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Small extracellular vesicles from mesenchymal stromal cells: the next therapeutic paradigm for musculoskeletal disorders

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ABSTRACT

Musculoskeletal disorders are one of the biggest contributors to morbidity and place an enormous burden on the health care system in an aging population. Owing to their immunomodulatory and regenerative properties, mesenchymal stromal/stem cells (MSCs) have demonstrated therapeutic efficacy for treatment of a wide variety of conditions, including musculoskeletal disorders. Although MSCs were originally thought to differentiate and replace injured/diseased tissues, it is now accepted that MSCs mediate tissue repair through secretion of trophic factors, particularly extracellular vesicles (EVs). Endowed with a diverse cargo of bioactive lipids, proteins, nucleic acids and metabolites, MSC-EVs have been shown to elicit diverse cellular responses and interact with many cell types needed in tissue repair. The present review aims to summarize the latest advances in the use of native MSC-EVs for musculoskeletal regeneration, examine the cargo molecules and mechanisms underlying their therapeutic effects, and discuss the progress and challenges in their translation to the clinic.

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Introduction

Musculoskeletal disorders, with osteoarthritis (OA) and low back pain identified as the major conditions, are the leading cause of disability worldwide, resulting in loss of productivity and high socio-economic costs [1]. The prevalence and disability due to musculoskeletal disorders are expected to continue to rise with an increasingly obese and aging population. OA is the most common form of arthritis and one of the leading causes of disability, affecting over 500 million people worldwide [2]. Although pharmacological therapies such as non-steroidal anti-inflammatory drugs are generally effective for symptomatic relief in musculoskeletal disorders, none of these treatments are curative. Consequently, if poorly managed, surgery is the only solution at the end stage of the disease. Thus, there is a need to explore new therapeutic options for better management of musculoskeletal disorders.

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In the past decade, mesenchymal stromal/stem cells (MSCs) have emerged as a promising therapeutic agent for a wide variety of injuries and diseases, with > 1400 registered clinical trials (<https://clinicaltrials.gov/>). As multipotent progenitor cells, these cells are able to differentiate into multiple connective tissue cell types, including adipocytes, chondrocytes and osteocytes [3]. MSCs are plastic-adherent under standard conditions, express CD105, CD73 and CD90, but not CD45, CD34, CD14 or CD11b, CD79 α or CD19 and HLA-DR surface molecules, and under appropriate conditions can differentiate to adipogenic, chondrogenic and osteogenic lineages [4]. Although the use of MSCs was originally predicated on their potential to differentiate and replace lost cells in injured or diseased tissues, it is now accepted that MSCs mediate tissue repair through paracrine secretion, particularly extracellular vesicles (EVs).

EVs are a heterogeneous class of bi-lipid membrane particles released by all cells and are broadly divided into the major subtypes known to date; namely, exosomes, microvesicles and apoptotic bodies [5]. Being of endosomal origin, exosomes are released through fusion of multivesicular bodies with the plasma membrane and are about 40–150 nm in diameter. Microvesicles (also known as ectosomes or microparticles) are formed through budding of the plasma

membrane and range from 100 nm to 1000 nm in size. Apoptotic bodies range from 50 nm to 5000 nm and are formed by blebbing of apoptotic cells. The molecular composition and bioactive cargo of different EV subtypes can include various combinations of lipids, proteins, nucleic acids and metabolites depending on the type and state of the cell, as well as the biogenesis pathway. Much of the EV cargo is located inside the vesicles. However, EVs may also acquire a protein corona through diverse molecular interactions of the EV surface with the proteins present in the surrounding interstitial fluid or blood plasma [6].

Various isolation methods, ranging from ultracentrifugation, filtration and size-exclusion chromatography to polyethylene glycol/polymer-based enrichment and antibody-based methods, have commonly been used for EV isolation [7]. However, isolation of EV subpopulations is presently challenging because of profound EV heterogeneity, with similar or overlapping biochemical or biophysical properties, and lack of definitive markers to unambiguously identify an EV subtype [7,8]. In view of these challenges, the International Society for Extracellular Vesicles, in an effort to promote standardization, reproducibility and rigor in EV research, has recommended that the EV nomenclature, isolation and characterization follow the minimal information for studies of EVs 2018 guidelines [9]. Minimal information for studies of EVs 2018 recommends use of the collective term “EVs” as well as operative terms such as “size” (small or large) to enhance the understanding of homogeneity of EV preparation. Information on sample type, culture conditions, collection and pre-processing and experimental details of EV separation, concentration and characterization should also be provided.

Although EVs were originally thought to collect and dispose of cellular waste, it is now recognized that EVs mediate intercellular communication through the delivery of bioactive cargoes to elicit biological responses in recipient cells. With MSC-EVs, many of these biological responses translate to a therapeutic outcome in injured or diseased cells. Increasingly, native MSC-EVs have replicated the wide-ranging therapeutic efficacy of their parental MSCs in several injuries and diseases, including musculoskeletal disorders. Essentially, native MSC-EVs are EVs secreted by MSCs where the EVs or secreting MSCs are not modified or engineered to modify EV composition. In this review, we will summarize the latest advances in the use of native MSC-EVs for the treatment of various musculoskeletal disorders, examine the cargo molecules and mechanisms underlying their therapeutic effects, and discuss the progress and challenges in their translation to the clinic.

MSC-EVs

For many years, the search for active trophic factors in MSC secretions mainly focused on growth factors, cytokines and chemokines. However, it became evident that no single factor could sufficiently account for all of the observed therapeutic effects of MSCs in tissue repair [10]. In 2009, Bruno *et al.* [11] first attributed the therapeutic effects of MSC-conditioned medium in a mouse model of glycerol-induced acute kidney injury to 80- to 1000-nm EVs, which at that time were called microvesicles. In 2010, Lai *et al.* [12] showed by size fractionation studies that 50- to 200-nm EVs, which were originally called exosomes, were efficacious against myocardial ischemia–reperfusion injury. In head-to-head comparisons in different animal models, EVs were shown to be therapeutically equivalent to their parental MSCs [13,14]. To address the issue of heterogeneity of MSC-EVs, Bruno *et al.* [15] performed differential ultracentrifugation to separate different MSC-EV subpopulations and found that a 100,000 g exosome-enriched EV population but not a 10,000 g microvesicle-enriched EV population was renal protective. More recently, Xu *et al.* [16] showed that EVs with a mean size of approximately 150 nm had more beneficial effects on tendon repair in a rat model of

Achilles tendinopathy than EVs with a mean size of approximately 230 nm.

Today, it is widely accepted that MSCs exert their therapeutic effects through the release of EVs, particularly small EVs (sEVs) with a size range of 50–200 nm [8]. This identification of sEVs as the active agent underpinning the therapeutic effects of MSCs provided the rationale to transform living MSC therapy into non-living MSC-sEV therapy, with many inherent advantages [17]. Unlike living MSCs, which have the capacity to engraft and replicate, MSC-sEVs are non-living and non-replicative and thus have a reduced risk of tumor or aberrant tissue formation. Their small size also renders MSC-sEV preparations amenable to sterilization by filtration and administration via different routes with reduced risk of embolism. MSC-sEV production is also more scalable and amenable to process optimization. For instance, the cell source for sEV production can be selected and immortalized to generate a high sEV-yielding clonal cell line with infinite expansion potential to ensure reproducible and cost-effective production of sEVs on a large scale [18].

MSC-sEVs for Musculoskeletal Regeneration

Within the musculoskeletal system, MSC-sEVs have demonstrated therapeutic efficacy in a wide variety of conditions, ranging from cartilage and bone defects to debilitating conditions such as OA and intervertebral disk (IVD) degeneration. Here we will summarize key studies and highlight the MSC-sEV-associated cargo molecules that have demonstrated functional effects (Tables 1–3, Figure 1).

Articular cartilage

Focal articular cartilage defects

Being avascularized, articular cartilage has poor intrinsic ability to regenerate upon injury [19]. Current techniques, such as microfracture and autologous chondrocyte implantation (ACI), often yield an inferior fibrocartilage repair that is prone to degeneration [20]. With ACI, there are also issues of limited tissue availability, donor site morbidity and loss of chondrocyte phenotype upon *ex vivo* expansion [20,21].

In the past few decades, MSCs have emerged as an alternative cell type for cartilage repair. Several clinical studies have demonstrated the safety and efficacy of MSC therapies for cartilage repair [22]. Recently, a 10-year follow-up study concluded that autologous bone marrow MSC treatment resulted in clinical outcomes equivalent to ACI, with no apparent increased risk of tumor formation [23]. Based on these findings, it was postulated that MSC-sEVs could recapitulate the therapeutic effects of MSCs in cartilage repair.

We first demonstrated that weekly intra-articular injections of MSC-sEVs were efficacious in promoting osteochondral regeneration in rats [24], and this was mediated through a well-orchestrated mechanism of augmenting cellular proliferation, attenuating apoptosis, increasing matrix synthesis and enhancing anti-inflammatory M2 over pro-inflammatory M1 macrophage infiltration with concomitant suppression of synovial inflammation [25]. Of these EV-mediated cellular activities, MSC-sEVs enhanced chondrocyte proliferation, migration and matrix synthesis, partly through exosomal CD73-mediated adenosine activation of pro-survival signaling pathways; namely, protein kinase B (AKT) and extracellular signal-regulated kinase. Other studies have reported MSC-sEV restoration of mitochondrial function [26] and suppression of apoptosis and senescence in chondrocytes [27] relevant for osteochondral repair (Table 1).

To determine the functional competency of cartilage repair, further studies were performed using larger animal models, including rabbits [26,28] and pigs [29,30]. As a prelude to a clinical trial, our group recently demonstrated the safety and efficacy of MSC-sEVs for repair of critical-size osteochondral defects in a clinically relevant porcine model [29]. Specifically, we showed that MSC-sEVs in

Table 1

Overview of studies using MSC-sEVs for articular cartilage repair.

MSC origin	<i>In vivo/in vitro</i> model	Key outcomes	Pathway (EV molecule)	Author
H-EMB	Rat osteochondral defect	Enhanced osteochondral repair.	–	Zhang [24]
H-EMB	Rat osteochondral defect/Rat chondrocytes	Enhanced osteochondral repair. Increased cell proliferation, migration and matrix synthesis.	AKT/ERK (CD73)	Zhang [25]
H-EMB	Rabbit osteochondral defect	Enhanced osteochondral repair with improved mechanical competence.	–	Wong [28]
H-EMB	Pig osteochondral defect	Enhanced osteochondral repair with improved gross appearance, MRI, histology and mechanical competence.	–	Zhang [29]
H-EMB	Pig chondral defect with BMS/Human chondrocytes	Impaired cartilage repair because of osseous ingrowth but enhanced subchondral bone healing. Improved chondrocyte proliferation.	–	Hede [30]
Ms-BM	Rabbit osteochondral defect/IL-1 β -treated chondrocytes	Restored chondrocyte mitochondrial function, increased matrix synthesis and enhanced synovial M2 macrophage infiltration.	Mitochondrial biogenesis (–)	Chen [26]
UC	Rat osteochondral defect/IL-1 β -treated chondrocytes	Promoted chondrocyte migration, matrix secretion and suppression of apoptosis and senescence.	miR-29b-3p/FoxO3 (lncRNA H19)	Yan [27]
H-EMB	Rat TMJ OA/IL-1 β -treated rat chondrocytes	Enhanced TMJ repair with reduced pain and inflammation. Restored chondrocyte matrix homeostasis.	AKT/ERK/AMPK (CD73)	Zhang [33]
H-AF	Rat MIA-induced knee OA/THP1 macrophages	Reduced pain and enhanced cartilage repair with increased matrix deposition and M2 macrophage polarization.	– (TGF- β)	Zavatti [36]
H-MSC	Rat collagenase II-induced knee OA/IL-1 β -treated rat chondrocytes	Enhanced cartilage repair. Increased chondrocyte proliferation and migration and reduced apoptosis.	– (lncRNA KLF3-AS1)	Liu [37]
H-IPFP	Mouse DMM-induced knee OA/IL-1 β -treated chondrocytes	Improved cartilage repair and gait performance. Enhanced matrix synthesis, reduced apoptosis and decreased expression of catabolic factors.	mTOR/autophagy (miR-100-5p)	Wu [38]
H-BM	Mouse PT OA model/Chondrocytes	Attenuated cartilage degeneration. Enhanced chondrocyte migration and matrix synthesis.	ELF3 (miR-136-5p)	Chen [39]

AF, amniotic fluid; AMPK, adenosine monophosphate-activated protein kinase; BM, bone marrow; BMS, bone marrow stimulation; DMM, destabilization of medial meniscus; ELF3, E74-like factor 3; EMB, embryonic; ERK, extracellular signal-regulated kinase; H, human; IPFP, infrapatellar fat pad; lncRNA, long non-coding RNA; MIA, monoiodoacetate; MRI, magnetic resonance imaging; Ms, mouse; mTOR, mammalian target of rapamycin; PT, post-traumatic; TMJ, temporomandibular joint; UC, umbilical cord.

combination with hyaluronic acid administered at a clinically acceptable frequency of three intra-articular injections at weekly intervals were able to promote functional osteochondral repair at the weight-bearing area of medial femoral condyles, with significantly improved morphological, histological and mechanical outcomes. Notably, the newly repaired tissues associated with MSC-sEV and hyaluronic acid combination treatment yielded mostly hyaline cartilage and exhibited Young modulus and stiffness that approximated those of the native cartilage. In terms of safety assessment, we observed no adverse effects with the use of human MSC-sEVs in immunocompetent animals. This was substantiated by the blood serum analysis, which showed levels of blood urea nitrogen, creatinine, albumin, aspartate aminotransferase, alanine aminotransferase and total bilirubin (for liver and kidney function) within the healthy reference value range for pigs.

The therapeutic outcome of tissue repair not limited to cartilage repair is likely influenced by several factors, including the defect model, dosage, scaffold, frequency, timing and route of administration that require further investigations. A recent study performed in mini-pigs observed that MSC-sEVs applied in conjunction with bone marrow stimulation failed to enhance cartilage repair over bone marrow stimulation alone, but instead promoted subchondral bone healing [30]. Thus, there is a need to determine the dosing regimen of MSC-sEVs for optimal cartilage repair under different clinical scenarios.

Osteoarthritis

Unlike focal cartilage defects, OA is a degenerative joint disease associated with excruciating pain and degradation of cartilage and subchondral bone with varying degrees of synovitis and damage to other joint structures, including ligaments, menisci

and muscles [31]. Several pre-clinical studies have reported the therapeutic efficacy of MSC-EVs in OA [32]. Using a rat model of temporomandibular joint OA, we demonstrated that intra-articular injections of MSC-sEVs alleviated pain and promoted temporomandibular joint repair through a well-coordinated mechanism of attenuating inflammation and enhancing proliferation and matrix synthesis while reducing apoptosis and matrix degradation to restore homeostasis and promote overall repair and regeneration [33]. In chondrocyte cultures, MSC-sEVs counteracted the effects of interleukin (IL)-1 β on matrix degradation and nitric oxide production, partly through CD73-mediated adenosine activation of pro-survival and homeostatic signaling pathways; namely, AKT, extracellular signal-regulated kinase and adenosine monophosphate-activated protein kinase.

In addition, the anti-inflammatory molecules, such as transforming growth factor (TGF)- β 1, present in MSC-sEVs can also exert protective effects. TGF- β is critical for cartilage maintenance, and lack of TGF- β or abnormal TGF- β signaling results in OA-like changes [34]. Given the important role of TGF- β in cartilage homeostasis, the chondroprotective effects of MSCs were attributed to the presence of TGF- β 1 in secreted EVs [35], and resolution of OA damage in rodents was positively correlated with the EV content of TGF- β [35,36].

Other groups have also associated the beneficial effects of MSC-EVs in OA animal models with RNA activity [37–39]. Inhibition of miR-100-5p, miR-136-5p or long non-coding RNA KLF3-AS1 was found to attenuate the chondroprotective effects of MSC-EVs against matrix degradation in rodent models of OA [37–39]. For example, miR-100-5p-containing MSC-sEVs were found to protect articular cartilage from damage and ameliorate gait abnormality in OA mice, possibly through miR-100-5p-regulated inhibition of the mammalian target of rapamycin/autophagy pathway [38].

Table 2
Overview of studies using MSC-sEVs for bone regeneration.

MSC origin	<i>In vivo/in vitro</i> model	Key outcomes	Pathway (EV molecule)	Author
H-EMB	Rat calvarial defect/Human BM-MSCs, endothelial cells (EA.hy926) and rat alveolar macrophages (NR8383)	Enhanced bone regeneration. Increased proliferation, angiogenesis, osteogenesis and M2 over M1 macrophage polarization.	–	Chuah [42]
H-BM	Mouse femur fracture	Accelerated fracture healing by enhancing callus formation and bone union.	–	Furuta [46]
H-iPSC	Rat calvarial defect	Enhanced bone regeneration. Increased BM-MSC proliferation, migration and osteogenesis.	(miR-4532, miR-125b-5p, miR-338-3p) PI3K/Akt (–)	Zhang [45]
H-UC	Rat femur fracture/Neonatal mouse osteoblasts, HUVECs	Promoted angiogenesis and bone healing. Enhanced osteoblast differentiation and HUVEC migration and tube formation.	HIF-1 α /VEGF (–)	Zhang [43]
H-AD	Rat calvarial defect/LPS/IFN- γ -treated M1 macrophages (U937)	Increased M2 over M1 macrophage infiltration and promoted bone healing. Promoted polarization of M1 macrophages toward M2 phenotype and suppressed inflammation.	miR-451a/MIF (miR-451a)	Li [52]
H-BM	Rat calvarial defect/Human BM-MSCs	Enhanced bone regeneration. Promoted BM-MSC migration, osteogenesis and angiogenesis.	– (VEGF)	Takeuchi [47]
R-BM	Rat femur fracture/Mouse osteoblasts (MC3T3-E1), HUVECs	Accelerated bone healing. Enhanced cell proliferation, migration and HUVEC tube formation.	HIF-1 α /VEGF BMP2/Smad1/RUNX2 (–)	Zhang [49]
H-UC	Rat calvarial defect/Endothelial progenitor cells	Promoted bone healing by enhancing angiogenesis and osteogenesis. Enhanced endothelial cell proliferation, migration and tube formation.	NOTCH1/DLL4 (miR-21)	Zhang [44]
H-BM	Mouse femur fracture	Promoted fracture healing. Enhanced osteogenic differentiation of H-BM-MSCs.	WWP1/Smurf2-mediated KLF5/ β -catenin (miR-19b)	Huang [51]
Ms-BM	Mouse femur fracture/Mouse osteoblasts (MC3T3-E1)	Promoted fracture healing. Enhanced osteoblast proliferation and differentiation	LRP4/Wnt/ β -catenin (miR-136-5p)	Yu [53]
Ms-BM	Mouse femur fracture/HUVECs	Enhanced fracture healing. Increased HUVEC proliferation, migration and tube formation.	PTEN/PI3K/AKT (miR-29b-3p)	Yang [50]
H-BM	Rat calvarial defect/Human osteoblasts (hFOB 1.19)	Enhanced bone regeneration. Promoted differentiation and proliferation of osteoblasts.	– (miR-196a)	Qin [48]

AD, adipose; BM, bone marrow; EMB, embryonic; H, human; HUVEC, human umbilical vein endothelial cell; IFN- γ , interferon gamma; iPSC, induced pluripotent stem cell; LPS, lipopolysaccharide; LRP4, low-density lipoprotein receptor-related protein 4; MIF, macrophage migration inhibitory factor; Ms, mouse; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; R, rat; UC, umbilical cord.

Table 3
Overview of studies using MSC-sEVs for IVD regeneration.

MSC origin	<i>In vivo/in vitro</i> model	Key outcomes	Pathway (EV molecule)	Author
H-BM	Rat IVD degeneration/Rat disk explants Human NP cells	Alleviated IVD degeneration with improved disk height, MRI and histological grading. Promoted NP cell migration, proliferation and anabolic activity.	Notch1 (vasorin)	Liao [66]
H-BM	Rat IVD degeneration/AGE-induced ER stress in human NP cells	Alleviated IVD degeneration with improved disk height, MRI and histological grading. Suppressed ER stress-related apoptosis in NP cells.	AKT/ERK (–)	Liao [71]
H-BM	Rat IVD degeneration/TNF- α -induced apoptosis in human NP cells	Reduced NP cell apoptosis and IVD degeneration with improvements in MRI and histology.	PTEN/PI3K/AKT (miR-21)	Cheng [69]
Ms-BM	IL-1 β -induced inflammation and apoptosis in mouse NP cells	Suppressed IL-1 β -induced NP cell inflammation and apoptosis.	MLK3/MAPK (miR-142-3p)	Zhu [67]
H-BM	Mouse IVD degeneration/LPS-induced pyroptosis in mouse NP cells	Reduced inflammation and IVD degeneration by inhibiting NLRP3-mediated pyroptosis.	NLRP3 inflammasome (miR-410)	Zhang [68]
H-BM	TNF- α -induced apoptosis in human NP cells	Suppressed TNF- α -induced apoptosis in NP cells.	miRNA-194-5p/TRAF6 (miRNA-194-5p)	Sun [70]
Ms-BM	Rabbit IVD degeneration/H ₂ O ₂ -treated rat NP cells	Alleviated IVD degeneration with improvements in disk height, MRI and histology. Suppressed H ₂ O ₂ -induced inflammation, apoptosis and matrix degradation of NP cells.	NLRP3 inflammasome (–)	Xia [72]
H-UC	Human NP cells	Inhibited METTL14/NLRP3-mediated pyroptosis in NP cells.	METTL14/NLRP3 (miR-26a-5p)	Yuan [73]
H-U	Rat IVD degeneration/Human NP cells	Alleviated IVD degeneration with improved disk height and histological grading. Suppressed NP cell senescence while enhancing proliferation and matrix synthesis.	TGF- β /Smad/AKT (matrilin-3)	Guo [74]

AGE, advanced glycation end product; BM, bone marrow; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; H, human; H₂O₂, hydrogen peroxide; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; METTL14, methyltransferase-like 14; MLK3, mixed lineage kinase 3; MRI, magnetic resonance imaging; Ms, mouse; NLRP3, NLR family pyrin domain containing 3; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; TRAF6, tumor receptor-associated factor 6; U, urine; UC, umbilical cord.

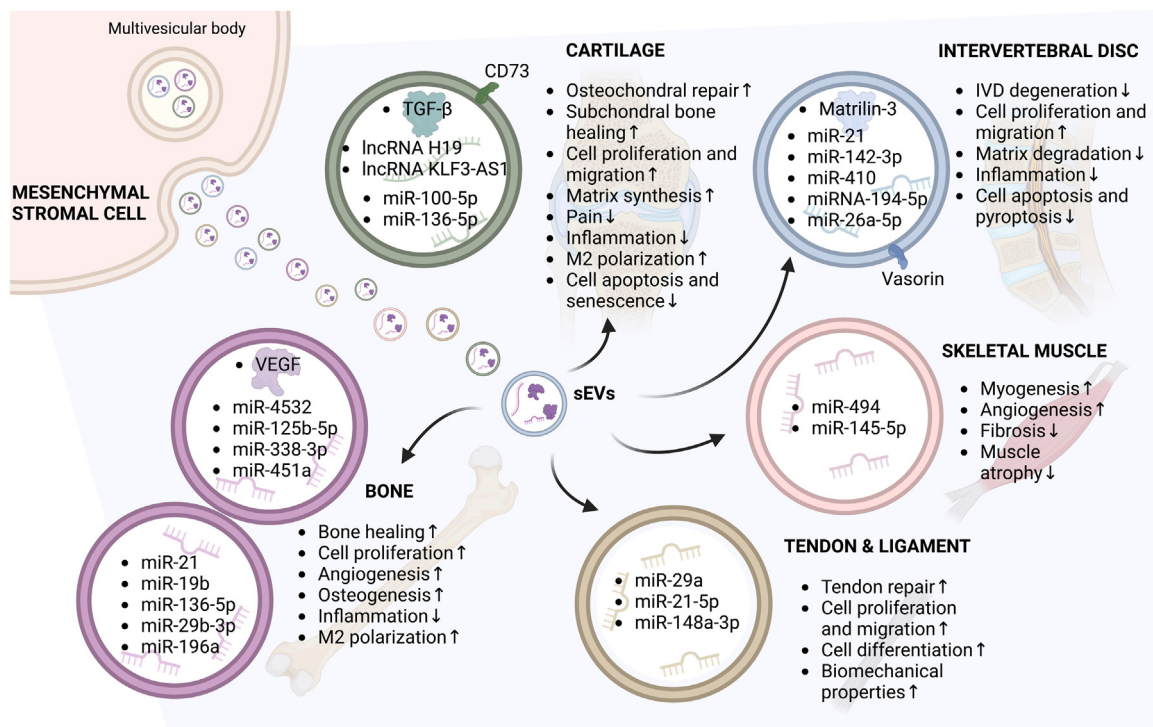


Fig. 1. Cargo molecules of MSC-sEVs and their associated therapeutic effects in the repair of musculoskeletal tissues. Different colored circles show the suggested cargo proteins and RNAs mediating the immunomodulatory and regenerative effects of MSC-sEVs in the repair of cartilage, bone, IVD, skeletal muscle, tendon and ligament following disease/injury. IncRNA, long non-coding RNA. Image is created with Biorender.com.

Bone

Critical-size bone defects and non-unions

The ability of bone to regenerate has long been recognized. However, spontaneous healing is limited in many conditions, such as in large defects of bone due to trauma, tumor and non-union. These conditions require transplantation of bone tissue or substitutes. However, current bone grafts are associated with significant drawbacks, including limited availability and donor site morbidity of autografts, risk of pathogen transmission with allografts and inferior healing and poor remodeling with synthetic grafts [40,41].

In recent years, several studies have reported the therapeutic effects of MSC-sEVs on bone regeneration in different animal models of bone defects (Table 2) [42–53]. In many animal studies, MSC-sEVs were found to promote new bone formation with supporting vasculature and displayed improved morphological, biomechanical and histological outcomes coupled with positive effects on cell survival, proliferation and migration as well as osteogenesis and angiogenesis [54]. Owing to their diverse cargo, MSC-sEVs have been reported to regulate these cellular processes through multiple signaling pathways, including phosphatidylinositol 3-kinase (PI3K)/AKT, BMP/Smad, and Wnt/ β -catenin.

Bone repair and remodeling involve the activation and complex interplay between angiogenic and osteogenic pathways. MSC-sEVs have been shown to enhance angiogenesis in bone healing through angiogenic proteins such as vascular endothelial growth factor (VEGF) [47] and microRNAs (miRNAs) such as miR-21 [44] and miRNA-29b-3p [50]. For example, Takeuchi *et al.* [47] showed that VEGF present in MSC-sEVs was critical for angiogenesis during bone healing. Specifically, rats treated with MSC-sEVs had increased amounts of newly formed bone, whereas rats treated with MSC-sEVs and anti-VEGFA antibody had significantly decreased amounts of newly formed bone, which was even lower than rats treated with saline. In the presence of anti-VEGFA, there was suppressed

angiogenesis with decreased numbers of VEGF- and CD31-positive endothelial cells, reduced mobilization of endogenous CD44-positive MSCs and reduced bone formation.

Other groups have also attributed enhanced osteogenesis by MSC-sEVs to miRNAs such as miR-196a [48], miR-19b [51] and miR-136-5p [53]. For instance, Yu *et al.* [53] showed that miR-136-5p-containing MSC-sEVs promoted mouse fracture healing and osteoblast proliferation and differentiation. Mechanistically, miR-136-5p was found to inhibit the expression of low-density lipoprotein receptor-related protein 4 to activate the Wnt/ β -catenin pathway, thereby stimulating bone growth and fracture healing.

To date, few studies have investigated the immunomodulatory effects of MSC-sEVs on bone repair [54]. Our group demonstrated that a single implantation of collagen sponge loaded with MSC-sEVs was sufficient to significantly improve bone healing in a rat calvarial defect model [42]. In addition to increased proliferation, vascularization and mineralization, we observed that MSC-sEV treatment enhanced M2 over M1 macrophage infiltration with concomitant suppression of inflammatory cytokines IL-1 β and TNF- α to promote bone healing. This preferential M2 over M1 macrophage infiltration could be attributed to the enhanced M2 over M1 macrophage polarization mediated by MSC-sEVs, possibly through anti-inflammatory M2-polarizing cytokines such as IL-10 [55] and matrix proteins such as collagen VI [56,57] present in the EV proteome, although this remains to be demonstrated. With the rich expression of different chemokine receptors, MSC-sEVs could also promote the infiltration of anti-inflammatory M2 macrophages while preventing the migration of pro-inflammatory M1 macrophages through interaction with chemokine ligands expressed on other cells and tissues [58,59]. More recently, Li *et al.* [52] attributed the augmented M2 over M1 macrophage polarization by MSC-sEVs during rat cranial bone repair to miR-451a. Using U937 cells, inhibition of miR-451a expression was found to reverse MSC-sEV effects on polarization of

macrophages through increased macrophage migration inhibitory factor (MIF) expression.

Spine

IVD degeneration

IVD degeneration is the leading cause of back pain. With an aging population, the prevalence of IVD degeneration is increasing drastically and is estimated to affect >90% of people over 50 years of age [60]. Most patients are managed conservatively with physiotherapy and anti-inflammatory/analgesic medication. If conservative management fails, patients may require invasive surgical procedures, such as decompression, fusion or disk replacement. Unfortunately, these approaches only relieve symptoms and do not repair the degenerated IVD.

MSC therapies offer considerable promise for early intervention of IVD degeneration and less invasive alternatives to spinal surgeries. In pre-clinical studies, transplanted MSCs were found to promote IVD regeneration by enhancing matrix production and increased disk height and hydration [61,62]. These pre-clinical data set the stage for multiple clinical trials for this indication [63–65]. Of note, a multicenter randomized controlled study conducted over a 3-year period in 100 subjects with chronic low back pain associated with moderate degenerative disk disease concluded that intradiscal injection of allogeneic bone marrow MSCs was safe and efficacious against IVD degeneration [65].

In recent years, several groups have reported the therapeutic efficacy of MSC-sEVs for IVD regeneration in animal models (Table 3) [66–74]. Using a rat disk degeneration model, Liao *et al.* [66] demonstrated that hydrogel delivery of MSC-sEVs effectively alleviated disk degeneration, with improvements in disk height, magnetic resonance imaging and histological scores. These beneficial effects of MSC-sEVs in disk degeneration could be attributed to vasorin-enhanced proliferation, migration and matrix synthesis of nucleus pulposus (NP) cells mediated through Notch1 signaling. In another study, MSC-sEV treatment was found to alleviate rat IVD degeneration, possibly through transfer of matrilin-3, which complemented the deficit in matrilin proteins in the degenerated IVDs [74]. Consistent with the *in vivo* observations, MSC-sEVs containing matrilin-3 suppressed NP cell senescence while enhancing proliferation and matrix synthesis *in vitro*. Other groups have also ascribed the therapeutic effects of MSC-sEVs in animal models of IVD degeneration to miRNAs such as miR-21, miR-142-3p, miR-410, miR-194-5p and miR-26a-5p [67–70,73]. For instance, MSC-sEV delivery of miR-21 reduced apoptosis of NP cells and alleviated IVD degeneration by targeting phosphatase and tensin homolog (PTEN) through PI3K/AKT pathway [69], whereas delivery of miR-142-3p attenuated NP cell apoptosis and IVD degeneration by suppressing mitogen-activated protein kinase (MAPK) signaling by targeting mixed lineage kinase 3 (MLK3) [67]. In other studies, miR-140 present in MSC-sEVs mitigated IVD degeneration by inhibiting pyroptosis via the suppression of the NLR family pyrin domain containing 3 (NLRP3)/caspase-1 pathway [68], whereas miR-194-5p inhibited tumor receptor-associated factor 6 (TRAF6), reducing NP cell apoptosis and attenuating IVD degeneration [70].

Other musculoskeletal tissue disorders

Apart from therapeutic applications in cartilage, bone and spinal disorders, there are a rapidly growing number of pre-clinical studies that have explored the use of MSC-sEVs for the treatment of other musculoskeletal tissue injuries/diseases, including skeletal muscle injuries [75–77], meniscus tears [78], growth plate defects [79,80] and tendon and ligament injuries [16,81,82].

Skeletal muscle loss

In a mouse model of skeletal muscle injury induced by cardiotoxin, local injection of MSC-sEVs improved muscle regeneration by enhancing myogenesis and angiogenesis through increased muscle fiber growth and capillary density accompanied by suppression of fibrosis [75]. These effects of MSC-sEVs on myogenesis and angiogenesis could be attributed at least in part to the transfer of specific cargo miRNAs such as miR-494. Using a non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mouse model of skeletal muscle defect, administration of MSC-sEVs in fibrin gel was found to promote skeletal muscle regeneration, with increased expression of myoblast determination protein 1 (MyoD) and myogenin. EV-treated animals also showed relatively preserved shapes and sizes of muscle bundles [76]. Additionally, MSC-sEVs have been shown to protect against skeletal muscle loss and atrophy. Using a mouse model of chemotherapy-induced muscle loss, MSC-sEV treatment was found to be effective in recovering body weight and skeletal muscle mass. Mechanistically, MSC-sEV treatment was found to counteract activin A-impaired myogenic differentiation by reducing the expression of activin A receptors ACVR2A and ACVR1B in a miR-145-5p-dependent manner [77].

Growth plate defects

Using a rat model of growth plate injury, we demonstrated that a single intra-articular injection of MSC-sEVs significantly enhanced physal repair and reduced limb length discrepancy but did not inhibit bone bridge formation [79]. Notably, EV-treated animals had an approximately 35% reduction in limb length discrepancy compared with vehicle-treated animals. The clinical implications of this reduction in limb length discrepancy include possibly avoiding invasive surgical procedures that are associated with a high risk of complications. Although the exact mechanisms remain to be fully elucidated, the therapeutic effects of MSC-sEVs on growth plate repair could be partly attributed to enhanced M2 over M1 macrophage polarization and increased anabolic activity of chondrocytes against inflammation [80].

Tendon and ligament injuries

MSC-sEVs have been explored in several studies for the treatment of tendon and ligament injuries [16,81,82]. For instance, Yu *et al.* [81] reported that MSC-sEVs embedded in fibrin significantly improved histological scores and enhanced the expression of tendon-related markers such as mohawk, tenomodulin and type I collagen, as well as mechanical properties of the neotendon. These beneficial effects of MSC-sEVs on tendon regeneration could be attributed to enhanced proliferation, migration and tenogenic differentiation of tendon stem/progenitor cells *in vitro*. Similarly, MSC-sEVs have been shown to enhance ligament healing in a rat medial collateral ligament injury model by reducing scar size, upregulating type I and III collagen expression and increasing collagen production and organization [82]. More recently, using a rat model of Achilles tendinopathy, Xu *et al.* [16] reported that MSC-EVs with a size of approximately 150 nm had superior efficacy in tendon repair over their EV counterpart with a size of approximately 230 nm, as evidenced by improved histological scores, lower fibril density, larger collagen diameter and better biomechanical properties. Further miRNA profiling analysis of the approximately 150-nm MSC-sEVs revealed enrichment of miRNAs such as miR-29a, miR-21-5p and miR-148a-3p, which have been reported to improve tendon healing [16,83].

Diverse Cargo of MSC-sEVs

As exemplified by several of the aforementioned studies, many musculoskeletal disorders display common pathological features of inflammation, oxidative stress, matrix degradation and apoptosis. Owing to their diverse cargo, MSC-sEVs have the ability to mount a

multifaceted mechanism of action (MoA) to elicit biological effects against these diverse pathological processes. However, elucidating the MoA of MSC-sEV preparations against an injury/disease remains challenging. In elucidating a multifaceted MoA where multiple pathological processes are modulated, only processes mediated directly by MSC-sEVs should be mapped to the specific attributes in the bioactive cargo of the MSC-sEV preparation. Currently, the MoA underpinning the therapeutic efficacy of MSC-sEVs is largely attributed to the cargo of RNA or proteins. On this note, the candidate RNA or protein must be present in the EVs in functional competent configuration and concentration to elicit the activity [84].

miRNA- versus Protein-Based MoA

In assessing the role of miRNAs in mediating the therapeutic effects of MSC-sEVs, it is imperative to note that miRNAs are only functional when associated with RNA-inducing silencing complexes (RISCs), and only pre-miRNA can be loaded onto RISCs. Despite several reports on miRNAs mediating the therapeutic effects of MSC-sEVs in the repair of musculoskeletal tissues, few had determined whether the miRNAs were pre-miRNAs or RISC-loaded mature miRNAs to be functional. A search of the publicly accessible databases ExoCarta (<http://www.exocarta.org/>) and Vesiclepedia (<http://www.microvesicles.org/>) revealed that RISC components, including Dicer and Argonaute, are not frequently present in EVs. Of the 41,860 protein entries in ExoCarta, there were only seven entries for Argonaute and none for Dicer. Similarly, of the 349,988 protein entries in Vesiclepedia, there was only one entry for Argonaute and two entries for Dicer. Interestingly, none of these entries were for MSC-EVs. This is consistent with an earlier report that Dicer and Argonaute are not present in MSC-sEVs [85]. Based on published data, we previously calculated that one MSC-sEV contains about 1.3 miRNA molecules [84]. With a large cargo of more than 100 miRNAs in MSC-sEVs, the probability of a sEV having a specific miRNA is about one in 100, and consequently, one cell would have to take up 100 sEVs for one specific miRNA molecule. Based on this probability, uptake of an miRNA molecule is likely not sufficient to elicit a cellular response. Our findings are consistent with an earlier study that tested EVs from diverse sources, including plasma, seminal fluid, dendritic cells, mast cells and ovarian cells, and observed far less than one molecule of a given miRNA per EV [86]. Similarly, in EVs isolated from human lymphoblastoid B-cell lines latently infected with Epstein–Barr virus, one miRNA molecule in 300–16,000 EVs was observed [87]. Increasing the doses of EVs engineered to fuse and deliver their miRNA cargoes to HEK293T cells had no measurable effect on the target mRNAs [87].

By contrast, many proteins in MSC-sEVs are enzymes or ligands for receptors. For example, the enzymatic activity of two adenosine triphosphate (ATP)-generating glycolytic enzymes present in our MSC-sEV preparation, namely, phosphoglycerate kinase (PGK) and pyruvate kinase m2 (PKm2) were determined to be 3.59×10^{-3} U/ μ g EV protein and 5.5×10^{-3} U/ μ g EV protein, respectively. Based on the assumption that one cell has approximately $2-6 \times 10^{-8}$ μ mol ATP, 1 μ g MSC-sEVs can generate in 1 min the same amount of ATP as in $1.5-4.6 \times 10^5$ cells. Alternatively, 1 μ g MSC-sEVs can meet the ATP consumption requirements of 1×10^5 cells/s [84]. Based on these considerations, we previously proposed that MSC-sEVs are more likely to work through a protein-based MoA.

There are also bioactive lipids and metabolites present in the diverse cargo of MSC-sEVs. Lipidomic analysis verified the presence of bioactive lipids such as glycolipids, sphingomyelin, free fatty acids and cardiolipins in MSC-EVs [88,89], whereas metabolite assays identified the presence of metabolites such as lactic acid and glutamic acid [88]. However, considering the molecular complexity of EVs, it remains challenging to attribute the effects of EVs to a specific lipid or metabolite [90].

Clinical Translation and Challenges

The promising therapeutic efficacy of MSC-sEVs in several pre-clinical studies has certainly increased the excitement regarding their translation to the clinic. To date, based on the search terms “extracellular vesicles” and “exosomes” (<https://clinicaltrials.gov/>; accessed on Oct 18, 2022), of 411 registered clinical trials using EVs for various diagnostic and therapeutic applications, 37 involve EVs derived from native MSCs. There are presently four clinical trials using MSC-EVs as therapeutics for various musculoskeletal injuries/diseases (Table 4). For example, a phase 1 trial (NCT05060107) will be conducted to evaluate the safety of EVs derived from allogeneic MSCs in the knees of patients with mild to moderate OA. Ten patients are expected to be enrolled and follow-up will be up to 12 months.

Despite the growing interest in MSC-EVs, the field is still in its infancy, and there are still several challenges that need to be addressed to achieve optimal translation to the clinic. Technical challenges represent a significant issue. Isolation and characterization of EVs and their subtypes are presently hampered by the lack of appropriate technologies and instrumentation to isolate and analyze EVs at nanoscale resolution. The scaling up of cell cultures for clinical manufacture of EVs is also challenging, as this requires optimizing the bioreactor system, defining the culture conditions and implementing the appropriate in-process controls to monitor and control the process [7,91].

Although the International Society for Extracellular Vesicles has recommended use of the collective term “EVs” to circumvent the lack of definitive markers and techniques for purifying a specific EV subtype according to their biogenesis pathway, there are still issues of EV heterogeneity that EV preparations derived from different MSC sources, culture conditions, and isolation protocols are likely to contain different EV subtypes with varying composition and properties. To overcome this and other limitations, immortalizing primary MSCs and establishing monoclonal MSC lines have been proposed and demonstrated to be a feasible strategy for reducing the heterogeneity of MSCs and their derived EVs. In this regard, Chen *et al.* [18] previously reported the transformation of human embryonic stem cell-derived MSCs using the MYC gene to immortalize the MSCs and generate the clonal cell line E1-MYC. The immortalized E1-MYC human embryonic stem cell-derived MSCs grew faster and had increased telomerase activity while retaining the parental karyotype. The produced EVs were therapeutically similar to those derived from the parental MSCs in their ability to reduce infarct size in a mouse model of myocardial ischemia–reperfusion injury. Importantly, EVs produced by these immortalized MSCs did not promote tumor growth in a mouse model of tumor xenograft, indicating that immortalization of MSCs for EV production is a viable strategy for large-scale production of safe EV preparations for therapeutic use [92].

Other parameters, such as culture conditions (i.e., culture medium, supplements, culture systems, oxygen concentration), may also affect EV yield and quality. Controlling these parameters in an EV manufacturing process is therefore necessary but remains

Table 4
Registered clinical trials using MSC-EVs/exosomes for musculoskeletal tissue repair.

NCT number	Title	Status
NCT05060107	Intra-articular Injection of MSC-derived Exosomes in Knee Osteoarthritis (ExoOA-1) (ExoOA-1)	Not yet recruiting
NCT05261360	Clinical Efficacy of Exosome in Degenerative Meniscal Injury (KNEEXO)	Recruiting
NCT04223622	Effects of ASC Secretome on Human Osteochondral Explants (ASC-OA)	Recruiting
NCT05520125	Treatment of Patients With Bone Tissue Defects Using Mesenchymal Stem Cells Enriched by Extracellular Vesicles	Not yet recruiting

challenging unless there are quality control metrics linked to a clearly defined MoA to guide and monitor the process. Consequently, several key identity and potency features of MSC-sEV preparations have been proposed as quality control metrics for measurement to ensure quality and batch reproducibility of the EV product before clinical testing or use. For more information on the proposed identity and potency metrics of MSC-sEVs, we refer readers to the recent reviews [8,93].

Several pre-clinical studies have demonstrated the therapeutic efficacy of MSC-sEVs for musculoskeletal tissue repair, at least for cartilage and bone defects, OA and IVD degeneration. However, most of these studies were performed using small animal models, which have very different sizes, biomechanics and healing capacities compared with humans. To enable clinical translation, clinically relevant large animal models would be necessary [94].

Another challenge involves optimization of the MSC-sEV dosing regimen. Variables such as dosage, frequency, timing, use of scaffold, and route of administration could affect the therapeutic outcome and remain to be systematically optimized for different injuries/diseases under different clinical scenarios. To optimize the dosing regimen, bioavailability and biodistribution studies should be performed but are currently limited by the lack of sensitive and specific labels. For example, lipophilic dyes (e.g., PKH26) that have been widely used for labeling cells and their EVs are frequently associated with labeling artifacts [95]. For large musculoskeletal tissue defects, MSC-sEVs are commonly used in combination with biomaterial scaffolds such as collagen sponges. However, biomaterial scaffolds could have profound effects on EV release, integrity and bioactivity that remain to be investigated, and optimal combinations of MSC-sEVs and biomaterial scaffolds should ideally be determined for each application.

Conclusion

Despite the ongoing challenges, significant advances have been made in the field of therapeutic MSC-EVs in recent years. Notably, an increasing number of pre-clinical studies have demonstrated the therapeutic potential of MSC-sEVs in various musculoskeletal disorders. To translate this potential to therapeutic applications, technical advancements in EV isolation and characterization will be required to define the identity and potency of MSC-sEV preparations. To elucidate the MoA of MSC-sEVs against an injury/disease, the pathological processes modulated by MSC-sEVs should be mapped to the MSC-sEV cargo molecule(s) as specific attribute(s), where critical considerations, including the biologically relevant concentration and functional configuration of the molecule(s), should be made.

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Declaration of Competing Interest

WST is a scientific advisory board member of Paracrine Therapeutics.

Author Contributions

Conception and design of the study: WST. Acquisition of data: KYWT, RT and KLW. Analysis and interpretation of data: KYWT, RT, KLW, DHWH and JHPH. Drafting or revising the manuscript: KYWT, RT and WST. All authors have approved the final article.

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