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Engineered Extracellular Vesicles for Cartilage Regeneration: Bridging Biological Complexity to Clinical Precision

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Abstract

Osteoarthritis (OA) is a common degenerative joint disease characterized by pain, stiffness, progressive cartilage loss, and reduced mobility. Current treatments primarily aim to relieve symptoms rather than restore damaged cartilage, and durable regeneration of native hyaline cartilage remains a major clinical challenge. Extracellular Vesicles (EVs), particularly those derived from Mesenchymal Stem Cells (MSCs), have emerged as promising cell-free therapeutic platforms because of their ability to modulate inflammation, regulate chondrocyte activity, and influence extracellular matrix metabolism. However, EV heterogeneity, source-dependent variability, limited targeting efficiency, inconsistent cargo loading, and lack of standardized manufacturing protocols continue to restrict their clinical translation. This review summarizes recent advances in engineered EV-based strategies for OA and cartilage repair, including parental-cell preconditioning, genetic modification, surface functionalization, cargo loading, artificial EV platforms, and biomaterial-assisted delivery. Importantly, we distinguish between *in vitro* findings, preclinical animal studies, and early clinical evidence to provide a balanced assessment of translational readiness. We also discuss key regulatory and safety challenges, including GMP-compliant production, batch-to-batch variability, quality-control criteria, potency assays, scalability, biodistribution, and long-term safety. By integrating EV engineering with translational and regulatory perspectives, this review highlights the potential of engineered EVs as future disease-modifying tools for OA while emphasizing that their clinical efficacy and capacity to restore durable hyaline cartilage remain to be demonstrated in robust human studies.

Keywords: Bioengineering, Clinical translation, Engineered EVs, Extracellular vesicles, Osteoarthritis, Regenerative medicine, Stem cells
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Introduction

Recent studies indicate that Osteoarthritis (OA) affects more than 500 million individuals, representing more than 3.3% of the world's population [1]. OA leads to chronic pain, progressive joint tissue damage, stiffness, and substantial loss of mobility. The economic complications are not only due to direct healthcare costs, but also because of productivity loss and long-term disability [2]. Because cartilage is a nonvascular tissue with limited self-repair capability, developing durable regenerative strategies remains a major clinical challenge. Existing therapeutic strategies for OA mainly focus on symptom management instead of structural regeneration of cartilage tissue. Conventional treatments, including Nonsteroidal Anti-Inflammatory Drugs (NSAIDs), cor-

ticosteroid injections, hyaluronic acid supplementation, and physical rehabilitation, may briefly improve pain and recover joint function; nevertheless, they fail to avert progressive cartilage degeneration. Surgical interventions such as microfracture, osteochondral grafting, and Autologous Chondrocyte Implantation (ACI) have established partial regenerative potential, yet regularly result in fibrocartilage formation rather than mechanically durable hyaline cartilage [3]. In advanced disease stages, total joint arthroplasty remains the decisive treatment option, in spite of concerns regarding implant longevity, revision surgery, and postoperative complications. Consequently, at present none of the available therapies consistently restores native cartilage architecture or effectively halts OA progression, highlighting the crucial need for disease-modifying regenerative strategies.

To overcome these limitations, regenerative medicine approaches based on cell therapy have been widely investigated. Mesenchymal Stem Cells (MSCs), induced Pluripotent Stem Cells (iPSCs), and autologous chondrocytes have shown potential to promote cartilage regeneration because of their differentiation capacity and paracrine activity. Among these, MSCs derived from bone marrow, adipose tissue, umbilical cord, and synovium are the most broadly studied because they can modulate inflammation, support extracellular matrix synthesis, and promote tissue repair [4,5]. However, several serious barriers continue to restrict the clinical translation of cell-based therapies, including low cell survival after transplantation, donor-to-donor variability, limited engraftment efficiency, phenotypic instability, risk of unwanted differentiation or tumorigenicity, and potential immunogenic responses. Challenges associated with large-scale manufacturing, storage, and regulatory standardization further complicate their clinical implementation. These limitations have increased attention toward alternative cell-free regenerative strategies capable of preserving therapeutic value while minimizing the risks related to live-cell transplantation.

Extracellular Vesicles (EVs) have recently emerged as promising cell-free therapeutic candidates for cartilage repair and OA management. EVs are membrane-bound nanoscale vesicles naturally secreted by nearly all cell types and play vital roles in intercellular communication through the transfer of proteins, lipids, and nucleic acids [6,7]. Among EV subtypes, exosomes have attracted particular attention because of their small size, intrinsic biocompatibility, and ability to deliver bioactive cargoes that regulate inflammation, apoptosis, and tissue regeneration. Collective evidence suggests that MSC-derived EVs can reproduce many of the beneficial paracrine effects of their parental cells while demonstrating lower immunogenicity, improved biosafety, and reduced tumorigenic risk compared with conventional cell therapies [8]. Preclinical studies have shown that EVs can reduce inflammatory signaling, enhance chondrocyte proliferation, stimulate extracellular matrix synthesis, and promote cartilage repair in OA models [4].

Despite these advantages, various significant limitations continue to delay the clinical translation of EV-based therapies. Natural EVs show considerable heterogeneity in their composition, biological activity, and therapeutic potency depending on donor cell source, isolation method, and culture conditions. Besides, challenges associated with inefficient targeting, rapid clearance, limited cargo-loading capacity, and lack of standardized manufacturing protocols limit their reproducibility and therapeutic efficacy. To address these barriers, growing attention has focused on engineering strategies designed to boost EV stability, targeting specificity, cargo delivery, and regenerative efficiency.

In this review, we critically examine engineered EV-based strategies for cartilage regeneration and OA therapy with a specific focus on their translational readiness. Unlike previous reviews that mainly summarize the biological effects of MSC-derived EVs, this article integrates EV source heterogeneity, engineering approaches, cargo-loading strategies, delivery platforms, and regulatory barriers into a single translational framework. We distinguish between *in vitro* evidence, preclinical animal data, and emerging early-stage clinical findings to avoid overestimating the current maturity of EV-based therapies. By emphasizing engineered EVs, precision-oriented source selection, manufacturing standardization, and clinical implementation challenges, this review aims to define the key scientific and regulatory milestones required before EV-based interventions can progress toward routine OA treatment.

Heterogeneity of EVs

EV Subtypes: EVs represent a diverse and heterogeneous group of membrane-bound particles released by cells. They are usually classified into three main categories, exosomes, microvesicles, and apoptotic bodies, based on their size, mode of biogenesis, and molecular characteristics (Figure 1) [9]. Exosomes (30–150 *nm*) originate from the endosomal pathway and are released *via* multivesicular bodies. Microvesicles (100–1000 *nm*) are formed through direct budding of the plasma membrane. Apoptotic bodies (500–2000 *nm*) are released during programmed cell death and contain cellular fragments.

Although EV subtypes share the ability to transport proteins, lipids, and nucleic acids, they differ substantially in biogenesis, molecular composition, and biological function. Exosomes are the most extensively investigated subtype in regenerative medicine because of their stability, small size, and enrichment in signaling molecules involved in tissue repair and immunomodulation. In contrast, microvesicles exhibit greater heterogeneity because they directly originate from plasma membrane budding and often reflect the physiological state of the parental cell. Apoptotic bodies are less commonly explored for therapeutic applications because

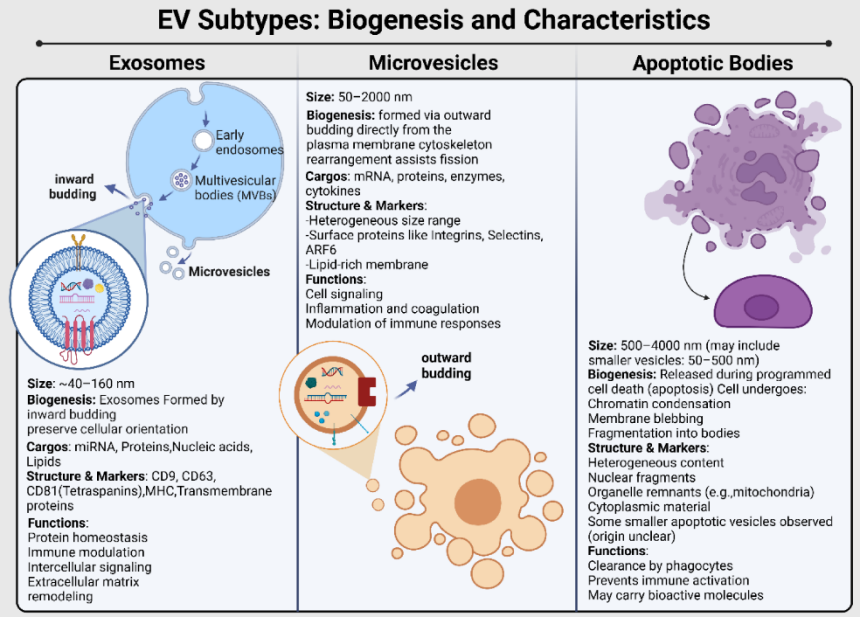

Figure 1. Heterogeneity and biogenesis of extracellular vesicle (EV) subtypes.
Schematic representation of the major EV subtypes, including exosomes, microvesicles, and apoptotic bodies, categorized according to their approximate size ranges, biogenesis pathways, and characteristic molecular composition. Exosomes originate from the endosomal pathway through multivesicular body formation and release, whereas microvesicles are generated by outward budding of the plasma membrane. Apoptotic bodies are released during programmed cell death and typically contain cellular organelles and fragmented biomolecules. The figure highlights the structural and functional heterogeneity of EV populations and their important roles in intercellular communication, tissue homeostasis, and regenerative medicine applications.

they may contain fragmented intracellular components associated with cell death and inflammatory signaling.

The selection of a source for therapeutic EVs should be practical and sustainable to support clinical applications. However, because EV composition and therapeutic activity are strongly influenced by donor-cell origin, culture conditions, and isolation methods, there remains no consensus regarding the optimal EV source for regenerative applications.

Functional Variations across EV Sources: EV therapeutic activity varies substantially depending on donor-cell origin, as cellular source influences EV cargo composition, immunomodulatory properties, and regenerative potential. In OA research, MSC-derived EVs have received particular attention because MSCs can be obtained from multiple tissues (Table 1), including adipose tissue [10], bone marrow [11], umbilical cord [12], synovium [13], dental follicles [14], induced Pluripotent Stem Cells (iPSCs) [15], amniotic fluid [16], human progenitor endothelium [17], and endometrial stem cells [18]. These EVs can serve as carriers for bioactive molecules involved in cartilage regeneration and tissue repair. Recent studies have shown that MSC-derived EVs perform better than do MSCs themselves in reducing cartilage breakdown, joint inflammation, and pain, even though the overall effects are moderate. Interestingly, treatment with MSCs alone sometimes leads to worse joint outcomes, especially in patients with metabolically driven OA. These findings, which are mainly derived from preclinical and early translational studies, suggest that MSC-EVs may offer a safer cell-free alternative to MSC transplantation; however, their superiority in human OA remains to be confirmed in adequately powered clinical trials [19]. Furthermore, EVs can be obtained from a variety of other sources, such as macrophages [20]; biological fluids, such as urine or semen; chondrogenic progenitor cells; synovial fluid; fibroblast-like synoviocytes; bovine milk; marine organisms; and plant-derived EVs [21].

In addition to MSC-derived EVs, tissue-specific EV populations have attracted attention because they may better preserve cartilage-associated signaling properties and microenvironmental compatibility. Unlike TGF-β treatment, Chondrocyte-derived EVs (CC-EVs) enhance the chondrogenic differentiation and proliferation of *Human Umbilical Cord Mesenchymal Stem Cells* (HUCMSCs) without inducing hypertrophy or fibrosis. CC-EVs increased key chondrogenic markers, including ACAN, COL2A, and SOX-9, and aided in cartilage repair. The increase in autophagosomes indicates that CC-EVs facilitate cartilage regeneration through autophagy activation, underscoring their potential in cell-free articular cartilage repair strategies [22].

EVs from synovial cells, including Cartilage Progenitor Cell-derived EVs (CPC-EVs), protect IL-1β-stimulated chondrocytes by promoting cartilage matrix production and reducing inflammation, mainly by inhibiting STAT3 activation. Proteomic analysis revealed that CPC-EVs contain STAT3 regulatory proteins that are transferable to chondrocytes. To enhance therapeutic effectiveness, CPC-EVs were modified with a cationic peptide. The modified EVs significantly reduced OA while assisting in matrix repair in *ex vivo* cartilage explants [23].

Table 1. Summary of *in vivo* and *in vitro* studies using MSC-derived EVs for OA and cartilage repair

EV source	Therapeutic effect	Model used	Origin	Method (EVs isolation, characterization, dosage, *etc*.)	Ref.
Bone Marrow MSC-EVs	BM-MSC-EVs decreased inflammation and increased proteoglycan and type II collagen production in OA chondrocytes	TNF-α-stimulated OA chondrocytes (*in vitro*)	Human	Isolation by differential centrifugation, characterization by Western blot (CD9, CD63), NTA, and immunoelectron microscopy. Dosage: Equivalent of 500x10^3 BM-MSC cells (up to ~1.8x10^9 particles per dose). Injection: In vitro culture and coculture with OA chondrocytes	[1]
	Equine BM-MSC-EVs reduced MMP-13 expression in chondrocytes under pro-inflammatory conditions	Equine chondrocytes (*in vitro*)	Equine	Isolation by ultracentrifugation, characterization by TEM, NTA, CD9 immunostaining. Dosage: 13,333 EVs per chondrocyte	[2]
	BM-MSC-EVs modulate macrophage polarization, lower oxidative stress, and protect mitochondria via PINK1/Parkin pathway	Rat OA model	Human	Isolation by ultracentrifugation, characterization by TEM, nanoparticle tracking analysis (NTA), Western blot for protein markers (PINK1, Parkin, Akt), JC-1 staining. Dosage: 100 $\mu g/ml$ for macrophage treatment, 50 μl for rat intra-articular injection	[3]
	LIPUS enhances MSC exosome release via autophagy, improving chondrocyte function and cartilage repair	Rat bone marrow MSCs and rat OA knee model	Rat	MSC Isolation: Bone marrow from SD rats cultured in DMEM +10% FBS. Autophagy: Activated by rapamycin (10 μM), inhibited by 3-MA (10 μM). Exosome Isolation: Ultracentrifugation. LIPUS: 3 MHz, 50 mW/cm^2, for 20 mins/day. Coculture: MSC + OA chondrocytes. Histopathology: Safranin-O staining, Mankin scoring. Injection: MSC + GW4869 (10 μM). Analysis: Western blot, NTA	[4]
	Cryogel (0.3% HA) + BM-MSC-Exos enhanced ECM production, GAG synthesis, and cartilage repair	Rabbit cartilage defect model	New Zealand rabbit	Exosome isolation by ultracentrifugation, Characterized by NTA and TEM, Dosage: 10^6 particles/*ml*, Cryogel seeding, Injection into cartilage defects	[5]
	Reduced MMP13, increased COL2, improved behavior, lowered CGRP/iNOS in DRG	OA Rat Model	Rat	Exosome isolation via ultracentrifugation, Characterization by TEM, Zetasizer, Western blot, Dosage: 40 μg/week, Injection into knee joint cavity, Fluorescence imaging for uptake	[6]
	Exosomes and MPs reduced joint damage, restored ECM markers, suppressed inflammation	CIOA Mouse Model	Mouse	EV isolation via differential centrifugation, Characterization by DLS, Nano Tracking Analysis, Size: 96 *nm* for Exos, 223 *nm* for MPs, Dosage: Exosomes (250 *ng*), MPs (500 *ng*), Injection into joint cavity	[7]
	Improved pain, suppressed abnormal nerve/vessel formation in the LBP mouse model	LFJ OA Mouse Model	Mouse	EV isolation via differential ultracentrifugation, Characterization by TEM and Western Blot (CD9, CD63, TSG101 markers), Dosage: 200 μg exosomes, injection *via* the tail vein	[8]
	Combined therapy improved histological and mechanical repair vs. HA alone	Rabbit osteochondral defect model	Rabbit	Exosomes isolated from MSCs (isolation and characterization method N/A), combined with HA, three intra-articular injections at 0, 7, and 14 days, evaluated at 6 and 12 weeks, macroscopic, histologic, and biomechanical analysis	[9]
	Improved function in 132 joints of Navy veterans with no adverse events	OA in the Navy SEALs	Human	Sample Size: 33 Navy SEAL veterans Joints Treated: Knee (n=58), Shoulder (n=32), Elbow (n=16), Hip (n=12), Ankle (n=8), Wrist (n=6) Injection Method: 2 *ml* of XoFlo (extracellular vesicle isolate) injected IA Follow-up: 12 *hr*, 24 *hr*, 48 *hr*, 2 weeks, 6 weeks, 3 months, 6 months, 1 year	[10]
	A single EVIP dose yielded 75% improvement in a grade III OA athlete	Grade III OA patient	Human	Patient: 51-year-old male athlete with a history of increasing left medial and patellofemoral knee pain, previously failed treatments Injections: Single 2 *ml* intra-articular injection (ExoFlo). Follow-up: 6-week and 3-month follow-ups	[11]

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EV source	Therapeutic effect	Model used	Origin	Method (EVs isolation, characterization, dosage, *etc.*)	Ref.
Adipose MSC-EVs	ADSC-EVs enhance BMSC migration, proliferation, and chondrogenic/osteogenic differentiation	Rat MSCs (*in vitro*), murine model	Rat	MSC Isolation: ADSCs, BMSCs, and SMSCs were isolated from rat adipose, bone marrow, and synovium, respectively. *In vitro* Studies: Assessing migration, proliferation, and differentiation of MSCs. *In vivo* Studies: Xenograft model to assess cartilage and bone regeneration	[12]
	AD-MSC-EVs and UC-MSC-EVs repaired cartilage and bone in the pig OA model; AT-MSCs were most effective	Humanized pig OA model	Human	ISOLATION: Ultracentrifugation, CHARACTERIZATION: NTA, Flow Cytometry Intra-articular Injection	[13]
	Human AD-MSC-EVs reduce cartilage degradation by miR-376c-3p targeting WNT3/WNT9a and inhibiting WNT/β catenin	*In vitro* and *in vivo* models	Human	Exosome Isolation from hADSCs, Bioinformatics, Luciferase Reporter Assay, Safranine O-Fast Green Cartilage Staining, Masson Staining, Immunohistochemistry, Immunofluorescence	[14]
	H-ApoEVs from hypoxia-treated adipose MSCs enhance proliferation and M2 macrophage polarization, improving cartilage regeneration with scaffolds	3D-printed ECM scaffold *in vitro* and *in vivo*	Human	ApoEVs and H-ApoEVs isolated from adipose MSCs under normoxic and hypoxic conditions, characterized by TEM, DLS, Western Blotting, NTA, and DiO-labeling; injection via Gel/ECM scaffold in the knee joint cavity	[15]
	Combined IA therapy in sheep reduced lameness and improved OA scores	Meniscectomy Sheep Model	Sheep	Exosomes isolated from Ad-MSCs, characterized by flow cytometry (CD63, CD81 positive), particle size (88.7 *nm*), zeta potential (-1.4 mV), and ultracentrifugation; injection via IA route at specified intervals (HA and exosomes)	[16]
Umbilical Cord MSC-EVs	hUCMSC-EVs boost BMSC proliferation and chondrogenesis via miR-181c-5p, suppressing SMAD7 and enhancing BMP2 signaling	*In vivo* cartilage repair model	Human	EVs isolated by ultracentrifugation from hUCMSCs; characterized by TEM, NTA, and Western blot (CD9, CD81, CD63); miR-181c-5p delivered via EVs to BMSCs	[17]
	miR-7704-modified EVs promote collagen II and reduce MMP13, preserving cartilage and motor function in OA mouse model	Mouse OA model	Human	EVs were isolated from HUCMSCs cultured in serum-free medium using ExoQuick. Characterization by Western blot (CD9, CD63, CD81), NTA, and TEM. Injection of EVs (1×10^7 particles/mL) into OA mice *via* intra-articular route	[18]
	hWJMSC-EVs deliver ITGB1 to activate TGF-β/Smad2/3 pathway, enhancing cartilage repair after microfracture	Rabbit model	Human	*In vitro*: Coculture with hBMSCs and chondrocytes treated with hWJMSC-EVs at a concentration of 50 *μg/ml*. *In vivo*: Injection of hWJMSC-EVs at various concentrations into rabbit joints post-MF surgery. Evaluation via micro-CT, histology, immunohistochemistry, and Western blot	[19]
	Combining PRP with hUCMSC-EVs improves cartilage regeneration and motor function by increasing collagen II and reducing IL-1β	Mouse OA model	Human	*In vivo*: EVs extracted from HUCMSCs cultured with 5% PRP were injected into the OA mice model. *In vitro*: Differentiation and characterization of HUCMSCs treated with PRP. Evaluation of motor function, cartilage integrity, and protein expression (type II collagen, aggrecan, IL-1β) using rotarod test, histology, ICRS scoring, and immunohistochemistry	[20]
	3D-culture-derived exosomes had better biological activity and stability than 2D-derived exosomes	Chondrocytes *in vitro*	Human	Isolation: U-MSCs were cultured in a hollow-fiber bioreactor, conditioned medium collected, and exosomes purified via ultracentrifugation. Characterization: Transmission electron microscopy for cup-shaped morphology; Western blot for exosome markers (CD63, CD81, TSG101); Nanosight analysis for particle size (~120 *nm*). Dosage: 10 *μg/ml* of exosomes used in in vitro assays; 500 *μl* of exosome suspension (1×10^{10} particles/*ml*) for in vivo injections. Injection: Exosomes were injected IA into the rabbit knee joints with cartilage defects	[21]

Beyond mammalian cell-derived EVs, various emerging nontraditional EV sources are currently being investigated for regenerative and anti-inflammatory applications. For instance, plant-derived EVs are safe, natural, and eco-friendly nanocarriers with significant potential in regenerative medicine. They offer advent-

Contd. Table 1. Summary of *in vivo* and *in vitro* studies using MSC-derived EVs for OA and cartilage repair

EV source	Therapeutic effect	Model used	Origin	Method (EVs isolation, characterization, dosage, *etc*.)	Ref.
Synovial MSC-EVs	Sy-MSC-EVs deliver miR-26a-5p to reduce inflammation and apoptosis, targeting PTEN	Chondrocytes and OA mouse model	Human	Isolation: not specified Characterization: not specified Dosage: SMSC-EVs (miR-26a-5p-loaded) were added to SW1353 cells at an appropriate concentration	[22]
	Sy-MSC-EVs promote chondrocyte proliferation and reduce apoptosis and inflammation via miR-130b-3p targeting LRP12 and AKT/β-catenin pathway	Chondrocytes and OA mouse model	Human	Isolation: EVs were isolated from SMSCs and identified. Characterization: miR-130b-3p was identified *via* microarray analysis, and its effects were explored through rescue experiments. LRP12 mRNA was confirmed as a target of miR-130b-3p. Dosage: EVs were administered to IL-1β-induced chondrocytes *in vitro* (dosage details not provided)	[23]
	Exosomes reversed IL-β-induced downregulation of COL2A1 and ACAN, inhibited MMP13/ADAMTS5, and corrected autophagy dysfunction	Chondrocytes *in vitro*	Human	Isolation using Ribo™ Exo isolation reagent from culture supernatants of SMSCs grown in Exo-free medium for 72 hours. After centrifugation at 2000 *g* and 1500 *g*, the exosome pellets were resuspended in PBS. Exosome characterization was done by TEM and DLS. Western blot confirmed the presence of exosome markers CD63 and HSP70 and the absence of Calnexin. In vitro, exosomes were applied to IL-1β-induced mouse chondrocytes at a concentration of 10 *μg/ml* for 24 hours. For in vivo studies, 10 *μl* of SMSC-Exo (100 *μg/ml*) was injected into the knee joints of OA mice for three consecutive weeks	[24]
	miR-140-5p-enriched SMSC-Exos enhanced chondrocyte proliferation, migration, and ECM maintenance	Chondrocytes *in vitro*, Rat *in vivo*	Human	Isolation after miR-140-5p transfection using ultracentrifugation. Exosomes were characterized by DLS, TEM, and Western blot analysis, identifying key markers such as CD63, CD81, and Alix. *In vitro*, exosome treatment was performed on articular chondrocytes (ACs) to assess proliferation, migration, and ECM secretion. *In vivo* cartilage OA rat protection was assessed through histology, Safranin-O and Fast Green staining, and immunohistochemical analysis	[25]
	Reduced ECM breakdown and cartilage degradation in the OA rat model	Rat OA model	Rat	(SMSC-Exo) isolated via ultracentrifugation. Exosome Characterization via TEM, DLS, and Western blot	[24]
Dental Follicle Cell EVs	DFC-sEVs improved TMJ chondrocyte activity and protected cartilage by downregulating HIF-1Î±/2Î± and catabolic markers	Rat TMJ-OA model	Human	DFC-sEV were isolated from DFC using ultrafiltration and characterized by TEM, NTA, and Western blot for surface markers (CD9, CD81, HSP90, TSG101). The size range was 50-200 *nm*. For *in vivo* treatment, 50-100 *μg/ml* DFC-sEV was injected intra-articularly into TMJ-OA rats for 4-8 weeks, showing therapeutic effects	[26]
Embryonic Stem Cell EVs	ESC-MSC-EVs reduce cartilage damage and matrix degradation by upregulating COL II and suppressing ADAMTS5	DMM OA mouse model	Mouse	Exosome isolation from ESC-MSCs	[27]
	ESC-MSCs and their EVs improved cognition and mobility in older dogs without adverse effects	Dog	Human	Isolation: TFF, concentrated ~10x. Characterization: NTA, TEM (spherical morphology), Flow cytometry (CD9, CD63, CD81). Dosage: 1.0×10^7 cells for 3-7 *kg* dogs, 2.0×10^7 cells for 7-12 kg dogs (IV, one injection)	[28]
iPSC-EVs	IPSC-EVs showed higher effectiveness than SM-MSC-EVs in reducing joint damage and enhancing chondrocyte repair	*In vitro* and in vivo OA models	Human	Isolation: Ultrafiltration method, characterized by Tunable Resistive Pulse Sensing (TRPS), TEM, Western blot (CD9, CD63, TSG101). Dosage: 8 µl (1.0×10^{10}/*ml*) intra-articular injection	[29]
	IPSC-EVs suppress T cells and enhance M2 macrophage polarization, similar to the regenerative impact of hUCMSC-EVs	*In vitro* comparative study	Human	Isolation: Conditioned medium from iMSCs, differential centrifugation, TFF Characterization: NTA, Western Blot (CD81, Alix), Cryo-EM, ExoView (tetraspanins CD9, CD63, CD81). Dosage: Varies by assay (*e.g*., 1:6,500 ratio for macrophage polarization)	[30]
MSC-Exos + HA	Improved cartilage and bone regeneration in porcine OA model; safe and effective	Porcine osteochondral defect	Human	Isolation: Exosomes isolated from MSCs. Characterization: MRI, histology, biomechanical tests, and micro-CT. Dosage: 2 *ml* IA injection on day 0, 8, and 15 postsurgery	[31]

Contd. Table 1. Summary of *in vivo* and *in vitro* studies using MSC-derived EVs for OA and cartilage repair

EV source	Therapeutic effect	Model used	Origin	Method (EVs isolation, characterization, dosage, *etc*.)	Ref.
Amniotic Fluid Stem Cell EVs	Induced hyaline-like cartilage, improved pain, and modulated macrophages *via* TGF-β	MIA-induced OA model	Human	Exosome Isolation: Commercial kit. Characterization: Identified markers, HGF, TGF-β, IDO. Dosage: Exo injection into the knee joints of animals, followed by histology and behavioral scoring up to 3 weeks	[32]
	Improved joint space and pain in OA and spine degeneration patients	OA & spinal degeneration	Human	Isolation, Characterization: not specified Dosage: 4 trillion exosomes in 1.5 *ml* matrix (Case 1 and Case 3: half IA + half IV; Case 2: all IV)	[33]
hPESC-derived EVs	Reduced pain and improved MRI cartilage quality in OA patients	Grade II/III knee OA patients	Human	Isolation: Conditioned medium (CM) from hPESCs. Characterization: GFs, CKs, and EVs. Dosage: 2 *ml* IA injection per patient	[34]

Abbreviations: BM-MSC: Bone marrow–derived mesenchymal stem cell, ADSC: Adipose-derived stem cell, UC-MSC: Umbilical cord–derived mesenchymal stem cell, WJMSC: Wharton's jelly–derived mesenchymal stem cell, Sy-MSC: Synovial-derived mesenchymal stem cell, BMSC: Bone marrow stem cell, SMSC: Synovium-derived mesenchymal stem cell, DFC: Dental follicle cell, ESC: Embryonic stem cell, iPSC: Induced pluripotent stem cell, hPESC: Human progenitor endothelial stem cell, EV: Extracellular vesicle, sEV: Small extracellular vesicle, Exo: Exosome, MPs: Microparticles, ECM: Extracellular matrix, HA: Hyaluronic acid, DRG: Dorsal root ganglion, OA: Osteoarthritis, LBP: Low back pain, LFJ: Lumbar facet joint, CIOA: Collagenase-induced OA, LIPUS: Low-intensity pulsed ultrasound, HGF: Hepatocyte growth factor, TGF-β: Transforming growth factor beta, BMP2: Bone morphogenetic protein 2, MMP: Matrix metalloproteinase, COL2: Type II collagen, ACAN: Aggrecan, miR: MicroRNA, NTA: Nanoparticle tracking analysis, TEM: Transmission electron microscopy, DLS: Dynamic light scattering, TFF: Tangential flow filtration, IV: Intravenous, IA: Intra-articular, PRP: Platelet-rich plasma, MF: Microfracture.

ages over synthetic nanoparticles, including lower toxicity and enhanced cellular uptake. The anti-inflammatory and immunomodulatory effects of these vesicles make them promising therapeutic agents. For example, EVs derived from grapefruit juice [21], tomatoes, and lemons [24] have demonstrated potential in treating OA.

EVs from Antler Blastema (deer) Progenitor Cells (ABPC-EVs) demonstrate emerging regenerative potential. EVs from deer stem cells have been shown to slow the aging of human mesenchymal stem cells and enhance cartilage repair. Treatment with these EVs reversed senescence-related changes in MSCs and modulated the expression of aging-associated genes, suggesting their potential in regenerative and antiaging therapies [25].

Bacterial Extracellular Vesicles (BEVs) have recently emerged as promising nanocarriers for OA therapy, primarily owing to their unique ability to modulate the gut–joint axis. Owing to their nanoscale size, low toxicity, high drug-loading capacity, and excellent biocompatibility, BEVs can efficiently deliver bioactive molecules to regulate both the intestinal and joint microenvironments, opening new opportunities for OA treatment [26].

Source selection is one of the most important determinants of EV reproducibility and translational feasibility. MSC-derived EVs are the most widely investigated because of their immunomodulatory and chondroprotective properties, but their composition varies according to tissue origin, donor age, health status, passage number, and culture conditions. Chondrocyte- and cartilage progenitor cell-derived EVs may provide more cartilage-specific signals, yet their scalability and donor availability remain limited. Platelet-Rich Plasma (PRP)-derived EVs are clinically attractive, because PRP is already used in orthopedic practice, but their composition is highly patient-dependent and may vary with platelet preparation protocols. Plant- or food-derived EV-like nanoparticles may offer advantages in scalability and oral delivery, but their mechanisms, molecular identity, and regulatory classification remain less defined. Therefore, the optimal EV source for OA therapy should not be selected only according to regenerative potency, but also according to reproducibility, safety, scalability, manufacturing feasibility, and compatibility with regulatory quality-control requirements.

Engineering Strategies for EV-Based Cartilage Repair and Artificial EV Platforms

Although native EVs exhibit considerable regenerative potential, their therapeutic application remains limited by insufficient targeting specificity, rapid clearance, restricted cargo-loading capacity, and biological heterogeneity [27]. To address these limitations, multiple engineering strategies have been developed to enhance EV stability, targeting efficiency, cargo delivery, and regenerative performance. These approaches generally include donor-cell engineering, direct surface modification, cargo-loading techniques, and the development of artificial or hybrid EV platforms [28].

Donor-cell engineering represents an indirect strategy in which parental cells are genetically or environmentally modified to alter EV cargo composition and biological activity. One study increased the number of EVs from human urine-derived stem cells *via* the overexpression of miR-140. Compared with standard EVs, the engineered version more effectively improved chondrocyte function and increased the production of key cartilage matrix proteins by targeting Vascular Endothelial Growth Factor type A (VEGFA). These EVs promote cartilage repair and subchondral bone regeneration, highlighting their therapeutic potential [29].

Hypoxic preconditioning of MSCs is a promising method for enhancing the therapeutic potential of their EVs in OA models. Hypoxic-derived EVs (Hypo-sEVs) are more effective than normoxic sEVs in enhancing chondrocyte growth, migration, and survival. This improved effect is associated with increased levels of miR-216a-5p in Hypo-sEVs, which regulate the JAK2/STAT3 pathway involved in cartilage health. The role of HIF-1α in this process highlights hypoxic preconditioning as a powerful, nongenetic method to increase EV bioactivity [30].

In parallel, therapeutic enhancement can also be achieved through incorporation of bioactive molecules or drugs into EV systems to improve regenerative and anti-inflammatory efficacy. Another innovative approach combines curcumin with small EV (sEVs) from adipose-derived MSCs to create sEV-CUR, a hybrid therapy. Compared with free curcumin or unmodified sEVs, sEV-CUR had more substantial protective effects on OA models. It enhances chondrocyte survival under oxidative stress and, in mice, reduces joint damage and cell death, highlighting its potential for cartilage protection and regeneration [31].

Beyond natural EV modification, artificial and hybrid EV platforms have emerged as alternative strategies designed to improve scalability, targeting precision, and cargo-loading flexibility. While exogenous Hyaluronic Acid (HA) injections relieve pain in OA patients, their effects are short-lived. A new approach utilizes engineered, pH-responsive EVs that carry Hyaluronan Synthase 2 (HAS2) to reprogram chondrocytes to produce high-molecular-weight HA internally [32].

Although PRP is widely used for healing due to its growth factors, PRP-EVs may promote angiogenesis during tissue repair, thereby supporting nutrient and oxygen delivery to injured areas and facilitating waste removal. In orthopedic conditions such as knee OA, PRP-derived EVs show therapeutic potential. They increase chondrocyte proliferation and reduce apoptosis by activating the Wnt/β-catenin pathway, which is essential for joint stability [33].

Another recent study examined the limitations of EVs in treating temporomandibular joint OA, suggesting that Artificial Cell-Derived Vesicles (ACDVs) may offer a superior alternative. When created from stem cells *via* extrusion techniques, ACDVs resemble EVs in structure but provide a greater yield of particles and proteins [34].

A stable hybrid system that integrates EVs and lipid nanoparticles through ethanol-mediated fusion retains the bioactive properties and targeting capabilities of both elements. The final hybrid particle contained nicotinamide-loaded, Col2A1 antibody-modified liposomes alongside TGF-β1-overexpressing EVs. This hybrid particle exhibited significant cartilage-protective and anti-inflammatory properties, featuring precise targeting and prolonged retention at OA sites [35].

Surface Modification for Targeted Delivery

One major strategy for improving EV therapeutic efficacy involves surface functionalization with targeting ligands that enhance tissue-specific delivery [36]. This strategy is particularly important in cartilage-targeted therapies, where natural EVs face a significant delivery barrier due to their negatively charged membrane. This leads to electrostatic repulsion from the anionic cartilage matrix, thereby reducing its retention and uptake [37].

In response, charge-reversal engineering strategies have been developed through the surface decoration of EVs with positively charged motifs of avidin or peptide carriers rich in arginine. These surface-modified EVs were then loaded with Interleukin-1 Receptor Antagonist (IL-1RA), a disease-modifying agent for OA. These EVs exhibited increased retention in joints and enhanced therapeutic delivery, indicating an essential advantage in penetrating cartilage barriers [38]. In another strategy, bio-conjugated EVs with a Cartilage-binding Peptide (CAP) are loaded with MMP13-targeting siRNA, an enzyme responsible for cartilage matrix degradation. This engineered delivery system, known as CAP-Exo/siMMP13, effectively targeted chondrocytes, inhibited MMP13 expression, and induced the expression of cartilage-specific markers in an OA rat model. This outcome further underscores the potential of EV-mediated gene silencing for the safe and therapeutic treatment of OA [39]. Another study used a collagen-binding domain peptide attached to the exosomal membrane protein LAMP-2B to bind miR-21-loaded EVs to collagen-I scaffolds. This helps improve the local retention of vesicles at sites of cartilage lesions, enhancing therapeutic efficacy [40].

Furthermore, dendritic cell-derived EVs tend to be distributed throughout the body, with notable accumulation in the kidney. To enhance site-specific delivery, a chondrocyte-affinity peptide that effectively delivers miR-140 into cartilage was fused to the EV surface, significantly improving its retention within the articular cavity. This modification not only confines the EVs to the joint space, but also promotes their penetration into cartilage, enabling targeted delivery to chondrocytes and thereby enhancing therapeutic efficacy [41]. One study incorporated a glycosylation motif at the N-terminus of the peptide–LAMP–2B construct to increase its stability. Additionally, a peptide was introduced for targeted delivery to Synovial Fluid-derived Mesenchymal Stem Cells (SF-MSCs). EVs engineered in this way and produced from dendritic cells were subsequently loaded with kartogenin. When applied *in vivo* and *in vitro*, these engineered EVs selectively targeted SF-MSCs and significantly increased the differentiation of MSCs into chondrocytes and promoted cartilage regeneration [42].

Compared with unmodified EVs, surface-engineered vesicles generally demonstrate enhanced cartilage targeting, prolonged intra-articular retention, and improved therapeutic efficacy. However, these modifica-

tions may increase manufacturing complexity, production costs, and potential immunogenicity, which remain important considerations for large-scale clinical translation.

Loading of Different Cargo Types for Enhanced Therapeutic Efficacy

Among the molecules delivered by EVs, RNA-based therapeutics, such as microRNAs (miRNAs), small interfering RNAs (siRNAs), and long noncoding RNAs (lncRNAs), are significant [43]. Additionally, short hairpin RNAs (shRNAs) and circular RNAs (circRNAs) have gained considerable attention for their ability to modulate gene expression and their potential application in treating a wide range of medical conditions (Table 2) [44].

miRNAs are small, noncoding, single-stranded RNAs, typically 19–24 nucleotides in length, that regulate gene expression at the post-transcriptional level. They are essential for normal physiological development and are implicated in various biological processes [45]. miRNAs originate from precursor molecules that form a characteristic hairpin-shaped loop structure [46]. Circular RNAs (circRNAs) are conserved, single-stranded RNA molecules with a closed-loop structure. Found across many species, they play essential roles in gene regulation by acting as transcriptional regulators, microRNA sponges, and, occasionally, templates for protein synthesis [47]. Like those of microRNAs, the expression patterns of specific circular RNAs (circRNAs) have also been associated with the progression of OA [48]. Long noncoding RNAs (lncRNAs) represent a significant class of RNA transcripts that are longer than 200 nucleotides and do not encode proteins. Although over 18,000 lncRNAs have been discovered, only a small number of them have established functions. Charac-terized lncRNAs participate in various regulatory processes, including gene expression, chromatin remodeling, and protein activity. Importantly, they demonstrate high specificity to cell types and play a crucial role in immune regulation [49].

Proteins, in addition to recombinant protein drugs, have good therapeutic potential because they adjust inflammatory pathways, balance cartilage catabolism and anabolism, and support tissue repair. However, their rapid clearance and short intra-articular half-life pose significant barriers to their clinical application, highlighting the need for effective and sustained drug delivery systems to improve therapeutic outcomes [50]. Advancements in genetic engineering have expanded the use of protein-based therapeutics, which are valued for their high efficacy, specificity, and low toxicity. However, their immunogenicity can limit their clinical application, which may trigger unwanted immune responses and allergic reactions [51]. In addition to the other cargoes discussed previously, EVs can be engineered to carry synthetic small molecules and common related drugs, offering a versatile platform for targeted drug delivery in regenerative medicine.

Different loading strategies offer distinct advantages. Passive loading approaches are technically simple and better preserve EV membrane integrity; however, they often exhibit poor encapsulation efficiency and limited control over cargo concentration. In contrast, active loading methods provide greater loading efficiency and cargo control but may compromise EV structural stability and biological activity. Therefore, selecting an appropriate loading strategy requires balancing loading efficiency, vesicle integrity, cargo type, and scalability requirements.

Overall, these studies demonstrate that engineering strategies can significantly enhance EV targeting, cargo delivery, and therapeutic efficacy, although differences in methodology and lack of standardization remain important challenges.

Engineered EVs exert their therapeutic effects through coordinated modulation of key signaling pathways involved in cartilage homeostasis. For instance, EV-mediated delivery of miRNAs and proteins frequently targets pathways such as JAK/STAT, Wnt/β-catenin, and TGF-β/Smad, which regulate chondrocyte proliferation, apoptosis, and extracellular matrix synthesis. Furthermore, suppression of inflammatory mediators such as IL-1β and TNF-α appears to be a central mechanism through which EVs attenuate cartilage degradation. These findings highlight that the therapeutic efficacy of engineered EVs is not merely dependent on cargo delivery, but on their ability to reprogram cellular signaling networks toward a regenerative phenotype.

EV Loading, Cargo Release, and Cellular Uptake

Therapeutic agents can be loaded into EVs through either active or passive encapsulation methods, each providing different levels of efficiency and cargo stability within the vesicles (Figure 2). Passive cargo-loading techniques provide a straightforward approach for incorporating therapeutic agents into EVs. This process involves culturing cells with the drug of interest, allowing the cells to naturally take up the drug, and then integrating it into the resulting EVs. The resulting drug-loaded EVs are then released into the culture medium. However, it usually results in low loading efficiency and offers limited control over the amount of drug packaged into the EVs. It tends to be more effective for lipophilic compounds that readily cross cellular and vesicular membranes. Nonetheless, this method may not be suitable for all therapeutic agents [52]. An alternative to passive loading is active loading, where drugs are introduced directly into pre-isolated EVs. This approach typically involves techniques that temporarily increase EV membrane permeability, including sonication, heat shock, electroporation, detergent incubation, or the use of saponins. Active loading generally increases drug encapsulation efficiency and offers more precise control over the amount of drug incorporated. However, it can be technically demanding and time-consuming, and improper handling may compromise the structural integrity of the

Table 2. Representative EV cargos and their mechanisms in promoting cartilage repair

Cargo type	RNA/Protein	EV Source	Target/Pathway	Therapeutic Effect	Ref.
miRNA	miR-206	BMSC-derived exosomes	ELF3	Enhances osteoblast proliferation, reduces inflammation, and apoptosis	[1]
	miR-148a	Milk-derived EVs	Catabolic and inflammatory pathways	Protects cartilage by maintaining homeostasis	[2]
	miR-214-3p	SFB-derived exosomes	NF-κB signaling pathway	Reduces inflammation and apoptosis, protects cartilage	[3]
	miR-9-5p	Bone marrow MSC-derived exosomes	Syndecan-1 (SDC1)	Reduces inflammation, oxidative stress, and cartilage degradation	[4]
	miR-127-3p	MSC-derived exosomes	CDH11, Wnt/β-catenin pathway	Suppresses cartilage degradation and joint inflammation	[5]
	miR-31	Synovial MSC-derived exosomes (SMSC-EVs)	KDM2A/E2F1/PTTG1 axis	Enhances chondrocyte function and alleviates cartilage damage and inflammation	[6]
	miR-136-5p	MSC-derived exosomes	ELF3	Protects against chondrocyte degeneration after joint injury	[7]
	miR-100-5p	Infrapatellar fat pad MSC-derived exosomes	mTOR signaling pathway	Protects cartilage and improves gait in OA models	[8]
	miR-129-5p	Human synovial MSC-derived exosomes	HMGB1	Reduces inflammation and apoptosis in chondrocytes	[9]
	miR-130b-3p	EVs (unspecified)	LRP12/AKT/β-catenin signaling	Protects against inflammation and ECM degradation in chondrocytes	[10]
	miR-181c-5p	Bone marrow MSCs	SMAD7	Promotes BMP2-induced cartilage repair	[11]
	miR-135b	MSC-derived exosomes	Sp1 transcription factor	Enhances chondrocyte proliferation	[12]
	miR-92a-3p	MSC-derived exosomes (overexpressing)	WNT5A	Enhances chondrogenesis and reduces cartilage degradation	[13]
	miR-150-3p	H–FLS–derived EVs	Trim14/NF-κB/IFNβ axis	Maintains joint homeostasis and reduces inflammation	[14]
	miR-320	MSC-derived exosomes	MMP-13, IL-1β	Regulates ECM remodeling and inflammation in chondrogenesis	[15]
	miR-140-5p	Human synovial MSC-derived exosomes (overexpressing)	Not specified	Promotes cartilage repair and delays OA progression	[16]
	miR-143	Exosomes from curcumin-treated MSCs	NF-κB, ROCK1	Enhances chondrocyte viability, reduces apoptosis, and restores miR-143 expression	[17]
	miR-124	Exosomes from curcumin-treated MSCs	NF-κB, ROCK1	Restores miR-124 expression, suppresses inflammation, and cartilage degradation	[18]
circRNA	circRNA_0001236	Exosomes	Sox9, MMP13 *via* miR-3677-3p	Facilitates chondrogenesis and alleviates OA symptoms	[19]
	circSERPINE2	Exosomes	miR-495/TGFBR2 pathway	Suppresses apoptosis and inflammation in OA	[20]
	hsa_circ_0005567	Exosomes	miR-495/ATG14 axis	Restores autophagy and protects cartilage	[21]
	circHIPK3	Exosomes	miR-124/SOX8	Regulates chondrocyte behavior	[22]
lncRNA	PCGEM1	Synovial fluid-derived exosomes	Not specified	Biomarker and stage indicator in OA	[23]
	KLF3-AS1	hMSC-derived exosomes	KLF3-AS1/miR-206/GIT1 axis	Reduces chondrocyte apoptosis and promotes cartilage repair	[24]
	TRAF1-4:1	RA-FLS-derived exosomes	miR-27a-3p/CXCL1	Impairs cartilage by ECM degradation and inflammation	[25]
	H19	Umbilical cord MSC-derived exosomes	miR-29b-3p/FoxO3	Enhances osteochondral regeneration and reduces apoptosis	[26]
	H19	Fibroblast-like synoviocyte-derived exosomes	miR-106b-5p/TIMP2	Mitigates OA progression	[27]

Contd. Table 2. Representative EV cargos and their mechanisms in promoting cartilage repair

Cargo type	RNA/Protein	EV Source	Target/Pathway	Therapeutic Effect	Ref.
Protein	scSOX9	Cell-penetrating recombinant protein	Not specified	Promotes hyaline-like cartilage formation in a rabbit model	[28]
	FGF18 (*via* CRISPR activation)	Targeted exosomes (CAP/FGF18-hyEXO)	FGF18 gene activation	Enhances cartilage regeneration, reduces inflammation, and ECM degradation	[29]
	Cas9	Chondrocyte-targeted exosomes fused with liposomes (CAP-Exo)	MMP-13	Reduces cartilage breakdown *via* gene editing	[30]
	MBPs	MSC-derived exosomes	Not specified	Support cartilage repair and attenuate inflammation in osteoarthritic joints	[31]
Synthetic molecules and drugs	Dexamethasone sodium phosphate	Folic acid–modified exosomes (FPC-Exo/Dex)	Inflamed joints in RA	Anti-inflammatory, preserves bone/cartilage in CIA model	[32]
	Kartogenin	E7-peptide engineered exosomes (E7-Exo)	MSC targeting, enhanced KGN intracellular dispersion	Promotes cartilage regeneration in OA	[33]
	Curcumin	Exosomes from curcumin-treated MSCs	NF-κB and ROCK1 (*via* miR-143 and miR-124)	Enhances chondrocyte viability and reduces apoptosis in OA models	[17]

Abbreviations: BMSC: Bone marrow–derived mesenchymal stem cell, MSC: Mesenchymal stem cell, SMSC: Synovial mesenchymal stem cell, hMSC: Human mesenchymal stem cell, UC-MSC: Umbilical cord–derived mesenchymal stem cell, FLS: Fibroblast-like synoviocyte, RA-FLS: Rheumatoid arthritis fibroblast-like synoviocyte, H–FLS: Human fibroblast-like synoviocyte, EV: Extracellular vesicle, Exo: Exosome, OA: Osteoarthritis, RA: Rheumatoid arthritis, ECM: Extracellular matrix, BMP2: Bone morphogenetic protein 2, MMP: Matrix metalloproteinase, NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells, ROCK1: Rho-associated protein kinase 1, CAP: Cartilage-affinity peptide, CAP-Exo: Chondrocyte-targeted exosome, CAP/FGF18-hyEXO: Cartilage-targeted exosome engineered for FGF18 activation, CIA: Collagen-induced arthritis, scSOX9: Single-chain SOX9 protein, MBPs: Matrix-binding proteins, FPC-Exo/Dex: Folic acid–modified exosome loaded with dexamethasone, E7-Exo: E7-peptide–engineered exosome, KGN: Kartogenin, miR: MicroRNA, circRNA: Circular RNA, lncRNA: Long non-coding RNA, Sox9: SRY-box transcription factor 9, SMAD7: Mothers against decapentaplegic homolog 7, HMGB1: High mobility group box 1, ATG14: Autophagy-related protein 14, TGFBR2: Transforming growth factor beta receptor 2, FoxO3: Fork-head box O3, TIMP2: Tissue inhibitor of metalloproteinases 2, GIT1: G-protein-coupled receptor kinase-interacting protein 1, IFNβ: Interferon beta, LRP12: Low-density lipoprotein receptor-related protein 12, SDC1: Syndecan-1, ELF3: E74-like ETS transcription factor 3, mTOR: Mechanistic target of rapamycin.

EVs. Regardless of the loading method, removing unincorporated drugs is essential and is usually achieved through purification techniques such as ultracentrifugation, size exclusion chromatography, or ultrafiltration [53]. Additionally, genetic engineering provides a strategy for incorporating specific genetic materials into EVs. This involves modifying the donor cells to express the desired RNAs or proteins, which are then packaged into EVs during biogenesis. Although this technique allows the targeted delivery of nucleic acids or proteins, it relies on advanced molecular biology tools and may not be practical for all therapeutic applications. Each approach presents advantages and limitations, and selecting the most suitable method depends on variables such as the drug's properties, the EV source, and the therapeutic target [52].

Strategies for loading bioactive cargo into EVs can be broadly classified into pre-loading (endogenous) and post-loading (exogenous) approaches. In the pre-loading approach, parental cells are exposed to therapeutic molecules (*e.g.*, proteins, RNAs, plasmids, drugs), which are naturally incorporated into EVs during biogenesis and subsequently secreted. In the post-loading approach, isolated EVs are engineered to carry cargoes through techniques such as transfection, electroporation, incubation, sonication, freeze–thaw cycling, extrusion, detergent permeabilization using agents such as saponin, or fusion with liposomes to form chimeric EVs. These complementary strategies enhance the versatility of EVs as delivery vehicles for nucleic acids, proteins, and small molecules in both experimental and therapeutic applications.

Consequently, the efficient therapeutic use of EVs depends on effectively loading functional molecules into vesicles and delivering them precisely to target cells. To increase the bioavailability and performance of EV-based treatments, cargo-loading strategies and cellular uptake mechanisms must be optimized to maximize their effectiveness. Once released, EVs can interact with recipient cells through several pathways, including endocytosis, direct fusion with the plasma membrane, or ligand and receptor interactions that trigger cell-to-cell signaling. Endocytosis is the most common uptake mechanism and is mediated by various routes, such as clathrin-dependent and caveolin-dependent pathways, macropinocytosis, and phagocytosis [54]. A fusion-based mechanism enables the rapid delivery of exosomal contents into the cytoplasm and is considered more efficient than the endocytic route [55]. Nonetheless, *via* the uptake route, EV cargo must ultimately be released into the cytoplasm to perform its biological functions. Otherwise, cargoes delivered through endocytosis can be degraded in lysosomes or removed from the cell, thus diminishing therapeutic efficacy [56]. Finally, while EV–cell interac-

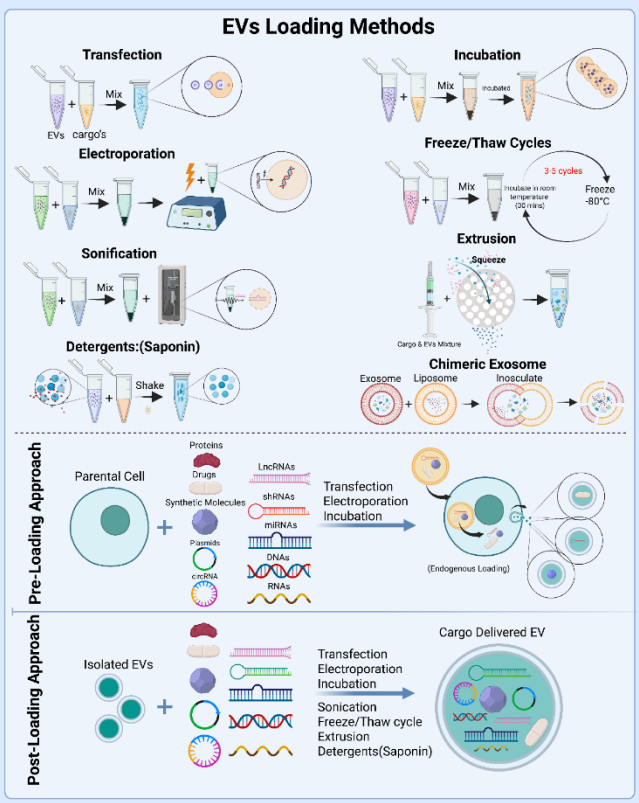

Figure 2. Endogenous and exogenous strategies for cargoes loading into EVs.
Overview of the principal approaches used for engineering EVs as therapeutic delivery systems. In endogenous (pre-loading) strategies, parental cells are modified or exposed to therapeutic agents, including proteins, RNAs, plasmids, or small-molecule drugs, which are subsequently incorporated into EVs during biogenesis and secretion. In exogenous (post-loading) strategies, isolated EVs are directly engineered using methods such as electroporation, transfection, passive incubation, sonication, extrusion, freeze–thaw cycling, detergent-mediated permeabilization, or liposome fusion to generate cargoes-loaded or chimeric vesicles. These engineering approaches improve the versatility of EVs for targeted delivery of nucleic acids, proteins, and bioactive compounds in regenerative and therapeutic applications.

tions depend primarily on the surface features of both vesicles and recipient cells, the exact nature and specificity of these interactions remain a subject of ongoing investigation. It is still debated whether EV uptake is a directed process or occurs randomly [57].

In the studies reviewed, dosing strategies for EV-based therapies vary widely and are often determined based on preliminary cytotoxicity assays or prior literature. However, the lack of standardized dose–response frameworks and inconsistent reporting of IC_{50} or therapeutic thresholds remains a major limitation in the field. Establishing dose standardization will be essential for improving reproducibility and clinical translation.

2D and 3D culture

The biological activity and therapeutic efficiency of EVs are strongly influenced by the microenvironment in which parental cells are cultured. Conventional two-Dimensional (2D) culture systems fail to precisely replicate the native cartilage microenvironment, whereas three-Dimensional (3D) culture platforms better mimic physiological cell–matrix interactions, and mechanical stimuli. Consequently, increasing attention has focused on 3D culture systems and biomaterial-based scaffolds to enhance EV production and regenerative potency.

A recent study revealed that cryogels made from extracellular matrix components such as gelatin, chondroitin sulfate, and hyaluronic acid can promote cartilage repair, particularly when combined with EVs from BMSCs. Among the formulations tested, cryogels containing 0.3% HA provided the optimal balance of strength and cell compatibility. Laboratory and animal studies confirmed that EVs and EV-infused cryogels enhanced cartilage matrix production. However, EV-loaded cryogels result in the most organized tissue structure and effective regeneration, positioning them as promising tools for cartilage repair [58]. Additionally, in another study, a bilayer cryogel scaffold featuring a cartilage-like layer (chitosan-gelatin-chondroitin sulfate) and a bone-like layer (nanohydroxyapatite-gelatin) was developed to repair osteochondral defects. Chondrocyte-derived EVs enhanced regeneration, with approximately 80% released within 72 *hr*, while chondroitin sulfate was gradually released over a week. The combination of EVs and scaffold extract improved chondrocyte growth and migration *in vitro*, indicating potential for osteochondral tissue repair [59].

A study examined umbilical-derived EVs under conventional 2D culture and 3D microgravity conditions for cartilage repair. Both types of EVs promoted chondrocyte proliferation, migration, and matrix synthesis and reduced apoptosis. However, EVs derived from 3D culture exhibited superior biological activity, higher yields, and an enhanced ability to maintain chondrocyte phenotypic stability. This effect was at tributed mainly to improved regulation of matrix homeostasis through the TGF-β1/Smad2/3 signaling pathway, suggesting that 3D-derived U-MSC-EVs may offer greater therapeutic efficiency in cartilage regeneration (Figure 3) [60]. Recent advances in the 3D bioprinting of hydrogels, especially those loaded with U-MSC-EVs, have shown potential for improving tissue regeneration and wound healing. The combination of EVs with 3D bioprinted hydrogels (3D-BPH-Exos) provides a novel platform for OA treatment by reducing the levels of inflammatory mediators such as IL-1β, TNF-α, and IFN-γ; preserving mitochondrial function; and enhancing chondrogenesis in joint-resident cells. Loading these EVs with chondrogenic agents, such as kartogenin, may offer a more effective, localized, and safer alternative to conventional anti-inflammatory or cell-based therapies. This emerging strategy emphasizes the potential of 3D-BPH-Exos as a next-generation immunomodulatory and regenerative approach for OA management [61]. A different study revealed that culturing U-MSCs in a 3D environment with human platelet lysate significantly enhances the produc-

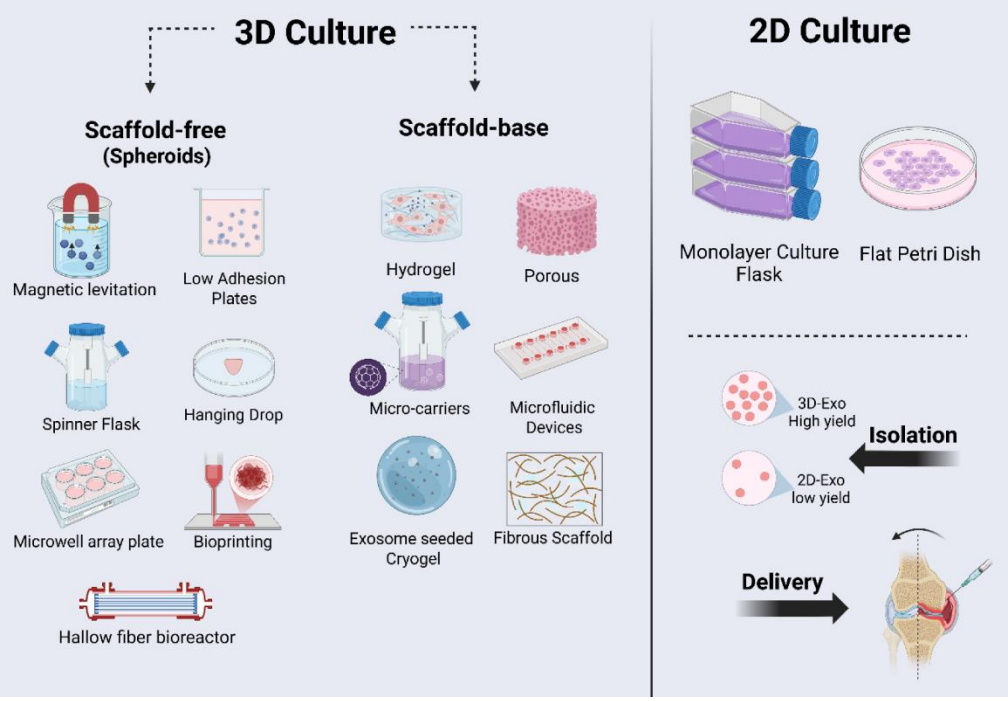

Figure 3. Comparison of two-dimensional (2D) and three-dimensional (3D) culture systems for EV production.
Schematic comparison between conventional 2D monolayer culture systems and advanced 3D culture platforms used for EV production and research. In 2D cultures, cells are grown on flat surfaces such as flasks or Petri dishes, resulting in limited cell–cell and cell–matrix interactions and relatively lower EV yield. In contrast, 3D culture systems more closely mimic the native *in vivo* microenvironment and support enhanced cellular communication, physiological behavior, and EV secretion. Scaffold-free 3D approaches include magnetic levitation, hanging drops, spinner flasks, low-adhesion plates, microwell arrays, bioprinting, and hollow-fiber bioreactors, whereas scaffold-based systems include hydrogels, porous scaffolds, microcarriers, fibrous matrices, cryogels, and microfluidic platforms. The figure emphasizes the advantages of 3D culture systems in generating more physiologically relevant EVs with improved therapeutic potential for regenerative medicine and intra-articular delivery applications.

tion and therapeutic efficacy of 3D-EVs. Compared with 2D-derived EVs, 3D-EVs promote the proliferation of MSCs and chondrocytes, suppress inflammatory and catabolic responses, and improve cartilage repair *in vivo* when administered through a hydrogel. The regenerative benefits were associated with increased synthesis of type II collagen and decreased expression of MMP13, underscoring 3D-EVs as a promising approach for effective EV production and cartilage regeneration [62].

Translational challenges, regulatory considerations, and future perspectives

While engineered EVs have established promising regenerative and immunomodulatory effects in preclinical OA models, numerous biological, manufacturing, regulatory, and economic barriers continue to limit their clinical translation. The effective integration of EV-based therapies into orthopedic practice will necessitate scalable manufacturing systems, and clear regulatory frameworks. Additionally, patient heterogeneity and disease-stage variability require important considerations that may significantly influence therapeutic sensitivity.

An important challenge involves the biological heterogeneity of OA itself. OA is increasingly recognized as a multifactorial and heterogeneous disease with distinct inflammatory, metabolic, traumatic, and age-associated phenotypes. Consequently, therapeutic responsiveness to EV-based interventions may differ significantly among patient populations. Recent evidence suggests that early-stage or moderate OA, where viable chondrocytes and partially preserved cartilage architecture remain present, is more likely to benefit from regenerative EV-based therapies.

In contrast, advanced OA characterized by extensive cartilage loss, severe subchondral bone remodeling, and chronic inflammation may require combination strategies involving biomaterials, surgical intervention, or joint replacement. Furthermore, obesity-associated and metabolically driven OA phenotypes may respond differently because of different inflammatory microenvironments. Therefore, future clinical translation will likely depend on personalized therapeutic approaches to improve patient satisfaction.

A significant challenge in harnessing EVs for clinical applications lies in their isolation and characterization, as current methods vary considerably in terms of efficiency, yield, and purity. These factors directly affect reproducibility across studies and therapeutic reliability. Techniques such as ultracentrifugation, size-exclusion chromatography, and polymer-based precipitation offer unique advantages and limitations; however, none have yet been universally accepted as the gold standard [63].

On the other hand, owing to their nanoscale size, EVs are too small to be visualized through conventional light

microscopy, and reliable characterization of EVs is essential for evaluating their size, concentration, and molecular content, which can significantly influence their therapeutic efficacy. Comprehensive physical, proteomic, and genomic analyses are therefore required to accurately assess EV bioactive cargoes and their functional properties. Variability in these parameters can lead to diverse clinical outcomes, highlighting the critical need for robust quality-control standards to ensure that only high-quality, reproducible EV preparations are employed in therapeutic settings [64].

EVs offer benefits over whole-cell therapies, including reduced immunogenicity, a lower risk of tumor formation, and improved stability in storage. However, progress toward approved OA treatments is slow due to challenges such as low yield, limited scalability, and high heterogeneity, which were discussed above. To improve clinical application, standardized purification methods are essential, along with the cautious selection of EV sources to ensure consistent therapeutic quality. Additionally, understanding the biodistribution of EVs and their effects on non-cartilaginous tissues is crucial for safety and targeting. Addressing scientific and regulatory challenges and conducting thorough preclinical and clinical studies are vital for integrating EV-based therapies into OA treatment [65].

Regulatory authorities, including the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA), currently classify human EV-based products as biological therapeutics, requiring compliance with stringent safety, quality, and efficacy criteria [66]. Regulatory standards for EV-based therapies vary worldwide, with differences in product classification, extraction methods, manufacturing requirements, and quality-control expectations across regions such as the United States, Europe, Japan, South Korea, and Taiwan. Despite these regional differences, the shared objective is to ensure that EV products meet rigorous standards for quality, safety, and efficacy. These regulations emphasize the importance of detailed documentation, traceability, and control throughout the product lifecycle, from raw material sourcing to final distribution and clinical use [67].

From a practical translational perspective, engineered EV products must satisfy several manufacturing and quality-control requirements before clinical implementation. GMP-compliant production requires standardized donor-cell sourcing, serum-free or xeno-free culture conditions, validated isolation methods, sterile processing, and reproducible storage protocols. Batch-to-batch variability remains a major barrier because EV yield, size distribution, cargo composition, and biological potency can change according to donor characteristics, passage number, culture conditions, and purification methods. Therefore, quality-control panels should include particle concentration, size distribution, morphology, protein and RNA markers, purity indicators, sterility, endotoxin testing, residual DNA or reagent assessment, and stability after storage. In addition, potency assays must be functionally linked to the intended therapeutic mechanism, such as suppression of inflammatory mediators, reduction of MMP13 or ADAMTS5 activity, enhancement of COL2A1 and ACAN expression, or promotion of chondrocyte survival under inflammatory stress. Without validated potency assays and release criteria, regulatory approval and inter-study reproducibility will remain limited.

Long-term safety evaluation is also essential because EVs can interact with multiple joint and non-joint cell populations. Potential concerns include undesired immune modulation, off-target biodistribution, profibrotic responses, altered synovial activity, ectopic tissue effects, and unknown consequences of repeated intra-articular dosing. These risks may be amplified in engineered EVs carrying exogenous RNAs, drugs, targeting peptides, or hybrid nanoparticle components. Therefore, future studies should evaluate biodistribution, retention time, degradation, repeat-dose toxicity, immunogenicity, and long-term joint structural outcomes before clinical translation.

Economic concerns may also influence the extensive acceptance of EV-based therapies. Compared with conventional treatments such as corticosteroid injections, hyaluronic acid supplementation, or PRP, engineered EV production currently requires sophisticated isolation technologies, quality-control systems, and specialized manufacturing facilities, which considerably increase treatment costs. In addition, the cost-effectiveness of EV-based therapies compared with existing treatments remains unclear and may represent a barrier to large-scale clinical implementation.

Cartilage repair is rapidly evolving through bioengineering, precision medicine, and combinatorial strategies like EVs, bioactive scaffolds, and gene therapies. While these advances show great promise for true regeneration, rigorous clinical trials, standardized protocols, and regulatory clarity are still needed to translate innovations into safe, patient-specific treatments [68].

At present, most EV-based therapies remain in preclinical or early clinical trial stages. Widespread clinical application is likely to require several years, depending on the resolution of current challenges related to standardization, safety, and regulatory approval.

The future of OA treatment holds significant promise through the incorporation of EVs and cutting-edge technologies such as machine learning and artificial intelligence to facilitate diagnosis. In recent studies, machine learning models have been used to diagnose OA at its earliest stages *via* untargeted metabolomics data from EV isolates obtained *via* liquid chromatography–tandem mass spectrometry. This innovative approach highlights the possibilities of combining EV-based therapies with advanced AI-driven diagnostics, paving the way for more effective and timely interventions in the fight against OA [69].

Although engineered EVs offer significant regenerative potential, overcoming current scientific, manufacturing, and regulatory limitations will determine whether these therapies can successfully transition from experimental platforms to routine clinical practice.

Conclusion

OA is a highly prevalent and disabling joint disorder for which current therapeutic strategies mainly provide symptomatic relief rather than restoring damaged cartilage. This limitation has driven growing interest in regenerative approaches capable of targeting the causal pathological mechanisms of disease progression. Among developing strategies, EVs, particularly those derived from MSCs, have gained significant attention as a promising cell-free regenerative platform. Through their immunomodulatory, anti-inflammatory, and pro-regenerative properties, EVs may help restore chondrocyte homeostasis, enhance anabolic activity, and promote extracellular matrix synthesis, thus supporting cartilage repair and joint tissue protection.

Recent advances in bioengineering have further expanded the therapeutic potential of EV-based therapies. As mentioned, surface engineering strategies improve tissue targeting and retention. Moreover, cargo-loading approaches enhance therapeutic efficacy through the delivery of bioactive molecules, while cellular preconditioning methods strengthen the biological activity of EVs under pathological microenvironments. Collectively, these innovations represent a vital step toward more accurate and effective regenerative interventions for OA.

A key outcome measure for future EV-based OA studies should be the quality of regenerated tissue. Many interventions described as "cartilage regeneration" may actually produce fibrocartilage-like repair tissue rather than native hyaline cartilage. Therefore, future studies should distinguish between symptomatic improvement, matrix deposition, fibrocartilage repair, hyaline-like cartilage formation, and restoration of native cartilage architecture using histological, biomechanical, and molecular criteria.

Nevertheless, several critical challenges continue to hinder clinical translation. Variability in EV source, isolation, purification, and characterization contributes to significant heterogeneity and limits reproducibility across studies. In addition, uncertainties regarding optimal dosing, biodistribution, long-term safety, storage stability, and scalable manufacturing remain major barriers to regulatory approval and widespread clinical application.

Although preclinical studies have demonstrated encouraging regenerative and chondroprotective effects, clinical evidence remains limited, and the long-term therapeutic efficacy of engineered EVs in human OA has yet to be fully established. Future progress in this field may focus on standardized production protocols, deeper mechanistic insights, and robust clinical trials designed to evaluate safety, efficacy, and translational viability. Integration of EVs with biomaterials, tissue engineering platforms, and advanced nanobiotechnology may further enhance their regenerative capacity and therapeutic precision.

In conclusion, engineered EVs represent a rapidly developing and promising frontier in cell-free regenerative medicine for OA. While important scientific, manufacturing, and regulatory challenges remain uncertain, continued advances in EV engineering and translational research may ultimately support the development of more targeted disease-modifying therapies for OA if current translational barriers are resolved.

Data availability statement

No data was used for the research described in the article.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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