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Extracellular vesicles derived from mesenchymal stem cells: A platform that can be engineered

Bo Qin[1], Qi Zhang[2], Dan Chen[2], Hai-yang Yu[2], Ai-xiang Luo[2], Liang-peng Suo[2], Yan Cai[3], De-yang Cai[2], Jia Luo[2], Ju-fang Huang[2,4] and Kun Xiong[2,4]

[1]Hubei Polytechnic University School of Medicine, Huangshi, Hubei, [2]Department of Neurobiology and Human Anatomy, School of Basic Medical Science, [3]Department of Histology and Embryology, Xiangya School of Medicine, Central South University, Changsha, Hunan and [4]Hunan Key Laboratory of Ophthalmology, Changsha, Hunan, China

Summary. Mesenchymal stem cells play an important role in tissue damage and repair. This role is mainly due to a paracrine mechanism, and extracellular vesicles (EVs) are an important part of the paracrine function. EVs play a vital role in many aspects of cell homeostasis, physiology, and pathology, and EVs can be used as clinical biomarkers, vaccines, or drug delivery vehicles. A large number of studies have shown that EVs derived from mesenchymal stem cells (MSC-EVs) play an important role in the treatment of various diseases. However, the problems of low production, low retention rate, and poor targeting of MSC-EVs are obstacles to current clinical applications. The engineering transformation of MSC-EVs can make up for those shortcomings, thereby improving treatment efficiency. This review summarizes the latest research progress of MSC-EV direct and indirect engineering transformation from the aspects of improving MSC-EV retention rate, yield, targeting, and MSC-EV visualization research, and proposes some feasible MSC-EV engineering methods of transformation.

Key words: Mesenchymal stem cells, Extracellular vesicles, Engineering, Targeting, Translational Medicine, Loading

Introduction

Stem cells are a type of cell that can renew themselves through symmetrical division and differentiate into a special cell type through asymmetric division (Shi et al., 2018). Among the different types of stem cells, mesenchymal stem cells (MSCs) have been most extensively studied, especially bone marrow-derived mesenchymal stem cells (BMSCs), which have received widespread attention and application in regenerative medicine and tissue engineering (Brooke et al., 2007; Lin et al., 2018; Qiu et al., 2018). Initially, researchers believed that MSCs could migrate and be transplanted into target tissues to repair damaged tissues or organs. However, subsequent studies have shown that MSCs are less efficient at reaching target tissues by the systemic route of administration, suggesting that the biological effects observed after systemic administration of MSCs may be due to the paracrine effects of the secreted factors, i.e., stem cells (Lucas-Ruiz et al., 2019). MSCs can play a role in affecting cell behavior through biologically active factors secreted by paracrine or endocrine mediators (Caplan and Dennis, 2006; Mukhamedshina et al., 2019), such as neuroprotection, neurogenesis, myocardial protection and inflammation inhibition (Lai et al., 2010; Yagi et al., 2010; Hsieh et al., 2013; Lin et al., 2018).

Extracellular vesicles (EVs) are a class of nano-sized vesicles (30-1,000 nm), surrounded by multi-membrane-bound phospholipids and derived from the interior of cells (Heijnen et al., 1999; Mittelbrunn and Sanchez-Madrid, 2012; Tetta et al., 2013). Almost all known cells secrete EVs, and the two main categories of EVs are exosomes and microvesicles (Deatherage and Cookson, 2012). Exosomes (30-150 nm) are intraluminal vesicles formed by the invagination of the membrane of multivesicular endosomes. The multivesicular endosomes that are formed can be fused with the plasma membrane of the cells and release small bubbles to the extracellular space in a manner that forms exosomes. Microvesicles (50-1,000 nm) are produced and secreted by the germination of the plasma membrane and they are a type of highly heterogeneous EVs (de Abreu et al., 2020). At present, size is still an important parameter for the classification of EVs. Based on this, EVs can be

divided into small EVs, medium EVs and large EVs (Thery et al., 2018). In this review, the term EVs refers to samples rich in small EVs, including exosomes but not microvesicles.

EVs are involved in many physiological and pathophysiological processes, including regulation of immune responses, maintenance of homeostasis, blood coagulation, inflammation, angiogenesis, and cancer progression (Ludwig and Giebel, 2012; Yanez-Mo et al., 2015). Stem cell-derived EVs can mediate cellular functional recovery and regulate inflammation. Studies have shown that mesenchymal stem cell -derived extracellular vesicles (MSC-EVs) have biological effects comparable to those of MSCs themselves and can mediate the paracrine effects of MSCs (Nawaz et al., 2016; Shao et al., 2018; Baek et al., 2019). A comparison of the therapeutic effects of MSC-EVs and MSCs shows that MSC-EVs are better able to act on damaged tissue, while MSCs appear to be a vehicle for these therapeutic effects (Moon et al., 2019). Furthermore, studies have shown that MSC-EVs play a bidirectional role between damaged cells and their progenitors: EVs from damaged cells stimulate stem cell differentiation, while EVs released from MSCs promote

regenerative mechanisms in viable cells after injury (De Jong et al., 2014; Zhang et al., 2020). MSC-EVs are generally biocompatible, have low immunogenicity and are non-cytotoxic, with a high loading capacity to cross the cytoplasmic and blood-brain barriers (BBB) (Zhuang et al., 2011; Yeo et al., 2013; Natasha et al., 2014) (Fig. 1). Also, MSCs can be obtained from peripheral blood, umbilical cord, bone marrow, and adipose tissue, so these advantages will help lay a solid foundation for MSC-EVs as a therapeutic option for tissue engineering and regenerative medicine (Timmins et al., 2012; Roura et al., 2015).

Currently, there are numerous reports indicating that engineered modifications (genetic manipulation, various physicochemical stimuli, loading of nanomaterials or drugs) can further enhance the efficacy of MSC-EV (Gowen et al., 2020). This review summarizes recent progress in the direct engineering and indirect engineering of MSC-EVs. As direct engineering modifications, the strategies are membrane penetration, surface functionalization or drug loading immediately after fresh extraction or thawing. Indirect engineering modifications are molecular or genetic strategies applied to the parent cells that secrete vesicles. By presenting the
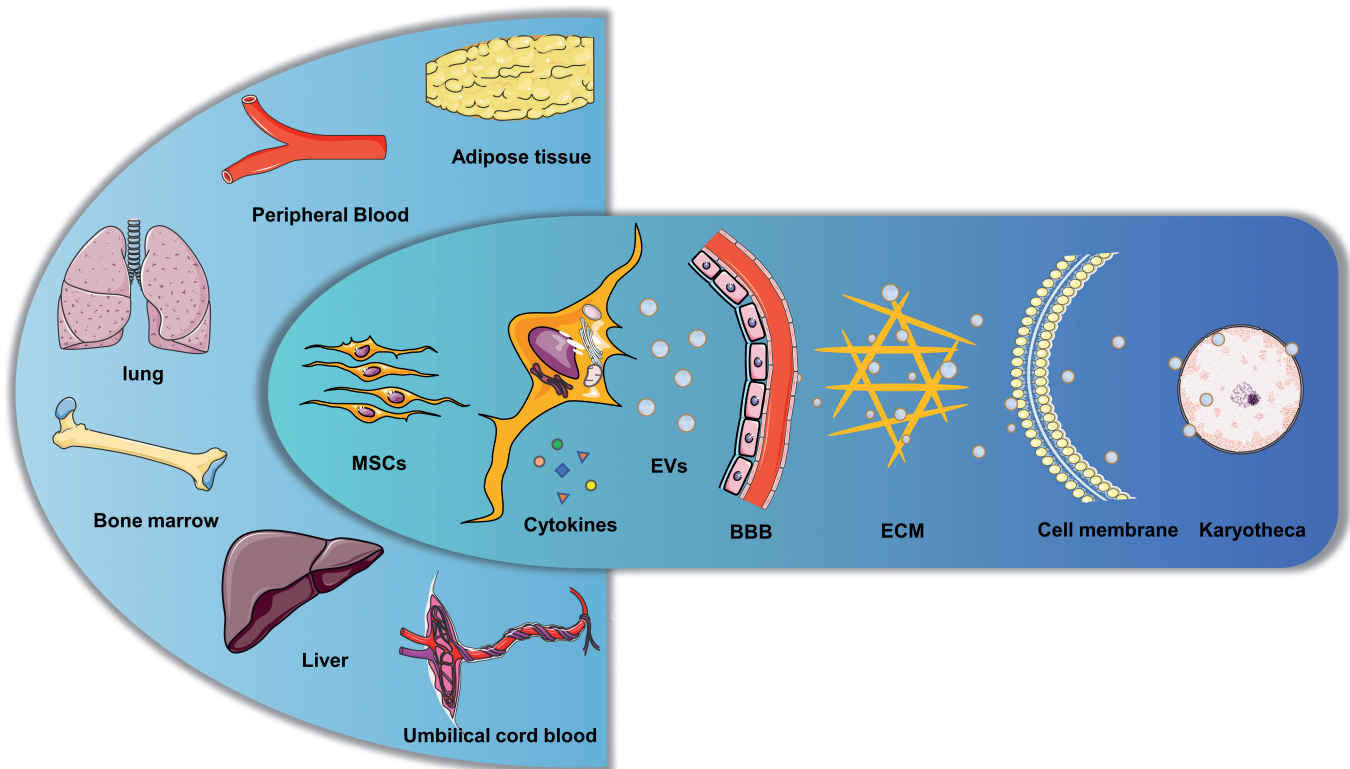

Fig. 1. Origin of MSCs and biological characteristics of MSC-EVs. MSCs are found in many tissues, such as bone marrow, adipose tissue, muscle or bone, brain, spleen, liver, kidney, lung, thymus, and pancreas. MSCs produce a variety of cytokines, growth factors, and EVs that influence other cells to produce inflammatory responses, promote proliferation of progenitor cells, improve tissue repair, and reduce infection. Among these, MSC-EVs play an important role in these repairs. MSC-EVs can cross the blood-brain barrier (BBB) and extracellular matrix (ECM), and finally reach target cells and enter the plasma and nuclear membranes.

latest results of engineering EVs, the stages of engineering transformation of MSC-EVs are clearly defined, which indicates the direction for the clinical application of MSC-EVs.

EVs derived from mesenchymal stem cells

Cargoes loaded by MSC-EVs

MSC-EVs can contribute to the repair of tissue injury by maintaining their stemness, inducing a regenerative phenotype, inhibiting apoptosis, and immunomodulation (Yin et al., 2020). These effects are mainly related to the biological molecules they carry (Fig. 2A). MSC-EVs have the signature proteins Alix and TSG101(tumor suppressor gene 101 protein), tetraspanin proteins, CD9 and CD81 (Lai et al., 2010, 2016), and also embed some proteins involved in the corresponding biological effects. These proteins are involved in cell-to-cell communication, structure, inflammation, biogenesis, development, tissue repair and

regeneration, and metabolism (Lai et al., 2012). For example, Wnt3a transported by BMSC-EVs binds to the LRP6 receptor and promotes dermal fibroblast proliferation *in vitro* (McBride et al., 2017). Similarly, MSC-EVs differentiated from embryonic stem cells can deliver CD73 protein *in vitro*, activate AKT and ERK signaling, and increase chondrocyte viability (Zhang et al., 2018a). MSC-derived exosomes enhance transcription of IϰBα via MZF1 and inhibit phosphorylation of IϰBα via metallothionein-2, thereby suppressing macrophage inflammation and NF-ϰB activation. In addition, all enzymes involved in glycolytic ATP synthesis (glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglucan mutase (PGM), enolase (ENO), and pyruvate kinase m2 subtype (PKm2)) and the rate-limiting glycolytic enzyme PFKFB3, which upregulates fructose phosphokinase, were found in MSC-derived exosomes.

The RNA carried by mesenchymal stem cells-EVs has attracted widespread research interest because EVs
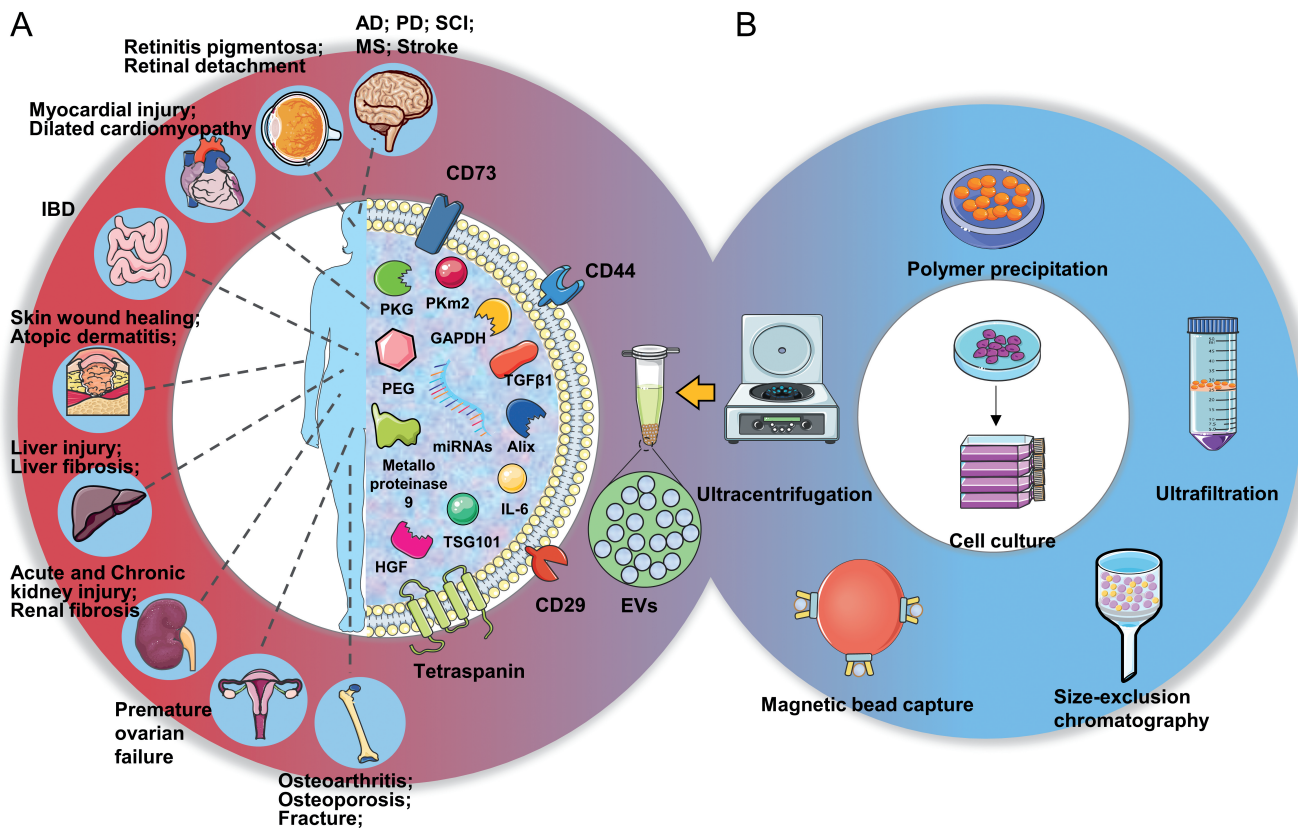

Fig. 2. Separation and purification methods of MSC-EVs and its content and application of disease model. **A.** MSC-EVs in addition to the general EV marker proteins (tetraspanin, CD44, CD29, CD73), there are glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglucose mutase (PGM) and some special miRNAs. The treatment based on MSC-EVs has been successfully applied to various diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), spinal cord injury (SCI) and inflammatory bowel disease (IBD) etc. **B.** MSC-EVs can be obtained by ultracentrifugation, ultrafiltration, magnetic bead-based immunocapture, size-exclusion chromatography, and polymer sedimentation. Obtaining sufficient cell culture supernatant to obtain EVs by culturing cells in large quantities;

reportedly carry mRNAs and microRNAs (miRNAs) that can be transferred to other cells, where the RNAs can induce biological responses in the recipient cells (Valadi et al., 2007; Skog et al., 2008). Mathiyalagan et al. found that exosomes derived from CD34+ MSCs can transport miR-126-3p to vascular endothelial cells *in vitro*, regulating the expression of VEGF (vascular endothelial growth factor), angiogenin 1/2, matrix metalloproteinase and thromboxane 1 (Mathiyalagan et al., 2017). In the rat optic nerve crush model, intravitreal injection of BMSC-derived exosomes can promote retinal ganglion cell survival and axon regeneration through miRNA transfer, thereby promoting the restoration of visual function (Mead and Tomarev, 2017).

Fully understanding the characteristics of nucleic acids carried by MSC-EVs is the prerequisite for their reasonable application in clinical treatments. Studies have shown that most of the RNA carried by MSC-EV on agarose or polyacrylamide gels are less than 300 nucleotides (Chen et al., 2010). Compared with other cell-derived EVs, 18s or 28sRNA was not detected in MSC-EVs, and it can be seen that these RNAs are shorter (Smalheiser, 2007; Valadi et al., 2007). Only a small fraction of miRNAs found in MSCs were reported to be secreted in MSC exosomes, suggesting that the secretion of miRNAs by MSCs is a regulatory process. Notably, many miRNAs are pri-miRNAs and pre-miRNAs, which are immature miRNAs (Toh et al., 2018). Also, after deep sequencing, the RNA of MSC-derived exosomes was found to be a highly heterogeneous RNA population, approximately 100 nts (Lai et al., 2016). In MSC exosomes, RNA produced a total of 151.13 million read operations, of which only 1.4 million read operations (0.9%) were miRNA (Pritchard et al., 2012). Exosomes, a subtype of EVs, carry RNAs with an average length of 100 nt. These RNAs (except miRNAs) are essentially their mapped RNA fragments and are unlikely to affect biological function (Toh et al., 2018).

MSCs-EVs as a 'cell-free' therapeutic

Previous studies have shown that MSC-based therapies are safe, but in recent years, reports have indicated that there are potential risks associated with the treatment of MSCs, such as microvascular occlusion, transformation of MSCs into cancer cells, and proarrhythmic side effects (Chang et al., 2006; Togel et al., 2007; Furlani et al., 2009; Jeong et al., 2011). For example, it has been reported that the differentiation of BMSCs into chondrocytes and osteocytes can cause tissue heterotopic ossification or calcification (Breitbach et al., 2007). However, MSCs-EVs do not suffer from these problems, and the techniques and processes for the separation and purification of EVs are scalable, and MSC-EVs can be engineered.

MSC-EVs have been used *in vivo* for tissue regeneration and treatment of several disease types, such as respiratory, kidney, liver, nerve, musculoskeletal and cardiovascular systems (Fig. 2A/Table 1) (Martin-Jaular et al., 2016; Zhang et al., 2016b; Eirin et al., 2017; Haga et al., 2017). Although most of the research on MSC-EVs is still in the preclinical model stage or early clinical stage (Lener et al., 2015; Zhang et al., 2018b; Gowen et al., 2020), the therapeutic results generated by

Table 1. Application of MSC-EVs in disease models.

EVs source	Separation method	Disease model/Target tissue	Dose	References
Animal species				
Mouse BMSCs	Differential centrifugation	Heart/IR	50 µg MSC-exosome	Zhao et al., 2019
BMSCs of adult wistar rats	Ultracentrifugation	Brain/Axonal compartment	3×10^8 MSC-exosome	Chang et al., 2018
Mouse BMSCs	ExoQuick-TCTM	Cancer/Breast cancer	100-200 µg of MSC-derived exosomes	Lee et al., 2013
Rat BMSCs	ExoQuick	Heart/Infarct	4×10^6 MSCs	Yu et al., 2015
Rat BMSCs	Ultracentrifugation	Kidney/Gentamycin	100 µg	Reis et al., 2012
Mouse BMSCs	PEG-S200	Lung/Hypoxia	0.1-10 µg	Lee et al., 2012
Rat BMSCs	ExoQuick	Brain/TBI	100 µg	Zhang et al., 2015b
Rat BMSCs	Exosome isolation reagent	Heart /IR	10 μg/mL	Liu et al., 2017a
Human				
BMSCs	Ultracentrifugation	Cancer/Pancreatic Cancer	0.4×10^5 MSCs	Wu et al., 2019
BMSCs	Ultracentrifugation	Bone/IVD	1.5×10^6 particles	Cheng et al., 2018
BMSCs	Differential centrifugation	Brain/Stroke	2×10^6 MSCs	Doeppner et al., 2015
UCMSCs	Ultrafiltration	Bone/Stabilized fracture	100 µg/mL	Zhang et al., 2019
BMSCs	Ultracentrifugation	Skin/Chronic wounds	10 µg/mL	Shabbir et al., 2015
BMSCs	PEG	Brain/Ischemia	$2\times2\times10^7$ MSCs	Ophelders et al., 2016
hiPSC-MSCs	Ultracentrifugation	Skin/Wounds healing	100 µg/mL	Zhang et al., 2015a
UCMSCs	Differential centrifugation	Eye/Macular holes	50 µg or 20 µg	Zhang et al., 2018b
BMSCs	Ultracentrifugation	Cancer/Breast cancer	80 µg/mL	Pakravan et al., 2017
UCMSCs	Ultracentrifugation	Gastrointestinal tract/IBD	200 µg	Ma et al., 2019

PEG: Polyethylene glycol; PDAC: Pancreatic ductal adenocarcinoma; IVD: intervertebral disc degeneration; TBI: traumatic brain injury; IR: Ischemia-reperfusion; BMSCs: Bone Marrow Mesenchymal Stem Cells; hiPSC-MSCs: human induced pluripotent stem cell-derived mesenchymal stem cells; UCMSCs: umbilical cord MSCs.

MSC-EVs are promising. For example, MSC-EVs carrying miR-146a-5p inhibit the levels of type 2 congenital lymphocytes and the infiltration of pneumocytes, thereby reducing Th2 cytokine levels and airway hyperresponsiveness in mice with asthma (Fang et al., 2020). At the same time, exosomes derived from umbilical cord mesenchymal stem cells can increase the expression of vascular endothelial growth factor and hypoxia-inducible factor-1α, promote the proliferation, migration and microtubule formation of human umbilical vein endothelial cells, as well as encourage the differentiation of osteoblasts (Zhang et al., 2019). In another study on osteoblasts, Yang et al. found that exosomes derived from bone marrow-derived mesenchymal stem cells mediate the miR-34c/SATB2 axis via lncRNA MALAT1 to enhance osteoblast viability in osteoporotic mice (Yang et al., 2019). MSC-EVs have shown beneficial therapeutic effects in many different diseases, but MSC-EVs have two effects on tumors: tumor-promoting and antitumor effects (Lin et al., 2016; Wu et al., 2019). So far, MSC-EV-based treatment has not seen any side effects, but further research is needed to confirm its clinical safety (Lener et al., 2015). In order to fully consider the advantages of cell-free therapies, in addition to tools to determine their concentration in samples (quantification) (Hartjes et al., 2019), molecular size, and molecular composition (characterization), the therapeutic dose of MSC-EVs needs to be further investigated.

Challenges faced by MSC-EVs applications

Applications of stem cell therapy have focused on the differentiation of transplanted bone marrow MSCs and the potential of BMSCs to suppress antitumor immune responses, as well as their potential to act as vascular progenitor cells that may promote tumor growth and metastasis. Additionally, the tumorigenicity, immunogenicity, and genomic variability of MSCs hinder their development (Furlani et al., 2009; Jeong et al., 2011). Fortunately, MSC-EVs are not affected by the above restrictions (Toh et al., 2018). EVs do not have the characteristics of these problems that, for many researchers, make them attractive candidates for therapeutic agents.

It has been reported that the uptake of exosomes saturates with dose and time and achieves a transduction efficiency close to 100% at picomolar concentrations (Heusermann et al., 2016). In the study of retinal ischemic therapy, MSC-EVs also show dose-dependent characteristics (Mathew et al., 2019), so we need to further determine the optimal clinical dose of MSC-EVs. Although there are many methods to isolate EVs, there is a lack of isolation methods that can meet the needs of large-scale clinical practice. There are five main methods for the separation of EVs. (1) differential centrifugation, (2) density gradient ultracentrifugation, (3) size-exclusion chromatography, (4) polymerization precipitation, and (5) immunocapture (Gardiner et al.,

2016; Coumans et al., 2017; Doyle and Wang, 2019; Hartjes et al., 2019; Ludwig et al., 2019) (Fig. 2B). Standard differential centrifugation requires 4 - 5 consecutive centrifugation steps to extract EVs, and the non-standardization of these methods severely hinders the yield of EVs (Haraszti et al., 2018). When MSC-EVs are used for functional studies by injection, they can be removed from the circulatory system or removed from the target area because the EVs in these aqueous solutions are difficult to contain in the target area, and the low retention rate, poor stability, and duration of action of MSC-EVs affect their clinical translation and application (Imai et al., 2015). Meanwhile, rapid and accurate methods of quantification and characterization of EVs, accurate characterization of the content of the load, pharmacokinetics and targeting are all obstacles to the effective clinical application of MSC-EVs (Gowen et al., 2020).

Engineering of MSC-EVs

Currently, the most common strategies for engineering EVs are direct modification of isolated and purified EVs, or modification of EV-secreting parent cells using nanotechnology (Susa et al., 2019). By modifying the isolated EVs, specific parts of the EVs' surface are physically or chemically functionalized to improve their targeting or biodistribution ability and facilitate tracing *in vivo* and *in vitro* (Armstrong et al., 2017). EVs can be successfully designed as carriers for transporting different types of cargo (drugs, active molecules, nucleic acids, nanoparticles, etc.) for imaging, tracking, or treatment of other diseases such as cancer (Fig. 3) (Mentkowski et al., 2018).

Currently, there are several methods for incorporating drugs into EVs. The method of loading exogenous EVs requires first separating the vesicles and then following different procedures to load the drug sequentially (Antimisiaris et al., 2018; Bunggulawa et al., 2018). Common loading methods include co-culture of EVs with the required content (Fuhrmann et al., 2015), electroporation (Tian et al., 2014; Nakase and Futaki, 2015), freeze-thaw cycles (Haney et al., 2019), and ultrasound (Haney et al., 2015). Electroporation is based on the application of an electric field to an EV solution that creates nanometer-sized pores in the phospholipid membrane of the vesicle, allowing diffusion of the desired drug, small interfering RNA (siRNA), or DNA, and maintaining the biological activity of the cargo (Alvarez-Erviti et al., 2011; Wahlgren et al., 2012; Lamichhane et al., 2015). However, electroporation can change the physical properties of EVs. It is only suitable for small molecules, and small molecules can also aggregate and adhere to the surface of EVs (Mentkowski et al., 2018). A series of freeze-thaw cycles of EVs ruptures their membranes, which can be used as an exogenous loading method. Due to the formation of ice crystals, membrane rupture or deformation induces the encapsulation of relatively large

molecules, such as proteins and nanoparticles, without affecting their biological activity (Haney et al., 2019). An alternative method of loading hydrophilic compounds into EVs is ultrasound. The agent is mixed with exogenous molecules through vesicles and exposed to ultrasound. By disrupting the lipid membrane, molecular binding will occur within the EVs as the membrane is automatically rebuilt (Jiang and Gao,

2017), and this method prevents the aggregation of sensitive contents such as siRNA (Lamichhane et al., 2016).

MSC-EVs have both the characteristics of most EVs and their deficiencies. The most obvious is that their residence time in the damaged area is not long, and they are easily cleared by the body (Imai et al., 2015). Additionally, the secretion of EVs from MSCs in
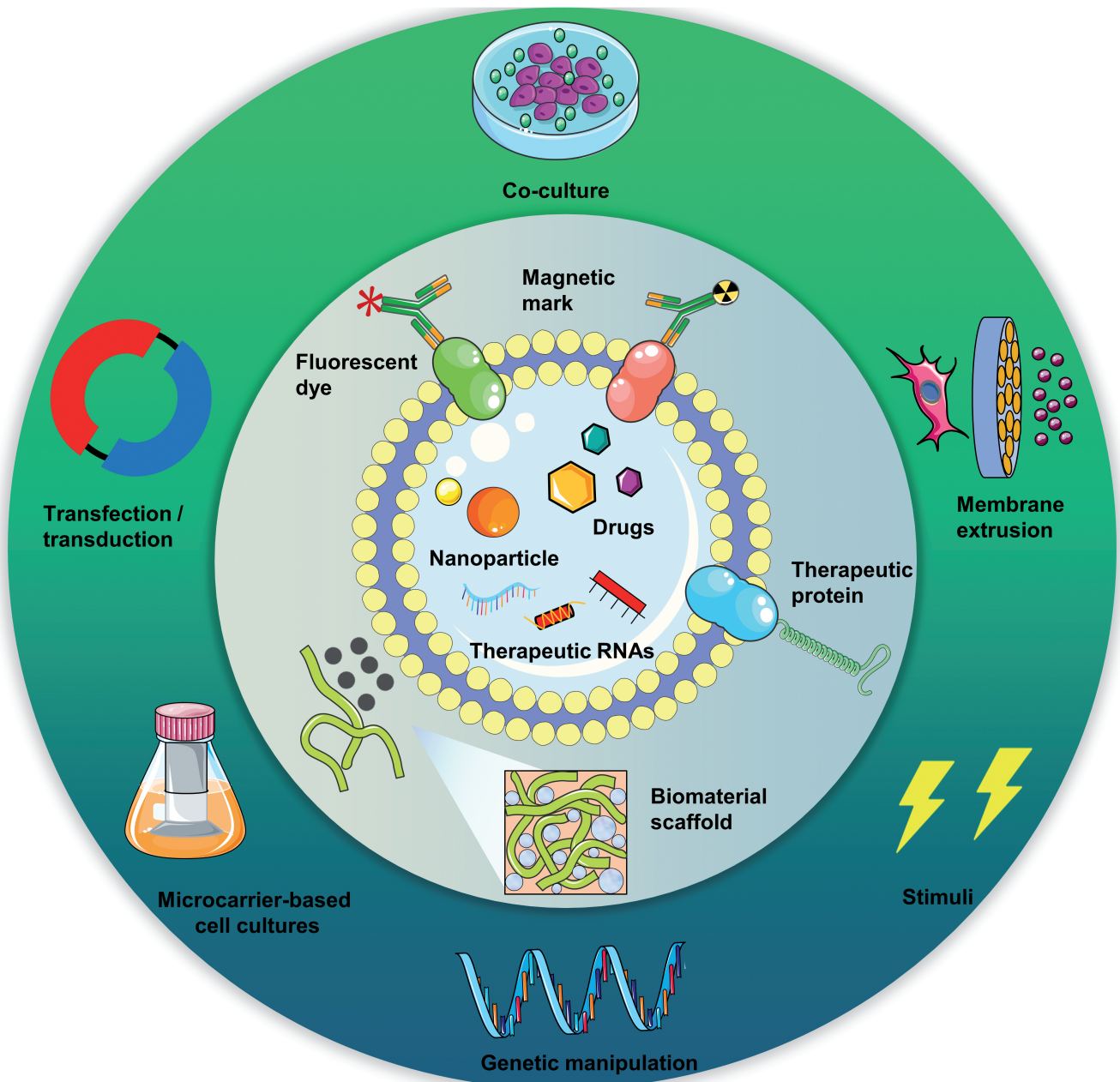

Fig 3. Strategies for MSC-EV engineering transformation. Obtaining optimized MSC-EVs through indirect engineering (parent cell engineering). Common methods include co-cultivation, membrane extrusion, transfection, genetic manipulation, microcarrier-based cell culture, and various physical and chemical stimulations. Also, MSC-EVs can be loaded directly with therapeutic nucleic acids, proteins, nanoparticles, or biological scaffolds.

Progress on the engineering of extracellular vesicles derived from mesenchymal stem cells

efficient differentiation generation has therapeutic effects, and the isolation and purification steps of EVs cannot be expanded. Therefore, the low yield of MSC-EVs is a fact that cannot be ignored. Furthermore, MSC-EVs have poor targeting properties. It has been pointed out that after MSC-EVs are introduced into animals, a large proportion of EVs are concentrated in the liver and spleen, but are distributed in target tissues (Wen et al., 2019). Based on these deficiencies of MSC-EVs, researchers have engineered MSC-EVs or parent cells to compensate for these deficiencies and are therefore better suited for the development of "cell-free" therapies (Table 2).

Improving retention rate and stability of MSC-EVs

Due to the high intracardiac pressure, treatment of EVs still presents a problem of temporary retention and escape into the pulmonary circulation (Gallet et al.,

2017; Liu et al., 2018). Many studies have shown that the mononuclear phagocyte system confers a short half-life (1-6 hours) on EVs due to its opsonization and accumulation in the spleen and liver (van der Meel et al., 2014; Armstrong and Stevens, 2018). Besides, since the regeneration process usually takes a long time, the activity of EVs cannot be maintained and their function may be impaired. Therefore, the development of biocompatible scaffold materials that can maintain the function and sustained release of EVs is essential for the clinical application of MSC-EVs. These biomaterials are biodegradable, have low immunogenicity, and relatively small pore size, making them suitable for drug and vaccine carriage and encapsulation. The more common biomaterials at present are fibrin glue (Blazquez et al., 2018), surgical grid (Blazquez et al., 2018), bioscaffold (Chen et al., 2019), hydrogel (Mardpour et al., 2019; Zhou et al., 2019) or modified hydrogel (Lv et al., 2019).

Hydrogels are a commonly used biomedical

Table 2. Overview of MSC-EV engineering transformation and its application.

Engineering purpose	Engineering method	Loading drug	Engineering type	Application	Reference
Increase retention rate	Electrostatic interaction	Multifunctional poly-saccharide-based dressing	Direct engineering	Wound Healing/ Skin Reconstruction	Wang et al., 2019c
	Encapsulate	Functional Peptide Hydrogels	Direct engineering	Cardiac Repair	Han et al., 2019
	Embedding	Photoinduced imine crosslinking hydrogel glue	Direct engineering	Articular Cartilage Regeneration	Liu et al., 2017b
	Ultrasonic concussion	Bio-Ppy-Ti	Direct engineering	Osteoinductivity	Chen et al., 2019
	Embedding	Fibrin glue	Direct engineering	Tendon regeneration	Yu et al., 2020
Improve targeting	Covalently conjugated	LJM-3064 Aptamers	Direct engineering	Bone regeneration	Hosseini Shamili et al., 2019
	Co-incubation	Venofer	Direct engineering	Tumor cell ablation	Altanerova et al., 2017
	Co-transfection	miR-222 / 223	Parent cell engineering	Breast cancer treatment	Bliss et al., 2016
	Co-incubation	Phosphatase and Tensin Homolog siRNA	Direct engineering	Spinal Cord Injury	Guo et al., 2019
	Co-transfection	miRNA-379	Parent cell engineering	Breast cancer treatment	Guo et al., 2019
	Gene knockdown	p53	Parent cell engineering	Gastric cancer	Mao et al., 2017
Biological distribution	Co-incubation	Near-infrared dyes	Direct engineering	Acute kidney injury	Grange et al., 2014
	Co-incubation	DiD	Direct engineering	Radiation injury	Wen et al., 2019
	Co-incubation	PKH-26A	Direct engineering	Crush injury	Bucan et al., 2019
	Co-incubation	DIO	Direct engineering	Neointimal hyperplasia	Wang et al., 2019b
	Membrane extrusion	Gadolinium, or a near infrared dye	Direct engineering	Osteosarcoma	Abello et al., 2019
Large-scale production of MSC-EVs	Co-culture	3D graphene scaffold	Parent cell engineering	Alzheimer's Disease	Yang et al., 2020a
	Co-culture	microcarrier	Parent cell engineering	Huntingtin disease	Haraszti et al., 2018

material. Due to the typical three-dimensional cross-linked network with high drug/cell embedding rate, hydrogels can act as a natural matrix barrier, locking EVs and preventing their rapid loss, thus significantly increasing the utilization of MSC-EVs (Lv et al., 2019). The research of Han et al. further proves this point. When human umbilical cord-derived MSC-EVs (UCMSCs-EVs) were loaded into functional peptide hydrogels, a sustained release profile and heart regeneration could be observed (Han et al., 2019). Specifically, EVs/hydrogel complexes improve myocardial function in rats by reducing inflammation, fibrosis, and apoptosis and promoting angiogenesis in the infarct marginal zone (Han et al., 2019). Also, it was found that when BMSC-EVs were loaded into matrix metalloproteinase-2 (MMP2)-sensitive self-assembling peptide (KMP2) hydrogels, EV-loaded KMP2 hydrogels had better efficacy than KMP2 or EVs alone in promoting endothelial cell proliferation and angiogenesis, subsequently reducing chronic renal fibrosis in I/R mice (Zhou et al., 2019).

When human embryonic MSC-EVs are embedded in PEG (polyethylene glycol) hydrogels, they have better anti-apoptotic, anti-fibrotic, and regenerative properties than conventionally injected EVs (Mardpour et al., 2019). However, conventional hydrogels generally lack kinetic properties such as self-healing and injectability, and these hydrogels are not ideal scaffolds for reconstructing damaged areas. Therefore, researchers developed a composite hydrogel consisting of Pluronic F127, oxidized hyaluronic acid, and EPL (denoted as FHE hydrogel) with strong water retention, biocompatibility, and adhesion properties (Silva et al., 2018; Wang et al., 2019a). Another study endowed hydrogels with good ultra violet shielding properties and showed the regulation of the release of EVs through pH (Wang et al., 2019c).

In addition to hydrogels, the application of EVs with other types of biomaterials has also shown promising therapeutic effects. For example, using ultrasonic vibration to anchor MSC-EVs to the surface of biotin-doped polypyrrole titanium (Bio-Ppy-Ti), EV-Bio-Ppy-Ti showed enhanced cytocompatibility and osteoclast osteoconductive properties *in vitro*, with anti-apoptotic capacity in an ectopic bone formation mode (Chen et al., 2019). And some organic biomaterials can also serve as biological scaffolds for MSC-EVs. Zhang et al. activated the PI3K/Akt signaling pathway by loading BMSC-EVs on tricalcium phosphate scaffolds to promote bone healing in cranial bone defects (Zhang et al., 2016a). It has also been reported that the loading of MSC-EVs onto heparin-modified electrospun poly(ε-caprolactone) can effectively inhibit thrombosis and calcification, thereby improving the patency of vascular grafts (Wei et al., 2019). In order to solidify the biomaterial in the target area, the researchers modified Poly (lactic-co-glycolic acid) (PLGA) with polydopamine to achieve the purpose of slowly releasing exosomes derived from human adipose-derived stem cells (Li et al., 2018).

Targeting and drug loading of MSC-EVS

From recent MSC-EVs engineering transformation results, the most common approach is to directly engineer the isolated and purified EVs. Another strategy is to engineer MSCs to produce modified component EVs, one of the goals of which is to make them targetable, especially in cancer research. The cyclic (Arg-Gly-Asp-D-Tyr-Lys) peptide is a well-known targeting ligand for cancer chemotherapy with high affinity for the surface overexpressed αvβ3 integrin receptor (Wang et al., 2017; Jia et al., 2018). It was found that Arg-Gly-Asp (RGD) peptide modification of isolated exosomal membranes by a donor cell-assisted membrane modification strategy exhibited enhanced targeting of blood vessels (Wang et al., 2017). MSC-EVs also face the problem of poor targeting, short residence time, or insufficient residence time in target tissues. In the direct engineering of EVs, the researchers covalently coupled a myelin-specific DNA aptamer (LJM-3064) with the amine group on the surface of MSC-derived exosomes and inhibited the inflammatory response of the nervous system, and reduced areas of demyelinating lesions (Hosseini Shamili et al., 2019). The c (RGDyK) has a high affinity for αvβ3 integrin receptors that are overexpressed on the surface (Jia et al., 2018). Based on this knowledge, the surface of exosomes derived from embryonic stem cells was modified with c (RGDyk) peptides, which can be used for the treatment of gliomas (Zhu et al., 2019).

Apart from the direct engineering of stem cell-derived EVs, several indirect engineering strategies have emerged. The most common approach is to engineer the parent cell to load drug-loading molecules or to complete membrane functionalization. Cellular engineering approaches, such as genetic and metabolic modifications and exogenous drug delivery, can alter the surface expression and drug-loading capacity of newly generated EVs, thereby improving their biocompatibility, targeting, and therapeutic capacity (Mentkowski et al., 2018). For example, ion-treated MSCs use extrusion to produce exosome-like vesicles loaded with iron oxide nanoparticles. Vesicles can transport these engineered exosomes to the area of spinal cord injury under the action of external magnetic navigation, thereby promoting angiogenesis of endothelial cells, anti-apoptosis of neuronal cells, and stimulating therapeutic growth of astrocytes. Factors and polarization resist macrophage inflammation from inflammatory M1 to tissue repair M2 (Kim et al., 2018). Also, ion-treated MSC-EVs tend to be richer in more therapeutic growth factors (Kim et al., 2020). In the study of tumors, the researchers found that MSCs expressing the yeast cytosine deaminase and phosphoribose transferase suicide fusion gen (yCD :: UPRT) can be labeled with venofer (iron oxide carbohydrate nanoparticles). These exosomes labeled with venofer can be effectively internalized by tumor cells, and then use an external alternating magnetic field

to induce hyperthermia, thereby promoting the ablation of targeted tumor cells through magnetically induced high temperature (Altanerova et al., 2017).

Co-transfection of target genes into MSCs is also a common method for indirect engineering of EVs. MiR-379, a potent breast cancer suppressor, was transfected into MSCs by lentiviral transfection to promote secretion of miR-379-rich EVs from MSCs, which inhibit tumor formation and growth, in part by regulating COX-2 (O'Brien et al., 2018). In another breast cancer study, transfection of miR-222/223 inhibitors into MSCs promoted the secretion of exosomes containing miR-222/223 inhibitors from MSCs. This prevented certain subpopulations of tumor cells from entering a quiescent state, thereby increasing the sensitivity of tumor cells to the chemotherapeutic agent carboplatin (Bliss et al., 2016).

There have been several reports on the production of EVs for the purpose of altering the culture environment of MSCs or pretreating MSCs with drugs. Studies have shown that pretreatment of human bone marrow MSCs with hypoxia can improve their bioactivity *in vitro* (Hu et al., 2014), and hypoxia preconditioning improved the effectiveness of MSCs transplantation for the treatment of myocardial infarction in nonhuman primates without increasing the occurrence of arrhythmogenic complications (Hu et al., 2016). Interestingly, hypoxia has a positive effect on the efficacy of secretory EVs. Hypoxia-induced human bone marrow MSC-EVs (1% O_2 or 72 h) showed higher myocardial regeneration than rat bone marrow MSC-EVs isolated under normoxia in a rat model of myocardial infarction. The mechanism is by increasing angiogenesis in the infarct area (Bian et al., 2014). Furthermore, hypoxic repair of mice and rats BMMSC-EVs (1% O_2 or 72 h or 0.5% O_2 or 24 h) prevents cardiomyocyte apoptosis by enrichment of miR-125b-5p-EVs and miR-210-EVs. The related mechanism is to inhibit the expression of pro-apoptotic gene p53 and BCL2 antagonist/killing factor 1 (BAK1), and increase the recruitment of cardiac progenitor cells in the infarcted heart (Zhu et al., 2018a,b). In addition to regulating O2, there are also reports that NO stimulation can also have a beneficial effect on MSC-EVs (Du et al., 2017). In conclusion, a specific microenvironment designed for *in vitro* stem cell culture, such as a microenvironment containing biologically active substances, will help develop customized vesicles.

Large-scale production of MSC-EVs

Current methods of isolating and purifying EVs result in low yields and are not scalable, especially in the case of MSCs, and methods for isolating EVs need to be easily scalable to support large-scale production. Therefore, current technical challenges also hinder the effective evaluation of MSCs-EVs-based preclinical therapies (Reiner et al., 2017; Colao et al., 2018). Two-dimensional (2D) culture techniques are currently the most commonly used method for cell maintenance and expansion. Long-term by producing a constant number of EVs may result in the loss of cloning and differentiation capacity of the cells. It has been found that 10^6 cells can produce 1-4 μg of exosomes per day, whereas MSCs form only a few exosomes (Katsuda et al., 2013). Other studies have shown that each mouse usually requires 10^9-10^{11} vesicles to obtain biological results (Didiot et al., 2016; Wen et al., 2016, Kamerkar et al., 2017; Pi et al., 2018). Therefore, there is an urgent need to develop reliable MSC or EV expansion methods for clinical application. The current expansion of MSC culture is labor-intensive, such as mass culture of MSCs. Common strategies are (1) traditional tissue culture techniques in flasks (Nekanti et al., 2010; Oliver-Vila et al., 2016) and (2) three-dimensional (3D) culture bioreactors that are constructed using polysulfone hollow fibers with semi-permeable membranes (Mennan et al., 2019). Unfortunately, the current production methods of EVs result in low production and cannot be expanded, which hinders the progress of preclinical and clinical applications of EVs as therapeutic drugs. Large-scale production of EVs uses large or multi-layer culture flasks, stirred tank bioreactors, or perfusion reactors for continuous production (Colao et al., 2018). Compared with traditional planar cell culture in flasks, most of these methods aim to increase the yield of EVs by maximizing the culture surface area (Haraszti et al., 2018).

A 2D culture system is a basic model for studying tissue physiology and complex biological activities from cell differentiation to tissue morphogenesis, and is generally regarded as the "gold standard" for cell model culture (Lampe et al., 2010). Monolayer cells under 2D culture conditions have aspects such as cell morphology, cell-cell interaction, growth behavior, and interaction with the extracellular matrix. Due to the different nature of the microenvironment in tissue structures (such as mechanical and biochemical properties), the cell monolayer cannot represent the physiology of living tissues or organs (Thippabhotla et al., 2019). 3D cell culture is considered to mimic cell behavior *in vivo*. The approach to 3D cell culture involves not only cell extension and interaction from various angles, but also cell and ECM interaction, rather than only through cell-cell interactions at the edge (Pampaloni et al., 2007).

3D cell culture technology not only isolates vesicles similar to EVs secreted by *in vivo* cells, but also greatly increases the yield of EVs. Combined with conventional differential ultracentrifugation, 3D culture produces 20% more exosomes than 2D culture. When 3D cell culture was combined with tangential flow filtration, the yield of Waldensium-derived exosomes was 7 times higher than that of regular 3D culture (Thippabhotla et al., 2019). Two recent studies have shown that mesenchymal stem cell and cardiac progenitor cell-derived EVs can be prepared on a large scale from bioreactors or HyperStack systems as vesicular preparations for medicinal products for future clinical translation (Andriolo et al., 2018; Mendt et al., 2018). Both studies show that neither the

cells nor the phenotype of EVs change during cell culture, but when extended to HyperStack systems and bioreactors, they can meet short-term clinical needs. Finally, PEG precipitation has been used in clinical studies of MSC-EV purification (Kordelas et al., 2014). Although the precipitation method is simple and is only used to precipitate EVs rather than purify them, precipitation is still a viable option for large-scale production. In addition to cell culture in a 3D environment to obtain more EVs, it has been reported that nitrogen cavitation (a physical force) and cellular nanoporation can significantly increase the yield of EVs or exosomes (Gao et al., 2017; Yang et al., 2020b). Although the EVs obtained by these methods are not derived from MSCs, the results of these methods are considerable in terms of increasing yield and imparting functionality. Perhaps this approach can also be applied to increase the production of MSC-EVs.

Visualization and biodistribution of MSC-EVs

As effective carriers for cell-free therapies, it is necessary to monitor the distribution of MSC-EVs in living organisms. There are many methods for labeling EVs, including lipophilic dyes and membrane-penetrating compounds. Common lipophilic dyes are PKH-67/PKH-26, DiI/DiD/DiO/DiR (Abello et al., 2019; Chew et al., 2019; Wang et al., 2019b); and permeable compound markers are carboxy fluorescein diacetate succinimide ester (van der Vlist et al., 2012) and calcein-acetyl methyl ester (Gray et al., 2015). Also, there are ways to label the sulfhydryl groups on the surface of EVs, such as C5-maleimide-Alexa 488/C5-maleimide-Alexa 633 (Roberts-Dalton et al., 2017), and there are reports of using radioactive material 99mTc-HMPAO to label EVs (Hwang et al., 2015).

Researchers have used different dyes to track the biodistribution of EVs after administration, and Wen et al. used lipid dye-labeled MSC-EVs to assess their distribution under different conditions in mice. DiD-labeled MSC-EVs were predominantly distributed in the liver and spleen, and to a lesser extent in the bone marrow of the spine, femur, and tibia (Wen et al., 2019). In a mouse model of acute kidney injury, the researchers labeled MSC-EVs and blasts with near-infrared (NIR) dye (Grange et al., 2014). They found that EVs could be detected in whole-body images and dissected kidneys using optical imaging techniques, and they also found that MSC-EVs accumulated in the kidneys of mice with acute kidney injury but not in controls, and that EVs labeled directly with NIR dyes had higher and brighter fluorescence than those secreted by labeled MSCs. The main reason is that MSCs can be recruited to target tissues through receptor-mediated interactions (Herrera et al., 2007), and MSC-EVs lipid membranes have membrane receptors that are the same as parent cells themselves, so these vesicles can be recruited through the same mechanism injury site (Grange et al., 2014).

In another report, the researchers used gadolinium or near-infrared dye to label human umbilical cord (HUC) MSC-EVs. Systemic HUC-MSC exosomes injection will continue to accumulate in the tumor after 24-48 hours. In contrast, the synthesized lipid nanoparticles only accumulated in the tumor in the first 3 hours after injection (Abello et al., 2019). Bucan et al. evaluated the effect of rat fat-derived MSC-EVs by PKH-26A on sciatic nerve regeneration and axon growth and found that rat fat-derived MSC-EVs gathered in the target area and promoted regeneration *in vivo* after sciatic nerve injury (Bucan et al., 2019).

To further achieve the visualization effect, the engineering transformation of MSC-EVs can better display their distribution in the target area. Glucose-coated gold nanoparticles can be absorbed and secreted into exosomes by MSCs, while 5 nm gold nanoparticles can improve exosome labeling. Intranasal administration leads to better brain accumulation than intravenous injection (Betzer et al., 2017). Furthermore, a study by Moon, et al. investigated the biodistribution, therapeutic effect, and mode of action of MSC-EVs in a preclinical stroke rat model. In this study, PKH-26 or 5- (and-6) - carboxy fluorescein diacetate succinimide ester labeled EVs were used for *in vivo* tracking (Moon et al., 2019). Flow cytometry was used to identify and count EVs, and nanoparticle tracking analysis was used to measure size and morphology. They found that MSC-EVs migrated to the infarcted brain. Although MSC-EVs accumulate in the infarcted brain in a dose-dependent manner, as the dose increases, the injected MSCs accumulate in the lungs and liver, again stressing that MSCs rarely reach the target tissue (Gao et al., 2001).

Conclusion

EVs are not only mediators of cell-to-cell communication, but can also be used as biomarkers or therapeutic tools in the diagnosis of certain diseases. This is because EVs can be targeted to transport nucleic acids and other organisms without activating immunity (O'Brien et al., 2020). MSC-EVs have been used to treat a variety of diseases and systemic disorders. Current clinical trials and preclinical studies of MSC-EVs are based on the following strategies. (1) direct transplantation of MSCs, which can release EVs on site; (2) injection of purified, unengineered MSC-EVs; (3) injection of directly engineered MSC-EVs; and (4) injection of these target-rich and/or target-enhanced EVs after MSCs have been engineered. Engineering of MSC-EVs is a promising, cell-free, customizable translational approach to maximize the advantages of cell-free therapies. Overall, targeted technologies to improve MSC-EVs accumulation, lower required doses, and strategies to enrich EVs content with specific biomolecules may be key to their successful clinical application.

Prospects

The use of MSC-EVs has a number of advantages,

such as their use as a cell-free alternative therapy, which provides patients with an easier route of drug delivery and may require less recovery time. However, there are certain limitations, one of which is the isolation and purification of MSC-EVs. The products produced are still unstable and contain few non-EV contaminants or vesicles for processing. Although the biological effects of MSC-EVs are based on paracrine function, this mechanism is still unpredictable. Therefore, any treatment with MSC-EVs requires extensive safety and efficacy evaluations prior to clinical trials. Also, in order for MSC-EVs to enter clinical trials and industrial production, the entire process must comply with current good manufacturing practices (cGMP) (Elsharkasy et al., 2020). There have been studies to incorporate electroporation-based siRNA loading into the GMP workflow, emphasizing the possibility of implementing foreign cargo loading of EVs in a GMP-compliant manner (Mendt et al., 2018). It should be pointed out that cGMP needs to be combined with a personalized EV storage solution. EV storage and protection involve many parameters, and the temperature will affect the quality and stability of EVs (Qin et al., 2020). Finally, the development of stable strategies in large-scale separation and purification of EVs is a prerequisite for meeting resource-intensive clinical needs. For example, Wharton gum in human umbilical cords can be used to isolate a considerable number of MSCs (Didiot et al., 2016; Haraszti et al., 2018).

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