

Mesenchymal stem cells-derived extracellular vesicles as 'natural' drug delivery system for tissue regeneration

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Abstract: Mesenchymal stem cells (MSCs) have abilities to mediate tissue protection through mechanisms of anti-apoptosis, anti-oxidative stress and anti-fibrosis as well as tissue regeneration through mechanisms of cell proliferation, differentiation and angiogenesis. These effects by MSCs are mediated by a variety of factors, including growth factors, cytokines and extracellular vesicles (EVs). Among these factors, EVs, containing proteins, mRNA and microRNAs (miRNA), may carry their contents into distant tissues with high stability. Therefore, the treatment with MSC-derived EVs may be promising as 'natural' drug delivery systems (DDS). Especially, the treatment of MSC-derived EVs with the manipulation of specific miRNAs expression has been reported to be beneficial under a variety of diseases and tissue injuries. The overexpression of specific miRNAs in the EVs might be through pre-loading method using the gene editing system by plasmid vector or post-loading method to load miRNA mimics into EVs by electroporation or calcium chloride-mediated transfection. Despite current several challenges for clinical use, it should open the next era of regenerative medicine for a variety of diseases. In this article, we highlight the therapeutic potential of MSC-derived EVs as 'natural' DDS and current challenges.

Main Text

Mesenchymal stem cells (MSCs) are multipotent cells with the capacities to differentiate into mesodermal, endodermal and ectodermal lineages as well as the abilities of self-renewal and regeneration (Pittenger *et al.*, 1999). MSCs have been shown to ameliorate multiple tissue injuries through the direct replacement into injured area (Tsuji *et al.*, 2020b). In addition, MSCs have also been shown to induce regeneration indirectly through the tissue-protective secretome, including cytokines, chemokines, growth factors and extracellular vesicles (EVs) containing proteins, mRNAs and microRNAs (miRNAs) (Tsuji *et al.*, 2020a). Recent evidence revealed that paracrine effect of MSCs is the dominant contributor of regenerative ability of MSCs, therefore the secretome from MSCs may provide a novel cell-free regenerative option in a variety of diseases. The mechanisms by which secretome mediates tissue protection and regeneration include suppression of pro-inflammatory response, modulation of immune system, inhibition of cell apoptosis and fibrosis as well as the stimulation of cell proliferation and tissue-intrinsic

progenitor cells differentiation (Tsuji *et al.*, 2020b). Although the therapy using secretome from MSCs is an attractive option for regenerative medicine, the quality control of the beneficial secretome is quite difficult (Allelein *et al.*, 2021; Giebel, 2017) especially because beneficial effects may be mediated comprehensively with many beneficial factors.

Among the secretome from MSCs, EVs have been reported to be the main mediators to induce tissue regeneration (Park *et al.*, 2019). EVs are small membrane vesicles released by a variety of cell types and can carry several factors into recipient cells and mediate cell-cell communications (Lee *et al.*, 2012). EVs mediate high stability of their contents and carry contents into distant tissues, thus sometimes act as organ-to-organ communication. In this point, EVs are a kind of *in vivo* 'natural' drug delivery system (DDS). The DDS requires the delivery of their contents of appropriate amount toward appropriate position at appropriate timing with good biocompatibility. Various types of DDS such as adeno-associated virus vectors and nanoparticles, including liposomes, lipid nanoparticles, polymer micelles and polymeric nanoparticles have been reported and some of them are under trials (Deng *et al.*, 2021; Prabhu *et al.*, 2015). However, there are several concerns, including toxicity, immunogenicity, biocompatibility, ease of production, transfection efficiency, transfer to the target tissue

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and cellular uptake. Since EVs are the natural DDS derived from cells, thus free from the concerns of toxicity, immunogenicity and biocompatibility, it would be the promising DDS. Indeed, EVs treatment as DDS has been reported since 2010 (Alvarez-Erviti *et al.*, 2011; Sun *et al.*, 2010). While EVs secreted from MSCs may mediate beneficial effects, recent focus of the analysis is shifting to strengthen the effect by overexpressing specific factors.

Manipulation of MSC-derived EVs has emerged as a novel therapeutic option against several diseases and tissue injuries. Among the content of EVs, recent evidence using knockdown of the ribonuclease III Droscha gene, essential for the miRNAs production, implied that miRNAs are the main contributor of therapeutic effect, since knockdown of miRNAs abolished the therapeutic effect with MSC-secretome (Collino *et al.*, 2015). miRNAs are the noncoding and single-stranded RNAs, which silence their targets genes through the binding to the 3'-UTR (Selbach *et al.*, 2008). More than 200 mammalian miRNAs have been found currently and each miRNA may regulate several gene transcriptions. The prediction of the target gene of each miRNA is available in several public website, such as miRBase (Hsu *et al.*, 2006). To explore specific miRNAs might be useful as disease biomarkers as well as novel therapeutic approaches. Indeed, some miRNAs are under clinical trials. For example, AntimiR of miR-122 (Miravirsen) to type C hepatitis (Ottosen *et al.*, 2015), and MRX34 to regulate miR-34 against tumors are under clinical trials (Zhang *et al.*, 2019b). Although the detail mechanisms as well as targeted genes of each miRNA are not fully uncovered, there are several miRNAs reported to mediate tissue beneficial effects, that may be divided into two aspects, tissue protection and regeneration. The protective mechanisms include anti-apoptosis, anti-oxidative stress and anti-fibrosis while the regenerative mechanisms include cell proliferation, differentiation and angiogenesis. miRNAs may regulate both aspects. For example, it is reported that let-7c may target TGFBR1, thus inhibit the progression of tissue fibrosis (Park *et al.*, 2014) and miR-21 may regulate phosphatase and tensin homolog (PTEN) and protein kinase B (AKT)/mammalian target of rapamycin (mTOR)

signaling, thereby mediating anti-apoptosis effect (Song *et al.*, 2018), both of which are the tissue protective effects. In addition, miR-26a-5p has been reported to target Toll-like receptor 4 (TLR4), thereby inactivating the NF- κ B pathway and protecting against diabetic nephropathy (Duan *et al.*, 2020). On the other hand, it is reported that miR-210 may promote angiogenesis through the activation of vascular endothelial growth factor (VEGF) pathway in ischemia/reperfusion-induced acute kidney injury (Zhang *et al.*, 2019a), which is the regenerative aspect of the miRNA effects. Since MSCs may secrete these beneficial miRNAs-containing EVs, the overexpression of these beneficial miRNAs by way of miRNA mimic or gene editing systems might be potent to mediate therapeutic effects (Lv *et al.*, 2020; Yang *et al.*, 2020; Zhang *et al.*, 2017). Important point is that the types of beneficial miRNAs are depending on the disease type. For example, recent analysis revealed that let-7 family therapy ameliorated diabetic kidney disease (Park *et al.*, 2014) while overexpression of miR-21 protected against ischemia reperfusion-induced kidney injury via anti-apoptotic mechanisms (Song *et al.*, 2018). We may need to choose the specific miRNAs depending on the patients' diseases and conditions as the personalized medicine.

Despite these promising effects, there are still several challenges for the clinical use. First, we need to standardize the way to obtain EVs. Because of the quality fluctuation under the isolation of EVs, The International Society for Extracellular Vesicles (ISEV) proposed Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines in 2014 and updated in 2018 (Thery *et al.*, 2018). It is required to optimize the way to isolate EVs and at least follow the guideline. Second, we need to explore how to transfer these EVs into particular target organs at high concentration. While MSCs have the ability of the homing by which MSCs may accumulate to the injured tissue in which MSCs may mediate therapeutic effect (Deak *et al.*, 2010), EVs do not have the homing effect. It is reported, using fluorescent-labeled EVs, that injected EVs were localized mainly in liver, spleen, lung and kidney (Takahashi *et al.*, 2013; Wiklander *et al.*, 2015; Lai *et al.*, 2014), suggesting that DDS approach using EVs is more likely to be advantageous in liver, lung

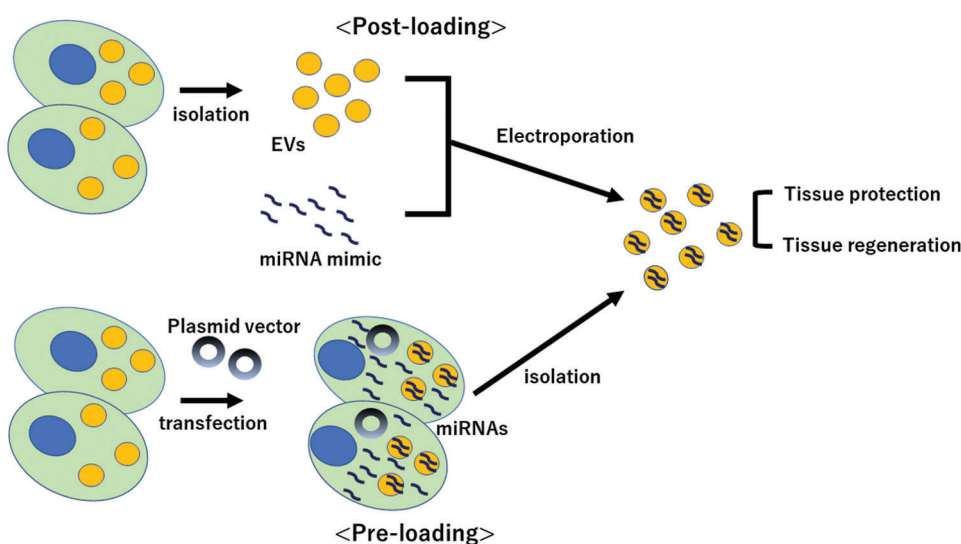


FIGURE 1. Scheme of two methods of loading miRNAs into extracellular vesicles. EVs; extracellular vesicles.

and renal diseases. In addition to this natural localization of injected EVs, it is also important to elucidate how to enhance EVs delivery to specific organs. For example, the method of mounting specific molecules on the surface of exosomes to increase delivery toward specific tissue was reported (Alvarez-Erviti *et al.*, 2011). Third, we need to clarify how to load and enclose the beneficial miRNAs in EVs. At present, there are two options, pre-loading method and post-loading method (Fig. 1). Pre-loading method is to use the gene editing system by plasmid vectors, in which modified cells strongly express miRNAs of interest and these miRNAs are enclosed in the secreted EVs. It is reported that the cargo of RNAs reflects the levels and types of cytoplasmic contents (Abels and Breakefield, 2016) and cells that overexpress specific miRNAs using plasmid vectors secrete EVs rich in that miRNAs (Kosaka *et al.*, 2013) and mediated regeneration (Yang *et al.*, 2020). Post-loading method is to load miRNA mimics into EVs for example by electroporation (Lv *et al.*, 2020) or calcium chloride-mediated transfection (Zhang *et al.*, 2017). On the other hand, for the pathogenic miRNAs, in which high expression may cause or worsen the tissue injury, the antisense of miRNAs may be useful to interfere the pathogenic miRNAs. Optimization of efficient loading of miRNAs is required.

In summary, the EVs treatment as ‘natural’ DDS might be the promising and novel options for a variety of diseases. To establish these therapies, we need to deepen the understanding of the properties and roles of EVs. Especially for clinical applications, there are several challenges about how EVs should be isolated, what sources of EVs should be used, how to enclose the contents, and how to add target specificity. Despite current limitations, it should open the next era of regenerative medicine for a variety of diseases.

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