane outward, and is a subunit of EV with a typical diameter of 100-1000 nm. They express predominantly annexin A, a cytoskeletal component such as actin and microtubules. Apoptotic bodies have a diameter of about 50-5000 nm and are a programmed cell death phenomenon, and are released into the extracellular space in the form of active amphisome and passive cell death by hydrostatic pressure. In general, it has double-stranded DNA or histones as markers (16, 17). The diameter of stem cells is about 15 μm , whereas the capillary is less than about 8 μm and becomes clogged when intravenous cell transplantation is performed. However, since EVs containing exosomes and MVs have a diameter of 40-1000 nm, they have the advantage of penetrating to the periphery (Fig. 1).

Constituents

Since the formation and transport of exosomes are regulated by the endosomal sorting complexes required for transport (ESCRT) signaling pathway, signal-regulating proteins such as Alix, TSG101, HSC70, and HSP90 β are found in exosomes. Also known as exosome marker proteins, tetraspanin family membrane proteins (CD63, CD51 and CD9) are their components (17, 18). Although tetraspanin proteins were originally thought to be specific markers of exosomes, these proteins have been recently identified in MVs and apoptotic bodies (16, 18). MVs contain cytoskeletal proteins, integrin family proteins of adhesion function, and proteins responsible for translational

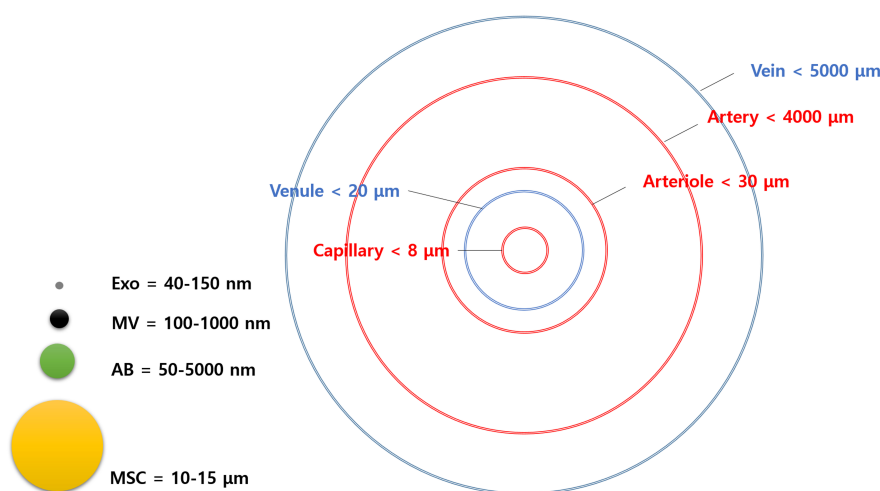


Fig. 1. Size comparison of extracellular vesicles.

modification. Specifically, glycan-binding protein responsible for interaction with other cells and Annexin A1 and A2, an abundant membrane-associated protein classified as an exosomal constituents, is recognized as a novel content of MV. Apoptotic bodies contain Annexin A5 protein that specifically binds to the negative curvature-specific lipid phosphatidylserine, and since it contains some organelles, proteins related to the nucleus, mitochondria, or endoplasmic reticulum can be observed (histones, heat shock protein, and glucose-regulated protein) (18, 19).

IMPROVEMENT OF EXTRACELLULAR VESICLE

Cell-derived EVs have already been studied to contain many physiologically active substances (20, 21). It has been reported that certain substances showing potential therapeutic efficacy are contained through proteomic profiling synthesized in EVs. However, for induction of cell-derived EVs containing a lot of tissue-specific or target-specific substances, extrinsic substances must be expressed at high concentrations and constant levels in EVs. In the context of regenerative medicine, RNA modification of cell-derived EVs can participate in genetic communication and transfer genetic information to target cells, while simultaneously altering target gene regulatory networks to accelerate disease recovery.

Strategies of enhancing information within the extracellular vesicle

Among many types of RNAs, mRNAs contain new protein sources and information necessary for tissue regeneration. mRNAs of less than about 1 kb in length can be included and delivered to EVs (22). Cuesta *et al.* suggested that human pulmonary artery smooth muscle cells-derived EVs overexpressing TGF- β 1 and BMP4 may contribute to vascular remodeling and endothelial-mesenchymal transition (EndoMT) during pulmonary hypertension development (23). To efficiently produce glial cell line-derived neurotrophic factor (GDNF) which is excellent for improving kidney damage, mRNA was inserted into MSCs through a lentiviral transfection system and EVs were isolated. With hypoxia/serum deficiency (H/SD) model, GDNF-enhanced MSC-derived EVs showed a cytoprotective effect on human umbilical vein endothelial cells (HUVECs) against damage by stimulating migration and angiogenesis and conferring resistance to apoptosis (24). GLO-1, the major rate-limiting enzyme of the glyoxalase system, is known to reduce the excessive accumulation of toxic end products due to oxidative stress in cells through glycolysis. GLO-1 was overexpressed to produce GLO-1-enhanced ASC-derived EVs. In *in vitro* and *in vivo* conditions, GLO-1-enhanced EVs improved type 2 diabetes via activation of the eNOS/AKT/ERK/P-38 signaling pathway, inhibition of AP-1/ROS/NLRP3/ASC/Caspase-1/IL-1 β , and increased secretion of various growth factors (25).

Unlike mRNA, transfecting miRNAs into EVs has the advantage of being able to simultaneously target a variety of cellular signals.

miRNA is a short noncoding and single-stranded RNA molecule that regulates the expression of protein-coding genes by promoting degradation and interfering with translation based on the mRNA-miRNA complementary sequence (26, 27). Although miRNAs cannot inhibit 100% protein coding like siRNA, they can cover about 60% of inhibition with a similar mechanism, and have the advantage of achieving efficient transformation due to their small size and low weight (28). The reason that miRNAs play a key function from the point of view of regenerative medicine for disease treatment is that miRNAs released from EVs internalized in donor cells can access neighboring genes and affect their expression with influencing cell-cell communication as cell-free therapy (29). To our knowledge, this function implies direct or indirect influence on neighboring cells or tissue microenvironment like stem cell paracrine effect at the transplanted site.

Izarra *et al.* observed highly expressed miRNA-133a through analysis of purified exosomal fraction after transfecting miRNA-133a, which is involved in cardiac development and pathophysiology, into SCA-1 + Lin⁻ adult cardiac progenitor cells (CPCs). miRNA-133a-enriched CPC clearly improved cardiac function in a murine myocardial infarction model by reducing fibrosis and hypertrophy, and increasing vascularization and cardiomyocyte proliferation. Despite non direct treatment of EVs, this study might correlate with upregulated expression of several relevant paracrine factors and cooperative secretion of miRNA-133a via EV secretion (30). Also, other studies demonstrated the effects of exogenous miRNA in endothelial cells (ECs) in regulating smooth muscle cell (SMC) turnover. It was confirmed that biotinylated miR-126 transfected into EC completely affected SMC co-cultured in the same space, and neointimal lesion formation of carotid arteries by cessation of blood flow was inhibited by regulating FOXO3, BCL2, and IRS1 through direct EC-to-SMC transmission (31, 32).

In addition, there is an increasing number of experimental studies demonstrating the relevance of direct miRNA delivery using EVs in various physiological environments. For example, in neuronal to astrocyte signaling, EV-mediated miRNA-124a modulated glutamate transporters, especially excitatory amino acid transporter 2 (EAAT2, rodent analog GLT1), by using SOD G93A mice, an end-stage animal model of amyotrophic lateral sclerosis (ALS). It was recognized that miRNA-124a controls perisynaptic function through the characterization of neuron-astrocytic communication (33). Furthermore, Salvucci *et al.* showed that adipose tissue macrophages (ATMs) from obese mice secreted miRNA-155-containing exosomes (Exos) that induce glucose intolerance and insulin resistance when administered to lean mice through a mechanism involving direct inhibition of the target gene peroxisome proliferator-activated receptor (PPAR) γ . This suggests that these EV regulatory systems can be delivered to insulin target cell types through paracrine or endocrine regulatory mechanisms that strongly influence insulin sensitivity and overall glucose homeostasis (34).

An antisense oligonucleotides (ASO) construct antagonizing

oncogenic miR-125b was induced in red blood cells (RBCs), and the potential for treatment of leukemia cells through the RBC-EVs was presented. Because RBCs lacking nuclear and mitochondrial DNA were treated with ASO-miRNA-125b, a safe and scalable platform without the risk of horizontal gene transfer was demonstrated (Table 1) (35).

Methodologies of loading constituents into extracellular vesicle

To our knowledge, two approaches can be used to generate EVs containing miRNAs that induce multiplexed targeting. The first is to induce overexpression by directly injecting miRNA into cells. The easiest and simplest method for loading miRNAs is to incubate them with miRNA-secreting cells. Migration to intracellular vesicles by concentration gradient can be a natural integration process that does not pose any threat to cells. However, this strategy has the disadvantage of not being able to predict the concentration of miRNAs that diffuse into EVs in cells, and low efficiency due to miRNA instability.

A method for stably inducing miRNA into EVs is the design of chemical transfection. As mentioned earlier, stem cells are basically loaded with paracrine molecules, and EVs inside the cells can be the best biogenesis plant and delivery tool that can over-produce and deliver desired miRNAs. It is to transduce miRNA itself or a specific plasmid/virus-based construct designed so that miRNA can be activated in the cell, and induce it to be expressed ectopic, and naturally package it in EVs (36, 37).

Physical force can be used to induce transient micropores in EV membranes or membrane recombination sites to promote miRNA incorporation. Sonication and electroporation are examples of methods that researchers can actively utilize. Ultrasound has been focused on methods that can shape solid images using sound waves. However, it is possible to apply a mecha-

nical shear force that can increase the temporary membrane permeability by using low physical energy. Lamichhane et al. found that miRNA capable of oncogene knockdown was introduced into EVs about more than 3 times by sonication, which showed much better results than siRNA and single-strand RNA (38). Electroporation has been widely used for gene transduction into cells, but it is also a strategy that can be used for miRNA EV transduction. Liang et al. used electroporation (1000 V, 10 ms and 2 pulses) while simultaneously treating cells with miRNA-21 and chemotherapeutics to control drug resistance in colon cancer. EV-derived miR-21 induced cell cycle arrest and apoptosis by simultaneously regulating phosphatase and tensin homolog (PTEN) and mutant DNA mismatch repair (hMSH2) (39). However, since the extracellularly ejected EV or the miRNA derived from the EV may aggregate or be destroyed, it is necessary to calculate the appropriate parameters before proceeding with the experiment. In addition, since it is difficult to distinguish between the result of direct packaging of miRNA by biogenesis in EV and the result of overexpression of miRNA by external processing, it is necessary to understand these approaches (40, 41).

Second, it is a direct method to isolate EVs from stem cells, concentrate them, and transfer miRNAs. Zhang et al. newly constructed a modified calcium chloride-mediated transfection method to produce miRNA-15a-loaded EVs. After mixing EVs isolated from macrophages with miRNA, calcium chloride and heat shock were applied to the samples, confirming that they were functional in recipient cells (42). Naseri et al. isolated EVs from bone marrow-derived mesenchymal stem cells, and LNA-antimiRNA-142-3p was transfected into EVs by electroporation. LNA-antimiRNA-142-3p-incorporated EVs reduced colonization and tumor formation in breast cancer stem cells (43). Although these techniques yield high results in efficiency

Table 1. A summary of enhancing information for cell-derived extracellular vesicles

Types of RNAs	Target	Cell source of EVs	Effects	Reference
mRNA	TGF-β1	Human pulmonary artery smooth muscle cells	Vascular remodeling EndoMT	23
	BMP-4			
	GDNF	Human adipose mesenchymal stem cells	Angiogenesis Decrease of renal fibrosis	24
	GLO-1	Adipose derived stem cells	Angiogenesis Improvement of T2DM	25
miRNA	miRNA-133a	Adult cardiac progenitor cells	Indirect EV treatment Anti-apoptosis Improvement of cardiac function	30
	miRNA-126	Endothelial cells	Indirect EV treatment SMC turnover Atheroprotective laminar shear stress	31
	miRNA-124a	Neuron	Neuron-astrocyte communication Improvement of ALS	33
	miRNA-155	Adipose tissue macrophage	Glucose tolerance Insulin sensitivity	34
	ASO-miRNA-125b	Red blood cells	Treatment of leukemia cells	35

compared to those that transfect miRNAs into cells, the difficulty of sorting the mechanistic details of packaging miRNAs within EVs also remains a significant limitation (40, 41). Therefore, a recent study has reported that protons of EVs to generate a pH gradient across the EV membrane can be used to enhance the vesicle loading of nucleic acids containing miRNAs. This is one of the new strategies that can be achieved without any external energy introduction (44).

REGENERATIVE MEDICINE FOR TISSUE REPAIR

A study that examines the improvement in regenerative medicine by introducing a stem cell-mediated miRNA into EVs present inside the cell has been recently published. This chapter represents to examine the case of multiplexed targeting of various signals related to cell/tissue improvement by stem cell-derived EVs with increased miRNA used in heart and brain diseases (Table 2).

Coronary artery diseases, a representative cardiovascular disease, are caused by atherosclerosis and are known to cause arrhythmias and myocardial infarction. In order to maintain the homeostasis of various cells (cardiac cells, fibroblasts, lymphocytes, mast cells, and macrophages) present in the cardiovascular system and the difficulties in cardiac regeneration and repair (14), so stem cell-derived EV therapy is essential, especially the special treatment of EV-derived miRNAs functioned as multiplexed targeting.

Song *et al.* conducted a multiplexed targeting study that showed a positive effect on cardiac and vascular cells while maintaining the advantages of stem cells, but a negative effect on myocardial fibroblast proliferation. Using a lentivirus, miRNA-EV system was constructed that can stably produce miRNA-210, which plays a pivotal role in cardiovascular cells, in adult stem cell

EVs. It was shown that miRNA-210 in stem cell-derived EVs controlled cardiac cell death by targeting protein tyrosine phosphatase 1B (PTP1B) and death-associated protein kinase 1 (DAPK1), and also controlled angiogenesis of vascular cells by targeting Ephrin A (EFNA3), with no effect on cardiac fibroblasts (14). Similarly, substances targeted by miRNA-133a expressed in adult CPC were discovered through bioinformatic prediction including BMF (Bcl-2 modifying factor) and BIM (Bcl2l11), which are potent proapoptotic factors, a serine threonine kinase STK4 (formerly Mst1) activated by oxidative stress, and Foxo1 that is a critical promotor of cardiomyocyte survival upon oxidative stress conditions. EVs derived from miR-133-CPC have been shown to protect cardiac function and hypertrophy and to increase vascularization (30). Peng *et al.* also discovered exosomal miRNA-25-3p targeting the proapoptotic protein Enhancer of zest homologue 2 (EZH2). Bone marrow MSC-derived miRNA-25-3p commonly targets phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and Fas ligand (FasL), which are proapoptotic genes, and showed cardioprotection effect by EZH2. The anti-cardioprotective effect and inflammation control of induced cardiomyocytes were confirmed under oxygen-glucose deprivation (OGD) condition (45). Ferguson *et al.* found multi-target candidates for inducing cardiomyocyte proliferation after isolating MSC exosomes in a virus-free miRNA loading procedure based on a miRNA prediction system. Results suggested a total of 22 targeting miRNA-199a, including Retinoblastoma protein 1 (RB1), which leads to cell cycle arrest, Liver kinase B1 (LKB1), specific loss of which results in cellular proliferation, and NEUROD1, which associated with cell cycle arrest (46).

As with cardiovascular disease, stem cell-derived EV-derived miRNA research that performs multiplexed targeting in CNS disease has not been conducted to a large extent. In order to control Rett syndrome (RTT), one of the neurodevelopmental

Table 2. A summary of stem cell-derived EV therapies mediating multiplexed targeting by miRNAs

Disease	miRNA	Target	Cell source	Effects	Reference
Ischemic heart	miRNA-210	Ptp1b Dapk1 Efn3	Human adipose-derived stem cells	Regulation of apoptosis and angiogenesis Heart regeneration	14
	miRNA-133a	Bmf Bim Stk4 Foxo1	Cardiac progenitor cells (SCA-1 + Lin ⁻)	Anti-apoptosis Improvement of cardiac function	30
	miRNA-25-3p	Fasl Pten Ezh2	Bone marrow mesenchymal stem cells	Cardioprotection Anti-inflammation	45
Heart	miRNA-199a	22 genes included Rb1 Lkb1 Neurod1	Mesenchymal stem cells	Cell death/proliferation Cell cycle regulation (Target Prediction)	46
Rett syndrome	miRNA-21-5p	EphA4 Tek	Urine-derived stem cells	Facilitation of early nerve formation Neurogenesis	47
Spinal cord injury	miRNA-126	Spred1 Pik3r2	Mesenchymal stem cells	Angiogenesis Neurogenesis	50

disorders, Pan *et al.* demonstrated that miRNA-21-5p was transfected into urine-derived stem cells and the association with EVs was confirmed. This study was also confirmed that miRNA-21-5p directly targeted Eph receptor A4 (EphA4) after treatment of EVs into neuroal stem cells and inversely correlated with TEK receptor tyrosine kinase (TEK or Tie2) receptor signaling (47). Spinal cord injury (SCI) due to loss of motor and sensory function has been mostly studied as stem cell therapy (48). In order to effectively improve SCI while having the paracrine effect of stem cells, Huang *et al.* confirmed the EV effect after loading miRNA-126 into MSC. In the case of miRNA-126, the functional recovery effect of direct treatment was already confirmed in the same research group (49). In MSC-derived MV, miRNA-126 was promoted by increasing angiogenesis and neurogenesis and inhibiting apoptosis with multi-targeting process of sprouty-related EVH1 domain-containing protein 1 (SPRED1) and phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2). Therefore, this result was recognized as a therapeutic study on the multifunctional targeting effect of EV-derived miRNA-126, which confirmed the stem cell effect (50).

CONCLUSION

Therapeutic research for the regenerative medicine with stem cells has made a lot of progress. Researches for stem cell-derived EV are also currently in progress, however it is necessary to secure therapeutic efficacy and safety in vivo. In the case of stem cell-derived EVs with increased specific miRNAs, it is expected that it will be a powerful cell-derived therapeutic agent that can overcome the limitations of existing studies and has the advantages of stem cells and multiplexed targeting of miRNAs.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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