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MINIREVIEW

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Mesenchymal stem cell–derived small extracellular vesicles and bone regeneration

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Abstract

Mesenchymal stem cells (MSCs) and MSC-derived small extracellular vesicles (sEVs) are promising candidates for cell-based and cell-free regenerative medicine, respectively. By virtue of their multiple lineage differentiation capacity, MSCs have been implicated as an ideal tool for bone and cartilage regeneration. However, later observations attributed such regenerative effects to MSC-secreted paracrine factors. Exosomes, endosomal originated sEVs carrying lipid, protein and nucleic acid cargoes, were identified as components of the MSC secretome and propagated the key regenerative and immunoregulatory characteristics of parental MSCs. Here, exosome biogenesis, the molecular composition of exosomes, sEV-cell interactions and the effects on key bone homeostasis cells are reviewed. MSC-derived sEVs show to promote neovascularization and bone and cartilage regeneration in preclinical disease models. The mechanisms include the transfer of molecules, including microRNAs, mRNAs and proteins, to other key cells. MSC-derived sEVs are interesting candidates as biopharmaceuticals for drug delivery and for the engineering of biologically functionalized materials. Although major exploratory efforts have been made for therapeutic development, the secretion, distribution and biological effects of MSC-derived sEVs in bone and cartilage regeneration are not fully understood. Moreover, techniques for high-yield production, purity and storage need to be optimized before effective and safe MSC-derived sEVs therapies are realized.

KEYWORDS

Bone regeneration, Cell-cell communication, Exosomes, Mesenchymal stem cells, small extracellular vesicles

1 | CELL-CELL COMMUNICATION AND ROLES OF MSCs DURING BONE REGENERATION

Bone and cartilage are the main tissues of the skeletal system and consist of multiple cellular and molecular components. The interaction and coordination of these cellular and molecular components is critical for maintaining homeostasis and successfully regenerating bone and cartilage.[1,2] Fundamental cellular components in the regeneration process are the bone- and cartilage-forming cells, particularly the mesenchymal stem cell (MSC)-osteoblast-osteocyte

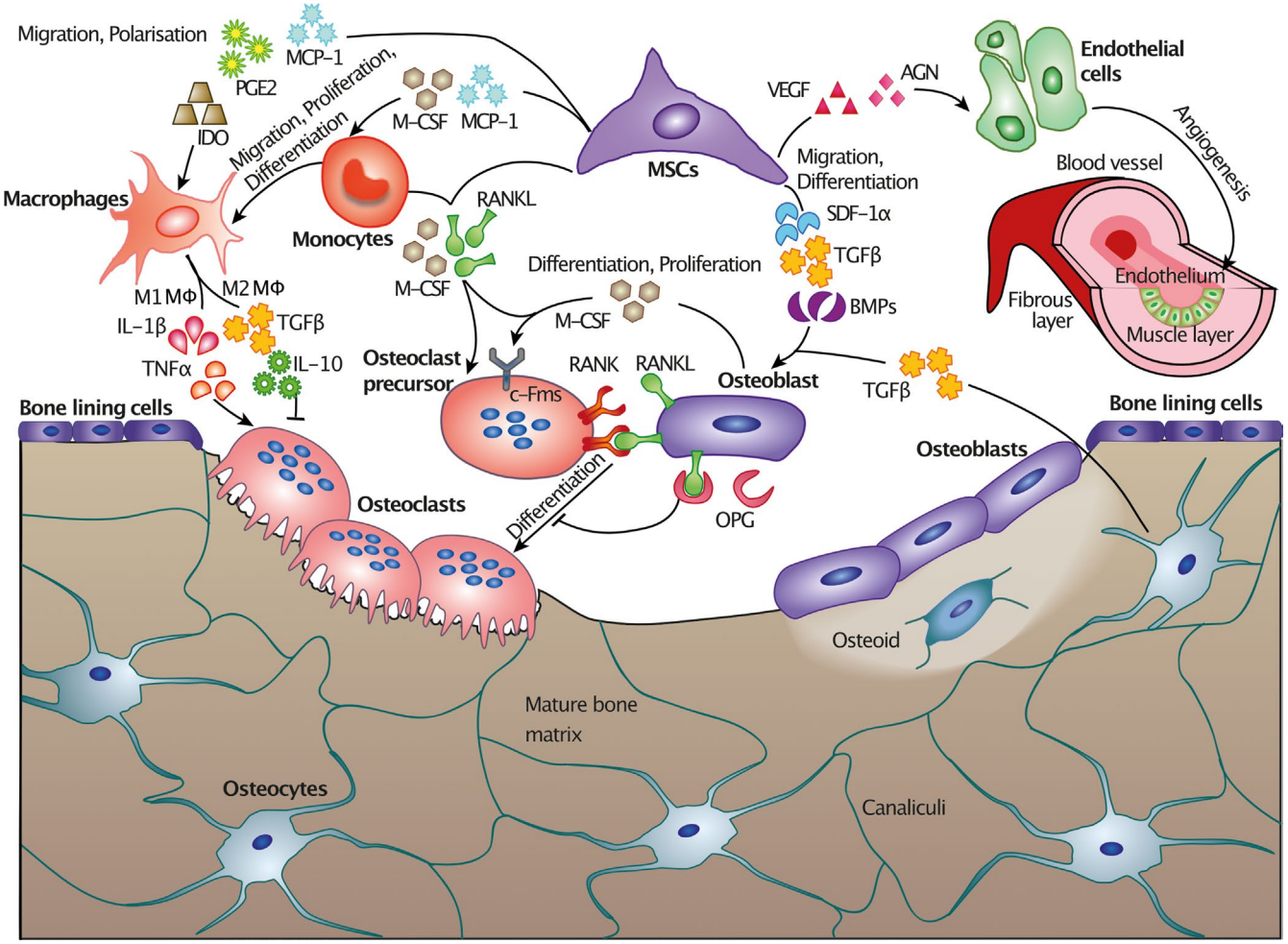

FIGURE 1 Multiple cellular and molecular interactions during bone regeneration. Some of the representative cellular interactions and responsible molecules are illustrated in the figure. (1) MSCs—OB/osteocytes: MSCs commit to osteoblasts and terminally differentiate to osteocytes. The secretion of SDF-1α, TGFβ and BMPs promotes the migration and differentiation of osteoblastic progenitor cells. (2) MSCs—Mo/Mϕ: MSCs regulate migration, proliferation, differentiation and polarization of monocytes/macrophages via secretion of MCP-1, M-CSF, PGE2 and IDO. (3) MSCs/OB—Mo/OC: MSCs/osteoblasts interact with the osteoclastic li—neage via secretion of M-CSF, RANKL and OPG, which regulate the proliferation, differentiation and activation of osteoclasts. (4) Mo/MΦ—OC: Macrophages differentially influence the activity of osteoclasts via secretion of pro- or anti-inflammatory cytokines, depending on the Mϕ phenotypes. (5) MSCs—EC: MSC-secreted VEGF and AGN promote angiogenesis via increased proliferation, migration and tube formation of endothelial cells. OB, osteoblast; OC, osteoclast; Mo, monocyte; Mϕ, macrophage; M1 Mϕ, proinflammatory Mϕ; M2 Mϕ, anti-inflammatory Mϕ; EC, endothelial cell; SDF-1α, stromal cell-derived factor 1α; TGFβ, transforming growth factor β; BMPs, bone morphogenetic proteins; M-CSF, macrophage colony-stimulating factor; RANK, receptor activator of nuclear factor-κB; RANKL, RANK ligand; OPG, osteoprotegerin; MCP-1, monocyte chemoattractant protein-1; PGE2, prostaglandin E2; IDO, indoleamine 2,3-dioxygenase; IL-1β, interleukin 1β; IL-10, interleukin 10; TNFα, tumour necrosis factor α; VEGF, vascular endothelial growth factor; AGN, angiostatin. The figure is adapted from Elgali [3] (Figure 2) (with permission from Dr Cecilia Graneli, Sweden).

and MSC-chondroblast-chondrocyte lineages, endothelial cells and the monocyte-macrophage-osteoclast lineage that regulates inflammation and bone resorption (Figure 1). These multiple cell types communicate with each other in a bidirectional or multi-directional fashion. In the current review, we focus on the central roles of MSCs in this complex intercellular communication network by virtue of their multi-potency and immunomodulatory capacity.

MSCs are capable of committing to osteogenic or chondrogenic progenitors, osteoblasts or chondroblasts, and terminally differentiate into the most abundant cells, osteocytes or chondrocytes, in bone and cartilage, respectively.[4] In addition to their roles in osteogenic differentiation and bone formation, MSCs function as linkages of bone formation to bone resorption via communication with osteoclast precursors through the prevailing dogma RANK/RANKL coupling to influence the formation and activity of osteoclasts, which consequently regulate bone resorption.[5] Bone injury is often accompanied by the disruption of local blood vessels, potentially impairing the sequential stages of bone regeneration.[6] MSCs interact with endothelial cells via the secretion of pro-angiogenic factors, resulting in the promotion of angiogenesis.[7]

The functional features of MSCs are shaped in response to the surrounding niches and are thus able to regulate the resolution of inflammation by interacting with inflammatory cells, such as monocytes/macrophages.[8] Injury-induced inflammation is essential to initiate tissue repair. Components of the MSC secretome promote migration, proliferation and differentiation of monocytes/macrophages, resulting in a boost of the inflammatory response indicated by peak secretion of proinflammatory cytokines in the early stage of the healing process.[9] On the other hand, MSCs are influenced by the inflammatory niche and, in turn, promote the polarization of macrophages to an anti-inflammatory phenotype.[10] Such a feedback loop potentially accelerates the transition from the inflammatory phase to the regeneration phase, consequently promoting bone regeneration. Taken together, MSCs possess multiple functional features, including osteogenic differentiation capacity, regulation of osteoclast activity, promotion of angiogenesis and immunoregulatory effects, and thus play pivotal roles in the bone regeneration process.

On the basis of the above functional features, several strategies have been developed to apply MSCs as a tool for bone regeneration. The primary strategy was to administer autologous MSCs at the injury site with the goal of engraftment and differentiation to form new bone. To further enhance their effects, MSCs were manipulated prior to injection by priming with specific cytokines or genetic engineering for carrying therapeutic molecules.[11] Secondly, to further improve the engraftment and function of native or manipulated MSCs, MSCs were implanted with various types of bioscaffolds, which function as cell carriers, support cellular activity and serve as templates for bone regeneration.[12] Both of these strategies attempt to achieve bone regeneration by osteogenic differentiation of transplanted MSCs. However, the observation that MSC-conditioned medium (CM) or MSC-secreted factors alone were capable of stimulating beneficial effects similar to those of MSCs shed light on the regenerative effects mediated by MSC-secreted paracrine factors.[13] Based on the paracrine effects of MSCs, a novel strategy to apply components of the MSC secretome as a regenerative tool was therefore proposed.

2 | THE PARACRINE EFFECTS MEDIATED BY MSC-SECRETED SOLUBLE FACTORS

The secretome is the set of factors/molecules, including soluble factors and different subtypes of extracellular vesicles (EVs), secreted into the extracellular space. MSCs were shown to secrete a broad spectrum of soluble factors, such as growth factors (GFs) and cytokines, which have been implicated in both tissue regeneration and immunoregulation.[7,14] Trophic factors, such as FGF, IGF, HGF and PDGF, were detected in MSC-CM and improved cell survival at the site of injury via their anti-apoptotic effects and promotion of cell proliferation.[7,15] Moreover, some of the MSC-secreted GFs, such as SDF-1, GM-CSF, VEGF and HGF, were shown to possess chemotactic effects and are capable of promoting progenitor cell migration.[7,15] Migration and homing of progenitor cells to the injury site is a critical initial step for successful tissue regeneration. Although transplantation of MSCs may not directly differentiate into tissue-specific cell types, MSCs transfected with BMP2 showed substantial secretion of BMP2, which stimulated osteogenic differentiation and promoted bone formation.[16,17] In addition, MSCs secrete angiogenic factors, VEGF and AGNs, which promote regeneration of blood vessels and consequently contribute to the restoration of the blood supply and the repair of injured tissues.[7] In summary, it is suggested that MSCs promote a regenerative microenvironment by secreting GFs at the site of injury.[7,18]

In addition to GFs, MSCs secrete a variety of cytokines that mediate immunoregulatory effects. These immunoregulatory cytokines include 1) chemokines, such as MCP-1, that are able to simulate the migration of immune cells and 2) pro- or anti-inflammatory cytokines, such as IL6, TNFα, IL10, PGE2 and IDO, which are able to regulate the proliferation and phenotypic polarization of various immune cells, including neutrophils, macrophages and T cells.[7,9,14] The secretion of immunoregulatory cytokines enables MSCs to partially control the inflammatory response during tissue repair. The tissue repair process consists of overlapping phases of inflammation and regeneration. Rational control of inflammation facilitates the transition of the inflammatory phase to the regeneration phase, which is proposed as a novel strategy to enhance bone regeneration.[19]

Taken together, the soluble factors secreted by MSCs consist of cytokines, which were suggested to be able to regulate inflammation in a phase-dependent manner,[9] as well as GFs and other trophic factors, which established a microenvironment favouring tissue regeneration.[7,18] Therefore, early research mainly attributed the paracrine effects of MSCs on tissue regeneration to MSC-secreted soluble factors. However, growing evidence has shed light on another part of the MSC secretome, EVs/exosomes, and accumulating evidence indicates that EVs are strong mediators of MSC paracrine effects on tissue regeneration.

3 | BIOGENESIS AND MOLECULAR COMPOSITION OF EXOSOMES

EVs are defined as particles that are naturally released from the cell and delimited by a lipid bilayer, carrying intravesicular components from the cytosol but not from the nucleus of the secreting cells.[20] An early evidence of EVs playing a role in physiological processes was the observation that

EVs, which were named "matrix vesicles," localized in the extracellular matrix (ECM) and associated with calcification in 1969.[21] Initially, EVs were assumed to be secreted by the outward budding of the plasma membrane of cells and functioned as "waste bins" of the cells to dispose of unwanted components. In 1983, the endosomal pathway was demonstrated as an alternative way for EV secretion; afterwards, a new term "exosome" was proposed for the EVs that originated from the endosome.[22–24] EVs/exosomes were further recognized as cell-to-cell communicators following the discovery of their capability to transfer genetic materials.[25] To date, accumulating evidence reveals the complexity of EV biogenesis and the heterogeneity of EVs.

Classically, EVs are categorized based on their biogenesis into three broad groups: apoptotic bodies, microvesicles/ectosomes and exosomes.[26] Apoptotic bodies are EVs with the most heterogeneous size (200 nm–5 μm) and are secreted by a dying cell,[26,27] while microvesicles/ectosomes (100 nm –800 nm) [28] and exosomes (30 nm–150 nm)[22–24] are secreted by viable cells through outward budding of the plasma membrane or intracellular endosomal pathways. In reality, due to the difficulty of experimentally obtaining solid evidence of the endosomal origin of the studied vesicles and the technical limitation of clearly distinguishing different subpopulations of EVs, terms including microvesicles/ectosomes, exosomes and EVs may be used in the current literature. In the present review, we highlight the most studied subtype of EVs, exosomes, by providing readers a general background of their biogenesis and molecular compositions. We use the general term small EVs (sEVs),[29,30] which is defined as exosome-like vesicles with size < 200 nm and may be referred to as exosomes, microvesicles/ectosomes and EVs in the literature, when summarizing their therapeutic effects, mechanism of action and potential application.

As the most studied subtype of sEVs, the complexity of exosome biogenesis is reflected by the existence of various regulatory machineries and the findings that the same machinery can play similar roles either at the plasma membrane or in the intracellular endosomal compartments.[31–36] Although the mechanism of exosome biogenesis is not yet fully unravelled, current knowledge has implicated the endosomal sorting complex required for transport (ESCRT)–dependent and ESCRT-independent machinery (Figure 2). Endocytosis initiates the intracellular formation of early endosomes, which further mature and form multi-vesicular bodies (MVBs) by inward budding of the late endosomal membrane to assemble intraluminal vesicles (ILVs). The formation of ILVs and MVBs is demonstrated to involve various mechanisms, among which the ESCRT-dependent machinery is the most described. The human ESCRT consists of four complexes, ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III, which are assembled by 33 proteins and are numbered according to the order they act in the pathway and play distinct roles (reviewed by Hanson and Cashikar[37]). In brief, ESCRT-0 together with clathrin recognize and sequester ubiquitinated transmembrane proteins in the endosomal membrane. Following the sequential recruitment of ESCRT-I and ESCRT-II to ESCRT-0, the complexes together initiate the local budding of the endosomal membrane with sorted cargo; subsequently, ESCRT-III participates in protein deubiquitination and drives vesicle scission.[20,28,37] This results in the formation of ILV-loaded MVBs, which may undergo two different fates: (a) fusion with lysosomes leading to the discharge and digestion of their ILVs in the lumen of lysosomes and (b) fusion with the plasma membrane and subsequent release of exosomes into the extracellular space. In addition to the endosomal pathway, TSG101 and VPS4, key components of the ESCRT machinery, were

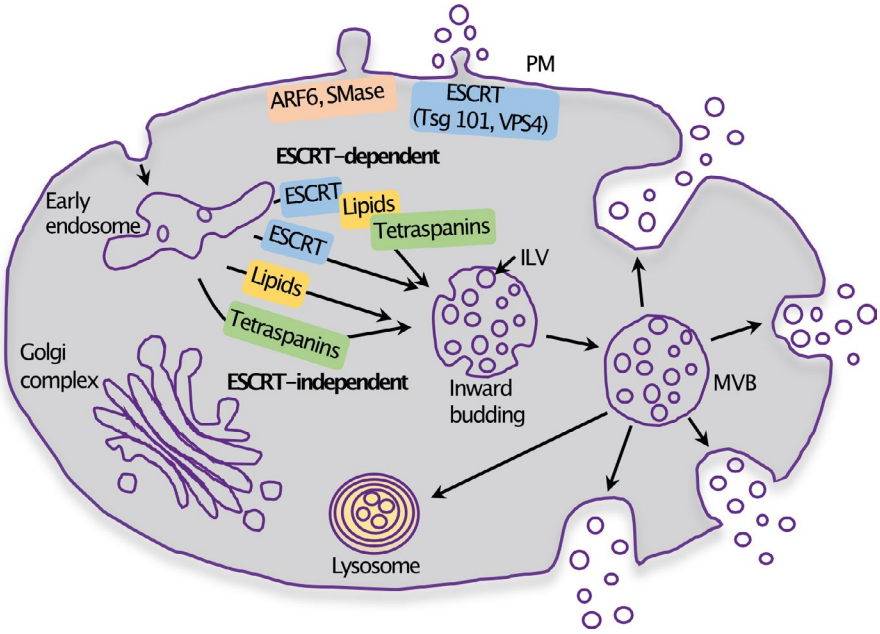

FIGURE 2 Biogenesis of exosomes. The biogenesis and secretion of exosomes is regulated by both ESCRT-dependent and ESCRT-independent machinery. ESCRT, endosomal sorting complex required for transport; ARF6, ADP ribosylation factor 6; SMase, sphingomyelinase; Tsg101, tumour susceptibility gene 101 protein; VPS4, vacuolar protein sorting-associated protein 4; ILV, intraluminal vesicle; MVB, multivesicular body; PM, plasma membrane. From Wang [39]; reprinted with permission.

also shown to be involved in the direct budding of small EVs at the plasma membrane.[32,38]

On the other hand, the ESCRT-independent machinery mediated by lipids, tetraspanins and small GTPases has been implicated in the regulation of exosome biogenesis. Lipid metabolism, enzymes and metabolic products, such as neutral sphingomyelinase (nSMase) and ceramide,[40–42] and phospholipase D2 (PLD2) and phosphatidic acid,[34,43] were shown to influence cargo sorting, inward budding of ILVs and exosome secretion. Evidence that overexpression of the tetraspanin CD9 or CD82 induced secretion of exosomal β-catenin[40] and depletion of CD63 affected the size of ILVs[44] indicates the roles of tetraspanins in cargo sorting and exosome formation. Several Rab proteins, including RAB11, RAB35, RAB7 and RAB27A/B, were suggested to play roles in endosome maturation and exosome secretion (reviewed by Stenmark[45]). Another small GTPase, ARF6, together with its effector PLD2, was found to affect the budding of ILVs into MVBs, indicating their roles in the regulation of exosome formation.[34] In comparison with the endosomal compartments, some evidence also suggests the involvement of SMase[31] and ARF6[35] in vesicle secretion at the plasma membrane. For example, overexpression of ARF6 promoted depolymerization of the actin cytoskeleton, which triggered the release of vesicles at the plasma membrane.[35]

The molecular composition of exosomes has been intensively studied, showing that exosomes contain lipids, proteins and nucleic acids (Figure 3). Comprehensive data of exosomal contents have been collected in databases, such as EVpedia (http://evpedia.info) and Vesiclepedia (http://microvesicles.org/). Exosomes contain both cell type–dependent contents,

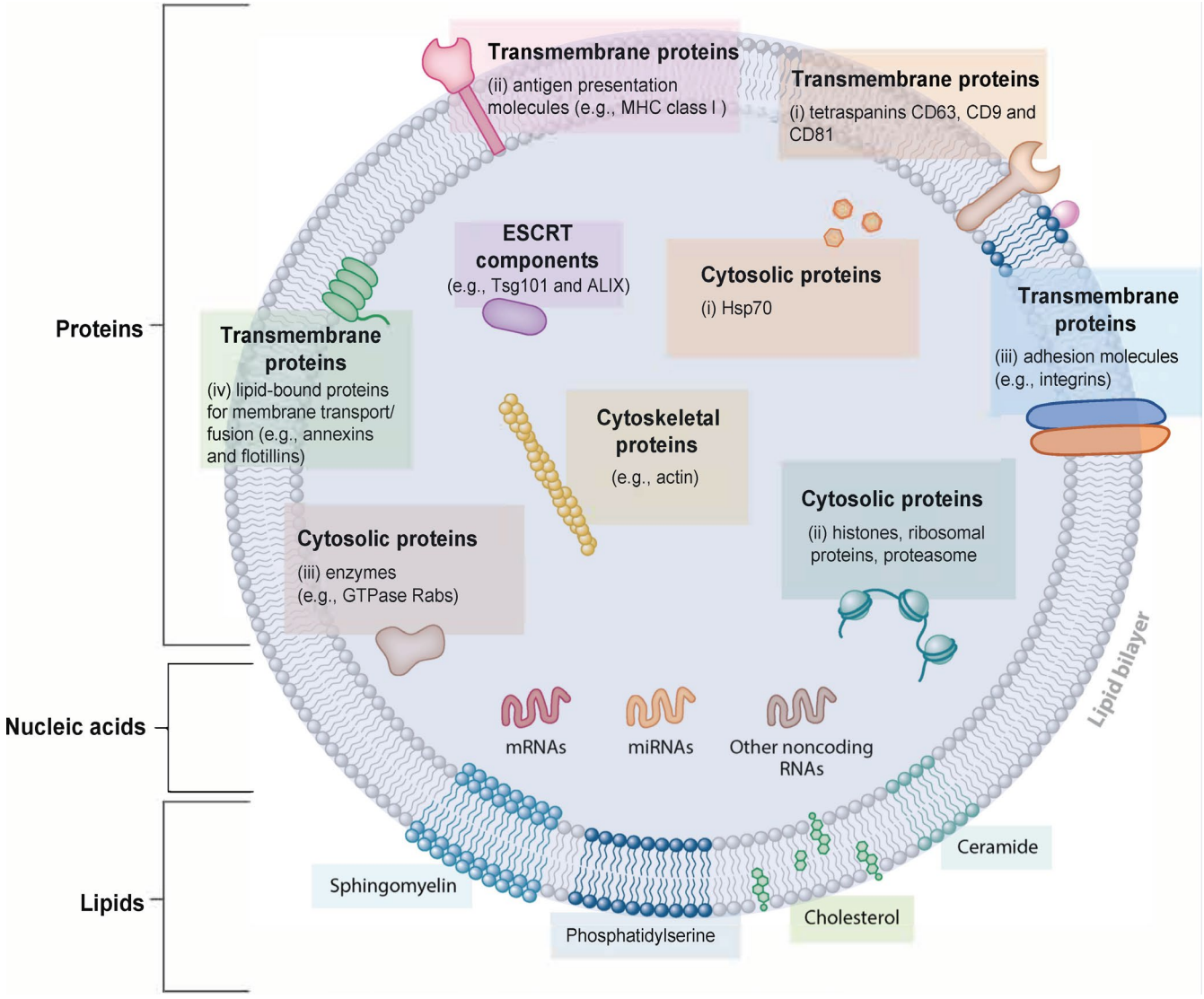

FIGURE 3 Molecular composition of exosomes. Exosomes have a molecular composition that includes numerous lipids, proteins and nucleic acids. The figure is adapted and republished with permission of Annual Review of Cell and Developmental Biology, from Biogenesis, secretion and intercellular interactions of exosomes and other extracellular vesicles, Colombo Marina; Raposo Graça; Théry Clotilde, Vol 30, 2014[20]; permission conveyed through Copyright Clearance Center, Inc

reflecting their parental cell sources and secretion conditions, and cell type–independent contents, compositions that are enriched in exosomes and reflect the common features shared among exosomes from diverse origins. For instance, several lipid species, cholesterol, sphingomyelin (SM), phosphatidylserine (PS) and saturated fatty acids, were enriched in exosomes compared with the total cell membrane.[33,46] The exploration of the lipidomes of exosomes secreted from Huh7 hepatocellular carcinoma cells and MSCs revealed a similarity in the exosomal lipid components.[47] In addition, exosomes also enrich cell type-independent proteins, some of which are often involved in the machinery of exosome biogenesis. The typical enriched exosomal proteins include (1) transmembrane proteins such as (i) tetraspanins CD63, CD9, CD81 and CD82, (ii) integrins and (iii) lipid raft-bound proteins (ie flotillin and annexins); (2) cytoskeleton proteins (ie actin); and (3) cytosolic proteins such as (i) components of the ESCRT machinery (ie TSG101 and ALIX), (ii) GTPase Rabs, (iii) heat shock 70-kDa protein (HSC70) and (iv) proteasomes (reviewed in[20,48]). Because of their common presence in exosomes, these proteins often serve as exosomal markers.[49] The strong interest in exosomes was further stimulated by the discovery of bioactive RNA, particularly miRNA and mRNA,[25] inside exosomes. Afterwards, several other species of non-coding RNAs, including vault RNA, Y RNA and tRNA, have been identified in exosomes.[50] In addition to RNAs, recent studies have also shown the presence of DNA inside or on the surface of exosomes.[51]

In addition to the cell type-independent contents, a growing number of studies have demonstrated that exosomes secreted by different cell types[47,49] or by cells undergoing various differentiation stages[52,53] or those in various environmental conditions, that is pathological or healthy conditions,[54,55] carry specific contents, thereby reflecting their origin.[56] These observations proposed the selective packaging of compositions into exosomes, which may be mediated by specific molecules and mechanisms.[57] The cell type–dependent exosomal contents may implicate the therapeutic application of exosomes, that is in tissue regeneration, and the discovery of exosomes as biomarkers for diseases, that is cancer development or degenerative diseases.[58,59]

4 | sEV-CELL INTERACTIONS AND THE MECHANISMS OF sEV-MEDIATED EFFECTS

The targeting of specific cells and the interactions between sEVs and cells are crucial initial steps for biological effects. The results suggest that such targeting and interaction are likely cell type-specific and organotropic. For instance, bone marrow dendritic cell (DC)–derived sEVs were shown to favour targeting to splenic DCs in comparison with plasmacytoid DCs, B cells, macrophages and T cells.[52] Similarly, sEVs derived from organotropic human breast and pancreatic cancer cell lines are specifically distributed to the organs to which the cell line of origin primarily metastasizes, particularly the lungs and liver, respectively.[60] These observations suggested that cancer cell–derived sEVs exhibit organ tropism, which recapitulates the organ specificity of their cell origin. Consistent with this finding, another study demonstrated that sEVs from three different mouse cell sources (a muscle cell line, a melanoma cell line and primary DCs) were distributed differentially in vivo, where DC-derived sEVs predominantly targeted the spleen.[61] Interestingly, in an acute kidney injury (AKI) model, MSC-derived sEVs preferentially accumulated in the injured kidney area unlike in healthy control mice.[62] This observation corresponded to the regenerative effects of MSC-derived microvesicles, which were exosome-like sEVs recovered at 100,000 x g, in AKI,[63] indicating the importance of sEV biodistribution for the exertion of therapeutic effects.

The cell type–specific and organotropic targeting and interaction of sEVs is dependent on not only the cell origin of sEVs but also the features of the recipient cell. One of the explanations for this specificity is the coupling of ligands and receptors presented on the surface of sEVs and recipient cells. Many of these ligands and receptors are adhesion molecules, such as tetraspanins, integrins, components of the ECM and proteoglycans, which provide an opportunity for sEVs to attach to the cell surface. Tetraspanins are small transmembrane proteins with considerable functional importance in the regulation of multiple cellular processes, including cell adhesion, migration, proliferation and signalling (reviewed in[64]). Several tetraspanin members, such as CD9, CD63 and CD81, are well known to be enriched in exosomes, a subtype of sEV, and often serve as exosomal markers.[29] Nine tetraspanins (TSPAN3, TSPAN5, TSPAN6, TSPAN9, TSPAN14, CD9, CD63, CD81 and CD151) have been detected in the MSC-derived sEV proteome.[65] Among these detected tetraspanins, CD81 and CD151 have been shown to markedly influence the strength of integrin-dependent adhesion via association with various integrins and regulation of post-ligand binding events.[63]

Integrins are also key receptors mediating adhesion by binding to ligands such as components of the ECM. Interestingly, various integrins and their ligands, including ECM proteins (collagens, laminins, fibronectin and vitronectin), ICAM1 and ICAM2, were both identified in the MSC-derived sEV proteome.[64] These findings indicate that sEVs and their recipient cells may interact in a bidirectional manner: sEV integrins bind to their corresponding ligands in recipient cells, and cellular integrins may bind to ECM proteins on sEVs. This bidirectional integrin-ECM protein interaction may result in the selective interaction of sEVs and specific cell types. This hypothesis is supported by the correlation

between the sEV integrin expression profiles and the organotropic distribution of sEVs.[60] Cancer sEVs abundant with integrins $\alpha_6\beta_1$ (ITG$\alpha_6\beta_1$) and ITG$\alpha_6\beta_4$ preferentially accumulated in laminin (ligand for ITG$\alpha_6\beta_1$ and ITG$\alpha_6\beta_4$)-enriched regions in the lungs, while ITG$\alpha_v\beta_5$-enriched sEVs preferentially distributed in the liver regions with abundant fibronectin, a ligand for ITG$\alpha_v\beta_5$.[60] Similarly, sEV-cell targeting can also be mediated by the bidirectional interaction of proteoglycans and lectins.[66] Proteoglycans, such as CD44, have previously been identified in the MSC-derived sEV proteome.[65] Blocking sEV-carried CD44 by soluble hyaluronic acid (a ligand for CD44) or anti-CD44 inhibited the incorporation of MSC-derived sEVs in recipient cells,[63] indicating the importance of sEV CD44 for sEV-cell interactions.

Phosphatidylserine (PS), an enriched lipid component in sEV, is displayed on the surface of sEVs and recognizes its receptors on the recipient cell surface. Wei et al[67] showed that pretreatment of MSC-derived sEVs with exogenous Annexin-V (specific binding to PS) markedly inhibited the internalization of sEVs in recipient human umbilical vein endothelial cells (HUVECs). Further, pretreatment of HUVECs with small interfering RNAs targeting the PS receptor or an antibody against the PS receptor greatly blocked the incorporation of MSC-derived sEVs into HUVECs.[67] Furthermore, this blockage of sEV-cell interaction via inhibition of PS-PS receptor binding abrogated the effects of sEVs on promoting tube formation of HUVECs.[67] Another cellular receptor, TIM4, was identified as a PS receptor, and the PS-TIM4 interaction was suggested to be associated with sEV-mediated intercellular signalling.[68] On the basis of the PS-TIM4 interaction, a novel affinity-based method has been successfully developed for sEV isolation.[69]

Taken together, these observations suggest that the interactions between sEV and cellular surface molecules play important roles in sEV-cell targeting and for sEVs to exert their effects. The interaction of sEV and cellular surface molecules consequently results in the docking and binding of sEVs on the surface of recipient cells and may further stimulate the activation of intracellular signalling in the recipient cells, depending on the functional features of the interacting surface molecules. The docking of sEVs also facilitates the subsequent membrane fusion or internalization of sEVs, leading to the transfer of sEV contents into the recipient cells. This transfer is mainly achieved via internalization of sEVs through various endocytosis pathways. A variety of inhibitors have been utilized to block the internalization of sEVs, where various functional effects have been observed. Readers interested in the details of routes for sEV internalization are referred to the recent review by Mulcahy et al.[70] Internalization of sEVs results in the intracellular release of sEV cargos, both proteins and nucleic acids, in the recipient cells and provides opportunity for the functional cargos to mediate their effects intracellularly. Previous studies showed the biological effects induced by the sEV contents, which indirectly confirm the intracellular release of sEV contents. For example, a recent study demonstrated that epidermal growth factor and androgen receptor were transferred via EVs to the nuclei of recipient cells and activated the respective nuclear pathways in the recipient cells.[71] In addition, sEV mRNA was shown to translate in the recipient cells,[25] and sEV microRNA regulated target gene expression in the recipients.[72] Nevertheless, the detailed intracellular fate of the transferred sEV cargos is not yet fully understood. Further, the mechanism of action, protein-based or nucleic acid-based, which predominantly mediates the biological effects induced by sEVs is not determined. Readers interested in the summary and comparison of these two mechanisms of action are referred to two recent reviews.[73,74]

In summary, the mechanisms by which sEVs mediate their effects depend on the sEV-recipient cell interaction. sEVs may mediate their effects via surface receptor-ligand binding to trigger intracellular signalling in recipient cells or via intracellular release and transfer of sEV contents to regulate the biological activities of recipient cells.

5 | THE EFFECTS OF MSC-DERIVED sEVs ON BONE AND CARTILAGE REGENERATION

Bone formation and bone resorption are processes that require interactions between cells via paracrine signalling and interactions with the ECM. The network of cells is under the influence of systemic and local hormonal regulation and mechanical stimuli. A recent study demonstrated that mechanical stimulation of osteocytes triggered a Ca^{2+}-dependent release of EVs carrying bone regulatory proteins, specifically RANKL, OPG and sclerostin.[75] This observation indicated that EV release and regulation of RANKL, OPG and sclerostin secretion via EVs may be a potential mechanism for osteocyte to coordinate tissue-level bone adaption and regulate bone metabolism in response to mechanical stimuli.[75] Nevertheless, the role(s) of sEVs in general and MSC-derived sEVs specifically during physiological conditions and bone homeostasis is not understood, as few studies have been conducted, but needs increased attention. In fact, the majority of sEVs studies in the musculoskeletal system have focused on phenotypic and molecular changes either in in vitro culture experiments or in in vivo animal disease models after sEV administration.

MSC-derived sEVs are an important component of the MSC secretome and mediate interactions with many different cell types to exert their effects on tissue regeneration. Here, we itemize the outcome of MSC-derived sEVs in preclinical models of bone fracture,[76–78] bone defects,[79–85] bone diseases (particularly osteogenesis imperfecta [86] and steroid-induced

avascular necrosis of the femoral head [87]), osteochondral defects,[88–90] osteoarthritis (OA)[91–94] and periodontitis.[95,96] Furthermore, we summarize our current understanding of how MSC-derived sEVs interact with various cell types involved in bone and cartilage regeneration.

5.1 | The functional outcome of MSC-derived sEVs in bone and cartilage regeneration

A growing body of studies has shown that MSC-derived sEVs promote neovascularization and bone and cartilage regeneration in different animal disease models using different approaches to administer sEVs, different vehicles for carrying sEVs and different sEV modifications (Table 1).

Among these studies, the most commonly used strategy of sEV treatment is a local injection of native sEVs in suspension[76,88,89,91–95] with one exception using a systematic tail vein injection.[86] Alternatively, native sEVs conjugated to scaffolds such as β-TCP,[79,80] PLA[81] and decalcified bovine bone matrix (DBM) scaffolds [97] via various sEV-immobilization protocols or embedded in hydrogel[77,82,90] or collagen sponges[96] have been administered at the injury site. Moreover, it has been shown that engineered sEVs via pre-modification or post-modification approaches were capable of augmenting functional outcome. Pre-modification of sEVs can be achieved by transfecting parental MSCs for overexpression of molecules, such as HIF-1α[85] or miR-375[82]; or by preconditioning parental MSCs, for example, inducing osteogenic differentiation[83] or prestimulating the MSCs with the cytokines IFNγ and TNFα[98] or with hypoxia.[78,99] Further, engineered sEVs can be obtained after isolation by coating the sEVs with crosslinking reagents such as PEI.[84,85]

Furthermore, in an attempt to unravel how MSC-derived sEVs result in the observed functional outcome, efforts have been made to investigate their effects on various cell types involved in bone and cartilage regeneration (Figure 4). MSC-derived sEVs propagate the properties of the parental cells and exhibit both regenerative and immunomodulatory effects, depending on the targeted cell type and microenvironment.

5.2 | The regenerative effects of MSC-derived sEVs on bone- and cartilage-forming cells

Emerging data have shown that MSC-derived sEVs contribute to bone and cartilage regeneration directly through interaction with bone and cartilage-forming cells: cells of the MSC-osteoblast-osteocyte and MSC-chondroblast-chondrocyte lineages. Stimulation with MSC-derived sEVs promotes the proliferation, migration, osteogenic differentiation and mineralization of MSCs from various sources.[53,79–84,87,100] Interestingly, recent studies have shown that MSC-derived sEVs increase the expression of VEGFA and VEGFR2 in MSCs and may consequently benefit both osteogenesis and angiogenesis during bone regeneration.[81,85]

Cell adhesion is one of the first crucial steps for cell-surface interactions and the regenerative process of bone associated with implanted prostheses.[101,102] Implant surface-immobilized MSC-derived sEVs accelerated MSC adhesion on titanium surfaces and affected the behaviour of adherent MSCs.[65] Moreover, under compromised conditions, MSC-derived sEVs elicit protection of bone-forming cells against damage. Ren et al[103] reported that MSC-derived sEVs diminished hypoxia and serum deprivation (H/SD)–induced apoptosis in osteocytes. In an in vitro OA model, MSC-derived sEVs attenuated IL-1β-induced senescence and the inflammatory response in osteoblasts.[104] In line with the results observed for the MSC-osteoblast-osteocyte lineage, accumulating evidence has been shown for the pro-chondrogenic effects of MSC-derived sEVs on chondrocytes. Chondrocytes treated with MSC-derived sEVs exhibited enhanced migration, proliferation, chondrogenic differentiation and matrix synthesis.[86,89,104] In addition, several recently published studies have reported that MSC-derived sEVs restored matrix homeostasis, ameliorated apoptosis and attenuated the proinflammatory effects of chondrocytes in OA mimic models with TNFα[105] or IL-1β[91–94] stimulation.

Several components of the sEV cargo as well as the targeted molecules and signalling pathways in the recipient cells have been explored in search of the molecular mechanisms of MSC-derived sEV-mediated regenerative effects on bone and cartilage-forming cells. sEVs derived from MSCs under various stages of osteogenic differentiation carry altered microRNA contents, with a specific set of osteogenesis-related microRNAs enriched in sEVs from the late stages of osteogenic differentiation.[53] The pro-osteogenic microRNAs miR-10b and miR-21[106,107] were increased, while the anti-osteogenic microRNAs miR-31, miR-144 and miR-221[108–110] were decreased in sEVs from late-differentiated MSCs, which corresponded to the induction of osteogenic differentiation and mineralization under sEV treatment.[53] This result indicated that MSC-derived sEVs induced osteogenic differentiation at least partially via the transfer of osteogenesis-related microRNAs carried by sEVs. Further, sEVs up-regulated the expression of pro-osteogenic and pro-angiogenic miRNAs, miR-2861 and miR-210, respectively, in recipient MSCs, corresponding to the increased expression of VEGF and the osteogenic master transcription factor RUNX2 and enhanced osteogenic differentiation.[91] In addition, the transfer of HIF-1α[86] and miR-375[82] via MSC-derived sEVs improved

TABLE 1 MSC-derived sEVs in bone and cartilage regeneration

sEVs and delivery routes		MSC source	Pretreatment of MSCs or sEVs	Experimental model	sEV administration	Functional outcome	Ref.
Native sEVs	Injection of suspension	Human BM	—	Femoral shaft fracture in C57BL/6 wild-type and $CD9^{-/-}$ mice	Local injection into the fractured part at 1 and 8 days after fracture	Promoted fracture healing in wild-type mice and rescued impaired fracture healing in $CD9^{-/-}$ mice	76
		Murine BM and human BM	—	Osteogenesis imperfecta animal model in G610 mice	Tail vein injection weekly for 4 weeks	Improved bone growth as indicated by increased bone length in both femora and tibiae	86
		AT	—	Ligature-induced periodontitis model in male albino Wistar rats	Local injection into pockets as an adjunctive treatment	sEV treatment showed promising periodontal regeneration as indicated by formation of highly organized proliferating periodontal ligament tissue and well-formed dense healthy bone in the periodontal ligament space	95
		Human ESCs	—	Critical-sized osteochondral defect on trochlear grooves of the distal femurs in female SD rats	Intra-articular injection weekly for up to 12 weeks	Promoted osteochondral regeneration as indicated by complete restoration of cartilage and subchondral bone	88
		Human ESCs	—	Critical-sized osteochondral defect on trochlear grooves of the distal femurs in female SD rats	Intra-articular injection weekly for up to 12 weeks	Accelerated cartilage repair, increased cellular proliferation and M2 macrophage infiltration, reduced cellular apoptosis and inflammatory cytokine secretion, and enhanced matrix synthesis	89
		Murine BM	—	Collagenase-induced OA model in C57BL/6 mice	Intra-articular injection at day 7	Protected cartilage and bone from degradation induced by collagenase as indicated by increased cartilage thickness and bone volume	91
		Human ESCs	—	Destabilization of the medial meniscus surgery induced OA model in C578L/6J mice	Intra-articular injection every 3 days for 4 weeks	Promoted recovery of cartilage destruction as indicated by lower OARSI score and increased expression of Col II	92
		Infrapatellar fat pads from OA patients	—	Induced OA model by surgical destabilization of the medial meniscus in male C578L/6 mice	Intra-articular injection twice per week for 4 or 6 weeks	Protected articular cartilage from damage and ameliorate gait abnormality as indicated by reduced OARSI score, reversed increase in COL2 expression and down-regulated ADAMTS5 and MMP13 expression	93
		Human ESCs	—	Induced TMJ OA model by injection of monosodium iodoacetate in SD rats	Intra-articular injection weekly for up to 8 weeks	Suppressed pain and modulated early gene expression changes in TMJ condylar cartilage tissue, reversed TMJ degeneration as indicated by restored matrix synthesis, alleviated subchondral bone deterioration as indicated by restored bone volume and architecture, suppressed inflammation as indicated by reduced IL-1β^{+} and $iNOS^{+}$ cells, promoted proliferation and reduced apoptosis as indicated by increased $PCNA^{+}$ cells and decreased $CCP3^{+}$ apoptotic cells	94

(Continues)

TABLE 1 (Continued)

sEVs and delivery routes	MSC source	Pretreatment of MSCs or sEVs	Experimental model	sEV administration	Functional outcome	Ref.
Material carrier	Human iPSCs	—	Critical-sized calvarial bone defects in osteopenic animal model using ovariectomized SD rats	Implantation of β-TCP scaffold with lyophilized sEVs in the defect site	Dose-dependently promoted bone regeneration and neovascularization in the defect site as indicated by an increased ratio of BV/TV, BMD, area of new bone formation and mineralization, area and number of vessels, and expression of the osteogenic markers OCN and OPN and the angiogenic marker CD31	79
	Human iPSCs	—	Critical-sized calvarial bone defects in SD rats	Implantation of β-TCP scaffold with lyophilized sEVs in the defect site	Dose-dependently enhanced bone regeneration in the defect site as indicated by an increased ratio of BV/TV, BMD, area of new bone formation and mineralization, and expression of the osteogenic marker OCN	80
	Human gingiva tissue	—	Cortical calvaria bone tissue damage in male Wistar rats	Implantation of 3D printed PLA scaffold enriched with sEVs or both sEVs and MSCs to cover the damaged area	Combination of 3D-PLA with enriched sEVs and MSCs improved bone healing as indicated by higher positive staining of calcium, increased vascularization, higher rate of regeneration and integration level at the damaged site	81
	SD rat BM	—	Subcutaneous bone formation model in nude mice	Subcutaneous implantation of sEV functionalized DBM scaffold pre-coating with fibronectin and with or without seeding of osteogenic-induced MSCs	sEV functionalized DBM showed pro-angiogenic activity as indicated by increased CD31 + vessel formation. However, only combination of sEV functionalized DBM and MSCs showed enhanced bone regeneration as indicated by increased staining of new bone, BV and ratio of BV/TV	97
	Human AT	—	Calvarial defect in male SD rats	Implantation of sEV-loaded hydrogel in the defect site	Promoted bone regeneration as indicated by micro-CT analysis and histology examination	82
	Human umbilical cord	—	Stabilized fracture in femur of male Wistar rats	Injection of sEV-embedded HyStem-HP hydrogel near the fracture site	Enhanced bone healing and angiogenesis at the fracture site as indicated by increased callus formation, BMD, BV, BV/TV, vessel volume, number of CD31 + blood vessels, maximum mechanical load and bending stiffness	77
	Human iPSCs	—	Surgically created periodontal intrabony defect in SD rats	Implantation of sEV-loaded collagen sponge in the defect site	Promoted periodontal regeneration with enhanced bone growth, increased functional PDL length and increased cellular infiltration and proliferation at the defect site as indicated by histological examination and micro-CT analysis	96
	Human iPSCs	—	Articular full-thickness cartilage defect in New Zealand rabbits	Injection of sEV suspension or sEV-embedded in situ–formed or in vitro–preformed hydrogel glue to the defect site	In situ–formed hydrogel glue containing sEVs had best performance promoting cartilage repair and cartilage-hydrogel integration as indicated by uniform and well-organized articular cartilage structure, distribution of abundant chondrocytes in newly formed tissue, positive staining of Safranin O and Col II.	90

(Continues)

TABLE 1 (Continued)

sEVs and delivery routes		MSC source	Pretreatment of MSCs or sEVs	Experimental model	sEV administration	Functional outcome	Ref.
Modified sEVs	Injection of suspension	Rabbit BM	Transfection for expression of mutant HIF-1α	Steroid-induced avascular necrosis of femoral head in rabbits	Local injection of single-dose sEVs into the femoral head	Promoted bone regeneration and neovascularization in the necrotic region as indicated by massive trabecular tissue generation, increased staining of new-born cartilage and increased density of $CD31^+$ micro vessels	87
		Human umbilical cord	Hypoxic condition	Femoral fracture model in mice	Local injection of single-dose sEVs near the fracture	Promote bone fracture healing primarily via enhanced angiogenesis indicated by increased volume of callus bridging the fracture gap, vessel volume, vessel number, expression of endothelial markers CD31 and endomucin and proliferation marker Ki67	78
	Material carrier	Human AT	Osteoinduction for 2 days	Critical-sized calvarial bone defects in male BALB/c mice	Implantation of an sEV-immobilized PLGA/pDA scaffold in the defect site	Increased bone formation and recruitment of $SSEA\text{-}4^+$/$CD45^-$ MSCs at the defect site as indicated by increased new bone volume, collagen expression and expression of the osteogenic markers RUNX2 and OCN	83
		Human AT	Transfection for overexpression of miR-375	Calvarial defect in male SD rats	Implantation of sEV-loaded hydrogel in the defect site	Enhanced bone regeneration as indicated by increased ratio of BV/TV, BMD, staining of new bone and expression of the osteogenic markers OCN and BMP2	82
		Human gingiva tissue	Coating sEVs with branched PEI by using non-covalent layer-by-layer protocol	Cortical calvaria bone tissue damage in male Wistar rats	Implantation of 3D printed PLA scaffold carried with native sEVs or PEI-coated sEVs to cover the damaged area	Improved bone healing as indicated by new bone formation inside the scaffold structure and presence of blood vessels around the new bone deposition area. PEI-engineered sEVs showed better healing effects	84
		Human PDL tissue	Coating sEVs with branched PEI by using non-covalent layer-by-layer protocol	Cortical calvaria bone tissue damage in male Wistar rats	Implantation of 3D collagen membrane carried hPDLSCs and enriched with native sEVs or PEI-coated sEVs to cover the damaged area	Both native and PEI-engineered sEVs promoted vascularization as indicated by expression of the angiogenesis markers VEGFA and VEGFR2, however, PEI-sEVs improved bone regeneration and integration as indicated by the distribution of OB and OC in the naïve bone, and quantification of bone parameters including BV, BS, BV/TV and BS/TV	85

Abbreviations: ADAMTS5, a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif 5; AT, adipose tissue; BM, bone marrow; BMD, bone mineral density; BMP2, bone morphogenetic protein 2; BS, bone surface; BV/TV, bone volume/total bone volume; CCP3, cyclic citrullinated peptide 3; Col II, type II collagen; ESC, embryonic stem cell; HIF-1α, hypoxia-inducible factor-1α; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; iPSC, induced pluripotent stem cell; MMP13, matrix metallopeptidase 13; OA, osteoarthritis; OARSI score, osteoarthritis research society international score; OB, osteoblasts; OC, osteoclasts; OCN, osteocalcin; OPN, osteopontin; PCNA, proliferating cell nuclear antigen; PDL, periodontal ligament; RUNX2, runt-related transcription factor 2; SD rat, Sprague-Dawley rat; TMJ, temporomandibular joint; VEGFA, vascular endothelial growth factor A; VEGFR2, vascular endothelial growth factor receptor 2.

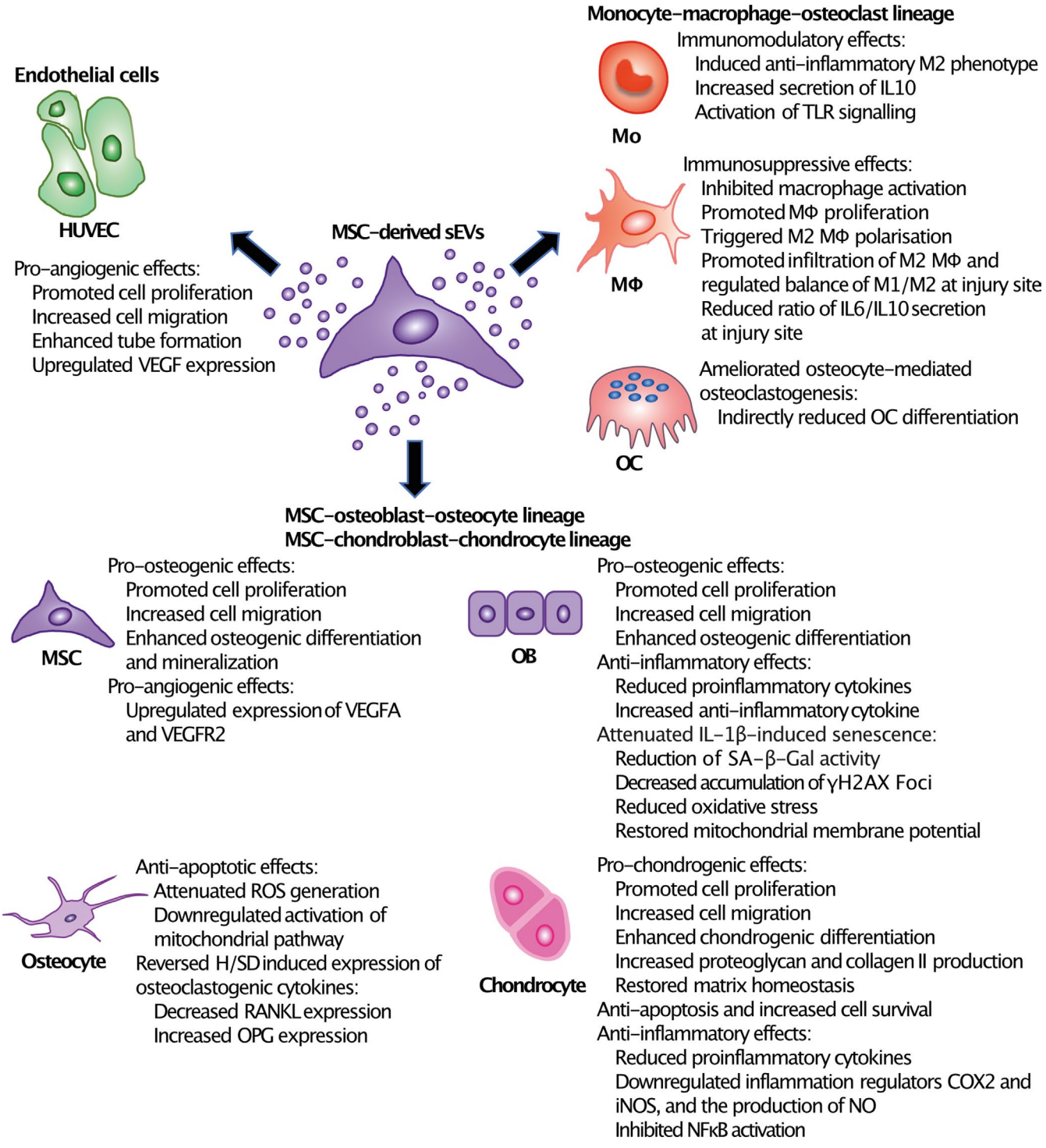

FIGURE 4 Effects of MSC-derived sEVs on multiple cell types involved in bone and cartilage regeneration. HUVEC, human umbilical vein endothelial cell; Mo, monocyte; Mϕ, macrophage; OC, osteoclast; MSC, mesenchymal stem cell; OB, osteoblast; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; ROS, reactive oxygen species; H/SD, hypoxia and serum deprivation; RANKL, receptor activator of nuclear factor-κB ligand; OPG, osteoprotegerin; IL6, interleukin 6; IL10, interleukin 10; TLR, Toll-like receptor; M1/M2, pro- and anti-inflammatory macrophage phenotypes; IL-1β, interleukin 1; SA-β-Gal, senescence-associated β-galactosidase; γH2AX, phosphorylated H2A histone family member X; COX2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NFκB, nuclear factor-κB.

the osteogenic differentiation of MSCs. Another study partially attributed the pro-osteogenic effects of MSC-derived sEVs to the enrichment of Wnt3a in sEVs and targeting of the Wnt signalling pathway, one of the most well-known signalling pathways regulating osteogenic differentiation, in the recipient human primary OBs.[111] Zhang et al[80] showed that

MSC-derived sEVs enhanced the osteogenic differentiation of MSCs by activating the PI3K/Akt signalling pathway, which has been reported to play critical roles in osteoblast differentiation and bone formation.[112,113]

Recent studies suggested that the therapeutic effects of MSC-derived sEVs on cartilage and periodontal regeneration were mediated through the activation of the pro-survival AKT, ERK and AMPK signalling pathways.[89,94,96] The activation was attributed at least in part to the sEV-carried CD73-mediated enzymatic activity.[89,94,96] It was implicated that sEV CD73 functioned as a key enzyme to hydrolyse AMP to adenosine, which further activated the adenosine receptor and enhanced adenosine receptor-mediated rapid phosphorylation of AKT, ERK and AMPK in chondrocytes[89,94] and periodontal ligament cells.[96] This cascade of reactions induced by MSC-derived sEVs consequently resulted in the stimulation of diverse cellular responses, such as cellular survival, proliferation, differentiation and migration, which were beneficial for tissue repair.[80,89,94,96,114] An alternative mechanism of MSC-derived sEV-mediated cartilage protection by targeting mTOR signalling in chondrocytes was recently reported.[93] It was demonstrated that MSC-derived sEVs down-regulated mTOR signalling, leading to enhanced autophagy in IL-1β-treated chondrocytes, thus inhibiting cell apoptosis and regulating cellular metabolism for matrix production. The effect was related to the transfer of one of the most abundant sEV-carried microRNAs, miR-100-5p, which targeted mTOR mRNA and decreased its expression.[93]

5.3 | The regenerative effects of MSC-derived sEVs on blood vessel-forming cells

Importantly, MSC-derived sEVs possess regenerative effects when interacting with blood vessel-forming cells such as HUVECs, contributing to bone and cartilage regeneration. Vascularization and restoration of blood supply at the injury site are crucial steps during tissue repair and can facilitate tissue regeneration.[6] MSC-derived sEVs promote the proliferation and migration of HUVECs, increase the tube formation capacity and up-regulate angiogenesis-related genes such as VEGF and HIF-1α.[77,78,87,97,100,115–117] MSC-derived sEVs exert a pro-angiogenic effect via the transfer of the sEV-carried microRNAs miR-30b and miR-210, which target DLL4 and EFNA3, respectively, in recipient HUVECs.[115,116] In addition to the sEV-carried microRNA-mediated effects, the transfer of sEV-carried Wnt4 to HUVECs to activate β-catenin has been shown to promote angiogenesis.[117] Further, Li et al[87] reported that mutant HIF-1α-modified MSCs secreted sEVs carrying the HIF-1α protein, which was expressed and was stable under normoxic conditions. Such enrichment of the sEV-carried HIF-1α protein further boosted the pro-angiogenic effects of MSC-derived sEVs. In line with this finding, a recent study showed that hypoxia preconditioning activated HIF-1α in MSCs, which promoted sEV release and induced enrichment of sEV-carried miR-126.[78] Hypoxic MSC sEVs transferred miR-126 that targeted SPRED1, a suppressor of Ras/ERK pathway, in the recipient HUVEC and consequently resulted in the activation of Ras/ERK cascades to promote proliferation, migration and angiogenesis of HUVEC.[78]

5.4 | The immunomodulatory effects of MSC-derived sEVs on inflammatory cells

In addition to the regenerative effects, MSC-derived sEVs possess immunomodulatory functions by interacting with immune cells, including monocytes/macrophages, B cells and T cells, consequently influencing both the innate and adaptive immune responses. The immunomodulatory effects of MSC-derived sEVs have been studied in autoimmune disease models such as colitis[118] and graft-versus-host disease (GVHD).[119] On the other hand, given the importance of the regulation of inflammation for promoting tissue regeneration and the crucial roles of monocytes/macrophages in the inflammatory response, studies have explored how MSC-derived sEVs affect monocytes/macrophages in both in vitro and in vivo tissue injury models.[89,91,99,120] For example, MSC-derived sEVs activated TLR signalling, particularly TLR4, resulting in MyD88-dependent nuclear translocation of NFκB in the monocytic THP1 cell line.[120] Moreover, MSC-derived sEVs induced an anti-inflammatory M2 phenotype in THP1 cells as indicated by high expression of the anti-inflammatory cytokine IL10 and low expression of the proinflammatory cytokines IL1β and IL12P40.[120] In line with this result, two other studies showed similar anti-inflammatory effects of MSC-derived sEVs on macrophages derived from the bone marrow and spleen, respectively.[91,99] Lo Sicco et al[99] revealed that MSC-derived sEVs promoted macrophage proliferation and triggered macrophage polarization towards the M2 phenotype by showing increased expression of the M2 surface markers, CD36, CD51 and CD206, and a decrease in the M1 surface markers, specifically Ly6C, CD11b, CD40 and CD86. Consistently, Cosenza et al[91] showed that MSC-derived sEVs significantly inhibited LPS-induced activation of macrophages by abrogating the increased expression of M1 surface markers. These results obtained from in vitro studies were further confirmed in in vivo models. MSC sEVs promoted the infiltration of M2 macrophages to the site of skeletal muscle injury after the initial inflammatory response, which was characterized by an increase in the expression of the M2 markers Arginase 1 and Chitinase

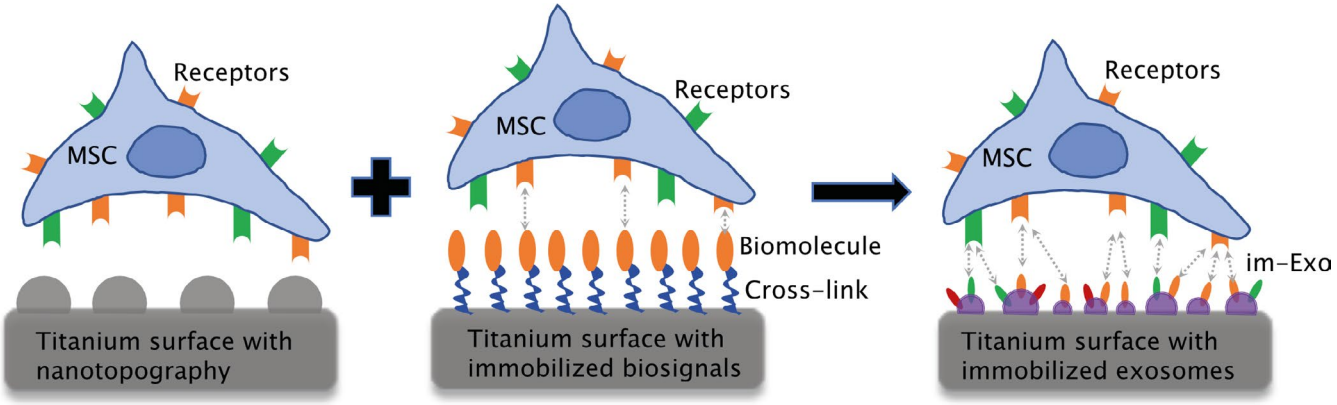

FIGURE 5 Functionalized titanium implant surface by immobilization of exosomes. The exosome-immobilized titanium surface may offer combined advantages to modify the surface nanotopography and provide biosignals on the surface by the bioactive molecules presented on the immobilized exosomes. im-Exo, immobilized exosomes. From Wang[128]; reprinted with permission.

3-like 3, and a reduction in the M1 marker nitric oxide synthase 2.[99] MSC sEVs therefore influenced the balance of M1/M2 macrophages at the injury site, resulting in a reduced IL6/IL10 ratio and an increased CD206/Ly6c ratio.[99] In agreement with this observation, MSC-derived sEVs increased $CD163^+$ cells and reduced $CD86^+$ cells in both the cartilage and synovium, concomitant with a decrease in IL1β and TNFα production, during the course of cartilage repair.[89] Taken together, MSC-derived sEVs appear to have a modulatory effect on macrophages, particularly the M1/M2 phenotype at the injury site, tentatively contributing to a microenvironment that favours tissue regeneration.

6 | CURRENT CHALLENGES AND FUTURE PERSPECTIVES

In light of the prominent biological effects of MSC-derived sEVs described in the literature, several potential applications of MSC-derived sEVs have been proposed. Similar to therapeutic peptides, proteins and nucleic acids, MSC-derived sEVs may be candidates for biopharmaceuticals. The sEV components strongly reflect their cellular origin and, as a consequence, determine the functions of sEVs. MSC-derived sEVs have been shown to carry therapeutic molecules, such as (a) enzymatic proteins CD73 [93] and neprilysin,[121] (b) mRNA for translation of growth factor or growth factor receptor including HGF, keratinocyte growth factor (KGF) and insulin growth factor receptor (IGFR),[122–124] and (c) various microRNAs, such as miR-223 and miR-100, with anti-apoptotic and anti-inflammatory effects.[93,125] MSC-derived sEVs propagate the regenerative and immunomodulatory characteristics of MSCs. While hundreds of preclinical studies have been performed in various disease models, 10 clinical studies are currently registered in the US-NIH clinical trial database (https://clinicaltrials.gov/) when searching with terms of "MSC exosomes" or "MSC EVs." Most of these studies aimed to utilize MSC-derived sEVs in patients in need of tissue regeneration or autoimmune diseases.

MSC-derived sEVs may also be interesting candidates for the engineering of biologically functionalized materials. Efforts have been made to embed unmodified or modified MSC-derived sEVs in hydrogels for site-specific injection, thereby accelerating defect healing.[77,82,90] A recent example is titanium surface-immobilized sEVs, which accelerate MSC adhesion, promote MSC growth and potentially augment the osteogenic differentiation of MSCs.[65] MSC-derived sEVs may offer combined advantages to modify the surface nanotopography and present biosignals on the surface, which are beneficial for cell-to-material interactions and consequently promote tissue regeneration around implants (Figure 5). Moreover, the possibility of modifying sEV contents provides the flexibility to customize functionalized implants for specific microenvironments, such as compromised tissues with reduced regenerative capability.

MSC-derived sEVs have also been suggested as potential vehicles for drug delivery. sEVs possess several similarities to liposomes, the first clinically approved nano-based drug delivery vehicle (reviewed in[126]). MSC-derived sEVs may have several advantages due to their unique physical and biological features, which may be able to overcome the challenges of liposome delivery systems, such as insufficient targeting efficiency and potential risk for immunocompatibility. Their nano-size provides them the ability to cross biological barriers, such as the blood-brain barrier and the blood-cerebrospinal fluid barrier, and passively diffuse through tissues. The nature of their membrane, originating from the cellular membrane and harboured with proteins, is beneficial for their efficient interaction with and internalization by cells. Therefore, these features together may potentially improve the drug targeting efficiency. In addition, efforts have been made to functionalize the surface properties of MSC-derived sEVs, for example conjugating with the c(RGDyK) peptide

on the sEV surface, which was shown to improve their targeting capacity.[127] Drugs and other therapeutic molecules can be loaded in sEVs for systemic or site-specific delivery. For example, MSC-derived sEV-encapsulated β-glucuronidase converted prodrug curcumin glucuronide to anti-inflammatory curcumin when loaded in hydrogel, resulting in anti-inflammatory effects comparable to those with enzyme-loaded liposomes.[128] This concept allows the protection of prodrugs, local synthesis and release of drugs and meanwhile augments the therapeutic effects of sEVs. Another example is the registered phase I clinical study, iExosomes in treating participants with metastatic pancreatic cancer with KrasG12D mutation, using MSC-derived sEVs loaded with KrasG12D siRNA to investigate the dose, effects and safety of these siRNA-loaded sEVs.

Nevertheless, although promising results on the therapeutic effects of MSC-derived sEVs on cartilage and bone regeneration have been obtained from preclinical studies, these results have to be verified in clinical studies. However, multiple translational challenges still exist. For example, it is not yet understood which specific cargo contents (lipids, proteins and nucleic acids) are responsible for the observed effects. While identification of sEV components with disease-modifying effects is of prime interest, the therapeutic efficacy of various cargos and the safety of an effective dose should be taken into consideration when applying sEVs as a therapeutic agent. Non-specific targeting is another factor that could result in side effects since MSC-derived sEVs have been shown to target multiple cell types. It is not yet fully understood how sEVs select their preferential recipients, especially in the in vivo environment. Moreover, the constitutive secretion of sEVs, their local and systemic distribution and cellular targets during physiological and pathophysiological conditions are important to decipher. Here, methodological challenges exist for the isolation and characterization of EVs in solid tissues *vs* liquids.

Drug loading efficiency is another challenge to overcome for the application of MSC-derived sEVs as drug delivery vehicles. Efforts should be made in future studies to develop drug loading techniques that are capable of loading a sufficient dose of drugs into sEVs while maintaining the physical integrity and biological activity of sEVs. Clearly, it is essential for clinical translation to obtain knowledge on the pharmacokinetics of native sEVs and drug-loaded sEVs.

To provide a robust therapy, the scalable production of sEVs with high purity is judged to be very important. Due to technical limitations, it is currently not achievable to obtain completely purified sEVs; however, it is essential to select the optimal isolation method for a specific application. Further development of techniques to improve isolation efficiency to obtain sEVs with high purity and production yield is still the long-term goal. In addition, it is necessary to determine the stability and shelf life, including optimal buffers and temperature, for the storage of sEVs.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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