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Harnessing extracellular vesicles as an emerging diagnostic and therapeutic strategy for osteoporosis

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Osteoporosis (OP) is a prevalent chronic bone disorder that causes reduction of bone mass, deterioration of bone microarchitecture, and increase of fragility and fracture risk. Current therapeutic strategies mainly alleviate these pathological features but often fail to fully restore bone quality. Extracellular vesicles (EVs) are nanoscale mediators of intercellular communication and have recently emerged as groundbreaking candidates for restoring bone homeostasis. This review systematically explores the multifaceted potential of EVs as therapeutics, diagnostic biomarkers, and drug delivery systems for OP. EVs from diverse biological sources (*e.g.*, mammals, plants, and microbial species) are critically evaluated as innovative modulators of bone metabolism. EVs carry dynamic biomarkers of OP progression which not only possess diagnostic value but also provide novel insights into disease mechanisms. Moreover, EVs could be further bioengineered for bone-targeted drug delivery. Indeed, preclinical studies validate the transformative potential of EVs, although challenges remain in clinical translation. We report current advancements, identify translational barriers, and emphasize the need for interdisciplinary collaboration to accelerate the transition from basic research to clinical applications.

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1. Introduction

Osteoporosis (OP) is a common bone metabolic disease with high rates of morbidity and high disability.[1] OP is usually categorized as primary OP including senile OP and postmenopausal OP, secondary OP including hyperthyroid OP and diabetic OP, and idiopathic OP of unknown etiology. OP affects 19.7% of the global population,[2] and the number of osteoporotic fractures is expected to rise to 5.99 million by 2050.[3] The rapid increase in the global aging population emphasizes OP as a significant burden on world healthcare systems. The current drugs primarily include anti-resorptive agents (*e.g.*, bisphosphonates, denosumab, and raloxifene) and bone anabolic agents (*e.g.*, teriparatide and romosozumab), but their long-term use is limited by significant side effects.[4] Therefore, there is a great need for new, effective and safe anti-OP therapies.

Extracellular vesicles (EVs) are traditionally defined as the nucleus-free and nonreplicable particles from cells, while recent findings uncover the role of EVs as novel mediators of cell–cell interactions in various physiological and pathological processes.[5] Indeed, a wide range of cells from plants, animals, and microorganisms release EVs in three main forms (*i.e.*, exosomes, 30–150 nm; microvesicles, 50–1000 nm; and apoptotic bodies, 1000–5000 nm) for regulating different cellular processes like cell trafficking and waste disposal. Exosomes and microvesicles are derived from the endosomal system and the budding of the plasma membrane,[6] whereas apoptotic bodies are formed through cell membrane infolding during programmed cell death. Exosomes and microvesicles contain many functional molecules, such as proteins, lipids, mRNAs, and microRNAs.[7,8] EVs transport different functional molecules to the recipient cells to regulate specific signaling pathways and alter the cell phenotypes.[9,10] Consequently, EVs exhibit sufficient stability, biocompatibility, low immunogenicity, and cytotoxicity, modulating the development and progression of various diseases.[11,12]

On the other hand, EVs have recently been targeted for the management of OP.[13–16] Indeed, EVs from bone cells, endothelial cells, muscle cells, stem cells, immune cells, and gut microbes play important roles in the regulation of bone remo-

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deling and exhibit potent anti-OP effects.[12] Research effort is currently focusing on two areas: (1) to evaluate these vesicles and the contents as biomarkers for diagnosing the disease and (2) to engineer EVs as drug carriers for enhancing the targeting and bioavailability of drugs in bone tissues. In this review, we first introduce the biogenesis, characterization, and functions of EVs derived from diverse sources, including mammalian, plant, and microbial. The therapeutic potential of both natural and engineered EVs in OP treatment is then discussed, emphasizing their promise as a novel therapeutic delivery system. Finally, we delve into the role of EVs as emerging biomarkers in the context of OP. This paper comprehensively reviews current research findings and provides an in-depth analysis of the role of EVs in OP, highlighting their significant potential as both diagnostic and therapeutic tools.

2. Sources of EVs

2.1. Discovery of EVs

EVs were initially discovered as platelet-derived particles (Fig. 1A) from human plasma in 1946,[17] and fully recognized after similar vesicles were detected in the Gram-negative bacteria *Escherichia coli*[18] and plant carrot cells[19] in the mid-1960s. The contents, composition, and function of these vesicles remained largely unknown. Harding[20] and Johnston[21] observed that the intraluminal vesicles were released from reticulocytes in rats and sheep in 1983. These vesicles originated from the endosomal pathway, and were therefore officially termed "exosomes" by Johnstone in 1987.[22]

However, for a long time, EVs were primarily considered to be transporters of metabolic waste products. In 1996, Raposo *et al.* discovered that B lymphocytes released EVs with the capacity of presenting antigen.[23] Exosomes have emerged as a key player in preclinical and clinical conditions. More studies demonstrated that EVs carried out the functional transfer of nucleic acids,[8] and facilitated cell–cell communication.[24] In 2013, James E. Rothman, Randy W. Schekman, and Thomas C. Südhof were awarded the Nobel Prize in Physiology and Medicine for their discovery of vesicle transport within cells. Scientists and clinicians strive for a better understanding of EVs by different new techniques such as nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), cryo-scanning electron microscopy (Cryo-SEM), flow cytometry, and Raman spectrometry.[25,26] Since the establishment in 2014, the International Society for Extracellular Vesicles (ISEV) has been

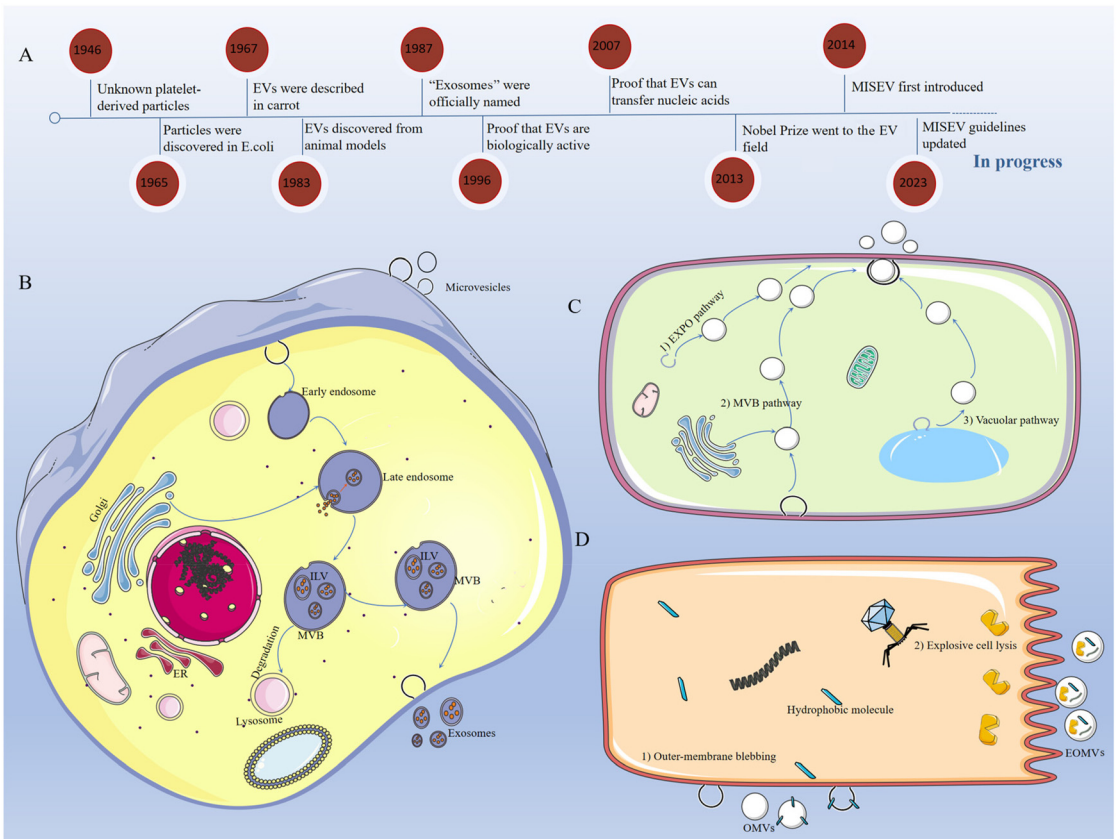

Fig. 1 The research milestones and biogenesis of EVs. (A) The research timeline of EVs; (B) the process of multivesicular body (MVB) pathway for exosome formation in mammalian cells; (C) three mechanisms for the release of EVs from plant cells; (D) two main routes for EV formation in Gram-negative bacteria. MVB, multivesicular bodies; EXPO, exocyst-positive organelle; OMV, outer membrane vesicles; and EOMV, explosive outer membrane vesicles.

optimizing the guidelines and providing standardized methodologies for the production, isolation, and characterization of EVs from diverse biological sources, while MISEV2023 was most recently released.[5]

2.2. EVs from mammalian cells

Mammalian EVs, commonly known as exosomes with diameters ranging from 30 to 150 nm, are ubiquitously present in diverse body fluids such as urine, plasma, saliva, semen, amniotic fluid, ascites, and cerebrospinal fluid.[27] As illustrated in Fig. 1B, these EVs are predominantly formed through the multivesicular body (MVB) pathway *via* three steps from inward budding of the plasma membrane to early endosome production, incorporation of various cargo into intraluminal vesicles (ILVs), and formation of MVBs. At the final stage, mature MVBs fuse with the plasma membrane to release exosomes.[28]

Mammalian exosomes vary in their contents with the cell types and thereby exhibit different biological functions. Lipids, proteins, and nucleic acids are three primary components that determine the functions of exosomes. Lipids are rich in the membrane of exosomes and also contribute to the biogenesis and cargo sorting.[29] Proteins and nucleic acids are probably key players in exosomal intercellular communications. It is now known that the exosome-mediated cellular signaling pathways are important for unraveling the pathogenesis of complex diseases. The exosomal proteins and microRNAs are implicated in the epigenetic regulation of diverse diseases, offering a new avenue for the diagnosis and treatment of different diseases.[30,31] Moreover, surface protein biomarkers such as CD9, CD63, and CD81 are widely used to identify mammalian-derived EVs[32] while other methods are also available.[33]

2.3. EVs from plants

Plant EVs are secreted into the extracellular apoplast space and contain a rich source of plant secondary metabolites, nucleic acids, and proteins.[34] Although initially overlooked, plant EVs were found to play a role in plant defense in 2006.[35] However, due to the absence of a consensus terminology system, a plethora of terms such as exosomes, nanovesicles, microvesicles and exosome-like vesicles exist. This issue was addressed by a group of botanists. And nomenclature, separation, and characterization were then standardized.[36] As a result, plant EVs are used to describe the vesicles isolated from the extracellular apoplast, whereas plant-derived nanovesicles (PDNVs) describe the vesicles generated through disruptive processes like blending or juicing plant materials. The biogenesis of plant EVs involves three potential pathways including the MVB pathway,[24] the exocyst-positive organelle (EXPO) pathway,[37] and the vacuolar pathway[38] (Fig. 1C). The MVB pathway dominates plant EV formation, while the EXPO pathway is a unique unconventional secretion pathway in plants.

2.4. EVs from microbes

EVs from Gram-negative bacteria are essentially membrane vesicles (MVs) originating from the bubbling of the outer membrane.[39] MVs are also formed in Archaea.[40] MVs with sizes ranging from 20 to 400 nm in diameter bear nucleic acids, toxins, signaling molecules, enzymes, and antibiotic resistance factors, indicating a crucial role in bacterial survival, communications, infection and other processes.[41,42] Fig. 1D depicts membrane blebbing and explosive cell lysis for the formation of MVs.[43,44] Membrane blebbing produces classic outer membrane vesicles (OMVs) due to disturbances in the cell membrane, whereas explosive cell lysis results in explosive outer membrane vesicles (EOMVs) due to cell wall degradation by phage-derived endolysins.[45] Consequently, OMVs are free of cytoplasmic contents, whereas EOMVs may contain cytoplasmic components. Gram-positive bacteria lacking the outer membrane may produce cytoplasmic membrane vesicles (CMVs) through the cell explosion pathway. CMVs were found to induce cell death, although the precise mechanisms remain largely unexplored.[46]

3. Roles of EVs in the therapy of OP

The current anti-OP treatments are limited by slow progression, undesired side effects, and a tendency of recurrence.[47] EVs hold promise for disease management due to their excellent biocompatibility and low cytotoxicity.[48] Different EV proteins and nucleic acids are responsible for the anti-OP effects.[49] Thus, we focus on the anti-OP properties of EVs derived from mammals, plants, and microbes as illustrated in Fig. 2.

3.1 Anti-osteoporotic effects of mammalian EVs

Mammalian EVs are currently categorized by sources into three groups: cell-derived EVs, body fluid-derived EVs, and tissue-derived EVs. Specifically, EVs from various cells, including MSCs, bone cells, endothelial cells, muscle cells, and immune cells, are important participants in bone modeling and remodeling (Fig. 3). It is expected that these EVs may improve the symptoms of OP through transporting their internal cargoes to exhibit osteogenic, osteoclastic, angiogenic, and immunomodulatory effects. The anti-osteoporotic effects of these EVs are discussed as follows.

3.1.1. Cell-derived EVs

3.1.1.1 Mesenchymal stem cell (MSC)-derived EVs. MSCs are adult stem cells with high self-renewal and differentiation potential. MSC-derived EVs are evaluated for the therapeutic effects for the treatment of OP,[50]including bone marrow-derived mesenchymal stem cells (BMSCs), adipose-derived mesenchymal stem cells (ADSCs), and umbilical cord mesenchymal stem cells (UMSCs) for the treatment of OP.

The miRNAs in EVs play pivotal roles in regulating the key signaling pathways in bone metabolism (Fig. 4). For instance, BMSC-derived exosomal miR-27a and miR-196a enhance osteoblastogenesis by activating the Wnt/β-catenin pathway through distinct or complementary mechanisms.[51,52] miR-27a directly suppresses the expression of Dickkopf2 (DKK2) and secreted frizzled-related protein 1 (SFRP1), which are two endogenous

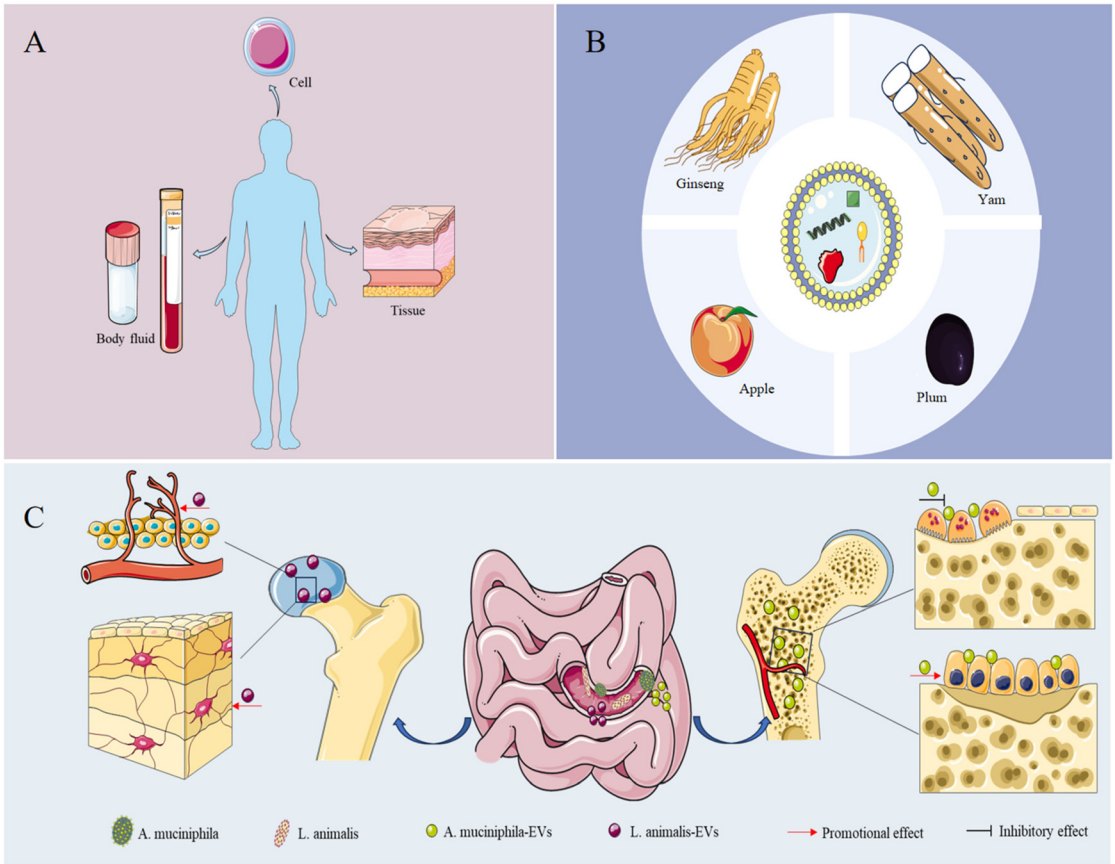

Fig. 2 Biotherapeutic applications of different EVs in OP management. (A) EVs from mammalian cells, body fluids, and tissues; (B) EVs from various plants (ginseng, yam, apple, and plum); (C) EVs from gut microbes (*Lactobacillus animalis* and *Akkermansia muciniphila*).

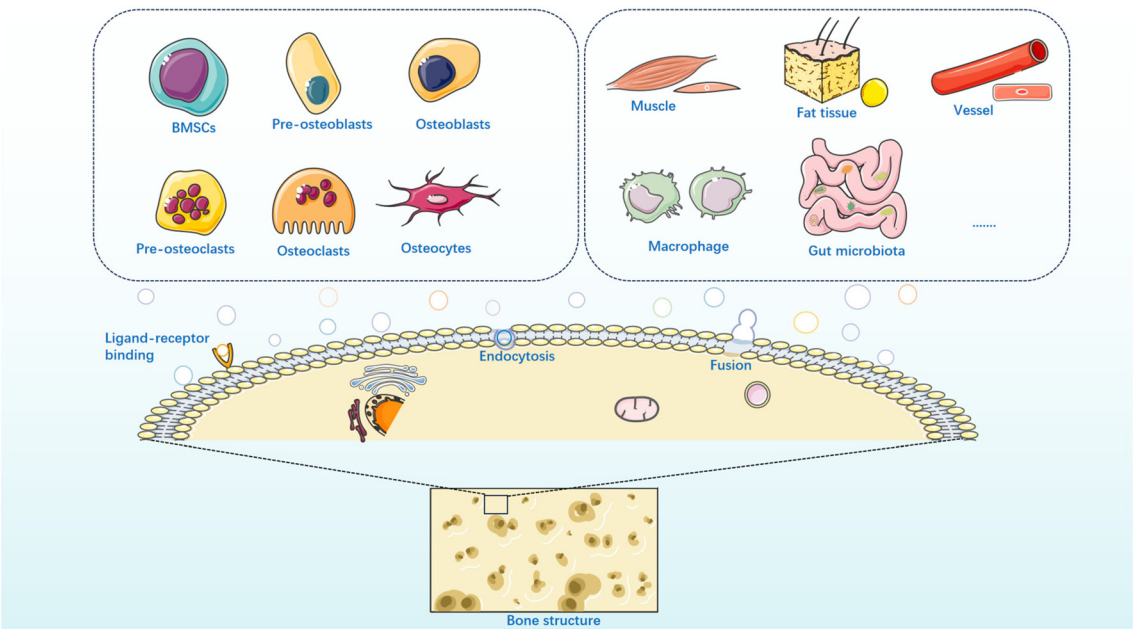

Fig. 3 A scheme illustrating the involvement of different cell-derived EVs in bone metabolism. EVs are released from different cells including bone cells, endothelial cells, skeletal muscle cells, adipose-derived stem cells, and immune cells and the gut microbiota. These EVs participate in bone modeling and remodeling *via* targeting recipient cells through binding to cell surface receptors, endocytosis or fusion.

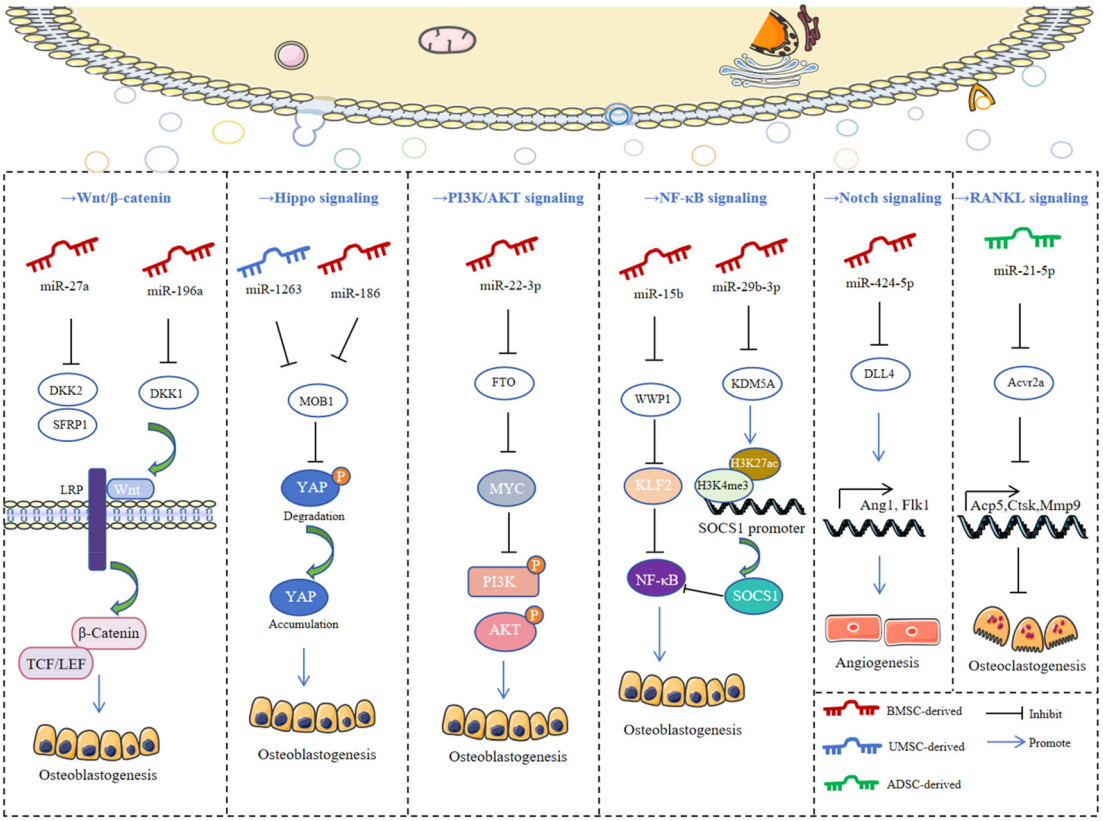

Fig. 4 Regulation of bone metabolism by representative miRNAs from different MSC-derived EVs.

Wnt antagonists, thereby supporting the Wnt ligand–receptor interactions.[51] miR-196a, on the other hand, specifically downregulates another potent Wnt pathway inhibitor Dickkopf1 (DKK1), thereby upregulating the LRP5/6 co-receptors to facilitate Wnt signaling.[52] It is now known that the stabilized β-catenin is translocated into the nucleus and binds to TCF/LEF transcription factors to upregulate osteogenic master regulators such as Runx2 and Osterix, ultimately driving osteogenic differentiation.

Conversely, miR-1263 and miR-186 regulate bone homeostasis by targeting the Hippo signaling pathway.[53,54] In disuse-induced OP, human UMSC-derived exosomal miR-1263 directly binds to the 3′-UTR of Mob1, suppresses its gene expression, and thereby disrupts the canonical Hippo signaling.[53] Consequently, YAP is not effectively phosphorylated and instead accumulates in the nucleus, where it not only inhibits BMSC apoptosis but also enhances osteogenic signaling, thus playing a dual protective role in bone maintenance. In postmenopausal OP, BMSC-derived exosomal miR-186 promotes osteogenesis *via* a mechanism analogous to YAP activation. Specifically, miR-186 downregulates Mob1, leading to upregulated YAP expression, thereby enhancing bone formation and mitigating osteoporotic bone loss.[54]

Moreover, miR-22-3p regulates osteoblast differentiation through the FTO (FTO α-ketoglutarate dependent dioxygenase)-mediated PI3K/Akt pathway.[55] Mechanistically, miR-22-3p directly targets the m6A demethylase FTO, disrupting MYC mRNA stability, suppressing PI3K/AKT signaling and ultimately promoting osteogenic differentiation. Other studies demonstrated that miR-15b and miR-29b-3p exert anti-osteoclastic effects by suppressing NF-κB signaling.[56,57] miR-15b in BMSC-secreted EVs targets WWP1 (WW domain-containing E3 ubiquitin protein ligase 1) to inhibit the ubiquitination and degradation of KLF2. By stabilizing KLF2, this pathway inactivates NF-κB signaling in BMSCs, thereby promoting osteogenic differentiation.[56] Downregulated miR-29b-3p in osteoporotic BMSC-derived EVs inhibits KDM5A, increasing H3K4me3 and H3K27ac histone marks at the SOCS1 promoter. SOCS1 upregulation further suppresses NF-κB signaling, establishing a self-reinforcing loop that enhances osteogenic differentiation.[57]

Previous studies also showed that EVs promote angiogenesis through the HIF-1α/VEGF[58] and DLL4/Notch[59] signaling pathways to control bone repair. miR-424-5p in EVs appears to enhance angiogenesis by suppressing DLL4,[59] while UMSC-derived EVs contain a potent pro-osteogenic and anti-osteoclastic protein CLEC11A to simultaneously enhance osteogenic differentiation and inhibit osteoclast formation.[60] Furthermore, ADSC-derived EVs exhibit multifaceted roles in bone regeneration. Specifically, miR-375 is transported to target IGFBP3 (insulin-like growth factor binding protein 3) and stimulate osteogenesis.[61] Other components such as

osteoprotegerin (OPG) and miR-21-5p suppress RANKL-induced osteoclastogenesis.[62] On the other hand, ADSC-derived EVs demonstrate immunomodulatory effects in OP treatment.[63] As a potential mechanism, the use of miR-146a attenuates inflammasome activation and bone loss by downregulating pro-inflammatory cytokines including TNF-α, IL-18, and IL-1β in diabetic OP models.

3.1.1.2 Bone cell-derived EVs. OP is primarily caused by the imbalance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Large multinucleated osteoclasts not only degrade bone collagen but also secrete EVs containing actins and integrins for establishing the sealing zone and facilitating cell adhesion and signaling in bone resorption.[64,65] Notably, osteoclast-derived EVs also carry miRNAs that specifically regulate osteogenic differentiation. For instance, miR-324 promotes osteogenic differentiation of BMSCs by targeting osteogenic differentiation inhibitor ARHGAP1.[66] However, osteoclast-derived miRNAs may play a different role. Li *et al.*[14] and Sun *et al.*[67] demonstrated that miR-214-3p and miR-214 from osteoclasts were absorbed by osteoblasts and subsequently inhibited osteoblastogenesis. Similarly, Yang *et al.*[68] found that miR-23a-5p in osteoclast-derived exosomes suppresses osteogenic differentiation by targeting Runx2.

Osteoblast-derived vesicles also mediate the intercellular communication between osteoblasts, osteoclasts, and their progenitors in bone remodeling. For instance, osteoblast-derived miR-218 enhances osteogenic differentiation through downregulating SOST, DKK2, and SFRP2 in the Wnt signaling pathway.[69] Conversely, osteoblasts secrete exosomal miR-133 and miR-135 to inhibit osteogenesis and attenuate osteoprogenitor differentiation by targeting two key BMP signaling regulators, Runx2 and Smad5.[70] Additionally, osteoblast-derived exosomal miR-503-3p suppresses osteoclast differentiation by targeting heparanase (Hpse),[71] while EVs also transfer RANKL protein to osteoclast precursors to activate RANKL-RANK signaling in osteoclast formation.[72] Osteocyte-derived EVs appear to participate in osteoblastic bone formation. Li *et al.* found that osteocyte-derived exosomal miR-124-3p inhibited osteoblastogenesis under high-glucose conditions by targeting galectin-3.[73] Emerging evidence suggests that the role of EVs in the communication between bone-related cells represents a novel regulatory mechanism for bone homeostasis, offering potential therapeutic targets for OP (Fig. 5).

3.1.1.3 Immune cell-derived EVs. The immune system is critical for bone metabolism.[74] EVs derived from M2 macrophages enhance osteogenesis. Specifically, miR-378a promotes BMSC differentiation by inhibiting PPARα (peroxisome proliferator-activated receptor alpha),[75] and miR-5106 facilitates osteogenesis by downregulating SIK2/3 (salt-inducible kinase 2/3).[76] Conversely, M1 macrophage-derived EVs inhibit osteogenesis. For example, miR-155 downregulates key osteogenic markers (BMP2, BMP9, RUNX2)[77] and miR-98 disrupts differentiation by targeting dual specificity phosphatase 1 (DUSP1) and activating JNK signaling[78] (Fig. 5). Moreover, dendritic cell-derived exosomal miR-335 enhances BMSC proliferation and osteogenic capacity by inhibiting the Hippo pathway *via* LATS1 suppression,[79] while exosomal osteopontin (OPN) and matrix metalloproteinase-9 (MMP-9) promote the recruitment and migration of BMSCs.[80] Regulatory T cells

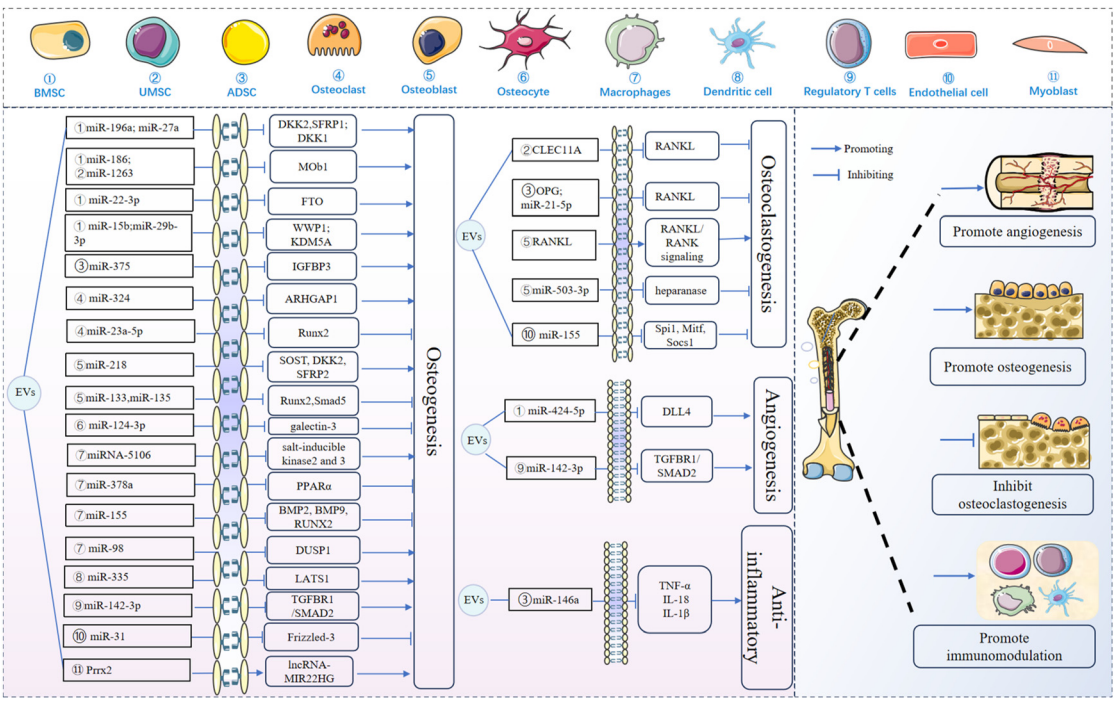

Fig. 5 EVs mitigate osteoporosis through osteogenic, osteoclastic, angiogenic, and immunomodulatory effects mediated by their cargo.

(Tregs) also promote bone repair and accelerate fracture healing through exosomal miR-142-3p.[81] It is proposed that miR-142-3p promotes angiogenesis and osteogenesis by downregulating TGFBR1/SMAD2 signaling in BMSCs and endothelial cells (Fig. 5).

3.1.1.4 Endothelial cell-derived EVs. Blood vessels deliver oxygen, nutrients, and hormones and transport immune cells and precursor cells to maintain bone homeostasis.[82] In particular, H-type endothelial vessels promote bone formation by coupling angiogenesis with osteogenesis in the murine skeletal system.[83] These results underscore the critical role of endothelial cells in bone homeostasis. Interestingly, endothelial cell-derived EVs (EC-EVs) modulate the activities of osteoclasts and osteoblasts in bone homeostasis for their superior bone-targeting capability and anti-osteoporotic effects compared to exosomes from other bone cells, while exosomal miR-155 contributes to the effective inhibition of osteoclast differentiation and function in OVX mice.[84] Mechanistically, miR-155 disrupts osteoclastogenesis by targeting key regulators including Spi1, Mitf, and Socs1 in bone marrow-derived macrophages. As for the osteogenic functions, EC-derived EVs enhance osteoblast viability and function by inhibiting ferroptosis and preventing osteoporotic bone loss.[85] Senescent EC-derived exosomal miR-31 conversely suppresses MSC osteogenic differentiation by downregulating a Wnt5A receptor protein Frizzled-3.[86] Such cargo- and cell state-dependent regulation of bone metabolism may be targeted for the development of new anti-OP treatments (Fig. 5).

3.1.1.5 Muscle cell-derived EVs. Skeletal muscles are anatomically adjacent to bones, implying that EVs may mediate functional crosstalk within the musculoskeletal system. Myoblast-derived EVs (Myo-EVs) exhibit dual osteogenic and pro-senescence effects on muscle–bone crosstalk in bone metabolism. It was recently shown that Myo-EVs enhanced osteogenic differentiation of BMSCs through Prrx2-mediated activation of MIR22HG transcription, while miR-128 promoted YAP expression and nuclear translocation to alleviate OVX-induced OP.[87] On the other hand, Myo-EVs deliver senescence-associated miRNAs (*e.g.*, miR-34a) that reduce Sirt1 expression in BMSCs, inducing cellular senescence and potentially contributing to age-related OP.[88] Moreover, Myo-EVs significantly inhibit osteoclastogenesis by suppressing RANKL-induced osteoclast formation in both mouse macrophages and RAW264.7 preosteoclastic cells. Myo-EVs may target multiple pathways including downregulation of osteoclast-specific markers (TRAP, cathepsin K, and NFATc1), inhibition of mitochondrial energy metabolism, and reduction of oxygen consumption.[89] Therefore, Myo-EVs represent promising therapeutic targets for regulating bone formation and resorption in musculoskeletal homeostasis and osteoporosis treatment (Fig. 5).

3.1.2 Body fluid and tissue-derived EVs. EVs derived from various bodily fluids and tissues may regulate bone metabolism through diverse mechanisms. Biofluid-derived EVs, particularly those from human umbilical cord blood (UCB-EVs), exhibit dual osteogenic and anti-osteoclastic properties.[90] In particular, UCB-EVs may promote BMSC osteogenesis while inhibiting osteoclastogenesis in a miR-3960-dependent manner. Similarly, dietary bovine milk EVs exhibit promising effects against bone loss in osteoporotic models.[91] Current evidence supports the potential of muscle-derived EVs (Mu-EVs) in the regulation of bone homeostasis. Mu-EVs could rebalance bone remodeling by simultaneously enhancing BMSC osteogenic differentiation through lactate dehydrogenase A-mediated glycolysis[92] and suppressing monocyte-derived osteoclast formation.[93] EVs derived from adipose tissue also exhibit anti-osteoporotic properties.[94] Nevertheless, EVs from tissues or tissue explant cultures remain underutilized.

3.2 Anti-osteoporotic actions of plant-derived EVs

EVs derived from plants, such as edible fruits, vegetables, and Chinese herbs, are extensively investigated for safety, biocompatibility, biodegradability, and easy preparation. Plant-derived EVs exhibit antioxidant, anti-inflammatory, and regenerative activities[95,96] and demonstrate therapeutic potential against various diseases, including gastric cancer,[97] kidney stones,[98] gut microbe dysregulation,[99] colitis,[100] breast cancer,[101] and liver dysfunction.[102] Thus, we will discuss the anti-OP actions of plant-derived EVs (Fig. 2B) as follows.

Plant-derived EVs are known to harbor active metabolites. Ginseng-derived EVs (GEVs) contain ginsenosides Rb1 and Rg1 and impede osteoclastogenesis and mitigate lipopolysaccharide-induced bone loss *in vivo*, partly through the inhibitory effects of ginsenosides on osteoclast differentiation.[103,104] It is a surprise that GEVs were tenfold greater in the anti-osteoclastogenesis efficacy compared to individual or mixed ginsenosides.[105] Presumably, the spherical lipid membrane structure of GEVs and other potent microRNAs or proteins may contribute to such high efficacy. Yam-derived EVs (YEVs) have been shown to stimulate osteoblast proliferation, differentiation, mineralization, and bone remodeling in OVX-induced osteoporotic mice.[106] However, YEVs do not contain the osteogenic compounds diosgenin and dioscin.[107] Further study suggested that YEVs might promote osteogenesis by activating the BMP-2/P-P38-dependent Runx2 pathway. Plum-derived EVs were found to suppress osteoclast differentiation and simultaneously promote osteoblast proliferation, differentiation, and mineralization in MC3T3-E1 cells or BMSCs.[108] Apple-derived EVs promoted osteoblastogenesis in MC3T3-E1 cells by regulating the BMP2/Smad1 pathway.[109] These findings suggest that plant-derived EVs are promising therapeutic agents in bone modeling and remodeling.

3.3. Anti-osteoporotic effects of microbial EVs

The term "osteomicrobiology" has been recently proposed to describe the action of gut microbiota on bone metabolism.[110–112] Previous studies on the differences in bone mass between normal and germ-free mice have demonstrated the role of gut microbiota in regulating bone quality.[113] Furthermore, significant variations in gut microbiota populations have been observed between OP patients and healthy individuals.[114,115] However, little is known about the impact of

gut microbiota on bone. Gut microbiota likely secretes EVs as messengers to communicate with bones.[116,117] The abundance of *Pseudomonas panacis*-derived EVs increased in the stool of mice on a high-fat diet. Upon oral administration, these stool-derived *P. panacis* EVs were absorbed by insulin-responsive tissues (*e.g.*, liver, adipose tissue, and skeletal muscle) and caused insulin resistance and glucose intolerance in the mice.[118] These results highlight the possibility that bacterial EVs mediate the intercellular crosstalk between the gut microbiota and the host.

Moreover, gut bacteria EVs may contribute to the management of OP.[119] It was found that cohousing with healthy mice or fecal microbiota transplantation (FMT) from normal mice reversed glucocorticoid-induced OP, likely due to the accumulation of gut *Lactobacillus animalis*. *L. animalis* secreted EVs to mitigate glucocorticoid-induced OP *via* promoting angiogenesis and osteogenesis while inhibiting apoptosis. In another similar study, Liu *et al.* found that gut bacterium *Akkermansia muciniphila* was reduced in OVX mice, whereas OVX mice on *A. muciniphila* supplementation showed less loss of osteoporotic bone.[120] Mechanistic studies revealed that the anti-OP activity of *A. muciniphila* was dependent on EV secretion. EVs were effectively infiltrated and accumulated in bone tissue to improve bone quality by promoting bone formation and inhibiting bone resorption (Fig. 2C).

FMT is now recognized as an important method for restoring the intestinal microbiota and a novel strategy for the prevention and treatment of OP.[121] Microbial EVs from parental strains encapsulate a variety of key bioactive contents and may exert similar functions. However, the gut microbiota should be carefully manipulated to exclude certain risks, such as chronic disease or pathogen transmission.[122] Cell-free nanocarriers of EVs derived from commensal bacteria in healthy hosts could provide an alternative to FMT (Table 1).

4. Engineered EVs for OP treatment

Liposomes are well established as an effective drug delivery method, while synthetic liposomes have inherent limitations, including membrane toxicity and low compatibility.[132] EVs are currently evaluated as potential carriers for drugs. EVs offer several advantages over synthetic liposomes in drug delivery systems. Firstly, EVs are naturally secreted biological liposomes by cells, and bear minimal immunogenicity and high safety for therapeutic applications. Secondly, EVs possess a unique ability to cross the blood–brain barrier to achieve the efficacy of intracranial drug delivery.[11,133] Additionally, many EVs exhibit inherent tumor-homing properties,[134] and facilitate high permeability and retention at solid tumor sites.[135] Importantly, EVs may be engineered to express specific molecules or enhance bone-targeting capability.[136] Indeed, two primary engineering strategies have been employed to prepare bone-targeting EVs: modification in parent cells or on EVs (Fig. 6).

Parent cell-based modification is to engineer EV-producing cells by genetic engineering techniques for EVs to carry specific molecules. The cells are transfected with a recombinant plasmid containing the desired genes for the expression of the desired proteins on the EV surface. LAMP-2B is a common exosomal surface protein in the EVs of mammalian cells and is thereby employed to display targeting motifs. Liang *et al.* generated chondrocyte-targeting exosomes by transfecting dendritic cells with a CAP (chondrocyte-affinity peptide)-Lamp2b fusion plasmid, enabling efficient delivery of miRNA-140 to chondrocytes and achieving bone tissue-specific drug targeting.[137] In another study, researchers developed CXCR4 (C–X–C motif chemokine receptor 4)-engineered exosomes that leverage the natural SDF1 (stromal cell-derived factor-1)/CXCR4 homing axis to achieve precise bone marrow targeting. When loaded with osteogenic antagomir-188, these modified exosomes effectively promoted osteogenesis while suppressing adipogenesis in BMSCs, demonstrating significant therapeutic potential for age-related bone loss.[138] These studies highlight the promise of targeted exosome therapy for OP and other bone disorders.

Bacterial extracellular vesicles (BEVs) could be readily engineered to express cell-targeting. Li *et al.*[139] and Cheng *et al.*[140] ingeniously introduced an efficient "Plug and display" system for presenting exogenous proteins on the surface of BEVs by fusing exogenous proteins with the ClyA protein, a pore-forming membrane protein in most bacteria.[141] Along this line, Liu *et al.* developed a tailored formulation termed BEVs-hCXCR4-SOST siRNA (BEVs-CSs) for OP treatment. In this formulation, the exogenous protein hCXCR4 was fused with the ClyA protein to guarantee bone-targeting of the BEVs. SOST siRNA was loaded into the BEVs-hCXCR4 as the therapeutic cargo to deliver SOST siRNA to the bone marrow, thereby mitigating OVX-induced OP.[142] BEVs are alternatively used for targeted peptide delivery. For instance, *E. coli Nissle* 1917-derived BEVs are engineered to express pre-osteoclast fusion protein DC-STAMP (BEV-DCS) for delivering the osteoclast-inhibiting peptide FRATtide.[143] Such a strategy protected FRATtide from degradation and enabled targeted delivery to pre-osteoclasts. Indeed, *in vivo* experiments validated that FRATtide-loaded BEV-DCS effectively targeted bone, limited bone loss, and showed excellent safety. Therefore, bioengineered BEVs possess natural biocompatibility and precise targeting capacity compared with current OP therapies.

As for EV-based modification, the engineering process takes place on the isolated EVs. Targeting molecules are often conjugated to EVs through click chemistry or hydrophobic insertion approaches. Wang *et al.* developed chemically modified EVs by coupling anti-OP drug alendronate with alkynyl groups by azide–alkyne cycloaddition and achieved enhanced EVs' bone-targeting capability *via* alendronate/hydroxyapatite binding.[144] Alternatively, hydrophobic interactions offer another innovative chemical modification strategy for bone-targeting EVs. In this approach, a phospholipid–polymer conjugate, DSPE-PEG, is widely used to assemble targeting motifs on EV membranes. Cui *et al.* engineered bone-targeting EVs by incorporating hydrophobic diacyl lipids for OP treatment. These EVs were synthesized by anchoring osteoblast-targeting peptide (SDSSD) onto exosome

Table 1 Effects of EVs from different types of cells on osteoporosis

EV sources	Recipient cells	Actions	Bioactive compounds	Ref.
BMSCs	BMSCs	Promote osteogenic differentiation by activating Wnt/β-catenin signaling	miR-196a; miR-27a	51 and 52
	BMSCs	Inhibit apoptosis/promote osteogenesis of BMSCs by activating Hippo signaling	miR-1263; miR-186	53 and 54
	BMSCs	Promote osteogenic differentiation *via* FTO inhibition	miR-22-3p	55
	BMSCs	Promote osteogenic differentiation by inhibiting NF-κB signaling	miR-15b; miR-29b-3p	56,57
	Osteoblasts/BMSCs	Enhance osteoblast proliferation and differentiation	miR-935; miR-150-3p	15 and 123
	HUVECs	Enhance osteogenesis and angiogenesis	—	58
	HUVECs	Promote angiogenesis by directly targeting repression of VASH1	miR-29a	124
	HUVECs	Promote angiogenesis by regulating the DLL4/Notch signaling pathway	miR-424-5p	59
ADSCs	Osteoclasts	Inhibit osteoclast differentiation	OPG; miR-21-5p; let-7b-5p	62
	Osteoblasts	Enhance osteogenic effects by inhibiting IGFBP3	miR-375	61
	Macrophages	Reduce bone resorption	miR-146a	63
UMSCs	Osteoblasts	Augment osteogenesis through activating AKT signaling	—	125
	BMSCs; RAW246.7	Enhance osteogenic differentiation and inhibit osteoclast formation	CLEC11A	60
Osteoblasts	BMSCs	Promote osteogenic differentiation and inhibit sclerostin	miR-218	69
	Osteoclasts	Inhibit osteoclast differentiation	miR-503-3p	71
	Osteoblasts	Contribute to bone matrix mineralization	TRIP-1	126
	BMSCs	Promote osteogenic differentiation of BMSCs	miR-324	66
Osteocytes	Osteoblasts	Decrease osteoblastic differentiation	miR-218	127
	Osteoblasts	Inhibit osteoblast differentiation	miR-124-3p	73
Macrophages	MSCs	Promote osteogenic differentiation of MSCs	miR-378a	75
	BMSCs	Facilitate osteogenic differentiation of BMSCs by downregulating salt-inducible kinase 2 and 3 genes	miR-5106	76
	BMSCs	Promote osteogenic differentiation	miR-21a-5p	128
	BMSCs	Promote osteogenic differentiation	miR-486-5p	129
	BMSCs	Facilitate osteogenesis and reduce adipogenesis	MiR-690	130
DCs	BMSCs	Enhance the proliferation and osteogenic differentiation of BMSCs	miR-335	79
	BMSCs	Promote the recruitment and migration of BMSCs	Osteopontin; MMP-9	80
Tregs	HUVECs; BMSCs	Promote both angiogenesis and osteogenesis	miR-142-3p	81
ECs	RAW246.7	Inhibit the differentiation and bone resorption of osteoclasts	miR-155	84
	MC3T3-E1	Enhance the function of osteoblasts and inhibit osteoblast ferroptosis	—	85
Muscle cells	BMSCs	Promote osteogenic differentiation of BMSCs	LDHA glycolytic enzymes	92
	RAW246.7	Inhibit osteoclast formation	—	89
Breast milk	Macrophages	Improve bone loss	TGF-β	131
Umbilical cord blood	BMSCs	Promote osteoblastic differentiation of BMSCs	miR-3960	90
Yam	MC3T3-E1	Promote osteogenesis by activating the BMP-2/P-P38-dependent Runx2 pathway	—	106
Ginseng	Macrophages	Inhibit osteoclastogenesis	Ginsenosides	105
Plum	Macrophages; BMSCs/MC3T3-E1	Suppress osteoclast differentiation and promote osteoblast proliferation, differentiation, and mineralization	—	108
Apple	MC3T3-E1	Stimulate osteoblastogenesis by regulating the BMP2/Smad1 pathway	—	109
L. animalis	HUVECs; BMSCs/MC3T3-E1	Promote angiogenesis and osteogenesis and inhibit apoptosis	—	119
A. muciniphila	BMSCs/RAW246.7	Promote bone formation and inhibit bone resorption	—	120

membranes. Subsequently, small interfering RNA (siShn3) was loaded into exosomes using electroporation technology to create the engineered bone-targeting EVs, BT-Exo-siShn3. Due to its high affinity for hydroxyapatite and calcium phosphate on the bone-forming surface, SDSSD enables BT-Exo-siShn3 to selectively deliver siRNA into osteoblasts.[145]

In fact, engineered EVs combine surface-targeting modifications with therapeutic payload encapsulation, solving the delivery challenges of conventional nucleic acids (miRNAs/siRNAs) and peptides that suffer from poor stability and tissue penetration. This makes EVs ideal carriers for bone-targeted RNA or protein therapies (Table 2).

5. Diagnostic role of EVs in OP

EVs are present in different biological fluids as intercellular messengers. Owing to their easy accessibility, good stability,

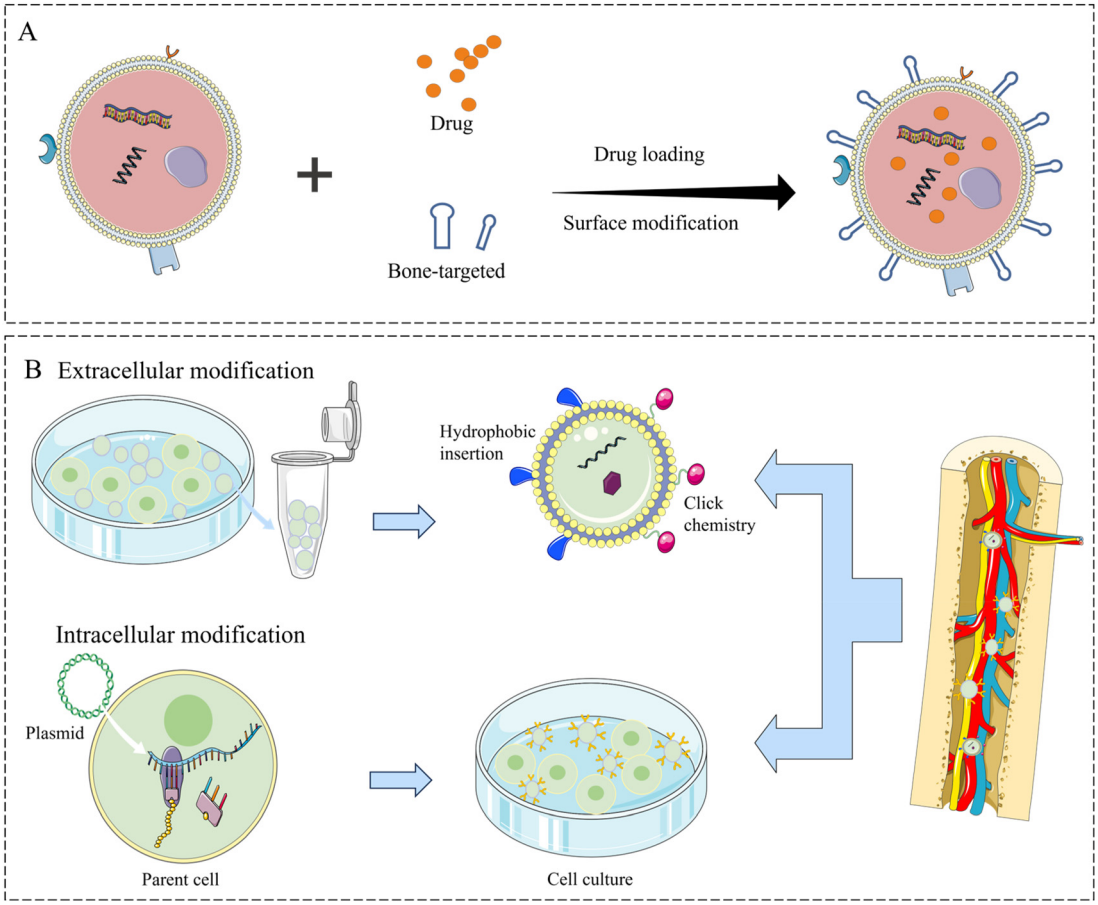

Fig. 6 Engineering strategies for achieving bone-targeted EVs. (A) Cargo loading and surface modification. (B) Surface modification and internal modification of EVs.

Table 2 The characterization and application of EVs from different sources

Sources of EVs	Mammalian EVs	Plant EVs	Microbe EVs	Artificial EVs
Biogenesis	MVBs	MVBs; EXPO; and vacuolar	OMVs; EOMVs	Extracellular modification; intracellular modification
Size	30–150nm	50–500 nm	20–400 nm	—
Contents	Lipids; nucleic acids; proteins	Lipids; nucleic acids; proteins; metabolites	Nucleic acids; toxins; signaling molecules; enzymes; and antibiotic resistance factors	—
Advantages	Superior biocompatibility; low immunogenicity	Less toxicity or immunogenicity; environmentally friendly; low cost; and free of human pathogens	Intrinsic immunomodulatory properties; ease of modification; and ease of industrialization	Selective targeting ability; controllability; and uniformity
Disadvantages	High cost; low extraction yield	Lack of characterization techniques; *in vivo* distribution is unclear; and high heterogeneity	Lack of standardization; potential biosafety; ambiguous contents	Low biocompatibility; material-related toxicity
Applications	Disease diagnosis; therapeutic drugs; and drug carriers	Biotherapeutic agents; drug carriers	Vaccines; cancer immunotherapy agents; drug carriers; antibacterial agents; and diagnostics	Targeted therapy; drug carriers
Ref.	146	147 and 148	149 and 150	136 and 151

and high accuracy, EVs can be readily developed as biomarkers in liquid biopsies for diagnosis. In fact, OP is currently diagnosed by examining bone density and detecting serum bone metabolism markers, such as procollagen I N-terminal extension peptide (P1NP) and C-telopeptide breakdown products (CTX).[152] However, bone density examination is not sensitive

enough to identify the early stages of the disease, whereas serum bone turnover markers are unstable and inaccurate.[153] Therefore, EVs are robust new biomarkers for monitoring OP progression. EVs bear bioactive proteins and nucleic acid molecules as disease biomarkers[154,155] (Fig. 7).

5.1 miRNAs

Exosomal miRNAs may be potential biomarkers for diagnosing postmenopausal OP (PMOP) and associated fractures. By studying 54 PMOP patients, 48 osteopenic (a state between normal bone density and osteoporosis) subjects, and 44 healthy controls, miR-1246 and miR-1224-5p were identified as the most upregulated and downregulated miRNAs in the EVs of osteoporotic patients.[156] Specifically, miR-1246 enhances RANKL-induced osteoclast formation, promoting osteoclastogenesis.[157] In contrast, miR-1224-5p suppresses ADCY2-dependent Rap1 signaling, inhibiting osteoclastogenesis while promoting osteoblastogenesis.[158] Exosomal miR-1246 was elevated in patients with Gorham-Stout disease (a rare disease characterized by progressive bone destruction or bone resorption), suggesting that miR-1246 could serve as a promising biomarker for bone loss-related diseases.[156] Ciuffi *et al.* reported that miR-21-5p levels were significantly higher in OP patients compared to healthy controls, regardless of fracture status, whereas miR-23a-3p levels were lower in osteoporotic patients compared to osteopenic and healthy individuals, while miR-320a-3p levels increased in osteoporotic patients with fractures.[159]

By analyzing a total of 139 serum and 134 plasma samples from 161 recruited participants using PCR arrays and RT-qPCR, the levels of miR-122-5p were found to be decreased in patients with OP, particularly in those with fractures. Additionally, the levels of miR-4516 were reduced in plasma samples from patients with OP.[160] Shi *et al.* demonstrated that miR-324-3p, miR-766-3p, and miR-330-5p were downregulated, while miR-1247-5p and miR-3124-5p were upregulated in PMOP with fragility fractures.[161] Three miRNAs (miR-324-3p, miR-766-3p, miR-1247-5p) were associated with BMD of the lumbar spine and hip, while miR-330-5p and miR-3124-5p were linked to femoral neck and hip BMD. Functional analysis revealed that miR-330-5p promoted, whereas miR-3124-5p suppressed, alkaline phosphatase (ALP) activity in bone marrow stromal cells. According to a large-scale clinical study involving a total of 448 participants, serum levels of miR-30c-2-3p, miR-497-5p, miR-550a-5p, and miR-654-5p were increased, while the levels of miR-199a-5p, miR-654-5p, miR-1260b, miR-1260b, miR-663a, and miR-1299 were decreased in patients with OP.[162] Taken together, due to convincing alterations in OP with or without fragility fractures, exosomal miRNAs may serve as potential diagnostic biomarkers for OP and consequent fracture risk.

5.2 Proteins

EVs also contain proteins as biomarkers for monitoring the pathological processes of OP. By analyzing the content of EVs from healthy individuals and OP patients, β1 and β3 integrins and CD34, as positive regulatory proteins of bone remodeling, were found to be lower in patients with OP.[163] The integrin family proteins regulate bone metabolism by modulating mechano-sensation, proliferation, differentiation, adhesion, and migration of bone-related cells.[164,165] $CD34^{+}$ cells are important to promote fracture healing *via* angiogenesis and

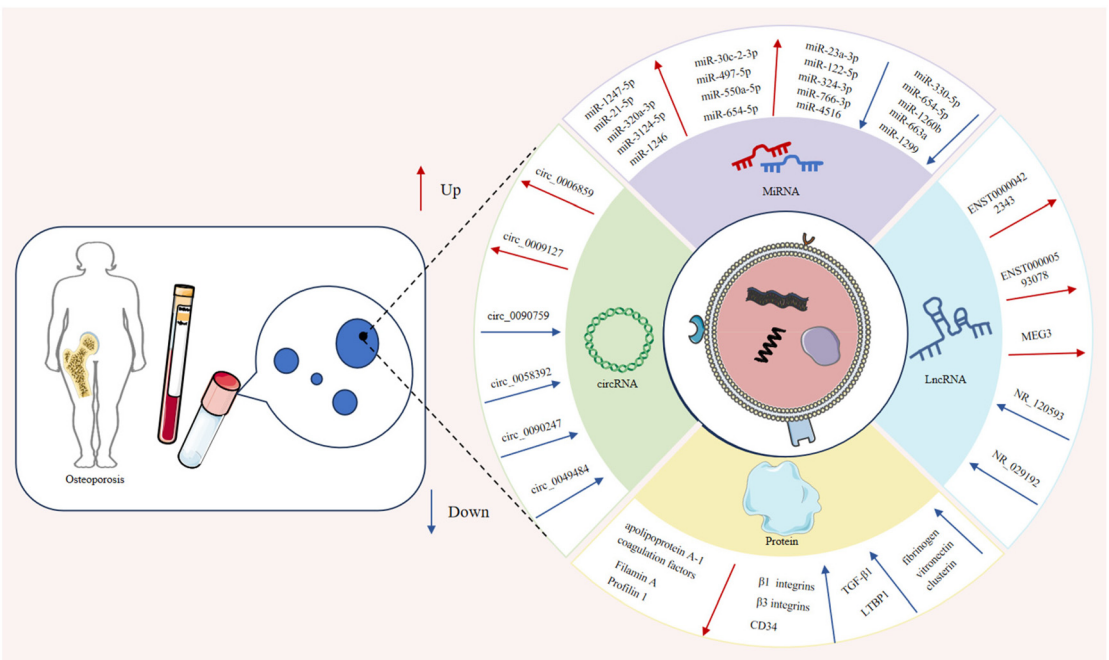

Fig. 7 EVs as biomarkers for OP diagnosis. Proteins and nucleic acids derived from EVs are subject to upregulation or downregulation during OP development, holding promise as potential biomarkers for clinical diagnosis.

osteogenesis.[166] TGF-β signaling-associated proteins, such as TGF-β1 (transforming growth factor-β1) and LTBP1 (latent-transforming growth factor beta-binding protein 1), were also decreased in patients with OP, indicating a discount in bone formation. However, the levels of cytoplasmic ribosomal proteins were increased, indicating the upregulation of RANKL-induced osteoclast differentiation.[167] The expression levels of Filamin A and Profilin 1 were significantly higher in serum EVs from patients with OP than those without OP.[168] Filamin A is an important stimulator of osteoclastogenesis,[169] whereas Profilin 1 is a negative osteoblast functional cytokine for inhibiting BMP-induced differentiation of osteoblasts and causing bone loss.[170]

Proteomic analysis of plasma samples from 146 participants revealed that the levels of fibrinogen, vitronectin, and clusterin were decreased, while the levels of coagulation factors and apolipoprotein A-1 were increased in EVs from PMOP patients and osteopenic subjects.[156] von Willebrand factor (VWF) was lacking whereas fibrinogen levels were reduced in osteoporotic patients, indicating the dysregulation of bone remodeling and the higher risk of fracture.[171,172] Apolipoprotein A-1 plays a positive regulatory role in osteoblast function but appears to be upregulated in EVs from OP patients.[173] Vitronectin may limit osteoblast differentiation and boost osteoclast activity, which was reduced in EVs from patients with OP.[174] These findings highlight that proteins in serum EVs may be potential biomarkers for bone remodeling and OP development.

5.3 LncRNAs and circRNAs

Other types of short RNAs such as long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) are also important components of EVs and should be evaluated as potential markers for the clinical diagnosis of OP.

LncRNAs are a class of RNA molecules with a transcript length of >200 nt and may regulate gene expression through epigenetic, transcriptional, and post-transcriptional mechanisms. In OP, particularly PMOP, various exosomal lncRNAs are dysregulated. ENST00000422343, ENST00000593078, and MEG3 were significantly upregulated, while NR_120593 and NR_029192 were downregulated in exosomes derived from BMSCs of PMOP patients.[175] ENST00000422343 may regulate osteogenic differentiation *via* competitive interactions with hsa-miR-214 against endogenous RNA (ceRNA), affecting the PI3K/Akt signaling pathway.[176] NR_120593 interacts with hsa-miR-221 to inhibit osteogenesis by targeting Runx2.[177] MEG3 overexpression in BMSC-derived EVs facilitates osteoblast proliferation and differentiation by targeting miR-3064-5p.[178]

CircRNAs are characterized by their covalently closed circular structures and the length from hundreds to thousands of nucleotides. circRNAs act as miRNA sponges to mitigate miRNA-mediated suppression of target gene expression and modulate various cellular processes. CircRNAs-containing EVs were documented to regulate osteoblastogenesis and osteoclastogenesis, suggesting a critical role in bone homeostasis.[179] Several exosomal circRNAs are potential biomarkers for diagnosing OP. For example, exosomal hsa_circ_0006859 is one of the most significantly elevated circRNAs in OP. Mechanistically, has_circ_0006859 functions as a sponge for miR-431-5p, inhibits osteoblastic differentiation and enhances adipogenic differentiation in BMSCs.[180] RNA analysis of the exosomes derived from BMSCs of 20 PMOP patients and corresponding healthy controls revealed that one circRNA (hsa_circ_0009127) was upregulated, while four circRNAs (hsa_circ_0090759, hsa_circ_0058392, hsa_circ_0090247, and hsa_circ_0049484) were downregulated in PMOP. The dysregulation of these circRNAs may affect autophagy, PI3K-Akt signaling, FoxO signaling, and MAPK signaling through a circRNA-miRNA-mRNA interaction network.[181] Taken together, these findings highlight the potential role of lncRNAs and circRNAs in the diagnosis of OP.

6. Perspectives and conclusion

The complex interplay of the immune system,[182] blood vessels,[183] intestinal flora,[184] and hormone levels[185] in maintaining bone homeostasis underscores the need for multifaceted therapeutic approaches. EVs, with their diverse bioactive cargo, inherent biocompatibility, and remarkable stability, have emerged as promising diagnostic and therapeutic tools for OP. Therapeutic delivery routes and mechanisms of action in OP models are summarized in Fig. 8.

Although recent preclinical studies show promising results, clinical translation faces significant challenges. For example, EV manufacturing is not well standardized. Conventional ultracentrifugation suffers from limited yields, while alternative approaches like size-exclusion chromatography require further validation.[186] The ISEV has attempted to establish guidelines to guarantee the quality including purity, potency, and sterility in the production of therapeutic EVs.[187] Secondly, EVs are affected by pharmacokinetic uncertainties. Current research primarily focuses on the systemic intravenous delivery of therapeutic EVs. As a result, the biodistribution of EVs is predominantly localized in the liver, lungs, kidneys, and spleen across most tested sources, size ranges, and animal models. EV accumulation peaks in these organs within 1–2 hours, while skeletal tissues exhibit delayed kinetics and active uptake of EVs. EVs are detectable in bone/bone marrow at background levels during early phases (<12 h), reaching moderately high levels by 24 h.[188] To overcome this limitation, RGD peptide modification has emerged as a promising strategy to enhance bone-specific delivery,[189] although the half-life and off-target distribution of EVs should be comprehensively characterized. Thirdly, long-term biosafety profiles of EVs should be thoroughly evaluated. Although EVs derived from non-tumorigenic sources (*e.g.*, MSCs) are generally considered safe,[190] their membrane composition, particularly enrichment with "self" markers such as CD47,[191] may further reduce immunogenicity risks compared to synthetic nanoparticles. Nevertheless, potential biosafety concerns persist regarding off-target effects, as systemic administration typically results in

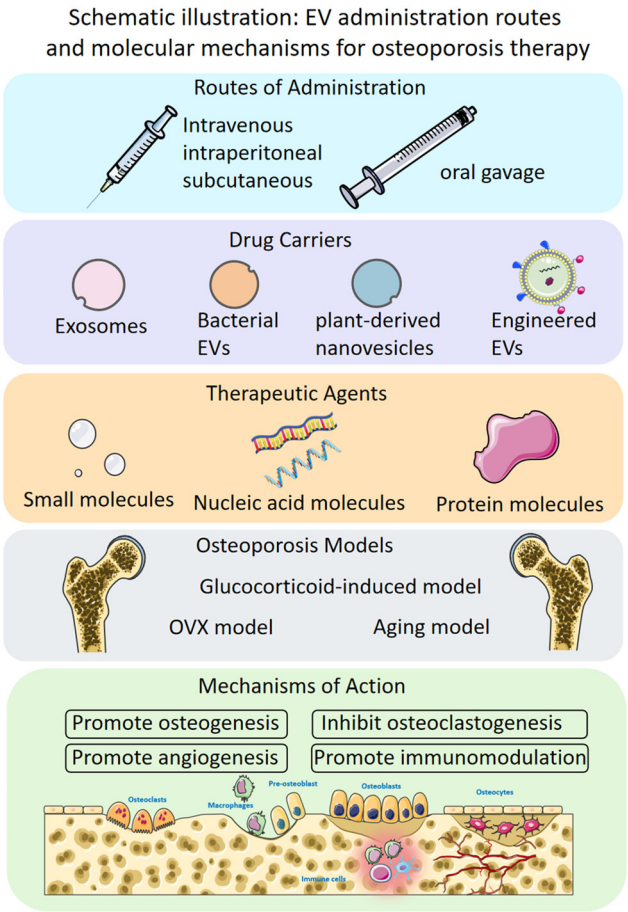

Fig. 8 Therapeutic delivery routes and the mechanism of action in OP animal models.

predominant liver and spleen accumulation, which may alter physiological functions in these organs over prolonged exposure. Complementing our discussion, these translational barriers have been thematically reviewed elsewhere,[192–194] with particular focus on manufacturing standardization, pharmacokinetic optimization, and safety validation.

Artificial intelligence (AI) greatly facilitates EV optimization through deep learning models to predict ideal osteogenic cargo combinations and simulate bone-targeting ligand interactions.[195] In addition, microfluidic sorting systems[196] and single-vesicle analysis technologies[197] are important high-throughput screening platforms for accelerating the identification of the most therapeutically potent EV subpopulations from heterogeneous mixtures. It is anticipated that the integration of AI-driven real-time quality control systems with automated high-throughput platforms will address critical challenges in the scalability of EV manufacturing to ensure batch-to-batch consistency and potency. Cutting-edge technologies open new avenues to translate EV biology into precision medicine. For example, coupling patient-derived multi-omics data with AI models (*e.g.*, graph neural networks) helps the design of EVs for specific molecular subtypes of OP, such as high bone turnover or sclerostin-dominant phenotypes. High-content screening platforms that correlate EV surface signatures (*e.g.*, CD63/CD81 ratios) with therapeutic responses could enable real-time treatment adjustment through bioinformatics-driven monitoring. Furthermore, the combination of engineered EV therapies[198,199] with conventional treatments enables the rational design of delivery systems, thereby yielding synergistic effects and minimizing adverse reactions. Comprehensive validation should include 12-month immunogenicity studies in non-human primates (NHPs), quantitative PET-MRI tracking of targeting efficiency in large animal models, and interlaboratory standardization of potency assays. By addressing these challenges and leveraging the unique properties of EVs, researchers may unlock novel, personalized approaches to combat OP, ultimately improving patient outcomes and reducing the global burden of this debilitating disease.

Author contributions

Gaiyue Yue: writing – original draft, investigation, formal analysis, visualization and conceptualization. Xuan Dai: investigation and formal analysis. Hanfen Shi: investigation and formal analysis. Jin Shen: investigation and formal analysis. Haochen Guo: investigation. Ruiqiong Liang: investigation. Zhengze Dai: investigation. Yongqi Li: investigation. Sihua Gao: writing – review & editing. Guangtong Dong: writing – review & editing. Jianhui Rong: writing – review & editing, supervision, and project administration. Lili Wang: writing – review & editing, supervision, and project administration. Dongwei Zhang: writing – review & editing, supervision, project administration, and conceptualization.

Conflicts of interest

There are no conflicts of interest to declare.

Data availability

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

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