Review Article

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The existing evidence for the use of extracellular vesicles in the treatment of osteoporosis: a review

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Abstract

Osteoporosis is a systemic metabolic bone disease characterized by decreased bone mass, microstructural deterioration, and increased fracture risk. The crucial role of extracellular vesicles (EVs) in the occurrence and development of osteoporosis has garnered attention, with vesicle-based treatments showing significant promise. Compared to conventional osteoporosis medications, EVs possess characteristics of naturalness, selectivity, and adaptability, and more importantly, they have negligible side effects. Hence, this review discusses the applications of natural and engineered EVs in osteoporosis are comprehensively outlined. Unfortunately, the absence of consensus on the extraction, purification, characterization, and storage of EVs has resulted in a lack of clinical evidence supporting their application in patients with osteoporosis. Although significant progress is still needed before the clinical use of EVs can be achieved, their substantial potential remains undeniable. Moreover, considering the complexity of bone metabolism in osteoporosis and the heterogeneity of EVs, further investigation into the functional subpopulations of different exosomes will facilitate their application.

Keywords: bone, exosomes, extracellular vesicles, osteoporosis

Introduction

Osteoporosis is a systematic bone metabolism disease characterized by chronic loss of trabecular bone as well as increased susceptibility to bone fracture[1]. Women face a greater risk of developing osteoporosis due to bone loss caused by the sharp decline in estrogen levels during menopause[2]. Individuals over 50 years old, particularly women, are more susceptible to fractures in the hip, vertebral body, and wrist. Moreover, prolonged glucocorticoid treatment also be a contributory factor to osteoporosis[3]. With the progressive aging of the general population, osteoporosis has emerged as a worldwide medical and socioeconomic issue[4]. Currently, a large number of drugs that both inhibit bone resorption and promote bone formation have emerged in clinical practice. However, multiple side effects and low patient compliance derived from long-term administration limit their application and curative effect[5]. Recent evidence suggests that bisphosphonates may contribute to atypical femoral fractures, while selective estrogen receptor modulators are associated with an increased risk of thrombosis[6]. Additionally, Denosumab has been linked to significant hypocalcemia[7]. Hence, it is necessary to develop more effective rehabilitative strategies for osteoporosis.

HIGHLIGHTS

- EVs serve as effective drug delivery systems, whether they are natural exosomes or engineered exosomes.
- Stem cells are the most widely used source of extracellular vesicles in osteoporosis.
- How to appropriately apply EVs for different stages of bone metabolism or prepare EVs with temporal and spatial characteristics is a challenge that engineered modifications should overcome.

Cell-based therapies have great potential in the field of regenerative medicine. Different cell types and their derivatives (especially about the extracellular vesicles (EVs)) have been used to treat diseases, such as cardiovascular disease, cancer, neurological disorders, and endocrine disorders[8,9]. EVs, including but not limited to exosomes and microvesicles, are lipid vesicles secreted by cells[10]. EVs ferry bioactive cargoes between cells to facilitate intercellular communication, which played a prominent role in the development and progression of osteoporosis[11-13]. Moreover, EVs, characterized by their naturalness, selectivity, and adaptability[14] – qualities that traditional osteoporosis medications lack – have the potential to serve as safe and effective alternatives to conventional treatments, thus minimizing the associated side effects.

In recent decades, the biomimetic simulation of exosomes has garnered significant attention as a form of nanomedicine because of its distinct advantages in enhancing therapeutic efficacy. Nanomedicine can be achieved through the utilization of

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self-assembled nanomaterials as drug carriers or the reconfiguration of drug structures via supramolecular chemistry to achieve nanoassembly. Following FDA's approval of the initial nanomedicine in 1995, numerous nanomedicines for cancer treatment have been authorized worldwide, with lipid nanoparticles (NPs) serving as the most commonly employed formulation. Lipid NPs have been extensively utilized as formulations, with liposomal doxorubicin receiving FDA approval for treating HIV-related Kaposi sarcoma and polymeric micelle paclitaxel receiving approval in Korea for the treatment of breast cancer and non-small-cell lung cancer[15]. Various forms of EVs have demonstrated remarkable therapeutic potential for osteoporosis. However, the diverse sources, administration methods, and modification strategies pose challenges and hinder their clinical application. Therefore, this review summarizes the applications of natural and engineered EVs in osteoporosis and aims to provide insights for future research. In this review, we conducted a comprehensive search of the PubMed, Web of Science, and Clinical Trials databases using keywords such as osteoporosis, bone density reduction, bone metabolism, exosomes, and nanovesicles, ultimately identifying 80 studies.

Changes of EVs in osteoporosis

Overview of EVs

EVs are cell–released NPs involved in intercellular communication and are promising as biomarkers for disease states, endogenous therapeutics, and drug delivery platforms[16-18]. Currently, according to the formation process of EVs, they can be divided into three categories: exosomes, microvesicles, and apoptotic bodies[19]. Exosomes formed by the interior budding of endosomal membranes to form large multivesicular bodies, which in the 30–150 nm range. EVs may be produced by budding from the extracellular membrane yielding particles from 100 to 1000 nm known as microvesicles, shedding microvesicles or microparticles. Apoptotic vesicles are formed by large-scale plasma membrane blebbing, released during apoptotic cell death and are generally larger (100–5000 nm in diameter). EVs are known to have a unique signature of lipids, proteins and nucleic acids, reflecting their cell and tissue of origin. Protein composition has been the most extensively studied. EVs are enriched for proteins in the tetraspanin family (CD63, CD9, CD81), which are thought to contribute to membrane remodeling to form the EVs structure[20] (Figure 1).

Currently, the identification of EVs mainly relies on both morphological and physicochemical properties. Strategies for exosome separation are sequential ultracentrifugation, gradient ultracentrifugation, ultrafiltration, size-exclusion chromatography, polymer precipitation, immunoaffinity capture, and microfluidics-based techniques[21]. There have been numerous reviews on the occurrence, extraction, and identification of EVs. The focus of this article is on the latest insights and applications of EVs in osteoporosis.

Interactions of EVs from different sources in bone metabolism

After being released from source cells, EVs can adhere to the extracellular matrix and adjacent cells or travel to distant organs via blood, lymph, and other bodily fluids. Upon interacting with cells, EVs facilitate intercellular signaling through two main mechanisms:[1] by directly contacting surface ligands to relay information to recipient cells or[2] by being internalized by

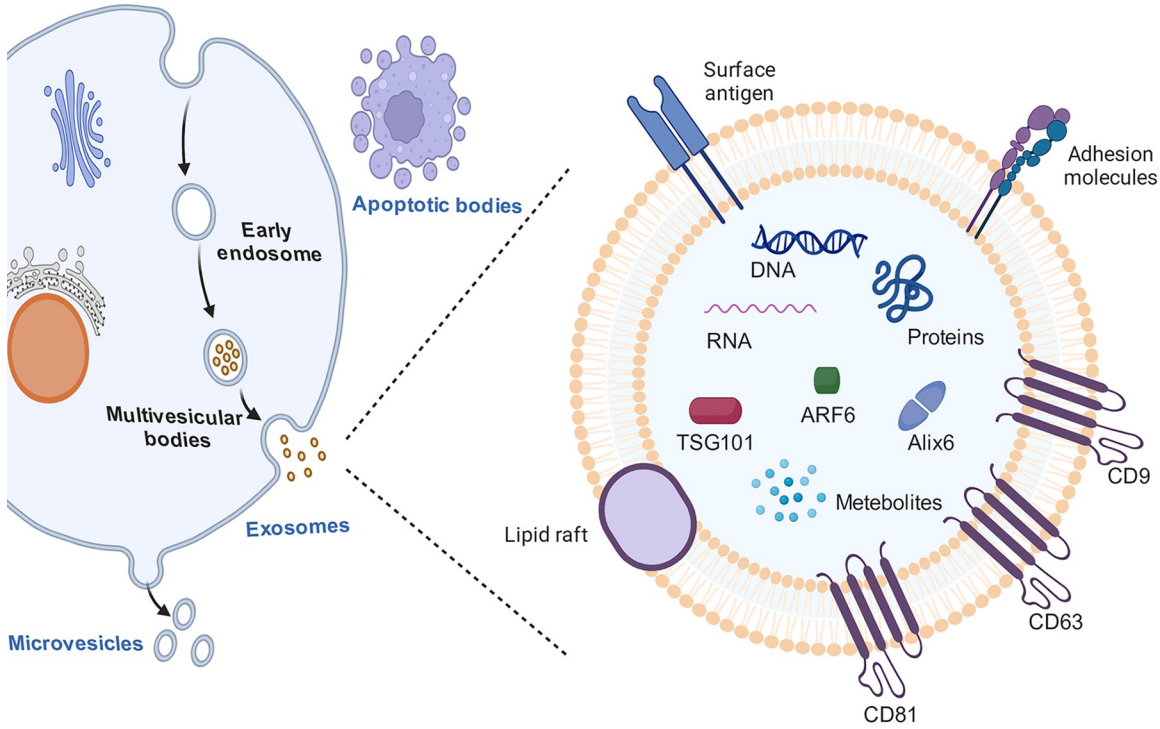

Figure 1. Structural characteristics of extracellular vesicles. TSG101: TUMOR susceptibility gene 101; ARF6: ADP-ribosylation factor 6; Alix6: Apoptosis-linked gene 2 interacting protein X, isoform 6; CD81: cluster of differentiation 81; CD9: cluster of differentiation 9; CD63: cluster of differentiation 63.

recipient cells, thereby transferring their contents to target cells[22]. EVs released by adjacent tissues or remote organs can impact the regulation of bone homeostasis, which is maintained through a delicate equilibrium of osteoblast-mediated bone formation and osteoclast-mediated bone resorption. The dynamic interplay among osteocytes, osteoblasts, and osteoclasts is a central focus of current research in the field. Osteoblasts, as terminally differentiated cells, are responsible for bone formation, while the bone marrow stroma harbors multipotent cells capable of differentiation into various mesenchymal lineage cells, including osteoblasts, chondrocytes, and adipocytes[23,24]. Hence, EVs derived from bone marrow mesenchymal stem cells play an essential role in bone metabolism of osteoporotic patients[25-27] (Figure 2).

EVs derived from bone marrow mesenchymal stem cells

On one hand, EVs derived from bone marrow mesenchymal stem cells of osteoporotic patients play a crucial role in bone metabolism by delivering miR-424-5p and miR-21 to target WIF1 and SMAD7, respectively, regulating the Wnt pathway to suppress osteogenesis[26,27]. On the other hand, EVs from bone marrow mesenchymal stem cells of osteoporotic patients transport to osteoclasts and release miR-143/145, targeting Cd226 and Srgap2, leading to increased osteoclastic activity[28]. Additionally, exosomes from osteoblasts in osteoporosis patients hinder the expression of the alp gene in BMSCs while increasing caspase 3/7 activity[29].

EVs derived from aged cells

The osteogenic activity of bone marrow-derived mesenchymal stem cells (BMSCs) is also influenced by various EVs. Aged bone matrix-derived EVs (AB-EVs) transmit miR-483-5p to the bone marrow, stimulating the expression of PPARγ in BMSCs. This, in turn, promotes adipogenic differentiation over osteogenic differentiation, leading to imbalanced bone-fat composition and ultimately osteoporosis[11]. In aging mice, exosomes derived from muscles and serum exhibit elevated levels of miR-34a, which accelerates aging by inhibiting Sirt1 and dampening BMSCs function[30]. In patients with osteoporosis, the circulating exosomal circFAM63B inhibits postmenopausal osteoporosis bone regeneration by modulating the miR-578/HMGA2 axis[31]. Elevated expression of SIRT2 in aging liver cells promotes bone loss[32]. EVs derived from the brain transport miR-483-5p to promote bone-fat imbalance in Alzheimer's disease

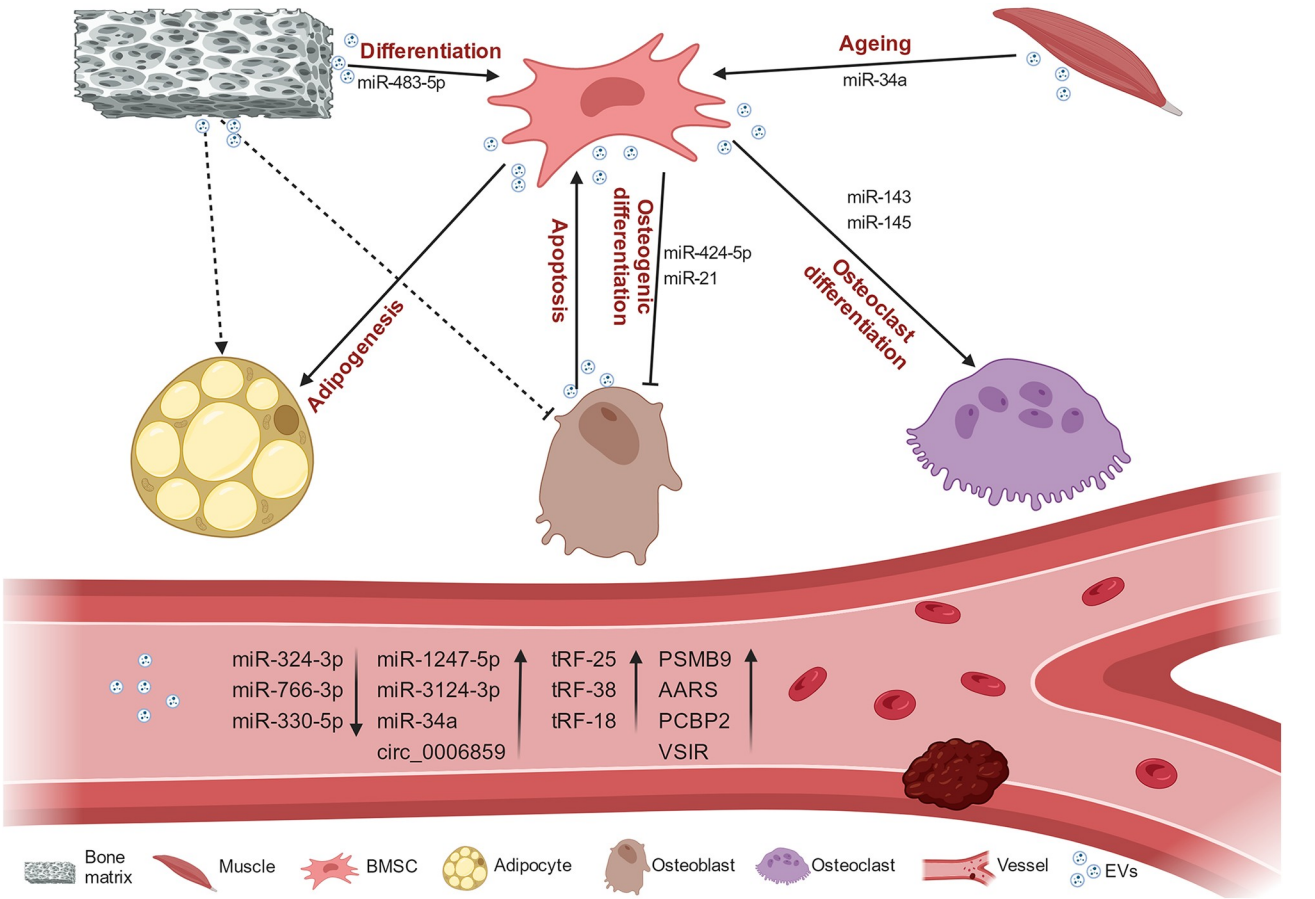

Figure 2. Interactions of extracellular vesicles from different sources in bone metabolism. In bone metabolism, EVs play the role of messengers between tissues. Extracellular vesicles derived from osteoblasts, myocytes, and mesenchymal stem cells influence osteogenic differentiation, osteoclastic differentiation, and adipogenic differentiation processes by delivering various substances. The content of circulating extracellular vesicles shows many specific changes in osteoporosis. EVs: extracellular vesicles; miR: microRNA; circ: circRNA; tRF: tRNA-derived fragments; PSMB9: proteasome subunit beta 9; AARS: alanyl-tRNA synthetase; PCBP2: Poly(rC) binding protein 2; VSIR: V-set and immunoglobulin domain containing 1.

patients[33]. The precise sources and mechanisms by which exosomes impact bone metabolism remain incompletely understood. The utilization of single-cell sequencing technology holds the potential to revolutionize the identification and categorization of exosome origins, filling a crucial knowledge gap in the field.

Changes in circulating EVs in osteoporosis

Circulating EVs harbor a wealth of information reflecting the overall condition of the body. The onset of menopause in women triggers significant alterations in circulating EVs[34]. Individuals with osteoporosis exhibit elevated serum EVs concentration and notable content variations compared to healthy counterparts[25,35,36]. Current research predominantly delves into non-coding RNAs, encompassing miRNAs, lncRNAs, and circRNAs. Numerous studies have highlighted the pivotal roles of aberrant changes in these non-coding RNAs in osteoporosis development, potentially contributing to postmenopausal osteoporosis diagnosis, prognosis, and treatment[25,37-41]. Noteworthy examples include miRNAs such as Mir-324-3p, Mir-766-3p, micr-1247-5p, Mir-330-5p, and Mir-3124-5p, associated with bone mineral density (BMD), serving as prospective diagnostic biomarkers[39]. Moreover, hsa_circ_00068529 emerges as a novel therapeutic target for osteoporosis, while tRF-25, tRF-38, and tRF-18 in plasma exosomes may also serve as diagnostic indicators for osteoporosis[40,42]. Additionally, the evolving landscape encompasses changes in the protein composition of circulating EVs, garnering substantial interest. Chen *et al* identified 45 differentially expressed proteins in the circulating EVs of osteoporotic patients, including PSMB9, AARS, PCBP2, and VSIR which are pertinent to osteoporosis development[43]. Despite the existing evidence spotlighting alterations in circulating EVs of osteoporosis patients, with a primary focus on non-coding RNAs, further comprehensive research is imperative to explore the full spectrum of content modifications. This endeavor will contribute to a more profound comprehension of the characteristics and pathogenesis of osteoporosis.

Extracellular vesicle therapy

EVs primarily govern the functionality of recipient cells through three distinct mechanisms. First, transmembrane proteins present on EVs membranes engage with corresponding receptors on the cellular membrane, thereby triggering signaling cascades that impact target cells. Second, EVs merge with the cell membrane, facilitating the ingress of bioactive components into the cytoplasm, consequently modulating or altering intracellular signaling pathways. Lastly, cells uptake EVs via endocytosis, ultimately releasing their cargo into specific organelles. Given the intricate involvement of EVs in bone metabolism, numerous researchers have sought to leverage EVs from diverse origins for osteoporosis therapy. This chapter delves into an in-depth analysis of the utilization of EVs derived from stem cells, muscles, serum, milk, plants, and other sources in addressing osteoporosis. Figure 3.

EVs derived from stem cells

Stem cells possess the remarkable capability to self-renew and differentiate into diverse cell types, rendering them a focal point in regenerative medicine. Stem cell transplantation has emerged as a promising therapeutic avenue extensively investigated across various diseases. Despite its potential benefits, stem cell transplantation entails inherent risks of immunogenicity, which could elevate the likelihood of organ failure and neurodegenerative conditions[44,45]. Of particular concern is the tumorigenic potential associated with long-term in vitro culturing of stem cells, culminating in the accumulation of karyotypic anomalies, copy number variations, and loss of heterozygosity[46]. Consequently, the spotlight has shifted towards EVs derived from stem cells. Stem cell-generated EVs, in comparison to stem cells themselves, offer numerous advantages such as ethical accessibility, plentiful sources, low immunogenicity, and reduced tumorigenicity[47-50]. Hence, stem cell-derived EVs are increasingly recognized as a safer and more effective strategy in the realm of regenerative medicine (Table 1).

OVX: ovariectomy; BMDM: bone marrow-derived macrophages; GIOP: glucocorticoid-induced osteoporosis; DOP: dioctyl phthalate induced osteoporosis; BMSCs: bone marrow stromal cells; HUVECs: human umbilical vein endothelial cells.

Adipose-derived stem cells

Recent years have seen a surge in attention towards the intricate relationship between bone and fat, both at the cellular level[86]and in clinical applications[87]. Adipose tissue emerges as a crucial reservoir of stem cells, serving as an alternative source to bone marrow mesenchymal stem cells. Studies have elucidated the dedifferentiation potential of adipocytes across various species, showcasing advantages like robust proliferative capacity, high uniformity, and versatile multi-lineage differentiation capabilities. These attributes position adipocytes as promising candidate progenitor cells for bone tissue engineering[88]. Moreover, adipocytes play a pivotal role in bone remodeling by secreting vital cell factors such as adiponectin, leptin, and IL-6[89]. The intricate interplay and reciprocal regulation of multiple signaling molecules governing the bone-lipid equilibrium constitute the foundational regulatory framework. EVs derived from adipose tissue-based stem cells, known as ASC-EVs, operate through analogous regulatory pathways, rendering them a focal point in osteoporosis therapeutics. The principal mechanisms through which ASC-EVs from adipose tissue-derived stem cells combat osteoporosis involve inhibiting osteoclast differentiation, facilitating bone marrow mesenchymal stem cell migration, curbing cellular oxidative stress, and dampening inflammation. ASC-EVs are enriched with growth factors and cytokines crucial in bone metabolism, including insulin-like growth factor-1 (IGF-1), interleukin-17 (IL-17), bone morphogenetic proteins (BMP-6 and BMP-7), osteoprotegerin (OPG), and osteopontin (OPN). Notably, OPG acts as a natural antagonist of the nuclear factor-kappa B ligand receptor activator (RANKL), significantly impeding osteoclast differentiation from macrophages. ASC-EVs also express cell factors instrumental in recruiting and mobilizing MSCs towards bone resorption sites, such as monocyte chemoattractant protein-1 (MCP-1), matrix metalloproteinase-2, MMP-3, and tumor necrosis factor-alpha (TNF-α). The abundance of these bioactive factors facilitates the homing of BMSCs. Additionally, miRNAs encapsulated within ASC-EVs actively contribute to the regulation of osteoclastogenesis. For instance, miR-21-5p downregulates Acvr2a to impede osteoclast differentiation, whereas let-7b-5p significantly suppresses the RANK-RANKL signaling pathway to hinder osteoclastogenesis[51].

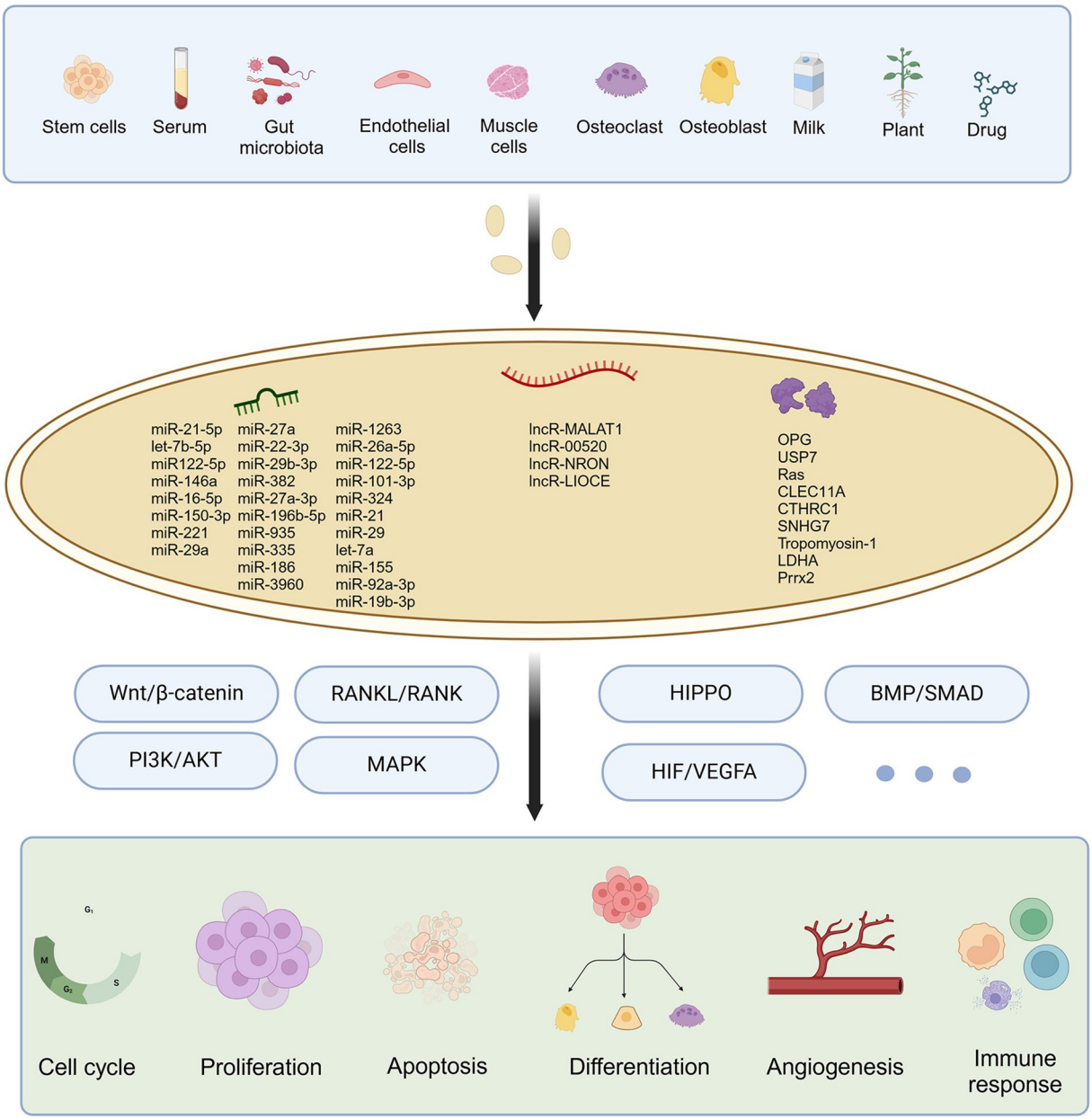

Figure 3. Applications of extracellular vesicles in osteoporosis. Extracellular vesicles from different sources primarily exert signaling regulatory roles by carrying miRNAs, lncRNAs, and proteins. MiRNAs are short non-coding RNAs that mainly regulate gene expression by binding to mRNAs and inhibiting their transcription. For example, exosomes derived from bone marrow mesenchymal stem cells release miR-16-5p, which targets and inhibits the transcription of Axin2, thereby activating the Wnt/β-catenin pathway to promote osteogenic differentiation. LncRNAs are long non-coding RNAs that can act as molecular sponges for miRNAs, often exerting their regulatory functions by competitively binding to miRNAs. For instance, exosomes from bone marrow mesenchymal stem cells release LncR-MALAT1, which competitively binds to miR-34c, promoting the transcription of the miR-34c target gene SATB2 and enhancing osteogenic differentiation. The role of proteins in exosomes is even more diverse; on one hand, they can exert effects through ligand-receptor binding, and on the other hand, they can act as transcription factors regulating the transcription of target genes. For example, exosomes derived from adipose stem cells can deliver OPG, which directly binds to its ligand RANKL, inhibiting the RANKL/RANK signaling pathway and thereby suppressing osteoclast differentiation. Furthermore, exosomes from myoblasts can deliver the transcription factor Prrx2, which directly binds to the promoter of lncRNA-MIR22HG to promote its transcription, thereby activating the HIPPO signaling pathway and promoting osteogenic differentiation. Ultimately, these exosomes from different sources regulate various biological processes, including cell cycle, proliferation, apoptosis, differentiation, angiogenesis, and immune responses, by delivering distinct genetic information. OPG: osteoprotegerin: USP7: ubiquitin-specific protease 7; Ras: rat sarcoma; CLEC11A: C-type lectin domain family 11 member A; CTHRC1: collagen triple helix repeat containing 1; SNHG7: small nucleolar RNA host gene 7; LDHA: lactate dehydrogenase A; Prrx2: Paired related homeobox 2.

Table 1

Application of extracellular vesicles from stem cell sources in osteoporosis

Source	Model	Cargo	Function	Target	Ref
Adipose Stem Cells	OVX	OPG, miR-21-5p, let-7b-5p	Inhibiting osteoclast differentiation and promoting the migration of bone marrow mesenchymal stem cells.	Acvr2a, Rankl	[51]
	BMDM cells	miR122-5p	Inhibiting osteoclast differentiation.	P38	[52]
	GIOP	NA	Inhibiting cell apoptosis and oxidative stress.	Nrf2/HO-1	[53]
	DOP rat	miR-146a/NA	Inhibiting inflammation.	NA	[54,55]
Bone marrow mesenchymal stem cells	OVX mice	NA	Enhancing osteoblast proliferation and differentiation.	NA	[56,57]
		miR-16-5p		Axin2/Wnt/ β -catenin	[58]
	OVX rat	miR-150-3p		NA	[59]
	OVX mice	miR-27a	Promoting osteogenic differentiation and inhibiting osteoclast differentiation.	DKK2/Wnt/ β -catenin	[60]
		USP7		YAP1/Wnt/ β -catenin	[61]
	OVX mice	miR-22-3p	Enhancing osteoblast differentiation.	FTO/MYC/PI3K-AKT	[62]
		miR-29b-3p		KDM5A/SOCS1/NF-κ B	[63]
	MG63 cells	miR-382		SLIT2	[64]
	hBMSCs	NA		NA	[65]
	OVX rat	miR-27a-3p, miR-196b-5p		NA	[66]
		miR-935		STAT1	[67]
	Fracture mice	miR-335		VapB/Wnt/ β -catenin	[68]
	DOP mice	miR-221	Regulating bone-fat imbalance.	NA	[69]
	OVX rat	NA	Regulating the cell cycle of osteoblasts.	ERK/ER α	[70]
	hFOB1.19 cells	miR-21-5p	Enhancing osteoblast proliferation.	KLF3	[71]
		NA		MAPK, GLUT3	[56,72]
		LncR-MALAT1		microRNA-34c/SATB2	[73]
		LncR-00520		NA	[74]
	OVX rat	miR-186		Mob1/yap/hippo	[75]
	OVX mice	Ras		Ras/Raf1/Mek/Erk	[76]
	Radiation rat	NA		Wnt/ β -catenin	[77]
	HUVECs	miR-29a		VASH1	[78]
Umbilical cord stem cells	OVX mice	CLEC11A	Enhancing osteogenic differentiation, inhibiting adipogenic differentiation, and osteoclast differentiation.	NA	[79]
	16 months old mice	miR-3960	Promoting osteogenic differentiation and inhibiting osteoclast differentiation.	NA	[80]
	DOP rat	miR-1263	Inhibiting cell apoptosis.	Mob1/Hippo	[81]
Urinary stem cells	OVX mice	CTHRC1, OPG	Promoting osteogenic differentiation and inhibiting osteoclast differentiation.	NA	[82]
	DOP rat	miR26a-5p	Promoting osteogenic differentiation and inhibiting osteoclast differentiation.	HDAC4/HIF-1 α / VEGFA	[83]
Dental pulp stem cells	OVX mice	NA	Regulating telomerase activity	NA	[84]
		NA	Promoting osteogenic differentiation	ERK1/2 Signaling Pathway	[85]

Inflammation and oxidative stress are pivotal factors influencing the differentiation of osteoclasts and osteoblasts. Inflammatory triggers and oxidative stressors promote osteoclast differentiation while inhibiting osteoblast differentiation through the induction of inflammatory mediators and reactive oxygen species (ROS). Exosomes derived from adipose-derived stem cells counteract apoptosis and oxidative stress in osteoblasts by enhancing the Nrf2/HO-1 expression, thereby mitigating the progression of glucocorticoid-induced osteoporosis[53]. Moreover, exosomes from adipose tissue-derived mesenchymal stem cells (AD-MSCs-Exos) dampen the NLRP3 inflammasome activation in osteoclasts, diminish the levels of pro-inflammatory cytokines such as TNF-α, IL-18, IL-1β, and impede bone resorption, offering a potential therapeutic avenue for diabetic osteoporosis[90]. In summary, EVs from adipose tissue-derived stem cells are accessible, secure, and efficacious bioactive entities. The utilization of EVs from distinct adipose stem cell types in addressing bone loss remains an unexplored area in current research, posing as a promising avenue for future investigations seeking enhanced exosomal therapies.

Bone marrow mesenchymal stem cells

BMSCs are pivotal actors within the bone marrow, acting as the shared precursors of osteoblasts and adipocytes. Nevertheless, aging processes suppress osteoblast formation while accentuating adipocyte generation, culminating in osteoporosis development. EVs derived from BMSCs (BMSCs-EVs) represent the principal bioactive byproducts of BMSCs, possessing inherent functions that facilitate osteoblast generation and differentiation. These vesicles enhance the expression of bone-related genes in osteoblasts, thereby fostering bone formation and underpinning the regulatory role of BMSCs-Exos in bone metabolism[91]. Numerous studies highlight the regulatory

influence of BMSCs-Exos, mediated through specific miRNAs, on diverse physiological and pathological aspects of osteoporosis. These nanostructures modulate bone remodeling, osteoblast proliferation and differentiation, bone-lipid equilibrium, endothelial cell maturation and differentiation, immune responses, and inflammatory reactions in bone tissues. Consequently, BMSCs-EVs are anticipated to emerge as a leading therapeutic modality for clinical management of osteoporosis.

BMSC-EVs contain a plethora of miRNAs that facilitate osteogenesis. Notable examples include miR-22-3p[62], miR-16-5p[58], miR-382[64], miR-150-3p[59], miR-186[75], miR-27a[60], miR-29b-3p[63], miR-27a-3p[66], miR-196b-5p[66], miR-29a[78], miR-935[67], and miR-335[68], all of which play a role in promoting osteogenic differentiation. These miRNAs primarily exert their effects by activating the Wnt/β-catenin pathway, suppressing the PI3K/AKT pathway, and modulation of the NF-κB pathway[54,58,77,90,92]. The canonical and non-canonical Wnt/β-catenin pathways play an indispensable role in osteogenic differentiation. Mutations in Wnt ligands within osteoblasts can lead to impaired osteogenic differentiation. For instance, mutations in Wnt1 result in deficiencies in osteogenic mineralization, while mutations in Wnt3a can reduce β-catenin activity, inhibiting osteogenesis and promoting programmed cell death in osteoblasts[93]. BMSC-EVs carrying miR-16-5p, miR-27a, and miR-335 can target and suppress Axin2, DKK2, and VapB, respectively, thereby activating the Wnt/β-catenin pathway to promote osteogenesis[58,60,68]. Additionally, there is crosstalk between the Wnt signaling pathway and other pathways, such as PI3K/AKT, during osteogenic differentiation[94]. MiR-22-3p carried by BMSC-EVs can target FTO to inhibit PI3K/AKT activation, thereby promoting osteogenic differentiation[62]. Moreover, miR-221[69] demonstrate an ability to impede adipogenic differentiation, whereas miR-21-5p[71], miR-150-3p[59], and miR-935[67] enhance osteoblast proliferation. In addition, BMSC-EVs foster osteogenesis and angiogenesis by activating key pathways such as the ERK-Erα pathway, MAPK pathway, BMP/Smad pathway, and HIF-1α/VEGF pathway[56,70,72,76].

The therapeutic effects of EVs from human dental pulp stem cells (DPSCs) should not be overlooked. DPSCs, a type of MSCs, are easily accessible and possess high proliferation capacity, thereby playing a significant role in osteoporosis treatment. EVs derived from stem cells from human exfoliated deciduous teeth (SHED) can inhibit bone loss in osteoporotic mice by restoring telomerase activity in mesenchymal stem cells[84]. Additionally, apoptotic vesicles from SHED promote bone formation through the ERK1/2 signaling pathway[85].

Umbilical cord stem cells

Umbilical cord mesenchymal stem cells (UC-MSC) offer various advantages compared to stem cells from alternative sources. They are easily accessible, obtained through non-invasive means, possess robust immune-modulatory capabilities, and demonstrate notable self-renewal characteristics. Mounting evidence supports the notion that UC-MSC play a pivotal role in bone formation and osteoclast inhibition by releasing beneficial factors via EVs. This mechanism stands to enhance new bone formation at injury sites by activating host cells and leveraging endogenous repair mechanisms[55]. UC-MSC-derived EVs have shown efficacy in delivering CLEC11A[79], miR-1263[81], and miR-3960[80] to orchestrate various regulatory processes in bone metabolism, safeguarding against bone loss through distinct signaling pathways. Notably, CLEC11A, abundantly expressed in the bone marrow, acts as a potent osteogenic factor. Research has illustrated that HUCMSC-EVs facilitate osteogenesis by delivering the osteogenic protein CLEC11A, prompting the transformation of BMSCs from fat accumulation to osteogenic differentiation while inhibiting bone resorption[79]. Among the array of beneficial factors harbored within HUCMSC-EVs, microRNAs have garnered significant attention. HUCMSC-EVs transport miR-3960 to bolster the osteogenic differentiation of BMSCs and curb osteoclast formation in RAW264.7 cells[80]. Furthermore, UC-MSC can impede Mob1 and activate Yap1, subsequently initiating the Hippo signaling pathway by delivering miR-1263, thereby mitigating apoptosis of BMSCs in disuse-related osteoporosis[81].

Urinary stem cells

Urine-derived stem cells (USCs) present a non-invasive and straightforward cell culture reservoir with a relatively modest cost, attracting significant attention for their proliferation capacity and differentiation aptitude[95]. Exploration into EVs derived from USCs (USC-EVs) is still at a nascent stage. Studies have evidenced that USC-EVs influence osteoporosis progression by modulating diverse signaling pathways through the transmission of recombinant proteins, osteoactivin, and miRNAs. USC-EVs facilitate osteoblast generation and inhibit osteoclast formation through the delivery of CTHRC1 and OPG[82]. By transporting miR26a-5p to curb HDAC4 expression and activate the HIF-1α/VEGFA axis, USC-EVs augment osteogenic differentiation, suppress osteoclastic function, and ameliorate diabetic osteoporosis[83]. USCs represent a novel non-invasive cellular reservoir with significant potential for cell therapy and tissue regeneration. Nonetheless, given the limited comprehension of the biological attributes of USCs, further investigation is imperative to pave the way for their clinical utilization[95].

EVs involved in drug intervention

Drug interventions can modulate cell function and disease progression by impacting the release of EVs. Current research on drug interventions predominantly centers on the bioactive constituents of individual Chinese herbs, such as icariin, artemether, and polysaccharides sourced from epimedium, artemisia, and radix morindae. These compounds have the capability to influence the composition and function of EVs through distinct pathways, thereby exerting pharmacological effects. For instance, icariin intervention in endothelial cell-derived EVs shows promise in ameliorating glucocorticoid-induced vascular endothelial cell damage, enhancing cellular viability, and promoting angiogenesis[96]. The formulation "Wenshen Tongluo Zhitong Tang" is found to hinder osteoclastogenesis by delivering miR-122-5p to suppress SPRY2 and activate the MAPK signaling within adipocyte-derived exosomes, showcasing anti-osteoporotic properties[97]. Chinese herbal medicine Guilu Erxian Glue inhibits osteoclast formation and activity via mc3t3-derived EVs in vitro[98]. Zhuang-Gu-Fang promotes osteoblast differentiation via myoblasts and myoblast-derived exosomal miRNAs[99]. Artemether intervention in bone marrow mesenchymal stem cell-derived exosomes facilitates the transfer of SNHG7 to activate the TAF15-RUNX2 pathway, thereby

fostering osteogenesis[100]. Moreover, polysaccharides from radix morindae demonstrate the ability to alleviate osteoporosis symptoms and enhance bone healing in glucocorticoid-induced osteopenic rats by upregulating miR-101-3p and downregulating PTGS2 in exosomes derived from rat bone marrow mesenchymal stem cells[101]. Furthermore, certain active substances and mechanical stimuli have found application in exosome investigations. For instance, bioactive glass NPs stimulate the expression of lncRNA NRON in BMSC-EVs, curtailing osteoclast differentiation and osteoporotic bone loss[102]. Exosomes derived from bone marrow mesenchymal stem cells exposed to cyclic mechanical stretch impede RANKL-induced osteoclast formation via the NF-κB signaling pathway[103]. These studies unveil novel mechanisms of drug intervention in EVs for orchestrating cell communication and disease management, thus offering fresh perspectives on leveraging EVs as therapeutic targets for osteoporosis.

Other types of EVs

Osteoclasts and macrophages

In addition to the aforementioned sources of EVs, a plethora of other EVs types originating from diverse cell sources like serum, gut microbiota, vascular endothelial cells, and muscle cells significantly impact the function of bone-related cells by carrying specific molecules, including miRNA and proteins. Activated osteoclasts release abundant small EVs (sEVs) during bone remodeling, with miR-324 within these sEVs transferring to MSCs to notably enhance their in vitro osteogenic differentiation and mineralization by targeting ARHGAP1, a negative regulator of osteogenic differentiation[104]. Inflammatory osteoclast-derived exosomes selectively convey lncRNA LIOCE to osteoblasts, where it interacts with osterix to stabilize osterix and foster bone formation[105]. M2 macrophages secrete glutamate-containing EVs to alleviate osteoporosis by reshaping osteoclast precursor fate[106].

Osteoblasts, osteocyte, and endothelial cell

Notably, EVs from osteogenic differentiated human BMSCs bolster the viability and proliferation capacities of newborn hBMSCs while inhibiting apoptosis. Enhanced expression of COL1A1 and OPN genes signifies that EVs from hBMSCs in the late osteogenic differentiation stage are more proficient at fostering the osteogenic capabilities of newborn hMSCs compared to EVs from early-stage hBMSCs[107]. Additionally, EVs derived from the mid-to-late stage of osteoblast differentiation significantly enhance osteogenesis both in vitro and in vivo[108]. Enriched with miR-21, miR-29, miR-221, and let-7a, Wharton's Jelly mesenchymal stem cell-derived EVs may confer bone-protective effects through BMP and PI3K/AKT signaling pathways[109]. Young osteocyte-derived EVs facilitate osteogenesis by transferring tropomyosin-1[110]. While the bone-protective effects of osteoblast-derived EVs and bone marrow mesenchymal stem cell-derived EVs are well-recognized, endothelial cell-secreted exosomes (EC-Exos) exhibit enhanced bone-targeting properties compared to osteoblast-derived or bone marrow mesenchymal stem cell-derived exosomes, primarily through the transportation of miR-155, exerting bone-protective effects[111].

Muscle and myoblast

Furthermore, Muscle-derived EVs (Mu-EVs) also demonstrate the ability to inhibit osteoclastogenesis[112]. Mu-EVs promote glycolysis in BMSCs by delivering lactate dehydrogenase A, thereby enhancing osteogenesis[113]. Myoblast-derived exosomes carrying Prrx2 bind to the miR-22HG promoter to enhance its transcription, leading to increased YAP expression and nuclear translocation by absorbing miR-128. This activation of the Hippo pathway promotes BMSCs osteogenic differentiation and alleviates osteoporosis[114]. Furthermore, Myoblast-derived exosomes under mechanical stress can promote osteogenic differentiation via the miR-92a-3p/PTEN/AKT signaling pathway[115].

Milk

Osteoporosis patients have traditionally been advised to prioritize the consumption of milk and protein-rich foods. Recent research has bolstered the foundation of this recommendation with insights from the realm of nanovesicles. Consuming exosomes derived from milk can enhance bone health by fostering the proliferation and differentiation of osteoblasts, potentially attributable to the growth factors they induce, such as bFGF, IGF-1, TGF-β, and VEGF[116,117]. Furthermore, oral milk-derived EVs could inhibit osteoclast differentiation and improve osteoporosis in OVX mice by modulating the gut microbiota, enhancing SCFAs, and reducing pro-inflammatory cytokines and osteoclast differentiation-related factors[118].

Plant

Plant-derived nanovesicles, termed exosome-like nanovesicles (PENs), are emerging as a focal point in research. These PENs are not only safe, biocompatible, and biodegradable, with no adverse impact on intestinal barrier function or organ toxicity, but are also scalable in production. Furthermore, PENs exhibit intrinsic biological characteristics like antioxidant, anti-inflammatory, and regenerative properties[119]. Recent investigations highlight that Yam-derived exosome-like nanovesicles (YNVs) stimulate osteoblast proliferation, differentiation, and mineralization, exerting bone-protective effects by activating the BMP-2/p-p38-dependent Runx2 pathway, hence mitigating osteoporosis in mice[120]. *Pueraria lobata*-derived exosome-like nanovesicles promote the differentiation and function of hBMSCs by elevating autophagy via the degradation of TMAO[121]. *Morinda officinalis*-derived extracellular vesicle-like particles promote osteoblast proliferation by modulating the MAPK signaling pathway[122].

Serum and gut microbiota

Researchers have been attempting to reverse skeletal aging through blood transfusions or fecal microbiota transplants, and EVs from young blood or feces have provided support for this approach. Serum exosomes from young rats improve osteogenic differentiation of bone marrow mesenchymal stem cells in elderly osteoporotic rats by delivering miR-19b-3p to target inhibit PTEN[123]. The gut microbiota of young mice can communicate with host bone cells by secreting EVs, directly regulating osteoblast bone formation and osteoclast bone resorption. *Akkermansia muciniphila* seems to be a major source of these EVs[124].

Applications of engineering EVs in osteoporosis

Despite the certain effects of exosomes from different sources on osteoporosis, their low yield, limited function, and unstable biological activity have been limitations hindering their practical application. In recent years, researchers have attempted to achieve mass production and precise targeted delivery of exosomes through the construction of engineered nanovesicles[125]. Based on the mechanism of exosomes, their functional enhancement is mainly achieved through two strategies: surface modification and functional molecule encapsulation. Surface modification involves designing functional ligands on the surface of EVs to target receptor cells and activate immunity, while molecular encapsulation refers to loading specified small molecule drugs, nucleic acids, and proteins into the EVs, transforming them into functional carriers for therapeutic delivery. In this section, the discussion will primarily focus on the two strategies of surface modification and functional molecule encapsulation. Table 2.

Surface modification

In order to enhance the targeting ability of EVs and enrich them in osteoblasts, osteoclasts, or immune cells, researchers typically express the target protein in fusion with endogenous membrane proteins to modify the target protein on the outer surface of the vesicle membrane[142]. Surface modification methods for EVs can generally be categorized into pre-extraction modification and post-extraction modification. Pre-extraction modification involves editing the protein expression of cells through genetic engineering methods, typically inducing high expression of targeting membrane proteins to allow the secreted EVs to express high levels of target membrane proteins indirectly. Hu used the CMV-MCS-PGK-Puro lentivirus packaging system to introduce the CXCR4 gene into NIH-3T3 cells to obtain CXCR4 + EVs, making them more marrow-targeted[133]. On the other hand, post-extraction modification involves directly inserting lipid molecules onto the surface of EVs through lipid insertion modification, covalent crosslinking modification, or click chemistry modification. Lipid-soluble molecules can anchor into the membrane of EVs, such as cholesterol or synthetic phospholipids (e.g., DSPE, DMPE, DOPE) as the most common lipid-soluble crosslinkers, can be modified on the surface of EVs by connecting to polyethylene glycol (PEG) via PEG-conjugated targeting molecules. Cui used the property of DSPE-PEG efficiently inserting into the phospholipid monolayer to bind the osteoblast-targeting peptide SDSSD to the exosome membrane, making it bone-targeted[134]. Xu constructed GMNPE-EVs by loading Fe3O4@SiO2PEG-CHO into EVs and found that they can effectively deliver miR-15b-5p to osteoclasts. This process resulted in the downregulation of GFAP expression and suppression of osteoclast differentiation[128]. Xu discovered that GMNPE-EV loaded with MEG3 can target miR-3064-5p to enhance mitochondrial autophagy, osteoblast proliferation, and differentiation[129]. Gui and He utilized DSPE-PEG-COOH as a linker to attach the bone-targeting peptide (Asp-Ser-Ser)6 ((DSS)6) to bone marrow mesenchymal stem cell-derived apoptotic EVs for bone-targeted delivery[135]. Zheng also utilized DSPE-PEG to bind alendronate (ALN) onto the surface of PL-derived exosomes (PL-exo) to form bone-targeting PL-exo-ALN[126]. The in vitro hydroxyapatite binding affinity and in vivo bone targeting aggregation of PL-exo were significantly enhanced after ALN modification. Besides directly modulating the osteogenic and angiogenic differentiation of BMSCs and endothelial progenitor cells (EPCs), respectively, PL-exo-ALN also facilitates their coupling under glucocorticoids' stimulation[126]. A bifunctional peptide, TBP-CP05, binds to both the CD63 on red blood cell-derived EVs (RBCEVs) and receptors on osteoclasts, serving as a guide. TBP-CP05 binds with RBCEVs through CP05, displays the TRAP-binding peptide (TBP) on the surface of EVs, and endows RBCEVs with osteoclast-targeting capability both in vitro and in vivo[137]. Luo constructed a BMSC-specific aptamer (5′-ACGAGGTGATATGCAAGGTCATGCACGAGTCAGAGG-3′) and coupled the aptamer with bone marrow stromal cell (ST)-derived exosomes (STExos) via aldehyde modification. This aptamer can transport STExos, which previously could not reach the bone marrow, to promote osteogenic differentiation in bone marrow mesenchymal stem cells, increase bone mass in ovariectomized (OVX) mice, and accelerate bone healing in a femoral fracture mouse model[127]. Liu utilized bioengineering to prepare *Escherichia coli*-derived exosomes modified with BMP-2 and CXCR4, targeting bone to enhance bone formation[139].

Functional molecule encapsulation

EVs primarily function as mediators of signal transduction by transporting cargo. Therefore, the type and function of the loaded cargo are crucial. Cargo loading involves mainly incorporating biologically active molecules with therapeutic properties, such as proteins, small molecules, or nucleic acids (e.g., miRNA, siRNA), into EVs through electroporation, plasmid transfection, or incubation with permeabilizing agents[142]. Huang used GPNMB-overexpressing BMSC-EVs obtained through lentiviral transduction to significantly stimulate the proliferation and osteogenic differentiation of BMSCs by activating the Wnt/β-catenin signal, thereby alleviating bone loss[131]. Hu utilized miR-21-overexpressing adipose tissue-derived mesenchymal stem cell exosomes obtained through lentiviral transduction to effectively alleviate spinal osteoporosis in ankylosing spondylitis mice[130]. Zhang's study demonstrated that microRNA-935-modified bone marrow mesenchymal stem cell-derived exosomes deliver miR-935 to osteoblasts, inhibiting STAT1 levels and promoting proliferation and differentiation of osteoblasts in osteoporotic rats[67]. Gui transduced RNF146 into BMSCs via adenoviral vectors to obtain EVs with elevated RNF146 expression, consequently enhancing osteoblast differentiation[135]. Yang discovered that overexpressing EphA2 on exosomes is an effective biological approach for targeted delivery of METTL14 into osteoclasts to inhibit osteoclast formation[143]. Cao found that EVs derived from BMSCs modified with circular RNA Rtn4 can target miR-146a to attenuate TNF-α-induced cytotoxicity and apoptosis in mouse MC3T3-E1 cells[144]. Wang constructed an lnc-KCNQ1OT1 overexpression vector using pcDNA-3.1 and obtained exosomes from adipose tissue-derived mesenchymal stem cells, which significantly inhibited TNF-α-induced cytotoxicity and apoptosis in primary osteoblasts[132].

In the construction of engineered exosomes, researchers often combine Functional molecule encapsulation with Surface modification to achieve targeted delivery of specific active substances. After constructing CXCR4 + exosomes, Hu fused CXCR4 + exosomes with liposomes carrying antagomir-188 using extrusion technology to generate hybrid nanoparticles. These NPs can specifically

Table 2

Application of engineering extracellular vesicles in osteoporosis

Modification strategy	Tool	Source	Cargo	Function	Ref.
Surface modification	Exo was conjugating with alendronate (ALN) grafted PEGylated phospholipid (DSPE-PEG-ALN) to establish a bone-targeting PL-exo (PL-exo-ALN)	Platelet lysates	NA	Enhancing bone targeting, promoting osteogenic and vascular differentiation.	[126]
	Aldehyde modification coupled with exosome.	Bone marrow mesenchymal stem cells.	NA	Targeting mesenchymal stem cells to promote osteogenesis.	[127]
	Fe3O4@SiO2PEG-CHO is loaded into extracellular vesicles.	Bone marrow mesenchymal stem cells.	NA MEG3	Inhibiting osteoclast activity. Enhancing mitochondrial autophagy, osteoblast proliferation, and differentiation.	[128] [129]
Functional molecule encapsulation	Transduction with lentiviral vectors.	Adipose-derived mesenchymal stem cells.	miR-21	Increasing bone density.	[130]
	Transduction with lentiviral vectors.	Bone marrow mesenchymal stem cells.	GPNMB	Facilitating proliferation and osteogenic differentiation of BMSCs.	[131]
	Transfection with pcDNA-3.1 plasmid.	Adipose-derived mesenchymal stem cells.	lnc-KCNQ1OT1	Inhibiting osteoblast apoptosis.	[132]
Surface modification and functional molecule encapsulation	$CXCR4^+$ lentivirus packaging system and Liposome hybridization.	NIH-3T3 cells	Antagomir-188	Enhancing bone marrow targeting, promoting osteogenic differentiation, and inhibiting adipogenic differentiation.	[133]
	Modification of Exo with bone-targeting peptide (DSPE-PEG-Mal-Cys-SDSSD) and loading siShn3-Cy3 by electroporation	Human iPSCs (cell line DYR0100)	siShn3	Targeting osteoblasts for siShn3 delivery to promote osteogenic differentiation and formation of type H vessels.	[134]
	Modification of Exo with bone-targeting peptide (Asp-Ser-Ser)6 ((DSS)6) and loading ubiquitin ligase RING finger protein146 by adenovirus transduction	BMSCs	RNF146	Targeting osteoblasts to promote osteoblast differentiation.	[135]
	Modification of Exo with bone-targeting peptide (AspSerSer)6 and loading Maytansinoids by DSPE-PEG-Mal	Macrophages	DM1	Clearing senescent bone cells.	[136]
	Non-covalent protein–peptide interactions and loaded with anti-miR-214 through electroporation	Red blood cell	Anti-miR-214	Targeted delivery of anti-miR-214 to osteoclasts to inhibit osteoclast activity.	[137]
	Transfection of pCMV6-AC-GFP plasmids modified with magnetic nanoparticles.	Bone marrow mesenchymal stem cells.	miR-150-5p	Targeting osteoblasts to promote osteoblast differentiation.	[138]
	Modification of Exo with Nissle 1917-pET28a-ClyA-BMP-2-CXCR4	*Escherichia coli*	NA	Targeting bone to promote bone formation.	[139]
Other	Calcium sulfate/nanohydroxyapatite-based nanocement (NC) as a carrier for the exosomes derived from recombinant human bone morphogenetic protein-2 (BMP-2), zoledronic acid (ZA), and BMSCs	Bone marrow mesenchymal stem cells.	NA	Promoting bone formation and healing, reducing the dosage of BMP.	[140]
	Loading exosomes onto classical porous β-TCP scaffolds.	Mesenchymal stem cells.	NA	Stimulating bone formation and vascularization.	[141]

aggregate in the bone marrow and release antagomir-188, which promotes bone formation and inhibits adipogenesis of bone marrow stromal cells, thereby reversing bone trabecular loss in aged mice and reducing cortical bone porosity[133]. Cui modified the surface of exosomes with bone-targeting peptides and used electroporation to load small interfering RNA (siShn3) into exosomes to construct bone-targeted engineered exosomes BT-Exo-siShn3. BT-Exo-siShn3 enhanced osteogenic differentiation, reduced autocrine RANKL expression, inhibited osteoclast formation, increased SLIT3 production, and promoted vascular formation, particularly the formation of type H vessels[134]. Xu's osteoclast-targeted red blood cell-derived EVs (OT-RBCEVs) can carry anti-miR-214 antibodies to target and inhibit osteoclast activity[137]. The EVs derived from bone marrow mesenchymal stem cells loaded with magnetic NPs (GMNP-BMSC-EV) constructed by Xu can carry miR-150-5p to target and inhibit MMP14, activate Wnt/β-catenin signaling to induce proliferation and maturation of osteoblasts, thereby slowing down the progression of diabetic osteoporosis[138].

Others

Qayoom used calcium sulfate/nanohydroxyapatite-based nanocement (NC) as a carrier for the exosomes derived from recombinant human BMP-2, zoledronic acid, and BMSCs to enhance bone formation and defect healing in a rat femoral neck defect model of osteoporosis. Similarly, Deluca suggests that exosomes

may be used as an enhancer, ultimately reducing the dose of BMP[140,145].

Qi extracted exosomes secreted by MSCs derived from human induced pluripotent stem cells (hiPSCs, hiPSC-MSC-Exos) and dropped them onto classical porous β-TCP scaffolds (5 mm diameter and 2 mm depth) with an average pore size of 500 μm and 75% porosity to construct hiPSC-MSC-Exos + β-TCP scaffold. The study shows that hiPSC-MSC-Exos + β-TCP can enhance angiogenesis and osteogenesis in a rat ovariectomy model, promoting bone regeneration in critical skull defects[141]. Luo developed a novel bioactive hybrid PLA scaffold (MDs-NFATc1/PLA-Exo) by incorporating MDs-NFATc1-silencing siRNA for targeting osteoclast to modulate osteogenic differentiation[146].

Yin developed an orthopedic implant with exosome targeting, featuring a nutrient coating. The utilization of a high-zinc atmosphere as a local microenvironmental cue not only inhibits bone resorption by suppressing osteoclasts but also induces reprogramming of senile osteogenesis and osteoclast dialogue through exosome modification[147].

Challenges and prospects

Numerous studies have shown that EVs from different sources play important roles in bone metabolism, drawing increasing attention to therapeutic approaches based on EVs. EVs serve as effective drug delivery systems, whether they are natural exosomes or engineered exosomes. On one hand, EVs possess excellent biocompatibility, low immunogenicity, and nano size, enabling effective drug delivery. On the other hand, engineering modifications make EVs more targeted and stable. However, there are still some unresolved issues regarding the clinical application of EVs.

How to deal with the heterogeneity and diversity of EVs?

With the advancement of technology, the classification and physicochemical properties of EVs are gradually being explored and demonstrated to be diverse. Based on the formation process and size of EVs, they can be divided into exosomes, microvesicles, and apoptotic bodies. EVs from different sources have specific membrane surface markers, which also give them different targeting capabilities. The cargo carried by EVs is crucial for the information exchange in bone metabolism. However, the information carried by EVs from different sources also varies greatly. According to the review, EVs from different sources and with different modifications have therapeutic effects on osteoporosis. Particularly, research on EVs derived from stem cells is the most concentrated. The therapeutic dosage of EVs is primarily characterized by protein mass and vesicle quantity, which complicates the comparison between the two methods. Therefore, the unification and conversion of these two parameters will be one of the key focuses of future research. Therefore, the comparison of different EVs should be a focus of future research, including but not limited to the source, dosage, administration method, and modification of EVs.

Moreover, from a therapeutic perspective, EVs as biological products and drugs require clear regulation, including but not limited to their manufacturing, characterization, and storage. To ensure the consistency and stability of EVs, selecting appropriate sources is critical. Stem cells with pluripotency and stability of replication appear to be suitable sources of EVs[148], while plants and milk serve as more cost-effective sources for EVs extraction.

How to solve the issues of large-scale extraction and preservation of EVs?

There are various methods for extracting EVs, including differential ultracentrifugation, density-gradient ultracentrifugation, polyethylene glycol precipitation, size exclusion chromatography, and antibody-conjugated agarose beads. These methods are commonly utilized for EVs extraction, but may have issues with low purity and low yield to varying degrees. Differential ultracentrifugation is currently regarded as the gold standard for the isolation and purification of EVs. In vivo experiments, the effective dose of EVs is 10–500 μg/mice, and in clinical trials, the number of extracellular vesicle particles reaches $0.5–1.4 \times 10^{11}$[149-151]. However, a consensus on strategies for large-scale isolation and purification of EVs has yet to be established[152]. To increase the yield of EVs and reduce costs, researchers have focused on technologies that hollow fiber bioreactors, and stirred tank bioreactors[153]. Through these techniques, the production efficiency of EVs can be increased by 5 to 10 times[151,154]. In addition to needing to meet parameters such as integrity, yield, purity, scalability, and cost of EVs, ensuring the stability of its efficacy is crucial[155]. Hence, the composition of the culture medium, production system, and production mode need to be optimized for each cell line. This also requires further characterization of EVs to identify the optimal subgroups for the treatment of osteoporosis. The storage of EVs is also a key issue for drug development. −80°C is currently recognized as the best storage method, but the associated storage costs are prohibitive. Cryoprotectants such as trehalose, which have been used in vaccines, can prevent EVs aggregation and freeze damage, making them a more favorable option for the storage of EVs[156]. Due to limitations in storage conditions, the administration methods of EVs pose challenges, and the development of microneedle systems may bring us surprising results.

How to ensure the safety of engineered EVs?

In order to enhance therapeutic effectiveness, different engineering modifications are applied to EVs. However, potential interference with the original content of EVs after genetic engineering or drug loading is a challenge that must be addressed. Therefore, a comprehensive evaluation of the safety, shelf life, and potential impacts of different engineering strategies and long-term use of extracellular vesicle products still requires further scrutiny.

How to optimize the application of EVs in osteoporosis?

Bone metabolism in osteoporosis is complex and variable, with different types of osteoporosis exhibiting distinct bone metabolism characteristics. For example, postmenopausal osteoporosis patients have increased bone turnover, with the rate of bone resorption increasing faster than the rate of bone formation, whereas in elderly osteoporosis patients, bone turnover is decreased, with the rate of bone resorption decreasing slower than the rate of bone formation. Additionally, bone metabolism follows time and spatial patterns. How to appropriately apply EVs for different stages of bone metabolism or prepare EVs with temporal and spatial characteristics is a challenge that engineered modifications should overcome. Unlike the currently used monoclonal antibodies for osteoporosis, EVs have a multi-target mechanism of action, which can enhance therapeutic effects through multiple pathways, but this may also lead to the potential off-target effects. Therefore, it is essential to

explore EVs with different functions, and future research should comprehensively investigate the integrated roles of EVs in bone metabolism, rather than being limited to osteoblast differentiation or osteoclast differentiation. To ensure the stability and consistency of EVs, it is one of the future directions to conduct a multifaceted comparison of different preparation methods, purification techniques, and storage conditions for EVs. Additionally, to ensure the safety of EVs applications, the development of both natural and engineered EVs requires comprehensive guidelines and regulations, along with appropriate clinical studies. EVs have clinical applications in certain diseases; for example, EVs derived from placental mesenchymal stromal cells and bone marrow mesenchymal stem cells can reduce the mortality risk in COVID-19 patients[157,158], and platelet-derived EVs can promote wound healing[159]. These studies provide sufficient confidence for the clinical application of EVs in osteoporosis, but caution is still needed before taking this step.

In conclusion, the treatment of osteoporosis based on EVs holds great potential and promising prospects. However, the aforementioned issues need to be addressed at least to advance their clinical application.

Ethical approval

Not applicable

Consent

Not applicable.

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