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Mesenchymal Stem Cell (MSC)-Derived Extracellular Vesicles: Potential Therapeutics as MSC Trophic Mediators in Regenerative Medicine

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ABSTRACT

Mesenchymal stem cells (MSCs) are pluripotent progenitor cells with the capabilities of self-renewing, differentiating into multiple lineages, and achieving trophic effects during tissue repair. MSCs can secrete extracellular vesicles (EVs) including exosomes and microvesicles, which mediate their trophic effects on other cells. Carrying a variety of intracellular molecules of MSCs including lipids, proteins, RNA (mRNA and noncoding RNA), and DNA, EVs deliver them into other cells to regulate tissue regeneration process. The therapeutic effects of MSC-derived EVs have been observed in a number of animal disease models. In this review, we focus on the current state and future directions of MSC-derived EVs in regenerative medicine. Anat Rec, 303:1735–1742, 2020. © 2019 American Association for Anatomy

Key words: extracellular vesicles; exosomes; mesenchymal stem cells; regenerative medicine

Extracellular vesicles (EVs) are heterogeneous small vesicles with a bilayer of phospholipids. EVs are secreted by almost all cell types and found in a variety of biological fluids (blood, urine, saliva, cerebrospinal fluid, breast milk, etc.). Once released into extracellular environment, they act on nearby recipient cells through paracrine regulation. In addition, they can also act on recipient cells far from the parental cells through systemic circulation.

As early as the 1960s, membrane vesicles in the extracellular space were observed, but their importance was unclear (Behnke, 1968). As RNAs including microRNAs were observed in EVs (Ratajczak et al., 2006), EVs have attracted a great deal of renewed attention as mediators of intercellular communication (Valadi et al., 2007). According to the size, composition, and origin, EVs can be classified into three types: (a) apoptotic bodies, (b) microvesicles/ectosomes, and (c) exosomes (Cocucci et al., 2009; Thery, 2011). Apoptotic bodies are released when plasma membrane blebbing occurs during apoptosis (Collino et al., 2015). These specialized EVs during cell death are not included in this review. The difference between the two remaining types of vesicles is mainly based on size: the range of exosomes is 30–150 nm, whereas the range of microvesicles is 100–1,000 nm. In addition, the origin of these two types of vesicles is also different: exosomes originate from endosomal

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membrane invagination, whereas microvesicles originate from the surface of cell membrane (Fig. 1).

Mesenchymal stem cells (MSCs) secrete various factors extensively to alter microenvironment following tissue damage. These factors affect regenerative processes including cell migration, proliferation, differentiation, and matrix synthesis (Meirelles Lda et al., 2009). In addition, these trophic factors suppress the local immune system, inhibit fibrosis and apoptosis, enhance angiogenesis, and stimulate mitosis and differentiation of reparative cells (Caplan, 2007). Most of the initial efforts were made to find bioactive therapeutic factors secreted by the MSC including cytokines, chemokines, and growth factors (Vizoso et al., 2017). Although there are many promising candidates, none could adequately explain the trophic mediator role of MSCs. As the demonstration that microvesicles secreted by MSCs could prevent acute renal tubule epithelium injury (Bruno et al., 2009), more and more evidence have emerged regarding the role of MSC-derived EVs as bioactive therapeutic factors during tissue regeneration (Akyurekli et al., 2015). Exosomes derived from different types of stem cells have been demonstrated to facilitate tissue repair in the skin (Zhang et al., 2015c), limbs (Hu et al., 2015), heart (Lai et al., 2010), joint (Zhu et al., 2017), and other tissues.

In this review, we focus on the current state of MSC-derived EVs including exosomes and microvesicles in

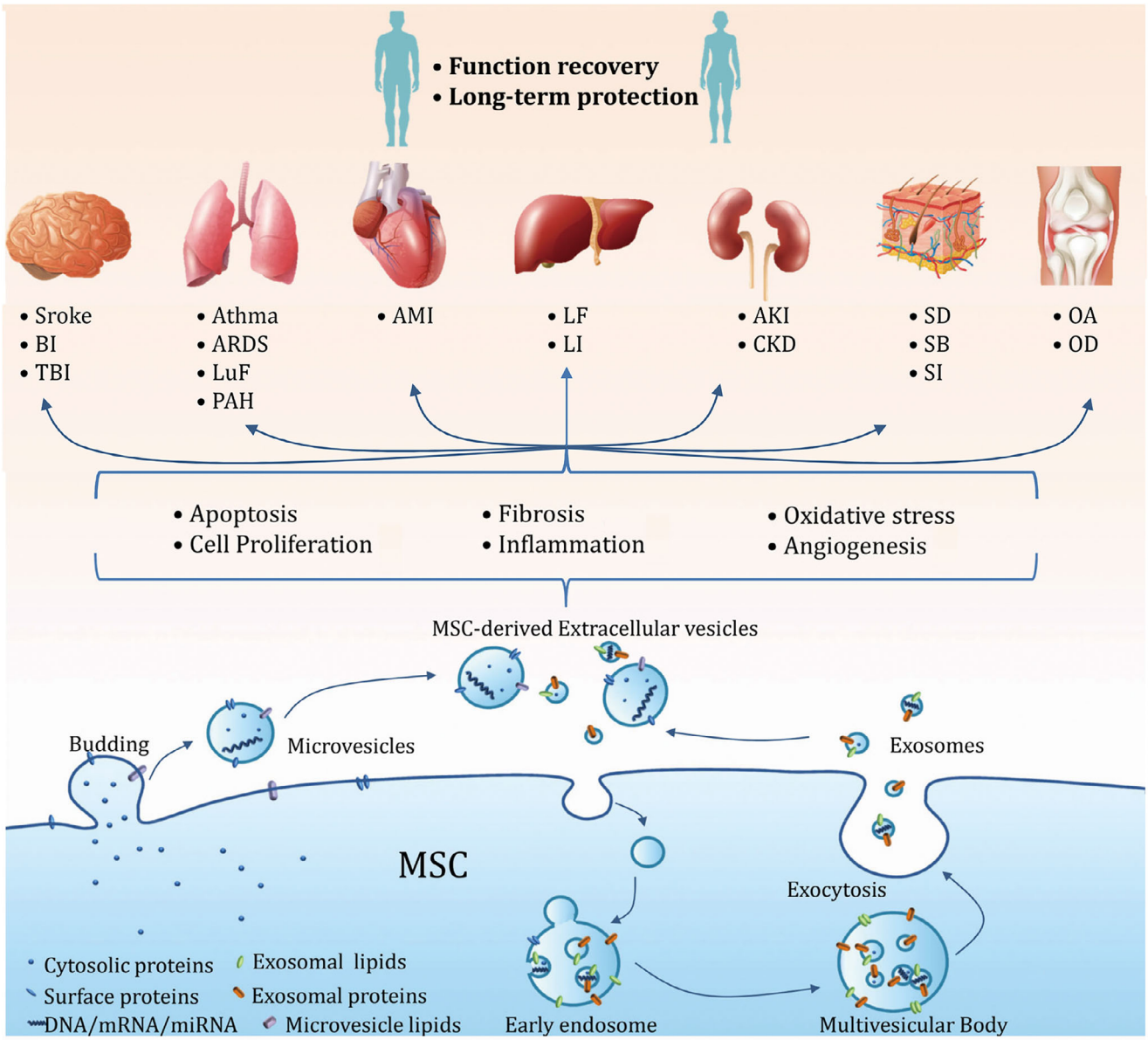

Fig. 1. Summary of biogenesis, biological components, and therapeutic potential of MSC-derived EVs in a variety of biological events. AD, adipose derived; AKI, acute kidney injury; ALI, acute lung injury; AMI, acute myocardial ischemia; ARDS, acute respiratory distress syndrome; BI, brain injury; CKD, chronic kidney disease; MSC, mensenchymal stem cell; LI, liver injury; LF, liver fibrosis; LuF, lung fibrosis; OA, osteoarthritis; OD, osteochondral defects; PAH, pulmonary arterial hypertension; SB, skin burn; SD, skin defect, SI, skin incision; TBI, traumatic brain injury.

regenerative medicine. We conducted search of the following key words in the Web of Science database: extracellular vesicles, microvesicles, exosomes, and stem cells.

General Characteristics of EVs

Biogenesis. The assembly of both exosomes and microvesicles involves the accumulation of components in the small membrane domains, which sprout and fall off the membrane (Fig. 1). Nevertheless, the initial processes leading to the generation of exosomes and microvesicles are largely different.

Exosomes are formed by reverse germination and thus contain cytosol (Thery et al., 2002). The biogenesis of the exosomes begins with the formation of early endosomes from endocytoses of the plasma membrane with its cargos. The early endosomes become multivesicular bodies containing intraluminal vesicles. They are either degraded by lysosomes or released as exosomes (Alenquer and Amorim, 2015).

In contrast, the initial process leading to microvesicles assembly is largely different from exosomes assembly. During microvesicle assembly, cargos are trafficked to the plasma membrane regions named cholesterol-rich microdomains, which are full of ceramide and lipid rafts. These membrane regions with their cargos are then protruded or budded to the outside of the cells to produce microvesicles (Nawaz et al., 2014).

Isolation methods. There are numerous techniques to isolate EVs, although they have not been standardized. The original EVs isolation and purification method was differential centrifugation based on the size of the EVs. Thery et al. introduced a detailed protocol of this method (Thery et al., 2006). However, EVs vary in size, origin, and molecular compositions, and different EVs populations overlap considerably in their size and phenotype. According to a large survey of current worldwide practices for the isolation of EVs (Gardiner et al., 2016), ultracentrifuge method is still the most commonly used method (81%), followed by density gradient centrifugation (20%), filter (18%), size exclusion chromatography (15%), and precipitation (14%). Ninety-one percent of respondents used two or more methods. Up to now, no consensus on the "gold standard" method of EVs isolation and purification has been reached.

Size and morphology. In whole-mount samples under electron microscope (EM), exosomes display cup-shaped appearance. However, this feature, produced by the fixation and contrast procedure that leads to exosome membrane shrinking, may be an artifact of exosomes. The true appearance of exosomes is a round shape, which is observed under cryo-EM and verified (Conde-Vancells et al., 2008). The diameter of the exosomes obtained by high-speed centrifugation is 30–150 nm, whereas that of microvesicles is 100–1,000 nm. These observations are consistent across different studies (Baietti et al., 2012).

Physical features. EVs can be separated by density gradients. Obtained by ultracentrifugation, exosomes densities range from 1.15 to 1.19 g/ml, whereas microvesicles range from 1.12 to 1.20 g/ml in sucrose (Robbins and Morelli, 2014). Such a range of densities indicates the heterogeneity of vesicles. Thus, various subpopulations exist in the preparations, and separate analysis of these subpopulations is needed.

MSCs IN REGENERATIVE MEDICINE

Biochemical Components of MSC-Derived EVs

MSC-derived EVs contain a large number of biochemical components including proteins, lipids, and nucleic acids. Several databases were built to facilitate transmission of these data. Exocarta contained the database of mammalian exosomes (Mathivanan et al., 2012), which has been incorporated into Vesiclepedia (http://microvesicles.org), a more comprehensive database. Another database (http://evpedia.info) contains information of nonmammal EVs (Kim et al., 2013).

Proteins. There are three groups of proteins, namely common proteins, enzymes, and signaling molecules, according to their specific functions in MSC-derived EVs. Approximately 2,000 proteins in MSC-derived exosome come from MSC membranes, Golgi, nuclei, and cytoplasm, and occasionally from ER or mitochondria (Choi et al., 2013). In MSC-derived exosomes, the common membrane proteins include CD9, CD63, CD82, CD81, HSP70, MHC-I, and MCH-II, whereas cell structure and motility proteins include tubulin, myosin, and actin. MSC-derived exosomes contain five important enzymes that play a key role in the glycolysis process (Lai et al., 2011). Signaling molecules such as chemokines, cytokines, interleukins, and growth factors were also found in MSC-derived exosomes (Lai et al., 2010; Formiga et al., 2014). It is worth noting that the exosomes protein composition reflects the physiological and pathological status of host cells, which can change in response to stress signals in the microenvironment (Salomon et al., 2013).

Lipids. Lipids are involved in EVs biogenesis. An important protein ESCRT can interact with lipids and enzymes related to lipid metabolism during EVs biogenesis (Wang et al., 2005). Moreover, Hsc-70 specifically binds to phosphatidylserine in the endosomal membrane, followed by ESCRT-III binding to polyglycerol phospholipids (monoglycerides) phosphate (Mobius et al., 2003). In addition, production of exosomes also needs the participation of cholesterol, oxysterols, ceramides, and lipid transporters (Record et al., 2011). Many bioactive lipids, as well as lipid metabolism-related enzymes, are transported by exosomes (Subra et al., 2010). Once ingested by the target cell, the bioactive lipids of exosomes are delivered, which can affect the signaling pathways and lipid homeostasis of target cells (Howcroft et al., 2011).

Nucleic acids. MSC-derived EVs contain mRNAs of various sizes and small RNAs including microRNAs. However, the levels of ribosomal 18S and 28S RNA are low or undetected. RNAs are not randomly included in exosomes. Different sequences are either secreted preferentially or retained in cells (Ratajczak et al., 2006; Valadi et al., 2007). Exosomes are enriched with miRNAs, most of which are in the form of pre-miRNAs that are inactive before they are transformed into mature miRNAs (Chen et al., 2010). Exosomal miRNAs are secreted in a strict regulatory process, which is affected by the source and development stage of host cells. A number of miRNAs have been identified in MSC-derived exosomes, which can function in recipient cells (Halkein et al., 2013; Bang et al., 2014; Wang et al., 2014b).

Thus, exosomal miRNAs may play an important role in mediating intercellular communication. The type and amount of miRNAs encapsulated into exosome are affected by pathophysiological stress stimuli and the state of the microenvironment (Jelonek et al., 2016).

Therapeutic Potential of MSC-Derived EVs

The effect of MSC-derived EVs on treatment of diseases in various organs has been studied. They are summarized as follows.

Brain disease. Potential regenerative effect of MSC-EVs has been observed for treatment of neurological and neurodegenerative diseases. In a rat stroke model, intravenous administration of MSC-EVs enhanced neurite remodeling, neurogenesis, and angiogenesis and improved functional recovery (Xin et al., 2013). MSC-EVs have also been tested in traumatic brain injury (TBI) models. By promoting endogenous neurogenesis and angiogenesis, MSC-EV improved recovery of brain function after TBI (Zhang et al., 2015d). Mechanistically, MSC-EV acts by improving the recovery of the impairment of both spatial learning and pattern separation (Kim et al., 2016) and by inhibiting neuroinflammation (Zhang et al., 2015d) (Zhang et al., 2015d; Kim et al., 2016).

The impact of MSC-EVs on Alzheimer's disease was also investigated. EVs from human adipose tissue-derived MSCs (ADSCs) decreased the levels of both secreted and intracellular β-amyloid peptide in the neuroblastoma cells (Katsuda et al., 2013). This effect might be achieved by neprilysin in the ADSCs, an important enzyme during degradation of β-amyloid peptide (Katsuda et al., 2013).

Lung diseases. In allergic asthma mice model, ADSCs and their EVs relieved inflammation, reduced eosinophil counts, and regulated airway rebuilding (de Castro et al., 2017). In silicotic mice model, MSCs-derived EVs decreased pulmonary fibrosis through inflammation remission and collagen deposition reduction in lung parenchyma (Choi et al., 2014b). In pulmonary arterial hypertension (PAH) mice model, MSC-exosomes were found to reverse PAH through increasing the levels of anti-inflammatory, antiproliferative miRNAs (Chen et al., 2014).

Cardiac injury. In mouse myocardial ischemia/reperfusion injury (IRI) model, exosomes from human embryonic stem cell (ESC) remarkably reduced the size of myocardial infarct (Lai et al., 2010; Arslan et al., 2013). In acute myocardial infarction rat model, EVs from hypoxic human bone marrow (BM)-MSCs *via* intramyocardial injection markedly enhanced blood flow recovery and reduced infarct size (Bian et al., 2014). Among human BM-MSCs, ADSCs, and endometrium-derived MSCs (EnMSCs), EnMSCs were most effective in infant size reduction, cardiac function restoration, and angiogenesis in ischemic zone (Wang et al., 2017).

Liver injury. In a mouse liver fibrosis model, administering exosomes from human umbilical cord-derived MSCs (hUCMSC) improved liver fibrosis and restored liver function by inhibiting the epithelial–mesenchymal transition of hepatocytes and collagen production (Li et al., 2013b). By transferring miR-122 into hepatic stem cells (HSCs), exosomes from ADSCs regulate collagen maturation and cell proliferation of HSCs through inhibiting the target genes of miR-122 (Li et al., 2013a).

Kidney injury. In IRI-induced acute kidney injury (AKI) mouse model, MSC-EVs protected mouse from AKI and restored kidney function by inhibiting apoptosis and stimulating tubular epithelial cell proliferation (Gatti et al., 2011; Wang et al., 2014a; Zhang et al., 2016b). Interestingly, these reno-protective effects were also found in the EVs isolated from kidney resident MSCs (Choi et al., 2014a).

In AKI mouse models induced by drug three cisplatin (Bruno et al., 2012) or glycerol (Bruno et al., 2017), systemic administration of MSC-EVs ameliorated tubular injury and restored renal function. Such EVs induced renoprotection was abolished if the internal mRNAs of EVs were degraded. It suggested that these EVs-derived mRNAs play an important role in renal protection.

Skin disease. In a full-thickness skin defect rat model, subcutaneous administration of exosomes derived from human induced pluripotent stem cells accelerated re-epithelialization, reduced scar widths, and promoted collagen maturity and angiogenesis (Zhang et al., 2015c). ADSCs-derived exosomes also protected skin by enhancing cell proliferation and increasing collagen content in a dose-dependent manner after ingestion by skin fibroblasts (Hu et al., 2016).

In a second-degree burn rat model, subcutaneous injection of exosomes from hUCMSC accelerated re-epithelialization and increased Type I collagen deposition (Zhang et al., 2015a; Zhang et al., 2015b). Mechanistically, these exosomes enhanced skin repair by accelerating the Wnt/beta-catenin signaling pathway and by inhibiting the YAP signaling pathway (Zhang et al., 2016a).

Joint disorder. MSC-derived EVs enhanced cartilage repair and inhibited cartilage degeneration related to osteoarthritis (OA) (Zhang et al., 2016c; Cosenza et al., 2017; Tao et al., 2017; Zhang et al., 2018b). In collagenase-induced OA mice model, single intra-articular administration of exosomes or microvesicles from murine BM-MSC inhibited chondrocyte apoptosis and macrophage activation (Cosenza et al., 2017). In femoral groove osteochondral defect rat model, weekly intra-articular injection of human ESCs-derived exosomes increased cell proliferation and matrix synthesis, enhanced the regenerative immune phenotype, and repaired cartilage defects (Zhang et al., 2016c; Zhang et al., 2018b). In a trauma-induced OA rat model, exosomes from synovial MSCs overexpressing chondroprotective miR-140-5p facilitated cartilage regeneration and inhibited OA pathogenesis (Tao et al., 2017).

FUTURE DIRECTION: REALIZING TANGIBLE PATIENT BENEFITS

As described above, many studies have shown that MSC-derived EVs were effective for regenerating injured or even degenerated tissues or organs, thereby restoring their functions (Fig. 1). Although multiple regenerative mechanisms could be involved potentially, some common features have emerged including antiapoptosis, inhibiting inflammation, and enhancing cell proliferation. These are the inherent properties of young and healthy tissue resident MSCs (Caplan, 2007). Through transmitting these

trophic effects of MSCs, MSC-derived EVs enhance tissue regeneration.

Despite these enormous progresses, the field of regenerative therapy using MSC-derived EVs is still in its early stage. No clinical trials have been published so far (http://www.clinicaltrials.gov/, accessed on May 2018). To date, there is only one interventional clinical trial of EV therapy, which demonstrated that immunotherapy of colorectal cancer using ascites-derived exosomes was safe and well tolerated. Although it was not a clinical trial using MSC-derived EV, it still instills the confidence about the future of clinical therapies using EVs.

A number of challenges need to be overcome to achieve the successful clinical translation of MSC-derived EV therapy. First, better treatment efficiency should be accomplished. Considering that MSC-derived EVs contain various bioactive cargos, such as mRNAs, microRNAs, and proteins, treatment efficiency may be improved by enhancing the active ingredient within EVs. MSCs and their EVs can be modified through overexpressing or knocking down a target gene to improve therapeutic efficacy. For example, cartilage regenerative effect is improved by overexpressing chondroprotective miR-140-5p in rat synovial MSCs-derived exosomes in a trauma-induced OA rat model (Tao et al., 2017). Second, target delivery is essential to reduce off-target side effect and toxicity. This can be accomplished by local delivery to target tissues such as subcutaneous injection to the skin and intra-articular injection to the joint. Alternatively, the surface molecules of the EVs can be tailored for binding to the specific receptors on recipient cells. This can be accomplished through modifying the membrane molecules such as peptides, lipids, or carbohydrates using cell engineering. For example, a brain-targeting peptide enables systemic exosomes delivery of siRNA to mouse brain (Alvarez-Erviti et al., 2011). Several subsequent studies confirmed the feasibility of this approach (Vader et al., 2016). Third, sustainability and longer half-life of EVs in tissues should be accomplished *in vivo*. Because of EVs small sizes in the nanometer to micrometer range, they are often cleared rapidly through blood vessels and lymphatic systems. Thus, it is crucial to understand the tissue transport and draining systems for EVs *in vivo* so as to develop more effective clinical treatment that evade clearance. In addition, the half-life of EVs can be improved by controlled and sustained release through a carrier. For instance, when MSC-derived exosomes were loaded with chitosan hydrogel, the stability and retention of exosomes were enhanced and the therapeutic effects for hindlimb ischemia were further augmented (Zhang et al., 2018a).

Last but not the least, EVs can be multifunctionalized to serve as not only therapeutic agents but also vehicles for drug delivery. For example, a novel carrier-in-carrier system was developed using MSC-derived EVs loaded with silk/curcumin nanoparticles (Perteghella et al., 2017). This drug delivery system combined beneficial effects of both regenerative cell therapies and pharmaceutical nanomedicine. Another example is the exosome–liposome hybrid nanoparticles, which could encapsulate large cargos more efficiently than exosomes alone (Lin et al., 2016). These hybrid nanoparticles have been used to deliver CRISPR/Cas9 system through endocytosis into MSCs and subsequent release of the encapsulated system within MSCs (Lin et al., 2018).

Based on these principles, we propose the following strategy for developing clinical application of MSC-derived EVs (Fig. 2). First, tissue donor should be selected and examined for MSC-derived EVs production. The donor can be autologous or allogeneic, and stem cell

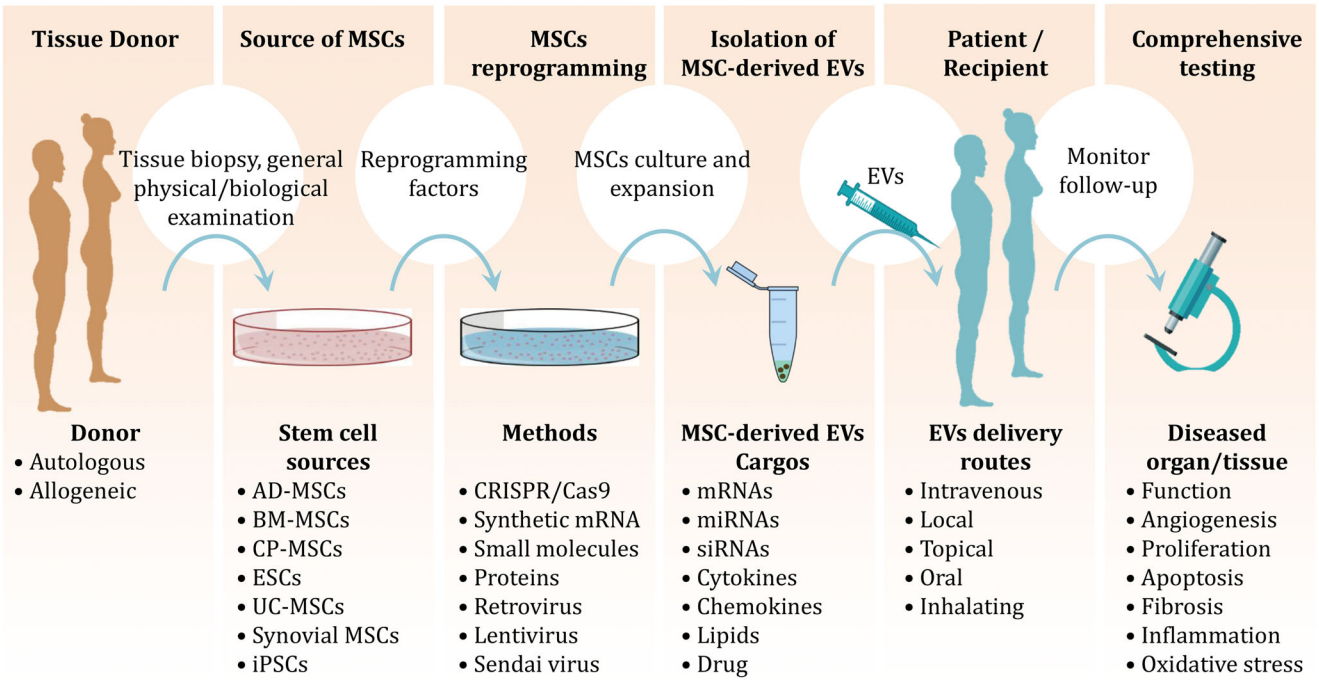

Fig. 2. A general strategy for clinical application of MSC-derived EVs. AD, adipose-derived; BM, bone marrow; CP, chorionic plate; ESC, embryonic stem cells; EV, extracellular vesicles; iPSC, induced pluripotent stem cells; MSCs, mensenchymal stem cells; UC, umbilical cord.

may be obtained from different tissues. Safety of the cell sources should be ensured by comprehensive cell and molecular analysis. Second, MSCs modification by bioengineering may be considered to improve therapeutic efficacy of MSC-derived EVs. For instance, the abundance of a specific gene product in the MSC-derived EVs can be changed through gene editing using CRISPR/Cas9 in MSCs. Third, MSC-derived EVs are isolated and maybe modified. Standardized protocol for EV isolation is preferred. Therapeutic effects of EVs may be further improved by modifying EVs, for example, by encapsulating miRNA or siRNA in EVs. Fourth, the route of MSC-derived EVs administration should be selected. The EVs delivery routes should be determined according to the tissue target of the specific disease. They can be systemic (intravenous, inhale, and oral) or local (topical or intra-articular injection). Lastly, safety and potential side effects should be monitored. Although positive regenerative effects of MSC-derived EVs were observed in a number of studies, few of the studies evaluated the safety of the MSC-derived EVs. Safety will be the key to the success of employing MSC-derived EVs as a clinical therapy in the clinic.

CONCLUSIONS

MSC-derived EVs showed favorable regenerative therapeutic effects under various disease conditions in preclinical models, although their composition and function are not yet clearly understood. Research in this field is still in its early stage and no clinical trial has been published so far. MSC-derived EVs are envisaged as a promising therapy in regenerative medicine, because they not only mediate the trophic effects of MSC but also provide a simpler alternative to the current cell-based therapeutics. Therapeutic efficacy of MSCs-derived EVs can be further improved through modification by bioengineering and genetic engineering, drug encapsulation, and nanomaterial science. There are still many obstacles to overcome before clinical use, including standardized isolation and purification, large-scale production at pharmaceutical grade, and safety.

AUTHOR CONTRIBUTIONS

Qi-ling Yuan: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. Yin-gang Zhang: Collection and assembly of data, final approval of manuscript. Qian Chen: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

CONFLICT OF INTEREST

No competing interests.

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