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Review article

The multifaceted roles of extracellular vesicles in osteonecrosis of the femoral head

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ABSTRACT

Osteonecrosis of the femoral head (ONFH) is a severe disease characterized by bone tissue necrosis due to vascular impairment, often leading to joint collapse and requiring surgical intervention. Extracellular vesicles (EVs) serve as crucial mediators of intercellular communication, influencing osteogenesis, angiogenesis, and immune regulation. This review summarizes the dual role of EVs in both the pathogenesis of ONFH and post-necrosis bone repair, highlighting the impact of various EV-mediated signaling pathways on bone regeneration and the potential crosstalk among these pathways. Additionally, EVs hold promise as diagnostic biomarkers or contrast agents to complement conventional imaging techniques for ONFH detection. By elucidating the role of EVs in osteonecrosis and addressing the current challenges, we aspire to establish a foundation for the timely identification and treatment of ONFH.

The translational potential of this article: This review comprehensively discusses the role of EVs in ONFH, providing innovative and promising insights for its diagnosis and treatment, which also establishes a theoretical foundation for the future clinical application of EVs in ONFH.

1. Introduction

Osteonecrosis of the femoral head (ONFH) is a debilitating disease characterized by subchondral bone necrosis and disrupted blood supply, often leading to joint collapse and loss of hip mobility [1]. Total hip arthroplasty (THA) remains an effective surgical option in the late stages of ONFH to alleviate pain and restore joint function [2]. With advancements in implant materials and surgical precision, contemporary studies report over 90 % implant survivorship at 15–20 years in selected populations [3]. However, younger patients or those with high physical demands may still face revision risks due to wear-related complications or aseptic loosening, imposing a cumulative socioeconomic burden over their lifetime [4,5]. This underscores the urgency of early ONFH diagnosis to delay or avoid arthroplasty. Nevertheless, early-stage ONFH is often asymptomatic, complicating timely detection [6]. While hypotheses such as hypercoagulability and lipid metabolism disorders have been proposed, the molecular mechanisms driving ONFH progression remain elusive, hindering targeted therapies [7].

Extracellular vesicles (EVs) are bilayered vesicles released by cells that carry proteins, lipids, and nucleic acids. Initially regarded as mere cellular waste [2], accumulating evidence now indicates that EVs are key mediators of intercellular communication, playing crucial roles in activating signaling pathways, modulating immune responses, and facilitating tissue regeneration [8]. Recent studies have demonstrated the therapeutic potential of EVs in various diseases, including myocardial fibrosis [9], pulmonary injury, malignancies [10], and osteoporosis [11]. Notably, bone marrow-derived EVs exhibit high biocompatibility and remarkable targeting ability toward damaged bone cells, making them promising carriers for drug delivery or exogenous bioactive factors [12,13]. By transporting bioactive molecules to target cells, EVs regulate bone metabolism [14–16] and lipid homeostasis [17] within the femoral head, influence microenvironmental dynamics and contribute to either the progression of ONFH or the repair of necrotic bone. Furthermore, EVs may serve as biomarkers for ONFH or act as contrast agents to assist

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in the diagnosis using traditional imaging techniques.

This review aims to comprehensively summarize the current understanding of EVs in ONFH pathogenesis, therapeutic potential, and diagnostic applications, highlighting their advantages and limitations while providing a theoretical foundation for future clinical applications.

2. Overview of EVs

2.1. Biogenesis

The biogenesis pathways of EVs vary depending on their subtypes, primarily including exosomes (exos), microvesicles (MVs), and apoptotic bodies(ABs). These vesicles are formed through distinct cellular mechanisms (Fig. 1) and carry specific molecular cargo from their parent cells, thereby playing essential roles in intercellular communication and pathophysiological regulation.

2.1.1. Exosomes

Exosomes, the smallest EVs (50–150 nm), are generated via the endocytic pathway [18]. Early endosomes (EEs) form through plasma membrane invaginations and can merge to create late endosomes [19]. These late endosomes bud inward to form intraluminal vesicles (ILVs), which are released as exosomes when multivesicular bodies (MVBs) fuse with the plasma membrane [19,20].

2.1.2. Microvesicles

MVs(100–1000 nm), also known as microparticles (MPs) or Ectosomes [21], are produced via the plasma membrane budding pathway, typically triggered by cellular stress. Environmental stimuli, such as hypoxia or inflammation, increase the production of reactive oxygen species (ROS), damage the cytoskeleton, and activate scramblase, leading to the outward flipping of the cell membrane [22,23]. Myosin light chain kinase (MLCK) promotes cytoskeleton remodeling and membrane contraction, driving the budding process [24]. As the membrane protrudes and buds, membrane-bound proteins, lipids, and Ribonucleic Acid(RNA) are enclosed in MVs. Eventually, the MVs detach from the parent cell and are released into the extracellular matrix [23].

2.1.3. Apoptotic bodies

Apoptotic bodies (500–5000 nm) are a subtype of EVs formed during programmed cell death [25], which can be generated through two distinct mechanisms. The first involves a budding and shedding process catalyzed by caspases, which cleave intracellular proteins, disrupt cellular structures, and induce apoptosis. This leads to the formation of spherical, membrane-bound structures containing cytoplasm, organelles, and nuclear fragments, which subsequently detach as apoptotic bodies [26,27]. The second mechanism involves the autophagic formation of apoptotic bodies, where apoptotic factors released from mitochondria enter the nucleus, trigger DNA fragmentation, and initiate cell apoptosis [28].

2.2. EVs composition

In terms of appearance, EVs originally possessed a spherical nanostructure; however, during artificial extraction and purification, the internal fluid evaporates, causing deformation. As a result, they appear as "cup-shaped" structures under scanning electron microscopy [29]. In terms of content, the cargo of exosomes, MVs, and ABs varies depending on their cellular origin [29]. Generally, it includes the following key molecules: The first category is lipids, which not only constitute the vesicular membrane but also participate in intercellular signaling and intracellular homeostasis regulation [6,21]. Exosomes are enriched in cholesterol, sphingolipids, and phosphatidylserine, which enhance their stability and cellular targeting properties [30]. MVs exhibit diverse lipid compositions, typically containing phosphatidylcholine, phosphatidylserine, and elevated levels of lipid-metabolizing enzymes [31]. In ABs, phosphatidylserine is exposed on the outer membrane surface, serving as a hallmark of apoptotic bodies [25]. The second key component is proteins, including membrane transport proteins, cytoskeletal proteins, and heat shock proteins. These proteins serve as crucial markers for EV identification, contribute to cargo transport and signaling functions, and maintain EV structural stability [32–34].

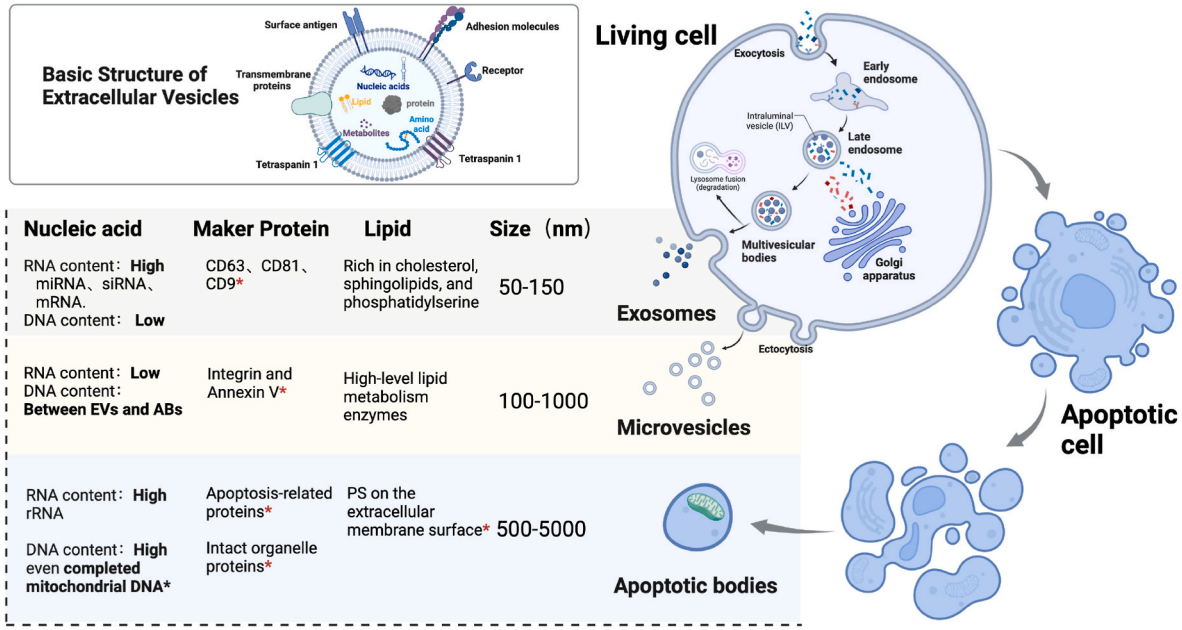

Fig. 1. The biogenesis of extracellular vesicles and the differences between their subtypes. Cells first produce endosomes through endocytosis. These endosomes exchange materials with other cellular organelles and load nucleic acids, proteins, and lipids from the cytoplasm into their lumen. Subsequently, the endosomal membrane invaginates to form small vesicles within the endosome. Eventually, these small vesicles are released when multivesicular bodies fuse with the plasma membrane, a process that results in the secretion of exosomes. In contrast, microvesicles are produced by ectocytosis, where vesicles are shed directly from the plasma membrane. Apoptotic bodies, on the other hand, are formed through a budding mechanism during the process of apoptosis. The red asterisks in the figure represent the characteristics of this type of EVs.

Exosomes are typically characterized by specific marker proteins such as Cluster of Differentiation 63(CD63), CD81, and CD9, whereas MVs and ABs lack universally defined markers [35]. However, certain MVs carry distinctive molecules like integrins and Annexin V [31], while ABs contain apoptosis-related proteins such as Caspase-3 and Poly (ADP-Ribose) Polymerase(PARP), along with intact organelle proteins [36]. The presence of whole organelles is a unique feature of ABs [37]. The third category is nucleic acids, which are present in all three types of EVs. Exosomes contain the most diverse range of nucleic acids [38], while MVs have relatively lower RNA content, mainly comprising MicroRNAs(miRNAs) and long non-coding RNAs [31]. Due to nuclear fragmentation during apoptosis, ABs are enriched with larger genomic DNA fragments and some transcriptional RNAs [39], including abundant rRNA and DNA fragments. miRNAs(miR) are the most abundant nucleic acid species in EVs and play critical roles in regulating gene expression in recipient cells, influencing various biological processes [40–42]. Additionally, EVs carry amino acids, sugars, mitochondrial DNA, and other bioactive molecules that collectively contribute to the metabolic regulation and signal transduction of target cells [43–45].

3. The role of EVs in the pathogenesis of ONFH

Although the vast majority of studies currently focus on the therapeutic effects of EVs, certain EVs can disrupt the balance between osteoblasts and osteoclasts, leading to abnormal adipogenesis, impaired angiogenesis, and dysregulated coagulation—key pathological events in ONFH. These processes are interconnected and mutually reinforcing (Fig. 2).

3.1. Disruption of lipid metabolic pathways

Previous studies have proposed the lipid metabolism disorder hypothesis of ONFH, in which dysregulated lipid metabolism involves adipocyte proliferation and hypertrophy, as well as abnormal fatty acid production [46]. Enlarged and proliferating adipocytes contribute to fat tissue expansion and increased intraosseous pressure, leading to perivascular fat deposition, enhanced immune cell infiltration, and elevated production of vasoconstrictive mediators [47]. Meanwhile, abnormally generated fatty acids can damage vascular endothelial cells, triggering aberrant coagulation [46], ultimately resulting in vascular occlusion, disrupted blood supply, and the onset of ONFH.

In fact, both Exos and MVs play pivotal roles in these pathological processes. Duan et al. found that an increase in M1 macrophages in both patients and mice with steroid-induced ONFH(SONFH) correlated with impaired bone repair. This was attributed to the presence of miR-1a-3p in exosomes secreted by M1 macrophages, which promotes the adipogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) by targeting the adipogenesis-related gene CCAAT/Enhancer-Binding Protein Zeta(C/EBPz) [17]. These proliferating adipocytes further secreted MVs, which were enriched in miR-148a, a microRNA that directly inhibited the Wnt5a/Ror2 signaling pathway [48]. The Wnt pathway is critically involved in BMSC differentiation, with Wnt5a activating the non-canonical Wnt signaling pathway to strongly suppress adipogenic differentiation [49]. The inhibition of Wnt5a by miR-148a further reinforced a pathological positive feedback loop that promoted adipogenesis while concurrently suppressing osteogenesis [48]. Under glucocorticoid (GC) exposure, this aberrant differentiation trend was

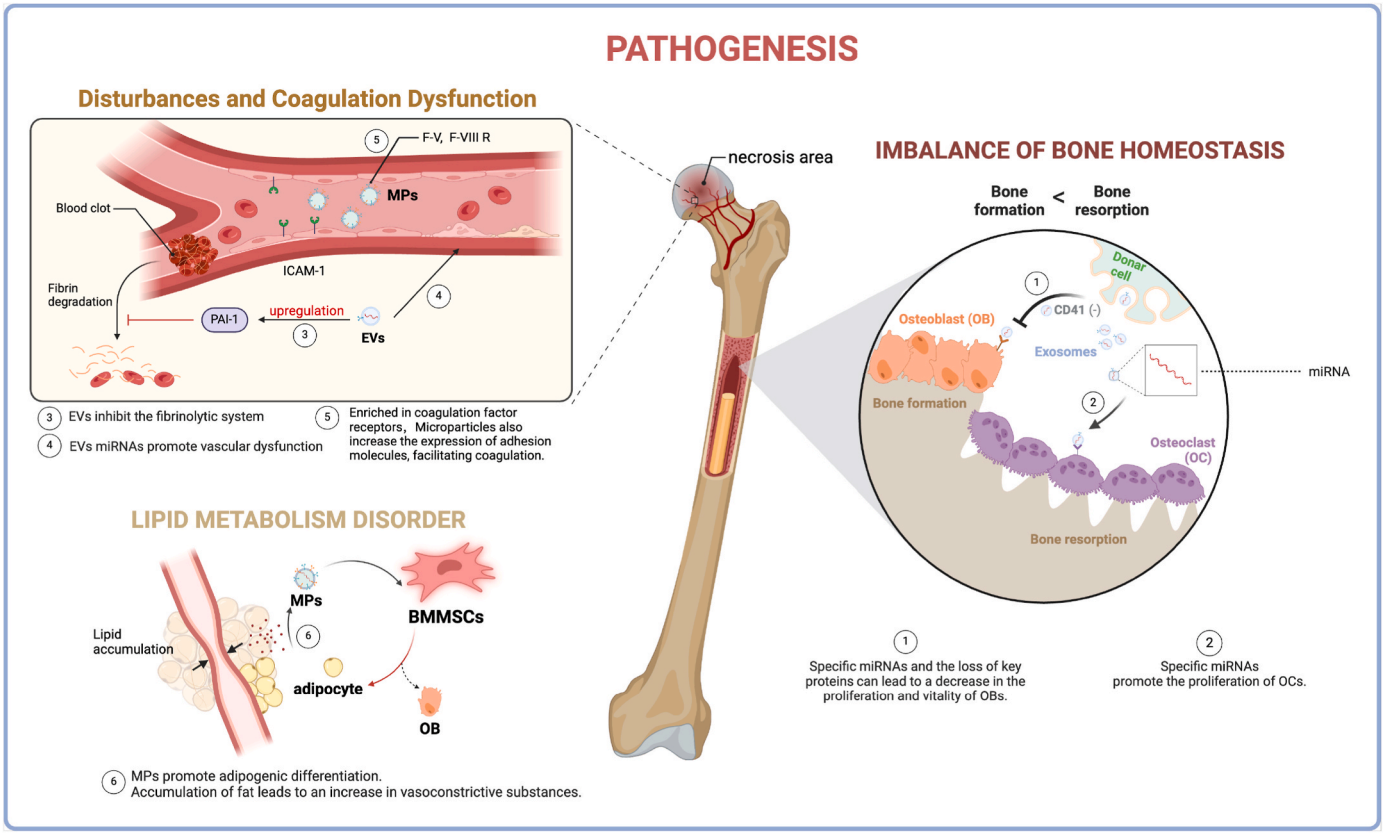

Fig. 2. Mechanisms of ONFH facilitated by extracellular vesicles. Exosomes absence of CD41 impede the proliferation and viability of osteoblasts, whereas particular RNAs, including miR-3133, miR-4693-3p, miR-4693-5p, miR-141-3p, and miR-182-5p, enhance the proliferation of osteoclasts and diminish the population of osteoblasts. The imbalance in the number of osteoblasts and osteoclasts leads to bone loss. Microparticles secreted by abnormally proliferating adipocytes can also enhance the adipogenic differentiation of BMMSCs, leading to the accumulation of fat around blood vessels and promoting the production of vasoconstrictor substances, which results in the femoral head's blood supply being decreased. In addition, extracellular vesicles can also promote blood clotting and inhibit the fibrinolytic system, leading.

exacerbated, ultimately leading to increased local bone pressure, dysregulated vascular homeostasis, and bone necrosis [50].

Furthermore, exosomes derived from necrotic femoral head tissue have a significant impact on lipid metabolism. Using chromatographic techniques, Guo et al. compared the lipid metabolic profiles of bone tissue-derived exosomes from patients with femoral neck fractures and ONFH, revealing significant differences in fatty acid-related metabolic pathways, such as glycerophospholipid and sphingolipid metabolism, between the ONFH and control groups [51]. Studies have shown that dysregulation of sphingolipid and glycerophospholipid metabolism disrupts intracellular homeostasis, leading to mitochondrial and endoplasmic reticulum damage and ultimately inducing apoptosis [52]. Further research by Guo et al. revealed that, in addition to glycerophospholipids and sphingolipids, the levels of other lipid metabolism-related metabolites, such as riboflavin and coenzyme A (CoA), were also significantly altered in exosomes from ONFH patients compared to normal controls [53]. Riboflavin deficiency has been shown to reduce bone mineral density in rats by impairing osteoblast function, leading to bone loss [54]. Moreover, as a crucial coenzyme in various metabolic reactions, riboflavin dysregulation may affect energy supply and oxidative stress in bone tissue, thereby influencing osteoblast function and survival. This aligns with the findings of Zhu et al., who reported riboflavin metabolic disturbances and abnormal oxidative stress responses in ONFH [55]. These metabolic abnormalities collectively contribute to the progression of ONFH.

3.2. Abnormalities in the number and function of osteocytes

Bone metabolism imbalance plays a crucial role in the pathogenesis of ONFH. Bone metabolism involves both bone formation and bone resorption, with the former primarily mediated by osteoblasts [56]. Under normal conditions, exosomes derived from BMSCs facilitate bone repair. However, during ONFH, exosomes originating from necrotic bone tissue can suppress osteogenic differentiation [14,57] and induce apoptosis in osteocytes and osteoblasts [58], leading to a reduced number of osteoblasts and diminished new bone formation. Additionally, exosomes circulating in the blood of some ONFH patients enhance osteoclastogenesis, contributing to bone resorption [59]. This imbalance in bone metabolism results in the destruction of bone tissue, ultimately leading to the structural and functional loss of the femoral head [56].

Runt-related transcription factor 2 (RUNX2) is a key regulator that ensures the differentiation of BMSCs into the osteogenic lineage and plays a crucial role in chondrocyte hypertrophy and maturation [60]. However, its expression is modulated by multiple pathways. Li et al. found that compared with patients with femoral neck fractures, patients with SONFH exhibited significantly lower levels of miR-182-5p in BMSC-derived exosomes [14]. miR-182-5p suppresses the activation of myeloid differentiation primary response protein 88 (MYD88), a key activator of the Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells(NF-κB) pathway. The downregulation of miR-182-5p leads to NF-κB pathway activation, resulting in decreased RUNX2 expression [14]. This finding aligns with the well-established role of the NF-κB pathway in promoting bone resorption and bone loss [61]. In in vitro experiments, exosomes derived from SONFH inhibited BMSC proliferation and migration while promoting apoptosis and suppressing osteogenic differentiation, thereby disrupting normal bone metabolism [14–16]. In vivo, tail vein injection of SONFH-derived exosomes in mice significantly exacerbated trabecular bone loss in the femoral head [14]. Additionally, exosomes from necrotic bone tissue are enriched in miR-100-5p [57] but exhibit reduced expression of CD41 [16]. The former downregulates the bone morphogenetic protein receptor type 2 (BMPR2), thereby suppressing its downstream Small Mothers Against Decapentaplegic (SMAD)1/5/9 signaling pathway. Meanwhile, CD41 deficiency impairs the activation of integrin β3-focal adhesion kinase (FAK), leading to Akt pathway inactivation. The suppression of these two pathways results in decreased RUNX2 protein expression [16,57], impairing MSC osteogenic differentiation and bone repair capacity, which further aggravates osteonecrosis.

Nuclear receptor coactivator 3 (NCOA3) is a transcriptional activator involved in cell proliferation, differentiation, and survival. In osteoblasts, NCOA3 expression is crucial for maintaining normal bone cell function and bone tissue integrity [62]. In an ONFH model, miR-532-5p expression was upregulated, inhibiting NCOA3 in osteoblasts, thereby reducing their viability and proliferation while inducing apoptosis. This process exacerbated the cytotoxic effects of steroids on osteoblasts. The role of miR-532-5p in osteoblast dysfunction was further validated by its knockout, which mitigated these detrimental effects [58].

Legg-Calvé-Perthes disease (LCPD) is an idiopathic ischemic necrosis of the femoral head that occurs in children [63]. While its precise etiology remains unclear, it shares similarities with adult osteonecrosis, being associated with coagulation abnormalities, vascular dysfunction, bone metabolism disorders, and local mechanical stress [64,65] Studies have shown that plasma exosomes in LCPD patients exhibit significantly elevated levels of miR-3133, miR-4693-3p, miR-4693-5p, miR-141-3p, and miR-30a. In vitro, the overexpression of these miRNAs resulted in a significant increase in tartrate-resistant acid phosphatase-positive (TRAP+) multinucleated cells, while concurrently reducing the total number of osteoblasts [59]. This imbalance, where bone resorption surpasses bone formation, leads to osteoporosis, a critical factor in the progression of osteonecrosis [66].

3.3. Microcirculatory disturbances and coagulation dysfunction

The disruption of blood supply is a critical factor in the pathogenesis of ONFH, characterized by hypercoagulability, hypofibrinolysis, and microvascular thrombosis [67]. Likewise, EVs play a pivotal role in exacerbating these pathological hematological conditions.

Wu et al. found that in a rabbit model of SONFH, plasma levels of coagulation and inflammatory factors were significantly elevated, along with increased levels of endothelial-derived microparticles (EMPs) and platelet-derived microparticles (PMPs), indicating a strong correlation between MPs and a hypercoagulable, inflammatory state [68]. Mechanistically, MPs not only harbor receptors for coagulation factors Va and VIII but also provide an optimal environment for thrombin generation. Thrombin, in turn, activates platelets, which release additional PMPs, further amplifying thrombin production in a positive feedback loop that exacerbates hypercoagulation. Moreover, PMPs upregulate the expression of intercellular adhesion molecule-1 (ICAM-1) in endothelial cells, which is closely linked to inflammatory responses and promotes the formation of neutrophil extracellular traps (NETs) [69]. NETs, in turn, capture platelets and red blood cells, facilitating thrombus formation and vascular occlusion [70]. CD31 expression increases during endothelial cell apoptosis, while CD42b serves as a platelet-specific marker [71]. Compared to CD31+/CD42b + EMPs, CD31+/CD42b− EMPs are more accurate indicators of endothelial dysfunction. Based on this, Li et al. reported a significant increase in CD31+/CD42b− EMPs in the plasma of children with LCPD, which correlated positively with interleukin-6 (IL-6) levels. In vitro, IL-6 stimulation of endothelial cells promoted the release of CD31+/CD42b− EMPs [71]. These EMPs further induced the expression of the pro-inflammatory cytokine IL-1β in plasma [68], creating a vicious cycle wherein increased inflammatory cytokines exacerbate endothelial dysfunction, inhibit angiogenesis, and promote endothelial cell apoptosis [71].Beyond MPs, certain miRNAs enriched in exosomes from LCPD patients' plasma are significantly elevated compared to healthy individuals. Notably, miR-3133, miR-4644, miR-4693-3p, and miR-4693-5p disrupt tight junctions between endothelial cells and induce endothelial dysfunction when overexpressed in vitro [59].

Hypofibrinolysis, another key factor in thrombosis formation, is typically mediated by an increase in plasminogen activator inhibitor-1 (PAI-1) levels [72]. As a critical regulator of the plasminogen-plasmin system, PAI-1 inhibits plasminogen activation, reducing fibrinolysis

and increasing the risk of thrombus formation [72,73]. EVs derived from BMSCs contain two key microRNAs that regulate PAI-1 balance: miR-451-5p, which indirectly enhances PAI-1 expression by inhibiting the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathway, and miR-133b-3p, which directly targets the 3′-UTR of PAI-1 mRNA to downregulate its expression [74]. Li et al. demonstrated through in vitro experiments that exosomes derived from glucocorticoid-treated BMSCs (GB-Exo) significantly upregulated PAI-1 expression. Compared with normal BMSC-derived exosomes, GB-Exo exhibited increased levels of miR-451-5p and decreased levels of miR-133b-3p. The dysregulation of these miRNAs contributed to PAI-1 imbalance, and their regulatory effects on PAI-1 upregulation were further validated by in vivo injection of their respective mimics [74].

4. The role of EVs in the treatment of ONFH

Traditional stem cell therapy has several limitations, including highly demanding extraction and culture techniques [75]. Additionally, the survival rate of transplanted cells is often low, further restricting therapeutic efficacy [76]. In contrast, as a cell-free therapeutic approach, EVs offer advantages such as high biocompatibility and low immunogenicity [76]. In recent years, EVs have shown potential in treating ONFH. EVs can enter target cells via membrane fusion or receptor-mediated endocytosis, subsequently releasing their cargo, such as RNA or functional proteins, into the cytoplasm or nucleus of the target cells. This process activates downstream signaling pathways, leading to various biological effects. EVs can prevent ONFH by directly inhibiting osteoblast and vascular endothelial cell apoptosis [77–79] and promoting cell proliferation and migration [80–82]. Moreover, EVs can indirectly delay or even reverse osteonecrosis by enhancing the expression of osteogenic inducers [83,84] and angiogenic factors [82,85]. Simultaneously, they modulate the inflammatory environment by reducing pro-inflammatory cytokines (e.g., tumor necrosis factor-α (TNF-α), IL-6) [84] and increasing anti-inflammatory cytokines (e.g., IL-10) [86], thereby creating a microenvironment conducive to bone regeneration and repair(Fig. 3).

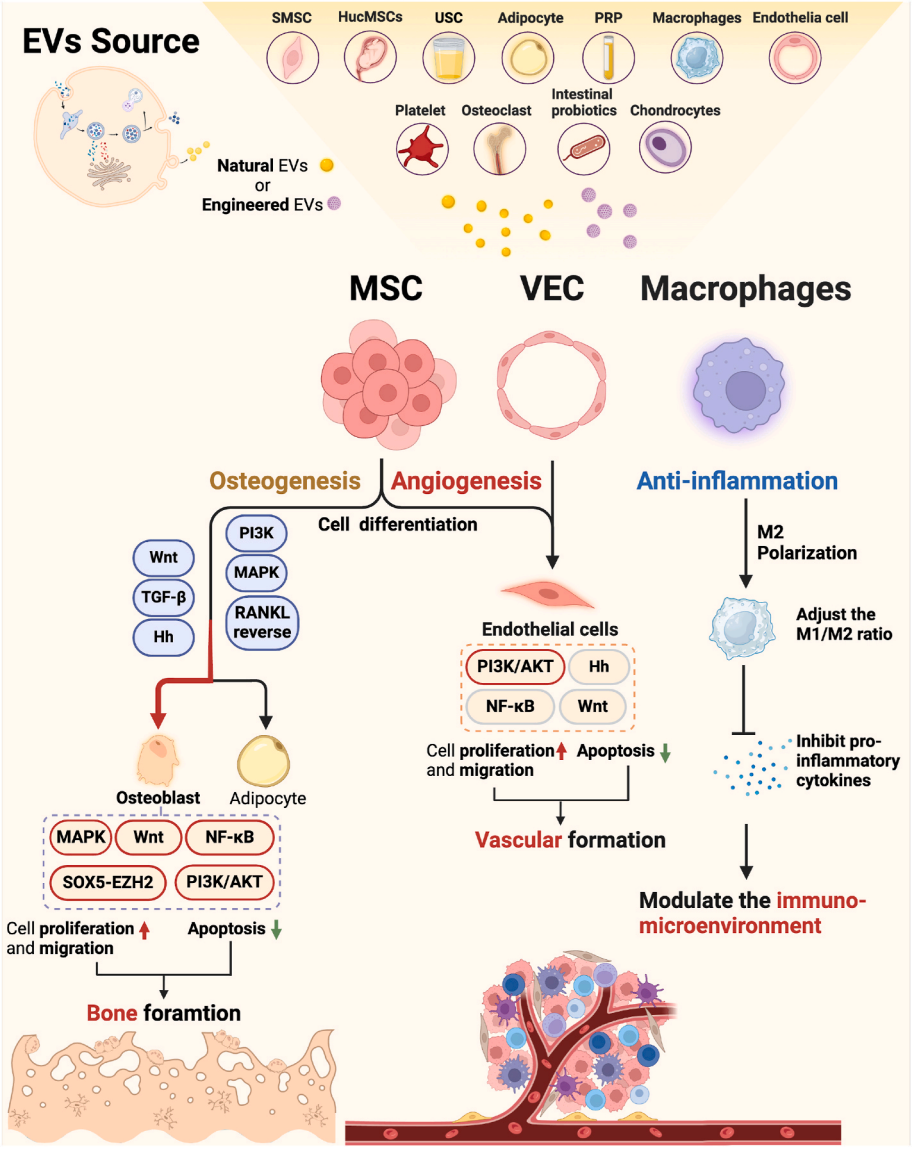

Fig. 3. Extracellular vesicles from different cell sources promote the repair of femoral head necrosis through distinct signaling pathways. EVs derived from various cell sources, whether natural or engineered, can act upon mesenchymal stem cells, vascular endothelial cells, and macrophages. They promote the proliferation and differentiation of osteoblasts and vascular endothelial cells through distinct signaling pathways such as Wnt, MAPK, and PI3K. Additionally, these EVs can inhibit apoptosis, enhance bone formation and angiogenesis, and create an immunomodulatory microenvironment conducive to bone regeneration, thereby facilitating bone repair.

Currently, an increasing number of researchers are striving to develop multifunctional EVs capable of coupling bone repair with vascular repair or even integrating bone repair, vascular repair, and bone microenvironment regulation. However, natural EVs often fail to meet such multifunctional demands. To overcome this limitation, researchers have developed engineered EVs by modifying their surface or altering their cargo, such as loading bioactive proteins or cytokines, to enhance their targeting ability and therapeutic efficacy [87]. For example, Zhou et al. constructed exosomes derived from miR-26a-overexpressing $CD34^+$ stem cells (miR-26a-CD34+-Exos). As vascular progenitor cells, $CD34^+$ stem cells secrete exosomes with significant angiogenic potential [83]. miR-26a enhances the expression of osteogenic proteins (e.g., Alkaline Phosphatase(ALP), BMP-2) through the Wnt and transforming growth factor-β (TGF-β) pathways [83]. Following treatment with these engineered exosomes, the osteogenic volume in rats increased nearly threefold compared to the ONFH control group.

4.1. Osteogenetic effects

Certain exosomes can promote osteoblast regeneration, thereby enhancing the load-bearing capacity of the femoral head and delaying the progression of collapse [88]. The osteogenic potential and bone-targeting ability of natural exosomes depend on their cellular origin. Notably, vesicles derived from stem cells exhibit a "homing" ability, allowing them to accumulate at bone injury sites and prolong their retention in bone tissue [12]. BMSCs originate from the bone marrow microenvironment and have strong biological relevance to bone tissue [13]. Studies have shown that compared to adipose-derived mesenchymal stem cells and human umbilical cord mesenchymal stem cells, BMSCs possess greater osteogenic potential and mineralization capacity [13]. Therefore, in the following discussion, BMSCs-EVs will be analyzed separately (Supplementary Table 1)

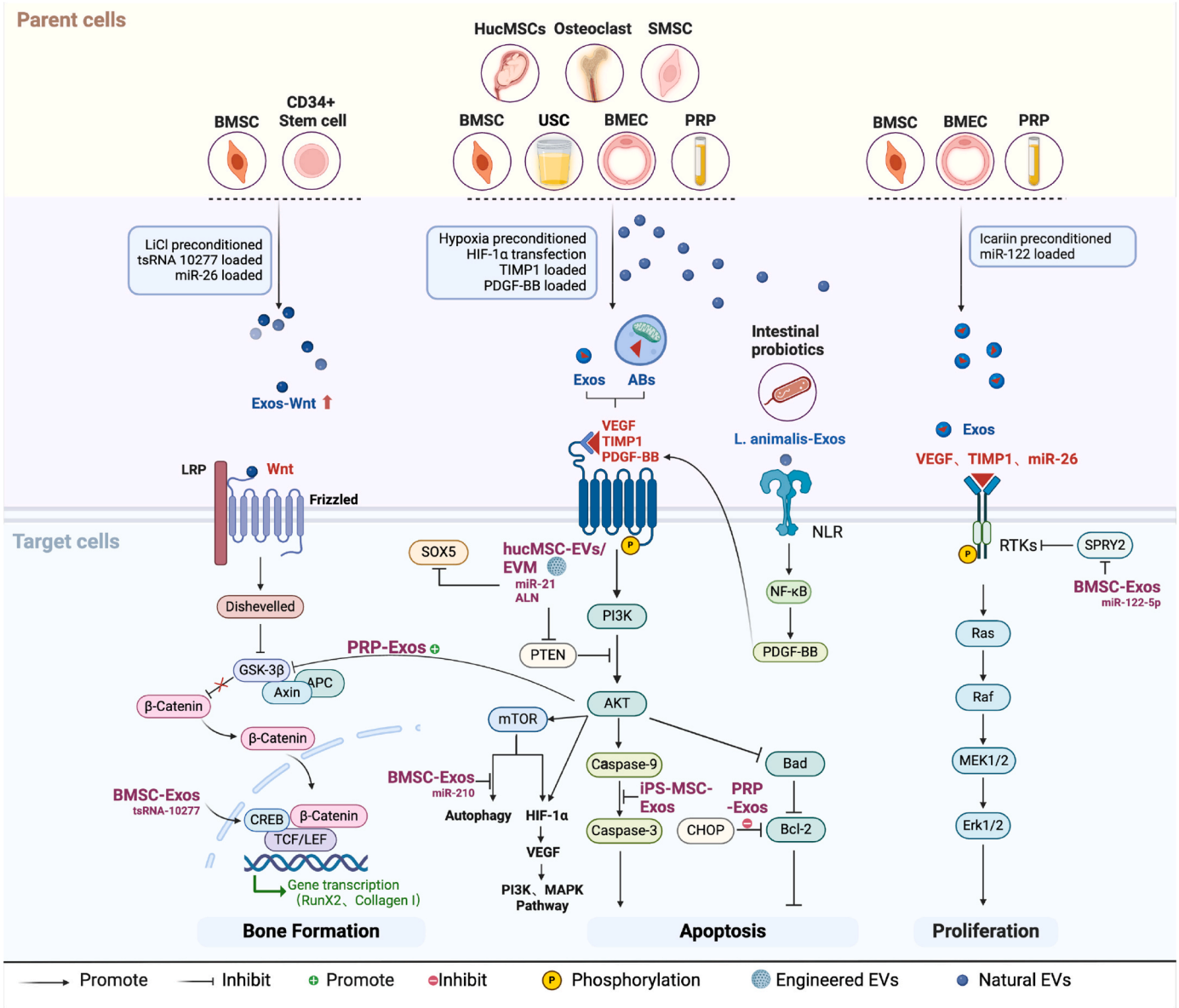

Fig. 4. The signaling pathways involved in the repair process of osteonecrosis mediated by extracellular vesicles. EVs derived from different cell sources or engineered EVs (such as those pretreated with LiCl or hypoxia, or loaded with functional substances) can activate Wnt, PI3K/AKT, NF-κB, and MAPK pathways to promote osteogenesis, angiogenesis, or the production of anti-apoptotic factors. The different signaling pathways can interact through their downstream products, and the cargo within EVs can directly inhibit various products in these pathways, thereby promoting bone and vascular regeneration.

4.1.1. BMSC-EVs

During the bone repair process following ONFH, ischemia and apoptosis-induced inflammatory responses drive osteoprogenitor cells to migrate to the damaged site and become activated [89]. At this stage, the key osteogenic transcription factor RUNX2 promotes the differentiation of these progenitor cells into mature osteoblasts by regulating downstream bone formation-related genes. Mature osteoblasts then accelerate inorganic salt deposition and bone mineralization by upregulating ALP expression. Subsequently, under the secretion and function of type I collagen (COL-I), a solid organic bone matrix is formed, providing structural support for subsequent mineralization. Ultimately, new bone formation and remodeling occur at the bone defect site [90], restoring the structure and function of the femoral head.

BMSCs-EVs themselves [91] and their cargo, such as miRNAs [92,93] and tRNA-derived small RNAs (tsRNAs) [94], can significantly promote the differentiation of BMSCs into osteoblasts while inhibiting adipogenic differentiation by suppressing the translation of target genes and upregulating factors such as ALP and RUNX2. They can counteract the inhibitory effects of glucocorticoid on osteoblast proliferation and migration, preserving vascular density and trabecular integrity in the femoral head. However, different EVs exert their effects through distinct signaling pathways (Fig. 4). For example, miR-148a-3p in mouse BMSCs-EVs targets Smad ubiquitination regulatory factor 1 (SMURF1) in the SMAD pathway, thereby preventing the degradation of downstream SMAD7 and promoting the expression of the anti-apoptotic protein BCL2. This enhances the viability and osteogenic potential of BMSCs [92]. In a rabbit model, miR-122-5p in BMSCs-EVs inhibits Sprouty2 (SPRY2), a receptor tyrosine kinase (RTK) inhibitor in the MAPK (mitogen-activated protein kinase) pathway, thereby increasing MAPK pathway activity. This enhances the expression of RUNX2 and COL-I, promotes osteoblast proliferation, and delays the progression of SONFH [93].

The classical Wnt/β-catenin signaling pathway is a key regulatory mechanism in bone formation [95]. When Wnt binds to receptors on the cell membrane, it activates intracellular Dishevelled protein (Dvl), which inhibits the β-catenin degradation complex composed of Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3 beta (GSK3β). This allows β-catenin to accumulate in the cytoplasm and translocate to the nucleus, where it binds to T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors, activating the expression of downstream osteogenic genes such as Runx2 and COL-I [95]. Studies have shown that upregulating tsRNA levels [94] or treating EVs with lithium ions [96] significantly enhances their osteogenic potential, both of which are mediated by the Wnt signaling pathway. tsRNAs are small non-coding RNAs derived from specific cleavage of tRNAs and play a broad role in post-transcriptional gene regulation [94]. Specifically, tsRNA-10277 targets and regulates cAMP response element-binding protein 1 (CREB1), a key component in the activation of β-catenin/TCF target genes in the Wnt signaling pathway. Upregulation of CREB1 promotes the activation of downstream Wnt pathway products [94]. Additionally, lithium-ion-treated BMSCs-derived vesicles stimulate bone regeneration in avascular areas of ONFH [97]. Further studies have revealed that lithium ions enhance the secretion of exosomes and their cargo, Wnt10a, through the MARK2 pathway, allowing these exosomes to deliver Wnt10a to bone injury sites [98].Unlike Wnt5a, which primarily activates the non-canonical Wnt signaling pathway to regulate BMSCs differentiation [99], Wnt10a activates the canonical Wnt/β-catenin pathway, promoting cell proliferation, inhibiting apoptosis, and enhancing the osteogenic capacity of BMSCs [98].

Furthermore, some researchers have transfected BMSCs with HIF-α to significantly increase the VEGF content in their secreted EVs [100], while others have directly transfected VEGF into EVs using plasmid transfection. VEGF activates the MAPK pathway, induces angiogenesis, and promotes osteoblast maturation and mineralization, significantly reducing the incidence of ONFH in a rat model [85].

4.1.2. Other sources of EVs

PRP-Exos activates the Akt signaling pathway, inhibiting GSK-3β activity and thereby stabilizing β-catenin. The accumulation of β-catenin promotes the expression of Runx2, which in turn maintains the osteogenic differentiation capacity of BMSCs [82]. PRP-Exos significantly increases the expression levels of osteogenic-related proteins, such as COL-I, ALP, and OCN (osteocalcin), in BMSCs and MC3T3-E1 cells, thereby promoting the formation of mineralized nodules and enhancing bone tissue formation. Additionally, PRP-Exos is rich in various growth factors (such as VEGF, bFGF, and PDGF-BB), which can activate the PI3K signaling pathway [82]. Cell survival requires active inhibition of apoptosis, a process regulated by the balance between pro-apoptotic and anti-apoptotic factors [101], with the activation of the PI3K/AKT signaling pathway playing a crucial role in this process [102]. Activated Akt can suppress the expression of pro-apoptotic factors, such as BCL2-associated agonist of cell death (Bad), BCL2-associated X protein (Bax), caspase-9, glycogen synthase kinase-3 (GSK-3), and Forkhead box O1 (FoxO1), while promoting the production of the anti-apoptotic factor B-cell lymphoma 2 (Bcl-2) [103]. However, GC can induce the inactivation of the PI3K/AKT signaling pathway, leading to increased apoptosis, which is a key factor in GC-induced ONFH. Peng [80] and Tao [82] demonstrated that intraperitoneal injection of human umbilical cord mesenchymal stem cell-derived EVs (hucMSC-EVs) in rats or intravenous injection of platelet-rich plasma-derived exosomes (PRP-Exos) could both counteract this effect of GC. Further studies on hucMSC-EVs attributed these protective effects to the presence of miR-21, which inhibits the expression of phosphatase and tensin homolog (PTEN) [79], a negative regulator of the PI3K/Akt pathway [104]. By downregulating PTEN, miR-21 enhances anti-apoptotic signaling to counteract bone necrosis. However, EVs derived from hucMSCs and similar cells suffer from issues such as a lack of targeting specificity and rapid clearance by the liver, which limit the therapeutic efficacy of miR-21. To address this problem, Jiang et al. developed a surface-modified EV mimetic by covalently conjugating alendronate (ALN) to silica–calcium–magnesium–strontium ion-based nanoparticles loaded with miR-21. Since ALN, a bisphosphonate, specifically binds to hydroxyapatite (HA), the primary inorganic component of bone tissue, this modification not only significantly enhances bone-targeting ability but also synergistically inhibits PTEN together with miR-21, thereby preventing osteoblast apoptosis and promoting angiogenesis [105]. In addition to inhibiting apoptosis, miR-21 negatively regulates the expression of SRY-box transcription factor 5 (SOX5) in osteoblasts, thereby suppressing EZH2 transcription. EZH2, a histone H3K27 trimethyltransferase, inhibits the expression of osteogenic genes such as RUNX2 and COL-I through histone modification. The suppression of EZH2 by miR-21 reverses osteogenic inhibition and apoptosis, as evidenced by increased ALP activity and the expression of osteogenic markers [77]. In contrast, SOX9 is positively associated with osteogenesis, though its exact mechanism remains unclear [91]. Beyond the aforementioned proteins, programmed cell death 4 (PDCD4) and caspase-3 are also key regulators of apoptosis. miR-135b inhibits their excessive expression, and Zhang et al. combined iPS-MSC-Exos with miR-135b to reduce osteoblast apoptosis, further enhancing the therapeutic potential of iPS-MSC-Exos [106].

EVs derived from human urine stem cells (USC-EVs) mitigate GC-induced cytotoxicity and prevent apoptosis in BMSCs and pre-osteoblast MC3T3-E1 cells. This protective effect is attributed to the tissue inhibitor of metalloproteinases-1 (TIMP1) contained within these EVs, which activates the PI3K and c-Jun N-terminal kinase (JNK) pathways in BMSCs, enhances parathyroid hormone-mediated trabecular bone formation, and effectively prevents GC-induced ONFH [78]. Similarly, EVs derived from synovial mesenchymal stem cells exhibit comparable therapeutic potential [1].

Apart from the PI3K signaling pathway, the Hedgehog (Hh) pathway also plays a crucial role in initiating cell proliferation and differentiation [107,108]. Nan et al. transfected miR-378 into adipose-derived stem

cells (ASCs), leading to the downregulation of the Hh pathway inhibitor suppressor of fused homolog (Sufu) and the upregulation of the Hh activators patched 1 (Ptch1) and glioma-associated oncogene homolog 1 (Gli1) in the EVs. These EVs reversed GC-mediated inhibition of bone formation and initiated osteogenic and angiogenic differentiation, exhibiting greater osteogenic efficacy than unmodified ASCs-EVs in a rat ONFH model [109].

4.2. Promoting angiogenesis

Angiogenesis is crucial for the repair of necrotic bone tissue. The blood supply provides essential nutrients for MSCs and other osteoprogenitor cells, as well as minerals necessary for bone mineralization and growth factors that regulate osteogenesis [110]. EVs and their cargo exhibit significant potential in enhancing angiogenesis, making them key contributors to vascular regeneration [111] (Supplementary Table 2).

4.2.1. The EVs that primarily promote VEGF

VEGF is perhaps one of the most important factors through which EVs promote angiogenesis and repair in osteonecrosis [112]. VEGF activates downstream signaling pathways, such as the PI3K/AKT pathway, which regulate cytoskeletal rearrangement and promote endothelial cell migration—a critical step in angiogenesis [113]. EVs can initiate vascular repair by either promoting VEGF expression or directly activating its downstream pathways. Hypoxia induces the activation of hypoxia-inducible factor-1α (HIF-1α), which binds to the promoter region of the VEGF gene and activates its transcription. Yuan et al. [114] preconditioned BMSCs under hypoxic conditions, while Li et al. [100] directly transfected BMSCs with HIF-1α via an adenovirus, thereby activating VEGF transcription. Both approaches significantly increased VEGF levels in exosomes. Similarly, Zhang et al. preconditioned bone marrow endothelial cells (BMECs) with Icariin, a traditional Chinese medicine with bone-protective properties. Icariin not only directly increased VEGF content in exosomes but also upregulated TGF-β1, which indirectly promoted VEGF expression [115]. Endothelial tip cells migrate in response to the VEGF gradient, forming new vascular sprouts [116].

Additionally, Xu et al. reported that miR-224-3p expression was significantly downregulated in BMSC-EVs derived from femoral heads affected by traumatic osteonecrosis, leading to the upregulation of the 200 kDa focal adhesion kinase family interacting protein (FIP200) [117]. This not only enhanced VEGF expression [117] but also facilitated the disassembly of focal adhesions, further reducing the resistance to endothelial cell migration and promoting angiogenesis [118]. Some EVs can also directly activate signaling pathways that promote vascular repair. Exosomes secreted by mesenchymal stem cells derived from human induced pluripotent stem cells (hiPS-MSCs) [81], urine-derived stem cells (USCs) [78], human urine-derived mesenchymal stem cells (hucMSCs) [80], and apoptotic bodies from immature osteoclasts (pOC-ABs) [119] have all been shown to activate the PI3K/Akt pathway, thereby promoting endothelial cell proliferation and migration. However, different EVs target distinct downstream signaling molecules upon PI3K/Akt activation, leading to divergent mechanisms in facilitating vascular repair within necrotic bone regions.

Mammalian target of rapamycin (mTOR) is a key downstream target of AKT that primarily regulates autophagy. GC can excessively activate the PI3K/AKT/mTOR pathway, leading to increased endothelial cell apoptosis. In in vitro studies, human umbilical vein endothelial cells (HUVECs) are commonly used as a model for endothelial cells due to their ease of culture. However, HUVECs do not exist in adult vascular systems, which limits the translational relevance of these studies. To improve experimental reliability, some researchers have opted to use bone microvascular endothelial cells (BMECs) or human microvascular endothelial cells (HMECs) [2]. Ma et al. demonstrated that BMSC-EVs inhibited the overactivation of the PI3K/AKT/mTOR pathway, reducing excessive BMEC apoptosis [120]. This effect was likely mediated by miR-210 contained within the EVs [2]. In addition to excessive autophagy, GC-induced apoptosis is closely associated with endoplasmic reticulum (ER) stress, which leads to the production of CCAAT/enhancer-binding protein homologous protein (CHOP). CHOP suppresses the expression of the anti-apoptotic protein BCL-2. PRP-Exos, however, can activate AKT, leading to the phosphorylation and inactivation of Bad, which in turn increases the expression of Bcl-2. Notably, PRP-Exos maintain stable Bcl-2 expression and anti-apoptotic activity even in the presence of CHOP [82]. Moreover, activation of the PI3K/AKT pathway stabilizes HIF-1α and enhances its transcriptional activity, leading to increased VEGF expression. As previously mentioned, VEGF can further activate the PI3K/AKT pathway, forming a positive feedback loop. In addition to miRNAs, TIMP1, a protein found in USC-EVs, can also activate this pathway [78].

4.2.2. Other EVs

The crosstalk between the NF-κB and PI3K/AKT signaling pathways may contribute to vascular regeneration in bone injury regions. Chen et al. found that *L. animalis*-EVs significantly increased vascular volume and reduced apoptosis in a mouse model of femoral head necrosis. Further investigation revealed that *L. animalis*-EVs are rich in proteins associated with the Nucleotide Oligomerization Domain(NOD)-like receptor signaling pathway, which can activate NF-κB, promote vascular repair, and upregulate the downstream signaling molecule PDGF-BB [121]. PDGF-BB, in turn, is also capable of activating the PI3K signaling pathway [119]. Additionally, functional proteins carried by *L. animalis*-EVs can directly reach the necrotic femoral head, alleviating vascular damage and promoting angiogenesis. Apart from being found in exosomes, PDGF-BB is also present in certain apoptotic bodies. Ma et al. revealed the differential effects of apoptotic bodies (ABs) derived from osteoclasts at various differentiation stages [119]. ABs from pre-osteoclasts (pOCs), which are rich in PDGF-BB, induce endothelial progenitor cells to differentiate into mature endothelial cells, enhancing nutrient supply at bone injury sites. In contrast, ABs from mature osteoclasts (mOCs) promote osteoblastogenesis via the RANKL reverse signaling pathway [119]. In the classical RANKL forward signaling pathway, RANKL secreted by osteoblasts binds to RANK on osteoclasts, stimulating osteoclastogenesis. Conversely, in the reverse signaling pathway, ABs containing RANK on osteoclasts interact with RANKL on osteoblasts, triggering osteoblast activation [122].

The Sonic Hedgehog (Shh) signaling pathway is another key pathway in angiogenesis. Exosomes secreted by adipose-derived stem cells contain miR-378, which suppresses the expression of Sufu, an inhibitor of the Shh pathway. This suppression subsequently promotes the production of VEGF and angiopoietin-1 (ANG-1), enhancing angiogenesis [109]. These findings suggest that EVs do not act on a single pathway to promote vascular repair but rather orchestrate multiple signaling molecules that activate one another, collectively driving vascular regeneration.

4.3. Anti-inflammatory effects

Necrotic bone tissue in ONFH attracts macrophages to the necrotic area, where local pro-inflammatory factors such as TNF-α exacerbate inflammation and recruit more macrophages to the injury site, polarizing them toward the pro-inflammatory M1 phenotype [123]. However, prolonged stimulation by necrotic tissue can lead to excessive inflammation and exacerbate damage. Therefore, timely promotion of M1-to-M2 macrophage polarization can effectively reduce osteocyte apoptosis [124].

Exosomes derived from chondrocytes containing miR-214-3p not only promote angiogenesis, but also facilitate M2 macrophage polarization via the activating transcription factor 7/Toll-Like Receptor 4 (ATF7/TLR4) axis, enhancing the production of anti-inflammatory mediators such as arginase-1 (Arg-1), interleukin-10 (IL-10), and

interleukin-13 [86]. Chen et al. stimulated BMSCs with lithium ions, enhancing the ability of their exosomes to promote M2 macrophage polarization [97]. Ultimately, M2 macrophages can also produce exosomes, regulating the M1/M2 macrophage ratio and forming a positive feedback loop that suppresses the expression of pro-inflammatory factors such as TNF-α and IL-6 [84]. This promotes osteogenesis and angiogenesis while reducing osteoclast formation, effectively alleviating bone necrosis in rats [84].

5. The role of EVs in diagnosing ONFH

In cases of ONFH, many patients experience hip discomfort at Association Research Circulation Osseous (ARCO) stage II or later, at which point conservative treatment is typically ineffective [6], necessitating joint replacement surgery. This underscores the urgent need for effective diagnostic tools to enable early detection, thereby preventing or delaying invasive procedures. Traditional imaging modalities such as radiography and computed tomography (CT) exhibit low sensitivity for early diagnosis [125],whereas magnetic resonance imaging (MRI) remains the gold standard for ONFH detection due to its near-perfect sensitivity (~100 %) and high specificity (96 %) in identifying early-stage bone marrow edema and subchondral fractures [126,127]. However, its application may be challenging in specific clinical scenarios. For instance, patients with Garden IV femoral neck fractures have a high risk (up to 44.4 %) of developing ONFH following internal fixation [128]. The presence of metallic implants, such as plates or screws used in femoral neck fracture surgery, induces local magnetic field inhomogeneities, leading to resonance frequency shifts and subsequent signal loss [129]. In internal fixation procedures, cannulated screws are inserted from the inferolateral femur into the central femoral head, where the resulting metal artifacts may obscure diagnostic features, increasing the risk of missed ONFH diagnoses. Furthermore, long-term MRI screening in high-risk populations faces practical challenges, including cost disparities in resource-limited settings and poor patient adherence to frequent imaging examinations [130,131]. These limitations highlight the need for adjunctive biomarkers. EVs, which encapsulate proteins, lipids, RNA, and DNA, accurately reflect the physiological and pathological states of their parent cells. Moreover, their protective lipid bilayer shields these molecular contents from enzymatic degradation in circulation, thereby extending their half-life and enhancing stability [132]. EVs can be isolated from biofluids such as blood, saliva, and urine, offering a minimally invasive alternative to tissue biopsy [133]. To date, EVs have been employed in the diagnosis of cancer, cardiovascular diseases, autoimmune disorders, diabetes, and Alzheimer's disease [134]. Increasing evidence suggests that EVs may serve as a non-radiative, implant-independent tool for dynamic monitoring and early risk stratification of ONFH [135].

Initially, research primarily focused on the correlation between EV levels and the occurrence of ONFH. Wu et al. first observed in a rabbit model that the serum levels of endothelial-derived microparticles (EMPs) and platelet-derived microparticles (PMPs) in the ONFH group were nearly 2- to 5-fold higher than those in the healthy controls [68]. Subsequently, Marsh et al. reported that plasma MPs levels in patients with sickle cell disease (SCD)-associated ONFH were 2.3 times higher than in non-ONFH SCD patients and 2.5 times higher than in healthy controls [136]. Consistent with the findings of Wu [68] and Kang [69], ONFH exhibited a strong association with elevated MP levels in circulation, which were linked to hypercoagulability, microthrombosis, and inflammation [68]. Conversely, compared with healthy individuals, ONFH patients demonstrated reduced serum exosome levels, with moderate diagnostic accuracy. However, no significant differences in serum exosome levels were observed across ARCO stages, suggesting that exosome levels may not predict disease severity [137]. With advancements in exosome isolation and purification techniques and a deeper understanding of EVs, research shifted towards the diagnostic potential of EV cargo, as alterations in their content may precede clinical manifestations [138]. Using chromatography and enzyme-linked immunosorbent assay (ELISA), Sung et al. identified significant upregulation of proteoglycan 4 (PRG4) and von Willebrand factor (vWF) in serum EVs from ONFH patients compared with healthy controls. While PRG4 exhibited limited sensitivity, elevated vWF reliably reflected hypercoagulability and endothelial dysfunction, which were exacerbated by glucocorticoid use [138]. Zhang et al. performed comprehensive sequencing of serum exosomal miRNAs in healthy individuals, systemic lupus erythematosus (SLE) patients, and SLE-associated ONFH patients [49]. They found that miR-135b-5p was elevated in SLE-ONFH patients compared with healthy controls, and quantitative real-time PCR (qRT-PCR) further confirmed its significantly higher expression in the SLE-ONFH group compared with both the healthy and SLE groups. However, no significant difference was observed between the SLE and healthy control groups [49]. The diagnostic specificity of miR-135b-5p is limited, as it is also upregulated in colorectal cancer [139] and pancreatic cancer [140], thereby diminishing its utility for ONFH diagnosis. Chen et al. demonstrated that urinary exosomal miR-200b-3p and miR-206 were upregulated in ONFH patients compared with those with hip osteoarthritis and healthy controls. These miRNAs were associated with angiogenesis inhibition and reduced osteoblast activity, exhibiting high diagnostic accuracy, with area under the curve (AUC) values of 0.938 and 0.926, respectively [141]. Additionally, Yang et al. conducted metabolomic profiling of urinary exosomes in ONFH patients, revealing significant deviations in amino acid and lipid metabolism compared with healthy individuals [142]. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated dysregulation of Wnt, MAPK, PI3K, and Hippo signaling pathways in ONFH samples [49,141]. These findings highlight endothelial and osteoblast-related abnormalities, suggesting that further investigation of these signaling pathways may aid in identifying effective biomarkers for ONFH diagnosis.

Despite these advances, several challenges remain, including the absence of long-term dynamic monitoring based on EV biomarkers, limited specificity, and substantial inter-individual variability (Table 1).

6. Conclusion and future perspective

The discovery of EVs dates back to 1946, when American biochemist Erwin Chargaff and physician Randolph West, while investigating coagulation factors, identified a highly procoagulant component in the sediment of centrifuged blood. They hypothesized that this component originated from fragmented blood cells. It was not until 1967 that British scientist Peter Wolf captured the first electron microscopy image of platelet-derived "cellular fragments," providing direct visual evidence of their existence [143]. In 1983, Canadian researcher Rose M. Johnstone formally introduced the concept of the "exosome," and in 1996, Raposo et al. demonstrated that EVs possess biological functions beyond being mere cellular metabolic waste [144]. This groundbreaking discovery fundamentally reshaped the perception of EVs and ignited a wave of intense scientific exploration.

A growing body of literature has documented the role of EVs in orthopedic research. EV-derived miRNAs and proteins regulate key signaling pathways crucial for bone homeostasis and vascular integrity, including AKT [77], Wnt [83], and MAPK [85]. These molecules modulate the expression of osteogenesis-related genes such as VEGF, RUNX2, and COL-I, thereby influencing osteoblast proliferation and differentiation. Additionally, EVs can control apoptosis in bone and vascular endothelial cells by affecting the expression of apoptosis-associated proteins, including Bax and Caspase-9. The pathogenicity of EVs largely stems from GCs altering their parental cells or from EVs originating directly from pathological tissues, as both sources exhibit significant changes in cargo composition compared to normal EVs. For instance, EVs secreted by M1 macrophages or adipocytes following GC treatment show markedly increased levels of miR-1a-3p [17]and miR-148a [48], which promote adipogenic differentiation of BMSCs, exacerbate lipid accumulation, and suppress osteogenesis.

Table 1
The diagnostic value and limitations of EVs from different sources.

Isolation method	Analytical approaches	EVs source	Results	Limitations	Reference
UC	NTA	Human Serum 10 SCD 10 SCD-ONFH 10 Control	Level of MPs∝abnormal blood conditions.	Significant inter-individual variability	[136]
UC	Flow Cytometry	Rabbit Serum 18 ONFH 9 Control			[68]#
/	NTA	Human Serum 85 ONFH 115 Control	1.Exos ↓ 2.Levels of exosomes ⊗ the staging of ONFH.	1.Limited specificity 2.Moderate diagnostic efficacy	[137]
Exo-Quick	NTA,Flow Cytometry	Human Serum 11 ONFH, 11 Control	VWF and PRG4 represent novel biomarkers	Significant inter-individual variability	[138]
Exo-Quick	NTA,Flow Cytometry	Human Serum 22 SLE, 27 SLE-ONFH 17 Control	Exo-miR-135b ↑	Significant inter-individual variability	[49]
Exo-Quick	NTA,Flow Cytometry	Human Urine 9 ONFH 9 HOA	Exo-miR-200b ↑ Exo-miR-206 ↑	The specificity of miR has not been studied.	[141]

The symbol "∝" means proportional, while the symbol "⊗ means unrelated. The hash "#" denotes animal studies; all others are clinical research findings. UC, Ultraspeed centrifugation. NTA, Nanoparticle Tracking Analysis. HOA, Hip Osteoarthritis. SCD, Sickle Cell Disease.

Similarly, exosomes derived from necrotic bone tissue exhibit reduced CD41 expression [16], while BMSC-derived EVs show significant downregulation of miR-182-5p [14] and miR-133b-3p [74]. These aberrant expression patterns contribute to the inactivation of Wnt and Akt signaling pathways, disrupting the osteoblast-osteoclast balance and vascular homeostasis.

Conversely, therapeutic EVs have been shown to upregulate these pathways. For example, hucMSC-derived EVs enriched in miR-21 activate the PI3K/Akt pathway, enhancing BCL-2 expression and increasing cellular resistance to apoptosis [79]. To achieve the goal of "one vesicle, multiple effects," researchers have introduced engineered EVs, broadly categorized into two strategies [87]. The first approach, cargo loading, involves introducing bioactive molecules into parental cells via gene transduction, electroporation, or ion stimulation to induce the secretion of EVs enriched with therapeutic agents. Alternatively, these molecules can be directly incorporated into EVs. Examples include the transfection of HIF-1α [100], tsRNA-10277 [94], VEGF [85], and miRNAs [83] into EVs, effectively counteracting the detrimental effects of GCs on bone. The second strategy involves modifying EVs through surface engineering, scaffold technologies, or synthetic EV-mimicking constructs. For instance, Jiang [105] developed engineered vesicle mimetics (EVMs) using osteogenesis-promoting metal ions and further enhanced their bone-targeting capabilities via surface modification with ALN. Similarly, Chen encapsulated EVs in hydrogels, improving the stability of therapeutic cargo release and prolonging treatment duration [97]. Despite these advancements, the mechanisms underlying EV-based therapy for osteonecrosis remain incompletely understood [111]. Moreover, the loading efficiency of exogenous molecules (e.g., miRNAs) remains unstable, and the long-term safety of EVs, including potential tumorigenicity, has yet to be fully clarified [88]. These challenges continue to hinder the translation of EV-based therapeutic strategies from laboratory research to clinical application.

In terms of diagnosis, EVs can assist in ONFH detection through two approaches. First, as previously mentioned, they can serve as biomarkers, a field currently under active investigation. MRI, the gold standard imaging modality for ONFH, can detect localized bone marrow edema even at the early stage when structural changes in the femoral head remain subtle. This indicates reduced blood flow and osteocyte damage—key early signals of ONFH [145]. However, MRI imaging can be compromised by artifacts from certain implants [129], and in the earliest stages of ONFH, no imaging modality may reveal positive findings despite the presence of necrotic tissue [146]. The cargo of EVs, including proteins such as vWF [138] and various miRNAs, accurately reflects the pathophysiological state of cells [137]. In such cases, monitoring EVs released from necrotic tissue as biomarkers may complement MRI in diagnosing osteonecrosis. The diagnostic utility of biomarkers depends on two key factors: sensitivity and specificity. High sensitivity is crucial, particularly for detecting ONFH at its earliest stage. However, most existing studies have included ARCO stage III or IV patients, and there is limited research on whether these biomarkers exhibit detectable changes during the subclinical phase, when imaging techniques remain inadequate. Specificity is another critical factor; for instance, although miR-200b-3p and miR-206 exhibit high sensitivity for ONFH diagnosis, their levels are also elevated in muscular dystrophy [147], pulmonary hypertension [148], and amyotrophic lateral sclerosis [149], potentially confounding ONFH diagnosis in patients with these conditions. Moreover, the considerable interindividual variability in EV expression underscores the need for long-term monitoring of EVs extracted from circulation as biomarkers, focusing on dynamic changes rather than relying solely on absolute EV counts to determine the presence of osteonecrosis. EVs derived from the femoral head constitute only a minute fraction of circulating EVs, and substantial protein contamination during centrifugation further complicates their isolation, making it extremely challenging to obtain high-purity EVs suitable for study [150]. Given the limitations of the "extract-and-detect" approach (using EVs as biomarkers), we may pivot toward an "inject-and-illuminate" strategy (utilizing EVs as contrast agents).

In animal models, contrast agents have been shown to enhance the sensitivity of MRI in detecting early-stage ONFH [151]. During ONFH progression, the capillary density and blood flow in the femoral head decrease. Conventional contrast agents, such as gadolinium-based agents, are water-soluble and relatively large in molecular weight (typically 500–1000 Da) [152], which limits their ability to freely penetrate capillaries and small blood vessels. This drawback prevents gadolinium-based agents from extravasating into the interstitial space beyond the vasculature, restricting their capacity to reflect the pathological state of necrotic tissue and cells [153]. However, Liao et al. successfully leveraged this characteristic to assess blood supply changes

in necrotic regions. Due to their small size and high biocompatibility, EVs can serve as carriers for contrast agents, not only penetrating blood vessels but also crossing various biological barriers [152]. For instance, Zhang et al. utilized EVs to deliver superparamagnetic nanoparticles across the blood–brain barrier for glioma and Parkinson's disease imaging [154]. Furthermore, an external magnetic field can be used to guide EVs carrying magnetic contrast agents precisely to the lesion site for imaging [155]. Currently, EV-based contrast agent delivery is primarily applied in tumor imaging, where abundant blood supply and large endothelial gaps facilitate contrast agent penetration [156]. However, ONFH is caused by thrombotic microvascular occlusion, leading to insufficient blood supply to the femoral head and creating an ischemic-hypoxic environment in necrotic bone and cartilage—fundamentally different from the tumor microenvironment. How can EVs accumulate in such low-blood-flow regions to enable imaging? Kim [157] and Mentkowski et al. [158] successfully enhanced EV targeting to ischemic myocardial tissue by modifying EV surfaces with cardiac-targeting peptides, significantly increasing EV accumulation in ischemic areas. Although certain natural EVs possess intrinsic bone-targeting properties, their targeting efficiency remains relatively weak, and they are prone to metabolic clearance [159]. Engineered EVs with bone or cartilage-targeting properties—such as cartilage-targeting EVs constructed by conjugating cartilage-affinity peptides with vesicle-associated proteins—can migrate into dense cartilage matrices even in the absence of blood supply [160]. Additionally, the inflammatory microenvironment of ONFH increases vascular permeability [161], further facilitating the extravasation of EV-based contrast agents. Moreover, as previously discussed, the cargo of EVs—including RNAs and proteins—exerts therapeutic effects in ONFH. Beyond delivering contrast agents, EVs can be engineered to carry therapeutic molecules, granting them dual diagnostic and therapeutic functionality. This dual-purpose approach may pave the way for novel strategies in ONFH diagnosis and treatment.

Unfortunately, research on the use of EVs as contrast agents for ONFH diagnosis remains scarce, and their role as biomarkers has yet to be fully explored. Several factors contribute to this limitation. First, the extraction and purification of EVs remain challenging, with high production costs, and ultracentrifugation is still the primary method for isolating EVs for ONFH diagnosis [14,15,136,137],which limits the purity of the extracted EVs. Additionally, EVs carrying contrast agents may accumulate in the body over time [162], and their long-term safety and efficacy in humans have yet to be established. Moreover, the potential immunogenic effects of engineered EVs remain unknown [163]. Nevertheless, we believe that with continuous advancements in EV isolation, purification, and detection technologies, EVs hold tremendous potential not only as biomarkers but also as carriers for MRI contrast agents.

CRediT authorship contribution statement

Hongxu Li: Investigation, Writing – original draft, Visualization, Writing – review & editing. **Haoyang Liu:** Writing – review & editing. **Yu Zhou:** Formal analysis. **Liming Cheng:** Supervision, Formal analysis. **Bailiang Wang:** Supervision, Funding acquisition, Resources. **Jinhui Ma:** Conceptualization, Project administration, Funding acquisition, Formal analysis.

Ethics approval and consent to participate

This article does not contain any studies with human or animal subjects.

Conflicts of interest

A conflict of interest occurs when an individual's objectivity is potentially compromised by a desire for financial gain, prominence, professional advancement or a successful outcome. The Editors of the *Journal of Orthopaedic Translation* strive to ensure that what is published in the Journal is as balanced, objective and evidence-based as possible. Since it can be difficult to distinguish between an actual conflict of interest and a perceived conflict of interest, the Journal requires authors to disclose all and any potential conflicts of interest.

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Appendix A. Supplementary data

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Glossary

Akt: Protein Kinase B
ALP: Alkaline Phosphatase
ARCO: Association Research Circulation Osseous
Arg-1: Arginase-1
ASCs: Adipose-Derived Stem Cells
AUC: Area Under the Curve
Bad: BCL2-Associated Agonist of Cell Death
Bax: BCL2-Associated X Protein
BCL-2: B-Cell Lymphoma 2
BMP: Bone Morphogenetic Protein
BMPR2: Bone Morphogenetic Protein Receptor Type 2
BMSCs: Bone Marrow Mesenchymal Stem Cells
CD: Cluster of Differentiation **C/EBPz:** CCAAT/Enhancer-Binding Protein Zeta
CoA: Coenzyme A
Collagen I: Type I Collagen
CREB1: cAMP Response Element-Binding Protein 1
CT: Computed Tomography
DMBT1: Deleted in Malignant Brain Tumors 1
ELISA: Enzyme-Linked Immunosorbent Assay
EMPs: Endothelial-Derived Microparticles
ERK: Extracellular Signal-Regulated Kinase
EVs: Extracellular Vesicles
FAK: Focal Adhesion Kinase
FIP200: FAK Family Interacting Protein of 200 kDa
FoxO1: Forkhead Box O1
FSTL1: Follistatin-Like 1
GCs: Glucocorticoids
Gli1: Glioma-Associated Oncogene Homolog 1
GSK-3β: Glycogen Synthase Kinase-3 Beta
H&E: Hematoxylin and Eosin
Hh: Hedgehog
HIF-1α: Hypoxia-Inducible Factor-1 Alpha
HOA: Hip Osteoarthritis
HUVECs: Human Umbilical Vein Endothelial Cells
ICAM-1: Intercellular Adhesion Molecule-1
IL: Interleukin
iPS-MSCs: Induced Pluripotent Stem Cell-Derived Mesenchymal Stem Cells
LCPD: Legg-Calvé-Perthes Disease
MAPK: Mitogen-Activated Protein Kinase
miRNA/miR: MicroRNA
MLCK: Myosin Light Chain Kinase
MRI: Magnetic Resonance Imaging
MSCs: Mesenchymal Stem Cells
MVBs: Multivesicular Bodies
MVs: Microvesicles
MYD88: Myeloid Differentiation Primary Response Protein 88
NCOA3: Nuclear Receptor Coactivator 3
NF-κB: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
ONFH: Osteonecrosis of the Femoral Head
PAI-1: Plasminogen Activator Inhibitor-1
PARP: Poly (ADP-Ribose) Polymerase
PDCD4: Programmed Cell Death 4
PDGF-BB: Platelet-Derived Growth Factor-BB
PI3K: Phosphatidylinositol 3-Kinase
PRG4: Proteoglycan 4
PRP: Platelet-Rich Plasma
PTEN: Phosphatase and Tensin Homolog
RANK: Receptor Activator of Nuclear Factor Kappa-B
RANKL: Receptor Activator of Nuclear Factor Kappa-B Ligand
RNA: Ribonucleic Acid
ROS: Reactive Oxygen Species
RUNX2: Runt-Related Transcription Factor 2
SCD: Sickle Cell Disease
siRNA: Small Interfering RNA
SMAD: Mothers Against Decapentaplegic Homolog
SMURF1: SMAD Ubiquitination Regulatory Factor 1
SLE: Systemic Lupus Erythematosus
SONFH: Steroid-Induced Osteonecrosis of the Femoral Head
SOX: SRY-Box Transcription Factor (e.g., SOX5, SOX9)
SPRY2: Sprouty RTK Signaling Antagonist 2
TGF-β: Transforming Growth Factor-Beta

TIMP1: Tissue Inhibitor of Metalloproteinases 1
TLR4: Toll-Like Receptor 4
TNF-α: Tumor Necrosis Factor-Alpha
TRAP+: Tartrate-Resistant Acid Phosphatase-Positive
tsRNA: tRNA-Derived Small RNA
UC: Ultracentrifugation
UPLC-MS/MS: Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry
USCs: Urine-Derived Stem Cells
VEGF: Vascular Endothelial Growth Factor
vWF: von Willebrand Factor
Wnt: Wingless/Integrated Signaling Pathway