Mesenchymal Stem Cell-Derived Extracellular Vesicles as Therapeutics and as a Drug Delivery Platform

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SUMMARY

Mesenchymal stem cells (MSCs) are one of the most easily accessible stem cells that can be obtained from various human tissues. They have raised considerable interests for their potential applications in tissue repair, anti-cancer therapy, and inflammation suppression. Stem cell-based therapy was first used to treat muscular dystrophies and has been studied intensively for its efficacy in various disease models, including myocardial infarction, kidney injuries, liver injuries, and cancers. In this review, we summarized the potential mechanisms underlying MSC-derived EVs therapy as a drug delivery platform. Additionally, based on currently published data, we predicted a potential therapeutic role of cargo proteins shuttled by EVs from MSCs. These data may support the therapeutic strategy of using the MSC-derived EVs to accelerate this strategy from bench to bedside. STEM CELLS TRANSLATIONAL MEDICINE 2019;8:880–886

SIGNIFICANCE STATEMENT

The future of exosome therapeutics has great potential, but several challenges, as discussed in the present study, must be overcome before exosome-based therapy will become an important option as a next-generation drug delivery system.

INTRODUCTION

Mesenchymal stem cells (MSCs), multipotent adult stem cells, are one of the most easily accessible stem cells that can be obtained from various human tissues. They have been widely tested as therapeutics due to their accessibility, therapeutic efficacy in various diseases, and tissue damage regeneration, their easy accessibility, and their availability from ethically acceptable tissues such as bone marrow aspirates and fat tissues [1].

MSCs have raised considerable interests for their potential applications in tissue repair, anticancer therapy, and inflammation suppression. Stem cell-based therapy was first used to treat muscular dystrophies [2] and has been studied intensively for its efficacy in various disease models including myocardial infarction [3], kidney injuries [4], liver injuries [5], and cancers [6].

Recent studies have suggested the possibility that the key therapeutic effects of MSCs in tissue repair are mediated mainly via paracrine mediators secreted from the MSCs and only partially from MSCs themselves. The "secretomes" of MSCs, including various secretory proteins such as growth factors,

cytokines, and chemokines and extracellular vesicles (EVs) such as microvesicles (MVs; 100–1,000 nm diameter) and exosomes (40–150 nm diameter), have been shown to induce many of the therapeutic properties of MSCs. For example, in an acute kidney injury (AKI) model, systemically injected MSCs induced significant recovery from cisplatin-induced kidney damage, despite the low permanent engraftment of MSCs within the kidney [7]. Further studies demonstrated that the paracrine factors collected from MSCs were sufficient to induce MSCs-mediated recovery from renal injury [8]. The beneficial effects of MSCs can be replicated through secretomes of conditioned media from MSCs, especially by exosomes [9].

Even though the precise mechanism of their paracrine effect is not clearly understood, EVs have been recognized as potent therapeutic vehicles that can transfer various proteins and regulatory genes to the targets. EVs are considered non-immunogenic nanovesicles, which can protect their cargoes from serum proteases and immune systems to transfer information and communicate with other cells [10].

In this review, we summarized the potential mechanisms underlying MSC-derived EVs therapy as a drug delivery platform.

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Additionally, based on currently published data, we predicted a potential therapeutic role of cargo proteins shuttled by EVs from MSCs. These data may support the therapeutic strategy of using the MSC-derived EVs to accelerate this strategy from bench to bedside.

EXTRACELLULAR VESICLES

EVs were initially identified as a quality control system used by cells to dispose of unwanted content [11]. After years of intensive research, the role of EVs as important cell-to-cell communication mediators, which deliver various bioactive molecules such as functional proteins, nucleic acids, and lipids, was identified [10, 12–14]. Depending on their size, cells or tissue of origin, biogenesis mechanisms, or proposed functions, EVs are generally classified as MVs, exosomes, or apoptotic bodies.

MVs, also known as ectosomes, shedding vesicles, or microparticles, are 100- to 1,000-nm vesicles released by budding from the plasma membrane. Exosomes, 40–150 nm in diameter, are generated from an inward budding of endosomes, forming intraluminal vesicles inside the endosomal compartment, which are called multivesicular bodies (MVBs). Exosomes are actively released from cells by membrane fusion of MVBs and the plasma membrane. Although the cargos of MVs are similar to the composition of parental cells due to simple diffusion, the cargos of exosomes differ from those of parental cells, suggesting selective cargo-sorting mechanisms. Cargo loading of exosomes is well controlled and well regulated, although the mechanism is not fully understood. The endosomal-sorting complex required for transport (ESCRT) family has been shown to play a key role in cargo loading and exosome biogenesis [15], but there are also ESCRT-independent pathways in cargo sorting [12]. Exosomes, unlike other synthetic nanoparticles such as liposomes, contain transmembrane and membrane-anchored proteins that may enhance endocytosis to facilitate delivery of their cargos [13, 14]. Even though the underlying mechanisms and pharmacokinetics are still not well understood, extensive studies have been directed toward utilizing exosomes as a therapeutic conveyor in various diseases, by using the exosome itself or engineering exosomes to increase stability, targetability, or loading efficiency of specific pharmaceutical cargos. Apoptotic bodies, which are larger than MVs or exosomes, are generally larger than 1 μm in diameter and are released as blebs from dying cells. They contain fragmented DNA and are further characterized by phosphatidylserine externalization [16, 17].

THERAPEUTIC POTENTIAL AND MANUFACTURING OF MSC-DERIVED EVS

Stem cells are currently the best candidate for treating intractable degenerative or genetic diseases because of their capacity to differentiate and produce new, healthy cells that can replace injured or diseased cells. In addition, nonhematopoietic tissue stem cells, such as the MSC, can similarly treat nonhematopoietic disorders by replacing diseased cells with newly generated cells [18–20].

Table 1. Comparison of MSC therapy and MSC-derived EVs therapy

	MSC therapy	MSC-derived EVs therapy
Therapeutic effects	Tissue regeneration, wound healing, antitumor effects, immunomodulation [29, 30]	Retains therapeutic effects of MSCs, and loading therapeutic cargo can increase their effects [2, 5, 25, 31, 32]
Homing to target tissue	Home to sites of injury and cancer, but less than 1% of MSCs result in engraftment [33]	Mostly homes to the liver and spleen, and surface engineering of exosomes can induce targeting ability [34–36] Can pass though the blood-brain barrier [37]
Rejection	Can induce allogenic immune rejection [38, 39]	Considered to be nonimmunogenic

Abbreviations: EV, extracellular vesicle; MSC, mesenchymal stem cell.

Of the stem cells that are currently in clinical trials, the most extensively used cell type is the MSC. Interestingly, sufficient MSC research exists to support alternative proposals that MSC exerts its therapeutic effects through a secretion and not through a differentiation mechanism [21]. MSC-conditioned culture medium has been suggested to recapitulate the efficacy of MSCs in cardio-protection [22, 23], renal tubular cell survival [8], and hepatic failure protection [24, 25], as well as relieve immune disease [26]. As MSC-conditioned culture medium is rich in EVs, a complex cargo of lipids, proteins, and RNAs in EVs is the most likely candidate of the therapeutic effects. In addition, MSC-derived EVs also have been shown to elicit an immunosuppressive phenotype and the therapeutic benefits of their parental cells. Therefore, the therapeutic potential of MSCs-derived EVs acting as a surrogate of parental cells has been rigorously investigated.

MSC-derived EVs are known to have various therapeutic effects such as tissue regeneration, wound healing, antitumor action, and immunomodulation. Tested in various disease models, EVs derived from MSCs of different organs have demonstrated similar or better therapeutic capacity compared with their parental cells. MSC-derived EVs have advantages in safety issues, because they are considered nonimmunogenic with a lower risk of allogenic immune rejection from the host [27]. In addition, exosomes can bypass the blood-brain barrier by transcytosis through the endothelial layers to deliver cargo biomolecules to the brain parenchyma [28] (Table 1).

Exogenous MSC EVs in Tissue Regeneration

Much research has shown the beneficial effects of MSC-derived EVs in healing a variety of stressed tissues (Fig. 1). Bruno et al. showed an improvement of recovery from glycerol-induced AKI by treating it with MSC-derived EVs, which contained mRNA associated with the mesenchymal phenotype [40]. Similarly, He et al. showed protection against kidney damage in the subtotal nephrectomy murine model of renal regeneration by decreasing the levels of uric acid, creathinine, fibrosis, and lymphocyte infiltration by treating MSC-derived EVs [31]. In another murine
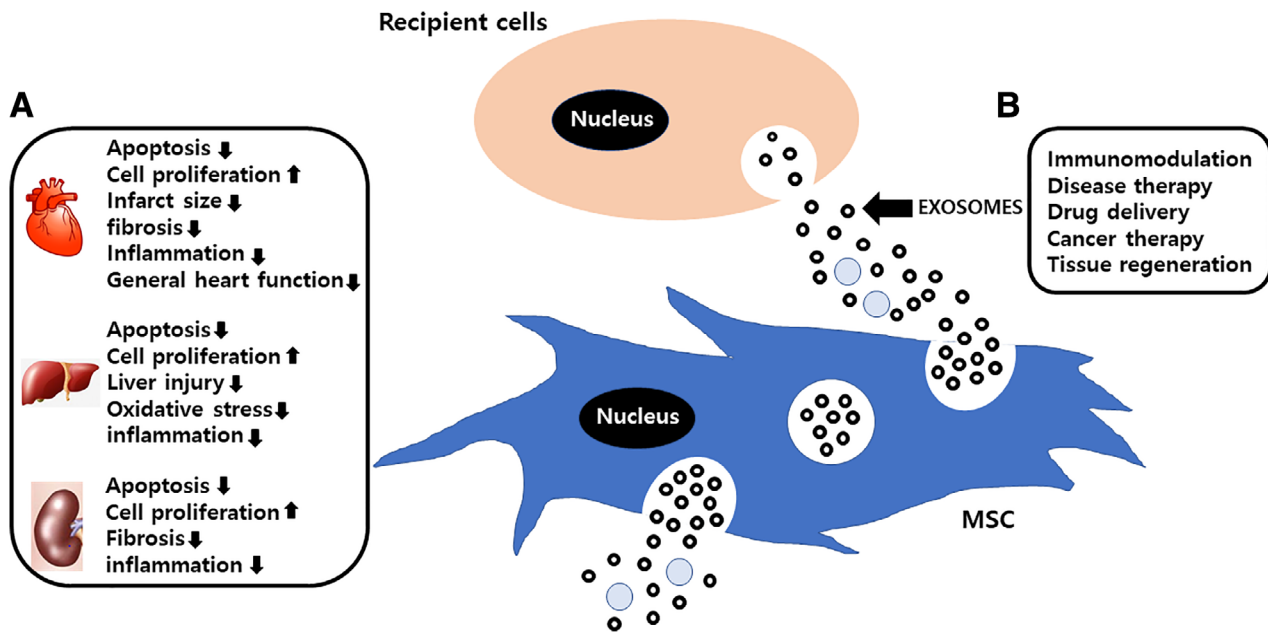
Figure 1. The characteristics of MSC-derived extracellular vesicles (EVs). **(A):** The function of the MSC-EV-mediated therapeutic effect studied in animal model such as the kidney, liver, and heart. **(B):** Secreted MSC-derived exosomes through fusion of multivesicular bodies with the cell membrane are mediated paracrine effects. Exosomes have the potential to exert various effects such as immunomodulation, disease therapy, and tissue repair in recipient cells. Abbreviation: EXPLOR, exosomes for protein loading via optically reversible protein-protein interaction; MSC, mesenchymal stem cell.

model of myocardial ischemia-reperfusion injury, MSC-derived EVs enhanced the recovery by increasing phosphorylated Akt and phosphorylated glycogen synthase kinase-3β and by decreasing phosphorylated mitogen-activated protein kinase 8 and oxidative stress [41]. Furthermore, the therapeutic potential of MSC-derived EVs was also verified in a murine model of fibrotic liver induced by carbon tetrachloride [5] and in a rat skin burn model [42]. Overall, these results indicate that MSC-derived EVs mediate the therapeutic effects in multiple diseases through multiple mechanistic pathways and provide a novel approach for the treatment of degenerative and acute injury-related diseases. However, the mechanism of action of MSC-derived EVs is still not fully understood. Further extensive investigation into the mechanism of action is essential to establish MSC-derived EVs as Food and Drug Administration-approved therapeutics. Besides the MSC-derived EVs, effects of neural stem cells (NSCs) and endothelial progenitor cells (EPCs) derived EVs were also studied. Grafted NSCs communicate with the host immune system via interferon gamma signaling mediated by EV-associated IFN gamma/Interferon gamma receptor 1 complexes [43]. Human EPCs-derived EVs delivered mRNA and microRNA, which activated the endothelial cell proliferation to support revascularization of injured murine tissue [44]. In addition, exosomes from human cardiac progenitor cells expanded ex vivo regenerated injured murine hearts by inhibiting apoptosis and increasing the proliferation of cardiomyocytes and endothelial cells [32].

Manufacturing Exosomes for Clinical Use

Most of exosomes are collected based on their size, and the most common way is to use differential centrifugation. However, centrifugation has a low recovery yield and low specificity due to nonexosomal or MV debris.

Unlike the strategies of isolating exosomes or MVs by size, the immunoaffinity-based approach sorts them via detecting the expression pattern of specific proteins on their surface. This approach has the advantage of isolating specific subpopulations of exosomes and simultaneously reducing copurification of cell debris and protein aggregates. One example of immunoaffinity-based sorting is the use of conventional magnetic-activated cell-sorting (MACS) columns [45]. The Taylor and Cercel-Taylor repurposed MACS to isolate exosomes from serum. In this study, exosomes with epithelial cell adhesion molecule (EpCAM) were incubated with anti-EpCAM magnetic microbeads and then the microbeads were trapped using a conventional MACS Separator [45]. However, applying this technique to clinical setting is doubtful because of difficulties in upscaling and automating such a process. It is necessary to develop an isolation method that can distinguish each type of exosome and facilitate a large-scale production of exosomes.

MSC was easily expanded relative to the other isolation methods using conventional tissue flasks and bioreactors, but their growth capacity in culture is limited and their biological properties can be changed with repeated passage. Certain strategies such as MSC immortalization by natural selection or by genetic modification could be used to overcome this limitation, although this would raise safety issues [46, 47]. Other approaches to scale up the amount of isolated exosomes could include using bioreactors to culture the MSCs [48]. However, it is important to determine whether bioreactor culture conditions could change exosome protein and RNA content, which may affect therapeutic efficacy [49]. There are many challenges associated with oxygen supply, shear stress, and pH balance by using bioreactor culture systems [50, 51]. Also, the impacts of these parameters may vary depending on the
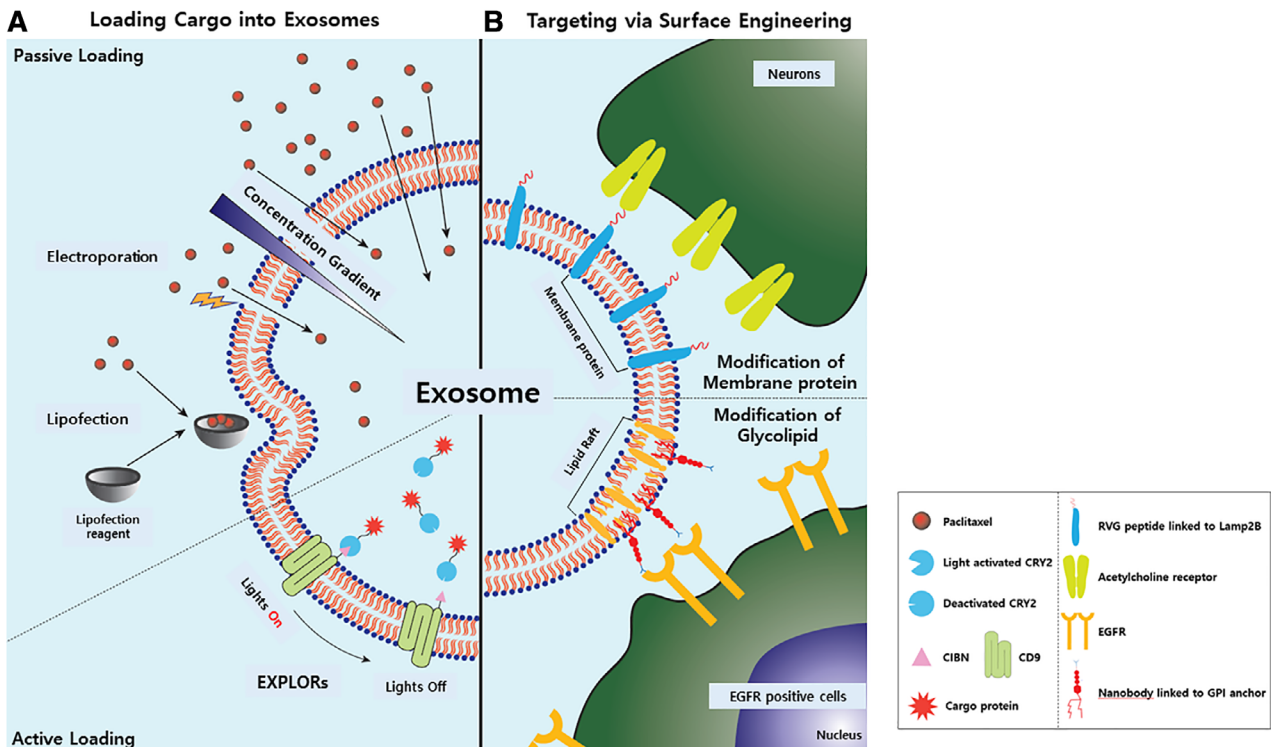
Figure 2. Engineering of exosomes. Exosomes are engineered to acquire therapeutic potential. Two different engineering methods are used. Exosomes are engineered **(A)** to load therapeutic cargos and **(B)** to acquire targeting abilities. Abbreviations: CIBN, truncated form of CIB(cryptochrome-interacting basic-helix-loop-helix); EGFR, epidermal growth factor receptor; RVG, rabies viral glycoprotein.

different cell types. In conclusion, to facilitate the production of large-scale MSC-derived exosome, new batches of MSCs should be periodically derived through testing and validation.

ENGINEERED EXOSOMES AS THERAPEUTICS

Due to the various therapeutic potential of exosomes, clinical trials are currently in progress. One clinical trial is testing the effects of naïve MSC-derived exosomes in promoting healing of large and refractory macular holes [52]. In contrast, the majority of other clinical trials currently in progress use engineered exosomes rather than naïve exosomes. For example, a clinical trial is in progress for promoting neurovascular remodeling and functional recovery after acute ischemic stroke using miR-124-loaded MSC-derived exosomes [53]. These engineered exosomes have a higher therapeutic potential when compared with naïve exosomes. There are mainly two different strategies that can improve the therapeutic potential of MSC-derived exosomes: loading cargo into the exosomes and targeting via exosomes (Fig. 2).

LOADING CARGO INTO EXOSOMES

Passive Loading

To enhance the therapeutic potential of naïve exosomes, researchers have developed several methods to load exogenous molecules into exosomes, involving passive and active cargo loading. Passive loading uses the concentration gradient of the molecules. By incubating exosomes with paclitaxel at 37°C for 1 hour, a small amount of paclitaxel was loaded into exosomes [54]. Cells were also incubated with paclitaxel to produce paclitaxel-loaded exosomes [55]. The loading capacity depended on the hydrophobic nature of the cargo molecules. Hydrophobic cargos bind to the lipid bilayer of exosomes and remain stable. However, the major downside of passive loading is the low loading capacity, even for hydrophobic cargos.

To compensate for the low loading capacity of concentration gradient-based methods, various physical and chemical techniques that can directly modify the exosomal membrane have been developed. Electroporation, sonication, extrusion, and freeze-thaw cycles are included in the physical methods, whereas lipofection, drug-associated loading, and click chemistry are examples of chemical methods [56]. However, these methods also have disadvantages, such as the aggregation of exosomes, compromise of exosomal membranes, toxicity to recipient cells, and excessive purification steps.

Active Loading

To overcome the low passive loading efficiency, researchers have targeted the membranes of exosomes during the exosome biogenesis processes. Fang et al. proposed a technology that uses plasma membrane anchors to allow highly oligomeric proteins to be targeted into exosomes because of the link between the cargo and anchor upon treatment to the recipient [57].

Exosomes are enriched in transmembrane proteins, such as tetraspanins including CD9, CD63, CD81, CD82 [58], lactadherin [59], and lysosome-associated membrane glycoprotein 2 (Lamp2B) [60]. By fusing transmembrane proteins to cargo molecules, transmembrane proteins have the potential to directly load cargo molecules into the exosomes in a manner similar to Fang's

approach. Yim et al. introduced a technology called exosomes for protein loading via optically reversible protein-protein interaction, which allows the cytosolic localization of cargo proteins. Upon treatment with recipient cells, the cargo proteins were not just restricted to membranes, but were scattered in the cytosol [61].

In addition to protein cargos, methods to load RNA into exosomes have also been developed. Hung et al. introduced a technology called TAMEL (Targeted and Modular EV Loading), which used Lamp2B as a fusion target. Hung et al. transfected cells with a Lamb2B-MS2 bacteriophage coat protein dimer (RNA binding domain) and MS2 stem loops fused to the cargo DNA. MS2 bacteriophage coat protein specifically recognizes the MS2 stem loop of RNA. Upon transcription, the RNA cargo is loaded inside exosomes due to recognition of the RNA loop [62].

TARGETING VIA SURFACE ENGINEERING

Since the discovery of exosomes, researchers have thoroughly studied their natural tropism. Many factors such as the cell source, route of administration, and dosage have effects on the biodistribution of EVs in vivo. Once administered exogenously, naïve exosomes are generally distributed to the liver, spleen, intestines, and lungs of mice, where the mononuclear phagocyte system (MPS) is active [63]. In macrophage-depleted mice, clearance of exosomes from the circulation was much slower compared with that in control mice, indicating the important role of macrophages in exosome biodistribution [64]. Engineering exosomes with targeting ability increases the chances of exosomes reaching target cells/tissues before being taken up by the MPS. Eventually this will decrease the off-target/side effects, leading to lower therapeutic dosages while maintaining therapeutic efficacy. In addition, surface modifications can be used to increase the delivery efficiency. Kim et al. introduced a membrane-editing technology by which vascular stomatitis virus-G protein-engineered exosomes increased the cargo delivery via low-density lipoprotein receptor targeting [65]. Here, we describe two different techniques that engineer exosome biogenesis processes to equip exosomes with increased targeting ability.

Modification of Membrane Protein (Lamp2B)

Alvarez et al. modified the N-terminus of Lamp2B with rabies viral glycoprotein (RVG). RVG specifically binds to acetylcholine receptors, which are rich in neuronal cells. By transfecting cells with Lamp2B fused to RVG, exosomes displaying RVG protein at the outer membrane were produced. Using electroporation, they loaded the RVG-exosomes with siRNA against BACE1, a protease that has an important role in Alzheimer's disease pathogenesis by cleaving the amyloid precursor protein. Upon treatment, RVG-exosomes specifically targeted neuronal cells compared with naïve exosomes, and successfully knocked down the BACE1 mRNA in wild-type mice [66]. However, targeting peptides fused to Lamp2B were vulnerable to degradation due to localization in the lumen of endosomes during exosome biogenesis [67].

Modification of Glycosylphosphatidylinositol (GPI)

Instead of modifying exosomal membrane proteins, Kooijman et al. modified a glycolipid that could be integrated into the exosomal membrane during exosome biogenesis. EVs are enriched in lipid raft-associated lipids and proteins. The glycosylphosphatidylinositol (GPI)-linked protein decay-accelerating factor (DAF) is one of the EV-rich proteins loaded during reticulocyte maturation. Kooijman et al. genetically engineered cells with DAF-derived GPI-linked peptides fused with nanobodies. They used specific nanobodies that targeted the epidermal growth factor receptor. Nanobodies were significantly enriched in exosomes compared with parent cells in fusion with GPI-anchors. It is suggested that GPI-anchoring could be used as a versatile tool to incorporate a variety of protein on exosomes, such as enzymes, antibodies, reporter proteins, and signaling molecules [68].

CONCLUSION

The future of exosome therapeutics has great potential, but additional challenges must be overcome. The following obstacles still must be adequately addressed: (a) exosome components and the mode of action must be fully understood. To be validated by the Federal Drug Administration as a drug, the safety and efficacy of exosomes should be thoroughly studied and the components of the preparations and modes of action must be validated. (b) A database of absorption, distribution, metabolism, and excretion (ADME) should be established. As previously mentioned, various parameters such as the cell source and the route of administration affect the ADME of exogenously administered exosomes. To reach the maximum therapeutic potential and establish the dosage, an ADME study is essential. (c) Exosome production efficiency must be increased. Even though MSCs are known to produce more exosomes than do other types of primary cells, it is necessary to develop culture methods that increase the production of exosomes or immortalized MSCs that produce validated exosomes for clinical applications. (d) Better targeting mechanisms should be developed. To reduce the off-target/side effects and avoid clearance, screening for targeting molecules is key. If these challenges are sufficiently addressed, exosome-based therapy will be an important option not only in the field of regenerative medicine but also as a next-generation drug delivery system.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

C.C. declare leadership position, patent holder, and stock interest with Cellex Life Sciences Incorporated and grant funding from Cellex to KAIST. The other authors indicated no potential conflicts of interest.

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