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The Treatment of Cartilage Damage Using Human Mesenchymal Stem Cell-Derived Extracellular Vesicles: A Systematic Review of *in vivo* Studies

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Damage to joints through injury or disease can result in cartilage loss, which if left untreated can lead to inflammation and ultimately osteoarthritis. There is currently no cure for osteoarthritis and management focusses on symptom control. End-stage osteoarthritis can be debilitating and ultimately requires joint replacement in order to maintain function. Therefore, there is growing interest in innovative therapies for cartilage repair. In this systematic literature review, we sought to explore the *in vivo* evidence for the use of human Mesenchymal Stem Cell-derived Extracellular Vesicles (MSC-EVs) for treating cartilage damage. We conducted a systematic literature review in accordance with the PRISMA protocol on the evidence for the treatment of cartilage damage using human MSC-EVs. Studies examining *in vivo* models of cartilage damage were included. A risk of bias analysis of the studies was conducted using the SYRCLE tool. Ten case-control studies were identified in our review, including a total of 159 murine subjects. MSC-EVs were harvested from a variety of human tissues. Five studies induced osteoarthritis, including cartilage loss through surgical joint destabilization, two studies directly created osteochondral lesions and three studies used collagenase to cause cartilage loss. All studies in this review reported reduced cartilage loss following treatment with MSC-EVs, and without significant complications. We conclude that transplantation of MSC-derived EVs into damaged cartilage can effectively reduce cartilage loss in murine models of cartilage injury. Additional randomized studies in animal models that recapitulates human osteoarthritis will be necessary in order to establish findings that inform clinical safety in humans.

Keywords: extracellular vesicle, mesenchymal stem cell, cartilage, tissue engineering, osteoarthritis

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INTRODUCTION

Damage to joints through injury or disease can result in cartilage loss, which if left untreated can lead to inflammation and ultimately osteoarthritis (OA) (Davies-Tuck et al., 2008). OA affects up to three out of 10 people over the age of 60 years (Woolf and Pfleger, 2003), and this is projected to increase substantially (Turkiewicz et al., 2014). There is currently no cure for OA and management is focused on symptom control (Mcalindon et al., 2014). Furthermore, the

search for Disease Modifying Osteoarthritis Drugs (DMOAD) has not been fruitful, and there are no approved DMOADs. End-stage OA can be severely debilitating and ultimately requires joint replacement in order to maintain function (Gillam et al., 2013). Joint replacement is costly and carries perioperative morbidity (Berstock et al., 2014) as well as unsatisfactory outcomes (Nilsdotter et al., 2003). Therefore, there is a need for innovative therapies to treat cartilage defects and in doing so, prevent OA.

The established treatment of microfracture for focal cartilage defects aims to encourage endogenous cells to repopulate areas of cartilage loss, but this has demonstrated limited effectiveness (Weber et al., 2018). A large number of studies have investigated tissue engineering and cellular regenerative approaches to treating cartilage defects (Negoro et al., 2018). Acellular biomaterial scaffolds are costly to develop and implantation of these scaffolds into cartilage defects exhibits a high failure rate (Vindas Bolaños et al., 2017). Certain cell-based approaches such as Autologous Chondrocyte Implantation (ACI) can be effective but cause donor-site morbidity (Reddy et al., 2007; Bexkens et al., 2017). Recently, there has been an increasing body of evidence to support the use of Mesenchymal Stem Cells (MSCs) in cartilage repair (Borakati et al., 2018).

MSCs are multipotent adult stromal cells that may be derived from a number of tissues including bone marrow, synovium, adipose, umbilical cord and dental pulp, and so are readily available for autologous harvest (Fernandes et al., 2018; Fabre et al., 2019). Ease of extraction and the potential for *ex vivo* expansion make MSCs an attractive option for tissue repair. To this end, studies have shown the therapeutic potential of MSC transplantation in promoting regeneration of tissues such as bone, cartilage, and nerve (Katagiri et al., 2017; Freitag et al., 2019; Masgutov et al., 2019). However, MSC transplantation is not without risks. Certain studies have revealed potential immunogenic complications related to repeated allogenic transplantation of MSCs (Cho et al., 2008) and others have reported possible tumorigenic properties (Beckermann et al., 2008). There is also *in vivo* evidence to suggest that, when transplanted in the presence of malignancy, MSCs may increase the risk of metastasis (Karnoub et al., 2007). Suboptimal engraftment and delocalization from the target site create difficulty in maintaining sustained benefit following transplantation, and suggest that observed long-term benefits may not result from MSC differentiation alone (Zwolanek et al., 2017).

Increasingly so, studies are focussing on the paracrine function of MSCs as the predominant mechanism of their regenerative effects (Linero and Chaparro, 2014; Xu et al., 2016). MSC-derived extracellular vesicles (MSC-EVs) are gaining interest as a cell-free therapeutic option for cartilage repair. As part of MSC secretome, EVs are nanovesicles ranging from 10 nm to several micrometers that contain various components including genetic material in the form of messenger RNA (mRNA), microRNA (miRNA), lipids and bioactive proteins (Di Vizio et al., 2012; Huang et al., 2017; Théry et al., 2018). MSC-EVs are characterized by cell-surface expression of generic EV markers such as CD9, CD81, CD82, TSG101, and Alix. Transplantation of MSC-EVs may carry certain advantages over cell-based therapies. Firstly, accurate quantification of number of transplanted MSCs may be difficult and their effects could also be less predictable than that of EVs within the recipient site, potentially making outcomes of clinical trials less reproducible. Production costs of EVs at a large scale could be lower than that of MSCs (Cha et al., 2018). Furthermore, EVs exert low potential for toxicity and immunogenicity with repeated transplantation (Zhu X. et al., 2017; Saleh et al., 2019), and therefore avoid the undesired immunogenic properties of MSCs (Gu et al., 2015). As a cell-free therapy, MSC-EVs can be stored by cryopreservation whereas MSCs cannot, and therefore have greater potential as an off-the-shelf treatment option (Vlassov et al., 2012).

Through mechanisms including direct receptor interaction, membrane fusion, and internalization, EVs are able to influence recipient cell behavior to promote a variety of effects relevant to cartilage repair. *In vitro* evidence shows that MSC-EVs exert anti-inflammatory effects through influencing IL-6 and TGF-β secretion by dendritic cells. Furthermore, MSC-EVs are found to contain miRNAs such as miR-21-5p which target the CCR7 gene for degradation (Reis et al., 2018) and non-coding RNA that mediate an anti-inflammatory response (Fatima et al., 2017). Co-culture of MSCs with chondrocytes is found to promote matrix production and chondrocyte chondrogenesis *in vitro*, and these effects appear to be EV-dependent (Kim et al., 2019). *In vitro* studies also suggest that chondrocytes take-up MSC-EVs that upregulate type II collagen production (Vonk et al., 2018). Finally, results of recent *ex vivo* studies have suggested that MSC-conditioned media is able to downregulate the expression of genes that promote extra-cellular matrix degradation such as MMP1, MMP13, and IL-1β in synovial explants (van Buul et al., 2012) facilitating cartilage repair, suggesting a role for EVs (Nawaz et al., 2018).

Recently, there has been increasing interest in the use of MSC-EVs in cartilage repair and some systematic reviews have examined the *in vitro* evidence for animal MSC-EVs. In this systematic literature review, we sought to explore the *in vivo* evidence for the use of human-derived MSC-EVs in murine models of cartilage repair.

MATERIALS AND METHODS

The methods used to conduct this review were according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement protocol (Moher et al., 2015). We carried out a literature search on PubMed, Scopus, and EmBase databases in January 2020, capturing articles starting from conception. The following search strategy was applied: (Mesenchymal stem cell OR MSC* OR Multipotent stromal cell OR Multipotent stem cell OR Mesenchymal stromal cell) AND (Extra-cellular vesicle OR extracellular vesicle OR EV* OR exosomal OR exosome) AND (osteoarthritis OR OA* OR osteochondral OR Cartilage). Following de-duplication, exclusion criteria was applied to studies not written in or translated into the English language. We excluded studies that only performed *in vitro* experiments. Studies that did not characterize or validate the cell populations as per the

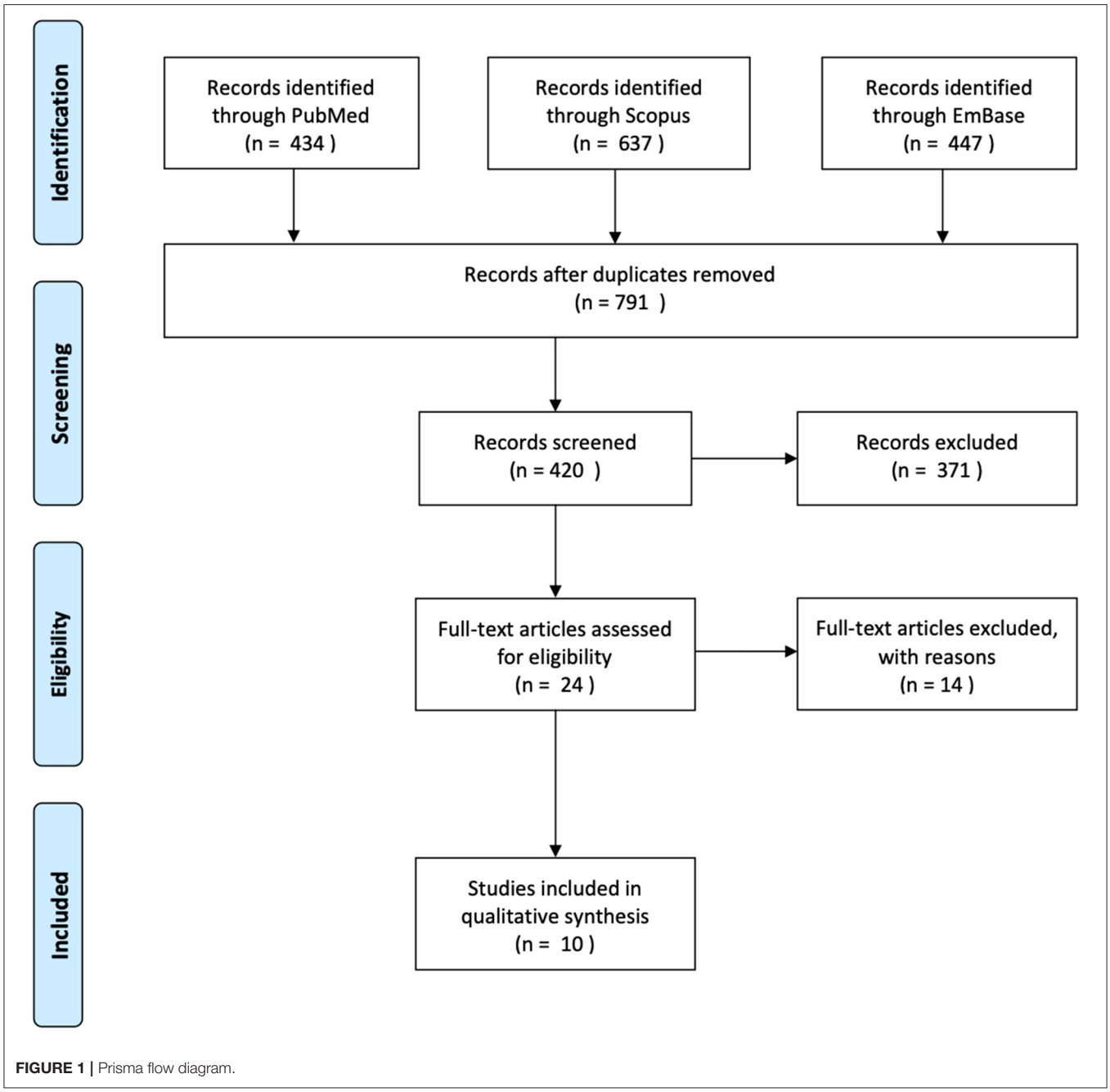

FIGURE 1 | Prisma flow diagram.

recommendations of the International Society for Cellular Therapy (ISCT) for MSC were excluded (Witwer et al., 2019). We included studies that examined the effects of human MSC-derived exosomes, studies examining animal MSC-derived exosomes were excluded. Studies that conducted characterization of EVs in accordance with The International Society for Extracellular Vesicles (ISEV) standards were included (Théry et al., 2018). We included case-control studies, randomized control trials, case series, and case reports with two or more subjects. After removing duplicates, a total of 727 studies underwent title screening (**Figure 1**). A total of 24 studies were examined in full text. Ten studies were included in our review.

Quality assessment was carried out independently KR and CM using the SYstematic Review Center for Laboratory animal Experimentation (SYRCLE) tool (Hooijmans et al., 2014), discrepancies in results were resolved by discussion.

RESULTS

MSC Characteristics

We identified 10 studies in our review, all of which were case-control studies with murine subjects. MSCs were derived from a variety of human tissue sources (**Table 1**). Four studies (Wang et al., 2017), three by the same group, used MSCs derived from

TABLE 1 | Method of MSC harvest and characterization.

References	Source	Cell harvest	Cell treatment	MSC characterization
Khatab et al. (2018)	Human	Heparinised femoral shaft bone marrow aspirate	Cultured in minimal essential medium alpha (α MEM), Fetal Calf Serum (FCS) and Invitrogen to third passage	Trilineage differentiation, Flow cytometry: CD73, CD90, CD105, CD166 +ve
Mao et al. (2018)	Human	Bone marrow aspirate from iliac crest	Cultured in [a]standard Mesenchymal Stem Cell (MSC) media and changed to chondrogenic media from third passage onwards. miR-92a-3p overexpressed in one group	Trilineage differentiation, Flow cytometry: CD11b, CD19, CD34, CD45, CD73, CD90, CD105, and HLA-DR –ve
Tao et al. (2017)	Human	Synovial membrane tissue	Cultured in standard MSC media to fifth passage. miR-140-5p overexpressed in one group	Trilineage differentiation, Flow cytometry: CD44, CD73, CD90, CD105, CD151 +ve
Wang et al. (2017)	Human	Embryonic stem cell-derived MSCs (obtained from third party)	Cultured in standard MSC media and cells between fourth and seventh passage were utilized	Trilineage differentiation, Flow cytometry: CD73, CD90, CD105 +ve
Wu et al. (2019)	Human	Infrapatellar fat pad obtained following total knee arthroplasty	Cultured in standard MSC media to confluence and used at first passage	Flow Cytometry: CD44, CD73, CD90 +ve. CD34, CD11b, CD19, CD45, HLA-DR present at low levels
Zhang et al. (2016)	Human	Cleavage and blastocyst- stage embryonic stem cells from *in vitro* fertilization	Cultured in standard MSC media. Further details not stated	Trilineage differentiation, Flow cytometry: CD105, CD24 +ve
Zhang et al. (2018)	Human	Immortalized E1-Myc 16.3 embryonic stem cell-derived MSC	Cultured in standard MSC media and passaged at 80% confluence until use. Grown in defined media for 3 days prior to exosome extraction	Trilineage differentiation, Flow cytometry: CD29, CD44, CD90, CD105 +ve, CD34, CD45, HLA-DR –ve
Zhang et al. (2019)	Human	Immortalized E1-Myc 16.3 embryonic stem cell-derived MSC	Cultured in standard MSC media and passaged at 80% confluence until use. Grown in defined media for 3 days prior to exosome extraction	Trilineage differentiation, Flow cytometry: CD29, CD44, CD90, CD105 +ve, CD34, CD45, HLA-DR –ve
Zhu Y. et al. (2017)	Human	Induced pluripotent stem cell-derived MSC (iMSC) induced from human umbilical cord iPS Synovial MSC (sMSC) from humans undergoing Anterior crucial efforts ligament (ACL) reconstruction	iMSC: iPS cultured for 5 days in mTESR1 (Stemcell) and then cultured in standard MSC media for 2 weeks. The cells were then passaged every 5–7 days until a fibroblastic morphology was adopted sMSC: cultured in standard MSC media, with media changed every 4 days	Trilineage differentiation Flow cytometry: iMSC: CD29, CD44, CD73, CD90 +ve. CD34, CD45, HLA-DR –ve sMSC: CD44, CD73, CD90, CD166 +ve. CD34, CD4, HLA-DR –ve
Jin et al. (2020)	Human	MSCs derived from bone marrow aspirate from the ilium of healthy subjects	Cultured to third to fifth passage with media changed every 48 h	Trilineage differentiation. Flow cytometry: CD29, CD44, CD71 +ve. CD34, CD45, and HLA-DR -ve

[a]*refers to individual culture condition protocol without addition of stimulating factors.*

embryonic stem cells (Zhang et al., 2016, 2018, 2019). Three studies obtained MSCs from bone marrow aspirate (Khatab et al., 2018; Mao et al., 2018; Jin et al., 2020), one from infrapatellar fat pad (Wu et al., 2019) and one from synovial tissue (Tao et al., 2017). One study compared EVs from Induced Pluripotent Stem Cell (iPS)-derived MSCs with synovium-derived MSCs (Zhu Y. et al., 2017). All MSCs were characterized using flow cytometry and trilineage differentiation. All MSCs expressed either CD44, CD90 or CD105, and most expressed low levels of HLA-DR.

EV Characteristics

Ultracentrifugation and ultrafiltration were the two most common methods for isolation of exosomes (**Table 2**). One study used polyethylene glycol precipitation as part of the purification process (Wu et al., 2019), and several used tangential flow filtration (TFF). EV dimensions were determined using Transmission Electron Microscopy (TEM) in all but one study (Khatab et al., 2018). EV size ranged from 30 to 200 nm, with a modal mean size of around 100 nm. Flow cytometry and western blotting were standard methods for characterizing EVs. CD9, CD63, CD81, and ALIX were the most common EV markers identified. Five out of 10 studies that determined the bioactive component of the EVs used with Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) or Western blot analysis. Three studies attributed the effects of EVs to various miRNA. Two studies determined that CD73-mediated protein kinase activation was responsible for the *in vivo* effects of EV transplantation (Zhang et al., 2018, 2019).

Animal Models

All studies used murine models; five studies induced osteoarthritis, including cartilage loss through surgical joint destabilization, two studies directly created osteochondral lesions and three studies used collagenase to cause cartilage loss. All but one study, which examined the TMJ, studied the knee joint. Follow-up duration ranged from 3 to 12 weeks

TABLE 2 | Method of EV purification and characterization.

References	Purification process	EV dimensions	EV marker	Imaging	Active component
Khatab et al. (2018)	Ultracentrifugation	Not determined	Not determined	Not utilized	Not determined
Mao et al. (2018)	Ultracentrifugation	50–150 nm	CD9, CD63, CD81, HSP70	Transmission electron microscopy (TEM)	miR-92a-3p
Tao et al. (2017)	Not specified	30–150 nm	CD63, CD9, CD81, ALIX	TEM, DLS	Not determined
Wang et al. (2017)	Ultracentrifugation	30–200 nm	CD63, CD9	TEM	Not determined
Wu et al. (2019)	Ultrafiltration and polyethylene glycol precipitation	30–150 nm, main peak at 125.9 nm	CD9, CD63, CD81	TEM	miR-199-3p, miR-99-5p, MiR-100-5p (targeting 3'UTR of mTOR)
Zhang et al. (2016)	Culture media concentrated by Tangential Flow Filtration (TFF) sequentially through membranes (1,000 kDa, 500 kDa, 300 kDa, and 100 kDa) then filtered through 0.2 um filter	Homogenously sized particles; modal size of 100 nm	CD81, TSG101, ALIX	TEM	Not determined
Zhang et al. (2018)	Conditioned medium was size fractionated and concentrated by TFF	Homogenously sized particles; modal size of 100 nm	CD81, TSG101, ALIX	TEM	CD73-mediated adenosine activation of MAPK signaling
Zhang et al. (2019)	Conditioned medium was sized fractionated and concentrated by TFF	Particles between 100 and 200 nm	CD81, TSG101, ALIX	TEM	CD73 mediated activation of MAPK signaling
Zhu X. et al. (2017)	Conditioned media concentrated by centrifugation and ultrafiltration	Tunable Resistive Pulse Sensing (TRPS): 50–150 nm TEM: 50–200 nm	CD9, CD63, TSG101	TEM, TRPS	Not determined
Jin et al. (2020)	Conditioned media extracted using size fractionation and filtration	50–100 nm	CD63, CD9, Hsp70	TEM	miR-26a-5p

after induction of cartilage injury. All studies delivered EVs via intra-articular injection. No significant side-effects were reported in any subjects. Varying amounts of EVs were used between studies, and the amount was quantified using different measures (**Table 3**). Some studies injected a given volume with a known concentration of EVs, whereas others determined the mass of EVs delivered.

In vivo Findings

Two studies measured pain scores and one conducted gait analysis following treatment with EVs, and all three showed improved functional scores. Zhang et al. (2019) used Micro-Computed Tomography (micro-CT) to assess cartilage morphology and found improved bone integrity from 8 weeks onwards. Gene-expression analysis undertaken in several studies. Mao et al. reported increased chondrogenic gene regulation after EV treatment. Using PCR, Zhang et al. (2019) and Jin et al. detected reduced regulation of pro-inflammatory cytokines in their studies.

Joint appearance was interrogated macroscopically, microscopically or using both approaches. All studies reported a reduction in cartilage loss after treatment with EVs. Microscopic histological analysis focused on assessing cartilage thickness and cartilage matrix appearance. Four studies conducted subjective quantification of appearance using the OARSI score. All studies found improved cartilage appearance or OARSI score in the treated groups, although Khatab et al. reported no treatment effect on subchondral bone volume at 3 weeks. Immunohistology was utilized in all studies and all reported increased collagen type II staining with several groups reporting reduced MMP3 immunostaining. Three studies reported reduced staining of apoptotic markers.

Quality of Studies

The SYRCLE tool was used to grade each study using 15 different parameters. Seven out of the 10 studies had a low level of concern overall and three studies had some concern toward risk of bias (**Table 4**). Blinding and detection bias constituted the main contributors to bias in the studies. There was little selection and reporting bias among the studies, but randomization of subjects were not mentioned in most studies. Overall, the studies included in this review were of high quality and low risk of bias.

DISCUSSION

All 10 studies in this review reported reduced cartilage loss following treatment with MSC-EVs. A variety of outcome measures were employed in each study to examine the impact of EVs on cartilage loss, but not all studies found improvements in every parameter measured. A total of 159 subjects were

TABLE 3 | Summary of findings from *in vivo* experiments.

References	Method of delivery	Injury model	Duration of follow up	Macroscopic appearance/ functional studies	Imaging and histology	Biochemical analysis
Khatab et al. (2018)	Intra-articular injection of secretome (Derived from 20,000 third passage MSCs suspended in 6 μl medium) on day 7, 9, and 11 after OA induction	Murine, Collagenase Induced Osteoarthritis (CIOA)	21 days	Greater pain reduction in OA-affected limb from day 7 in treated groups	Histological assessment revealed improved cartilage thickness but no treatment effect on subchondral bone volume	Immunostaining of iNOS, CD163, and CD206 did not demonstrate a difference between treated and untreated groups
Mao et al. (2018)	Intra-articular injection of 15 μl of MSC-derived exosomes or MSC-derived exosomes from a group pre-treated with miR-92a-3p-Exos On days 7, 14, and 21	Murine, CIOA	28 days	Improved cartilage appearance in treated groups	Improved microscopic cartilage matrix appearance	Greater COL2a1 and aggrecan staining in treated lesions. Increased regulation of WNT5A, COL2A1, and aggrecan mRNA expression
Tao et al. (2017)	Intra-articular injection of 100 μl of 10^{11} exosome particles/mL weekly from week 5 to 8 post-surgery	Murine, medial meniscus, and medial collateral ligament transection	12 weeks	Not undertaken	Histology: Less joint wear and cartilage matrix loss in the treated group undertaken. Improved Osteoarthritis Research Society International (OARSI) score	Immunostaining: greater type II collagen (Col II), aggrecan, and type I collagen expression
Wang et al. (2017)	Intra-articular injection of 5 μl of exosomes into Knee joint at week 4 and every 3 days thereafter	Murine, destabilization of medial meniscus (DMM)	8 weeks	Not undertaken	Improved OARSI score in treated group. Reduced microscopic appearance of OA in treated group	Greater Col II staining and weaker ADAMTS5 staining in the treated group
Wu et al. (2019)	Intra-articular injection of 10 μl of exosome (10^{10} particles/ml) weekly or biweekly	Murine, DMM	8 weeks	Improved gait; increased weight bearing on OA knee, swing speed and intensity in treated groups	Improved OARSI score in the treated group	Immunohistology showed increased Col II expression, decreased ADAMTS5 and MMP13 expression.
Zhang et al. (2016)	Intra-articular injection of 100 μg of exosomes weekly from surgery	Murine, surgically induced osteochondral defects on trochlear grooves of distal femur	12 weeks	Macroscopic: Moderate improvement at 6 weeks, near-complete neotissue coverage and integration with surrounding cartilage at 12 weeks in treated groups. Improved International Cartilage Repair Society (ICRS) score at 12 vs. 6 weeks	Histology: smooth cartilage in five out of six treated defects at 12 weeks	Immunohistochemistry: intense GAG staining (>80%), high level of T2Col and low level of T1Col in treated lesions. Lubricin +ve cells found in superficial and middle zones of neo-cartilage
Zhang et al. (2018)	Intra-articular injection of 100 μg of exosomes weekly from surgery	Murine, Surgically induced osteochondral defects on trochlear grooves of distal femur	12 Weeks	Improved Wakitani macroscopic score at 2, 6, and 12 weeks compared to controls	More neotissue formation compared to control	Increased GAG and T2Col staining in treated groups. Increased Proliferative Cell Nuclear Antigen (PCNA) and decreased Cleaved Caspase-3 (CCP3) at 12 weeks in treated groups

(Continued)

TABLE 3 | Continued

References	Method of delivery	Injury model	Duration of follow up	Macroscopic appearance/ functional studies	Imaging and histology	Biochemical analysis
Zhang et al. (2019)	Intra-articular injection of 100 μg of exosomes at weekly intervals starting from 2 weeks following OA induction	Murine, monosodium iodoacetate (MIA) induced cartilage loss in temporomandibular joint (TMJ) by inject of Monosodium iodoacetate (MIA) into upper compartment of the joint bilaterally	12 weeks	Reduced Pain behavior in exosome treated rats from 2 weeks onwards as indicated by higher Head Withdrawal Threshold (HWT) as stimulated by Von Frey fibers	Micro-CT: Improved condylar height, cartilage thickness, matrix deposition and subchondral bone integrity from 8 weeks post treatment Histology: Improved Mankin score in treated groups at 4 weeks onwards	Immunohistochemistry: Increased GAG and T2Col staining in treated groups. Increased Proliferative Cell Nuclear Antigen (PCNA) and decreased Cleaved Caspase-3 (CCP3) at 12 weeks in treated groups. PCR: reduced expression of IL-1B, BAX, alpha-SMA) and Substance P, Nerve Growth Factor (NGF), Tyrosine receptor Kinase A (TrkA). Increased TIMP2 expression. Decreased ADAMTS5 expression
Zhu X. et al. (2017)	Intra-articular injection with 8 μl (10^{10}/ml) of exosomes on day 7,14, and 21 following OA induction	Murine, CIOA	4 weeks	Improved ICRS score in both iMSC and sMSC group compared with OA group at endpoint	Improved OARSI score in all groups compared to untreated control. iMSC showed a greater improvement in OARSI score than sMSC	Greater Col II staining in treated groups. Greater Col II staining in iMSC compared to sMSC groups
Jin et al. (2020)	Intra-articular injection of 250 ng of exosomes in 5 μl	Murine, anterior crucial ligament, posterior cruciate ligament, medial cruciate ligament, lateral cruciate ligament, medial and lateral meniscus transection	8 weeks	Not undertaken	Improved microscopic appearance, greater synovial cell infiltration and fibrous tissue formation in treated groups	Reduced MMP-3 and MMP-13 expression in treated group. Reduced synovial cell apoptosis in treated group. Decreased IL-1B in treated groups. Downregulation of PTGS2

TABLE 4 | Summary of risk of bias analysis.

References		Selection bias in sequence generation	Selection bias in baseline characteristics	Selection bias in allocation concealment	Performance bias in random housing	Performance bias in blinding	Detected bias in random outcome assessment	Detected bias in blinding	Attrition bias in incomplete outcome data	Reporting bias	Number of Subjects (n =)	Number of Controls (n =)	Selection bias	Performance bias	Detection bias	Attrition bias	Reporting bias	Overall
Khatab et al. (2018)	Mice	No	No	No	No	Unclear	Unclear	No	No	No	11	11	No	No	No	No	No	No
Mao et al. (2018)	Mice	No	No	No	Unclear	Unclear	Unclear	yes	No	No	10	10	No	Unclear	yes	No	No	Some concerns
Tao et al. (2017)	rat	No	No	No	No	Unclear	Unclear	No	No	No	10	10	No	No	No	No	No	No
Wang et al. (2017)	Mice	No	No	No	Unclear	Unclear	Unclear	No	No	No	20	12	No	Unclear	No	No	No	No
Wu et al. (2019)	Mice	No	No	No	Unclear	Unclear	Unclear	yes	No	No	8	8	No	Unclear	yes	No	No	Some concerns
Zhang et al. (2016)	rat	No	No	No	Unclear	Unclear	Unclear	No	No	No	12	12	No	Unclear	No	No	No	No
Zhang et al. (2018)	rat	No	No	No	Unclear	Unclear	Unclear	No	No	No	36	36	No	Unclear	No	No	No	No
Zhang et al. (2019)	rat	No	No	No	Unclear	Unclear	Unclear	yes	No	No	32	14	No	Unclear	yes	No	No	Some concerns
Zhu X. et al. (2017)	Mice	No	No	No	Unclear	Unclear	Unclear	No	No	No	10	5	No	Unclear	No	No	No	No
Jin et al. (2020)	Mice	No	No	No	Unclear	Unclear	Unclear	No	No	No	10	10	No	Unclear	No	No	No	No

treated with MSC-EVs without significant or immunogenic complications. While all the MSC-EVs were derived from human MSCs, the subjects were all animal models of cartilage injury, and therefore only indirectly inform safety and effectiveness in human subjects.

The capacity for MSCs to expand *ex vivo* varies with cell source (Fazzina et al., 2016) and anatomical site (Davies et al., 2017). The ability of MSCs to undergo chondrogenic differentiation also appears to differ between cell source (Bernardo et al., 2007). The source of EVs varied significantly between studies as the MSCs were harvested from different tissues and anatomical donor sites. The influence of MSC source on the chondrogenic potential of EVs remains unclear, with some studies suggesting that certain MSC-EVs reduce type I and III collagen production (Li et al., 2013). There is evidence that the biological properties of EVs are dependent on MSC source (Kehl et al., 2019) and it is also apparent that certain MSCs, for example those derived from amniotic fluid, may produce a greater number of EVs than bone marrow-derived MSCs when controlled for cell number (Tracy et al., 2019). In this review, two studies used MSCs from immortalized cell lines (Zhang et al., 2018, 2019) this provides an advantage over autologous harvest as it is not invasive. Zhu et al. compared iPS-derived MSC-EVs with sMSC-derived EVs and found the former to be superior in cartilage repair. Relative cost and convenience of production will dictate which of these is favorable. These are important considerations in tissue engineering as the optimal source cell should achieve a balance between ease of harvest and acceptable EV production. MSC-EV bioactivity also appear to depend on cell-culture conditions, for example, the anti-apoptotic effects of adipose-derived MSC secretome can be affected by oxygen tension (An et al., 2015). Therefore, future studies will be required to delineate the relationship between MSC cell source and secretome in order to select the best source for optimal large-scale EV production.

Most studies in the literature examining EV function, in line with our findings, use ultracentrifugation as the main component of the isolation procedure (Gardiner et al., 2016). There is evidence that suggests that ultracentrifugation could increase the amount of contamination by macromolecules within the MSC culture media (Webber and Clayton, 2013). This may be of relevance in our interpretation as MSC-conditioned media is known to contain non-vesicular bioactive components that may promote chondrogenesis (Chen et al., 2018) and lead to an overestimation of EV effectiveness in cartilage repair. While this may make comparisons difficult, the augmented repair is not necessarily an undesired effect. Furthermore, while the effect of multiple washing stages that form part of the centrifugation process improves purity, it may decrease the total number of EVs obtained (Webber and Clayton, 2013) TFF was the next most commonly used method of concentrating EVs. Compared to ultracentrifugation, TFF achieves a greater EV yield, with a reduced amount of non-vesicular macromolecules contamination (Busatto et al., 2018). Other forms of flow-based purification such as Cross-flow isolation are also favorable over ultracentrifugation in terms of rate of production at large scale (McNamara et al., 2018). Ultimately, robust cost-effectiveness studies may be required to determine the optimal method

of purification. The transferability of this review may also be limited by the inconsistent methodologies used by the studies to characterize the EVs used. Apart from one study that did not report on any EV markers (Khatab et al., 2018), all other studies identified markers recognized by the Minimal Information for Studies of Extracellular Vesicles (MISEV) criteria (Théry et al., 2018). EV dimensions were found to range from 30 to 200 nm. The range of 50–150 nm was most commonly reported and may reflect the bias of measurement devices. Future studies should attempt to ascertain the main peak value within the detected range as Wu et al. (2019) did in their study.

Whilst all studies directly injected EVs intra-articularly, the dose delivered was highly varied. Although EV transplantation is yet to be tested in clinical studies, proof-of-concept human clinical trials of intra-articular injection of MSCs to treat cartilage lesions demonstrate a dose-dependent effect without increased risk of adverse effects (Jo et al., 2014). Similar studies will be required to establish such a relationship for EV transplantation. In MSC treatment of cartilage lesions, intra-articular injection appear to promote good engraftment rates with minimal off-site engraftment (Satué et al., 2019). In *in vivo* studies of anterior cruciate ligament repair, intravenous injection of MSCs concomitantly with intra-articular injection produced improved outcomes (Muir et al., 2016). Likewise, intravenous EV injection appear to produce a dose-dependent immunosuppressive effect that may be beneficial for treating arthritis (Cosenza et al., 2018), but the effect on cartilage repair remains unknown, and the potential for off-site engraftment of EVs is not yet characterized.

All animal studies to date on human-derived MSC-EVs have focussed on murine models. Articular cartilage repair can be studied in murine models in several ways. Firstly, the joint may be surgically destabilized such as in destabilization of medial meniscus (DMM) models, leading to altered weight-bearing and subsequently generalized OA changes, which includes cartilage loss. These models are reliable, reproducible, and have high disease penetrance. The time-course over which cartilage changes develop is delayed and therefore recapitulates the nature of human disease (Glasson et al., 2007). Cartilage can also be excised surgically through induction of an osteochondral defect. These defects may progress at different rates depending on the operative site (Haase et al., 2019), and so makes for difficulty in determining the optimal timing of EV treatment. In contrast, DMM models may progress to display cartilage loss at time points later than direct osteochondral injury and therefore may not benefit from EV treatment in the acute post-injury phase. The amount of lesion healing however appeared to be similar when comparing findings from Zhang et al. (2016) and Zhang et al. (2018), where the same treatment was given after DMM and direct osteochondral injury, respectively. Cartilage loss may also be induced using enzymes or chemicals that degrade the cartilage. Three studies induced chondral injury in murine models using collagenase and one study used monosodium iodoacetate (MIA). Chemical induction is less predictable and may also attenuate the effects of cell-based therapies mimicking its effects on tissue-native cells (Taghizadeh et al., 2018). One study focused on the temporomandibular joint (TMJ). As the pathoaetiology and epidemiology of these non-weight bearing joints typically differ, with radiographic TMJ cartilage loss often being asymptomatic (Schmitter et al., 2010), it may not be relevant to compare functional outcomes following treatment.

It is important to determine standardized methods of assessing outcomes relevant to cartilage repair. Several studies reported improvements in the macroscopic appearance of cartilage; while macroscopic appearance scoring is predictive of histological scoring (Goebel et al., 2017), it is unclear how this correlates with functional improvements. The three studies that used collagenase to induce cartilage loss, often termed collagenase induced OA (CIOA) models, assessed the highly clinically relevant outcomes of pain behavior, with both reporting reduction from early stages. It is difficult to draw conclusions from these results as cartilage loss and eventual OA in the murine model is typically late in onset and appears over 10 weeks following injury (Inglis et al., 2008). It is encouraging however, that all studies reported improved microscopic cartilage repair, with several studies employing various subjective quantitative scoring systems (Tao et al., 2017; Wang et al., 2017; Zhu Y. et al., 2017; Wu et al., 2019). It may be informative to conduct a pooled analysis of such outcomes, but it is unlikely to be meaningful in this study owing to the heterogeneity in scores used. Not all studies reported favorable outcomes when assessing markers of cartilage repair. Khatab et al. conducted an immunohistochemical analysis of iNOS and CD206 expression which are markers of inflammation and M2 macrophage, respectively (Fahy et al., 2014), and found no difference between the groups at the end of the study. Inflammation probably attenuates cartilage repair by interfering with chondrocyte activity and so could be of greater relevance in the acute stage (Tung et al., 2002). The majority of studies showed improved collagen immunostaining, and greater expression of chondrogenic genes in tissue samples. Several groups evaluated cell apoptosis as an outcome and found decreased expression of markers of apoptosis following treatment (Zhang et al., 2018; Jin et al., 2020). Indeed, chondrocyte apoptosis may be reflective of matrix depletion in the cartilage (Kim et al., 2001).

In addition to promoting chondrogenesis in chondrocytes, it is likely that EVs contribute to cartilage repair via effects on other cell types. Macrophages are postulated to be a key target for MSC-EVs, with evidence suggesting that EVs cause an M2 phenotype polarization that promotes resolution of inflammation and so promotes cartilage repair (Chen et al., 2019). This notion is supported by the fact that macrophages pre-conditioned with MSC-EVs, termed EV-educated macrophages (EEMs) support tendon healing *in vivo* to a greater extent than treatment with MSC-EVs alone (Chamberlain et al., 2019). Others suggest that MSC-EV secretome actually augments the immunomodulatory effects of MSCs via autocrine action. It appears that IL-1β-pretreated MSCs induce macrophages into an anti-inflammatory phenotype only when in the presence of EVs containing miR-146a (Song et al., 2017). Transplantation of circulating EVs from septic mice however, appear to encourage neutrophil migration and macrophage inflammation; this is attributed to certain miRNAs including miR-126-3p, miR-222-3p, and miR-181a-5p, suggesting that EV-dependent modulation of inflammation is content and context dependent (Xu et al., 2018). Further to this,

caution should be exercised with MSC-EV transplantation in certain patient cohorts, as MSC-EV have been shown to attenuate the ability of macrophages to suppress cancer cells, and in doing so promotes tumorigenicity (Ren et al., 2019).

CONCLUSION

Due to the plethora of pathways through which MSC-EVs can promote cartilage repair, a key step to studying their effects in animal models is to establish the roles of the different bioactive components within EVs. Similarly, outcome measures utilized in studies should complement this. We recommend that cartilage appearance and chondrogenic gene expression should be primary outcomes in addition to quantifiable and clinically relevant functional outcomes such as pain reduction or animal gait analysis. Likewise, it would be beneficial to establish a link between functional and histological outcomes, as the value of assessing histology may otherwise be minimal. In order to establish the optimal way to deliver clinical benefit using MSC-EVs, the most efficient MSC cell source, methodology of cell culture and EV purification should be investigated for the purposes of cartilage repair. Finally, randomized studies in animal models that recapitulates the human disease will be necessary in order to establish a dose-response relationship and therefore clinical safety before we proceed to human trials.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KT and WK conceptualized the review manuscript. KT wrote the manuscript under the supervision of FH and WK. KT and AK assembled study data and conducted literature searches. KR and CM conducted data analysis and risk of bias analysis. All authors contributed to editing and approving the manuscript.

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Extracellular vesicles in cartilage homeostasis and osteoarthritis

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Abstract

Purpose of review—Extracellular vesicles (EV) carry bioactive molecules that can be transferred between cells and tissues. This purpose of this review is to describe how EV regulate functions of cells in cartilage and other joint tissues. The potential application of EV in the treatment of osteoarthritis (OA) and as biomarkers will also be discussed.

Recent findings—EV are found in synovial fluid, in articular cartilage and in the supernatants of synoviocytes and chondrocytes. EV in cartilage have been proposed to be involved in cross talk between cells in joint tissues and to affect extracellular matrix turnover and inflammation. EV from arthritic joints can promote abnormal gene expression and changes in cartilage extracellular matrix, including abnormal mineralization. Promising results were obtained in the therapeutic application of mesenchymal stem cell derived EV for cartilage repair and experimental OA.

Summary—EV have emerged as vehicles for the exchange of bioactive signalling molecules within cartilage and between joint tissues to promote joint homeostasis and arthritis pathogenesis. As the molecular content of EV can be customized, they offer utility in therapeutic applications.

Keywords

extracellular vesicles; exosomes; cell-cell communication; chondrocytes; synoviocytes; osteoarthritis

INTRODUCTION

Osteoarthritis (OA) represents the most common musculoskeletal disorder [1]. It is a complex and multifaceted disease, characterized by the degradation of articular cartilage, subchondral bone remodeling, joint inflammation and changes in meniscus and ligaments [2]. Various risk factors for OA have been identified and these include aging, joint injury, excessive chronic

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mechanical stress, genetic factors, and metabolic disorders [3]. Although several pathogenesis pathways have been characterized [4], current knowledge is incomplete and has not led to effective approaches for prevention or treatment. These limitations can be overcome by advances in the understanding of molecular mechanisms that are involved in the maintenance of joint tissues which involves communication of cells within the different joint tissues. Cells are able to communicate with neighboring or distant cells through cytokines and hormones. In addition, extracellular vesicles (EV) that are released from cells have attracted attention as novel a mechanism of cell-cell communication. EV transfer bioactive molecules to recipient cells to modulate their activity [5]. In this review, we focus on the role of EV in cell-cell communication within and among joint tissues during homeostasis and OA pathogenesis and address the potential therapeutic application of EV.

EXTRACELLULAR VESICLES

Extracellular vesicles (EV) had previously been regarded as inert cellular debris, that was generated as a consequence of cell damage or the result of dynamic plasma membrane turnover. However, recently cell-cell communication via EV has become the center of attention in research of diseases and tissue repair. EV contain bioactive molecules, including proteins, mRNAs, microRNAs, lipids, and DNA [6]. EV have been classified depending on size and biogenesis pathway. Exosomes are small EV (30–150 nm in diameter) that are generated in multivesicular endosome (MVE)/multivesicular bodies (MVB) and are released when these compartments fuse with the plasma membrane. Microvesicles/microparticles (MVs) (50–1000 nm in diameter) are released by budding from the surface of the plasma membrane [7]. Exosome biogenesis is a very tightly regulated process governed by multiple signaling molecules, and begins with receptor activation that is unique to each cell type [8]. Detailed and conclusive characterization of the various types of EV has not yet been accomplished. The International Society for Extracellular Vesicles provided a minimal set of biochemical, biophysical and functional standards [9]. Size alone can not distinguish exosomes from MVs [10]. Furthermore, some proteins previously used as exosome markers, such as MHC class II or class I molecules, heat-shock proteins, flotillins, or actin, are present in all types of EV, and thus cannot be considered as either exosome or even EV-specific markers [11]. New specific markers of medium and large size EV (e.g., actinins), of endosome-derived exosomes (co-expressing three tetraspanins CD9/CD63/CD81 and including TSG101 and syntenin-1), and of non-endosomal EV (some ITGs) have been proposed [7, 11]. There remains a continuing need to better understand the molecular mechanisms of the biogenesis and release of EV and to discover better markers for the various types. The released EV have surface receptors/ligands of their cell source and have potential to interact with specific target cells [6]. EV directly stimulate target cells by receptor-mediated interactions or transfer of the enclosed bioactive molecules [8].

EV FROM CARTILAGE AND CHONDROCYTES

EV have long been known to be present in the pericellular matrix of articular cartilage and growth plate cartilage [12–17]. Various terms have been used to describe them, including matrix vesicles (MaV), articular cartilage-derived extracellular vesicles (ACEV) or apoptotic bodies but there is no conclusive distinction (Table 1). Originally, MaV were described in

growth plate as derived from budding or disintegrating cells that are associated with hydroxyapatite deposition. Alkaline phosphatase activity is abundant in MaV and is used as a marker for their identification [13]. MaV also contain pyrophosphate-generating nucleoside triphosphate pyrophosphohydrolase (NTPPH) activities. Matrix vesicles can be isolated from collagenase-digested articular cartilage and separated from chondrocytes by differential centrifugation and used for functional, biochemical, and ultrastructural studies [18]. Isolated MaV can incorporate calcium, hydrolyze ATP or other nucleotide triphosphates, and facilitate precipitation of hydroxylapatite [19]. Although MaV from different sources are heterogeneous [20], they are similar with respect to the capacity to mineralize matrix [12, 17]. ACEV have been shown to have a physiological function in endochondral bone development and pathologic role in calcium crystal deposition in articular cartilage [21]. The majority of the proteome was shared by EV isolated from normal and OA cartilage, but immunoglobulins and complement components were present only in OA ACEV which also contained lower levels of matrix proteoglycans [22]. Importantly, the ACEV proteome shares fewer similarities with exosomal proteomes. The heterotrimeric G proteins, HSP70 and 90 and members of the tetraspanin family such as CD9, CD63 and CD81 that are particularly characteristic of exosomes were not seen in ACEV [22]. CD9, CD63 and CD81 were previously considered to be specific markers for exosomes; however, in recent proteomics comparison, these proteins were observed in all EV including MV and apoptotic bodies [11, 23].

RNAs are also packaged in EV and are transferable genetic material from tissue to tissue and from human to human [5]. RNAs are protected from degradation by the lipid membrane of the EV. Coding and non-coding small RNAs in EV were in proportions that differed from parent cells with an enrichment of specific miRNAs suggesting that miRNAs are selectively packaged into EV. For example, small RNAs such as miRNAs were enriched in EV isolated from cultures of costochondral growth zone chondrocytes, while large RNAs such as 18S and 28S rRNA were not detected [24]. EV from normal articular cartilage contain full length mRNAs for factor XIIIA, type II transglutaminase, collagen II, aggrecan, ANKH and GAPDH. When transferred to chondrocytes, ACEV-derived RNA was internalized. This was associated with changes in the expression of alkaline phosphatase and osteopontin [25].

The mechanisms of ACEV formation include apoptosis which is increased in OA-affected cartilage [26]. Chondrocyte-derived apoptotic bodies contain alkaline phosphatase and NTP pyrophosphohydrolase activities, and can precipitate calcium [27]. A role of apoptosis in generating this type of ACEV has been demonstrated in experiments where apoptosis was induced by the nitric oxide donor sodium nitroprusside or antibody to the Fas antigen [27]. EV accumulate around apoptotic cells (Figure 1). The levels of pyrophosphate produced by apoptotic bodies were increased by pretreatment of the chondrocytes with transforming growth factor-β and decreased by IL-1β [27]. It has also been suggested that ACEV from primary articular chondrocytes can be generated through the autophagy pathway [28]. In normal but not in OA chondrocytes, rapamaycin, which induces autophagy by inhibiting mTOR signaling, increased the release of ACEV that contained LC3, a marker of autophagosomes [28]. Release of ACEV was inhibited by gene knock down of caspase-3, suggesting an involvement of apoptosis-related mechanisms [28]. Thus, ACEV include various types of EV that differ in mechanism of generation and apparently in molecular content.

More detailed information about calcium crystal deposition by ACEV is presented in a recent review [21]. A database of MaV proteins also provide comprehensive information on protein components of mineralization-related MaV [29].

EV IN COMMUNICATION AMONG JOINT TISSUES

The concept that EV can mediate communication among cells from different joint tissues has thus far only been tested in a limited number of examples. Exosomes from IL-1β stimulated synoviocytes significantly up-regulated MMP-13 and ADAMTS-5 expression in articular chondrocytes, and down-regulated COL2A1 and ACAN compared with synoviocyte derived exosomes [30] (Figure 2). Migration and tube formation activity were significantly higher in human umbilical vein endothelial cells treated with the exosomes from IL-1β stimulated synoviocytes, which also induced significantly more proteoglycan release from cartilage explants. Inflammatory cytokines, IL-6, MMP-3 and VEGF in exosomes were only detectable at low level. IL-1β, TNFα MMP-9 and MMP-13 were not detectable in exosomes. NanoString analysis showed that levels of 50 miRNAs were differentially expressed in exosomes from IL-1β stimulated synoviocytes compared to non-stimulated cells [30].

EV IN SYNOVIAL FLUID AS POTENTIAL OA BIOMARKERS

EV are abundant in synovial fluid, and can be derived from resident cells in joint tissues and from leukocytes that infiltrate arthritis-affected joints. Synovial fluid EV modulate the release of chemokines and cytokines in synoviocytes [31, 32]. The expression patterns of miRNAs in synovial fluid of OA were similar to miRNAs secreted by synovial tissues [33]. A recent microarray analysis of miRNAs in synovial fluid exosomes showed that in samples from female OA patients miR-16-2-3p was up-regulated and miR-26a-5p, miR-146a-5p and miR-6821-5p were down-regulated. In synovial fluid from male OA patients miR-6878-3p was down-regulated and miR-210-5p was up-regulated. Thus, synovial fluid exosomal miRNA content is altered in patients with OA and these changes are gender specific [34]. This is the first study to analyze exosomal molecules as biomarkers in OA. Future studies need to address the possibility of detecting joint-derived EV in blood and of identifying the cellular origin of EV. This has potential to detect tissue specific changes as biomarkers for OA.

EV IN THERAPEUTIC APPLICATIONS

Mesenchymal stem cells (MSC) have been used successfully in tissue engineering approaches to treat cartilage lesions and OA in animal [35] and human studies [36, 37]. These beneficial functions of MSC are at least in part mediated by paracrine effects of cytokines and growth factors that decreased inflammation, enhanced progenitor cell proliferation, improved tissue repair. In a mouse model of myocardial ischemia/reperfusion injury it was first demonstrated that the protective paracrine effect was mediated by secreting exosomes [38].

Since then additional studies have demonstrated that EV derived mesenchymal stem/stromal cells (MSC) have therapeutic effects [39–41]. We reported that MSC-derived EV promote

skeletal muscle repair and bone fracture healing in mouse models through acceleration of biological function such as angiogenesis and cell differentiation [32, 42].

Exosomes can be used in therapeutic approaches, either from specific cell types such as MSC or from cells that are transfected with genes that have therapeutic potential to enrich for RNA levels for the gene of interest. Exosomes derived from synovial membrane MSC promoted chondrocyte proliferation and migration but inhibited their secretion of extracellular matrix (ECM). Wnt5a and Wnt5b carried by exosomes activated the alternative Wnt signaling pathway and enhanced proliferation and migration of chondrocytes but significantly decreasing ECM secretion. We previously reported that miRNAs, in particular miRNA-140, one of the most abundant miRNAs in chondrocytes are important regulators of cartilage homeostasis [43, 44]. Exosomes were prepared from cells that were transduced with lentiviral miR-140-5p enhanced the proliferation and migration of articular chondrocytes and reduced OA severity in a rat model [45]. Human embryonic MSC exosomes promoted cartilage regeneration in a rat osteochondral defect model [46]. In that study, MSC exosomes accelerated neotissue filling and enhanced matrix synthesis of type II collagen and sulphated glycosaminoglycans. By the end of 12 weeks, exosome-treated rats displayed complete restoration of cartilage and subchondral bone.

Exosomes from conditioned culture media of embryonic stem cell derived MSC (ESC-MSC) maintained the chondrocyte phenotype by increasing collagen type II synthesis and decreasing ADAMTS5 expression in the presence of IL-1β. Intra-articular injection of ESC-MSC alleviated cartilage destruction and matrix degradation in the DMM model. Immunocytochemistry revealed colocalization of the exosomes and collagen type II-positive chondrocytes. Subsequent intra-articular injection of exosomes derived from ESC-MSC successfully impeded cartilage destruction in the DMM model. The exosomes from ESC-MSC exert a beneficial therapeutic effect on OA by balancing the synthesis and degradation of chondrocyte ECM, which in turn provides a new target for OA drug and drug-delivery system development [47]. This study demonstrated the utility of MSC exosomes as a ready-to-use and ‘cell-free’ therapeutic alternative to cell-based MSC therapy.

FUTURE PERSPECTIVES

The role of EV in joint homeostasis and OA pathogenesis is of great interest and further research on this topic has potential implications for the discovery of novel biomarkers and therapeutic approaches. Currently this field is at an early stage and the topic that seems most advanced is the use of EV as therapeutic vehicles. Key questions that need to be investigated are: regulation of the types, amounts and compositions of EV that are generated and released by cells; stability of EV in the various joint tissue environments and transport of EV through dense ECM structures such as in cartilage; mechanisms of recognition and internalization by cells; the role of EV in joint homeostasis and pathogenesis. Markers of EV in synovial fluid or blood that allow tracking their cellular origin and thus profiling the status of these cells.

CONCLUSION

EV are present in articular cartilage and synovial fluid and represent a heterogeneous mixture that varies in regard to mechanism of generation and molecular content. Synovial Fluid EV are potential new OA biomarkers. Composition of EV from OA cartilage appears to be altered and may contribute to abnormal mineral and crystal deposition. EV are released from synovial fibroblasts and affect gene expression in chondrocytes. The pattern of mRNAs and miRNAs in EV can be altered by stimulation or gene transduction of cells and thus be designed to specifically change the function of target cells for therapeutic use.

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KEY POINTS

- EV are released from chondrocytes through various mechanisms, including autophagy and apoptosis.
- EV from chondrocytes can play a role in abnormal articular cartilage mineralization.
- EV from mesenchymal stem cells can be enriched for certain bioactive molecules, such as miRNAs and used for tissue repair and in the treatment of OA.

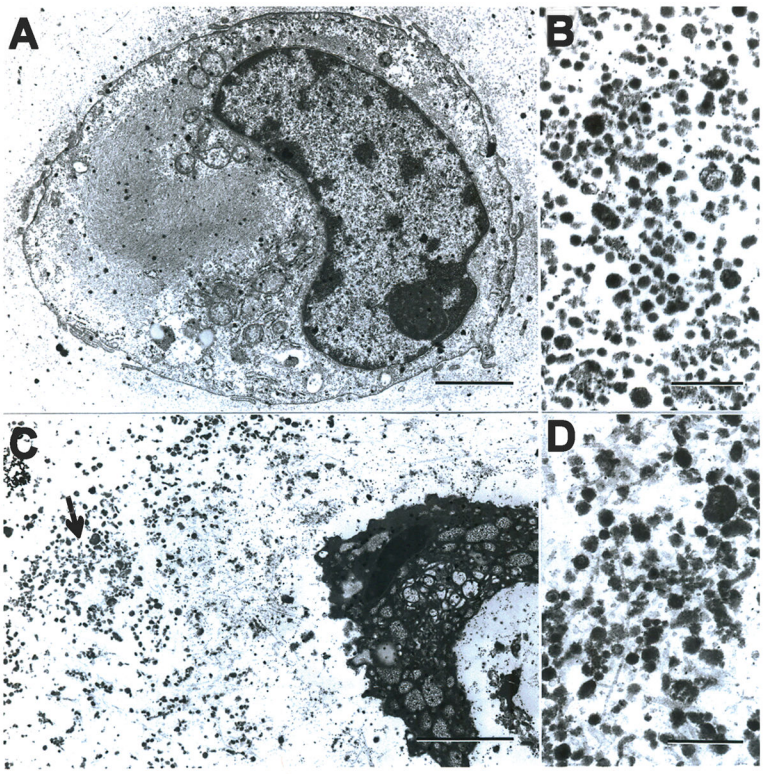

Figure 1. Electron microscopy of apoptotic chondrocytes, apoptotic bodies and matrix vesicles

(A) Electron micrograph of a chondrocyte from normal articular cartilage. Bar represents 2 μM.

(B) Isolated matrix vesicles from normal cartilage. Bar represents 0.5 μM.

(C) Electron micrograph of an apoptotic chondrocyte in cartilage treated with the NO donor SNP. The area indicated by the arrow is shown at higher magnification in (D). Bar represents 2 μM.

(D) High magnification view of the area indicated by the arrow in (C). Bar represents 0.5 μM.

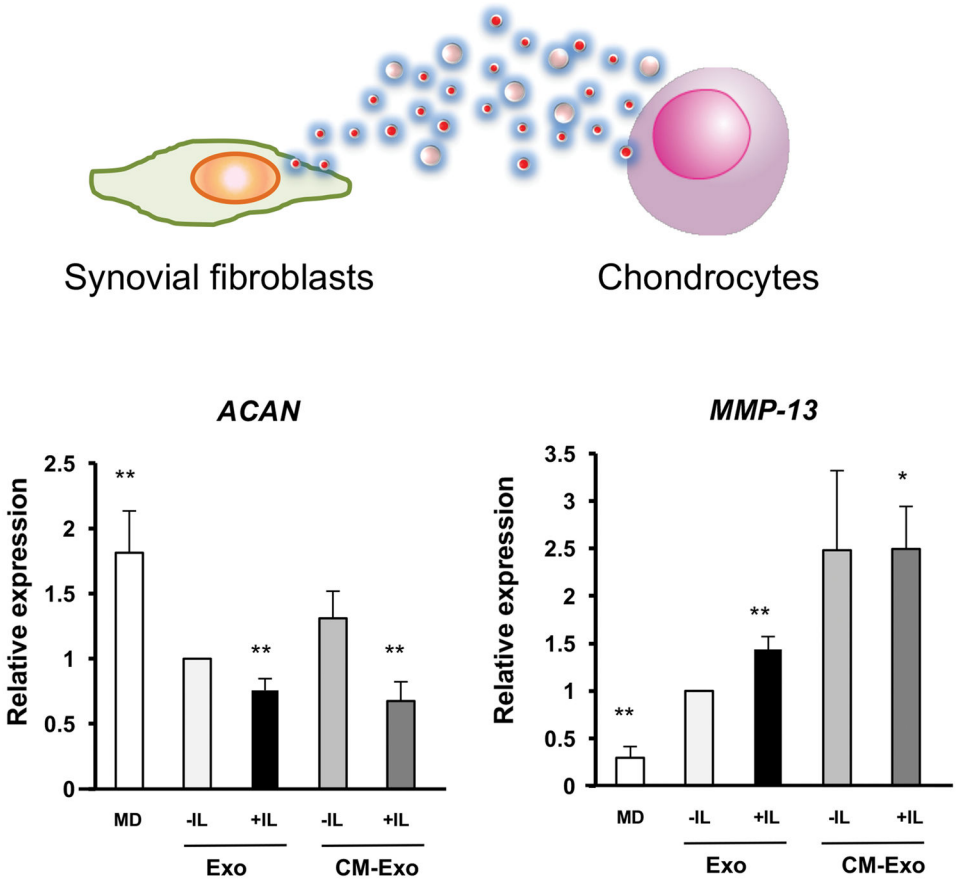

Figure 2. The effect of EV from IL-1β stimulated synovial fibroblasts on normal articular chondrocytes

Articular chondrocytes were treated with fresh-DMEM, exosomes from non-stimulated synovial fibroblasts (SFB) or exosomes from IL-1β stimulated SFB. The expression of OA-related genes was analyzed by real-time PCR. *MMP-13* was significantly up-regulated, and *ACAN* was significantly down-regulated by exosomes from IL-1β stimulated SFB. **P < 0.01 versus Exo -IL. MD; fresh-DMEM with 10% FBS, Exo -IL; exosomes from non-stimulated SFB, Exo + IL; exosomes from IL-1β stimulated SFB.

Table 1

Characteristics of Articular Cartilage-derived Extracellular Vesicle (ACEV).

	Exosome	Matrix vesicles/Microvesicle	Apoptotic body
Origin	Endocytic pathway Autophagic pathway	Budding off/fusion from the Plasma membrane Autophagic pathway	Plasma membrane in apoptotic cell
Size	30–150 nm	100 – 1000 nm	100 nm <
Marker	CD9, CD63, CD81, Flotillin-1, Alix, Tsg101, LC3 etc. No specific markers		
Content	mRNA, non-coding-RNA (microRNA etc), protein, lipid, DNA		
Isolation method	Differential centrifugation/Density gradient centrifugation Commercial kit		

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Extracellular vesicles in osteoarthritis of peripheral joint and temporomandibular joint

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Osteoarthritis (OA) is a disabling disease with significant morbidity worldwide. OA attacks the large synovial joint, including the peripheral joints and temporomandibular joint (TMJ). As a representative of peripheral joint OA, knee OA shares similar symptoms with TMJ OA. However, these two joints also display differences based on their distinct development, anatomy, and physiology. Extracellular vesicles (EVs) are phospholipid bilayer nanoparticles, including exosomes, microvesicles, and apoptotic bodies. EVs contain proteins, lipids, DNA, micro-RNA, and mRNA that regulate tissue homeostasis and cell-to-cell communication, which play an essential role in the progression and treatment of OA. They are likely to partake in mechanical response, extracellular matrix degradation, and inflammatory regulation during OA. More evidence has shown that synovial fluid and synovium-derived EVs may serve as OA biomarkers. More importantly, mesenchymal stem cell-derived EV shows a therapeutic effect on OA. However, the different function of EVs in these two joints is largely unknown based on their distinct biological characteristic. Here, we reviewed the effects of EVs in OA progression and compared the difference between the knee joint and TMJ, and summarized their potential therapeutic role in the treatment of OA.

KEYWORDS

osteoarthritis, TMJ, peripheral joint, extracellular vesicles, EV therapy

1 Introduction

Extracellular vesicles (EVs) are nanoscale sphere-like phospholipid bilayer particles secreted by cells in a physiological or pathological state (1, 2). EVs can inherit bioactive substances from their host cells, including proteins, lipids, DNA, micro-RNA (miRNA), and mRNA (3). Nevertheless, EVs are not only carriers of bioactive substances. They also

deliver their contents to target cells by specific ligand-receptor binding patterns or endocytosis (4). From this, EVs can mediate the communication between cells and affect the biological behavior of target cells (5). The diameter of EVs is generally 30-2000 nm. Based on particle size and generation pattern, EVs can be divided into three main types: exosomes, microvesicles, and apoptotic vesicles (apoVs) (6, 7). Exosomes exist in daily cellular activities and are usually considered to be intermediate between 30-150 nm. With the stage of endocytosis, sorting endosomes and multivesicular bodies, exosomes are finally assembled and released to specific tissue (8, 9). The diameter of microvesicles is larger than that of exosomes, which is about 200-1000 nm. Compared to exosomes formed by endocytosis, microvesicles shed directly from the cytoplasmic membrane (10). ApoVs are produced during the process of cell apoptosis, which include apoptotic bodies, apoptotic microvesicles, and apoptotic exosomes (11, 12). Different from other vesicles, the diameter of apoVs is variable and previous studies mainly focused on apoptotic bodies in 1-5 μm diameter (13–15). Recent studies show that apoptosis also encompass apoptotic microvesicles (100-1000 nm in diameter) and apoptotic exosomes (<150 nm in diameter) (16, 17). Emerging evidence shows EVs widely participate in cell activity and pathological processes.

As the population ages globally, joint trauma rate and the incidence of osteoarthritis (OA) are increasing yearly, affecting more than 240 million people and imposing a significant medical burden on society (18, 19). OA is a degenerative joint disease characterized by synovial inflammation, progressive cartilage degradation, and subchondral bone remodeling, leading to joint pain, deformity, and dysfunction (20). OA can affect both peripheral joints and temporomandibular joints (TMJ), which are synovial joints (21). The most affected peripheral joints are the joints of the fingers, knee, and hip joints. Their symptoms can interfere with work and normal daily activities (22). TMJ are the two synovial joints connecting the jawbone to the skull. Similar symptoms with peripheral and TMJ OA cause joint pain and movement limitation. Different from peripheral joint OA, TMJ OA is the terminal stage of TMJ disorder and often presents with abnormal jaw movement and restricted mouth opening, partly accompanied by tinnitus, headache, and other symptoms (23).

Traditional treatment emphasizes symptomatic treatment. However, it showed a limited effect in reversing the destruction of cartilage or subchondral bone (24). Although emerging stem cell therapies can promote cartilage tissue regeneration, shortcomings such as immune exclusion and tumorigenicity alarmed us (13, 25). The therapeutic effects of stem cells are mainly attributed to their paracrine effects, and EVs are one of the important components of paracrine secretion (26). Compared with stem cell therapy, EVs have many superiorities, including explicit pathways of effect, low immunogenicity, low tumorigenicity, easy preservation, and no need to consider cell survival and abnormal differentiation (27). So EVs possess a safer and greater tissue regeneration characteristic. Mesenchymal stem cell (MSC)-derived EVs have been shown to have notable effectiveness in OA on pain relief, inhibition of inflammation, immunomodulation, and cartilage tissue regeneration (28–31).

Furthermore, EVs carrying specific substances can be used as potential biomarkers for the diagnosis of OA and for monitoring the progression of OA (32). EVs, as a future cell-free therapy, show promising applications in joint diseases. It is expected to become a better alternative to MSC therapy in tissue regeneration. Although the significance of EVs in the pathogenesis and treatment of OA has been reviewed elsewhere (33–36), there is still lacking a review that summarizes the similarities and differences about EV function between peripheral joint and TMJ OA. This manuscript mainly compares the difference between the peripheral joints and TMJ and reviews the role of EVs in the pathogenesis and treatment of peripheral joints OA and TMJ OA. We also highlight the challenges facing EVs used as a conventional biologic agent for the treatment of OA and the significance of exploring next-generation EV-drug in the future.

2 The difference between peripheral joint and TMJ

2.1 The anatomy and development in TMJ and peripheral joint

The difference between the peripheral joint and TMJ is mainly generalized for anatomy and development. Peripheral joints include knee, hip, shoulder, elbow, wrist, and ankle joints, most of which are subordinate to synovial joints. The typical structure of synovial is comprised of two opposing skeletal elements and intermediate discs capsuled by synovial tissue, permitting a wide and low-friction movement (37). The knee is the largest synovial joint and is studied the most. Here, we used the knee joint as a representative to compare with TMJ, which both belong to synovial joints. As a typical single synovial joint, the knee comprises the patella, femur, tibia, fibula, meniscus, and surrounding ligaments and joint capsule (38). The TMJ also comprises the synovial joint capsule and ligaments, temporal fossa, articular tuberosity, mandibular condyle, and articular disc (39). Besides the typical synovial joint structures, TMJ is the only bilaterally linked joint characterized by sophisticated structure, precise regulation, and complicated functions (40). The other anatomical difference is the nerve distribution, which may be the basis for the distinct symptoms of TMJ OA. No sensory structures are included within the 3 cm diameter sphere centered on the meniscus of the knee. In contrast, several important anatomical structures and sensory nerves are within the 3 cm diameter sphere area centered on the TMJ disc, such as the cochlea, brain, trigeminal ganglion, mandibular nerve, and auriculotemporal nerve (41). The nerve-rich structure may be the reason that TMJ OA patients can be more susceptible to neurological symptoms (42).

The different growth pattern is the other reason for TMJ specificity. TMJ is derived from the cranial neural crest in the way of intramembranous ossification, whereas peripheral joints are mainly derived from cell migration of lateral plate mesoderm by endochondral ossification, which may contribute to the histological differences between the two joints (43). The articular cartilage type

on the surface of the condyle and the knee joints is distinguishable. It is known that the articular surface of the knee is covered by hyaline cartilage with varying proportions of type I and type II collagen, whereas the surface of TMJ is covered by fibrocartilage and is composed mainly of type I collagen (32). In addition, the growth pattern of condylar is different from the long bones. Condylar have ever been regarded as a semi-epiphyseal plate of long bone, but now it has been proven to be wrong (44). The condylar cartilage derived from the cranial neural crest is secondary cartilage, undergoes endochondral ossification, and exhibits characteristic developmental processes, whereas the cartilage of long bones directly originates from embryonic cartilage primordia (45, 46) (Figure 1).

2.2 Clinic manifestation in TMJ OA and peripheral joint-OA

OA is a common chronic degenerative joint disease worldwide, which is believed to be the fourth top cause of disability worldwide (22). OA is usually associated with genetics, trauma, old age, obesity, mechanical stress, mental elements, and other factors. However, the pathogenesis of OA is still unclear (47, 48). Typical clinical symptoms of knee OA are often joint pain and limitation of movement. Patients suffering from knee OA usually feel pain and transient morning stiffness at the early stage. With the progress of OA, a more severe lesion can be found, including subchondral bone cysts, bone marrow lesions, and osteophytes. These changes can be diagnosed by Cone Beam Computed Tomography (CBCT) and Magnetic Resonance Image (MRI) with osteophytes, bone marrow lesions, and meniscal tears (49). Despite similar joint symptoms, mental and biopsychosocial symptoms are also crucial in TMJ OA. TMJ OA presents hyperalgesia and neurological symptoms, including tinnitus, headache, and psychological disabilities (50). In addition, CBCT is relatively mild and often shows blurred and incomplete condylar cortex, localized bone resorption defects, bone redundancy formation, subchondral sclerosis, or cystic bone changes (51). The current goals for treating knee and TMJ OA are consistent, include: reducing pain in the joint area, ameliorating joint function, and slowing the progression of OA. Most patients are treated clinically by a combination of conservative and surgical treatment, which requires a personalized treatment plan that

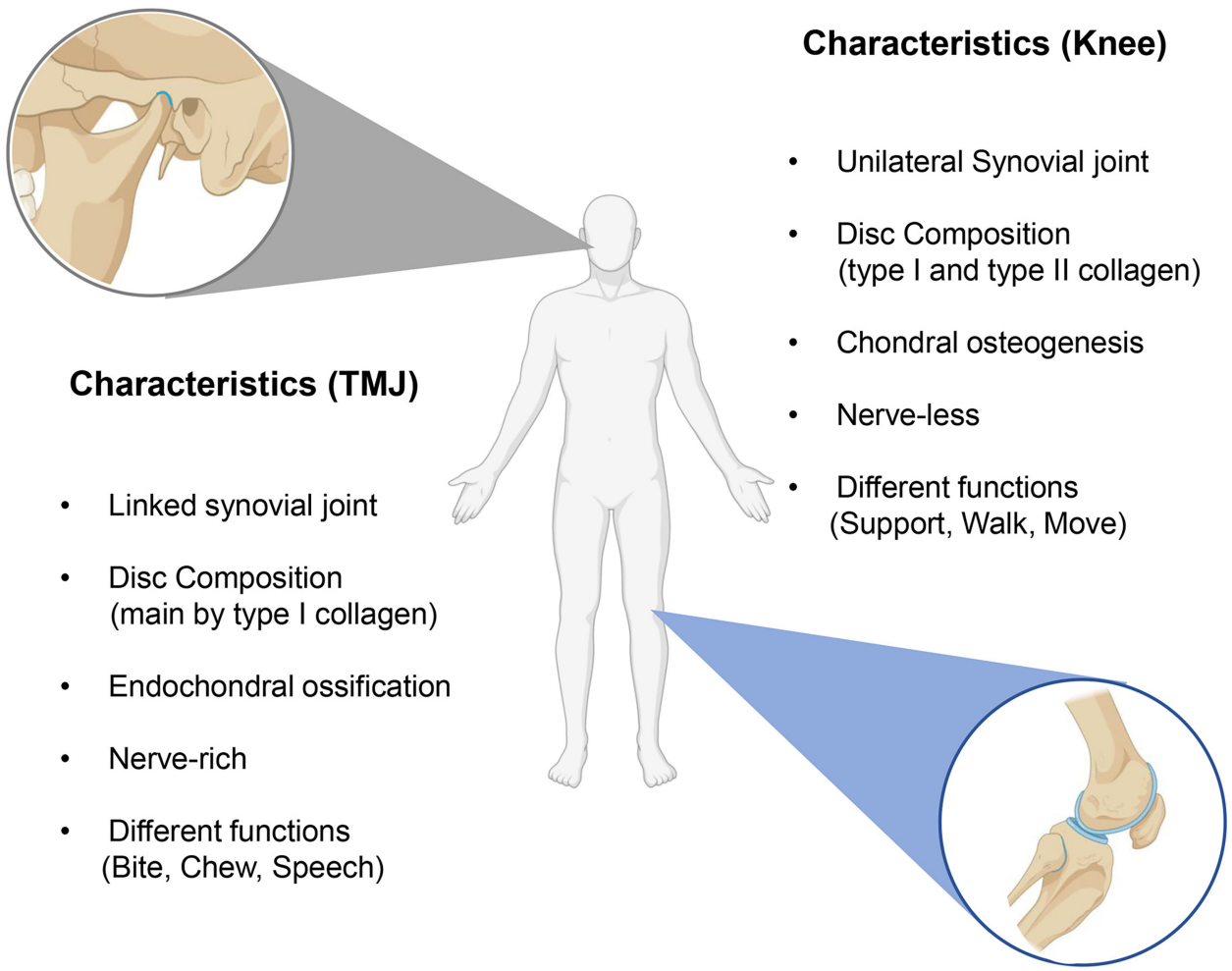

FIGURE 1
The differences between knee OA and TMJ OA. The main differences are the histological structure and functions.

considers the patient's situation, such as age and severity of the disease (52). In addition to traditional treatments, such as physical therapy, oral medications, intra-articular injections, minimally invasive arthroscopic surgery, and surgical operation, many emerging biological therapeutic approaches are currently under study, such as stem cell therapy, platelet-rich plasma, stromal vascular fraction, and EVs (53–56).

2.3 Animal models of OA and TMJ OA

OA is a multifactorial disease, mainly including aging, obesity, anatomical factors, mechanical loading, and genetic factors (Figure 2A). Despite all the above elements contributing to TMJ OA. Occlusion elements, mental and biopsychosocial factors also play an important role in the pathogenesis of TMJ OA (22, 50) (Figure 2B). Based on these risk factors, OA animal models are usually divided into induced, naturally occurring, and genetically modified models, while the knee is the most used joint for the animal OA model. Similar invasive approaches are suitable for both knee and TMJ, including surgical induction and chemical injection (monosodium iodoacetate, papain, collagenase). In comparison, the surgical methods to induce knee and TMJ OA are distinguished based on different anatomical structures (57). The differences between knee and TMJ OA models mainly display in non-invasive methods.

Although the high-fat diet model and mechanical loading can apply to both knee and TMJ, more specific methods are required for TMJ OA (58, 59), including but not limited to disordered bite, excessive mouth opening, and soft food induction (58, 60). Intriguingly, the sleep deprivation model can be used to reduplicate the model of TMJ OA, which further explains the relationship between mental factors and TMJ OA (61). In addition, naturally occurring and genetically modified models are also used to study the progression of OA to simulate aging and genetic factors, which have been reviewed in detail (57). OA is a multi-factor process, and most animal models are single-factor models. It reminds us that choosing the appropriate animal model needs strict consideration according to the purpose of the study.

3 The role of EV in the pathogenesis of OA and TMJ OA

The pathogenesis of OA is complicated and diversified. It is not only a single disease, but a common final stage of joint failure contacted with body status, environmental impact, and triggered by

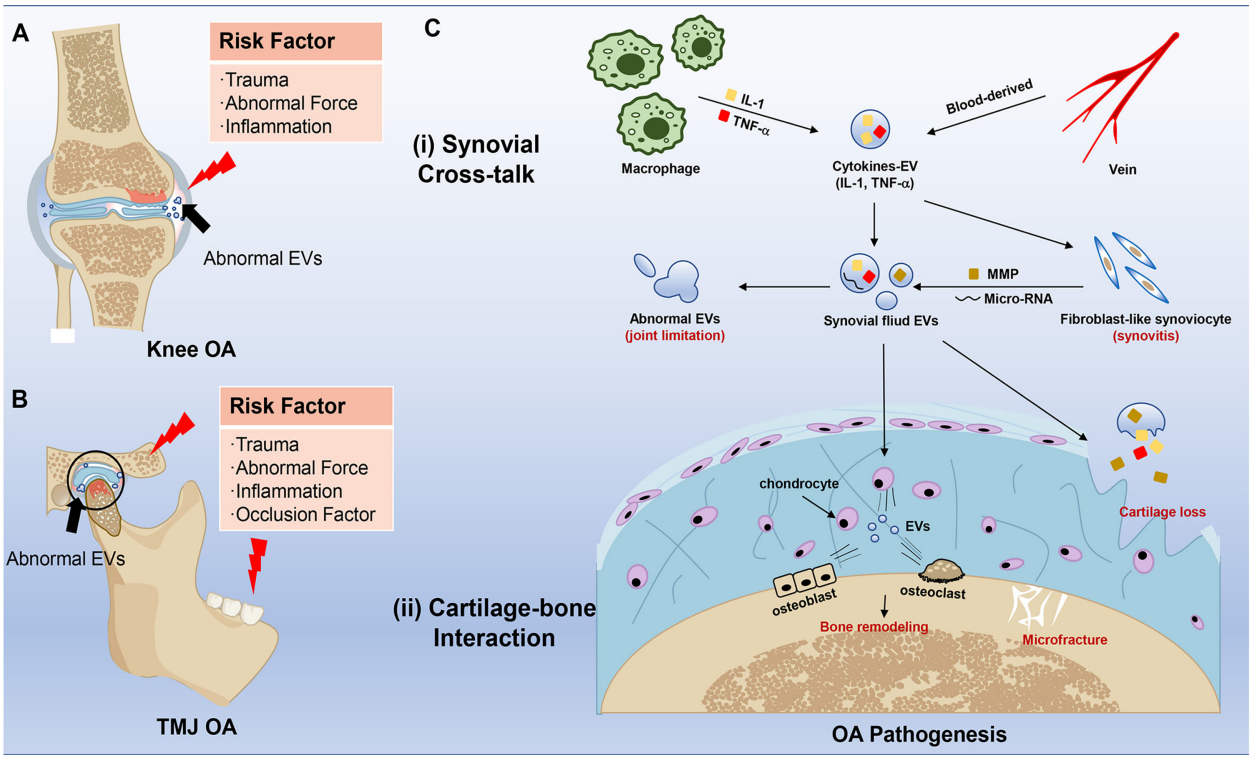

FIGURE 2
Risk factors and effects of EVs in knee OA and TMJ OA pathogenesis. **(A)** Risk factors in knee OA. The red pattern means inflammation and bone destruction, and the blue pattern means healthy joint with uniform EVs. The black arrow points to abnormal EVs in the joint. **(B)** Risk factors in TMJ OA. The red pattern means inflammation, and the black arrow points to abnormal EVs. **(C)** Schematic representation of EVs in the pathogenesis of OA. The red typeface stands for clinical change in OA. The major effects of EVs are mainly divided into two sites, including (i) Synovial Cross-talk and (ii) Cartilage-bone Interaction. The two sites also communicate with EVs.

other risk factors (62–64). In current views, OA is a degenerative disease characterized by the destruction of cartilage, synovial inflammation, and bone remodeling increase (64–66). These changes can be observed in the early stage of OA (49). OA usually needs several years to develop, meaning this disease needs long-term pathological stimulus and progresses to a worsened state.

EVs distribute in humor and tissues, deliver bioactive substances, and participate in cellular activity, which plays a vital role in the whole progression of OA. On the one hand, the serum-derived EVs carried bioactive substances like IL-1β which activated fibroblast-like synoviocytes (FLS) (67), and then these EV cargos can further raise monocytes and macrophages, which usually barely exist in normal synovial tissue. Then a low-grade inflammation can be continuous in synovial fluid and tissue in the whole stage of OA (68). On the other hand, synovitis is closely related to cartilage destruction (69, 70). Activated FLS can secret more EVs carrying inflammatory factors, micro-RNA, and matrix metalloproteinases (MMPs) to induce the apoptosis of chondrocytes and destroy the extracellular matrix (ECM) (71–73) (Figure 2C). So, it is essential to understand EVs' function in OA seriously.

3.1 Inflammation and cytokines

OA has been described as an inflammatory disease for a long time. Many individuals with OA have joint inflammation symptoms like stiffness and pain. In addition, low-grade inflammation can be observed in patients during the whole progression of OA (74). The previous study showed that OA patients have a high level of inflammatory plasma proteins such as albumin and acid glycoproteins in their blood and synovial fluid (75). Recent research showed that other inflammatory factors also maintain a high concentration in OA synovial fluid, including complement components and cytokines (IL-1β, TNF, IL-6, IL-8) (76–79). Furthermore, these inflammatory mediators can be produced or overproduced by chondrocytes and synovial cells in OA (78). These evidences showed a close relationship between inflammation and OA.

Undoubtedly, inflammation factors are crucial in OA progression. These cytokines possess little organizational penetration and stability, so EVs are needed for bioactive cytokines carry to penetrate into deep cells or tissues (34, 80, 81). As a cell secretory mediator, EV has been demonstrated to contain many cytokines, miRNA, and other bioactive substances. EVs can be endocytosed by chondrocytes, FLS, and macrophages which are the main cells in the joint cavity microenvironment (82–84). These biological characteristics are the basis of EV to be effective. In knee OA, a mass of EVs protein cargoes has been identified, including inflammatory cytokines (for example, IL-1, IL-6, and TNF-α), immunoglobulin, complement component, fibrinogen, apolipoprotein and transforming growth factor β (35). EVs derived from synovial fluid of severe knee OA contained higher levels of numerous cytokines (85). Among all the cargoes of EVs in OA, two major players, IL-1β and TNF-α, can induce cartilage destruction and inflammation (71). It was well known that IL-1β and TNF-α increased in synovial fluid, synovial tissue, and cartilage of OA patients (86). On the condition of OA, the plasma EVs can carry a mass of TNF-α to participate in OA progression (87). Similarly, the EVs in OA patients' synovial fluid also carry many inflammatory cytokines, including IL-1β and TNF-α (88). Subsequently, FLS strongly expressed IL-6, IL-8, and MMP, which can directly destroy the extracellular matrix stimulated by activated or apoptotic T cells and macrophages EV (89). This process demonstrated that the EVs from arthritis patients' synovial fluid could further induce the FLS, secreting inflammatory cytokines and chemokines (90). FLS and immunocyte-derived EVs formed a high-concentration EV microenvironment in synovial fluid. These EVs can further be endocytosed by chondrocytes, which trigger chondrocytes to produce more inflammatory cytokines and chemokines (33). The other experiments demonstrated that the healthy chondrocytes treated with OA-derived EVs displayed elevated expression of inflammatory genes (88). OA is the result of the cascade reaction of inflammation. Immunocytes, FLS, and chondrocytes can be activated by EVs with different components and finally induce the production of MMP and destruction of ECM. Overall, the progression of OA in different joints is similar. The changes in knee joint OA can also be detected in TMJ OA (91–94). More and more evidence prove the relation between OA histological changes and EVs. However, the mechanism between the biological changes of EVs and synovial inflammation and cartilage loss is still unknown.

3.2 MicroRNA

People have realized the effect of miRNA on OA progression in recent years. miRNAs are a family of approximately 21-nucleotide-long RNAs that can regulate genetic expression by combining the 3'-UTR (95, 96). MicroRNA gets involved in all the known cellular activity and widely participates in disease progression, including OA (95). It has been demonstrated that miRNA differential expression is the characteristic of OA and EV-microRNA is highly related to OA progression (35, 49, 88). Compared with healthy people, the EVs from synovial fluid of the OA knee joint showed a differential microRNA pattern (88, 97). Recent research showed that 142 microRNAs were differentially expressed between damaged or non-damaged articular cartilage in peripheral joint OA patients (hip and knee) (98). Moreover, researchers collected the human synovial fibroblasts from healthy knees to verify *in vitro*. Likewise, approximately 340 miRNAs upregulated in FLS treated by IL-1β; meanwhile, only 11 miRNA increased in FLS-derived EVs (99).

The synovial fluid-derived EV-miRNA can be endocytosed by chondrocytes and stimulate inflammation in cartilage (88). Since EV-miRNA penetrated into cells, they can directly bind to specific mRNA or proteins to regulate cellular behaviors. Present research mainly focused on knee OA. For example, miR-181a-5p is a critical mediator involved in the cartilage destruction by promoting inflammatory, catabolic, and cell death activity (100, 101). Notably, not all the OA patients have the same EV-miRNA changes; EV-miRNA in OA displays a gender-specific pattern (88). Only one miRNA (miR-504-3p) were upregulated in both

genders, which may promote cell apoptosis (88, 102). The other downregulated miRNAs were associated with cell adhesion molecules, whereas the upregulated miRNAs were related to biotin metabolism signaling. The female-specific EV-miRNAs most participate in estrogen pathway (35), which may explain the high incidence rate of OA in female (22).

TMJ OA also has similar mechanisms that microRNA can anchor target pathways to regulate OA progression, including Wnt, Smad/TGF-β, and PTEN (103–106). Like knee OA, TMJ OA also displays a high incidence rate in females, which may attribute to EV-miRNAs gender-specific expression. However, TMJ OA is different from knee joint OA, such as gender incidence rate and symptoms, and there is little research to detect the difference in TMJ OA.

3.3 Mechanical stimulation

It is undisputed that the mechanical factor is the key to OA progression. The joint is the force-bearing structure of our bodies. Owing to the existence of synovial fluid and disc, bone can move with very low friction. Even so, the response of different joints to force-loading is usually discrepant. For example, the knee usually bears a relatively large force, four times more than body weight in jogging, while TMJ can undergo a body weight when people bite (107, 108). Different function determines different mechanical adaptation, and overuse may cause joint structural changes. So, articular inflammation mainly concentrates on the functional joints like the knee, hip, hand, and TMJ (49). With further validation, we gradually realize that the mechanical factors are not just the physical "erosion" from the previous studies (109). It is a complicated system composed of mechanically sensitive pathways and functional proteins. In the early stage of OA, the microfracture began to emerge, and the cartilage was the first to appear in the microarchitectural changes with chondrocyte mitochondrial dysfunction (110, 111). The mechanical loading may further impact the chondrocyte, which secreted EVs containing mechanically sensitive substances like miR-221-3p to regulate cell communication in bone remodeling (112). However, the impact of mechanical is usually self-limiting. If we intervene timely, the cartilage dysfunction will recover and rebalance.

In the process of force loading, overuse or abnormal loading can cause cartilage loss and subchondral bone marrow lesions with meniscus degeneration or severe tears. Unlike the knee joint, TMJ are two linkage joints having a more precise mechanical system. Recent studies showed that increased loading force led to a thicker calcified cartilaginous layer of TMJ and caused osteochondral interface stiffness with mild disc displacement or perforation (110, 113). Although there is a difference in symptoms between the two joints, the initial mechanical response systems are both chondrocytes and ECM. EVs are one of the most important components in ECM and mediate cell-to-cell interaction in this system (114). In fact, it can detect a mass of apoVs in the ECM collected from knee OA (115, 116). ApoVs were also observed in the apoptotic chondrocytes of TMJ OA (117). Except for apoVs, the other EVs are mainly from articular cartilage that contains over 1700 bioactive substances, including type II transglutaminase, COL II, aggrecan, βig-H3 (TGFβ-induced protein), and GAPDH (80, 118). These EVs form the ECM and regulate the communication between cartilage and bones (119). In the process of knee OA, as the force loading, ECM-derived EVs respond to mechanical effects and are released by chondrocytes, and then interact with the surrounding cells by activating bone morphogenetic proteins (BMPs) and transforming growth factor-β (TGFβ). These changes will further activate downstream pathways, including Wnt and MAPK signaling pathways (109). Finally, these ECM-derived EVs can take away the ECM components, which contains lots of cytokines and cathepsin, such as MMP-2, causing cartilage loss and bone lesions (120). In addition, not only the cargos but EVs' mechanical properties also impact the progression of OA. Studies showed that EVs in OA become inhomogeneous and soft (121), which indicated that mechanical loading may change the mechanical properties of EVs derived from cartilage and bone.

The TMJ OA showed a similar pathological process with knee OA by abnormal mechanical stress stimulation. These EVs and changes can also be found in TMJ OA, including TGFβ, BMPs, Wnt, and FGF pathways (60, 122). However, there are still some differences between TMJ OA and knee OA. Considering they have different origins and growth patterns, the expressions of the same genes also cause different endings. For example, small leucine-rich proteoglycans (biglycan/fibromodulin) are one of the ECM components, participating in both knee OA and TMJ OA (123). Intriguingly, TMJ OA was observed four months later than knee OA in biglycan/fibromodulin double-deficient mice (124, 125). Although we have reviewed current research, the specific pathways of knee and TMJ OA are still unclear. Overall, more direct evidence should be presented in mechanical-induced OA.

3.4 Genetic factor

OA is regarded as an idiopathic disease; however, genetic factor is also an important reason for OA development. OA shows the characteristic of familial aggregation, and the heritability rate of OA is around 35%-65% depending on different joints (126, 127). OA is a polygenic disease that cannot attribute to any single gene. Gene detection was done to analyze the susceptible gene including growth differentiation factor 5 (GDF5) and human leukocyte antigen (HLA) class II/III locus (126, 128). A European genome-wide association study (GWAS) confirmed two genes were related to knee pain in 171561 people, which were close to GDF5 and COL27A1 (129).

Compared with the early industrial and prehistoric eras, the OA prevalence rate of modern people has doubled (130). These genes have so low odds ratios that they are less responsible for OA. However, the paradoxical evidences seem unreasonable to explain the familial aggregation and mother-daughter inheritance patterns in OA. Recent research showed that mothers suffering from OA were more likely to pass it on to their offspring compared with fathers (131). Mother-to-child transmission patterns contain mother-to-embryo, which mainly depends on EVs except for gene recombination. Since mothers are inflammation sufferers, EVs can carry these inflammatory cytokines (IL-1β, TNF-α) and environmental factors, pass through the placental barrier into the

fetus (132). These studies added EV regulatory factors to explain why OA presents as a matrilinear inheritance with a low-degree effect of susceptible genes. However, further studies are needed to elaborate.

4 The role of EVs in the treatment of peripheral OA and TMJ OA

The treatment of OA is always challenging. Currently, conservative and surgical treatments are used to treat OA, but there is no golden therapy for OA. The mild OA lesions can be treated with drugs or splints, while the severe ones may need surgical therapies. In 1957, the first hemiarthroplasty knee device was designed by McKeever, while Christensen designed a fossa-eminence prosthesis for TMJ hemiarthroplasty six years later (133, 134). Till now, knee arthroplasty and hip arthroplasty have been well developed, but TMJ arthroplasty rarely achieves satisfactory results (135, 136). With the progression of OA research, people have gradually realized the importance of biological factors in OA treatments. In 1985, IL-1 receptor antagonist (IL-1Ra) was first reported to find in human macrophages and synovial macrophages in OA patients (137). Autologous conditioned serum (ACS) therapy, mainly working through IL-1Ra, was studied in the mid-1990s (138). The preparation of autoserum is a mature technological process, and the autologous platelets can produce IL-1Ra and various kinds of cytokines in cell degranulation (139). People realize that IL-1Ra was not the only one in OA, and platelet-rich plasma (PRP) was studied subsequently (140).

Because of their preparation process, ACS and PRP retain lots of blood-derived EVs, which showed therapeutic effects in OA (141, 142). It was reported that blood-derived EVs partook the substance exchange and cell communication (141). Blood-derived EVs can inhibit inflammation and elicit chondroprotective gene expression (142). In addition, a recent study showed that blood-derived $CD34^+$ EVs were the potential therapeutic targets for OA. Blood-derived $CD34^+$ EVs were the only subpopulation that significantly correlated between plasma and synovial fluids containing lots of functional mitochondria and little pathogenic cytokines such as TNF-α and IFN-γ (143, 144). EVs from autologous blood-derived products may be the core components of treating OA. Although ACS and PRP showed significant effects in animal models, the complexity of autologous blood-derived products caused controversial clinical efficacy (145, 146). More biotherapies have been studied, including MSC therapy and EV therapy (34, 147). Compared with traditional stem cell therapy, EV administration does not have the risks of allogeneic and xenogeneic immunological rejection and malignant transformation, so it has opened a new era of stem cell therapy.

4.1 Stem cell therapy for OA

With the development of regenerative medicine, stem cell therapy was gradually studied. OA is a degenerative disease, so researchers mainly focused on tissue regeneration and immunoregulation, and stem cell meets requirements. MSCs have the characteristics of self-renew, multiple differentiation, and immune regulation, which contribute to tissue repair of OA (148–151). The clinical trial also showed that stem cell therapy could effectively treat knee OA (152). A recent multicenter randomized controlled clinical trial (phase I/II) demonstrated that bone marrow MSC (BMMSC) therapy could safely alleviate the symptoms of knee OA (153). In contrast, the successive clinical trial showed no significant difference between PRP therapy (154). In the treatment of TMJ OA, stem cells from different sources showed therapeutic effects and promoted condylar cartilage regeneration (24, 155–158). In addition, up to 2,021 randomized clinical trials of stem cell therapy showed a statistically significant superiority over hyaluronan in pain relief and mandible mobility (159). However, stem cell therapy has disadvantages, including immunogenicity and unstable phenotype, so it reminds us whether there is an alternative approach in biotherapy which can be more effective for OA (27).

4.2 EV treatment for peripheral joint OA

Compared to cells, EVs show lower immunogenicity. Therefore, it is safer to apply EVs in OA treatment than cells (160). EVs from a variety of sources, including synovial MSCs (SMSCs) (161–163), adipose-derived MSCs (ADMSCs) (164, 165), BMMSC (166), and human umbilical cord MSCs (hUMSCs) (167, 168), has been proved to be beneficial for peripheral joint OA treatment.

Perturbed balance of the local immune system is the key factor that causes clinical symptoms and organic damages in OA. During knee OA development, immunological cells exert proinflammatory factors, such as IL-1, IL-6, IL-8, and MMP-3, thereby inducing synovial inflammation and cartilage degradation (169). MSC-EVs have immunomodulatory effects in peripheral joint OA treatment. MSCs-EVs generate large amounts of anti-inflammatory cytokines, such as IL-10 and TGF-β1, and simultaneously suppress the production of the proinflammatory factors IL-1, IL-6, TNF-α, and IL-12. Moreover, MSC-EVs inhibit macrophage activation and induce the proinflammatory M1 phenotype to convert to the anti-inflammatory M2 phenotype. Together, MSC-EVs decrease local inflammatory reactions in knee OA (170).

Inflammatory response causes cartilage degeneration in peripheral joint OA, including cell death, matrix degradation, and finally, a loss of structure and function (160, 171). MSC-EVs prevent chondrocyte apoptosis (170) and facilitate cell migration and proliferation in peripheral joint OA (172). By mediating the expression of fibroblast growth factor (FGF)-2, survivin, and Bcl2/Bax, EVs directly promote chondrocyte proliferation or eliminate the inhibitory effect of pro-inflammatory factors like TNF-α and IL-1β (167, 170, 173, 174). Besides, MSCs-EVs can also control chondrocyte proliferation and migration by releasing miRNAs (164, 175, 176).

MSC-EVs induce the expression of matrix protein, while inhibit the expression of matrix-degrading enzymes, thereby promote ECM synthesis. MSC-EVs can reduce the expression of MMP-1, MMP-3, MMP-13, and ADAMTS-5 and increase COL II production (165). Previous research has elucidated that MSC-EVs regulate the expression of cartilage formation-related genes such as aggrecan,

SRY box gene-9 (SOX9), COL9A1, COL2A1, and cartilage oligomeric matrix protein (COMP) while decreasing the expression of COL10A1, Runt-related transcription factor 2 (Runx2), and MMP-13, with their cargos, including miRNAs and proteins (177). Besides the natural EVs, there are emerging types of engineered EVs. Engineered EVs are designed for targeted therapy or controlled release and have better therapeutic properties, which have drawn increasing attention of researchers (34, 178, 179) (Figure 3).

4.3 EV treatment for TMJ OA

Studies on TMJ OA are relatively stagnant compared with knee OA (41). To date, few studies have demonstrated the effect of EVs on TMJ OA treatment. Based on current understanding, the mechanisms of EV therapeutic effect on TMJ OA are rather similar to OA. BMMSC-derived EVs could induce cartilage reconstruction in TMJ OA *via* the autotaxin–YAP signaling axis in chondrocytes (180). MSC-EVs activate AKT and ERK signaling in chondrocytes and then enhance the proliferation and migration of chondrocytes. The EVs can also activate AMP-activated protein kinase (AMPK) signaling, which contributes to restoring and maintaining chondrocyte matrix homeostasis (181). Moreover, MSC-EVs promote s-GAG synthesis and COL II protein expression, and suppress NO and MMP-13 production, to restore stromal homeostasis in a TMJ-OA, enhancing cartilage regeneration in chondrocytes (182).

4.4 Limitations of EV therapy in OA

EV therapy in OA still has limitations, including but not limited to the unclear mechanism, targeting and stability, and EV production. Although the engineered EVs exhibit targetable and stable properties, it is still unclear whether they may cause other problems (183). Moreover, there are difficulties in the preparation and preservation of EVs. The contamination from the EV separation process reduces the purity of EVs, and these confounding factors hamper EV usage in clinical practice (184, 185). Solving these problems is challenging, and more efforts should be devoted to EV research.

5 Conclusion and perspective

In summary, researchers have gradually realized the crucial function of EVs in OA progression. The effect of EV in OA is multiplex. EV-carried bioactive substances mediate cell-to-cell communication, and so does inflammation. Inflammation is passed and aggravated by EVs. Differed from Rheumatoid arthritis, which has a heavier inflammatory burden stimulated by immunocomplex than OA. EVs in OA cannot cause much stronger inflammatory storms, which may be attributed to their relatively low immunogenicity. The changes in EVs' mechanical properties may limit joint movement. Meanwhile, the EV-carried bioactive substance may determine the fate of FLS and chondrocytes. This evidence

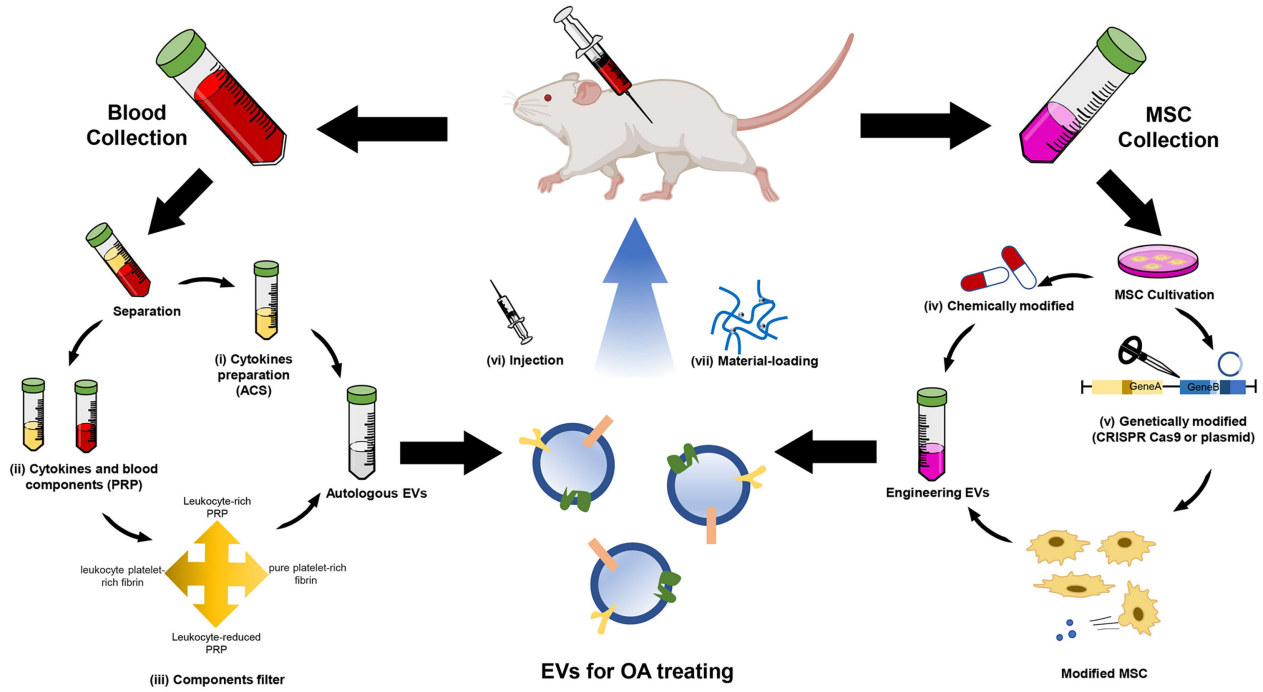

FIGURE 3
Schematic illustration of EV therapies for OA. EV therapy usually collects EVs from autologous EVs and engineering EVs. The autologous EVs are mainly derived from (i) Autologous conditioned serum (ACS) and (ii) platelet-rich plasma (PRP). The latter needs (iii) components filter and then produces four types of EV suspension. The engineering EVs are mainly derived from MSCs. Two strategies are used to modify the MSC-derived EVs, including (iv) chemical and (v) genetic modification. The modified MSC-secreted specific EVs contain miRNA and proteins in OA treatment. Autologous or engineering EVs are usually delivered by (vi) injection and (vii) material-loading.

suggests that OA is not only an inflammatory disease but also an EV-related disease.

There is no specific therapy for OA so reducing the risk factor is crucial to OA treatments. Although the joint replacement therapy succeeded in knee and hip, it was mainly for later period OA patients, and the treatment in TMJ failed. EV therapies may be a promising treatment for OA in peripheral joints and TMJ because of their low immunogenicity, low cytotoxicity, and long-term stability characteristics. Recently, the EVs from modified cells or loaded with drugs showed therapeutic effects in OA (182, 186, 187), which are attractive for this field. There are three forms of EVs, and the recent research of EV therapy mainly focused on exosomes, which showed therapeutic effects in OA treatment (188). Compared with exosomes, apoVs can be easier to obtain from tissues. It also contains more bioactive substances suitable for immunomodulation and regeneration (189, 190). Although there is currently no study in the treatment of OA with apoVs, considering its biological characteristics, we believe apoVs may be a potential candidate for OA therapy. However, the side effects of EVs are still unclear, which impels us to conduct a long-term safety assessment before the clinical transformation.

Author contributions

BY and XL wrote the initial draft. CF, WC, BM and YQ contributed to the manuscript revision and figure preparation. XK and QZ provided their conception, design and comments on the manuscript and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Review

Harnessing Tissue-derived Extracellular Vesicles for Osteoarthritis Theranostics

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Abstract

Osteoarthritis (OA) is a prevalent chronic whole-joint disease characterized by low-grade systemic inflammation, degeneration of joint-related tissues such as articular cartilage, and alteration of bone structures that can eventually lead to disability. Emerging evidence has indicated that synovium or articular cartilage-secreted extracellular vesicles (EVs) contribute to OA pathogenesis and physiology, including transporting and enhancing the production of inflammatory mediators and cartilage degrading proteinases. Bioactive components of EVs are known to play a role in OA include microRNA, long non-coding RNA, and proteins. Thus, OA tissues-derived EVs can be used in combination with advanced nanomaterial-based biosensors for the diagnostic assessment of OA progression. Alternatively, mesenchymal stem cell- or platelet-rich plasma-derived EVs (MSC-EVs or PRP-EVs) have high therapeutic value for treating OA, such as suppressing the inflammatory immune microenvironment, which is often enriched by pro-inflammatory immune cells and cytokines that reduce chondrocytes apoptosis. Moreover, those EVs can be modified or incorporated into biomaterials for enhanced targeting and prolonged retention to treat OA effectively. In this review, we explore recently reported OA-related pathological biomarkers from OA joint tissue-derived EVs and discuss the possibility of current biosensors for detecting EVs and EV-related OA biomarkers. We summarize the applications of MSC-EVs and PRP-EVs and discuss their limitations for cartilage regeneration and alleviating OA symptoms. Additionally, we identify advanced therapeutic strategies, including engineered EVs and applying biomaterials to increase the efficacy of EV-based OA therapies. Finally, we provide our perspective on the future of EV-related diagnosis and therapeutic potential for OA treatment.

Key words: Osteoarthritis, Extracellular vesicles, Controlled-release, Biomaterials, Biosensors

Introduction

Osteoarthritis (OA) is a highly aging-related disease that involves entire joint disorder and is typically associated with irregular chronic pain, which seriously affects the life quality of patients [1]. To some degree, almost all joint tissue abnormalities are involved in OA development. Articular cartilage as a pivotal part of joints plays an important role in OA progress [2]. Cartilage is a resilient, elastic, and hydrated tissue that supports and cushions the ends of long bones at joints and does not contain vascular and neural networks [3]. The primary cells found in cartilage are chondrocytes that produce extracellular matrix (ECM), including proteoglycans and collagens (Col). Cartilage can be divided into three major groups: fibrous cartilage, hyaline cartilage, and elastic cartilage in ascending order of ECM amount [4]. Hyaline cartilage is the most widespread cartilage type found in different organs such as synovial joints,

ribs, and trachea rings to tolerate bone loading and lubricate joint movement [5]. Type II collagen (Col II) accounts for 90-95% of total collagen molecules and forms filamentous structures with collagen IX (Col IX) to resist tensile, and shear stresses in hyaline cartilage. Hence, the maintenance of ECM integrity is crucial for the regular function of cartilage. Damage, degeneration, or distortion of ECM elements and composition are the main features of cartilage diseases [6]. Since chondrocytes are physically confined in lacunae and rely on diffusion to obtain nutrients due to the lack of blood supply [7], the matrix renewal process in cartilage is slow when compared to bone, and the damaged cartilage is easily susceptible to chronic diseases.

The degeneration of articular cartilage is referred to as one of the hallmarks of OA [2, 8]. Traumatic injuries, obesity, and congenital abnormalities are the clinically relevant causes of pathological conditions that undermine cartilage load-bearing capacity and lead to chronic diseases like OA [9]. Pathological conditions such as inflammation often perturb the microenvironment of cartilage ECM, resulting in dysfunction and apoptosis of chondrocytes, which further aggravates OA [10]. According to the Kellgren-Lawrence classification system (K-L score) by radiography, stages of knee osteoarthritis can range from (1) normal, (2) mild, (3) moderate, and (4) severe stages (**Figure 1A**) [11]. K-L scores 1-2 are defined as early-stage OA, and K-L scores 3-4 are late-stage OA [12]. During OA, the components of the joint tissues, including bone, joint capsule, synovial tissue, tendons, ligaments, and cartilage, fail in various ways, leading to joint instability [13]. One of the significant phenomena is that the cartilage surface progressively erodes, and joint inflammation becomes severe along with the increased stage level and is accompanied by a systemic low-grade chronic inflammation. However, the self-healing process of damaged articular cartilages is slow and limited, as mentioned previously. Hence, in the treatment of OA, articular cartilage has mainly been the focus of research. Current treatment options, including surgical (e.g., total knee replacement, TKP) and non-surgical (e.g., pharmaceutical treatment, viscosuppements) therapies, are associated with side effects and low-efficacies [14-18]. To overcome these limitations, cell-based therapies have been suggested to replace or stimulate endogenous regeneration of damaged cartilage tissue. Stem cells, including mesenchymal stem cells (MSCs) demonstrating multipotency, and embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) demonstrating pluripotency, can undergo differentiation into somatic cells of the organ that are critical to restoring and repairing injured tissues [19]. However, cell-based therapies may require operational surgery and high costs to maintain large cell numbers before the final delivery to patients. Moreover, previous studies have reported that engrafted stem cells in a diseased joint environment with inflammatory cytokines can intensify the inflammatory response and escalate disease progression [20-22]. Even though MSCs from the same tissue of origin have been characterized with strong immunomodulation and inflammatory suppression ability, they have demonstrated prodigious batch-to-batch and donor-to-donor variation that can influence MSC availability and function [23, 24]. The existence of MSC heterogeneity is potentially the reason for this variation that different subpopulations can show distinct expression profiles and functional properties from the same sample source [25-27]. Thus far, improperly purified MSCs may induce adverse immune effects upon injection to the OA site.

As an alternative, extracellular vesicles (EV) or exosomes that are produced by stem cells and contain potent cytokines, growth factors, and miRs may be powerful in mediating inflammation and enhancing progenitor cell proliferation [28]. The benefits of MSC-derived EVs in treating cardiovascular, respiratory, renal, and hepatic diseases [29-31] and cartilage regeneration are well-established [32]. EVs exhibit an increased capacity to escape degradation or clearance by the immune system [33], and MSC-derived EVs (MSC-EVs) have been shown to play substantial therapeutic roles in regulating intracellular pathways in different diseases, including inflammatory bowel disease [34, 35], neurodegenerative diseases [36, 37], and respiratory tract diseases [38, 39] or pneumonia infections related to COVID-19 [40], mainly due to their immunomodulatory effects, including suppression of inflammation.

Apart from building up effective treatment strategies, early diagnosis of OA is of pivotal importance to limit further progression of cartilage damage [41]. Therefore, investigating biomarkers of early-stage OA helps prevent the disease progression and potentially probes the initial molecular mechanisms that lead to OA initiation. In this review, we aim to introduce (1) the biological characteristics of body tissue-derived versus OA tissue-derived EVs, (2) synovial fluid-derived EVs and their OA biomarkers that can be detected by currently available biosensors, (3) therapeutic values of MSC-derived EVs for treating OA, and (4) highlight the cutting-edge technologies and discuss the current limitations of EV-based and biomaterial-based platforms toward optimizing cartilage/OA therapy.

The biological and pathophysiological characteristics of EVs

Extracellular vesicles (EVs) are membrane vesicles with diameters of 30-5000 nm secreted from various cells that communicate with each other *via* paracrine signallings [42]. The term EVs is often used as an umbrella term but can be further broken down into different terms associated with specific sizes. Apoptotic bodies are considered the largest EVs with diameters from 1000-5000 nm, extracellular microvesicles range in size from 100-1000 nm, while exosomes (also known as small EVs) are typically defined by diameters of 30-150 nm vesicles [43]. EVs can be generally separated by ultracentrifuge from body fluids/whole blood or the culture medium during cell culture. To obtain small EVs from the other EV subpopulations (e.g., apoptotic bodies), size-based separation methods such as filtration, flow field-flow fractionation, affinity-based techniques, and size-exclusion chromatography (SEC) have been adopted for the separation [44]. The studies featured in this review primarily focus on exosomes or small EVs, and we use EV(s) to describe them, according to minimal information for studies of extracellular vesicles (MISEV) recommended by the International Society for Extracellular Vesicles (ISEV) [45]. The paracrine signalling requires transferring donor (EV-secreting cells) cargo to recipient cells by exocytosis and endocytosis, respectively [42]. Specifically, EVs contain and protect useful biological information, including long non-coding RNAs (lncRNAs), messenger RNAs (mRNAs), regulatory microRNAs (miR), lipids, and proteins. Such information transportation facilitates non-contact intercellular communication, thereby regulating the behaviours of distant cells. Therefore, EVs are clinically significant for biological signal transmission and as promising natural nanocarriers for clinical application. The biogenesis and isolation procedures of EVs have been comprehensively reviewed by others and will not be discussed in detail in this review [42, 46-48].

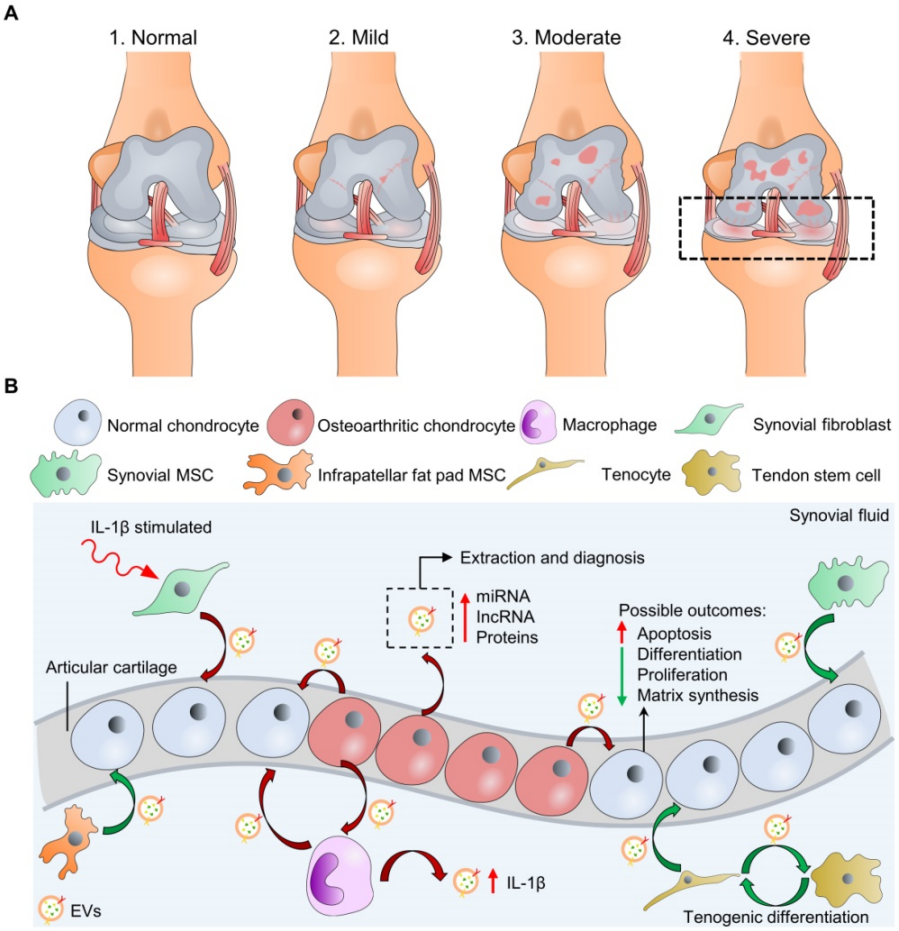

Figure 1 Macroscopic and microscopic illustration of osteoarthritis (OA). (A) Knee OA at four different stages can be evaluated by K-L scores, including "normal" at stage 1. Only cartilage degradation is shown for illustration simplicity instead of the whole-joint damages. (B) Microscopic exploration of normal and osteoarthritic chondrocytes interacting with other cell types through cell-cell communication (EV secretion) at stage 4 with possible biological outcomes. The synovial fluid-derived EVs can be extracted for OA diagnosis. Green/red curve arrows indicate cells secreting EVs with bioactive molecules that potentially are chondro-protective/chondro-destructive. Osteoarthritic chondrocytes may also secret EVs to stimulate inflammasome activation of cells in synovial space, including macrophages.

Overview of EV characteristics from cartilage and chondrocytes

EV-related materials in the pericellular cartilage matrix and growth plate cartilage have long been described in the previous literature [49, 50]. Generally, EVs bear surface markers CD9, CD63, CD81, LC3, tumour susceptibility gene 101 (TSG101), flotillin-1, and Alix, although a recent study reveals that CD63 is a more specific EV surface marker compared to others and non-CD63 bearing vesicles can be ectosomes [51]. The reported size and surface markers of human articular chondrocyte-derived EVs are similar to that of other cell-derived EVs. Although some reports highlighted that heterotrimeric G protein, HSP70 and 90, and members of the tetraspanin family such as CD9, CD63 and CD81 were not detected in the proteome of articular cartilage-derived EVs [52, 53], emerging research confirmed that CD9, CD63, and TSG101 proteins were expressed on chondrocyte-derived EVs [54-57]. Meanwhile, the content of EVs varies along with different types of tissues. In 1969, Anderson identified matrix vesicles (size from ~30 nm to 1 μm) containing hydroxyapatite and/or fluorapatite at all levels in the epiphyseal plate of calcified cartilage [58]. The matrix vesicles were later discovered to enrich in phosphatase that hydrolyzed a variety of nucleotide triphosphates, diphosphates, monophosphates, and other phosphate-containing substrates and metabolites to facilitate the precipitation of hydroxyapatite for calcification [59]. These findings demonstrated that cartilage calcification is associated with the deposition of apatite-like material, including the matrix vesicles, to bind calcium for endochondral bone development. Also, the results imply that EVs may possess a high tissue-penetration ability to diffuse deep into cartilage for delivery. Indeed, more than 1,700 proteins and mRNAs for factor XIIIA, type II transglutaminase, collagen II, aggrecan, ANKH, and GAPDH were identified in articular cartilage-derived EVs [52, 60]. The Articular cartilage-derived EVs have been shown to concentrate those enzymes (e.g., coagulation factor XIIIA and metalloproteinase), ions, and substrates necessary for mineral formation, implying that they can be considered as physiologic structures in articular cartilage [61]. Specifically, there are quantitative changes of matrix proteoglycans and TGF-β signalling pathway-related proteins in OA [62]. Nevertheless, these changes might contribute to the reactivation of ossification centres and matrix mineralization of articular cartilage, one of the hallmarks of OA [63].

Diagnostic value of EVs in OA

Recent research has illustrated the involvement of EVs in the pathological and physiological processes of OA [64]. Pathological EVs play a crucial role in inflammation and chronic pain diseases and have emerged as a potential marker in OA [65]. EV cargoes from OA pathological conditions may show distinct genomic and proteomic profiles for distinguishing pathological EVs from physiological EVs that help identify OA at an early stage. Moreover, previous studies have focused on the diagnostic significant and biological outcomes of endogenous EVs during OA (**Figure 1B**). For instance, evidence showed that OA chondrocytes (from OA patients undergoing total knee replacement, TKR) actively released EVs with enriched miR-372 lncRNA and a low level of a lncRNA, HULC (highly upregulated in liver cancer), while EVs from normal articular chondrocytes showed a reverse trend [66]. Similarly, OA chondrocytes (from OA patients undergoing TKR) produced EVs with high content of miR449a-5p to inhibit autophagy (a function that eliminates unwanted materials and suppresses inflammasome activation) and promote mature IL-1β production of macrophages [67]. This process was shown to aggravate synovitis in the destabilization of the medial meniscus (DMM) OA model in knee joints of 8-week-old male mice. Other cell types of joint tissues, including synovial fibroblasts, synovial MSCs, infrapatellar fat pad MSCs, or tenocyte/tendon stem cells, may also interact with normal/OA chondrocytes *via* releasing EVs in the synovial space. Ni et al. and Withrow et al. have summarized these interactions that can be referenced for probing OA pathogenesis [62, 68].

Several types of EV readouts, including the size, amount, and biological contents of EVs from the diseased sites, can be the representative biomarkers. Mustonen and colleagues recently showed that synovial fluid (SF) from the human knee joint with rheumatoid arthritis (RA) has a significantly higher proportion of hyaluronan (HA)-positive EVs at size range 101-200 nm but a much lower proportion of HA-positive EVs at size range >501 nm than those in OA and control groups [69]. This finding provides a valuable reference for the polydispersity of EVs size and surface bioactive moieties from different disease sites. Similarly, Xu et al. determined that SF from early-stage and late-stage OA in patients contained a higher amount of EVs than those in control groups [70]. Specifically, the expression level of lncRNA, PCGEM1, an OA-related marker (a sponge for miR-770 for stimulating the proliferation of OA synoviocytes) in EVs, was remarkedly higher in the late-stage OA group than those in the early-stage OA group, suggesting that different stages of OA can be distinguished by analyzing EV contents from SF.

Non-coding RNA is an important biologically active molecule in EVs, including miR, lncRNA, and circular non-coding RNA (circRNA) [14]. Increasing evidence proves that they play critical roles in regulating the occurrence and development of diseases [15, 16]. Proteins in EVs from the synovial fluid are another critical biomarker responsible for cell-to-cell communication in the OA microenvironment [71, 72]. These non-coding RNA and proteins are either upregulated or downregulated during the occurrence of OA or RA that deteriorates the normal functions of articular chondrocytes. Emerging evidence has suggested that these bioactive signals are transmitted *via* EVs. For instance, Liu and colleagues have demonstrated that synovial fibroblast secreted EVs containing miR-126-3p are responsible for suppressing apoptotic cell death, inflammation, and osteophyte formation in chondrocytes, and this miR expression was significantly reduced in OA patients [73]. Similarly, a lncRNA, the upregulation of PVT1 expression was shown in isolated EVs from whole blood of OA patients, and PVT1 regulated OA progression through the HMGB1/Tlr4/NF-κB signalling pathway [74]. Another study revealed that protein profiles of SF-derived EVs in RA, axial spondyloarthritis, gout, and OA patients were different [75]. For instance, haemoglobin and actin-related protein 2/3 complex subunit 3 in EVs were more abundant in the OA group compared to the other three groups. We have tabulated OA-related EV-derived biomarkers, including microRNA, lncRNA, and proteins, from the recent studies as useful references for OA detection (**Table 1**). Therefore, it is highly desirable to develop biosensors, especially with current advances in nanotechnology and biomaterials, for detecting OA-related EVs and the EV contents to probe the progression of OA [76].

Despite the biochemical contents of EVs, biophysical properties of OA-related EVs have been rarely explored. A recent report revealed the mechanical difference between non-malignant and malignant cell lines EVs by employing quantitative nanomechanical mapping atomic force microscopy (QNM AFM) [77]. The authors isolated EVs from human urothelial HCV-29 cells (non-malignant cells), human urothelial FL3 cells (malignant cells), and non-metastatic parental cell line T24 (malignant cells). Intriguingly, QNM AFM results showed that EVs derived from a non-malignant cell line (HCV-29: ~1527 MPa) were stiffer than those from malignant cell lines (FL3: ~280 MPa and T24: ~95 MPa). Similarly, malignant cell-derived EVs exhibited a higher adhesion force to the AFM tip than that of the non-malignant cell-derived EVs, suggesting an increased interaction between the tip and EV surface constituent. Consistently, the reduced stiffness in malignant cells (HCV-29 and T24)-derived EVs correlated with the reduced cell stiffness by order of magnitude that might contribute to the ability of EVs to transport across biological membranes [78]. Based on these findings, we may expect that EVs derived from osteoarthritic chondrocytes can be softer than that of normal chondrocytes for an increased tissue-infiltration property, leading to OA progression deteriorating. This postulation needs further justification.

Biosensors to detect EV-based biomarkers to monitor OA progression

Plain radiography is traditionally the gold standard for morphological assessment of OA knee with K-L score analysis of the images [12]. However, this method may only detect the cartilage change with >10% cartilage lost and cannot be able to visualize other soft tissues, including meniscus and ligaments in the joint. Magnetic resonance imaging (MRI) is one of the reliable methods to detect the damaged cartilage in OA anatomically with ~70% sensitivity and 90% specificity, compared to reference diagnosis by arthroscopy (invasive approach to observe joints) [83]. However, MRI techniques require expensive equipment, lengthy processing time and are not suitable for those patients implanted with metallic devices such as pacemakers. Also, the diagnostic standard of MRI-based OA needs further clinical validation. Thus, serological tests may provide an alternative option to detect biochemical changes in serum/SF of patients with OA/RA at the early stage [84, 85]. For molecular biomarkers in RA, anti-cyclic citrullinated peptide (CCP, plays a critical role in initiating inflammatory responses in autoimmune diseases, such as RA) is a biochemical marker for detecting early-stage RA and the reported sensitivity and specificity by serological tests were ~60% and ~90%, respectively [86]. A recent study developed an isotopic dilution analysis mass spectrometric method to analyze the concentration of citrullinated peptide (CP), anti-CCP, and 4-hydroxyproline (Hyp, a marker of bone turnover and resorption) by biochemical assay to discriminate the type of arthritis at the early stage (within five months of the onset of symptoms of inflammatory arthritis) and advanced arthritis stage in plasma/serum/synovial fluid of patients [87]. The key findings of this study show that the stage and the type of arthritis (OA or RA) can be identified by measuring the amount of CP and Hyp in serum and synovial fluid and match expression patterns to reported data from this study. Nevertheless, further research is required to investigate whether these markers can be found in EVs from OA sites.

Table 1 Biomarkers in EV-derived from the serum/SF of patients with joint arthritis.

EV-derived biomarkers	EV Source	Expression levels	Possible biological effects/reasons	Source of OA joints	Reference
microRNAs (miR)					
miR-126-3p	Human synovial fluid	Downregulation in OA patients	*In vivo*, rat SFC-derived EVs containing miR-126-3p could constrain chondrocyte inflammation and cartilage degeneration	SF from knees of OA patients undergoing TKR	[73]
miR-500b miR-720 miR-4454 miR-199b-5p miR-3154	Human synovial fibroblasts	Upregulation with IL-1β stimulation	All of them presented in IL-1β-stimulated SFB and EVs from IL-1β-stimulated SFB	Normal human knee synovial fibroblasts and chondrocytes	[111]
miR-504-3p miR-16-2-3p miR-210-5p miR-26a-5p miR-146a-5p miR-6821-5p miR-68678-3p	Human synovial fluid	Upregulation Upregulation Upregulation Downregulation Downregulation Downregulation Downregulation	miR-504-3p is the only common miR upregulated in both male and female OA patients, highly gender-specific.	Normal/OA SF was obtained from knee joints of patients undergoing arthrocentesis/ TKR	[112]
miR-372-3p	Human chondrocytes	Upregulation in OA chondrocytes	Promoted cell growth and proliferation GSK signalling pathway	Human cartilage specimens were obtained from patients undergoing TKR	[66]
miR-449a-5p	Human primary chondrocytes	Upregulation with IL-1β treatment	Inhibit ATG4B expression and autophagy in LPS-primed macrophages	Human cartilage specimens were obtained from patients undergoing TKR	[67]
miR-155-5p	Human synovial fluid	Upregulation	Potentially stimulate a positive feedback loop of TNF-α stimulated inflammation	SF was obtained from knee joints of patients (ages of 40-60) undergoing arthrocentesis	[113]
Long non-coding RNA (lncRNA)					
PVT1	Human serum	Upregulation	EV-derived PVT1 regulated OA progression by modulating the HMGB1/Tlr4/NF-κB pathway	Whole blood was extracted from 30 OA patients (ages range from 50-70 years old) and 30 healthy volunteers (ages range from 50-70 years old)	[74]
HULC	Human chondrocytes	Downregulation in OA chondrocytes	Suppressed cell growth and proliferation GSK signalling pathway	Human cartilage specimens were obtained from patients undergoing TKR	[66]
PCGEM1	Human synovial fluid	Late-stage OA > early-stage OA > Control	Distinguish the stage of OA. There was a positive relationship between EV-derived lncRNA PCGEM1 and WOMAC Index	Blood sample from the cubital vein and synovial fluid sample from knee joints: (1) 20 healthy people who suffered from incidental knee pain as a control group; (2) 20 patients with primary OA in the early stage; (3) 22 patients with primary OA in the progressive stage (late-stage)	[70]
Proteins					
Haemoglobin Actin-related protein 2/3 complex subunit 3	Human synovial fluid	Upregulation	More abundant in OA than those in RA, spondyloarthritis (axSpA), and gout	SF-derived EVs were isolated from RA, axSpA, gout, and OA patients	[75]
COL6A1 B-2glycoprotein I Complement component 5-variant Haptoglobin Alpha-1-acid glycoprotein Ceruloplasmin KIAA1466 CCDC101 PPARBP Apolipoprotein Anti-folate binding protein Anti-HER3 HRV Fab N27-VL C1QC	Human synovial fluid	Upregulation (Male) Upregulation (Male) Upregulation (Male) Upregulation (Female) Upregulation (Female) Upregulation (Female) Downregulation (Male) Downregulation (Male) Downregulation (Male) Downregulation (Female) Downregulation (Female) Downregulation (Female) Downregulation (Female) Downregulation (Female)	The upregulated or downregulated markers were gender-dependent in EV protein cargo from SF of non-OA and OA patients	Knee joint synovial fluid from both healthy and osteoarthritic knees was obtained from patients (8 non-OA females, 10 OA females, 7 non-OA male, and 7 OA male patients) undergoing arthrocentesis/total knee arthroplasty procedures.	[114]
Toll-like receptor 3 (TLR3)	Human serum	24- and the 17- to 18-kDA TLR3 showed ~6-fold higher intensity in the active RA group than in the other groups	The increased TLR3 expression in active RA patients might reflect the inflammatory conditions of fibroblast-like synoviocyte	Whole blood was extracted from 33 patients (12 with active RA, 11 with inactive RA, 10 with OA, and 10 healthy donors)	[115]

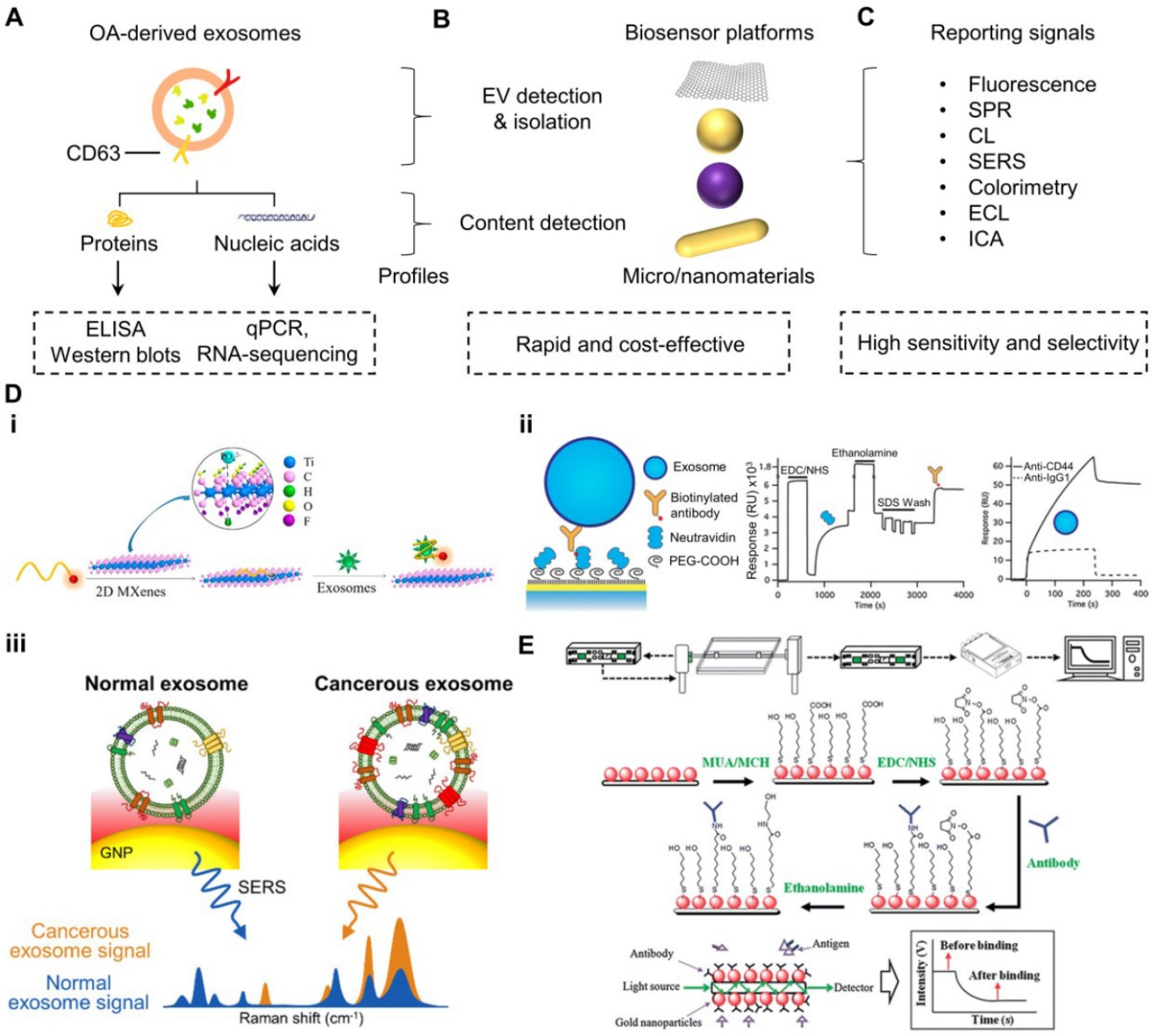

Figure 2 Overview of the process and principles of biosensors to detect EVs and OA biomarkers in EVs. (A) OA site-derived EV proteins and nucleic acids can be conventionally detected by ELISA-based and qPCR-based methods, respectively. (B) Recent advances in nanotechnology develop many rapid and cost-effective biosensors for detecting EVs and EV contents through (C) various techniques. (D) EVs can be probed by (i) fluorescence-based system on CD63-targeting Cy3-conjugated aptamer, which is initially quenched by MXene nanosheets and recovers the fluorescent signal upon binding to EVs in the solution [79]; (ii) SPR-based system with EV surface protein-specific antibodies to capture EVs that cause the evanescent surface plasmon wave at the sensor surface to generate differential optical signals for distinguishing normal or diseased EVs [80]; and (iii) SERS-based system to amplify the Raman profile of specific surface protein of EVs for distinguishing normal or diseased EVs [81]. (E) EV contents can also be detected by biosensors, such as the use of an SPR-based system consisting of specific antibodies immobilized on gold nanoparticles/optic fiber sensor to detect OA-related markers (such as TNF-α) from human knee SF [82]. The figures are reprinted and re-arranged with permission from Ref. [79-82]. Copyright American chemical society. (2018), Royal Society of Chemistry (2013), and SpringerLink. (2015).

A number of analytical assays have been employed to detect EV sizes by nanoparticle tracking analysis (NTA) and EV contents, including the mentioned biomarkers by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and RNA-sequencing (RNA-seq) for nucleic acids [88, 89], flow cytometry and magnetic bead-based isolation for EV isolation [90, 91], enzyme-linked immunosorbent assay (ELISA) and western blotting (WB) for proteins (**Figure 2A**) [92, 93]. Although these methods have been robust and highly reliable, limitations such as time-consuming operations and complicated procedures are noted [94]. Rapid and sensitive biosensors with simple handling steps are alternative and promising choices to probe OA biomarkers in EVs isolated from OA sites, such as SF.

Numerous studies applied nanomaterials typically conjugated with anti-CD63 antibody, CD63 aptamer, or EV-related surface marker capturing molecules to bind EVs and switch on particular physical or chemical signals, including fluorescence [79, 95, 96], surface-enhanced Raman scattering

(SERS) [97-99], surface plasmon resonance (SPR) [100, 101], colorimetry [102, 103], immunochromatographic assay (ICA) [104], chemiluminescence (CL) [105, 106], and electrochemiluminescence (ECL, **Figure 2B-D**). For instance, Zhan et al. developed a self-standard ratiometric fluorescence resonance energy transfer (FRET) nanoprobe, consisting of Cy3-CD63 aptamer adsorbed onto 2D MXene nanosheets (fluorescent quencher) *via* hydrogen bonds and metal chelate interactions for quantifying the EVs in the solution (**Figure 2Di**) [79]. The detecting mechanism was based on the initial "OFF" Cy3-CD63 aptamer fluorescent signal quenched by MXene, but the aptamer was specifically bound to CD63 protein of EV surfaces, thereby loosening the attachment to MXene and recovering fluorescent signal of Cy3-CD63 as "ON" state. The detection time only required 1 h. The reported limit of detection (LOD) for EVs by this platform was 1.4 x 10^3 particles mL^{-1}, which was 1000x lower than that of ELISA. This method offers a rapid and ultrasensitive approach to detect EVs. On the other hand, Shin and colleagues reported a SERS-based platform comprised of aggregated and positive charge gold nanoparticles (AuNPs) coated on a glass substrate for capturing the negative charge surface of EVs (**Figure 2Diii**) [81]. Their results showed that SERS fingerprints of the proteins on EV surfaces were intensified by the localized SPR of the substrate. Importantly, their findings demonstrated that nonsmall cell lung cancer-derived EVs dominantly expressed epidermal growth factor receptors on their surface but not on regular cell-derived EVs, resulting in unique Raman scattering profiles for cancer diagnosis. This platform can potentially be useful to distinguish OA chondro-derived EVs from the normal EVs, although limited reports suggest any surface proteomic difference between OA chondro-derived EVs and normal chondro-derived EVs. To develop an SPR biosensor specific to surface biomarkers of EVs, Grasso et al. constructed a real-time and label-free EV monitoring platform to identify the molecular profile of EVs from cultured cell lines or isolated from human biofluids (**Figure 2Dii**) [80]. This platform consisted of gold-coated sensor surfaces conjugated with antibodies specific to CD44, CD63, CD24, CD9, epithelial cell adhesion molecule (EpCAM), or human epidermal growth factor receptor 2 (HER2). The detection mechanism was based on the evanescent surface plasmon wave at the contacting dielectric region of the gold sensor surface. Thus, this biosensor quantified changes in the number of cancer cell-specific EVs from human blood at the sensor surface upon binding to the antibodies within 1 h. These studies demonstrate the importance of developing advanced biosensors to replace conventional methods that are time-consuming and multiple handling steps. Several excellent reviews have also comprehensively discussed the applications, strengths, and drawbacks of the nanomaterial-based biosensors for detecting EVs [98, 107, 108]. After those nanoplatforms identifying and isolating EVs, EV-derived biomarkers can be profiled by conventional methods.

Similarly, recent reviews have discussed various biosensors for detecting OA and RA biomarkers based on the mechanisms above for signal amplification [76, 109, 110]. For example, Huang and colleagues reported a label-free and real-time fiber-optic particle plasmon resonance sensing system, of which the fiber outer surface is coated with antibody-bearing AuNPs (**Figure 2E**) [82]. The optic fiber restricted light to pass as a multiple total internal reflection scheme that permitted a high contact chance between the light and AuNPs to enhance the SPR signal-to-noise ratio. The LODs achieved by this platform for TNF-α and metalloproteinase-3 (MMP-3) in human SF from knee joints of OA patients (12 patients) were 8.22 pg mL^{-1} (0.48 pM) and 34.3 pg mL^{-1} (1.56 pM), respectively. This platform was more sensitive and more rapid (< 10 min) than that of the conventional ELISA (cut-off LOD at ~100 pg mL^{-1} with handling time ~6 h). These findings demonstrate the possibility of probing OA biomarkers by rapid and sensitive biosensors. Point-of-care, non-invasive, and real-time biosensors can be highly attractive for clinical applications towards screening OA. However, very limited literature reports the usage of biosensors to detect the content of EVs from OA joints. Moreover, biosensors that can simultaneously probe both EV membranes and EV-associated contents in fluid samples in OA patients have been rare. We believe that biosensors with this detection ability are novel, attractive, and cost-effective without the need for EV isolation to understand the stage of OA, as OA-related markers can be concentrated in EVs.

The therapeutic value of non-bone marrow MSC-derived EVs for OA

MSC-EVs exhibit great therapeutic potential for treating OA, as previous literature shows that the paracrine factors of MSCs provoke chondrocyte proliferation [116]. The biology, preparation, characterization, and applications of MSC-EVs have been extensively discussed in previous reviews [14, 117-119]. We have enlisted several recent reports utilizing bone marrow MSC-EVs to treat OA in different animal models (**Table 2**). However, the isolation of bone marrow MSCs from other tissues requires invasive procedures that increase pain and

cost for the patients. Therefore, alternative sources for isolating EVs from other tissues, including synovial MSCs, platelet-rich plasma, infrapatellar fat pad, and umbilical cord-derived MSCs, have been emerging and showing promising tissue engineering results and treating/inhibiting OA symptoms. This section highlights and explores the recent findings of EVs derived from non-bone marrow MSC sources as potential options for treating knee OA and cartilage injuries.

Platelet-rich plasma-derived EVs

Platelet-rich plasma (PRP) is an autologous derivative of whole blood [120]. The blood can be centrifuged and separated into the following components: plasma, platelets, and leukocytes (the "buffer coat"), and erythrocytes from top to bottom layers. The preparation of PRP is generally based on its leukocyte and fibrin content ratio with four categories: (1) leukocyte-rich PRP (L-PRP); (2) leukocyte reduced PRP (P-PRP); (3) leukocyte platelet-rich fibrin; and (4) pure platelet-rich fibrin [121]. PRP is demonstrated to play critical roles in bone and soft tissue healing processes [122]. Numerous studies have reported regenerative and anti-inflammatory effects of PRP administration to the sites of advanced-stage diseases such as OA [123-125]. Mechanistic studies have revealed that activated platelets secret a high amount of growth factors (GFs) and cytokines to promote cell proliferation and inhibit the apoptosis of chondrocytes [126, 127]. This secretion can be mediated by delivering EVs that interact with chondrocytes for fusion and subsequent release of bioactive contents [128]. However, it is shown that leukocytes-containing PRP (e.g., L-PRP) can concentrate pro-inflammatory cytokines, thereby showing less effective OA treatment *in vivo* than that of P-PRP [126]. On the other hand, several leukocyte subsets, including M2 macrophages showing anti-inflammatory may initiate tissue repair and suppress fibrosis [129]. Thus, the inclusion of leukocytes in PRP formulations is debatable. Leukocytes can be removed from PRP to avoid complications.

Table 2. *In vivo* efficacy of MSC-EVs in animal cartilage/osteochondral models.

EV source	Dose/volume	Animal type	Disease model	Molecular mechanisms	Biological Outcomes	Reference
IPFP-MSCs*	10 µl 10^{10} particles mL^{-1}	Mice	Surgical destabilization of the medial meniscus (DMM)	Inhibition of mTOR	Prevent the cartilage destruction and partially improve the gait abnormality in the DMM mice model	[134]
human embryonic stem cell-derived MSCs	100 µg EVs per 100 µl of injection	Rat	Osteochondral defect model created on the trochlear grooves of the distal femurs	CD73-mediated adenosine activation of AKT and ERK signallings.	Improve the surface regularity and integration with host cartilages, improve the quality of osteochondral repair	[163]
hBMMSCs	15 µl 500 µg mL^{-1} in PBS	Mice	Collagenase VII-induced OA model	MiR-92a-3p directly targets the 3′-UTR of WNT5A mRNA	Inhibit the progression of early OA, prevent the damage to knee articular cartilage	[164]
mBMMSCs	250 ng per 5 µL	Mice	Collagenase VII-induced arthritis model	Re-induce the expression of chondrocyte markers while inhibiting catabolic and inflammatory markers	Higher bone volume (BV/TV parameter); less bone degradation	[165]
hBMMSCs	100 µL of 10^{11} particles mL^{-1}	Mice	Mouse model of traumatic OA in a mechanical test device	miR-136-5p target ELF3, downregulate its expression	Higher expression of collagen II and aggrecan inhibits early post-traumatic OA and prevents further damages to the knee cartilage.	[166]
mBMMSCs*	200 µg of EVs 200 in µl PBS	Mice	Lumbar facet joint (LFJ) osteoarthritis model	Suppressing RANKL-RANK-TRAF6 Signalling Pathway	Attenuate the articular Cartilage degeneration, promote cartilage and subchondral bone remodeling	[167]
hBMMSCs*	250 ng per 5 µL	Rat	left knee joints of the rats were opened to expose the joints, followed by skin suture	miR-26a-5p specifically target PTGS2	Alleviate synovial tissue proliferation, reduced inflammatory cells, and attenuated pathological changes of synovial tissues	[168]
Synovial MSCs	100 µL; 10^{11} EV particles mL^{-1}	Rat	Transecting the medial collateral ligament and the medial meniscus completely	YAP activation via the alternative Wnt signalling pathway	Slow the progression of early OA and prevented severe damage to knee articular cartilage	[135]
UCMSCs	100 µL; 1 mg mL^{-1}	Rat	A drill bit (1.5 mm diameter) was used to make cartilage defects on the distal femurs	UCMSC-EVs contain high content of lncRNA H19	Promote chondrocyte proliferation and matrix synthesis and inhibit apoptosis in vitro; promote cartilage repair *in vivo*	[148]

*represent the EVs are collected from modified MSCs. Abbreviations: murine bone marrow mesenchymal cells (mBMMSCs); infrapatellar fat pad (IPFP) MSCs; human bone MSC (hBMMSC); umbilical cord mesenchymal stem cells (UCMSCs).

A recent study demonstrated that activated PRP upregulated the expression of platelet-derived growth factor-AB (PDGF-AB), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF). These factors were secreted in PRP-EVs to promote cell proliferation (with reduced apoptosis) and cartilaginous matrix secretion *via* suppressing the Wnt/β-catenin signal pathway in interleukin-1β (IL-1β)-stimulated chondrocytes, which were harvested from the terminal of tibia and femur of 4-week-old New Zealand white rabbits [130]. Notably, this study reported intriguing findings that the accumulation of β-catenin and Wnt5a increased IL-1β-induced osteoarthritic chondrocytes but could be reversed by the presence of PRP-derived EVs (PRP-EVs) or activated PRP. Importantly, the PRP-EVs also reduced the expression level of tumour necrosis factor-α (TNF-α), a pro-inflammatory mediator of OA. The authors further showed that intra-articularly injected PRP-EVs induced more cartilage repair and OA inhibition than activated PRPs alone in a rat OA model (6-7 weeks post-surgery), which was created by cleavage of the medial collateral ligament and the anterior cruciate ligament with the excised medial meniscus in the left knee of the rabbits. This study demonstrates a novel strategy to utilize PRP as an EV source to treat damaged cartilage.

Infrapatellar fat pad MSC-derived EVs

Human infrapatellar fat pad (IPFP)-derived MSCs can generally be obtained by a single arthroscopy during knee arthroplasties [131]. Previous literature has reported that the three-dimensional co-culture of IPFP MSCs with articular chondrocytes (ACs) in the same cell number ratio promotes chondrogenic outcomes and prevents the inflammatory status of ACs and hypertrophic differentiation of MSCs [132]. For example, their results showed that the concentration of the secreted 1L-1β and MMP-13 declined during the co-culture, especially with the presence of chitosan/hyaluronic acid nanoparticles (NPs). However, a previous study illustrated that IPFP-derived MSCs or synovial fluid-derived MSCs expressed human leukocyte antigen-DR (HLA-DR) under interferon-gamma (IFN-γ) stimulation, which was particularly enriched in the OA microenvironment. This HLA-DR interacts with T-cells *via* MHC class II molecules that potentially trigger an alloresponse, rejecting foreign transplanted cells [131, 133]. Large-scale allogeneic therapies require a large cell number limited to IPFP-MSCs isolation from the donor and hence hampers the practical application of IPFP-MSCs for OA therapy.

Recently, Wu et al. reported that EVs derived from IPFP (IPFP-EVs) contained abundant miR-100-5p, as evident by RNA-sequencing (RNA-seq) [134]. Their findings showed that the injection of IPFP-derived EVs into the articular space of the DMM mouse model alleviated OA severity with low OARSI (Osteoarthritis Research Society International) grade compared to the PBS (phosphate buffer saline) control group, inhibited cell apoptosis, enhanced matrix synthesis (e.g., Col II), and reduced the expression of catabolic factors (e.g., MMP13) *in vitro* and *in vivo*. Moreover, their mechanistic study showed that miR-100-5p from IPFP-EVs was able to bind to the 3'-untranslated region (3'UTR) of mTOR that inhibited the autophagy activity signalling pathway, such as the expression of ADAMTS5 and MMP13 that are responsible for OA progression. Therefore, IPFP-EVs may provide an alternative therapeutic source for treating OA.

Synovial MSC-derived EVs

Synovial MSCs (SMSCs) have a remarkable proliferative and chondrogenic potential for cartilage repair [135]. SMSCs can be simply isolated from the synovium of human knee joints by fluorescence-activated cell sorting (FACS). It has been shown that the injured joint and OA knee induces the mobilization of MSCs into the synovial space [136]. The possible source of SMSCs is the synovium, although no direct evidence shows this process. The therapeutic value of SMSC-derived EVs (SMSC-EVs) has been frequently explored [137]. For instance, Lian and colleagues have demonstrated SMSC-EVs containing miR-31 that play inhibitory roles in the regulation of lysine demethylase 2A (KDM2A), which associates with the demethylation of histone H3 at lysine 4 (H3K4) at the secreted frizzled-related protein 2 (SFRP2) [138]. SFRP2 is shown to inhibit osteogenesis and induce the occurrence of OA [139]. Critically, their findings denoted that KDM2A suppressed proliferation and migration of articular chondrocytes through binding to the transcription factor E2F transcription factor 1 (E2F1), which promoted the expression of pituitary tumour transforming gene 1. Therefore, the delivery of SMSC-EVs into the OA model reduced cartilage damage with downregulation of IL-1β, IL-6, and TNF-α expression and activated E2F1/PTTG1 axis to prevent the occurrence of knee OA. This report has shed light on SMSCs-EVs mediated signalling pathways for treating OA.

To further enhance chondrocyte ECM secretion *via* SMSC-EV delivery, Wang et al. overexpressed SMSCs with miR-155-5p and harvested their EVs (SMSCs-155-5p-EVs) as the miR-155-5p enriched

cargoes [140]. This study illustrated that SMSCs-155-5p-EVs exerted an inhibitory effect on Runx2 expression and an elevation effect on the expression of ColII (collagen II) and SOX9 in the stimulated chondrocytes and OA model of BALB/C mice. A similar study showed that the overexpressed miR-140-5p in SMSCs-EVs targeted ras-related protein (RalA) to promote SOX9 and aggrecan translation [135]. These results imply that the delivery of EVs from the cells with overexpressing genes critical for miR biogenesis and processing can assist the activation of multiple signalling pathways to alleviate OA symptoms and promote cartilage repair [141]. Nevertheless, the limited number of mobilized SMCSs present in the synovial fluid restricts the contribution to repairing diseased injuries by natural processes [136] and hence leads to a low yield of EVs that refrains their translation research.

Umbilical cord tissue-derived EVs

Human bone marrow-derived MSCs (hBMMSCs) are the most commonly used MSCs for research and clinical purposes. However, there are some limitations of hBMMSCs. For example, the relative number of hBMMSCs in the marrow and their differentiation potential decreases significantly with the age of donors [142]. Also, the isolation procedure is painful and invasive that may cause complications and morbidity to donors [143]. Recently, umbilical cord tissue-derived mesenchymal stromal cells (UCMSCs) have been an emerging MSC source that overcomes these limitations, as the harvesting procedure is not invasive or painful and does not involve donor site morbidity, according to the isolation instructions (enzyme digestion or explant culture method) from Wharton's jelly umbilical cords [144]. Also, it is reported that the primary UCMSCs can be expanded ~300 times of the original cell number for more than seven passages without the loss of differentiation potential [145], thereby lowering the cost for yielding the same amount of EVs from hBMMSCs. Several studies also demonstrate the excellent potential of UCMSCs to differentiate into chondrogenic lineage for cartilage tissue engineering [146, 147]. Based on the previous descriptions of the advantages of umbilical cord-derived EVs over adopting MSCs alone, recent research has demonstrated the application of UCMSC-derived EVs (UCMSC-EVs) for osteochondral regeneration and joint arthritis.

Yan et al. have shown that a rotary cell culture system creates microgravity for the 3D culture of UCMSCs by cell aggregation [148]. Their findings illustrated that such 3D culture yielded more UCMSC-EVs than those in 2D culture. More importantly, the EVs contained a high level of lncRNA H19, a highly conserved sequence with ~2.3 kb in length to play an important role in the osteochondral activity, stem cell differentiation, and embryonic growth. In this study, the authors demonstrated that UCMSC-EVs promoted chondrocyte proliferation and matrix synthesis (e.g., collagen II and aggrecan) and inhibited apoptosis *in vitro* with IL-1β stimulation (osteoarthritic chondrocyte model). Also, they employed a rat cartilage defect model with a drill bit to assess the efficacy of UCMSC-EVs treating damaged cartilage tissues in vivo. Their results further showed that UCMSC-EVs treated model exhibited the highest score by International Cartilage Repair Society (ICRS) macroscopic assessment and the highest pain persistent level, compared to those in the PBS control group and H19 silencing UCMSC-EVs group. A similar study was reported by the same group employing a hollow-fiber bioreactor (cylindrical fibers) to simulate 3D culture for enhancing the production of UCMSC-EVs [149]. Thus, UCMSC-EVs show great potential for cartilage defects other than conventional BMMSCs.

Limitations

Although non-MSC sources can be considered as alternative options for harvesting regenerative EVs, several limitations arising from the cell source and methodology of EV isolation may restrict the application of these sources. For instance, the volume of autologous concentrates of PRP is limited, and the protocol of harvesting PRP lacks reproducibility as the separation methods may not be standardized [150]. Other confounding factors include donor variability, storage conditions, or the use of external activators, including calcium and thrombin can limit the clinical use [151-153]. Besides, it is reported that EV isolation methodology can influence the biological effects of PRP-EVs and their clinical translation. Specifically, lipoproteins usually accompany PRP-EV during the isolation process (e.g., ultracentrifugation as the traditional method) and hence decrease the PRP-EV purity as lipoproteins may impose pro-inflammatory effectors that cause undesirable effects [154]. In fact, other challenges to harvesting tissue-derived EVs are those EV-associated contaminants, such as high abundances of serum proteins, including globulins and albumins, and also non-EV lipid particles such as chylomicrons can influence particle counts and biomarker analysis [155]. These lipoproteins of different subpopulations (e.g., very low density, low density, small low density, and high-density lipoprotein) share sizes similar to those of EVs [44]. In terms of content, high-density

lipoproteins are shown containing miRs, which can disturb the nucleic acid profile of EVs [156]. Thus, alternative EV separation approaches such as density gradient centrifugation, SEC, and polymer-based precipitation, with each varying in yield of EVs, the depletion of lipoproteins and protein contaminants, labour-intensity, and cost of the procedure, have been adopted to improve purity ratios and yields of EVs [157]. Nevertheless, further study is required to investigate whether EVs from new isolation approaches remain the same therapeutic properties as the traditional one *in vivo*.

Engineering advanced therapeutic strategies in treating OA and cartilage injury

Injection of specific EVs alone into the articular space may not be optimal towards potential therapeutic outcomes due to low half-lives (<6 hours) *in vivo* [158]. Also, the cellular uptake of EVs through several pathways may not be specific to articular chondrocytes. Hence, immune cells can easily uptake the injected EVs, such as dendritic cells (DCs) and macrophages, and other cell types in the microenvironment [159]. Although several methods (e.g., 3D culture) have been adopted to enhance the yield of cell-secreted EVs for systematic administration, these limitations are inevitable [160-162]. To overcome these barriers, recent research has developed 3D biomaterials/scaffolds for EV retention and to achieve local sustained release. Besides, EVs can be engineered to enhance cell-targeting delivery through employing surface modifications or coupling with nanomaterials. Alternatively, donor cells, such as MSCs, can be pre-conditioned to improve the regenerative potential of the donor cells-derived EVs. This section highlights the advanced strategies that combine molecular biology, biomaterials, and/or nanotechnology to optimize the OA therapy/cartilage repair capacity.

Engineered EVs as therapeutic agents for joint arthritis

Recent research has attempted to incorporate therapeutic agents into EVs or EVs mimetics through passive or active loading. Passive encapsulation only requires a simple incubation of EVs with desired drugs or cells that can secrete desired drugs spontaneously through hydrophobic interactions [169]. However, this approach is low efficiency for loading drugs. Alternatively, active loading involves physical, chemical, and genetic/biological engineering to modify/insert EV contents or surface proteins of EVs (**Figure 3A**). Subsequently, the engineered EVs can carry an elevated level of specific contents or cell-targeting ligands to enhance the therapeutic effects and also insert imaging molecules for long-term tracking purposes. Several reviews have comprehensively described the details of these engineering strategies for targeted drug delivery [42, 170]. Nevertheless, many studies have overwhelmingly paid efforts to modify EVs for tumour therapy, probably because of the urgent need to tackle the fast-growing and high lethal rate of cancer in patients [171]. The engineered EVs for tumour therapy were also conjugated with targeting ligands and contained a high content of bioactive molecules such as miR, mRNA, and proteins that were shown to suppress tumour cell growth or trigger T-cell response to attack specific tumour cells [172]. Also, chemical drugs were loaded into EVs for chemotherapy [173]. We expect that more studies will focus on the emerging role of EVs in repairing joint-related diseases in the near future. In this session, we mainly explore a few examples of modified EVs for RA/OA.

As the infiltration of inflammatory cells plays a vital role in cartilage destruction and bone erosion, the modulation of the immuno-environment may help reverse OA progression [174]. Conventional methods adopt suppressing the inflammation process, including inhibiting inflammatory cytokines and depletion of M1 macrophages [175]. Several reports have employed nanomaterials to re-polarize macrophages from M1 to M2 [176-179], but limited studies of this field focus on OA treatment (**Figure 3B**). Recent approaches realize the conversion of dominant phenotype from M1 to M2 to foster a restorative environment in RA [174]. He et al. reported the promotion of M2 macrophage polarization by jaw bone marrow MSC (jBMMSCs)-derived EVs (jBMMSC-EVs) through the EV content, miR-223 targeting pknox1. Likewise, Cui et al. also showed that jBMMSC-EVs reduced expression levels of the pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α but increased IL-10 in synovial fluid for M2 macrophage generation [180]. To improve the biodistribution half-lives of EVs for the immunomodulation approach, Park and colleagues developed metabolically engineered adipose-derived stem cell EVs (ADMSC-EVs) to target activated macrophages [181]. The authors conjugated dextran sulfate to ADMSC-EVs by bio-orthogonal copper-free click chemistry to target the scavenger receptor class A (SR-A) of activated macrophages. The EVs were isolated at a high yield using tangential flow filtration. More importantly, the intravenously injected ADMSC-EVs effectively accumulated in the inflamed joints of mice with collagen-induced arthritis and at higher levels than bare EVs, thereby reducing the

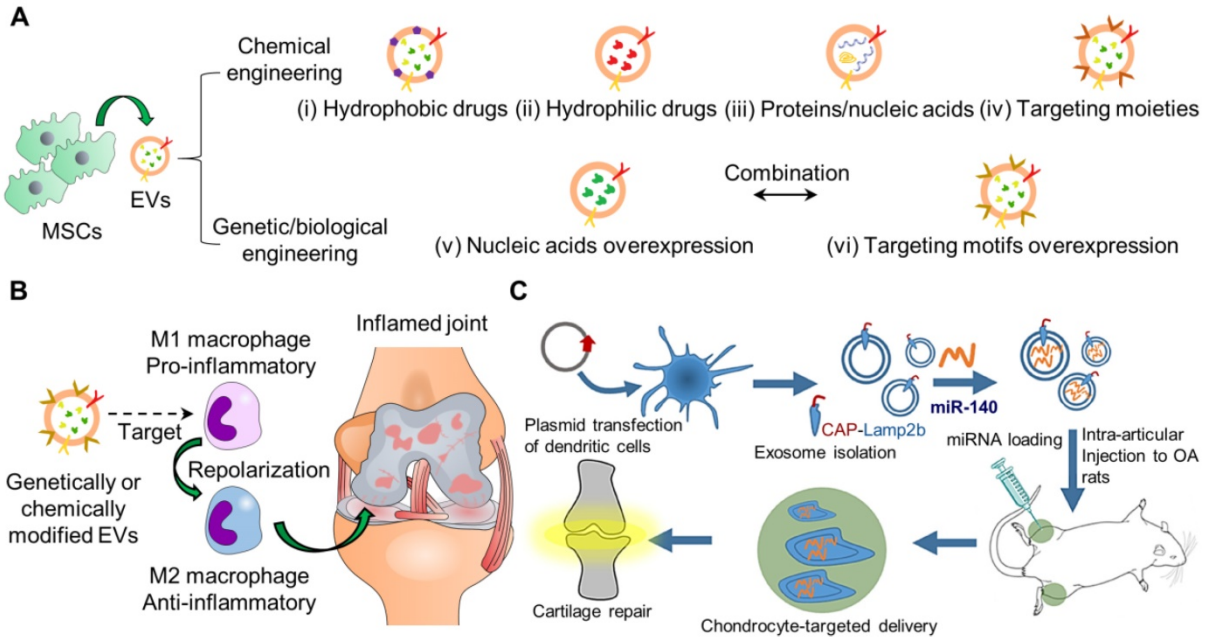

Figure 3 Schematic representation of engineering EVs for cargo delivery by various types of strategies for OA. (A) Chemical engineering strategies for the incorporation of (i) hydrophobic drugs, (ii) hydrophilic drugs, (iii) proteins/nucleic acids, and (iv) targeting moieties into EVs. Genetic/biological engineering strategies for (v) nucleic acids and (vi) targeting motifs overexpression in EVs. Multiple strategies may be combined to optimize therapeutic efficacy. (B) Genetically/chemically engineered MSCs-derived EVs can be used for targeting and repolarizing activated macrophages from M1 (pro-inflammatory) to M2 (anti-inflammatory) to treat OA as the recent novel therapeutic approach. (C) Genetically engineering synovial fluid MSCs-derived EVs to target chondrocytes for OA [184]. The figures are reprinted with permission from Ref. [184]. Copyright American chemical society (2020).

administrative dose 10 times for reprogramming of macrophages from M1 to M2. Thus far, engineering EVs for targeting and modifying the dominant phenotype of immune cells is promising for OA therapeutics.

Activated macrophages also express folic acid (FA) receptors (FRs), especially FRβ, on the membrane surface. Thus, FRβ can be a target to mediate cellular uptake [182]. Yan et al. reported a platform of RAW 264.7-derived EVs coupled with FA-bearing polyethylene glycol (PEG)-cholesterol (Chol) to encapsulate and control the delivery of dexamethasone (Dex), which is the most frequently used glucocorticoids to treat RA in the clinic. Dex can downregulate pro-inflammatory cytokines of macrophages [183]. The incorporation of Dex into EVs was achieved by electroporation. The modified EVs showed prolonged circulation and enhanced RA therapeutic efficacy compared to synthetic liposomes counterparts.

Similarly, EVs can be engineered to target chondrocytes. Xia and colleagues recently demonstrated a chondrocyte-targeted miR-140 delivery platform based on EVs (**Figure 3C**) [184]. The authors transfected dendritic cells with plasmids to overexpress chondrocyte-affinity peptide (CAP) with lysosome-associated membrane glycoprotein 2b (Lamp2b), utilized for EV isolation. The authors incorporated miR-140 into the isolated EVs by electroporation. Chondrocytes effectively endocytosed the EVs with increased intracellular miR-140 levels and decreased expression levels of MMP-14 and ADAMTS-5, the markers inhibiting the metabolic balance of chondrocyte matrix *in vitro*. Moreover, the intra-articularly injected EVs remained around the cartilage tissues within the DMM model ~1-fold more than the non-tagged EVs within 24 or 48 h. These findings demonstrate the possibility of genetically modified EV surfaces for prolonged retention in wound sites [185].

CRISPR/Cas9 system has been a promising and powerful gene therapy tool by genome editing [186]. It opens new avenues and possibilities for treating OA. Numerous literature reports the nullification or upregulation of different molecular targets of the host MSCs, chondrocytes, or the neighbouring osteoblasts by the CRISPR/Cas9 system to alleviate OA severity [187-194]. To tackle with inflammation microenvironment inhibiting cartilage formation, Brinchmann and colleagues knocked out the IL-1β receptor (IL1R1) of human articular chondrocytes and rendered the cells not amenable to secret inflammatory cytokines under IL-1β stimulation [195]. Thus, the gene-edited cells can be re-injected into the OA site for improved therapeutic effects. To enable *in vivo* gene editing, effective delivery of the CRISPR/Cas9 system to the target cells is necessary, especially utilizing EVs. However, incorporating large nucleic acids (e.g.,

CRISPR/Cas9 vectors) into EVs/exosomes is challenging. Lin et al. have developed exosome–liposome hybrid nanoparticles through merging CRISPR/Cas9 expression vectors-bearing liposomes with exosomes at 37 °C overnight to achieve membrane fusion [196]. Their results demonstrated that mBMMSCs successfully endocytosed the hybrid nanoparticles to express the CRISPR/Cas9 system for knocking out Runx2 expression. Potentially, the surface of the hybrid nanoparticles can be modified with receptor ligands for targeting specific cell types, such as articular chondrocytes and macrophages *in vivo*.

Besides the mentioned EV modification methods, MSCs can also be primed/pre-conditioned to produce EVs with desirable miR profile/contents useful for OA treatment [197]. For instance, curcumin is a natural polyphenol compound derived from turmeric and shows anti-osteoarthritic and anti-inflammatory effects [198]. However, several limitations such as low stability, hydrophobicity, and fast systemic elimination restrict its bioavailability. Li et al. have reported a strategy that curcumin-treated hBMMSCs can secret curcumin-containing EVs (Cur-EVs), which can be further harvested for the treatment [199]. Their results demonstrated that Cur-EVs were able to upregulate the expression of has-miR-126-3p in IL-1β-stimulated primary human articular chondrocytes with promoted viability, reduced apoptosis, and reduced phosphorylation of components of pro-inflammatory signalling pathways. The authors concluded that upregulated has-miR-126-3p suppressed the pro-inflammatory signallings by MAPK, NF-κB, and PI3K/Akt, which controlled the pathways participating in the progression of OA. These findings prove the anabolic effects of Cur-EVs derived from curcumin-treated hBMMSCs on OA. Similarly, Rong and colleagues reported that hypoxia (hypoxia-inducible factor-1α)-treated BMMSCs released EVs with a high expression level of miR-216a-5p to promote anabolism, migration, proliferation, and apoptosis inhibition of IL-1β-induced rat joints-derived chondrocytes *via* suppressing JAK2/STAT3 pathway, which was shown to play a pathological role in OA [200]. Their findings also showed that intra-articular injection of the hypoxic hBMMSCs-derived EVs effectively attenuated the cartilage degeneration in the DMM-triggered OA model. Therefore, rather than performing sophisticated engineering methods, the applications of EVs derived from those preconditioned/primed MSCs may provide a simple route to obtain a promising drug delivery vehicle of useful contents for the treatment of OA.

Biomaterials for EV retention and delivery

The therapeutic efficacy of EVs is directly related to the exposure time of EVs to targeted cells and surface receptors. Similar to the challenges with cell or soluble factor delivery, EV delivered intravenously, intraperitoneally, or *via* subcutaneous injection are rapidly cleared *in situ* by circulating innate immune cells and subsequently require repeated administration to obtain their desired effect [201]. Biomaterials have made a significant impact on facilitating the local and sustained delivery of therapeutic agents over the last 60 years (extensively reviewed in [202]), and have become a promising and attractive approach for mitigating the clinical barriers of EV translation *in vivo* [203].

Extracellular matrix-derived natural biopolymers and proteins found in native tissues such as hyaluronic acid, sulfated glycosaminoglycans, collagen, and fibronectin contain motifs have been exploited to retain EVs *via* binding or affinity-based mechanisms locally [204]. Previous work has shown that MSC-EVs bind to collagen and fibronectin *via* integrins and to hyaluronic acid *via* CD44 interacting with hyaluronan [73, 205]. While natural polymers have unique biomimicry and bioactivity, one of the main disadvantages to their use is that raw materials may have significant innate variability depending on tissue source/origin [206]. On the other hand, synthetic polymers tend to have less raw material variability related to supply availability or batch-to-batch consistency, are associated with lower costs, but lack inherent composition/structure to interact with cells as natural polymers do [207]. Synthetic and naturally-derived polymers can be chemically modified to facilitate material tunability with respect to architecture (shape, size, pore/mesh size, topography), degradability, bioactivity, biocompatibility, and mechanical behavior [208-210]. Each of these parameters plays a significant role in the therapeutic administration of the biomaterial (i.e., implant vs. injectable), is specific to the targeted tissue/injury site, and will subsequently impact EV delivery.

Biomaterials of various forms, including hydrogels, sponges, membranes/matrices, scaffolds, and decellularized tissues, have been used to incorporate and deliver EVs. Most commonly used biomaterials for EV release are hydrogels and ECM-based scaffolds/matrices; hydrogels are (synthetic or natural) polymer networks swollen in water with very well-established and diverse fabrication techniques, chemical modifications, drug delivery release/diffusion kinetics, while ECM-based materials (e.g., decellularized tissues, matrix fibers) retain native architectures and chemical compositions

and also have a variety of well-established fabrication methods to produce complex 3D structures and drug-loading. Natural or synthetic polymers can be combined to generate hybrid or composite biomaterials with more tunable functionality with respect to fabrication, physical or mechanical properties, or the addition of therapeutics. Chemical modifications of biopolymers for hydrogel formulation have been extensively reviewed [211]. Biopolymer modifications used for EV retention and delivery for cartilage tissue engineering applications, including the incorporation of biopolymers and carbodiimide crosslinking [212, 213], photocrosslinkable methacrylamide-modified gelatin (GelMA) [214, 215], modifications to support thiol-ene reactive Michael addition crosslinking [216], dynamic covalent crosslinking via reversible Diels-Alder reactions [217], and synthetic thermosensitive triblock copolymers (**Figure 4**) [218]. The use of various kinds of biomaterials for EV delivery for tissue regeneration in several applications has been thoroughly reviewed [203, 219-224]. In this section, we will provide a brief overview of ECM-based scaffolds and hydrogels that have been used for EV delivery, including the rationale for their use, along with a description of form and function with respect to therapeutic delivery in this session.

EVs-biomaterials for cartilage tissue engineering or treatment of OA

While there are numerous studies (and reviews) of EV delivery *via* biomaterials for bone regeneration [225-227], cardiac tissue remodeling following myocardial infarction [228], and traumatic brain injury [229], there are only a small number of studies that have specifically investigated EV delivery from biomaterials for cartilage tissue regeneration or treatment of osteoarthritis. A few major themes emerge when examining these studies collectively. Biomaterials are primarily fabricated in scaffold or hydrogel form, are derived from natural polymers such hyaluronic acid or gelatin (one exception where a synthetic triblock copolymer hydrogel was investigated), and are applied within animal models surgically as an implant or *via* intra-articular injection. Additionally, a full-thickness osteochondral defect in New Zealand rabbits is the most heavily used animal model to assess treatment efficacy for cartilage regeneration, followed by an osteoarthritis model transecting the anterior cruciate in combination with a medial meniscectomy in Sprague-Dawley rats. Lastly, all studies EVs derived from human cells (primary or cell line) plated on tissue culture plastic used ultracentrifugation for isolation, collection, and a combination of nanoparticle tracking analysis or dynamic light scattering, transmission electron microscopy (TEM), and western blotting or flow cytometry for EV characterization prior to use *in vivo*. Experimental details, including EV source, isolation technique, biomaterials, *in vivo* models, and outcomes, are summarized in **Table 3**.

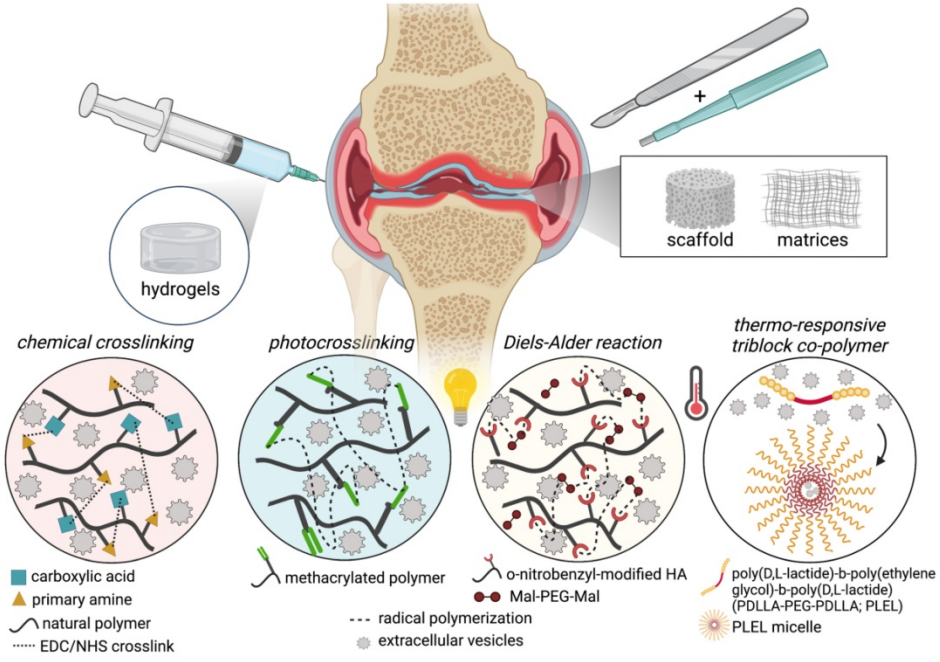

Figure 4 Summary of EV-biomaterials for cartilage tissue engineering or treatment of OA. Biomaterials for OA include implanted biomaterials and intra-articularly injected biomaterials into joints induced with OA (e.g., DMM or osteochondral defects). Biomaterials are generated from natural polymers/materials such as decellularized cartilage tissue, hyaluronic acid, gelatin, collagen, chitosan, nanoclay, or even synthetic polymers, including PEG. Studies feature various strategies to modulate biomaterial tunability and EV retention and releases, such as chemical crosslinking, chemical modification of natural polymers to enable photocrosslinking or thermoreversibility, and even synthetic thermoresponsive triblock copolymer that forms micelles at room temperature.

Table 3. Scaffold/Matrix and hydrogel biomaterials for EV/exosome delivery for cartilage tissue engineering or treatment of OA

EV Source + Isolation Method	Biomaterials	*In vivo* EV dose/volume	*In vivo* model/ Species	*In vivo* timepoints	*In vivo* outcomes	Ref.
Scaffold/Matrix Biomaterials (Implanted)						
Bone marrow derived MSCs + tissue culture plastic (TCP) 50-60% confluence + 1hr 100k xg ultracentrifugation (UC)	3D printed decellularized porcine cartilage/GelMA scaffold	200 µg in 200 µL (PBS Control); 200 µg/mL hydrogel	Osteochondral defect in patellar groove; 4mm diameter x 4mm deep; Rabbit	6 and 12 weeks	EV/hydrogel significantly increased ICRS macroscopic scores, COL2A1 expression, and decreased MMP13 expression after the 6 and 12 weeks compared to all controls.	[214]
Passage (p) 3-5 human umbilical cord Wharton's Jelly MSCs + TCP (60% confluence) + 2hr, 100k xg UC	Freeze-dried decellularized porcine cartilage ECM	25 µg/mL, supplementary EV-only injection once every 7 days for a total of 5 injections	Osteochondral defect in femoral trochlea; 3.5 mm diameter x 1.5 mm deep; Rabbit	12 and 24 weeks	EVs enhanced the effect of the scaffold and promoted osteochondral regeneration; EVs promoted the polarization of macrophages toward the M2 phenotype and inhibited the inflammatory response *in vivo*.	[212]
Hydrogel Biomaterials (Injected)						
Immortalized E1-MYC 16.3 human embryonic stem cell-derived MSCs Size-fractionated, concentrated 50× by tangential flow filtration (100kDa MWCO)	Hyaluronic acid hydrogel solution	200 µg of EVs in 1 mL intra-articular injection days 7 and 14 after wound closure	Osteochondral defect in femoral trochlear grooves; 4.5 mm diameter x 1.5 mm depth; Rabbit	6 and 12 weeks	The combination of MSC-EVs and HA *via* intra-articular injections (immediately post-surgery and after 7 and 14 days) promoted enhanced functional cartilage repair compared with HA alone.	[230]
Human articular chondrocytes + TCP + 2hr, 100k xg UC	Chitosan–hyaluronic acid hydrogel	30 µg EV + 1.5^6 MSCs + 100 µl hydrogel	Osteochondral defect in patellar groove; 4 mm diameter x 3 mm depth; Rabbit	4 and 24 weeks	EDC/NHS cross-linked CS–HA/EV/MSC, and CS-HA/MSC hydrogel enhanced cartilage repair compared to EV/MSC or CS-HA controls via MRI and histological analysis.	[213]
P4 human iPSC-MSCs + TCP (80% confluency) + 2hr, 100k xg UC	*o*-nitrobenzyl alcohol-modified hyaluronic acid and gelatin	1^{11} EVs/ml, 20 uL	Osteochondral defect in patellar groove; 4 mm diameter x 3 mm depth; Rabbit	12 weeks	Increased defect regeneration and well-organized articular cartilage structure in the EV/hydrogel group compared to gel alone and EVs alone.	[216]
Human umbilical cord MSCs + TCP + 70min, 100k xg UC	GelMA and nanoclay composite	1^9 EVs/mL, volume not specified	Osteochondral defect; 2.5 mm diameter x 1.5 mm depth; Rat	12 weeks	EV delivery increased collagen II stainings compared to controls *in vivo*.	[215]
p5-10 human iPSC-line C1P33 + TCP + 70min, 100k xg UC	Diels-Alder crosslinked hyaluronic acid/PEG (DAHP) hydrogel	1^{10} EVs/mL (100 uL), supplemental intra-articular treatment; Multi-*treatment* group received injections on 7, 14, 21, 28 days or a single injection at 7 days after surgery	OA model; transection of the anterior cruciate ligament in combination with partial medial meniscectomy; Rat	35 days	DAHP hydrogel improved the bioavailability and therapeutic efficacy of MSC-EVs for OA - with the lowest OARSI score following *in vivo* study.	[217]
Human synovial membrane stem cells + TCP (50-60% confluency) + 30% sucrose/D_2O cushion + UC, 1hr 100k xg	Thermoresponsive triblock PDLLA-PEG-PDLLA hydrogel (PLEL)	1^{11} EV/mL (200 µL) + 800 µL of hydrogel solution; Intra-articular injection performed every four weeks after surgery	OA model; transection of the medial collateral ligament, medial meniscus, and anterior cruciate ligament; Rat	24 weeks	PLEL@circRNA3503-OE-sEVs limited OA progression; Through multiple pathways, circRNA3503-OE-EVs alleviated inflammation-induced apoptosis and the imbalance between ECM synthesis and ECM degradation by acting as a sponge of hsa-miR-181c-3p and hsa-let-7b-3p.	[218]

Implantable scaffolds for EV delivery

Decellularized extracellular matrix continues to be a widely used and attractive raw material for biomaterial scaffold fabrication due to the retention of native proteins and matrix architecture while effectively eliminating cells or debris known to cause a detrimental immunological response *in vivo*. While there are challenges in working with decellularized materials as described above, combining decellularized tissues with synthetic or chemically modified natural polymers has enabled a high level of tunability, bioactivity, and manufacturability. For example, decellularized porcine cartilage combined with GelMA was used to generate a unique 3D printable bioink to support the controlled release of MSC-EVs [214]. Prior to evaluating a novel 3D printed material, Chen et al. investigated the efficacy of EVs in modulating chondrocyte behavior and found that isolated EVs alone promoted decreased expression of MMP-13 (a marker of cartilage degradation) and ADAMTS-5 while also increasing COL2A1 and aggrecan expression [214]. Proteomic analysis followed by subsequent gene ontology enrichment analysis and STRING showed that EVs were significantly enriched in various segments of the mitochondria, suggesting that these processes and pathways may be heavily involved in the chondroprotective function of the EVs. Using an inhibitor to induce mitochondrial damage to chondrocytes *in vitro*, EVs were found to provide unique mitochondrial proteins to rescue the damage [214]. Next, dye-labeled EVs were incorporated into the decellularized cartilage ECM-GelMA bioink and

3D printed using a desktop stereolithography technique in combination with visible light crosslinking initiated by LAP to fabricate high-resolution hydrogel scaffold discs with radially oriented channels and a pore size between 100-500 μm to facilitate optimal cell infiltration. A pilot subcutaneous investigation showed that EVs were retained within the 3D printed scaffold for up to 14 days promoted significantly fewer M1 macrophages (CD86 and CD3) and increased M2 macrophages (CD163 and Arg1) compared to scaffolds without EVs. Subsequent *in vivo* analysis in a rabbit osteochondral defect model showed that the EV-containing hydrogel significantly increased ICRS macroscopic scores (Safranin O and alcian blue) and immuno-histochemistry of COL2A1 and decreased MMP13 expression after the 6 and 12 week time points compared to all other groups, demonstrating a clear impact of local EV retention for cartilage tissue regeneration.

Surface modification of biomaterial scaffolds with EVs or employing a biomaterial scaffold as a reloadable depot for EV retention and release are other methods recently investigated for cartilage tissue engineering. EVs isolated from human umbilical Wharton's jelly MSCs (gelatinous substance on the inside of the cord that is rich in hyaluronic acid and chondroitin sulfate) intra-articularly injected weekly into rabbit osteochondral defects pre-implanted with freeze-dried, and EDC-crosslinked decellularized porcine cartilage scaffolds enhanced osteochondral regeneration compared to the scaffold alone [212]. A follow-up analysis was subsequently performed to investigate potential mechanisms of action of EVs compared to saline control in the osteochondral defect model and found that EVs did not significantly change the pro-inflammatory expression of TNF-α or IL-1β. However, the EVs show significantly increased IL-10 staining in the defect and synovium after 10 days, suggesting EVs promoted increased anti-inflammatory behavior [212]. Furthermore, EV treatment promoted significantly higher numbers of M2 macrophages (CD206+), lower numbers of M1 macrophages (CD86+), with no significant changes in MSC proliferation or endogenous recruitment after 10 and 20 days post-treatment.

Injectable hydrogels for EV delivery

HA hydrogels have recently been investigated in conjunction with EVs for enhanced cartilage repair and regeneration, including soluble high molecular weight HA viscosupplements alone. Treatment of critical-sized osteochondral defects in five-month-old New Zealand White rabbits immediately following wound closure with either 1mL intra-articular injections of high molecular weight HA (1100 kDa, 3 wt%) or 1mL of high molecular weight HA and 200 μg of EVs isolated from clonal immortalized E1-MYC 16.3 human embryonic stem cell-derived MSC line, with repeated injections performed 7 and 14 days post wound closure resulted in significantly increased ICRS score at 6 and 12 weeks [230]. Further, combined HA and EV treatment resulted in significantly increased toluidine blue (sulfated glycosamino-glycans) and decreased collagen I staining after 12 weeks (but no significant differences at 6 weeks) compared to HA alone, which also yielded more fibrocartilage rather than hyaline cartilage [230]. This work demonstrates that repeated intra-articular injection of high molecular weight HA combined with EVs improves cartilage tissue repair and regeneration compared to HA alone; however, it is unclear how long EVs are retained within the material or the dose at which they are released. Other studies have investigated the use of covalently crosslinked HA, modified HA and/or supplementing HA with other biopolymers to augment material functionalization and incorporate EV delivery.

Chemical crosslinking

Freeze-dried EDC/NHS-crosslinked chitosan and hyaluronic acid hydrogels (CS-HA) combined with EVs and adipose-derived MSCs injected into full-thickness osteochondral defects immediately following surgery promoted significantly increased ICRS scores of MRI imaging compared to the hydrogel alone, MSCs alone, MSC-EVs, CS-HA/MSC, or the CS-HA/MSC-EVs group after 4 weeks and 24 weeks [213]. Further, gross analysis and scanning electron microscopy of the joint surface, along with histological analysis (hematoxylin and eosin, Masson's trichrome, and ColII) after 24 weeks illustrated the CS-HA/MSC and CS-HA/MSC-EVs group produced cartilage repair with ICRS scores that were not statistically different from normal cartilage [213].

Photocrosslinkable hydrogels

HA modified with o-nitrobenzyl alcohol (HA-NB) generates aldehyde groups under light irradiation that can interact with amines on other biopolymers, enables *in situ* covalent crosslinking; this design was recently exploited by Liu et al. to entrap and deliver EVs derived from human induced pluripotent (cell line iPS-S-01)-derived MSCs to cartilage defects in rabbits [216]. Full-thickness cartilage defects filled with HA-NB, combined with gelatin and EVs (EHG), photo-irradiated for one minute at 395 nm integrated within the native cartilage matrix, promoted cell deposition, and

cartilage defect repair significantly better than the HA-NB/gelatin (HG) hydrogel alone, pre-cultured EHG implants, or intra-articular injections of EVs at the same dose alone. Importantly, this study examined EV retention by immersing 200 µL gels prepared with 2.45^{12} DiI-labeled EVs in fresh PBS daily; supernatants were analyzed with a particle analyzer and subtracted from the loaded EV total to determine percent retention [216]. There are limitations to this technique, as it is unclear if the temperature or mechanical agitation were used during the EV release study or if the physical properties of the hydrogel glue (e.g., degradation, swelling) influenced EV retention/release. In another study, authors used a BCA protein assay to quantify the release of small EVs isolated from human umbilical cord MSCs from photo-crosslinked (3 minutes, UV light) GelMA/laponite nanoclay hydrogels (nanoclay is a unique nanoparticle composite material containing layered silicates) [231]; the GelMA/nanoclay photocrosslinked hydrogel retained EVs for up to 30 days *in vitro* compared to crosslinked GelMA alone or GelMA/gelatin hydrogels [215]. Furthermore, the EV-containing hydrogel significantly improved cartilage repair in a full-thickness osteochondral defect in Sprague-Dawley rats compared to the Gel-nano hydrogels alone after 12 weeks, suggesting the retention and subsequent release of EVs over 30 days provided the enhanced therapeutic effect [215]. Hu et al. also performed extensively *in vitro* mechanistic analysis on their EVs; microarray analysis demonstrated that miR-23a-3p was highly enriched, and bioinformatic analysis suggested that miR-23a-3p may bind to the 3'UTR coding sequence of the gene PTEN. The authors then cultured miR-23a-3p with 293 T-cells transfected with luciferase reporter constructs containing the predicted 3'UTR of PTEN and found that luciferase activity was decreased and subsequently abolished when cultured with mutated 3'UTR of PTEN, confirming EV function via miR-23a-3p targeting PTEN [215]. A silencing assay with human bone marrow-derived mesenchymal stem cells further validated that miR-23a-3p attenuated the effects of EVs on COL2A1 and SOX9 gene expression, alcian blue staining, and impaired activation of protein kinase B (AKT) secretion *via* western blotting. Collectively, these two studies highlight the clinical efficacy of using photocrosslinkable networks for EV retention and delivery from hydrogels.

Thermo-responsive hydrogels

As previously described, covalent/supramolecular interactions can be exploited to entrap and control the delivery of biomolecules such as EVs. Here, Diels-Alder crosslinked hyaluronic acid/PEG hydrogels (DAHP) were developed using a furyl functionalized HA and a maleimide (Mal)-PEG-Mal crosslinker that enables orthogonal crosslinking with low reactivity with amine groups [232]. Diels-Alder crosslinked hydrogels are thermo-reversible polymer networks formed without any additional catalyst of toxic solvent, making them particularly advantageous for incorporating biomolecules [233]. Wang et al. found that DiO-labeled EVs isolated from human-induced MSCs derived from iPSC (C1P33) were released from the DAHP hydrogel for 16 days *in vitro* via a transwell assay (0.4 µm membrane, ~16% cumulative release) and nanoflow cytometric analysis. Furthermore, EV release kinetics were accelerated under increasing concentrations of hyaluronidase treatment, confirming a degradation-dependent control of EV release [232]. Using a model of OA in rats (transection of the anterior cruciate ligament in combination with partial medial meniscectomy), authors found that intra-articular injection of hydrogels containing EVs significantly improved cartilage repair (the lowest OARSI score) compared to the DAHP hydrogel alone and a single injection of EVs after 35 days *in vivo* [232]. Interestingly, the authors also found minimal differences in cartilage repair between the hydrogel delivery and weekly intra-articular injections of EVs, suggesting the good potential of hydrogels as a single-application therapeutic.

In addition to EVs being a potent and independent therapeutic for treating OA or promoting cartilage repair, EVs have also been shown to be unique bioactive carriers for nucleic acids [234]. Tao et al. showed that sleep-related circular RNAs (circRNA), which are more stable and less susceptible to rapid clearance than miR or linear RNAs [235], are enriched in melatonin-treated chondrocytes that are thought to play a role in OA pathogenesis [218]. The authors isolated EVs from synovium mesenchymal stem cells (SMSCs) overexpressing circRNA3503 and loaded them into a poly(D, L-lactide)-b-poly(ethylene glycol)-b-poly(D, L-lactide) (PDLLA-PEG-PDLLA; PLEL) triblock copolymer hydrogels. This thermosensitive triblock copolymer is particularly useful as an injectable vehicle for biotherapeutics due to its ability to self-assemble into micelles at room temperature and non-flowing structure under physiological load [218]. Using a model of OA in rats (transection of the medial collateral ligament, medial meniscus, and anterior cruciate ligament), the PLEL hydrogels loaded with circRNA-doped EVs demonstrated significantly enhanced cartilage repair after 24 weeks (injections performed after surgery and

every 4 weeks after OA-induced injury) compared to the PLEL hydrogel alone or PLEL hydrogel with SMSC-EVs *via* Safranin O/Fast Green and Toluidine Blue, and Col II staining [218]. EVs released from hydrogels were confirmed *in vitro* over 36 days performed on a rocker at 37 °C and detected using a CD63 ELISA kit. Mechanistic *in vitro* studies of the circRNA3503-overexpressed EVs highlighted their efficacy in attenuating inflammation-induced apoptosis and provided a balance between ECM synthesis and degradation by acting as a sponge for miRs that regulate expression of target functional genes such as MMPs and SOX9 [218].

Summary of biomaterials for EV/exosome delivery

Together, these studies showcase a wide variety of biomaterials for EV delivery to promote cartilage tissue repair and regeneration. Collectively and independently, each of these materials indicates potential for their biomaterial delivery vehicle to release EVs and subsequently enhance cartilage repair compared to the materials alone and in some cases compared to EV treatment alone. As discussed in many review articles, there are significant limitations to translating EV/biomaterial therapeutics to clinical application, first, in standardizing cell/tissue/species source for EV generation, scalability, isolation techniques, application techniques (e.g., single therapeutic application or application of an EV/biomaterial therapy followed by multiple injections of EVs at later time points) and in designing appropriate pre-clinical studies to thoroughly identify and qualify that the effects of EV-loaded materials are primarily driven by EVs. Second, there also remains challenges in standardizing techniques to evaluate EV release and subsequent synergistic effects of bioactivity of EVs released from biomaterials. Important to note that many of the specific functional outputs *in vitro* and *in vivo* be tissue- or application-specific (i.e., cartilage vs. cardiac vs. bone). Decoupling these effects will be critical to validate EV/biomaterial efficacy.

Lastly, based on several reviews and publications showing the robust effects of intra-articular delivery of EVs to promote cartilage repair and regeneration, studies here suggest that a single application of EV-loaded biomaterials may provide a similar therapeutic effect controlled *via* material degradation-mediated EV release or EV diffusion from biomaterials. It will be essential to elucidate the effects of EV/biomaterial degradation further and release with respect to timing and dosing to achieve a therapeutic effect for OA. One area of biomaterials that has shown promise in cartilage tissue engineering (and many other applications) that may be advantageous for EV delivery is granular hydrogels and microparticles [236, 237]. These materials are uniquely poised for various biopolymer compositions, fabrication methods, and tuning therapeutic delivery that can be applied in the joint using translational and minimally invasive surgical techniques.

Conclusion and Perspective

In summary, recent research has made significant progress in overcoming major barriers to using EVs as a delivery system and a marker for OA pathology diagnosis. EVs are ideal systems for delivering osteoarthritis therapeutics, owing to their size, surface expression profiles, low immunogenicity, low cytotoxicity, and long-term safety. EVs from modified cells or engineered EVs with drug loading technologies have been shown to improve the therapeutic effect. However, the side-effect of using EVs for OA therapy is unknown, and safety evaluation research is required before clinical translation. Recent advances in nanomaterials-based offer great sensitivity and rapid biosensors for detecting EVs. Tissue engineering techniques are also used in EV-based OA therapy/cartilage repair. Biological scaffolds, especially hydrogels, have been shown to have a good sustained EV-release effect in cartilage repair. We believe that theranostic platforms will be the main direction of EV-based OA therapy/cartilage repair in the near future.

The mentioned exosome-liposome nanohybrid system also inspires us to consider its potential application to detect both EVs and the content of EVs [196]. For instance, the liposome surface can be modified with EV binding peptide/aptamers that will turn on the signals upon binding with EVs. In parallel, biosensors for sensing EV contents, such as miR, can be initially encapsulated in the liposomes, and another physical/chemical signal will be switched on upon the liposomes fusing with EVs/exosomes. Thus far, this nanosystem can simultaneously probe both EVs and OA biomarkers in EVs. Nevertheless, this platform will require further research to validate efficacy.

Abbreviations

OA: osteoarthritis; MSCs: mesenchymal stem cells; ECM: extracellular matrix; HSP70/90: heat shock proteins 70/90; Col: collagens; Col II: Type II collagen; Col IX: collagen IX; ESCs: embryonic stem cells; EVs: extracellular vesicles; COVID-19: coronavirus disease 2019; miR: microRNAs; mRNAs: messenger RNA; TSG101: tumour susceptibility gene 101; RA: rheumatoid arthritis; HA: hyaluronan; SF:

synovial fluid; lncRNA: long non-coding RNA; circRNA: circular non-coding RNA; NTA: nanoparticle tracking analysis; aRT-PCR: quantitative reverse transcription-polymerase chain reaction; RNA-seq: RNA-sequencing; ELIA: enzyme-linked immunosorbent assay; WB: western blotting; SERS: surface-enhanced Raman scattering; SPR: surface plasmon resonance; ICA: immunochromatographic assay; CL: chemiluminescence; ECL: electrochemi-luminescence; PRP: platelet-rich plasma; PDGF-AB: platelet-derived growth factor-AB; TGF-β: trans-forming growth factor-β; VEGF: vascular endothelial growth; IL-1β: interleukin-1β; IPFP: infrapatellar fat pad; AC: articular chondrocytes; NP: nanoparticles; HLA-DR: human leukocyte antigen-DR; IFN-γ: interferon gamma; MMP-13: matrix metallopeptidase 13; ADAMTS5: a disintegrin and metalloproteinase with thrombospondin motifs; SMSCs: synovial MSCs; H3K4: histone H3 at lysine 4; KDM2A: lysine demethylase 2A; SFRP2: secreted frizzled-related protein 2; E2F1: E2F transcription factor 1; TNF-α: tumour necrosis factor-α; RalA: ras-related protein; BMMSCs: bone marrow MSC; ADMSC-EVs: adipose-derived stem cell EVs; FA: folic acid; PEG: polyethylene glycol; Chol: cholesterol; Dex: dexamethasone; CAP: chondrocyte-affinity peptide; Lamp2b: lysosome-associated membrane glyco-protein 2b; HA: hyaluronic acid; RHAMM: hyaluronan-mediated motility receptor; GelMA: methacrylamide-modified gelatin; EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl; n-hydroxysuccinimide (NHS); TEM: transmission electron microscopy; Arg-1: arginase-1; AKT: activation of protein kinase B (AKT); OARI: osteoarthritis research society international; PLEL: poly(D, L-lactide)-b-poly(ethylene glycol)-b-poly(D, L-lactide); OARSI: Osteoarthritis Research Society International.

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Competing Interests

The authors have declared that no competing interest exists.

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