Tissue Regeneration Capacity of Extracellular Vesicles Isolated From Bone Marrow-Derived and Adipose-Derived Mesenchymal Stromal/Stem Cells

Yuan Liu and Christina Holmes*

Department of Chemical and Biomedical Engineering, Florida A&M University-Florida State University College of Engineering, Tallahassee, FL, United States

Mesenchymal stem cell (MSC)-based therapies have demonstrated tissue repair and regeneration capacity in various preclinical models. These therapeutic effects have recently been largely attributed to the paracrine effects of the MSC secretome, including proteins and extracellular vesicles (EVs). EVs are cell-secreted nano-sized vesicles with lipid bilayer membranes that facilitate cell–cell signaling. Treatments based on MSC-derived EVs are beginning to be explored as an alternative to MSC transplantation-based therapies. However, it remains to be determined which MSC source produces EVs with the greatest therapeutic potential. This review compares the tissue regeneration capacity of EVs isolated from the two most common clinical sources of adult MSCs, bone marrow and adipose tissue, with a particular focus on their angiogenic, osteogenic, and immunomodulatory potentials. Other important issues in the development of MSC-derived EV based therapies are also discussed.

Keywords: mesenchymal stem cell, extracellular vesicle, bone marrow, adipose, angiogenesis, osteogenesis, immunomodulation, tissue regeneration

INTRODUCTION

Mesenchymal stem cell (MSC) transplantation has demonstrated great promise as a novel treatment for tissue repair and regeneration in several organ systems, including the central nervous system (CNS) (Azari et al., 2010), heart (Jeong et al., 2018), cartilage, skin, and bone (Mitxitorena et al., 2019). Displaying trophic and immunomodulatory effects upon transplantation, MSCs currently represent a critical part of clinical cell-based regenerative medicine. To date, over 950 clinical trials involving MSCs have been listed with the United States Food and Drug Administration and more than 10,000 patients have received MSC-based therapies (Pittenger et al., 2019). However, issues with MSC-based therapies, such as low cell survival rate upon transplantation, limited donor supply, donor-to-donor variability and storage issues, have prompted researchers to investigate alternative approaches. In recent years, extracellular vesicles (EVs) derived from MSCs have become the focus of much research as they exhibit many similar trophic and immunomodulatory functions.

Abbreviations: MSC, mesenchymal stem cells; EV, extracellular vesicles; MI, myocardial infarction; ICH, intracerebral hemorrhages; miRNA, microRNA; BMMSC, bone marrow-derived MSC; ADMSC, adipose-derived MSC; I/R, ischemia/reperfusion; SCI, spinal cord injury; Tregs, regulatory T cells.
In order to translate EV-based therapies to the clinic, the relationship between MSC cell source and EV therapeutic potential needs to be clarified.

MSCs in Tissue Repair and Regeneration
Mesenchymal stem cells are a heterogeneous subset of pluripotent stromal stem cells that are easily isolated from various tissues, including adipose tissue, peripheral blood, bone marrow, synovial fluid, muscle, placenta, umbilical cord, and dental pulp (Uccelli et al., 2008). The minimal criteria for defining MSCs are: the ability to self-renew and differentiate into classical mesodermal lineage cells such as osteoblasts, adipocytes, and chondrocytes in vitro and in vivo; a CD105+, CD73+, CD90+, and CD45−, CD34−, CD11b−, CD79α−, CD19−, and HLA class II- expression profile; a fibroblast-like morphology; and, adherence to tissue culture plastic in vitro (Horwitz et al., 2005; Dominici et al., 2006). Among the various MSC sources, bone marrow (BMMSCs) and adipose (ADMSCs) are the two most commonly used in preclinical and clinical tissue regeneration applications. While, umbilical cord-derived MSCs (UCMSCs) have also been widely employed in research and clinical trials, their use in many applications is limited since they are not practical for autologous administration in adults (Kern et al., 2006). Although BMMSCs were the first MSC type to be characterized and are the most widely used (Caplan, 1991), ADMSCs are an attractive alternative as they are higher in frequency, more easily obtained and cause less donor site morbidity (Reumann et al., 2018). Furthermore, ADMSCs display a higher proliferation rate than BM-MSCs in vitro and show a greater ability to maintain their stem cell characteristics, including self-renewal, proliferation, and differentiation potential, after repeated passaging (Zhu et al., 2008).

While both BMMSCs and ADMSCs have been successfully employed in preclinical tissue repair and disease models to promote angiogenesis (Jin and Lee, 2018; Zhang et al., 2019; Ryu et al., 2020), induce bone regeneration (Jin and Lee, 2018) and modulate the immune system (Tao et al., 2016; Zhao et al., 2016; Waldner et al., 2018), there appear to be several differences between cell types. In vitro studies have shown that BMMSCs exhibit significantly higher chondrogenic differentiation capacity (Noël et al., 2008; Mohamed-Ahmed et al., 2018), while ADMSCs show significantly higher adipogenic capacity in vitro (Mohamed-Ahmed et al., 2018). ADMSCs also display a higher endothelial differentiation capacity in vitro than BMMSCs (Fan et al., 2016), and superior angiogenic capacity in several preclinical ischemic injury models (Ikegame et al., 2011; El-Badawy et al., 2016). However, it remains unclear which MSC source exhibits greater osteogenic capacity or immunomodulatory potential. While some in vitro studies showed higher osteogenic differentiation in BMMSCs than ADMSCs (Park et al., 2012), others showed the opposite (Kang et al., 2012). More significantly, no significant differences in bone regeneration ability were observed in vivo between the two MSC types in rat cranial defect models (Wen et al., 2013) or canine radius defect models (Kang et al., 2012). Similarly, both MSC types showed comparable immunomodulatory potential in an immunocompetent myocardial infarction (MI) model (Paul et al., 2013), while BMMSCs displayed greater immunomodulatory potential in an endotoxic shock model (Elman et al., 2014), and ADMSCs demonstrated more effective immunosuppression of peripheral blood mononuclear cells and T-cells in vitro (Waldner et al., 2018).

EVs in Paracrine Signaling
While the therapeutic effects of transplanted MSCs were originally thought to be due to direct cell replacement (Friedenstein et al., 1968), research soon showed that intravenously administrated MSCs were largely caught in capillaries and/or cleared (Fischer et al., 2009), and that remaining MSCs contributed to short-term therapeutic effects (Caplan and Dennis, 2006). It is now widely theorized that the therapeutic effects of MSCs are mainly due to paracrine secretion of various growth factors, glycosaminoglycans, cytokines and EVs which modulate angiogenesis (Pankajakshan and Agrawal, 2014), apoptosis (Pan et al., 2012), proliferation (Di Nicola et al., 2002), differentiation (Chioussone et al., 2016), and the immune response (Dyer et al., 2014) to create a reparative microenvironment (Phinney and Pittenger, 2017). Secreted by the majority of cell types, EVs are phospholipid vesicles of different sizes, including micro-vesicles (MV) (200 nm–1 μm) and exosomes (50–200 nm), that transport proteins, lipids, and nucleic acids (Hunter et al., 2008). Exosomes are generated in multivesicular bodies by the endosomal compartment and express endosomal markers (CD9, CD61, CD83, ALIX, TSG101) (Cosenza et al., 2017) and surface molecules that allow them to be targeted to recipient cells (Mathivanan et al., 2010). Meanwhile, MVs are the outcome of direct outward budding of the cell plasma membrane and thus carry cytoplasmic contents (Heijnen et al., 1999). EVs are recognized and internalized by recipient cells through receptor-ligand interactions (Raposo et al., 1996), endocytosis and/or phagocytosis (Morelli et al., 2004), or they can fuse with the target cell membrane and deliver their contents into the cytosol (Tkach and Théry, 2016). Recent research suggests that the paracrine efficacy of MSC-based therapies can largely be attributed to EVs. For example, conditioned MSC culture media was found to have therapeutic effects similar to direct delivery of MSCs in rodent models (Gneccchi et al., 2005; Aslam et al., 2009). Subsequently, Timmers et al. (2008) demonstrated that it was the EVs within the conditioned media that actually were effective.

Extracellular vesicles can be harvested via a variety of methods from cell culture media or clinical samples such as blood plasma, urine, and saliva. The most frequently employed isolation methods include differential ultracentrifugation and density gradient ultracentrifugation, both of which involve centrifugal forces greater than 100,000 x g and can fractionate EVs from their liquid sample of origin into subsets based on size, density, and mass (Zarovni et al., 2015; Li et al., 2017). EVs harvested from different tissues display varying content profiles, depending on their origin, age, state and environment (Tkach and Théry, 2016). For example, the microRNA (miRNA) profile of MSC-derived EVs from myoplastic syndrome patients is significantly different compared to that of EVs from disease-free patients (Muntion et al., 2016). Among EV contents, the function of bioactive
lipids and proteins have been well-studied (Toh et al., 2018; Skotland et al., 2020). However, nucleic acid cargo, including mRNA, miRNAs, and other non-coding RNAs has become an increasingly hot topic in EV research. miRNAs, which are small (19–23 nucleotide) non-coding RNAs (Lau et al., 2001) that regulate gene expression via specific binding to messenger RNAs (mRNAs) (Lai, 2002), make up a large portion of the cargo within EVs (Valadi et al., 2007). miRNA transfer to recipient cells via EVs contributes significantly to paracrine signaling and has been found to be a main mediator of therapeutic effects in many preclinical studies. For example, miR-223 from BMMSCs-derived EVs contributed to cardioprotection in a surgically induced sepsis model (Wang X. et al., 2015).

The use of MSC-derived EVs in place of MSC transplantation in clinical treatments provides a number of potential advantages. EV therapies increase the accessibility of damaged tissues, since cultured MSCs are approximately 20 µm in diameter and thus tend to be caught and cleared by the circulation (Crop et al., 2010), whereas EVs are significantly smaller and have demonstrated transport through the pulmonary circulation and the blood-brain barrier (Batsali et al., 2020) (Bang and Kim, 2019). Unlike MSCs, which may undergo changes during in vitro culture that make them a clearance target of NK cells and macrophages (Eggenhofer et al., 2014), EVs are more likely to avoid immune rejection due to their low expression of membrane histocompatibility complexes (Lai et al., 2019). EVs are also more easily modified than MSCs to encapsulate desired therapeutic cargos, and are more easily stored than cells, since they are more stable when freezing and thawing (Lai et al., 2019). However, before the clinical application of MSC-derived EVs can be achieved, the optimal cell source for a given therapeutic application needs to be determined.

This review will compare the therapeutic effects of EVs isolated from BMMSCs and ADMSCs in various in vivo tissue repair and regeneration models (Figure 1). More specifically, the capacity of BMMSC- and ADMSC-derived EVs to induce angiogenesis, osteogenesis and immunomodulation will be investigated. EV cargos and any signaling pathways involved, where characterized, will also be detailed.

COMPARING THE THERAPEUTIC EFFICACY OF BMMSC-DERIVED AND ADMSC-DERIVED EVs

Angiogenesis

Studies employing BMMSC-derived EVs in preclinical models, including calvarial defects (Liang et al., 2019), myocardial infarctions (MI) (Teng et al., 2015; Xu H. et al., 2020), random pattern dorsal skin flaps (Xie et al., 2019), intracerebral hemorrhages (ICH) (Han et al., 2019b), fracture non-unions (Zhang et al., 2020), focal cerebral ischemia models (Doeppner et al., 2015), traumatic brain injury (TBI) models (Zhang et al., 2017), STZ-induced diabetic rat models (Yu M. et al., 2020), and subcutaneous implantation

FIGURE 1 | Schematic outline of the use of MSC-derived EVs within tissue regeneration models as analyzed in this review. EVs were isolated from the culture media of bone marrow-derived or adipose-derived MSCs and employed in various in vitro proliferation, differentiation, gene expression, and other assays, as well as within a variety of in vivo tissue regeneration studies, including preclinical animal models and human clinical trials.
models (Narayanan et al., 2016), generally demonstrated that EV treatment stimulated localized vasculogenesis and/or angiogenesis (see Table 1). Meanwhile, ADMSC-derived EVs promoted neovascularization and angiogenesis in fat grafting models (Han et al., 2019a), acute ischemic stroke models (Chen et al., 2016), acute kidney ischemia/reperfusion (I/R) models (Lin et al., 2016), and MI models (Xu H. et al., 2020) (see Table 1). With far fewer studies employing ADMSC-derived EVs than BMMSC-derived EVs, it remains unclear whether once source displays greater angiogenic potential than the other. In one study that directly compared the effects of EVs derived from both cell sources, human ADMSC-derived EVs displayed significantly increased therapeutic potential compared to BMMSC-derived EVs in a rat MI model, as indicated by improved cardiac function, reduced cardiomyocyte apoptosis and infarction area and increased microvessel density (Xu H. et al., 2020). By contrast, when comparing two separate brain ischemia model studies, BMMSC-derived EVs appeared to display increased angiogenic potential compared to ADMSCs-derived EVs, with the former exhibiting an approximately 4-fold increase in the number of endothelial cells compared to controls, while the latter showed a 1.5-fold change (Doepppner et al., 2015; Chen et al., 2016).

Few of these preclinical studies have investigated the mechanisms and signaling pathways underlying the observed increase in angiogenesis induced by MSC-derived EV therapies. In a calvarial defect model, enhanced angiogenesis due to BMMSC-derived EV treatment was coupled with endogenous MSC migration (Takeuchi et al., 2019). While, in a rat full-thickness skin wound model, human BMMSC-derived EVs accelerated angiogenesis and the cutaneous wound healing process via inhibition of the TGF-β/Smad signaling pathway, as verified by RT-qPCR and western blotting analysis (Yang et al., 2018). Similarly, in a STZ-induced diabetic rat model, BMMSC-derived EVs accelerated angiogenesis and the cutaneous wound healing process via inhibition of the AKT/eNOS pathway, as indicated by improved cardiac function, reduced cardiomyocyte apoptosis and infarction area and increased microvessel density (Xu H. et al., 2020). By contrast, when comparing two separate brain ischemia model studies, BMMSC-derived EVs appeared to display increased angiogenic potential compared to ADMSCs-derived EVs, with the former exhibiting an approximately 4-fold increase in the number of endothelial cells compared to controls, while the latter showed a 1.5-fold change (Doepppner et al., 2015; Chen et al., 2016).

In vitro experiments further showed that EVs isolated from both BMMSCs and ADMSCs possessed great potential for inducing angiogenesis and enabled more detailed study of the pathways underlying these effects. ADMSC-derived EVs enhanced angiogenic tube formation in human brain microvessel endothelial cells via increased expression of miR-181b-5p, which, in turn, directly targeted expression of the ion channel protein TRPM7 (Yang et al., 2018). Similarly, BMMSC-derived EVs induced angiogenic tube formation in HUVECs (Teng et al., 2015; Xie et al., 2017; Kang et al., 2020; Yu M. et al., 2020; Zhang et al., 2020) and enhanced expression of the angiogenesis-related genes VEGF, ANG1, and ANG2 in hBMMSCs (Takeuchi et al., 2019) and the mRNA expression of PDGF, EGF, and ANG1 in HUVECs (Yu M. et al., 2020). In a rare study that directly compared EVs derived from the three most commonly employed clinical MSC sources, i.e., BMMSCs, ADMSCs and UCMSs, ADMSC-derived EVs yielded the highest in vitro protein expression levels of VEGF, bFGF, and HGF in rat neonatal cardiomyocytes and also showed the strongest inhibitory effect on apoptosis (Xu H. et al., 2020).

In order to improve angiogenic therapeutic capacity, many studies isolated EVs from MSCs cultured in hypoxic conditions or in the presence of dimethylxalylglycine (DMOG), which enhances activation of HIF-1a. In a nude mouse model of fat grafting, for example, hypoxic ADMSC-derived EVs (hyp-ADSC-EVs) dramatically promoted neovascularization and increased the protein expression of VEGF/VEGF-R compared to EVs derived in normoxic conditions (Han et al., 2019a). Similarly, in other studies hyp-ADSC-EVs were found to express significantly higher levels of VEGF and VEGF-R2/R3 and promote increased HUVEC proliferation, migration and tube-formation in vitro (Xue et al., 2018; Han et al., 2019a), compared to EVs from cells cultured in normoxia. These hyp-ADSC-EVs also dramatically changed HUVEC expression levels of the angiogenic genes Angpt1, Flk1 and Vash1, and increased activation of the PKA signaling pathway (Xue et al., 2018). Interestingly, EVs from hypoxia-preconditioned ADMSCs exhibited increased diameters (by 59 nm) compared to those derived in normoxia (Han et al., 2019a). Meanwhile, EVs derived from DMOG-stimulated hBMMSCs increased HUVEC angiogenesis in vitro and decreased expression levels of PTEN (Liang et al., 2019), a tumor suppressor gene found to promote neovascularization by inducing HUVEC migration (Zhang et al., 2016). This decreased PTEN expression was further accompanied by increased expression levels of its corresponding downstream AKT/mTOR signaling pathway members, p-AKT, mTOR, and p-mTOR (Liang et al., 2019). Increased angiogenesis in a calvarial defect model treated with EVs isolated from DMOG-stimulated hBMMSCs was also observed (Liang et al., 2019).

**Osteogenesis**

Although both BMMSC- and ADMSC-derived EVs have been shown to promote osteogenesis *in vivo*, most preclinical studies use BMMSC-derived EVs to induce bone formation and fracture healing. BMMSC-derived EVs were shown to promote bone regeneration in rat calvarial bone defect models (Qin et al., 2016; Takeuchi et al., 2019), murine femoral fracture models (Furuta et al., 2016; Xu T. et al., 2020), rat models of distraction osteogenesis (Jia et al., 2020), and subcutaneous bone formation models in nude mice (Narayanan et al., 2016; Xie et al., 2017)
| EV cell origin | Method of EV isolation | EV Characterization (size, surface markers) | Amount of EVs delivered | In vitro effects | Model | Delivery mechanism | Amount of EVs delivered | In vivo effects | Pathway(s)/miRNA(s) involved | Ref. |
|----------------|------------------------|---------------------------------------------|-------------------------|------------------|-------|-------------------|------------------------|----------------|-----------------------------|------|
| Bone Marrow    | Centrifugation, filtration | 40–100 nm CD9+, CD63+, GM130+, TSG101+ | 25 µg/mL                | Reduced apoptosis| HBMSCs | Transwell assay, qRT-PCR, ELISA | 5 µg/mL                | Increased migration | Increased VEGF, ANG1, and ANG2 expression | N/A |
| Human BM       | UC                     | 80–182 nm CD9+, CD63+, GM130+, TSG101+ | 50 mg/mL                | Increased angiogenesis| HUVECs | Scratch wound, cell proliferation, and tube formation assays | N/A                | SD rats Calvarial defect model | Implanted via porous hydroxyapatite scaffold | PTEN; AKT/mTOR | Liang et al., 2019 |
| Human BM p6    | UC                     | 80–182 nm CD9+, CD63+, GM130+, TSG101+ | 100 µg                  | Increased bone formation | SD rats | Myocardial infarction (MI) model | 75 µg (+1.5 x 10⁶ cells) | Increased angiogenesis | N/A | Xu H. et al., 2020 |
| Human BM p3-p6 | UC                     | 80–182 nm CD9+, CD63+, GM130+, TSG101+ | 30 µg                   | Increased angiogenesis| HMSCs | Transwell assay, qRT-PCR | EVs from 0.5 x 10⁹ cells | Increased osteogenic differentiation | Increased RUNX2, Osterix, BMP9 and TGFβ1 expression (mRNA); and BMP2, TGFβ3, and PDGF expression (protein) | N/A | Takeuchi et al., 2019 |
| Human BM p0    | N/A                    | N/A                                         | N/A                     | N/A              | N/A | N/A | N/A | Increased osteogenic differentiation | Increased RUNX2, Osterix, BMP9 and TGFβ1 expression (mRNA); and BMP2, TGFβ3, and PDGF expression (protein) | N/A | Narayanan et al., 2016 |
| Human BM       | PEG 6000, UC           | N/A                                         | N/A                     | N/A              | N/A | N/A | N/A | Increased osteogenic differentiation | Increased RUNX2, Osterix, BMP9 and TGFβ1 expression (mRNA); and BMP2, TGFβ3, and PDGF expression (protein) | N/A | Doeppner et al., 2015 |
| Human BM     >p3 | ExoQuick kit          | CD9+, CD63+, CD81+                          | N/A                     | N/A              | N/A | N/A | N/A | Increased angiogenesis | Increased angiogenesis (CD31+ cells) | N/A | Zhang et al., 2017 |
| Human BM     p5 | Total exosome isolation reagent, centrifugation | CD63+, GM130+, TSG101+ | N/A                     | N/A              | N/A | N/A | N/A | Increased angiogenesis | Increased angiogenesis and xSMAC/D31, cardiac function (reduced left ventricular dilation and preserved systolic function) | N/A | Wang L. et al., 2017 |
| EV cell origin | Method of EV isolation | EV Characterization (size, surface markers) | In vitro | In vivo | Pathway(s)/miRNA(s) involved | Ref. |
|---------------|------------------------|-----------------------------------------------|----------|---------|------------------------------|------|
| Human BM p4   | UC                     | 30-150nm, CD105+, CD90+, CD73+, CD34-, CD45- | RAW264.7 cells ELISA, qRT-PCR, WB          | N/A     | Reduced IL-1β, TNF-α, IL-10, INF-γ, iNOS expression | PTEN/AKT | Liu et al., 2020b |
| Human BM p4   | UC                     | 80-120nm, TSG101+, Alix+, CD81+              | 50 µg/mL | Increased cell viability, mobility, VEGF secretion | N/A     | Accelerated wound closure | Yu M. et al., 2020 |
| Rat BM p2-p5  | UC                     | 122nm CD90+, CD29+, CD34-, CD11b/C-          | 1 × 10^{10} EVs | Increased proliferation, migration, and tube formation | N/A     | Increased angiogenesis at the fracture site | Zhang et al., 2020 |
| Rat BM p3     | ExoQuick-TC kit        | 50–100 nm CD63+                               | 10 µg/mL | Increased angiogenesis | N/A     | Increased angiogenesis (new capillaries and blood vessel density) | Teng et al., 2015 |
| Rat BM p0     | ExoQuick-TC kit        | N/A                                           | N/A      | N/A     | N/A                          | N/A     | Increased vascular density and angiogenesis | Han et al., 2019b |
| Rat BM p4–p6  | UC                     | TRPS: 50–150 nm, TEM: 50–100 nm CD9+, CD63+, TSG101+, GM130- | 1 × 10^{10} EVs | Increased proliferation, migration, and angiogenesis | 1 × 10^{10} EVs/mL | Increased angiogenesis | Zhu et al., 2019 |
| EV cell origin | Method of EV isolation | EV Characterization (size, surface markers) | In vitro | In vivo | Pathway(s)/miRNA(s) involved | Ref. |
|---------------|------------------------|---------------------------------------------|---------|--------|-------------------------------|------|
|               |                        |                              | Cell and assay type | Amount of EVs delivered | In vitro effects | Model | Delivery mechanism | Amount of EVs delivered | In vivo effects |            |      |
| Rat BM p4     | Exosome extraction kit (E1340, Wuhe Biology) | 80–100 nm CD9+, CD63+, TSG101+ | N/A | N/A | N/A | SD rats Random pattern dorsal skin flap model | Localized injection | 135 µg | Increased angiogenesis | Xie et al., 2019 |
| Adipose       | UC                     | normoxia: 75 ± 61 nm hypoxia: 130 ± 65 nm CD9+, CD63+, TSG101+ | Hypoxia Eves increased proliferation, migration, and tube formation | 25 µg | Increased expression of VEGF and CD34 | N/A |
| Human adipose | UC                    | Centrifugation, filtration | 30–100 nm CD63+, CD81+, CD9+ | Neonatal rat cardiomyocytes cultured in hypoxic conditions | ELISA | SD rats MI model | Localized injection | 75 µg (+1.5 × 10⁶ cells) | Increased microvascular density | Xu H. et al., 2020 |
| Human adipose | UC                    | 20–300 nm CD9+, CD63+, TSG101+ | HUVECs Capillary formation assay | 50 µg/mL | Reduced apoptosis | Increased VEGF, bFGF, and HGF expression | SC injection | 50 µg | Increased neovascularization | di Han et al., 2018 |
| Human adipose | UC                    | Total exosome isolation reagent, centrifugation | HUVECs Tube formation assay | Cardiac myocytes | TUNEL assay | Increased angiogenesis | Decreased apoptosis | SD rats MI model | Localized injection | N/A |
| Rat adipose   | SDS-PAGE | CD63+, TSG101+ | N/A | N/A | N/A | SD rats Acute kidney IR model | IV injection | 100 µg | Increased expression of CD31, vWF, and angiopeptin | Lin et al., 2016 |
| Pig adipose   | SDS-PAGE | N/A | N/A | N/A | N/A | SD rats Acute ischemic stroke model | IV injection | 100 µg | Increased protein expression of VEGF and CXCR4 Increased cellular expression of CXCR4 and SDF-1α and endothelial function integrity (vWF) | Chen et al., 2016 |

Centrifugation: <100,000 g; HBMSCs, human bone marrow-derived mesenchymal stem cells; HMSCs, human mesenchymal stem cells; HUVECs, human umbilical vein endothelial cells; IV injection, intravenous injection, no vein specified; MI, myocardial infarction; PEG6000, polyethylene glycol 6000; Ref, references; SC injection, subcutaneous injection; SD, Sprague Dawley; STZ, streptozotocin; UC, ultracentrifugation, ≥100,000 g. *Cells cultured in special conditions (e.g. hypoxia).
(see Table 2). By contrast, the bone regeneration capacity of ADMSCs-derived EVs has only been explored in two studies to date in calvarial defect models (Li et al., 2018); one of which involved EVs derived from hADMSCs engineered to overexpress miR-375 (Chen et al., 2019) (see Table 2). With so few studies of ADMSC-derived EVs, it is difficult to compare their osteogenic capacity to BMMSC-derived EVs. However, in separate rat calvarial defect studies, treatment with BMMSC-derived EVs led to a greater increase (fourfold) in bone volume [i.e., (BV)/(TV)] compared to controls (Qin et al., 2016), than that induced by ADMSC-derived EVs (approximately 1.33-fold) (Chen et al., 2019).

The mechanisms and pathways underlying the osteogenic effects of MSC-derived EVs have not been widely reported. In a non-union model, BMMSC-derived EVs enhanced osteogenesis via the activation of the BMP-2/Smad1/Runx2 signaling pathway (Zhang et al., 2020). In a femoral fracture model, BMMSC-derived EVs from young rats yielded increased bone formation and expression of Runx2, ALP, and Col I compared to those from older rats; with EV osteogenic capacity linked inversely to levels of miR-128-3p expression, which was found to negatively modulate Smad5 signaling (Xu T. et al., 2020). Significantly, additional studies also found that miRNAs played an important role in promoting EV-mediated bone regeneration, with miR-196a regulating differentiation of osteoblasts (Qin et al., 2016), while miR-375 stimulated osteogenic differentiation of MSCs by inhibiting IGFBP3 (Chen et al., 2019).

In vitro studies of MSC-derived EVs further illustrated their role in bone regeneration through promotion of MSC proliferation, migration, and osteogenic differentiation (Narayanan et al., 2016; Takeuchi et al., 2019). Many studies have observed that treatment with BMMSCs-EVs increased osteogenic differentiation and upregulation of related genes, including Runx2, Osterix, BMP9, TGF-β1, BMP2, TGF-β, OCN, ALP, Col I, and PDGF in BMMSCs (Narayanan et al., 2016; Xu T. et al., 2020) and osteoblastic MC3T3 cells (Zhang et al., 2020). Similarly, ADMSC-derived EVs were also found to promote osteogenic differentiation of human BMSCs in vitro by significantly increasing the expression of Runx2, ALP, and COL1A1 (Li et al., 2018; Chen et al., 2019). Interestingly, BMSC-derived EVs isolated from young (2-week old) SD rats promoted proliferation and enhanced the osteogenic capacity of older BMSCs, and significantly upregulated the expression of ALP, Runx2, and OCN (Jia et al., 2020). In human osteoblasts, BMMSC-derived EVs enhanced differentiation, likely due to differential expression of miR-196a (Qin et al., 2016). In another study, miR-26 was found to be crucial to the in vitro capacity of BMSC-derived EVs to induce osteogenic differentiation via silencing experiments (Luo et al., 2019).

**Immunomodulation**

**Effects on Macrophage Polarization**

In preclinical models, EVs from MSCs have shown a variety of immunomodulatory effects. EVs from both BMMSCs and ADMSCs can change macrophage polarization from the pro-inflammatory M1 type to the anti-inflammatory M2 type in vitro and in vivo (see Table 3) (Li et al., 2020; Wang J. et al., 2020). With very few in vivo studies analyzing the effects of ADMSC-derived EVs on macrophage polarization and no direct comparison studies, it is difficult to conclude whether one EV source is more effective than the other. However, looking at separate studies that used comparable metrics, BMMSC-derived EVs induced a dramatically increased change in the expression of the M2 polarization marker CD206 (3.2-fold) in a murine acute lung injury model (Wang J. et al., 2020) compared to that induced by ADMSC-derived EVs in a murine air pouch model (1.5-fold) (Liu et al., 2020b).

In the preclinical models reviewed here, M2 polarization stimulated by MSC-EV treatment was found to be associated with increased expression of anti-inflammatory cytokines, such as IL-10 and TGF-β (Garnier et al., 2018), and decreased secretion of IL-6 and TNF-α (Arora et al., 2018). In a murine model of acute respiratory distress syndrome BMMSC-derived EVs reduced lung damage and LPS-induced inflammation (Deng et al., 2020). As in previous studies demonstrating that metabolic reprogramming of glycolysis in macrophages contributes to M2 polarization (Zhao et al., 2020), EV-treatment in this model was associated with downregulation of glycolysis in lung macrophages and M2 polarization (Deng et al., 2020). Research in models of cutaneous wound-healing and spinal cord ischemia-reperfusion injury (SCIIRI) have also shown that BMMSC-EV induced macrophage M2 polarization was associated with the AK2-STAT6 signaling pathway (Sun et al., 2018), miR-223 (He et al., 2019), and miR-124-3p/Ern1 (Li et al., 2020). BMSC-derived EVs also inhibited M1 microglia activation and tissue neutrophil infiltration and reduced the expression of TNF-α, IL-1β, and IL-6 in a rat ICH model (Duan et al., 2020). Similarly, ADMSC-EV treatment in a murine model of LPS-induced lung injury mitigated injury, increased localized expression of miR-27a-3p, and induced M2 macrophage polarization via NFKB1 signaling (Wang J. et al., 2020).

Interestingly, hypoxic BMSC culture conditions were found to produce EVs with enhanced capacity to induce M2 macrophage/microglia polarization. For example, in a spinal cord injury (SCI) model, treatment with EVs from hypoxia preconditioned BMMSCs resulted in increased conversion of microglia from the pro-inflammatory M1 to the anti-inflammatory M2 phenotype, compared to EVs from BMMSCs cultured in normoxia (Liu et al., 2020a). This increased shift toward M2 polarization was found to be associated with increased expression of miR-216a-5p in hypoxic EVs. In another in vitro study of the microglia M1 to M2 phenotype change, hypoxic preconditioning of BMSCs enhanced secretion of EVs and increased the M2 polarization capacity of the BMSC secretome as compared to normoxic conditions (Yu H. et al., 2020).

**Effects on Fibrosis**

Several preclinical studies have shown that MSC-derived EVs can promote tissue regeneration over fibrosis, thus reducing the formation of scar tissue and other fibrotic processes that impair normal cellular and tissue function (Mutsaers et al., 1997) (see Table 4). For example, treatment with hBMMSC-derived EVs reduced interstitial kidney fibrosis by 80% in a mouse model...
| EV cell origin | Method of EV isolation | EV Characterization (Size, surface markers) | In vitro | In vivo | Pathway(s)/miRNA(s) involved | Ref. |
|---------------|------------------------|---------------------------------------------|----------|---------|----------------------------|------|
| **Bone Marrow** |                        |                                             |          |         |                            |      |
| Human BM p3-p5 | UC                     | CD63+                                       | Human osteoblasts | 5 µg/mL | SD rats Calvarial defect model | Qin et al., 2016 |
| Human BM p3-p6 | UC                     | 80–100 nm CD9+, CD63+, CD81+                | HBMSCs Transwell assay, qRT-PCR | 5 µg/mL | SD rats Calvarial defect model | Takeuchi et al., 2019 |
| Human BM p4-p6 | UC                     | 80 nm CD9+, CD81+, flotillin-1-             | N/A N/A | N/A | C57BL/6 mice (WT, CD9–/–) Femoral fracture model | Furuta et al., 2016 |
| Human BM p0    | N/A                    | N/A                                         | HMSCs qRT-PCR, immunoblotting cells | EVs from 0.5 × 10^6 cells | Athymic nude mice SC implantation model | Furuta et al., 2016 |
| Rat BM p4-p6   | UC                     | TRPS: 50–150 nm; TEM: 50–100 nm CD9+, CD63+, TSG101+, GM130- | BMSC Cell proliferation, migration, and osteogenic differentiation assays | 1 × 10^10 EVs/mL | BMSCs | Zhu et al., 2019 |
| EV cell origin | Method of EV isolation | EV Characterization (Size, surface markers) | In vitro | In vivo |
|----------------|------------------------|---------------------------------------------|----------|--------|
|                | Cell and assay type    | Amount of EVs delivered                     | Cell and assay type    | Model | Delivery mechanism | Amount of EVs delivered | In vivo effects |
| Rat BM p3      | UC                     | 100-1000 nm CD73+, CD105+, CD90+, CD44+, CD34+, CD45- | HUVECs Cell proliferation, scratch wound, and tube formation assays | 1, 20, or 50 µg/mL | Increased proliferation, migration, and pro-angiogenic potential | Implanted via DBM scaffold | 20 µg | Increased bone regeneration, bone volume and BV/TV |
| Rat BM p3-p5   | UC                     | TPRS: 60–130 nm TEM: 60–100 nm CD9+, CD63+, TSG101+ | BMSCs Proliferation assay, qRT-PCR | 0, 1 × 10^3, 5 × 10^3, or 1 × 10^4 EVs/mL | Increased proliferation, osteogenic differentiation | 15-month SD rats Distraction osteogenesis model | Localized injection | 1 × 10^10 EVs per week | Increased new bone formation, BV/TV Improvement in mechanical tests |
| Rat BM p2-p5   | UC                     | 122 nm CD90+, CD29+, CD34, CD11b/C-TEM: 1 × 10^10 EVs | MC3T3-E1Cs EdU incorporation assay, qRT-PCR | Increased proliferation migration, and osteogenic differentiation | Wistar rats Femoral fracture model | Localized injection | 1 × 10^10 EVs | Increased BV/TV Increased expression of BMP2, Smad1/5, Runx2, OCN, OPN and OCN |
| Rat BM p3-p5   | UC                     | 50–150 nm CD81+, CD63+ | hMSCs Osteogenic differentiation assay, qRT-PCR | Increased osteogenic differentiation | SD Rats Femoral fracture model | Localized injection | 200 µg | Increased fracture healing, BV/TV Increased expression of Runx2, ALP and CoI |
| Adipose        | UC                     | Human adipose p1 105 ± 72 nm CD63+, CD9+, tubulin-, histone1- | HBMSCs Cell proliferation, transwell, and osteogenic differentiation assays, qRT-PCR | 25 µg/mL | Increased proliferation, migration, and osteogenic differentiation | Implanted via PLGA/pDA scaffold | 165.72 ± 15.4 µg | Increased new bone formation, mature collagen formation, bone volume, recruitment of host MSCs, and expression of RUNX2 and OCN |
| Adipose        | UC                     | mRNA-375 over-expressing human ADSC 75 nm CD63+, CD9+, β-tubulin-, histone1- | HBMSCs Osteogenic differentiation assay, qRT-PCR | 50 µg/mL | Increased osteogenic differentiation | Implanted via hydrogel | 1 µg | Increased bone formation and BV/TV |

BMSC, bone marrow-derived mesenchymal stem cells; BV/TV, bone volume/total volume; DBM, decalcified bone matrix; HBMSCs, human bone marrow-derived mesenchymal stem cells; HMSCs, human mesenchymal stem cells; HUVECs, human umbilical vein endothelial cells; MSC, mesenchymal stem cells; pDA, polydopamine; PLGA, poly(lactic-co-glycolic acid); SC, subcutaneous; Ref, references; SD, Sprague Dawley; UC, ultracentrifugation, ≥100,000 g; WT, wild type.
### TABLE 3 | Preclinical studies employing BMMSC- and ADMSC-derived EVs to induce macrophage polarization.

| EV cell origin | Method of EV isolation | EV Characterization (Size, surface markers) | In vitro | In vivo | Pathway(s)/ miRNA(s) involved | Ref. |
|---------------|------------------------|---------------------------------------------|----------|---------|--------------------------------|-----|
| Bone Marrow   |                        |                                             |          |         |                                |     |
| Human jaw BM  | ExoQuick-TC kit        | Human PBMCs-derived macrophages              |          |         |                                |     |
| p2-p5         |                        | CD63+, CD81+                                | N/A      |         |                                |     |
|               |                        | Human PBMCs-derived macrophages              | N/A      |         |                                |     |
|               |                        | IHC, qRT-PCR                                | N/A      |         |                                |     |
|               |                        | 20–200 nm                                   | 50 µg/mL |         | Increased M2 polarization      |     |
|               |                        |                                             |          |         | Increased IL-10 expression, CD14|     |
|               |                        |                                             |          |         | and CD163 double-positive cells|     |
|               |                        |                                             |          |         | Decreased TNF-α expression     |     |
|               |                        |                                             |          |         |                                |     |
| Human BM*     | UC                     | RAW264.7                                    |          |         |                                |     |
| p4 cultured   |                        |                                             |          |         |                                |     |
| w/and w/o melatonin |                |                                             |          |         |                                |     |
|               |                        | CD105+, CD90+, CD73+, CD34–, CD45–          | N/A      |         | Increased M2:M1 ratio          |     |
|               |                        |                                             |          |         | Reduced IL-1β, TNF-α, IL-10,  |     |
|               |                        |                                             |          |         | Arg-1 and iNOS expression      |     |
|               |                        |                                             |          |         | Increased PTEN, AKT and p-AKT  |     |
|               |                        |                                             |          |         | expression Melatonin (MT)-EVs  |     |
| Rat BM        | Total Exosome isolation reagent; UC |                                             |          |         | Increased M2 polarization      |     |
|               |                        |                                             |          |         |                                |     |
| Rat BM        | UC, Invitrogen exosome isolation kit |                                             |          |         |                                |     |
| p4–p8         |                        |                                             |          |         |                                |     |
|               |                        | 30–120 nm                                   | N/A      |         |                                |     |
|               |                        |                                             |          |         |                                |     |

(Continued)
Table 3 | Continued

| EV cell origin | Method of EV isolation | EV Characterization (Size, surface markers) | Cell and assay type | Amount of EVs delivered | In vitro effects | In vivo Model | Delivery mechanism | Amount of EVs delivered | In vivo effects | Pathway(s)/miRNA(s) involved | Ref. |
|----------------|------------------------|---------------------------------------------|--------------------|------------------------|------------------|---------------|------------------|----------------------|------------------|----------------------------|------|
| Mouse BM       | UC                     | 35.21 nm NAUX+, TSG101+, CD9+, CD63+, TUNEL assay | 10 µg/µL           | Reduced apoptosis       | C57BL6 mice Dilated cardiomyopathy model | Tail vein injection | 300 µg         | Reduced IL-1, IL-6, and TNF-α expression in circulation | Reduced circulating macrophages, increased M2 polarization, number of anti-inflammatory macrophages and Ly6C<sup>low</sup> cells | AK2-STAT6 | Sun et al., 2018 |
| Mouse BM       | UC                     | 50–150 nm TSG101+, CD9+, CD63+, CD81+ BV2 microglia and primary microglia qRT-PCR | 200 µg/mL          | Increased M2:M1 ratio   | C57BL6/6 mice SCI model | Tail vein injection | 200 µg         | Increased M2:M1 ratio | miR-216a-5p | Liu et al., 2020a |
| Adipose        | UC                     | 50–150 nm CD63+, CD81+, CD105+, CD40+, CD44+, CD146+, HSP90+ Bone marrow-derived macrophages (BMDM) qRT-PCR | 100 µg/mL          | Increased M2 polarization | C57BL6/6 mice LPS-induced lung injury | Tail vein injection or intratracheal injection | 50 µg          | Increased M2 polarization | miR-27a-3p | Wang J. et al., 2020 |
| Adipose        | ExoQuick-TC kit        | ~100 nm TSG101+, CD9+, CD63+, HSP90+ Macrophages qRT-PCR | 10 or 20 µg/mL     | Increased M2 polarization | C57BL6/6 mice High-fat diet model | Intraperitoneal injection | 50 µg          | Increased WAT Beiging | N/A                         | Zhao et al., 2018 |

BV2: mouse, C57BL/6, brain, microglial cells; Centrifugation: <100,000g; DCM, dilated cardiomyopathy; ICH, intracerebral hemorrhage; IHC, immunohistochemistry; IV injection, intravenous injection, no vein specified; LPS, lipopolysaccharide; PBMCs, peripheral blood mononuclear cells; SC injection, subcutaneous injection; Ref, references; SCI, spinal cord injury; SD: Sprague Dawley; STZ, streptozotocin; UC, ultracentrifugation, ≥100,000 g; WAT, white adipose tissue; WB, Western blotting. *Cells cultured in special conditions (e.g. hypoxia).
| EV cell origin | Method of EV isolation | EV Characterization (Size, surface markers) | In vitro | In vivo | Pathway(s)/miRNA(s) involved | Ref. |
|----------------|------------------------|---------------------------------------------|---------|---------|----------------------------|------|
| Bone Marrow | UC | 162 ± 59 nm | N/A | N/A | N/A | NOD/SCID/IL2Rγ KO mice STZ-induced diabetic nephropathy model | Reduced tubular damage, interstitial and glomerular collagen deposition Reduced collagen I, TGF-β and α-SMA (mRNA) | Grange et al., 2019 |
| Mouse BM p2-p3 | UC | 100 nm | N/A | N/A | N/A | C57BL6/J mice 5/6 subtotal nephrectomy model | Reduced interstitial lymphocyte infiltration | He et al., 2012 |
| Adipose | ExoQuick-TC kit | CD9+, CD63+ | 100 µg/mL w/ or w/o 100 ng/mL LPS | Increased the expression ratios of collagen III to collagen I, TGF-β3 to TGF-β1, and MMP1 and −3 to TIMP1 | Intravenously injection | 1 × 10^10 EVs/week, 4 weeks, 5 injections total | N/A | Wang L. et al., 2017 |
| Human adipose p3 | UC | CD63+ | N/A | Reduced transformation of renal TECs into profibrotic phenotype Reduced α-SMA, Col-I, TGF-β1, and CTGF expression | Tail vein injection | 100 µg | TGF-β1/Smad3 | Zhu et al., 2017 |
| Pig adipose (autologous) | UC | 2/3 ~150 nm, 1/3 ~50 nm | CD9+, CD29+, CD63+ | N/A | N/A | Pigs Metabolic syndrome and renal artery stenosis model | Intrarenal injection | 1 × 10^10 EVs | Eirin et al., 2017 |

*IV injection, intravenous injection, no vein specified; Ref, references; UC, ultracentrifugation, ≥100,000 g.*
of STZ-induced diabetic nephropathy, while porcine ADMSC-derived EVs resulted in a 24.4% reduction in tubulointerstitial kidney fibrosis in a porcine model of metabolic syndrome and renal artery stenosis (Eirin et al., 2017; Grange et al., 2019). BMMSC-derived EVs were also studied in two different chronic kidney injury models resulting in decreased interstitial lymphocyte infiltration in a 5/6 subtotal nephrectomy model (He et al., 2012), and significantly improved renal function and histological parameters, and reduced apoptosis and fibrotic markers in a cyclosporine nephrotoxicity model (Ramírez-Bajo et al., 2020). While, the anti-fibrotic capacity of ADMSC-derived EVs was less studied in vivo, in vitro studies showed that they inhibited the proliferation of CD4+ and CD8+ T cells (Blazquez et al., 2014), increased the expression ratios of collagen III to collagen I, TGF-β3 to TGF-β1, and MMP1 and -3 to TIMP1 in dermal fibroblasts (Wang L. et al., 2017), and prevented the transformation of tubular epithelial cells to a profibrotic phenotype via activation of tubular Sox9 (Zhu et al., 2017).

### Effects on Immune Cell Infiltration

Modulation of immune cell tissue infiltration is another important target of EV-based therapies. EVs derived from both MSC sources significantly influenced immune cell migration at treatment sites in various preclinical models (see Table 5); although, again, it is difficult to make efficacy comparisons. In murine models of fat grafting (di Han et al., 2018) and high-fat diet (Zhao et al., 2018), ADMSC-derived EVs decreased inflammatory cell infiltration into adipose tissue, while, in a model of type-1 diabetes mellitus, they significantly increased the number of regulatory T cells (Tregs) (Nojehdehi et al., 2018). ADMSC-derived EVs also decreased the infiltration of mast cells, CD86+ cells, and CD206+ cells in skin lesions and reduced the mRNA expression levels of various inflammatory cytokines, such as IL-4, IL-31, and TNF-α in a murine model of atopic dermatitis (Cho et al., 2018). Similarly, BMMSC-derived EVs reduced the infiltration of CD45+ immune cells in a mouse model of aristolochic acid induced nephropathy (Kholia et al., 2020), reduced the number of GFAP+ astrocytes and CD68+ cells in a TBI model (Zhang et al., 2017) and decreased infiltration of leukocytes in a murine model of focal cerebral ischemia (Wang C. et al., 2020).

## Effects on Other Immune Cells and Processes

The immunomodulatory potential of both ADMSC- and BMMSC-derived EVs has also been explored in many other preclinical disease and injury models (see Table 5). For example, ADMSC-derived EVs were found to alleviate inflammation in a retinal laser injury model (Yu et al., 2016), attenuate complement levels in a SCI model (Zhao et al., 2019), and downregulate the expression of inflammatory biomarkers in an acute ischemic stroke model (Chen et al., 2016). Meanwhile, in two kidney I/R injury models ADMSC-derived EVs either upregulated the expression of tubular Sox9 (Zhu et al., 2017), or decreased the expression of inflammatory proteins, including TNF-α, NF-κB, IL-1β, MIF, PAI-1, Cox-2 (Lin et al., 2016). Similarly, ADMSC-derived EVs mitigated scar formation, inhibited granulation tissue formation, increased expression of TGF-β3 compared to TGF-β1 and increased the ratio of collagen III to collagen I in a skin wound healing model (Wang C. et al., 2017). BMMSC-derived EVs have been studied in an even wider array of preclinical injury models than those from ADMSCs (see Table 5 and below).

Extracellular vesicles from BMMSCs have been studied in a wide array of preclinical models where immunomodulation plays a key role. Localized injection of BMMSC-derived EVs decreased inflammation in both an acute MI model (Teng et al., 2015) and an ischemia/reperfusion (I/R) model (Arslan et al., 2013) of myocardial injury. In the I/R model, both local and systemic inflammation were significantly reduced via inhibition of the c-JUK signaling pathway (Arslan et al., 2013). TGF-β expression was also shown to be reduced by BMMSC-derived EVs in a muscle injury model (Iyer et al., 2020). Similarly, treatment with BMMSC-derived EVs reduced IL2 mRNA expression, increased expression of TGF-β and HGF, and increased the ratio of Treg to CD4+ cells among NPCs in a concanavalin A-induced liver injury model (Tamura et al., 2016). Meanwhile, in an allergic airway inflammation model induced by repeated exposure to Aspergillus hyphal extract, BMMSC-derived EVs reduced lung inflammation and airway hyperreactivity, and shifted the inflammatory response of Th2 and Th17 type T-cells (Cruz et al., 2015). In an osteoarthritis model, they reduced knee joint inflammation, mainly due to EV-expressed miR-9-5p directly targeting syndecan-1 (Jin et al., 2020). Finally, BMMSC-derived EV treatment in a renal allograft rejection model resulted in higher numbers of T- and B-cells, reduced NK-cell infiltration and significantly decreased TNFα expression (Koch et al., 2015). In vitro studies illustrate additional mechanism underlying the immunomodulatory effects of MSC-derived EVs. BMMSC-derived EVs significantly inhibited proliferation of CD3-stimulated T-cells (Teng et al., 2015) and increased expression of IL-10 and TGF-β1 in blood mononuclear cells, which induced Tregs differentiation and enhanced their immunosuppressive function (mo Du et al., 2018).

## Similarities and Differences in MSC-Derived EV Signaling and Therapeutic Capacity

In the reviewed studies, treatment with both ADMSC- and BMMSC-derived EVs activated several common signaling pathways related to cellular survival, proliferation and/or differentiation. For example, the VEGF pathway, which is involved in angiogenesis and thus wound healing, was observed to be modulated by EVs in fracture non-union (Zhang et al., 2020), fat grafting (Han et al., 2019a), and calvarial bone defect models (Zhu et al., 2019). Several AKT-related signaling pathways, including AKT/mTOR (Liang et al., 2019), AKT/eNOS (Yu M. et al., 2020), and PTEN/AKT (Liu et al., 2020b), were also induced by ADMSC- and BMMSC-derived EVs. This is not surprising given that AKT participates in a wide range of signaling pathways, including those involved in angiogenesis, osteogenesis, and immunomodulation. Meanwhile, signaling pathways involving Smad family proteins were also activated by
## Table 5
Preclinical studies employing BMMSC- and ADMSC-derived EVs to induce immunomodulation.

| EV cell origin | Method of EV isolation | EV Characterization (Size, surface markers) | In vitro | In vivo | Delivery mechanism | Amount of EVs delivered | In vivo effects | Pathway(s)/miRNA(s) involved | Ref. |
|----------------|------------------------|--------------------------------------------|----------|---------|-------------------|------------------------|----------------|--------------------------|------|
| Bone Marrow    |                        |                                            |          |         |                   |                        |                |                          |      |
| Human BM       | UC                     | 35–100 nm CD63+, CD29+, CD44+, CD49+, CD105+, CD146+, CD9+, CD81+, CD31+, CD326+, GM130- | Aristolochic Acid (AA)-treated murine tubular epithelial cells co-cultured with mouse kidney cortical fibroblasts | qRT-PCR | Decreased a-SMA, TGFβ1, and COL1A1 expression | 1 x 10^10 EVs/mL | Reduced infiltration of CD45+ cells | N/A | Kholia et al., 2020 |
| Human BM       | PEG6000, UC            | ZetaView: 99–123 nm; NanoSight: 133–138 nm | Syntenin+, CD63+, CD81+, CD9+, proliferation-, canexin- | Tail vein injection | CS7HL6/J mice Focal cerebral ischemia model | 16 µg EVs/Kg | Reduced infiltration of leukocytes (PMNs, monocytes/macrophages, lymphocytes) | N/A | Wang C. et al., 2020 |
| Human BM       | UC                     | N/A                                        | N/A      | N/A     |                   |                        |                |                          | N/A | Doepnner et al., 2015 |
| Human BM       | Chromatography         | N/A                                        | N/A      | N/A     |                   |                        |                |                          | N/A | Arslan et al., 2013 |
| Human BM       | ExoQuick kit           | CD9+, CD63+, CD81+                         | Tail vein injection | EV from 2 x 10^6 MSCs | Increased numbers of B-cells, natural killer cells, and T-cells in the peripheral blood | 100 µg, ~ 3 x 10^7 EVs | Reduced local and systemic inflammation, local neutrophil and macrophage infiltration, and circulating WBC count | N/A | Zhang et al., 2017 |
| Human BM       | UC                     | N/A                                        | Tail vein injection | EV from 3 x 10^6 cells | CS7HL6/J mice Allergic Airway Inflammation model | N/A | Reduced AHR, lung inflammation, and numbers of antigen-specific CD4 T-cell (Th2 and Th17 phenotype) | N/A | Cruz et al., 2015 |
| Rat BM p7–p9   | UC                     | N/A                                        | Tail vein injection | Reduced T lymphocytes | Reduced T lymphocytes | 80µg | Reduced T lymphocytes | N/A | Iyer et al., 2020 |
| Rat BM p3      | ExoQuick-TC kit        | CD3-stimulated T-cells Proliferation assay | SD rats Acute MI model | Localized injection | Reduced expression of TGF-β | 1 x 10^6 EVs | Reduced expression of TGF-β | N/A | Iyer et al., 2020 |

(Continued)
### TABLE 5 | Continued

| EV cell origin | Method of EV isolation | EV Characterization | In vitro | In vivo | Pathway(s)/miRNA(s) involved | Ref. |
|---------------|------------------------|---------------------|----------|---------|-----------------------------|------|
| **EV cell origin** | **Method of EV isolation** | **EV Characterization** | **In vitro** | **In vivo** | **Pathway(s)/miRNA(s) involved** | **Ref.** |
| Rat BM p5 | QEV kit | 20–130 nm CD9+, TSG101+, calnexin− | N/A | N/A | Wistar rats SCI model | Tail vein injection | 100 µg | Reduced complement levels and expression of NF-κB | Zhao et al., 2019 |
| Rat BM p2 | UC | 40–100 nm CD9+, CD63+, TSG101+, calnexin− | N/A | N/A | SD rats Osteoarthritis model | Intra-articular injection | N/A | Reduced inflammation | Jin et al., 2020 |
| Mouse BM p1–p3 | UC | 100–150 nm CD9+, CD63+ | N/A | N/A | CS7B6 mice ConcanaVail A-induced liver injury model | IV injection | 10 µg | Reduced expression of IL-2 (mRNA) increased percentage of Treg to CD4+ cells among NPCs, and expression of TGFβ and HGF | Tamura et al., 2016 |
| Mouse BM p3–p5 | UC | 80–150 nm CD63+, CD81+ | LPS-treated MH-S alveolar macrophage cells qRT-PCR | 10 µg/mL | CS7BL/6 mice LPS-induced acute respiratory distress syndrome model | Intratracheal instillation | 50 or 100 µg | Reduced LPS-induced inflammation, lung pathological damage, and lung tissue glycolysis | Deng et al., 2020 |
| Adipose | Human adipose | UC | 20–300 nm CD9+, CD63+, TSG101+, HUVECs | Capillary network formation assay | 50 µg/mL | BALB/c nude mice Fat grafting model | SC injection | 50 µg | Reduced infiltration of inflammatory cells | Han et al., 2018 |
| Human adipose ≤ p9 | Sequential filtration method | Most 200 nm CD9+, CD63+, TSG101+, CD81+ | N/A | N/A | NC/Nga mice Atopic dermatitis model | IV or SC injection | 0.14, 1.4, or 10 µg | Reduced mast cell infiltration number of CD88+ and CD206+ cells, serum IgE, and circulating eosinophils | Cho et al., 2018 |
| Rat adipose | SDS-PAGE | N/A | N/A | N/A | SD rats Acute kidney injury model | IV injection | 100 µg | Reduced expression of TNF-α, NF-κB, IL-1β, MIF, PAI-1, and Cox-2 | Lin et al., 2016 |
| Mouse adipose | UC | 40–100 nm 630 mg/mL protein | N/A | N/A | CS7BL/6 mice Type-1 diabetes mellitus model | Intraperitoneal injection | 50 µg, twice a week | Increased number of Treg cells | Nojehdehi et al., 2018 |
| Mouse adipose p3–p5 | UC | 40–100 nm CD9+, CD63+, CD81+ | N/A | N/A | CS7BL/6 mice Retinal laser injury model | Intravitreal injection | N/A | Reduced injury-induced inflammation and MCP-1 expression | Yu et al., 2016 |
| Pig adipose | SDS-PAGE | N/A | N/A | N/A | SD rats Acute ischemic stroke model | IV injection | 100 µg | Reduced expression of iNOS, TNF-α, NF-κB, IL-1β, MMP-9, and plasminogen activator inhibitor-1/RANTES | Chen et al., 2016 |

AHR, airway hyperreactivity; AKI, acute kidney injury; BSCB, blood–spinal cord barrier; HUVECs, human umbilical vein endothelial cells; I/R, ischemia/reperfusion; IV injection, intravenous injection, no vein specified; MHC, major histocompatibility complex; NPCs, non-parenchymal liver cells; PMNs, polymorphonuclear leukocytes; Ref, references; SC injection, subcutaneous injection; SD, Sprague Dawley; UC, ultracentrifugation, ≥100,000 g; WB, western blotting; WBC, white blood cells; w/, with; w/o, without.
BMMSC- and ADMSC-derived EVs, including those involving BMP-2/Smad1/RUNX2 (Zhang et al., 2020), TGF-β1/Smad3 (Zhu et al., 2017), and Smad5 (Xu T. et al., 2020). Observed differences between studies in activated signaling pathways are due to variations in study design and purpose, including species, in vivo model, cell type, cell passage number, cell state, and assay type. Importantly, overlapping miRNAs within EVs or in EV-activated signaling pathways were not observed between the studies reviewed here. This may be caused by real differences in miRNA expression or variations in the methods that the researchers used to study miRNAs, such as miRNA sequencing (to target wide range of differentially expressed miRNAs) vs. qRT-PCR (to target a much smaller group of miRNAs).

While EVs from both sources showed great potential in inducing angiogenesis, osteogenesis and immunomodulation in various preclinical tissue regeneration models, the therapeutic capacity of EVs derived from ADMSC has been far less widely studied than EVs from BMMSCs. This research imbalance combined with few direct comparative studies, in vivo or in vitro, makes it difficult to conclude which EV source is best for a given application. However, in vitro studies do provide insight into a few possible similarities and differences in ADMSC-derived vs. BMMSC-derived EVs. For example, although EVs from both MSC sources generally expressed CD63, CD9, and CD81, and were negative for expression of either CD45, CD34, or calnexin (Katsuda et al., 2013; Del Fattore et al., 2015; Gouveia et al., 2015; Gualerzi et al., 2017; Bari et al., 2019; Chance et al., 2019; Villatoro et al., 2019; Chance et al., 2020; Hoang et al., 2020), ADMSC-derived EVs expressed higher levels of CD63, phosphatidylycerine (Chance et al., 2019), and ceramides (Gualerzi et al., 2017), while BMMSC-derived EVs displayed more protein types and a higher protein content per cell (Villatoro et al., 2019). EV cargos and resulting in vitro effects were also observed to vary significantly depending on MSC-type. For example, ADMSC-EVS expressed higher levels of HGF, whereas BMMSC-derived EVs expressed higher levels of VEGFA, FGF-2, and PDGF-BB and thus induced greater proliferation in dermal fibroblasts (Hoang et al., 2020). In direct comparison studies, ADMSC-derived EVs were shown to promote more HUVEC tube formation (Chance et al., 2020) and display higher thrombogenic activity (Chance et al., 2019) than BMMSC-derived EVs. Meanwhile, BMMSC-derived EVs increased IL-10 secretion by a factor of 1.8 in phytohemagglutinin-activated peripheral blood mononuclear cells compared to ADMSC-derived EVs (Bari et al., 2019). Interestingly, treatment with MSC-derived EVs did not induce any effects in some in vitro studies, such as those on lymphocyte (Gouveia et al., 2015) and peripheral blood mononuclear cell proliferation (Villatoro et al., 2019).

FACTORS AND STRATEGIES AFFECTING EV THERAPEUTIC EFFICACY

The method and route of EV delivery, such as intravenous injection (IV), localized injection, subcutaneous injection (SC), intraperitoneal injection (IP), intra-arterial infusion (IA), intramuscular injection (IM), topical application, or carrier-based delivery, significantly affects EV biodistribution and thus therapeutic efficacy in vivo. For example, in one study, IV administration lead to significantly increased BMMSC-derived EV accumulation in the liver and spleen and decreased accumulation in the gastrointestinal tract compared to SC or IP delivery; whereas IP injection lead to more EVs in the pancreas compared to IV administration (Wiklander et al., 2015). Meanwhile, IP injection of BMMSC-derived EVs was more therapeutically effective in a hepatic failure model than IV injection, resulting in a better survival rate (Haga et al., 2017). However, as systemic delivery methods, such as IV or IP injection, tend to result in EV accumulation in the liver, spleen and lungs, regardless of the cell source, delivery route, or injury model being studied (Gatti et al., 2011; Wiklander et al., 2015; Eirin et al., 2017; Maudens et al., 2018), carrier-based EV delivery methods have been developed to localize and control release (Maudens et al., 2018).

Carrier-based EV delivery methods, such as hydrogel encapsulation and surface absorption on membranes or scaffolds, provide several advantages over systemic delivery methods. Localized delivery concentrates EVs in the vicinity of target tissue, potentially reducing the amount of EVs required for achieving a given therapeutic effect, and can also prolong EV release (Liu et al., 2018; Riau et al., 2019). For example, delivery of EVs isolated from ADMSCs via a pluronic F127/oxidized hyaluronic acid-poly-ε-lysine hydrogel accelerated wound healing, promoted neovascularization, and increased collagen I and III expression in a diabetic wound healing model over a 21-day period, compared to delivery of EVs alone (Wang C. et al., 2019). Similarly, EVs loaded onto poly(lactic co-glycolic acid)/polydopamine scaffolds exhibited continuous release in vitro, with 28.19 ± 9.2% EVs retained within the scaffold after 8 days, and resulted in significantly improved bone regeneration when implanted in vivo (Li et al., 2018).

EV cargo, and thus therapeutic efficacy, is strongly influenced by a variety of factors, including donor-to-donor variability (e.g., age, gender, health status), tissue and site of cell origin (e.g., vertebral vs. femoral bone marrow), cell passage number, culture microenvironment (e.g., mechanical, chemical, hypoxia vs. normoxia), and cell state (e.g., differentiation, metabolism). For example, melatonin-treated MSCs secreted smaller sized EVs that resulted in decreased inflammation and wound size and increased angiogenesis in a diabetic wound healing model compared to EVs from untreated MSCs (Liu et al., 2020b). Meanwhile, EVs isolated from rats with type 1 diabetes yielded less bone and blood vessel formation in a rat calvarial defect model than BMMSC-EVs from normal rats (Zhu et al., 2019). Further, BMMSC-EVs obtained from young donors induced increased fracture healing compared to EVs from old donors (Xu T. et al., 2020).

Many studies employ specific cell culture conditions and/or pretreatments to obtain EVs with desired cargo(s),
including hypoxic conditions, drug or growth factor treatments, genetic modification, and 3D culture. These treatments have been shown to increase the therapeutic potential of the resulting EVs in several models (Haque et al., 2013; Zhang et al., 2017; Zhu et al., 2018). For example, EVs isolated from 3D cultured MSCs yielded enhanced functional recovery and immunomodulation in a traumatic brain injury model compared to EVs derived from 2D culture (Zhang et al., 2017). Similarly, EVs obtained from hypoxia preconditioned MSCs exhibited increased neoangiogenesis and repair in a myocardial injury model compared to normoxia EVs (Zhu et al., 2018). In yet another study, IL-1β treatment increased miR-146a expression in MSCs and their corresponding EVs, which in turn resulted in increased miR-146a expression and M2 polarization in EV-treated macrophages (Song et al., 2017). However, care must be taken in using such strategies to enhance EV therapeutic efficacy as cellular pretreatment can also lead to adverse effects. For example, culturing MSCs in hypoxic conditions can interfere with differentiation and mitochondria biogenesis (Ejtehadifar et al., 2015), which, in turn, can affect the therapeutic efficacy of the resulting EVs, as mitochondria were shown to be transferred via MSC-derived EVs into recipient cells (Jackson et al., 2016; Konari et al., 2019).

Post-modification of EVs is also widely used in studies to modify therapeutic efficacy. EV surfaces can be modified to facilitate uptake by specific target cells. For example, MSC-derived EVs that were surface-modified with cationic pullulan displayed increased liver targeting and resulted in improved liver function compared to unmodified EVs in a rat model of liver damage (Tamura et al., 2017). Therapeutic miRNAs and other cargos can also be introduced into EVs to improve efficacy (Naseri et al., 2018). For instance, overexpressing miR-181a in MSCs-derived EVs resulted in decreased infarct size and area-at-risk in a myocardial IR injury model compared to control EVs (Wei et al., 2019). Surface modification strategies can also be used to improve EV stability within the circulation, as glycosylation of EV surface peptides was shown to EV delivery to neuroblastsoma cells (Hung and Leonard, 2015). However, post-processing of EVs can also lead to adverse effects. For example, post-processing EVs via mechanical extrusion or electroporation can result in loss of EV integrity, and biological activity (Shi et al., 2018).

OBSTACLES TO CLINICAL TRANSLATION OF MSC-DERIVED EV THERAPIES

There is a crucial need for studies which directly and systematically compare EVs derived from ADMSCs and BMMSCs to determine the optimal EV source for specific clinical applications. More importantly, transferring EV-based therapies to the clinic will require the development of reproducible approaches for high-yield production of EVs with well-defined properties and therapeutic potential. Standardized EV purity metrics and isolation and characterization methods will thus be critical to enable not only systematic comparison of therapeutic EV sources, also for validation of safety and efficacy. However, standardizing characterization of even a simple parameter such as EV size can be challenging. While a wide variety of methods have been used to characterize EV size, concentration, and polydispersity, including Transmission Electron Microscopy, Atomic Force Microscopy, Nanoparticle Tracking Analysis, Tunable Resistive Pulse Sensing, and Dynamic Light Scattering (Caponnetto et al., 2017), these methods can result in different size range and concentration determinations for the same EV samples (Zhu et al., 2019; Wang C. et al., 2020), even when employing different devices based on the technology (Bachurski et al., 2019; Wang C. et al., 2020). Furthermore, different EV isolation methods can also preferentially result in different EV subpopulations, exhibiting variations in EV size distribution, yield, purity, mRNA, and protein profile (Van Deun et al., 2014; Zlotogorski-Hurvitz et al., 2015).

Extracellular vesicles storage is another important issue in expanding clinical EV treatments. For example, the combination of lyophilization and cryoprotectants was found to maintain model enzyme activity within EVs to a greater extent than lyophilization alone, or storage at 4°C and –80°C (Frank et al., 2018). Further studies to systematically characterize the dynamic changes in EV content and number for varying storage periods and conditions will be required to extend EV therapeutic use.

CONCLUSION

The reviewed studies demonstrate that tissue regeneration therapies based on both BMMSC- and ADMSC-derived EVs show promise as alternatives to MSCs-based treatment. However, there is still limited evidence to determine which EV source is optimal for which tissue regeneration application, as there are significantly more studies which used BM-MSC-derived EVs than ADMSC-derived EVs, few comparative studies, and considerable variation in overall study design. There is thus a crucial need for more studies, particularly in vivo, which directly compare the therapeutic efficacy of EVs derived from ADMSCs and BM-MSCs. Optimization of donor sources, passage number, and culture conditions will also be essential to maximizing EV therapeutic capacity for specific applications. Establishment of thorough EV characterization standards, including size distribution, surface markers and cargo(s), as well as isolation and production standards will also be crucial in both systematic comparison of EV therapeutic efficacy as well as transferring EV therapies to the clinic.

AUTHOR CONTRIBUTIONS

YL performed the literature search and wrote the manuscript. CH revised the manuscript. Both authors reviewed the manuscript and approved the final version.
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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