


Potential of Exosomes as Cell-Free Therapy in Articular Cartilage Regeneration: A Review


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Abstract: Treatment of cartilage defects such as osteoarthritis (OA) and osteochondral defect (OCD) remains a huge clinical challenge in orthopedics. OA is one of the most common chronic health conditions and is mainly characterized by the degeneration of articular cartilage, shown in the limited capacity for intrinsic repair. OCD refers to the focal defects affecting cartilage and the underlying bone. The current OA and OCD management modalities focus on symptom control and on improving joint functionality and the patient's quality of life. Cell-based therapy has been evaluated for managing OA and OCD, and its chondroprotective efficacy is recognized mainly through paracrine action. Hence, there is growing interest in exploiting extracellular vesicles to induce cartilage regeneration. In this review, we explore the *in vivo* evidence of exosomes on cartilage regeneration. A total of 29 *in vivo* studies from the PubMed and Scopus databases were identified and analyzed. The studies reported promising results in terms of *in vivo* exosome delivery and uptake; improved cartilage morphological, histological, and biochemical outcomes; enhanced subchondral bone regeneration; and improved pain behavior following exosome treatment. In addition, exosome therapy is safe, as the included studies documented no significant complications. Modifying exosomal cargos further increased the cartilage and subchondral bone regeneration capacity of exosomes. We conclude that exosome administration is a potent cell-free therapy for alleviating OA and OCD. However, additional studies are needed to confirm the therapeutic potential of exosomes and to identify the standard protocol for exosome-based therapy in OA and OCD management.

Keywords: extracellular vesicle, exosome, chondrocyte, cartilage, osteoarthritis

Introduction

Osteoarthritis (OA) is a common disease linked to mobility-related disability. In 2020, an estimated 654 million people worldwide had knee OA.¹ Rising obesity and population aging are the main contributors to the increasing prevalence and incidence of OA.² OA is a chronic inflammatory disease that causes the deterioration of articular cartilage, which leads to joint pain and stiffness.³ OA is not confined to the articular cartilage, but also affects the synovium, subchondral bone, and joint ligaments.⁴ Osteochondral defect (OCD) develops when cartilage lesions caused by OA affect the subchondral bone.⁵ However, OCD can also be caused by traumatic injury.⁶

Currently, no treatment can halt the progression of OA. However, treatments are available for relieving the symptoms. Treatments for OA can be classified into non-pharmacologic, pharmacologic, surgical, and alternative therapies such as regenerative therapy. Non-pharmacologic treatments are recommended for OA caused by

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modifiable risk factors (eg obesity). Weight loss is associated with improvement in cartilage structure by reducing the joint load.⁷ Exercise has also been proven to relieve pain and improve muscle strength in OA patients.^{8,9} Nevertheless, these treatments are more effective for lower limb OA. Pain is the hallmark symptom of OA. Therefore, pharmacologic treatment is mainly used for managing pain. The classical pain-relieving treatments for OA include acetaminophen and non-steroidal anti-inflammatory drugs (NSAIDs).¹⁰ However, the usage of these drugs increases the risk of gastrointestinal and cardiovascular toxicity.¹¹ The surgical approach is preferred when conservative treatments are not effective. Although most patients have improved quality of life after surgery, potential complications such as pain, infection, and poorer knee function must be taken into consideration when opting for surgical intervention.¹² In addition, knee replacement implants have limited lifespans and may fail eventually.¹³ OCD treatment can be categorized into non-surgical and surgical. Non-surgical treatment includes rest, joint immobilization, and pain-relieving medication. OCD can also be corrected surgically by removing the lesion, microfracture drilling, retrograde drilling, antegrade drilling, cancellous bone graft implantation, osteochondral transplantation, and autologous chondrocyte implantation.¹⁴

Regenerative therapy is a rapidly growing approach used for treating OA and OCD due to conventional therapies being unsatisfactory, only managing to provide symptom control and short-term functional improvement. The conventional therapies fail to address the underlying problem of cartilage and osteochondral bone loss.¹⁵ Several preclinical studies have proven that intra-articular injection of mesenchymal stem/stromal cells (MSCs) enhances cartilage and meniscal tissue regeneration, and slows OA progression by attenuating synovial membrane inflammation.^{16,17} Similarly, a few preclinical studies have reported OCD regeneration by MSCs.^{18,19} Clinically, intra-articular injection of adipose-derived MSCs (AD-MSCs) is safe and produces significant functional improvement in OA and OCD patients.^{20–22} Recently published systematic reviews have shown that MSC therapy is safe and can improve pain and joint function significantly, but the improvement in cartilage regeneration, based on structural assessment by magnetic resonance imaging (MRI), is not statistically significant.^{23,24} Although no major adverse effects have been reported, we should remain aware of the possible risks

associated with cell therapies. These include differentiation into undesirable cell types or tissues and the pro-tumorigenic effect of MSCs.^{25–30} The transplantation of autologous AD-MSCs caused renal fibrosis and inflammatory cell infiltration in the interstitium of a patient with chronic kidney disease, and the reason might be the differentiation of the multipotent stem cells recruited by the MSCs into myofibroblasts or the differentiation of the transplanted MSCs into myofibroblasts.^{31,32}

A paradigm shift has recently occurred, as MSCs and their conditioned medium were found to have similar therapeutic effects.^{33–35} Many papers have summarized that the primary mechanism of action of MSC therapy is paracrine signaling via extracellular vesicles (EVs).^{36–38} The use of EVs can mitigate the risk of transdifferentiation of transplanted MSCs into the wrong cells in response to the local milieu while retaining the beneficial therapeutic effects exerted by MSC paracrine secretion. Furthermore, the use of EVs can also minimize the risk of rejection of donor stem cells and tumor formation (especially for embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)).

EVs can be categorized into three groups based on their biogeneses, ie microvesicles, exosomes, and apoptotic bodies.³⁹ Lai et al⁴⁰ found that the exosome is the active component in MSC-conditioned medium. Exosomes are nanosized intraluminal vesicles in multivesicular bodies (MVBs) secreted by various cell types upon MVB fusion with the plasma membrane.⁴¹ They play a major role in cell–cell communication by transferring their contents, including proteins, lipids, and nucleic acids.⁴² MSC-derived exosomes have drawn much attention recently for their broad therapeutic effect on various diseases such as myocardial infarction, liver fibrosis, cutaneous wound healing, and OA.^{43–47} A recent study reported that exosomes aid bidirectional signaling between MSCs and chondrocytes for chondrogenesis, ECM deposition, and cell proliferation, suggesting that they are important in communication between native heterogeneous cell populations of cartilage tissue, and thus are the crucial paracrine factors in cartilage repair.⁴⁸

Apart from identifying the biogenesis pathway, a concrete means of distinguishing the EV subtypes is absent due to the lack of techniques for purifying and specific markers for the EV subtypes.⁴⁹ Moreover, the size of EVs is heterogeneous and overlap among the subtypes.^{50,51} In fact, the generic term “extracellular vesicles” or “EVs” should be used.⁴⁹ In the present systematic review, the

term “exosomes” essentially refers to 50–200 nm EVs, without demonstration of their origin and/or purity, with regard to the nomenclature used by the authors.

Recently, several systematic reviews reported the potential of using MSC-derived EVs to promote cartilage regeneration in preclinical *in vivo* studies.^{52–54} However, EVs derived from other cell sources have also been used for promoting cartilage regeneration. Thus, the present review was aimed at summarizing the broad literature and evidence on the effects of exosome therapy derived from all cell sources on cartilage repair. Only results from preclinical *in vivo* studies were considered.

Literature Search, Article Selection and Data Extraction

The literature search was conducted from December 30, 2020, to January 6, 2021, on the PubMed and Scopus databases using the following keywords: (exosome OR extracellular vesicle) AND (cartilage OR osteoarthritis OR osteochondral). We included all preclinical and

clinical studies reporting the safety and efficacy of EVs on cartilage regeneration. Studies reporting only *in vitro* findings were excluded. We also excluded secondary literature, studies only available in abstract form, conference/proceeding papers, letters to the editor, theses, and articles written in languages other than English. Figure 1 shows the article identification and selection process. In brief, a total of 160 articles from PubMed and 284 articles from Scopus were identified. After deduplication and title and abstract screening, 79 articles were obtained for full-text screening. Finally, a total of 29 articles published between 2016 and 2021 were included in this review. All included articles reported *in vivo* findings without any clinical data. Two reviewers (C.Y.N. and J.Y.C.) conducted the literature search, article selection, and data extraction independently. The methodological quality of the studies was assessed using the Systematic Review Center for Laboratory Animal Experimentation (SYRCLE) risk of bias tool.⁵⁵ Meta-analysis was not performed due to the lack of quantitative data for pooling because of the

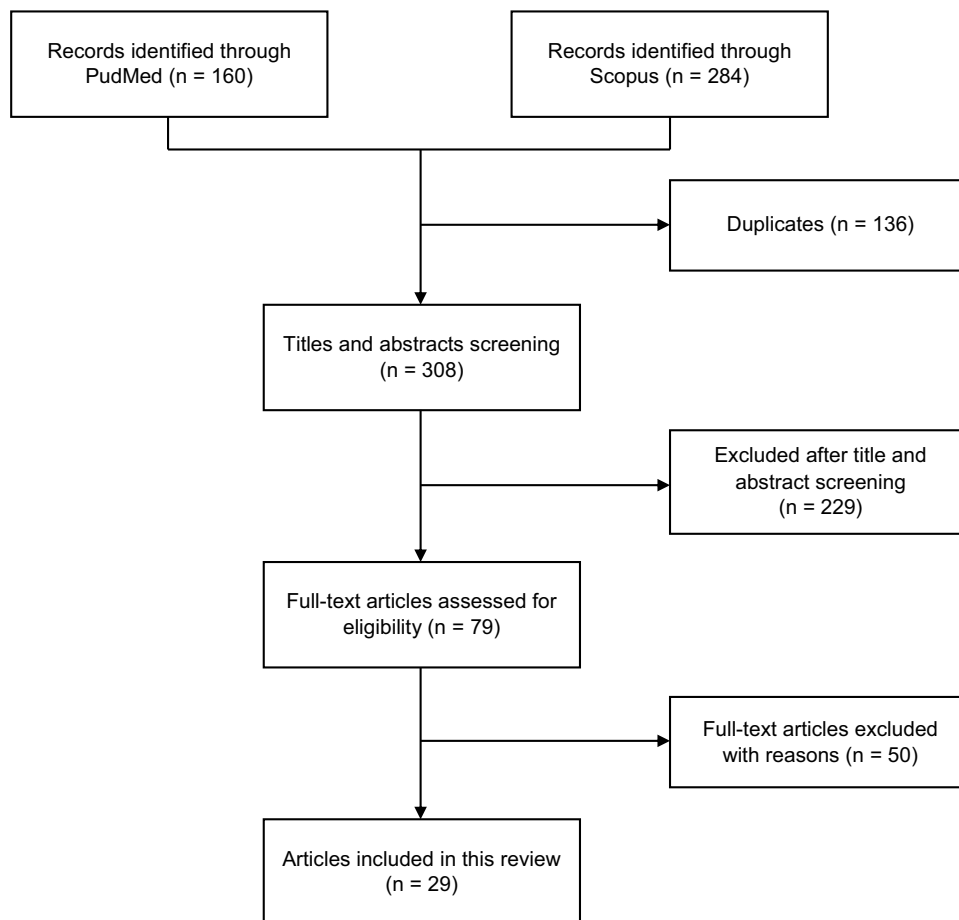


Figure 1 Flow diagram of article selection process.

heterogeneity in the scoring systems and exosome isolation methods used. Thus, the results extracted were analyzed qualitatively.

Results and Discussion

Quality of Studies

The included studies were graded using the SYRCLE risk of bias assessment tool.⁵⁵ Table 1 summarizes the risk of bias analysis. There is no high risk of bias among the 29 included studies. All the included studies have low risk of selective outcome for reporting bias and other sources of bias were not detected. However, only nine studies (31.0%) were assigned low risk of bias and 20 studies (69.0%) had unclear risk of bias for baseline characteristics (ie animal species, age, gender, and weight) in selection bias. Furthermore, all studies showed unclear risk in most of the domains, including sequence generation and allocation concealment for selection bias, random housing and blinding for performance bias, random outcome assessment and blinding for detection bias, and incomplete outcome data for attrition bias. The lack of detailed documentation in the included articles resulted in a high unclear risk of bias and ambiguous methodological quality.

Source of Exosomes

Exosomes were extracted from a variety of cell sources. Seventeen studies used human exosomes,^{56–72} nine studies used murine exosomes,^{73–81} one study used rabbit exosomes,⁸² and two studies did not mention the cell origin^{83,84} (Table 2).

Regarding cell type, 25 studies reported the use of exosomes from stem cells, namely ESC-derived MSCs (ESC-MSCs),^{61,63,68–70} iPSC-derived MSCs (iPSC-MSCs),^{58,72} amniotic fluid stem cells (AFSCs)⁶⁷ and other adult stem cells, including bone marrow-derived MSCs (BM-MSCs),^{56,57,59,71,73–76,78,83,84} synovial membrane-derived MSCs (SM-MSCs),^{60,62,72} infrapatellar fat pad-derived MSCs (IPFP-MSCs),⁶⁴ umbilical cord-derived MSCs (UC-MSCs),^{65,66} polydactyl BM-derived MSCs (pBM-MSCs),⁷¹ and commercial MSCs of unknown tissue origin.⁷⁹ Exosomes from primary chondrocytes,⁸¹ platelet-rich plasma (PRP),⁸² dendritic cells,⁷⁷ and serum⁸⁰ were also studied.

Exosomes have similar biological functions as parental cells.^{39,85} Most of the studies included in this review used MSCs as the source of exosomes due to the predominant therapeutic benefits of MSCs in tissue repair and

regeneration. MSCs have self-renewal, differentiation, anti-apoptotic, anti-fibrotic, pro-mitotic, anti-oxidative, and immunomodulatory properties.^{86–88} Besides, MSCs can be harvested from many tissue sources using minimally invasive techniques.⁸⁹ MSCs can also be expanded easily for many passages without significant changes in characteristics and functionality, and produce more exosomes.^{90,91} Currently, the most ideal cell source of exosomes for promoting cartilage regeneration remains ambiguous. In the present review, only two studies compared the efficacy of exosomes secreted by different cell sources.^{71,72} Zhu et al⁷² used exosomes secreted by iPSC-MSCs and SM-MSCs, and found that iPSC-MSC-secreted exosomes were more effective in supporting cartilage regeneration. Neotissue of the iPSC-MSC-derived exosome-treated group presented typical hyaline features and intense type II collagen staining in the superficial and deep zones of cartilage tissue, which were comparable to the healthy cartilage in the control group. On the other hand, the SM-MSC-derived exosome-treated group exhibited moderate cartilage repair and very weak type II collagen staining in the superficial cartilage zone, but the results were nevertheless better compared to the untreated cartilage in the OA group. Zhou et al⁷¹ reported that pBM-MSC-secreted exosomes were more potent in facilitating cartilage repair compared to those secreted by BM-MSCs, as demonstrated by the lower Osteoarthritis Research Society International (OARSI) scores. Exosomes secreted by MSCs of different tissue sources show distinctive therapeutic results, as the exosomal cargo varies according to the tissue origin.^{92–94} The therapeutic potential of exosomes secreted by MSCs isolated from different tissues has been summarized in the reviews of Álvarez-Viejo,⁹⁵ Nikfarjam et al,⁹⁶ Yin et al,⁹⁷ and Tang et al.⁹⁸

Modification of Exosomal Cargo for Enhancing Efficacy

Exosomes mediate cell–cell communication by transporting bioactive lipids, proteins, and RNAs, including mRNAs, and non-coding RNAs such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs).⁹⁹ We identified 15 studies that only used exosomes derived from naïve cells.^{58,61,63,67–75,81,82,84} All naïve exosomes promoted the repair and regeneration of damaged cartilage. Zhang et al^{69,70} reported that exosomal CD73 from naïve ESC-MSCs contributed to cartilage repair by inducing AKT and ERK phosphorylation in chondrocytes. Chen

Table 1 Summary of Risk of Bias Analysis Using SYRCLE Tool

References	Selection Bias			Performance Bias		Detection Bias		Attrition Bias		Reporting Bias
	Sequence Generation	Baseline Characteristics	Allocation Concealment	Random Housing	Blinding	Random Outcome Assessment		Blinding	Incomplete Outcome Data	
						Blinding	Blinding			
Zhang et al ⁶⁸	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Cosenza et al ⁷⁴	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Liu et al ⁵⁸	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Tao et al ⁶⁰	Unclear	Low risk	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Wang et al ⁶¹	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Zhu et al ⁷²	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Mao et al ⁵⁹	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Wang et al ⁷⁹	Unclear	Low risk	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Zhang et al ⁶⁹	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Zhang et al ⁷³	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Chen et al ⁷³	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Liu et al ⁸²	Unclear	Low risk	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Wu et al ⁶⁴	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Zhang et al ⁷⁰	Unclear	Low risk	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Zheng et al ⁸¹	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Chen et al ⁵⁶	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
He et al ⁷⁵	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Jin et al ⁷⁶	Unclear	Low risk	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Jin et al ⁵⁷	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Liang et al ⁷⁷	Unclear	Low risk	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Liu et al ⁷⁸	Unclear	Low risk	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Qiu et al ⁸³	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Wong et al ⁶³	Unclear	Low risk	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Yan and Wu ⁶⁶	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Zavatti et al ⁶⁷	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Zhang et al ⁸⁴	Unclear	Low risk	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Zhou et al ⁷¹	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Wang et al ⁸⁰	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Wang et al ⁶²	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk
Yan et al ⁶⁵	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low risk

Table 2 Cell Sources, Exosome Isolation, and Characterization

References	Cell Sources	Cell Types	Exosome Isolation	Exosome Characterization
Zhang et al ⁶⁸	Human	HuES9 ESC-MSCs	Size fractionation and TFF Exosomes were stored at -20°C until use	NTA: Homogenously sized with modal size of 100 nm Western blot: Positive for CD81, ALIX, and TSG101 RNA detection: Majority less than 300 nucleotides
Cosenza et al ⁷⁴	Murine	BM-MSCs from C57BL/6 mice	Ultracentrifugation	DLS: 96 nm NTA: 112 ± 6.6 nm Flow cytometry: Positive for CD9 and CD81
Liu et al ⁵⁸	Human	iPSC (iPSC-01)-MSCs	Ultracentrifugation and ultrafiltration	TEM: Spherical morphology; 30 to 60 nm TRPS: 50 to 150 nm Western blot: Positive for CD9, CD63, and CD81
Tao et al ⁶⁰	Human	Normal SM-MSCs and miR-140-5p-overexpressing SM-MSCs from knee joint synovial membrane tissue of 30 to 35 years old donors	Ultrafiltration and ultracentrifugation on 30% sucrose/D2O cushion Used freshly	TEM: Hollow spherical microvesicles DLS: 30 to 150 nm Western blot: Positive for CD63, CD9, CD81, and ALIX RT-qPCR: Increased miR-140-5p expression level in miR-140-5p overexpressed SM-MSC-Exos
Wang et al ⁶¹	Human	Male HI ESC-MSCs	Ultracentrifugation Exosomes were stored in PBS at -80°C	TEM: Round lipid bilayer vesicles; 38 to 169 nm Western blot: Positive for CD63 and CD9
Zhu et al ⁷²	Human	iPSC (C1P33)-MSCs	Ultrafiltration The exosomes aliquot was stored -80°C or used freshly	TEM: Cup or round-shaped TPRS: 50 to 150 nm Western blot: Positive for CD9, CD63, and TSG101
	Human	SM-MSCs from 3 donors (2 males and 1 female, age range 22 to 28 years)	Ultrafiltration The exosomes aliquot was stored -80°C or used freshly	TEM: Cup or round-shape TPRS: 50 to 150 nm. Western blot: Positive for CD9, CD63, and TSG101
Mao et al ⁵⁹	Human	Normal BM-MSCs and miR-92a-3p-overexpressing BM-MSCs from 6 donors (3 males and 3 females, ranged of 32 to 38 years old)	Ultracentrifugation	TEM: Cup-shaped or round morphology; 50 to 150 nm NTA: 50 to 150 nm. Western blot: Positive for CD9, CD63, CD81, and HSP70
Wang et al ⁷⁹	Murine	Normal MSCs, TGF- β 1 stimulated MSCs and miR-135b inhibited MSC from SD rats	Exosome extraction kit	TEM: Hollow-spherical morphology Western blot: Positive for CD63, CD9, and CD81; higher CD81 and ALIX levels in TGF- β 1-MSC-Exos RT-qPCR: Increased miR-135b expression level in TGF- β 1-MSC-Exos

(Continued)

Table 2 (Continued).

References	Cell Sources	Cell Types	Exosome Isolation	Exosome Characterization
Zhang et al ⁶⁹	Human	Immortalized E1-MYC 16.3 ESC-MSCs	Size fractionation and TFF Exosomes were stored at -20 °C until use	Size determination: Homogenously sized particles; 100 nm model size Western blot: Positive for CD81, ALIX, and TSG101 Flotation density: 1.10 to 1.19 g/mL
Chen et al ⁷³	Murine	BM-MSCs from articular cartilage from femoral condyles and tibial plateau of C57BL/6 mice	Ultrafiltration and ultracentrifugation on 30% sucrose/D2O cushion	TEM: Hollow spherical vesicles DLS: 40 to 110 nm in diameter Western blot: Positive for TSG101, CD9, and CD63
Liu et al ⁸²	Rabbit	PRP isolated from whole blood of New Zealand white rabbits	Membrane-based affinity binding step using exoEasy Maxi Kit Exosomes were stored at -80 °C until used	TEM: Round-shaped morphology NTA: 145.6 ± 50.4 nm Western blot: Positive for CD9, CD63, CD81, and HSP101
Wu et al ⁶⁴	Human	IPFP-MSCs from patients with primary knee OA	Precipitation (ExoQuick reagent kit) or ultrafiltration Exosomes were stored at -80 °C for further use	No significant differences between exosomes isolated from precipitation and ultrafiltration TEM: Sphere-shaped bilayer membrane structure about 100 nm in diameter NTA: 121.9 nm Western blot: Positive for CD81, CD9, and CD63 Flow cytometry with CiO-labeling: 30 nm to 150 nm
Zhang et al ⁷⁰	Human	Immortalized E1-MYC 16.3 ESC-MSCs	Size fractionation and TFF Exosomes were stored at -20 °C until use	Size determination: 100 to 200 nm Western blot: Positive for CD81, ALIX, and TSG101 Flotation density: 1.10 to 1.19 g/mL
Zheng et al ⁸¹	Murine	Primary chondrocytes from knee articular cartilage of 5 to 6 days old C57BL/6 mice cultured in standard or inflammatory environment (degenerative chondrocytes)	Ultrafiltration and ultracentrifugation with 30% sucrose/D2O	TEM: Hollow and spherical-like morphology DLS: 40 to 110 nm Western blot: Positive for TSG101, CD9, and CD63 Mass spectrometry: 2409 and 2077 proteins were identified in primary chondrocytes and degenerative chondrocytes, respectively
Chen et al ⁵⁶	Human	Normal BM-MSCs and miR-136-5p-overexpressed BM-MSCs from femur bone marrow of traumatized patients	Ultrafiltration and ultracentrifugation with 30% sucrose/D2O	TEM: Hollow spherical microvesicles DLS: 50 to 150 nm Western blot: Positive for CD63, CD9, CD81, and ALIX; but very low TSG101 RT-qPCR: Upregulated miR-136-5p expression level in miR-136-5p overexpressed BM-MSC-Exos

(Continued)

Table 2 (Continued).

References	Cell Sources	Cell Types	Exosome Isolation	Exosome Characterization
He et al ⁷⁵	Murine	BM-MSCs from SD rats	Ultracentrifugation	TEM: Oval in shape DLS: 153 nm Western blot: Positive for flotillin-1, TSG101, and CD63; negative for calnexin
Jin et al ⁷⁶	Murine	Normal BM-MSCs and miR-9-5p overexpressed BM-MSCs	Ultracentrifugation Exosomes were stored at -80 °C	TEM: Round or tea-shaped with outer membrane; 40 to 100 nm Western blot: Positive for CD9, TSG101, and CD63; negative for calnexin
Jin et al ⁵⁷	Human	Normal BM-MSCs and miRNA-26a-5p overexpressed-BM-MSCs from ilium of healthy volunteers	Precipitation (ExoQuick-TC reagent kit)	TEM: Round or oval-shaped; 30 to 100 nm DLS: 50 to 100 nm Western blot: Positive for CD63, Hsp70, and CD90 RT-qPCR: Upregulated miR-26a-5p expression level in miR-26a-5p overexpressed BM-MSC-Exos
Liang et al ⁷⁷	Murine	CAP-Lamp2b dendritic cells	Ultracentrifugation Exosomes were stored at -80 °C miR-140 mimic is introduced into exosomes by electroporation	TEM: Cup-shaped morphology; 40 to 200 nm NTA: 40 to 200 nm Western blot: Positive for CD63 and CD9 Confocal microscope: Fluorescence signals of CAP-GFP-Exos
Liu et al ⁷⁸	Murine	Normal BM-MSCs from SD rats and BM-MSCs treated with kartogenin	TFF and HPLC Exosomes were stored at -80 °C	TEM: Cup or round-shaped morphology NTA: 50 to 200 nm Western blot: Positive for CD63 and CD81
Qiu et al ⁸³	Not reported	Normal BM-MSCs and curcumin pre-treated BM-MSCs	Ultrafiltration	TEM: Round-shaped morphology; 50 to 150 nm Western blot: Positive for CD9, CD63, and CD81
Wong et al ⁶³	Human	E1-MYC 16.3 ESC-MSCs	Size fractionation and TFF Exosomes were stored at -20 °C until use	Size determination: 100 to 200 nm Western blot: Positive for CD81, ALIX, and TSG101
Yan and Wu ⁶⁶	Human	UC-MSCs cultured in 2D and 3D (hollow-fiber bioreactor)	Ultracentrifugation Exosomes were stored at -80 °C until used	TEM: Cup-shaped morphology NTA: 120 nm Western blot: Positive for CD63, CD81, and TSG101; negative for calnexin
Zavatti et al ⁶⁷	Human	AFSCs from pregnant women (mean age of 35.7) between the 16th and 17th weeks of gestation	Precipitation (total exosome isolation solution)	Western blot: Positive for CD9, CD63, CD81, and Rab5

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Table 2 (Continued).

References	Cell Sources	Cell Types	Exosome Isolation	Exosome Characterization
Zhang et al ⁸⁴	Not reported	BM-MSCs	Ultrafiltration and ultracentrifugation with sucrose cushion Exosomes were stored at -80 °C or used freshly	TEM: Cup-like shape NTA: 140 nm Western blot: Positive for CD63, CD81, and CD9; negative for cerulean
Zhou et al ⁷¹	Human	Polydactyly BM-MSCs	Precipitation (total exosome isolation kit)	TEM: 30 to 150 nm Western blot: Positive for CD63 and CD9
	Human	BM-MSCs	Precipitation (total exosome isolation kit)	TEM: 30 to 150 nm Western blot: Positive for CD63 and CD9
Wang et al ⁸⁰	Murine	OA serum from OA mice and sham serum from sham mice	Norgen's proprietary resin-based purification (Serum Exosome Purification Mini Kit) ATF4-OA-Exos were constructed by introducing ATF4 mRNA into OA-Exos through electroporation.	TEM: Spherical-shaped morphology NTA: 0 to 150 nm Western blot: Positive for ALIX and CD63 RT-qPCR: Highest expression level of ATF4 in ATF4-OA-Exos, followed by OA-Exos and sham-Exos
Wang et al ⁶²	Human	Normal SM-MSCs and miR-155-5p-overexpressing SM-MSCs	Not reported	TEM: Cup-shaped morphology NTA: 100 to 120 nm Western blot: Positive for CD63 and CD81; negative for TFIIIB RT-qPCR: 60-fold higher miR-155-5p expression in miR-155-5p overexpressed SM-MSC-Exos compared to SM-MSC-Exos
Yan et al ⁶⁵	Human	UC-MSCs cultured in static environment and rotary cell culture system	Ultracentrifugation Exosomes were stored at -80 °C	TEM: Cup-shaped morphology NTA: 120 nm Western blot: Positive for CD63, CD81, and TSG101; low calnexin RT-qPCR: Increased lncRNA H19 expression level in exosomes collected from cells cultured in rotary cell culture system

Abbreviations: AFSC, amniotic fluid stem cell; BM-MSC, bone marrow-derived mesenchymal stem/stromal cell; CAP-Lamp2b, chondrocyte-affinity peptide-lysosome-associated membrane glycoprotein 2b; D2O, deuterium oxide; DLS, dynamic light scattering; ESC-MSC, embryonic stem cell-derived mesenchymal stem/stromal cell; Exo, exosome; IPFP-MSC, infrapatellar fat pad-derived mesenchymal stem/stromal cell; iPSC-MSC, induced pluripotent stem cell-derived mesenchymal stem/stromal cell; lncRNA, long non-coding RNA; miR, microRNA; MSC, mesenchymal stem/stromal cell; NTA, nanoparticle tracking analysis; PBS, phosphate-buffered saline; PRP, platelet-rich plasma; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SD rat, Sprague Dawley rat; SM-MSC, synovial membrane-derived mesenchymal stem/stromal cell; TEM, transmission electron microscopy; TFF, tangential flow filtration; TGF- β 1, transforming growth factor beta 1; TPRS, tunable resistive pulse sensing; UC-MSC, umbilical cord-derived mesenchymal stem/stromal cell.

et al⁷³ suggested that BM-MSC-derived exosomes restored the mitochondrial dysfunction of degenerated chondrocytes by supplementing mitochondrial-related proteins. Another study noted that normal chondrocyte-derived exosomes carried proteins that involved in mitochondrial

function and immune system process, which are important in alleviating OA progression.⁸¹ On the contrary, 14 articles modified and loaded exosomes with specific therapeutic agents, and reported enhanced therapeutic efficacy of cartilage regeneration compared to naïve

exosomes.^{56,57,59,60,62,64–66,76–80,83} Exosomal cargo can be modified via preconditioning of the exosome-secreting cells; incubating the exosome-secreting cells with the therapeutic cargo (eg drugs, proteins, RNAs, nanomaterials); transfection of the exosome-secreting cells; and physical treatments such as electroporation, sonication, extrusion, surfactant treatment, dialysis, and freeze-thawing.¹⁰⁰

The genomic molecules within exosomes aid the regulation of gene expression.¹⁰¹ Valadi et al¹⁰² were the first to discover the presence of mRNAs and miRNAs in exosomes, and indicated that exosomes could modify the protein production and gene expression of target cells by transferring exosomal mRNAs or miRNAs. Wang et al⁸⁰ investigated the therapeutic effect of the activating transcription factor 4 (*ATF4*) gene in cartilage regeneration by introducing *ATF4* mRNA into exosomes via electroporation. The effects of exosomes derived from OA serum (OA-Exos) and *ATF4* mRNA-overexpressing exosomes (ATF4-OA-Exos) were compared, and showed that the ATF4-OA-Exos were more potent compared to the OA-Exos in preventing and alleviating cartilage degeneration via the stimulation of autophagy.

miRNAs play a critical role in the post-transcriptional regulation of a wide range of physiological processes, including cartilage homeostasis, and the pathological processes in disorders such as OA.^{103,104} In the past few years, there has been growing emphasis on determining the biological function of miRNAs in regenerative medicine.^{105,106} Given the eminent role of miRNAs in cartilage regeneration, six studies included here used transfection to upregulate specific miRNAs, ie miR-9-5p, miR-26a-5p, miR-92a-3p, miR-136-5p, miR-140-5p, and miR-155-5p, in exosomes.^{56,57,59,60,62,76} All miRNA-overexpressing exosomes showed superior therapeutic potential compared to the naïve exosomes by regulating the target genes and their downstream signaling pathways in the recipient cells. Mao et al⁵⁹ revealed that exosomal miR-92a-3p downregulated the Wnt signaling pathway via *WNT5A*, eventually causing lower ECM degradation. Comparing the results of exosome-treated groups with and without antagomir-miR-100-5p, Wu et al⁶⁴ reported that IPFP-MSC-derived exosomes ameliorated cartilage damage and the gait patterns of OA mice by delivering miR-100-5p into the recipient chondrocytes, leading to activation of the miR-100-5p-mediated mTOR autophagy pathway. In another study, miR-140 was loaded into exosomes through electroporation, and the miR-140-overexpressing exosomes were more effective compared to the

naïve exosomes in suppressing the progression of cartilage degeneration and in enhancing cartilage regeneration.⁷⁷ Additionally, miR-135b was increased in exosomes secreted by transforming growth factor (TGF)- β 1-stimulated MSCs.⁷⁹ The exosomal miR-135b downregulated Sp1 protein expression, leading to better cartilage regeneration in rats with OA.⁷⁹

LncRNAs are involved in the transcriptional and post-transcriptional regulation of many biological processes related to cartilage development, degeneration, and regeneration, and can be transferred by exosomes to recipient cells.^{107,108} Yan et al studied the role of exosomal lncRNA H19 in cartilage regeneration.⁶⁵ The exosomal lncRNA H19 played an important role in cartilage regeneration by promoting chondrocyte proliferation and matrix production whilst suppressing apoptosis. In vivo, injecting exosomes rich in lncRNA H19 led to greater improvement in cartilage repair, with uniform tissue, obscured boundaries, and lower T2 values on MRI analysis compared to that of cartilage tissue treated with exosomes secreted by UC-MSCs transfected with small interfering RNA (siRNA) against H19.⁶⁵ In that study, the 10-fold increase in exosomal lncRNA H19 was achieved by exposing the cells to mechanical stimulus via culture in a rotary cell culture system (RCCS).

These results clearly show that exosomal cargo can be modulated by modifying the culture condition. The changes in exosomal cargo, such as higher miR-135b expression in TGF- β 1-stimulated MSC-derived exosomes and higher lncRNA H19 expression in exosomes secreted by UC-MSCs exposed to mechanical stimulus, enhanced cartilage regeneration in vivo.^{65,79} Additionally, preconditioning BM-MSCs with kartogenin and curcumin improved the therapeutic potential of the secreted exosomes for treating OA.^{78,83} However, the studies did not examine the differences in the exosomal cargos of preconditioned and naïve cells.

Apart from modulating the exosomal cargo, cell preconditioning can also stimulate exosome secretion. Yan and Wu⁶⁶ collected exosomes secreted by UC-MSCs cultured in 2D and 3D (using a hollow-fiber bioreactor) conditions. The 3D culture had increased exosome yield (7.5-fold higher), and the exosomes secreted by 3D-culture UC-MSCs showed more potent therapeutic effects in promoting cartilage repair compared to the exosomes secreted by the 2D-culture UC-MSCs, as indicated by the greater surface regularity and better thickness of OA cartilage in vivo. Similarly, Yan et al⁶⁵ also found that UC-MSCs

cultured in 3D conditions using a RCCS secreted exosomes with increased yield and therapeutic potential in treating OA. Consistent with this, Yang et al¹⁰⁹ and Cao et al¹¹⁰ attained higher exosome yields from cells cultured in 3D conditions, and the exosomes showed improved therapeutic potential, albeit for different indications.

The above results demonstrate the importance of exosomal cargo modification for achieving better performance. Additionally, exosomal cargo profiling is crucial for elucidating the mechanism of action.

In vivo Delivery and Exosome Uptake

Cartilage is an avascular and alymphatic tissue.¹¹¹ Thus, unlike in other tissue injuries, exosomes are not suitable for infusing intravenously for treating cartilage defects. In fact, all of the included studies administered exosomes directly to the affected joint, as intra-articular administration increases bioavailability and reduces off-target effects, thereby improving the delivery of exosomes to the cartilage and decreasing the dose of exosomes needed.¹¹² The exosomes were either injected or encapsulated in a scaffold. Some studies used a single injection and others used multiple injections to treat cartilage injuries. Currently, it remains unclear if multiple injections are more effective compared to single injection for promoting cartilage regeneration, as none of the studies compared the therapeutic effect of single and multiple injections. Cosenza et al⁷⁴ and Wang et al⁸⁰ showed that the effect of exosomes from a single intra-articular injection may be month-long. This is most probably due to the direct exosome administration to the target site (cartilage is avascular and alymphatic; thus, the clearance of the injected exosomes is slower) and the exosomes might induce cellular reprogramming and remodel resident or injured cells by activating regenerative mechanisms by transferring bioactive molecules.^{113–115}

The findings of the 29 studies are summarized in Table 3 and illustrated in Figure 2. To date, direct injection appears to be a more popular cell delivery method, as only a few studies^{58,73,78} used a scaffold to deliver exosomes to the injured cartilage tissues. A scaffold is beneficial for sustaining the delivery of exosomes for a longer period and also aids exosome homing to the injury site. Additionally, combining exosomes and biomaterial can create a synergistic effect to promote cartilage regeneration. Liu et al⁵⁸ found that the implantation of in situ hydrogel glue with iPSC-MSC-derived exosomes was more potent for promoting cartilage regeneration

compared to in situ hydrogel glue implantation and iPSC-MSC-derived exosome injection, supported by the formation of a smoother surface with fully filled regenerated tissue that integrated with the surrounding cartilage. The histological findings were supported by the results of optical coherence tomography (OCT), which displayed a uniform and well-organized articular cartilage structure in the in situ formed iPSC-MSC-exosome-hydrogel tissue patch implantation group.

In vitro studies have shown that exosomes promote chondrocyte proliferation and migration, impede chondrocyte apoptosis and the expression of pro-inflammatory markers, and restore the balance between chondrocyte catabolism and anabolism in a dose-dependent manner.^{69,74,78,82,116} However, no article included in the present review reported on the in vivo response to exosome dosage.

We also noted that the studies used several exosome quantitation methods, ie nanoparticle analyzer, protein quantitation assay, and enzyme-linked immunosorbent assay (ELISA). The number of exosome particles (particles/mL) was detected using nanoparticle analyzers such as nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing (TRPS). Some of the studies used an ExoELISA kit to quantify exosomes based on the presence of exosome markers such as CD63. The exosome concentration was also determined by exosome protein mass concentration ($\mu\text{L}/\text{mL}$) using the bicinchoninic acid (BCA) or Bradford assay. Although all included studies reported that exosomes promoted cartilage repair and prevented lesion progression compared to the control group, none conducted in vivo study for identifying the optimal dosage and number of injections. Based on the different quantification methods used in the 29 included studies, the quantity of exosomes used ranged from 8×10^7 – 1×10^{10} particles and 7.5–200 μg in mouse models; 1×10^8 – 1×10^{10} and 1.25–100 μg in rat models; and 2×10^9 – 5×10^9 and 100–200 μg in rabbit models, and the injection number and time varied in each study.

Intercellular communication via exosomes mainly takes place through three mechanisms: (1) direct interaction of exosome ligands and receptors on target cells that activate the intracellular signaling cascades; (2) direct fusion; or (3) endocytosis. Direct fusion and endocytosis resulted in the release of exosomal contents into the cytosol.¹¹⁷ Wu et al,⁶⁴ He et al,⁷⁵ Liang et al,⁷⁷ and Zhang et al⁸⁴ monitored exosome uptake in joint tissues. Fluorescent labeled exosomes were mainly observed in the

Table 3 Summary of Efficacy and Safety Findings

References	Animal Models	Method of Delivery	Treatment Groups	Euthanasia	Efficacy Outcome			Safety Outcome
					Macroscopic and Functional Analysis	Imaging	Histological and Biochemical Analysis	
Zhang et al ⁶⁸	SD rats Surgically induced OCD	Multiple intra-articular injections after surgery and thenceforth weekly	1. Exo group: 100 µg/100 µL of ESC-MSC-Exos 2. Contralateral control: 100 µL PBS 3. Un-operated control	Week 6 or 12 post-surgery	<ul style="list-style-type: none"> Exo group demonstrated almost complete neotissue filling with good surface regularity and complete integration of neotissue with surrounding cartilage by 12 weeks 	Not reported	<ul style="list-style-type: none"> Exo group exhibited almost complete regeneration and bonding of cartilage and underlying subchondral bone after 12 weeks Distinctly higher modified O'Driscoll histological scores in the Exo group Exo group showed hyaline cartilage formation with high amount of GAG, Col II, and low amount of Col I 	No detrimental responses were observed in all animals
Cosenza et al ⁷⁴	C57BL/6 mice Collagenase-induced OA	Single intra-articular injection at day 7 after OA induction	1. Cell group: 2.5x10 ⁷ BM-MSCs/5 µL 2. MP group: 500 ng/5 µL of BM-MSC-microvesicles 3. Exo group: 250 ng/5 µL of BM-MSC-Exos 4. OA group 5. Healthy control	Day 42 post-collagenase induction	Not reported	<ul style="list-style-type: none"> CLSM showed structural improvement in articular cartilage which comparable with healthy control group in all treatment groups µCT showed higher bone volume, lower bone degradation, lower osteophyte formation and lower calcification of menisci and ligaments in all treatment groups compared to the OA group 	Exo group showed the greatest improvement with the lowest OA scores	Not reported
Liu et al ⁵⁸	New Zealand rabbits Surgically created OCD	Scaffold implantation or single intra-articular injection immediately after surgery	1. EHG group: 20 µL in situ formed EHG tissue patch containing 1 x 10 ¹¹ /mL iPSC-MSC-Exos 2. HG group: 20 µL in situ formed HG tissue patch 3. Pre-EHG group: 20 µL in vitro preformed EHG containing 1 x 10 ¹¹ /mL iPSC-MSCs-Exos 4. Inj-Exo group: 20 µL of 1 x 10 ¹¹ /mL iPSC-MSCs-Exos suspension 5. OA group: Saline rinsing	Week 12 post-surgery	<ul style="list-style-type: none"> EHG group showed the best repair with smooth surface with white regenerated tissue fully filled the defects and integrated with surrounding cartilage 	<ul style="list-style-type: none"> OCT displayed uniform and well-organized articular cartilage structure in EHG group 	EHG group has the highest ICRS score and the neotissue was almost entirely hyaline cartilage (strong safranin O and Col II staining, weak Col I staining)	Not reported

Tao et al ⁶⁰	SD rats Surgically induced OA	Multiple intra-articular injections on the first day of week 5 to 8 after surgery	1. Exo group: 100 μ L of 10 ¹¹ SM-MSC-Exos particles/mL 2. Exo-miR-140-5p group: 100 μ L of 10 ¹¹ miR-140-5p overexpressed SM-MSC-Exos particles/mL 3. OA group: Saline 4. Healthy control: Saline	Week 12 post-surgery	Not reported	Not reported	<ul style="list-style-type: none"> Exo-miR-140-5p group showed the lowest OARSI assessment scores Significant increased chondrocyte count, higher Col II and aggrecan expression, and lower Col I in the Exo-miR-140-5p group compared to the Exo and OA groups 	No adverse events occurred
Wang et al ⁶¹	C57BL/6 J mice DIMM induced OA	Intra-articular injections at week 4 after surgery (once for Cell and Cell-OA group; multiple injections every 3 days for 4 weeks for Exo and Exo-OA group)	1. Cell group: 5 μ L of 1 \times 10 ⁶ ESC-MSC suspension 2. OA group: 5 μ L of PBS 3. Sham control 1. Exo group: 5 μ L ESC-MSC-Exos 2. OA group: 5 μ L of PBS 3. Sham control	Week 8 post-surgery	Not reported	Not reported	<ul style="list-style-type: none"> Exo group exhibited similar regenerative effect as Cell group. Exo group revealed milder OA pathology compared to the OA group which was concomitant with lower OARSI scores and stronger Col II staining and weaker ADAMTSS and aggrecan neoptope staining 	Not reported
Zhu et al ⁷²	C57BL/6 J mice Collagenase-induced OA	Multiple intra-articular injections on day 7, 14 and 21 after collagenase administration	1. iPSC-MSC-Exo group: 8 μ L of 1 \times 10 ¹⁰ particles/mL iPSC-MSC-Exos 2. SM-MSC-Exo group: 8 μ L of 1 \times 10 ¹⁰ particles/mL SM-MSC-Exos 3. OA group: 8 μ L of PBS 4. Normal control: 8 μ L of PBS	Day 28 post-collagenase induction	No significant differences in ICRS macroscopic analysis scores among the normal, iPSC-MSC-Exo and SM-MSC-Exo groups, but significant higher than the OA group	Not reported	<ul style="list-style-type: none"> Neotissue of iPSC-MSC-Exo group presented smooth cartilage, regular cellular organization and normal proteoglycan content that similar to control group; SM-MSC-Exo exhibited moderate surface irregularity, superficial fibrillation in neotissue and lower proteoglycan in cartilage, but the results were better compared to the OA group No significant differences in OARSI scores between the iPSC-MSC-Exo and normal group, but notable lower than the SM-MSC-Exo and OA groups More intense Col II staining in the iPSC-MSC-Exo group compared to the SM-MSC-Exo group 	Not reported

(Continued)

Table 3 (Continued).

References	Animal Models	Method of Delivery	Treatment Groups	Euthanasia	Efficacy Outcome			Safety Outcome
					Macroscopic and Functional Analysis	Imaging	Histological and Biochemical Analysis	
Miao et al ⁵⁹	C57BL/10 mice Collagenase-induced OA	Multiple intra-articular injections on day 7, 14 and 21 after collagenase administration	1. Exo group: 15 μ L of 500 μ g/mL BM- MSC-Exos 2. Exo-miR-92a-3p group: 15 μ L of 500 μ g/mL miR-92a-3p overexpressed BM- MSC-Exos 3. OA group: 15 μ L of PBS 4. Healthy control: 15 μ L of PBS	Day 28 after collagenase induction	Not reported	Not reported	Not reported	<ul style="list-style-type: none"> Exo-miR-92a-3p group demonstrated significant reduced severity of cartilage matrix loss with higher Col II and aggrecan, and lower Wnt5a and MMP13 expressions in both gene and protein levels compared to the Exo and OA groups
Wang et al ⁷⁹	SD rats Surgically induced OA	Intra-articular injection	1. Exo group: 100 μ L of 1×10^{11} particles/mL MSC-Exos 2. TGF- β 1-Exo group: 100 μ L of 1×10^{11} particles/mL TGF- β 1-MSC-Exos 3. TGF- β 1-NC-Exo group: 100 μ L of 1×10^{11} particles/mL TGF- β 1-MSC-NC-Exos 4. TGF- β 1-miR135b inhibitor-Exo group: 100 μ L of 1×10^{11} particles/mL TGF- β 1-MSC-miR135b inhibitor-Exos	Week 12 post-surgery	Not reported	Not reported	Not reported	<ul style="list-style-type: none"> Significant lower OARSI scores in the TGF-β1-Exo group than the Exo group, with high OARSI scores in the TGF-β1-miR135b inhibitor-Exo group TGF-β1-Exo group showed elevated number of chondrocytes, while dropped in chondrocyte number was recorded in the Exo and TGF-β1-miR135b inhibitor-Exo groups

Zhang et al ⁶⁹	SD rats Surgically induced OCD	Multiple intra-articular injections immediately after surgery on weekly basis	1. Exo group: 100 µg/100 µL ESC-MSC-Exos 2. Contralateral control: 100 µL PBS	Week 2, 6 or 12	Not reported	Not reported	Not reported	Not reported
Chen et al ⁷³	New Zealand white rabbits Surgically induced OCD	3D printed scaffold implantation	1. ECM/GelMA scaffold 2. GelMA group: GelMA scaffold 3. ECM/GelMA/Exo group: 3D printed ECM/GelMA/BM-MSC-Exo scaffold 4. OA group: untreated	Week 6 or 12 post-surgery	ECM/GelMA/Exo group exhibited smooth and intact tissues and gave the highest ICRS macroscopic analysis scores	MRI scanning demonstrated smooth neo-cartilage and great defect filling in both ECM/GelMA and ECM/GelMA/Exo groups µCT displayed increased ratio of bone volume to tissue volume, trabecular thickness and ossified tissues in the subchondral bone of both ECM/GelMA and ECM/GelMA/Exo groups	ECM/GelMA and ECM/GelMA/Exo groups showed hyaline-like cartilage regeneration in the defect sites ECM/GelMA/Exo group has higher ICRS visual histological scores at week 12 Increased Col II and decreased of MMP13 expression in the synovial membrane of ECM/GelMA and ECM/GelMA/Exo groups ECM/GelMA/Exo group expressed the lowest MDA levels	No apparent pathological effects in myocardium, liver, and kidney 1 to 2 weeks after transplantation
Liu et al ⁸²	New Zealand white rabbits Surgically induced OA	Multiple intra-articular injections once a week	1. Exo group: 100 µg/mL PRP-Exos 2. PRP-As group: 100 µg/mL activated PRP 3. OA group: normal saline 4. Control: normal saline	Week 6 after surgery	Not reported	Not reported	Exo group exhibited more regular arrangement of chondrocytes, clearer tidal line, reduced hyperplasia on articular cartilage surface and lower OARSJ scores than the PRP-As and OA groups Exo group showed increased expression of Col II and RUNX2	No adverse events occurred
Wu et al ⁶⁴	C57BL/6 mice DMM induced OA	Multiple intra-articular injections twice a week, starting at week 4 after surgery	1. Exo group: 10 µL of 10 ¹⁰ particles/mL IPPF-MSC-Exos 2. OA group: 10 µL PBS 3. Sham control: 10 µL PBS	Week 8 post-surgery	Improved gait pattern (CatWalk gait analysis) after 6 weeks of exosome treatment	Obvious green fluorescent dots (DIO-labeled IPPF-MSC-Exos) can be found at the defect sites	Exo group showed integration of cartilage with smooth surface and lower OARSJ scores confirming cartilage lesion was healed Exo group showed higher expression of Col II as well as lower expression of ADAMTS5 and MMP13	Not reported

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Table 3 (Continued).

References	Animal Models	Method of Delivery	Treatment Groups	Euthanasia	Efficacy Outcome			Safety Outcome
					Macroscopic and Functional Analysis	Imaging	Histological and Biochemical Analysis	
		3 weeks of 10 µL antagomir pre-injection (once a week), starting at week 1 after surgery, followed by injection of Exos and antagomir twice a week for 4 weeks	1. PBS + antagomir-NC group: 10 µL PBS + antagomir-NC; 2. Exo + antagomir-NC group: 10 µL 10 ¹⁰ particles/mL IPPF-MSC-Exos + antagomir-NC 3. Exo + antagomir-100-5p group: 10 µL 10 ¹⁰ particles/mL IPPF-MSC-Exos + antagomir-100-5p	Week 8 post-surgery	Not reported	Not reported	<ul style="list-style-type: none"> Exo + antagomir-100-5p group reversed the results of the Exo group 	Not reported
Zhang et al ⁷⁰	SD rats MIA-induced TMJ-OA	Multiple intra-articular injections once a week, starting 2 weeks after OA induction	1. Exo group: 100 µg/50 µL of ESC-MSC-Exos 2. OA group: 50 µL of PBS 3. Sham control: needle pricks	Week 2, 4 or 8 post-treatment	<ul style="list-style-type: none"> HWT improved gradually in the Exo group and reached the baseline level of the sham group at week 5 	<ul style="list-style-type: none"> µCT showed Exo group restored subchondral bone volume and architecture at week 8 	<ul style="list-style-type: none"> Exo group revealed significant reduced gene expressions of pro-inflammation (IL-1β), apoptosis (BAX), fibrosis (α-SMA) and pain (Substance P, CGRP, NGF, P75NTR and TrkA) and upregulated TIMP 2 and downregulated ADAMTS5 in condylar cartilage tissues Significant lower Mankin scores at week 4 and 8 in the Exo group Exo group showed smoother cartilage surface, improved cellularity, reduced fibrous cartilage thickening, minimal depletion of s-GAG in condylar cartilage lesion at week 4 and marked restoration of TMJ condylar structure at week 8 Exo group has lesser MMP13+ cells in condyle region at week 4; lesser IL-1β+ and iNOS+ cells, higher proliferative PCNA+ cells and lesser CCP3 + apoptotic cells at week 4 and 8 	No adverse immune reactions observed
Zheng et al ⁸¹	C57BL/6 mice Surgically induced OA	Multiple intra-articular injections once per week, starting 10 days after surgery	1. Exo group: 200 µg of primary chondrocyte-Exos 2. OA group: 20 µL saline 3. Sham control: Untreated	Week 4, 6 or 8 post-surgery	Not reported	<ul style="list-style-type: none"> µCT demonstrated lower subchondral bone mineral density and smaller osteophyte formation at the joint margins in the Exo group 	<ul style="list-style-type: none"> Both femoral and tibia cartilage of the Exo group showed nearly complete preservation No obvious synovitis appearance in all groups Exo group expressed marked repression in MMP13 staining and elevated Col II staining in joint samples Significant lower OARSI scores in the Exo group Exo group exhibited higher level of M2 macrophages infiltration in the synovium and cartilage tissues 	Not reported

Chen et al ⁵⁶	C57BL/6 mice Mechanical load induced OA	Single intra-articular injection immediately after mechanical induction	1. Exo group: 100 µL of 10 ¹¹ particles/mL BM-MSC-Exos 2. Exo-miR-136-5p group: 100 µL of 10 ¹¹ particles/mL miR-136-5p overexpressed BM-MSC-Exos 3. OA group: Untreated 4. Normal control: Untreated	One hour after injury	Not reported	Not reported	Not reported	Not reported
He et al ⁷⁵	SD rats MIA-induced OA	Multiple intra-articular injections once a week, starting one week after OA induction	1. Exo group: 40 µg/100 µL BM-MSC-Exos 2. OA group: 100 µL of normal saline 3. Sham control: 100 µL of normal saline	Week 6 post-treatment	Not reported	Not reported	Not reported	Not reported
Jin et al ⁷⁶	SD rats Surgically induced OA	Intra-articular injection, starting two weeks after OA induction	1. Exo group: BM-MSC-Exos 2. OA group: Normal saline	Week 7 post-treatment	Not reported	Not reported	Not reported	Not reported

(Continued)

Table 3 (Continued).

References	Animal Models	Method of Delivery	Treatment Groups	Euthanasia	Efficacy Outcome			Safety Outcome
					Macroscopic and Functional Analysis	Imaging	Histological and Biochemical Analysis	
			<ol style="list-style-type: none"> 1. Exo group: BM- MSC-Exos 2. Exo-miR-9-5p group: miR-9-5p overexpressed BM- MSC-Exos 3. Exo-miR-9-5p inhibitor group: miR-9-5p inhibited BM- MSC-Exos 4. Exo-miR-mimic-NC group: miR-9-5p mimic NC BM- MSC-Exos 5. Exo-miR-inhibitor-NC group: miR-9-5p inhibitor NC BM- MSC-Exos 6. Liposomes miR-9-5p group: miR-9-5p embedded liposomes 7. Sham control 		Not reported	Not reported	<ul style="list-style-type: none"> • Exo-miR-9-5p group exhibited the lowest observation and Mankin scores among the treated groups • Lower OA infiltration in the Exos, Exo-miR-mimic-NC and Exo-miR-inhibitor-NC group, and improved cartilage regeneration in the exo-miR-9-5p group • Exo-miR-9-5p group has the lowest expression level of inflammatory factors, AKP content, and oxidative stress injury indicators, as well as highest level of SOD in synovial fluid among all the treated groups • Exo-miR-9-5p group showed the lowest gene and protein expressions of MMP13, OCN and COMP in cartilage tissue compared to all the treated groups 	Not reported
Jin et al ⁵⁷	Wistar rats Surgically induced OA	One-week intra-articular injection of treatments after surgery	<ol style="list-style-type: none"> 1. Exo-miR-26a-5p group: 250 ng/5 µL of miR-26a-5p overexpressed BM- MSC-Exos 2. Exo-miR-NC group: 250 ng/5 µL of miR-NC BM- MSC-Exos 3. OA group: Untreated 4. Sham control: Untreated 5. Healthy control: Untreated 	Week 8 post-surgery	Not reported	Not reported	<ul style="list-style-type: none"> • Exo-miR-26a-5p group has less pathological changes with the concomitant of reduced synovial tissue proliferation and suppressed inflammation • Exo-miR-26a-5p group has lower MMP3 and MMP13 expression and higher apoptotic index in synovial cells • Notably increased of miR-26a-5p expression and declined of PTGS2 expression in synovial tissue of the Exo-miR-26a-5p group • Exo-miR-26a-5p group showed reduced serum IL-1β levels 	Not reported

Liang et al ⁷⁷	SD rats DMM induced OA	Multiple intra-articular injections post-injury, once per week	1. Exo group: 100 µg of Exos particles in 100 µL PBS 2. Exo-miR140 group: 100 µg of Exo- miR140 particles in 100 µL PBS 3. CAP-Exo-miR140 group: 100 µg of CAP-Exo-miR140 particles in 100 µL PBS 4. OA group: Untreated 5. Sham control: Untreated	Week 8 post- surgery	Not reported	● Fluorescence microscopy showed that CAP-Exo-miR-140 mainly stayed in the articular cavity, while Exo-miR-140 distributed to other body parts and enriched in kidney	● CAP-Exo-miR140 group displayed smooth and flat cartilage surface, small joint space, proper cell alignment, normal subchondral bone and dense proteoglycan which was almost identical to the sham control and comparable OARS1 scores with sham control ● CAP-Exo-miR140 group suppressed the MMP13 and Adams5 protein levels in cartilage tissue ● Upregulation of miR-140 and downregulation of MMP13 gene expression in cartilage tissue in the CAP-Exo-miR140 group	Non-toxic to major organs (heart, liver, kidney, lung, and spleen)
Liu et al ⁷⁸	SD rats Surgically induced OCD	Intra-articular implantation	1. Exo group: Col- Tgel hydrogel with BM-MSC-Exos 2. KGN-Exo group: Col-Tgel hydrogel with KGN-BM-MSC- Exos 3. Gel group: Col- Tgel hydrogel 4. OA group: untreated 5. Normal control: untreated	Week 4, 6 and 8 post- surgery	● Better cartilage regeneration in the KGN-Exo group as indicated by smoother articular surface and better integration of newly formed cartilage with adjacent host cartilage ● KGN-Exo group exhibited higher ICRS macroscopic scores which were comparable with the normal group at 8 weeks	Not reported	● KGN-Exo group revealed better cartilage reconstruction with hyaline cartilage predominantly, corresponding to notable high ICRS visual histological scores ● KGN-Exo group showed increased s-GAG in cartilage tissue started at week 2 ● More lubricin and Col II positive cells and less Col I positive cells in KGN-Exo group compared to other groups except for the normal control group	Not reported
Qiu et al ⁸³	Mice Surgically induced OA	Not mentioned	1. Exo-Cur group: Curcumin pre- treated BM-MSC- Exos 2. Exo group: BM- MSC-Exos 3. OA group 4. Sham control	Not reported	Not reported	Not reported	● Exo-Cur group showed higher gene expression of miR-124 and miR-143 as well as lower protein expression of ROCK1, NF-κB and TLR9 that were similar to the sham control group ● Exo-Cur group has less apoptotic chondrocytes compared to the OA and Exo groups	Not reported

(Continued)

Table 3 (Continued).

References	Animal Models	Method of Delivery	Treatment Groups	Euthanasia	Efficacy Outcome			Safety Outcome
					Macroscopic and Functional Analysis	Imaging	Histological and Biochemical Analysis	
Wong et al ⁶⁵	New Zealand white rabbits Surgically induced OCD	Multiple intra-articular injections at day 0, day 7 and day 14 post-surgery	1. Exo + HA group: 1 mL of 3% (w/v) hyaluronic acid with 200 µg ESC-MSC-Exos 2. HA group: 1 mL of 3% (w/v) hyaluronic acid	Week 6 or 12, post-treatment	<ul style="list-style-type: none"> Significant improvement in ICRS scores of Exo + HA group at week 6 and 12, associated with marked improvements of neotissue integration at the border zone Exo + HA group displayed improved mean Young's moduli and stiffness of the repaired cartilage that approximated the normal tissue 	Not reported	<ul style="list-style-type: none"> Exo + HA group showed improvements in cartilage regeneration over time as confirmed by complete defect coverage by neotissue characterized by the presence of hyaline cartilage, normal cellularity and chondrocytic-like cells, high GAG and Col II deposition, and lower Col I deposition at week 12 Exo + HA group exhibited higher modified O'Driscoll scores than the HA group at week 6 and 12 	No adverse events
Yan and Wu ⁶⁶	New Zealand rabbits Surgically induced cartilage defect	Multiple intra-articular injections on a weekly interval	1. 2D-Exo group: 500 µL of 1×10^{10} particles/mL 2D cultured UC-MSC-Exos 2. 3D-Exo group: 500 µL of 1×10^{10} particles/mL 3D cultured UC-MSC-Exos 3. OA group: 500 µL PBS	Week 4 post-treatment	<ul style="list-style-type: none"> Significant improvement in ICRS macroscopic scores in the 3D-Exo group compared to the 2D-Exo and OA groups 3D-Exo group demonstrated more neotissue formation with smoother surface and better integration with the native hyaline cartilage at the surrounding 	Not reported	<ul style="list-style-type: none"> 3D-Exo group displayed partly hyaline cartilage and the defects showed greater surface regularity and better thickness of cartilage than the 2D-Exo group 3D-Exo group exhibited lower Wakitani score than the 2D-Exo and OA groups 	Not reported
Zavatti et al ⁶⁷	CD rats MIA-induced OA	Intra-articular injection 3 weeks after OA induction (once for Cell and OA groups; twice for Exo group with 10 days interval)	1. Cell group: 50 µL of 5×10^5 AFSCs 2. Exo group: 50 µL of 100 µg AFSC-Exos 3. OA group: 50 µL of glucose/PBS 4. Contralateral control	Week 3 post-treatment	<ul style="list-style-type: none"> Cell and Exo groups have higher pain tolerance, and the results were comparable to the normal control group 	Not reported	<ul style="list-style-type: none"> Exo group showed better cartilage regeneration as indicated by complete neotissue formation with good surface regularity compared to the cell-treated defects which exhibited few fissures on the cartilage surface Uniformed GAG distribution was demonstrated in the Exo group Both Exo and Cell groups exhibited improved OARSI scores Cell group displayed more intense Col II staining, whereas Exo group showed more regular distribution of Col II 	No adverse inflammatory responses were observed

Zhang et al ⁸⁴	SD rats Surgically induced OA	Intra-articular injection every 3 days for 4 weeks, starting at week 4 post-operation	1. Exo group: 10 µL 10 ¹⁰ particles/mL BM1-MSC-Exos 2. OA group: 10 µL PBS 3. Sham control	Week 4 post-treatment	Not reported	<ul style="list-style-type: none"> µCT showed less cartilage degradation, near-normal chondrocyte morphology and distribution, and less osteophyte formation around the joint treated with Exo DiI-labeled Exo were observed in the knee joint and taken up by the synovial cells 	<ul style="list-style-type: none"> Lower OARSI scores in the Exo group compared to the OA group Exo group demonstrated upregulated chondrogenic gene expressions and downregulated hypertrophic gene expressions Exo group has decreased synovial hyperplasia and cell filtration Exo group has less M1-positive cells and more M2-positive cells Exo group exhibited reduced pro-inflammatory cytokines (IL-1β, TNF-α) and increased anti-inflammatory cytokine (IL-10) in synovial fluid Exo group showed significant reduction in synovitis scores 	Not reported
Zhou et al ⁷¹	C57BL/6j mice Collagenase VII induced OA	Multiple intra-articular injections at day 7, 14 and 21 after collagenase induction	1. Exo group: BM-MSC-Exos 2. pExo group: polydactyl BM-MSC-Exos 3. OA group: Saline 4. Normal control	Day 28 post-collagenase VII injection	Not reported	Not reported	<ul style="list-style-type: none"> Exo group alleviated cartilage damage evidenced by the significant lower OARSI scores than the OA and Exo groups 	Not reported
Wang et al ⁸⁰	C57BL/6j mice Surgically induced OA	Single intra-articular injection at week 4 post-operation	1. Sham-Exo group: 200 µg sham-Exos 2. OA-Exo group: 200 µg OA-Exos 3. ATF4-OA-Exo group: 200 µg ATF4-OA-Exos 4. OA group 5. Sham control	Week 8 post-surgery	Not reported	<ul style="list-style-type: none"> µCT showed reduced osteophyte formation in Sham-Exo and ATF4-OA-Exo groups, while OA-Exo group displayed enlarged osteophytes 	<ul style="list-style-type: none"> Sham-Exo and ATF4-OA-Exo groups alleviated pathological injury of articular tissues observed in the OA group, whereby the ATF4-OA-Exo group exerted greater therapeutic effect and lesser proteoglycan loss ATF4-OA-Exo group exhibited lower Mankin scores than the Sham-Exo group, while OA-Exo group aggravated the cartilage damage and exhibited the highest Mankin scores among all groups ATF4-OA-Exo group gave stronger effect in upregulating Col II levels and downregulating MMP13 levels ATF4-OA-Exo group was more potent in decreasing inflammatory cytokine levels in the cartilage ATF4-OA-Exo group partially restored the impeded autophagy of the OA cartilage 	Not reported

(Continued)

Table 3 (Continued).

References	Animal Models	Method of Delivery	Treatment Groups	Euthanasia	Efficacy Outcome			Safety Outcome
					Macroscopic and Functional Analysis	Imaging	Histological and Biochemical Analysis	
Wang et al ⁶²	BALB/C mice 4°C water stimulated OA	Multiple intra-articular injection once a day, starting from day 20 after OA induction	1. Exo group: 30 µL of 10 ¹¹ particles/mL SM-MSC-Exos 2. Exo-155-5p group: 30 µL of 10 ¹¹ particles/mL miR-155-5p overexpressed SM -MSC-Exos 3. OA group: normal saline 4. Normal control	Two weeks post-treatment	Not reported	Not reported	<ul style="list-style-type: none"> Exo-155-5p group revealed lower OARSI scores and higher chondrocyte number in femoral condyle Exo and Exo-155-5p groups reversed the increased caspase-3 and decreased Col II expressions in OA femoral condyle sections, but Exo-155-5p was more effective 	Not reported
Yan et al ⁶⁵	SD rats Surgically induced cartilage defects	Multiple intra-articular injections on a weekly interval	1. S-Exo group: 100 µL of 1 mg/mL rotary cell culture system cultured UC-MSC-Exos 2. si-Exo group: 100 µL of 1 mg/mL rotary cell culture system cultured siRNA H19 transfected UC-MSC-Exos 3. OA group: 100 µL PBS	Week 4 or 8	<ul style="list-style-type: none"> S-Exo group revealed reduced pain with higher LWT at week 3 Defects covered with neotissue at week 4 and were filled with uniform tissue and obscured boundaries at week 8 in the S-Exo group S-Exo group showed the highest ICRS scores 	<ul style="list-style-type: none"> Defects of S-Exo group displayed similar intensity to the adjacent cartilage through MRI at week 8 S-Exo group exhibited the lowest T2 values at week 4 and 8 	<ul style="list-style-type: none"> S-Exo group has the best surface regularity, highest glycosaminoglycan, most orderly tissues and best subchondral bone repair among the groups S-Exo group has significant lower Waktani scores compared to the other groups S-Exo group displayed the highest matrix synthesis which consists mainly of Col II 	Not reported

Abbreviations: µCT, micro computed tomography; ADAMTS, ADAM metalloproteinase with thrombospondin motifs; AFSC, amniotic fluid stem cell; AKP, alkaline phosphatase; BAX, Bcl-2 associated X-protein; BM-MSC, bone marrow-derived mesenchymal stem/stromal cell; CAP, chondrocyte-affinity peptide; CCP3, cleaved caspase-3; CGRP, calcitonin gene-related peptide; CLSM, confocal laser scanning microscopy; col I, type I collagen; col II, type II collagen; COMP, cartilage oligomeric matrix protein; DMM, destabilization of medial meniscus; DRG, dorsal root ganglion; ECM, extracellular matrix; EHG, exosome encapsulating hydrogel; ELF3, E74-like factor 3; ESC-MSC, embryonic stem cell-derived mesenchymal stem/stromal cell; Exo, exosome; GAG, glycosaminoglycan; GelMA, gelatin methacrylate; HWT, head withdrawal threshold; ICRS, International Cartilage Repair Society; IL, interleukin; INOS, inducible nitric oxide synthase; iPPF-MSC, infrapatellar fat pad-derived mesenchymal stem/stromal cell; iPSC-MSC, induced pluripotent stem cell-derived mesenchymal stem/stromal cell; KGN, kartogenin; LWT, leg withdrawal threshold; MDA, malondialdehyde; MIA, monosodium iodoacetate; MMP, matrix metalloproteinase; MRI, magnetic resonance imaging; MSC, mesenchymal stem/stromal cell; NC, negative control; NGF, nerve growth factor; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International; OCD, osteochondral defect; OCN, osteocalcin; OCT, optical coherence tomography; P75NTR, p75 neurotrophin receptor; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PRP, platelet-rich plasma; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; RUNX2, runt-related transcription factor 2; SD rat, Sprague Dawley rat; SMA, smooth muscle actin; SM-MSC, synovial membrane-derived mesenchymal stem/stromal cell; SOD, superoxide dismutase; TGF-β1, transforming growth factor beta 1; TIMP, tissue inhibitor of metalloproteinase; TMJ, temporomandibular joint; TNF-α, tumor necrosis factor-alpha; TrkA, tropomyosin receptor kinase A; UC-MSC, umbilical cord-derived mesenchymal stem/stromal cell.

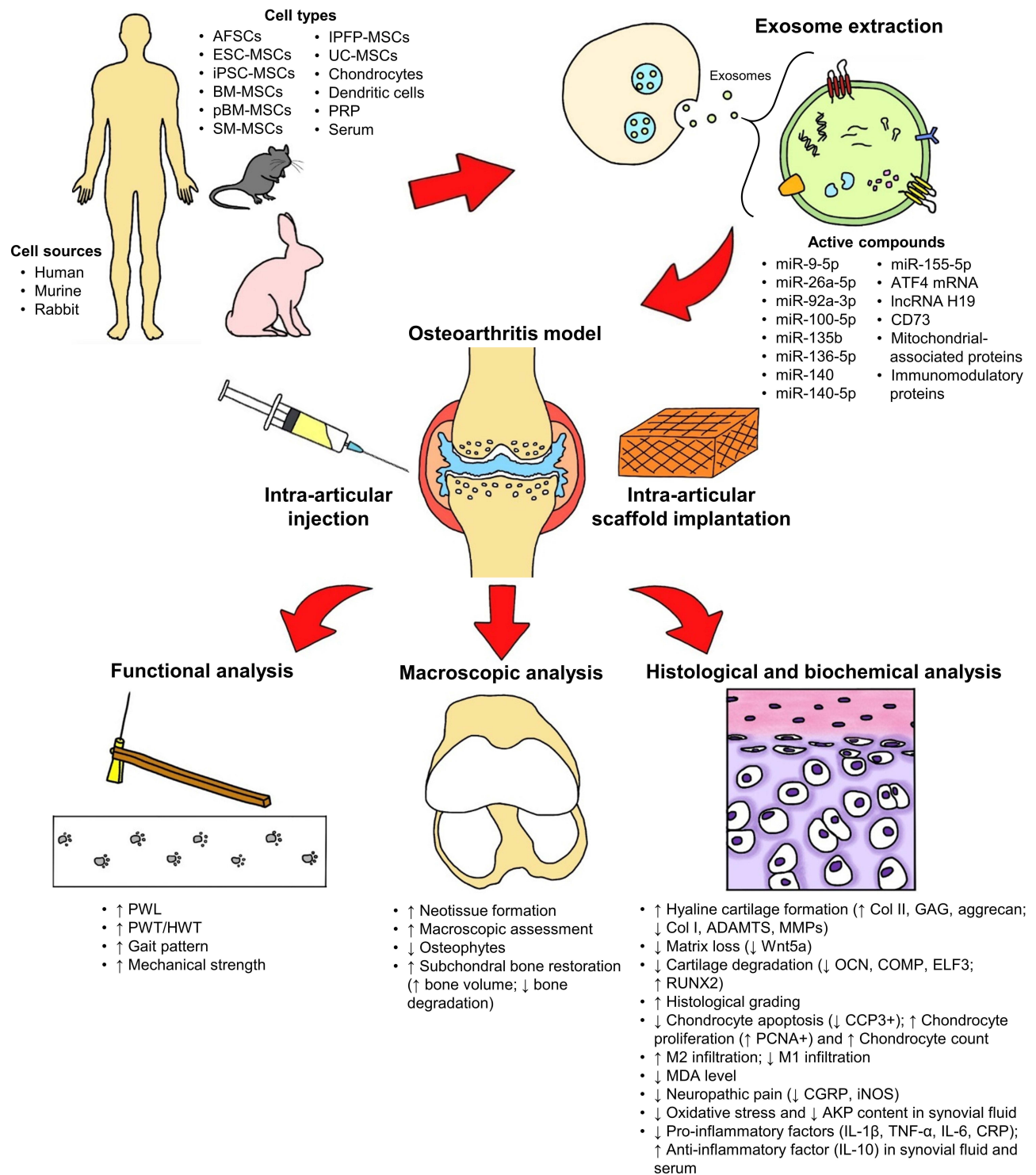


Figure 2 Overview of the studies. The exosomes tested in the included studies were derived from human, murine, or rabbit amniotic fluid stem cells (AFSCs), embryonic stem cell-derived mesenchymal stem/stromal cells (ESC-MSCs), induced pluripotent stem cell-derived MSCs (iPSC-MSCs), bone marrow-derived MSCs (BM-MSCs), polydactyl BM-MSCs, synovial membrane-derived MSCs (SM-MSCs), infrapatellar fat pad-derived MSCs (IPFP-MSCs), umbilical cord-derived MSCs (UC-MSCs), chondrocytes, dendritic cells, platelet-rich plasma (PRP), and serum. The exosomes were administered to the osteoarthritic joint through intra-articular injection or scaffold implantation. The exosomal bioactive compounds played an important role in cartilage and subchondral bone repair and regeneration. Overall, exosome therapy restored joint function, reduced joint pain, and improved the joint macroscopic, histological, and biochemical features.

articular cavity, suggesting that exosomes acted locally after intra-articular injection. Zhang et al⁸⁴ noted BM-MSC-derived exosomes could be taken up by synovial cells *in vivo*. Liang et al⁷⁷ reported that dendritic cell-derived exosomes were distributed to other body parts and enriched in the kidney. Exosome retention in the articular cavity was improved by fusing a chondrocyte-affinity peptide on the exosome surface. The peptide confined the exosomes to the articular cavity and facilitated exosome penetration into the middle zone of the cartilage tissue to achieve targeted delivery of exosomes to chondrocytes for better therapeutic efficacy.⁷⁷ Another study reported on the improved tissue-targeting ability of modified exosomes.¹¹⁸

Cartilage Gross Morphological and Morphometric Analysis

Macroscopic assessment of cartilage degradation in small animals, especially mice, is technically difficult due to their small size.¹¹⁹ Hence, most of the included studies used rat and rabbit OA models to conduct cartilage gross morphological analysis. Eight studies graded the gross appearance of treated joints according to the International Cartilage Repair Society (ICRS) macroscopic assessment scale.^{58,63,65,66,68,72,73,78} Higher ICRS macroscopic assessment scores indicated morphological improvement in exosome-treated defects. Generally, the cartilage defects of the negative control group exhibited incomplete neotissue coverage, surface irregularity, and distinct border areas even at 12 weeks. On the other hand, exosome-treated cartilage defects showed much better and improved gross appearance. For example, Zhang et al⁶⁸ stated that exosome-treated cartilage defects displayed almost complete neotissue filling, with good surface regularity and full integration with the surrounding cartilage at 12 weeks. Another study used confocal laser scanning microscopy (CLSM) analysis to evaluate cartilage defects, and reported improvement in articular cartilage volume and thickness, as well as lower cartilage degradation in collagenase-induced defects treated with BM-MSCs, BM-MSC-derived exosomes, and BM-MSC-derived microvesicles as compared to the untreated control defects.⁷⁴

Apart from gross morphological assessment, Wong et al⁶³ also performed biomechanical assessment of regenerated cartilage tissue. The mean Young's moduli and stiffness of the repaired cartilage at the center and periphery of the defects improved significantly over time in the

hyaluronic acid (HA) + ESC-MSC-derived exosome group in comparison to the HA group, suggesting that exosomes not only promote cartilage tissue morphology repair, but also contribute to functional improvement, ie better mechanical strength.

Cartilage Histological Analysis

Histological assessment is vital for determining tissue regeneration, repair, and pathological changes at the microscopic level. Various histological stains are used for identifying different cellular and tissue components.¹²⁰ Hematoxylin-eosin (H&E) staining is widely used for revealing cell and tissue structure, and has become a common practice in histological study. Safranin O/fast green, toluidine blue, and Alcian blue are used for examining cartilaginous and chondrogenic differentiated tissues by visualizing the proteoglycans.¹²¹ In all 29 included studies, exosome treatment contributed to notable improvements in cartilage regeneration at the histological level. The studies used multiple histological grading systems for joint repair to grade the defects, ie the OARSI,^{60–62,64,67,71,72,74,75,77,79,81,82,84} ICRS visual histological assessment scale,^{58,73,78} O'Driscoll histological cartilage repair score,^{63,68} Wakitani histological scoring system,^{65,66,69} and Mankin scoring system.^{70,76,80} Other than that, Jin et al⁵⁷ scored the pathological changes of condylar joint and synovial tissues using a 0–3 subjective grading system with the parameters of synovitis inflammation, synovial thickening, and subchondral bone erosion. All studies recorded improved histological scores after exosome treatment. For example, Zhang et al⁶⁸ reported that ESC-derived exosome-treated defects demonstrated good cartilage and subchondral bone restorations by week 6 post-surgery. Near complete regeneration and bonding of cartilage with the underlying subchondral bone, which was very similar to that of the age-matched native control, were recorded at week 12. The O'Driscoll histological cartilage repair scores were significantly higher at week 6 and 12 in the exosome-treated group compared to the phosphate-buffered saline (PBS)-treated group. Similarly, Liu et al⁸² reported that, at week 6, articular cartilage defects treated with PRP-derived exosomes had regularly arranged chondrocytes, clearer tidal line, reduced hyperplasia, and better OARSI scores compared to defects treated with activated PRP.

Immunohistochemistry is used for detecting specific antigens in tissue sections by incubating the tissue sections with the appropriate antibody. Anabolic and catabolic markers of cartilage metabolism in regenerated tissue are identified to analyze the cartilage regeneration properties.

Type II collagen and aggrecan, a major cartilage ECM component and a core proteoglycan of articular cartilage, respectively, are always determined for evaluating cartilage regeneration.^{122,123} Cartilage degradation is mostly indicated by key enzymes in cartilage matrix degradation: matrix metalloproteinases (MMPs) and ADAM metalloproteinase with thrombospondin motifs (ADAMTS).¹²⁴ Generally, exosome-treated cartilage defects had upregulated type II collagen and aggrecan expression and downregulated MMP13 and ADAMTS5 expression. Wong et al⁶³ treated surgically induced OCD with ESC-MSC-derived exosomes and found that the regenerated tissue was mainly hyaline cartilage with high glycosaminoglycan (GAG) and type II collagen, as well as low type I collagen deposition. The accumulation of type I collagen is a marker of fibrocartilage formation.

Zhang et al⁶⁹ reported increased proliferating cell nuclear antigen (PCNA)⁺ cells in reparative cartilage and synovium, and decreased cleaved caspase-3 (CCP3)⁺ apoptotic cells on reparative cartilage, suggesting that exosomes derived from ESC-MSCs mediate cartilage repair by promoting cell proliferation and suppressing apoptosis. The research team reported similar results in a subsequent study using ESC-MSC-derived exosomes for treating temporomandibular joint OA.⁷⁰ Chondrocyte apoptosis may abrogate cartilage homeostasis, eventually leading to cartilage degeneration.¹²⁵ Qiu et al⁸³ and Jin et al⁵⁷ used the TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay to examine apoptosis. Qiu et al⁸³ stated that curcumin-treated BM-MSC-derived exosomes were more effective in inhibiting chondrocyte apoptosis compared to naïve BM-MSC-derived exosomes. Jin et al⁵⁷ found that miR-26a-5p overexpression in BM-MSC-derived exosomes enhanced synovium fibroblast apoptosis to alleviate cartilage damage.

Furthermore, Zhang et al⁶⁹ indicated that MSC-derived exosomes mediate cartilage repair by modulating the pro-inflammatory environment in defects, as demonstrated by the increase in M2 macrophages (CD163⁺ cells) and reduction in M1 macrophages (CD86⁺ cells) in both cartilage and the overlying synovium in the ESC-MSC-derived exosome group, with a concomitant reduction in M1-associated cytokines, ie interleukin-1 beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α), in synovial fluid. Aside from this, exosomes induced regenerative M2 macrophage infiltration and attenuated inflammatory M1 macrophage infiltration in defect sites, evident in the higher abundance of CD163⁺ or arginase-1⁺ cells and scarcity of CD86⁺, inducible nitric

oxide synthase (iNOS)⁺ or IL-1 β ⁺ cells.^{67,70,81,84} The results are consistent with studies reporting that exosomes have anti-inflammatory and immunomodulatory functions for attenuating disease progression.^{126–129}

Subchondral Bone Regeneration Analysis

OA may lead to pathophysiological changes in subchondral bone and cartilage, also known as the bone–cartilage unit, that perform complementary work in the load-bearing of joints.^{130,131} Microstructural alterations of subchondral bone and the osteochondral junction, including osteophytes, subchondral sclerosis, subchondral cysts, or bone marrow lesions, are observed in the different stages of OA.¹³² Increasing evidence has shown that subchondral bone disturbance is associated with OA initiation and progression.^{133–136} There is molecular crosstalk between cartilage and subchondral bone, and cartilage is partly dependent on the neighboring subchondral bone for its nutrient supply.^{137,138} Subchondral bone migration is involved in OCD repair.¹³⁹

Among the 29 included studies, six evaluated subchondral bone regeneration.^{70,73,74,80,81,84} Bone parameters including bone volume (bone volume/tissue volume); bone degradation (bone surface/bone volume); osteophyte formation; meniscal or ligament calcification; and trabecular thickness, separation, and number were quantified. The six studies used micro-computed tomography (μ CT) to examine the joint microarchitecture and reported that exosome-treated groups showed a lower extent of osteophyte formation, whereas the groups treated with normal saline exhibited a rough joint surface with osteophytes on the bone edge.^{70,73,74,80,81,84} Interestingly, osteophytes formed in the OA serum-derived exosome-treated group were impeded by the introduction of *ATF4* mRNA into the OA serum-derived exosomes.⁸⁰ Apart from that, Cosenza et al⁷⁴ reported higher epiphyseal and subchondral bone volume, lower bone degradation, and decreased calcification of menisci and ligaments in the groups treated with BM-MSCs, BM-MSC-derived exosomes, and BM-MSC-derived microvesicles. Zhang et al reported notable differences in bone volume, trabecular thickness, trabecular separation, and trabecular number at week 8 post-treatment,⁷⁰ proving that ESC-MSC-derived exosomes restored subchondral bone volume and architecture in monosodium iodoacetate (MIA)-induced OA. Moreover, subchondral bone regeneration has been reported in cartilage defects treated with exosomes loaded in a scaffold. Chen et al⁷³ reported that more ossified tissues were

regenerated in OCD that received implantation of a 3D-printed cartilage ECM/gelatin methacrylate (GelMA)/BM-MSC-derived exosome scaffold.

Pain Behavior Analysis

Joint pain is the major symptom of OA. Recent studies indicate that sensory innervation in osteoarthritic subchondral bone or neuronal hypersensitivity as a result of aberrant subchondral bone remodeling during OA progression may contribute to arthritic pain.^{140,141} Hence, behavioral and functional assessments are important in OA evaluation. Pain cannot be measured directly in animals, but can be estimated through pain-like behaviors.¹⁴² Withdrawal response, a pain-like behavior towards a nociceptive stimulus, is the most commonly used method for examining pain in animals.¹⁴³ Yan et al,⁶⁵ Zhang et al,⁷⁰ and He et al⁷⁵ measured the withdrawal threshold of OA animals using the von Frey microfilament procedure, while Zavatti et al used a pressure application measurement device to do so.⁶⁷ He et al⁷⁵ also tested the paw withdrawal latency of rats treated with BM-MSC-derived exosomes using a plantar test and measured the thermal threshold. The results showed that exosome-treated OA animals have high pain tolerance levels and that the results were comparable to that of healthy control animals. Furthermore, increasing evidence supports the notion that neuropathic pain also contributes to OA pain.^{144–146} He et al⁷⁵ reported neuropathic pain in OA rats, and demonstrated that exosome injection relieved inflammatory and neuropathic pain, as shown by the decreased protein levels of calcitonin gene-related peptide (CGRP) and iNOS in the dorsal root ganglion tissue.

In addition, OA animals may have altered gait patterns to reduce the pain from movement or loading force.¹⁴⁷ Wu et al⁶⁴ performed gait analysis using the CatWalk method to measure motion-related pain in OA mice, and found that IPFP-MSC-derived exosomes ameliorated gait disturbance, indicated by the marked increase in duty cycle at week 10.

Safety Profile

Exosomes have low immunogenicity and potent immunoregulatory properties.^{148,149} So far, in vivo studies using single or repeated doses of exosomes have not reported severe immune reactions.^{150,151} Among the included studies, nine reported the safety outcome.^{60,63,67,68,70,73,77,82} No adverse events or inflammatory responses occurred in those studies, regardless of the cell source, ie MSC- or

non-MSC-derived exosomes, and method of administration, ie intra-articular administration in solution form or encapsulated within a scaffold. Liang et al⁷⁷ demonstrated via H&E staining that dendritic cell-derived exosome injections did not cause toxicity to major organs such as the heart, kidney, lung, and spleen. Similarly, no apparent pathological effects were shown in the myocardium, liver, and kidney 1–2 weeks after 3D printed ECM/GelMA/exosome scaffold implantation.⁷³

The majority of the included studies used human exosomes to promote cartilage repair in animal models. No study reported adverse events related to the use of xenogeneic and allogeneic exosomes. Thus, allogeneic exosomes may be considered safe for use in humans. Nonetheless, clinical trials are needed to validate the safety of allogeneic exosomes.

Cells or Exosomes?

Previously, stem cell therapy was believed to exert its tissue repair mechanism through the replacement of injured cells. However, many studies have shown that most of the MSCs injected are trapped and cleared in the blood circulation and that only a small fraction of the transplanted cells reach the target tissues.¹⁵² Despite the low cell homing to the injured tissues, the therapeutic effect of MSCs remains significant, indicating that MSCs support tissue repair and regeneration via the secretion of paracrine factors.¹⁵³

Several studies showed that exosomes have comparable therapeutic effects to cell-based therapy in managing OA. Wang et al⁶¹ reported that ESC-MSC-derived exosomes were as effective as ESC-MSCs for treating DMM (destabilization of medial meniscus)-induced OA animal models. However, the experiments were carried out sequentially, and the study compared single cell suspension injection versus multiple exosome injections. Thus, it might not be a fair comparison. According to Cosenza et al⁷⁴ and Zavatti et al,⁶⁷ cell therapy using BM-MSCs and AFSCs, respectively, promoted cartilage regeneration. However, more spectacular results were recorded when exosomes derived from these cells were used. Zavatti et al⁶⁷ stated that both AFSCs and AFSC-derived exosomes improved the pain threshold of OA rats, whereby the results were comparable to that of the healthy group. However, histological and immunohistochemical analyses showed that the exosome-treated group displayed better cartilage regeneration compared to the AFSC-treated group. Additionally, the lower OARSI grading scores and higher volume of cartilage tissue

with lower fibrous connective tissue composition in the neotissue in exosome-treated defects indicated that AFSC-derived exosomes were superior for repairing and regenerating cartilage tissue as compared to AFSC therapy. The AFSC-derived exosomes were also more potent for reducing osteoarthritic joint inflammation compared to AFSCs, as indicated by the presence of more M2 macrophages. Nonetheless, the OA animals received two intra-articular injections of exosomes whilst the AFSCs were only administered once, as the authors postulated that the transplanted cells may exert their therapeutic effects for a longer period, ie up to 3 weeks. Cosenza et al⁷⁴ reported that single injection of BM-MSCs, BM-MSC-derived exosomes, and BM-MSC-derived microvesicles exhibited similar chondroprotective effects. Yet, exosome treatment led to greater improvement in modified Pritzker OARSI scores and reduction in osteophyte formation compared to BM-MSC and microvesicle treatments. These results suggest that exosomes might be a more effective and safer alternative to cell-based therapy for OA. Nevertheless, more studies, especially clinical trials, are needed to validate the safety and efficacy of exosome therapy.

Nowadays, many researchers advocate the transition from cell-based therapy to cell-free therapy, driven by several factors. Generally, the heterogeneity of living cells often leads to inconsistent curative effects. Long-term *in vitro* expansion might lead to cell dedifferentiation and senescence, thus compromising the therapeutic potential.¹⁵⁴ Furthermore, stem cells carry the risk of mutation and tumor formation.¹⁵⁵ Tumor formation is a hurdle to overcome for the clinical use of ESCs and iPSCs.¹⁵⁶ Exosome therapy is a cell-free therapeutic option available for overcoming the drawbacks of cell-based therapy. Exosomes are easier to handle and store, and are less costly and less time-consuming to produce compared to cells.^{101,157} Furthermore, exosome dosage and potency are easier to optimize.³⁷ Apart from that, exosomes can be developed as nanosized carriers to deliver the desired therapeutic cargo to the target cells.^{158,159} However, the most prominent shortcoming of exosomes is that they cannot replicate or reproduce *in vivo*. Coupled with their short half-life, multiple dosages might be required to achieve the desired treatment results.^{160,161}

Conclusion and Perspective

The promising *in vivo* findings indicate that exosomes promote cartilage repair and regeneration, and modulate the pro-inflammatory environment, subchondral bone

regeneration, and pain behavior in both OA and OCD models. More importantly, none of the included studies reported adverse events, indicating the low immunogenicity and excellent immunomodulatory property of exosomes. The modification of exosomal cargo further enhanced the therapeutic efficacy of exosomes.

On the other hand, despite many preclinical studies reporting that exosomes enhance cartilage regeneration, research on the therapeutic efficacy of exosomes for OA is still in its infancy. The exact mechanism and detailed signaling cascade mediated by exosomes in cartilage repair and regeneration are not fully understood. Hence, there should be more studies examining the mechanism of action. Moreover, exosomes demonstrate dose-dependent therapeutic efficacy in terms of promoting chondrocyte migration and proliferation, reducing chondrocyte apoptosis and pro-inflammatory markers, and restoring cartilage anabolic–catabolic marker equilibrium *in vitro*. However, the optimum source and dose of exosomes for OA management have not been determined in animal models. Furthermore, multiple intra-articular injections of exosomes cause pain and discomfort to the recipients and increase the risk of other complications such as inflammation, arthritis, and neuropathy.¹⁶² Encapsulating exosomes within a scaffold might reduce the number of injections needed, as it allows the controlled release of exosomes. A scaffold also provides an ECM that mimics the native cartilage tissue to facilitate regeneration. However, more studies are needed to identify a scaffold with all the desired biological and physicochemical properties that could hasten the regeneration of damaged cartilage without causing adverse events. Finally, the term “exosome” used in the studies is controversial, as the identity of exosomes cannot be confirmed through size, density, or protein markers due to the lack of specific isolation methods and characterization techniques. “Exosome” is used mostly due to perceived popularity.¹⁶³ To be more precise, EVs should be defined based on their physical characteristics, biochemical composition, culture condition, and cell of origin.⁴⁹

The low yield of EV production with existing harvesting methods is a challenge for clinical application. Large-scale EV production can be achieved by manipulating the culture condition, such as with a microcarrier-based 3D culture system and human platelet lysate supplementation.^{164,165} Batch-to-batch biological variation of EV therapy also cannot be ignored. To address this issue, Chen et al¹⁶⁶ transformed human ESC-MSCs into immortalized cells, which enabled the consistent supply of therapeutic EVs or delivery vesicles. Besides, Lian et al¹⁶⁷ proposed a protocol for producing

human ESC-MSCs that are reproducible and able to generate consistent batches of cells and conditioned medium on a large scale. iPSCs, iPSC-derived MSCs, and iPSC-derived MSC-like mesenchymal progenitor cells could also be alternative inexhaustible EV production sources due to their unlimited proliferative potential.^{168,169} In addition, there is no standard protocol for ensuring exosome or EV quality.

In summary, studies examining the therapeutic efficacy of exosomes on cartilage regeneration remain limited to small-animal models. Therefore, studies using large-animal models that are more clinically relevant should be carried out in the future to validate the safety and efficacy of EV therapy. The standardization of EV therapy is needed to achieve consistent and optimum therapeutic outcomes. More efforts are required to identify the most ideal cell source, culture condition, exosome dosage, and frequency of administration, as well as the method of administration, to achieve the best therapeutic results without causing adverse events.

Abbreviations

2D, two-dimensional; 3D, three-dimensional; ADAMTS, ADAM metalloproteinase with thrombospondin motifs; AFSC, amniotic fluid stem cell; ATF4, activating transcription factor 4; BM-MSC, bone marrow-derived mesenchymal stem/stromal cell; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; ESC-MSC, embryonic stem cell-derived mesenchymal stem/stromal cell; EV, extracellular vesicle; GelMA, gelatin methacrylate; H&E, hematoxylin-eosin; ICRS, International Cartilage Repair Society; IL-1 β , interleukin-1 beta; iNOS, inducible nitric oxide synthase; IPFP-MSC, infrapatellar fat pad-derived mesenchymal stem/stromal cell; iPSC-MSC, induced pluripotent stem cell-derived mesenchymal stem/stromal cell; lncRNA, long non-coding RNA; miR/miRNA, microRNA; MMP, matrix metalloproteinase; MRI, magnetic resonance imaging; MSC, mesenchymal stem/stromal cell; MVB, multi-vesicular bodies; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International; OCD, osteochondral defect; PRP, platelet-rich plasma; RCCS, rotary cell culture system; SM-MSC, synovial membrane-derived mesenchymal stem/stromal cell; SYRCLE, Systematic Review Center for Laboratory Animal Experimentation; TGF- β 1, transforming growth factor beta 1; UC-MSC, umbilical cord-derived mesenchymal stem/stromal cell.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Author Contributions

C.Y.N. and J.Y.C. conducted the literature search, article selection, and data extraction. All authors contributed to data analysis, drafting, or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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