

Blood-derived exosomes: A novel cell-free approach in bone healing

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ABSTRACT

Current treatments for bone regeneration have shown limited efficacy, underscoring the need to explore new therapeutic methods. Among treatment options, autologous and allogeneic bone grafts, though standard, are sometimes associated with complications such as donor site morbidity, immune rejection, infection, and the need for secondary surgeries. In general, there are safety concerns regarding cell-based therapies, as mentioned earlier. Cell-free therapies, particularly exosome-based ones, have emerged as a promising strategy in regenerative medicine. Exosomes are nanosized extracellular vesicles (30–150 nm) that contain various molecules such as proteins, lipids, and RNAs that can be delivered to recipient cells or can be used in the design of molecule-delivery vehicles. By transferring molecules, exosomes can regulate proliferation, migration, differentiation, and immune responses. While exosomes can be derived from various tissues, blood and its components (plasma, serum, and blood cells) are an attractive source due to their accessibility, abundance, and translational potential. Preclinical studies demonstrate that red blood cell-, platelet-, and immune cell-derived exosomes can promote angiogenesis, osteogenesis, and immunomodulation, collectively contributing to bone repair *in vitro* and *in vivo*. This review highlights recent advances in the therapeutic application of blood-derived exosomes in bone regeneration, discusses their underlying mechanisms of action, and outlines current challenges such as large-scale production, heterogeneity, and safety evaluation. Finally, these insights may accelerate the clinical translation of blood-derived exosomes as a novel and effective therapy for bone regeneration.

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Introduction

Bone damage can lead to disability, causing pain and discomfort for many patients, with infections, trauma, and systemic diseases being major contributing factors (1). The “gold standard” for clinical treatment of these defects remains the transplantation of autologous, allogeneic, or synthetic bone grafts. However, this approach has drawbacks, including limited donor availability, immune rejection, infections, and poor integration with the bone, which can result in secondary surgeries caused by uneven scar formation, delayed healing, or even failure of the bone to unite (2). In this context, some researchers have suggested utilizing exosomes for cell-free therapy (3). Exosomes are extracellular vesicles (EV) secreted from cells. They have a diameter of almost 30–150 nm (4) and contain various types of components obtained from source cells, such as proteins, miRNAs, RNA, and DNA, which play a significant role in cell-to-cell interaction (5, 6). Exosomes are found in many biological fluids, including amniotic fluid, cerebrospinal fluid, breast milk, and serum, and they are common in a variety of cell types, including immune cells, platelets, and stem cells (7).

Many studies focus on the therapeutic application of exosomes isolated from cell culture media; however, this use is limited by the complexity of primary cell isolation and culture, insufficient production, and the challenge of ensuring functional stability and biosafety (8). Allogeneic cell exosomes have immunogenic properties, and isolating sufficient quantities of exosomes from autologous cell sources is costly, time-consuming, and necessitates a highly advanced GMP-level laboratory setup (9). In osteogenesis, bone marrow mesenchymal stem cells (BMSCs)-derived exosomes (BMSC-Exos) are known for enhancing BMSCs' osteogenic functions, including proliferation, migration, and differentiation. However, the clinical application of BMSC-Exos is obstructed by challenges such as donor scarcity and the difficulty of mass production (10). On the other hand, autologous exosomes can be accessed more conveniently and safely via plasma. Additionally, it is simpler to manage their production yield (8). Since blood is a safe and abundant source of exosomes, the widespread use of blood in the blood transfusion process indicates the high potential of allogeneic blood exosomes for use in medical therapies (11). Blood exosomes are a new source

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of biomarkers for disease diagnosis and prognosis and are crucial for cell-to-cell communication (12).

Despite significant advances in exosome-related research (3, 13-16), existing reviews have primarily focused on exosomes derived from stem cells and immune cells and have not comprehensively examined blood-derived exosomes as a distinct group with unique capabilities in bone regeneration. In contrast, reports on platelets, plasma, or serum have been mostly anecdotal and sporadic and have not yet been reviewed in a coherent analytical framework. Therefore, a review of blood-derived exosomes and their role in bone repair addresses an important gap in the literature and may provide new insights for the development of translational therapeutic strategies.

The terminology surrounding exosomes and EVs has evolved over time. Since 2004, "exosomes" has been the most commonly used term in published articles describing EVs. However, the International Society for Extracellular Vesicles (ISEV) recommends using "EVs" until a consensus on exosome nomenclature is reached, as outlined in MISEV2014 and MISEV2018 guidelines. These guidelines note that classifying EVs, including exosomes, remains challenging due to overlapping biophysical properties and the need for detailed reporting of their biogenesis and functional mechanisms (3, 17, 18). Therefore, for the benefit of readers, this review includes some classical research with "EV" as the key term in addition to compiling and evaluating articles that specifically use the term "exosome" as a keyword.

Here, we focus on the role of blood-derived exosomes in bone regeneration, emphasizing their contributions to cellular communication and therapeutic application. We will explore exosomes from plasma/serum, RBCs, platelets, and immune cells, highlighting their unique roles in promoting osteogenesis and modulating inflammation during bone healing.

Exosomes: Definition, characterization, and their significance in bone regeneration

Exosomes were first discovered in the mid-1980s by R.M. Johnstone and colleagues. During the maturation of mammalian reticulocytes, they observed that the transferrin receptor and other membrane-associated components are selectively released in circulating vesicles derived from multivesicular bodies (MVBs), which were subsequently named exosomes (19-21). Exosomes are tiny, membrane-bound, secreted organelles that are made up of proteins, lipids, and nucleic acids. Exosomes can be selectively absorbed by distant cells or transported to distant organs by bodily fluids. Neighboring cells can also absorb secreted exosomes. Bioactive compounds found in exosome cargo have the ability to modify signaling pathways in recipient cells, thereby reprogramming them (22). Exosomes are naturally occurring biological nanovesicles secreted by various normal cells, including macrophages, mesenchymal stem cells (MSCs), natural killer cells, and red blood cells (RBCs), as well as tumor cells (23). These acellular, non-replicative, nanosized EVs facilitate intercellular communication by delivering cargo such as growth factors (GFs), signaling lipids, and genetic material, all of which are safeguarded by their lipid bilayer (24). The exocytosis of MVBs is the foundation of exosome biogenesis. There are three phases to this procedure. The creation of endocytic vesicles from plasma membrane pits is the initial stage.

MVBs are created in the second stage when endosomal membranes bud inward. The cargo of MVBs is loaded at this point. The lysosome may then break down these MVBs during the third stage, or they may fuse with the cell membrane and be released as exosomes (25). Exosomes possess several beneficial characteristics for therapeutic delivery. Their nanoscale size allows them to infiltrate tissues deeply, while a mildly negative surface charge (zeta potential) promotes stability and extended blood circulation time. Furthermore, their natural composition helps them avoid immune detection and rapid clearance (23). From a biochemical view, exosomes are complex vesicles, and their membranes are rich in specific proteins involved in transport and fusion, tetraspanins (like CD9, CD63, CD81), and markers related to their endosomal origin, such as Alix and LAMP2. The structural core of exosomes also includes a lipid bilayer composed of cholesterol, sphingolipids, and phosphoglycerides. This molecular cargo is the medium through which exosomes facilitate intercellular signaling (26). A variety of methodologies are employed for the isolation of exosomes, with differential centrifugation widely regarded as the gold standard. Additional techniques include size exclusion, immunoaffinity isolation, polymeric precipitation, and microfluidic device-based approaches (27).

Due to their high biocompatibility, long-term stability, and low immunogenicity, EVs have attracted much attention and have been widely studied and used, especially in the field of bone regeneration (28). Exosomes play an important role in intercellular communication, including between different organs, in bone biology. These small vesicles carry a variety of molecular materials and play a role in bone remodeling, repair, and maintaining bone tissue balance. Exosomes are also involved in the communication of bone with tissues such as muscle, adipose tissue, gut microbiota, immune system, nervous system, and blood vessels. Disruption of these pathways can lead to diseases such as osteoporosis, osteoarthritis, femoral head necrosis, and bone metastasis (29). Exosomes also offer new insights into how heterogeneous cells communicate during bone remodeling. In healthy bone, these vesicles transmit messages that help both bone formation and bone breakdown. Exosomes also function by regulating the differentiation, recruitment, and further activity of cells involved in bone remodeling, and even play an active role in the mineralization of the extracellular matrix (27).

For example, Li *et al.* demonstrated that adipose-derived stem cell-derived exosomes (ADSC-Exos) can efficiently control bone immune metabolism and enhance bone healing, in part by immunely regulating miR-451a. This could offer a treatment avenue for bone restoration (30). Lin *et al.* found that exosomes derived from human umbilical vein endothelial cells (HUVECs-Exos) overexpressing PD-L1 promote osteogenic differentiation and enhance fracture healing by binding to PD-1 on T cell surfaces, thereby suppressing T cell activation as an immunosuppressive mechanism (31). Furthermore, Takeuchi *et al.* reported that exosomes from conditioned media of BMSCs promote bone regeneration by enhancing angiogenesis (32).

Collectively, these findings highlight the therapeutic potential of EVs in bone tissue engineering through diverse biological pathways. By integrating immunomodulatory functions and regenerative processes such as osteogenesis and angiogenesis, exosomes act as pivotal signaling hubs that orchestrate the complex interactions required for

effective bone regeneration.

Methods

A structured search was performed from database inception to June 29, 2026, in PubMed, Scopus, and Web of Science. The systematic search was based on three core concepts: (1) extracellular vesicles/exosomes; (2) blood derived sources (plasma, serum, whole blood); and (3) bone regeneration/osteogenesis. Keywords and MeSH terms included: "Extracellular Vesicles", "exosomes", "blood-derived exosomes", "plasma-derived exosomes", "serum-derived exosomes", "macrophage-derived exosome", "neutrophil-derived exosome", "Bone Regeneration", "Fracture Healing", osteogenesis, osteogenic differentiation, and bone defect.

Inclusion criteria: (1) preclinical or clinical studies evaluating blood derived extracellular vesicles or exosomes in osteogenesis, bone regeneration, bone defect repair (including calvarial bone), and senescence of BMSC.

Exclusion criteria: (a) nonEnglish publications; (b) grey literature; (c) studies focused on periodontal bone regeneration; (d) studies using stemcell-derived exosomes or cellculture-derived exosomes unless obtained from blood components; (e) studies evaluating periodontal defect models.

Therapeutic potential of plasma/serum-derived exosomes in bone regeneration

All body fluids and tissues are potential sources for EVs, but blood provides the richest one for EVs (33). Blood plasma, a light amber liquid component of blood, is devoid of blood cells but has a suspension of proteins and other components of whole blood. It accounts for roughly 55% of the body's blood volume (34).

The therapeutic use of exosomes isolated from cell culture media is the subject of numerous studies; however, this application is constrained by the challenging process of isolating and cultivating primary cells, the insufficient production, and the challenge of managing functional stability and biosafety. On the other hand, exosomes that are isolated from plasma are easier to obtain and safer. Controlling their production yield is also simpler (35).

The potential of plasma-derived exosomes (P-Exos) in diagnosis, treatment, and monitoring of various diseases such as colon cancer (36), neurodegenerative (37, 38), rheumatologic (39, 40), and even psychological diseases (41, 42) has been widely investigated.

Similar to plasma, serum is also a blood supernatant, but it is obtained by different methods. These differences in collection lead to distinct profiles of proteins and nucleic acids between plasma and serum (43). It has been reported that serum-derived EVs (S-Exos) generally contain additional EVs released from platelets. This may qualitatively and quantitatively alter EV profiles when serum is used as a source of blood EVs (43).

In the context of bone tissue engineering, exosomes have demonstrated remarkable osteogenic potential. For instance, a combination of porous zinc scaffolds with rabbit blood serum Exos (BF-Exos) and Poloxamer 407 hydrogels was investigated by B. Zhang and colleagues. Loading BF-Exos enhances the zinc scaffold's ability to promote osteogenic cell activity and suppress osteoclastic activity. It has been suggested that the presence of BF-Exos may reduce the number of M1 macrophages and promote vascular and bone formation, despite the prolonged M1 macrophage polarization with exogenous bone grafts. Further, loading

with highly bioactive serum exosomes was found to further promote the expression of p38 while inhibiting the expression of STAT1 (44).

The functional efficacy of P-Exos is also profoundly influenced by the physiological age of the donor, a phenomenon closely linked to cellular senescence. Research indicates that exosomes derived from young subjects (Y-Exos) confer superior osteogenic capacity on BMSCs compared with those from aged subjects (A-Exos). In an osteoporotic *in vivo* model, Y-Exos effectively alleviated osteoporosis by increasing bone metabolism, while A-Exos hindered bone regeneration. Notably, exosomal miR-217-5p was identified as a key factor in the osteoprotective effects of Y-Exos (10). In another study, the decreased osteogenic differentiation of BMSCs after fatigue loading in aged osteoporotic rats was successfully restored by S-Exos from young rats. These exosomes were isolated via a modified ExoQuick method and characterized as saucer-like structures with a diameter of approximately 71.35–100 nm, expressing typical markers such as CD9, CD81, and TSG101. Confocal fluorescence imaging confirmed the internalization of these exosomes by BMSCs, showing numerous green fluorescent particles localized around the nuclei. These exosomes significantly enhanced mineralization (Alizarin Red) and osteogenic markers (OCN, Collagen I, Runx2) (Figure 1). miRNA profiling identified miRNA-19b-3p as highly abundant in young exosomes. Functional assays demonstrated that miRNA-19b-3p mimics promoted osteogenesis by suppressing P-11N (45).

Building upon these mechanistic insights, the profiling of exosomal miRNAs in plasma has emerged as a transformative approach for clinical translation. Exosomal miRNAs play an important role in regulating bone homeostasis, and their detection in plasma is a promising approach for clinical applications. In particular, increased expression of miR-106b-5p and miR-181a-5p in patients with osteoporosis indicates impaired bone remodeling processes (46, 47). As liquid biopsy markers, these miRNAs could bridge the gap between molecular pathology and clinical diagnosis, providing a highly sensitive tool for detecting skeletal deterioration, even before a noticeable decrease in bone mass occurs.

Exosomes derived from blood cells: Mechanisms and applications in bone regeneration

Red blood cell-derived exosomes

Red blood cells (RBCs) are flat, biconcave, disk-shaped, anucleate cells, approximately 8 µm in diameter and 2 µm thick, and play a pivotal role in transporting oxygen from the lungs to peripheral tissues (48). As reticulocytes develop into mature erythrocytes, they undergo a selective elimination of specific proteins and membrane-associated enzymes. This is achieved through MVBs, which fuse with the plasma membrane and release their internal vesicles, called exosomes, into the external environment (49).

The biological properties and roles of RBC-derived EVs (RBCEVs) have drawn the attention of researchers in recent years. Mature red blood cells are considered biologically safe with minimal risk of causing cancer. This safety stems from their lack of a nucleus and other internal organelles, which are the primary carriers of genetic material. Consequently, when producing or studying EVs derived from these cells, there is no risk of transferring donor DNA to recipient cells. Despite having no DNA, RBC-derived EVs carry a diverse

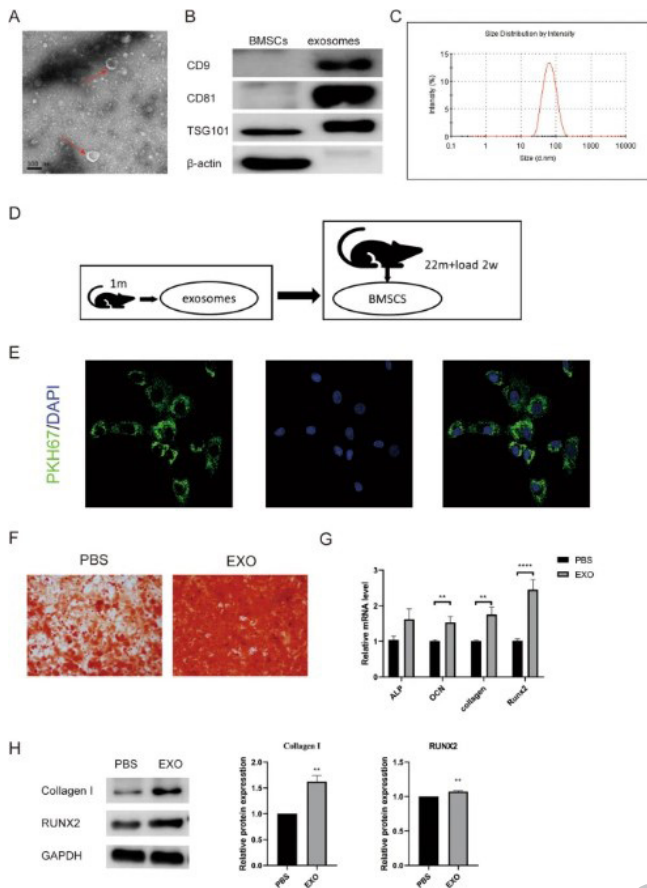


Figure 1. Young rat serum exosomes enhance BMSC osteogenesis in a model of aged osteoporosis (A) TEM image of exosomes. (B) WB for exosomal markers (CD9, CD81, TSG101). (C) DLS size analysis. (D) Schematic of BMSCs in a bone microdamaged model in rats induced by serum exosomes from young rats. (E) Confocal images of BMSCs and exosomes. (F) Alizarin Red staining for mineralization. (G) mRNA levels of osteogenic markers (ALP, OCN, Collagen I, Runx2). (H) WB analysis of Collagen I and Runx2 protein expression. (Reproduced from [45] under CC BY 4.0 license). BMSC: Bone marrow mesenchymal stem cells; TEM: Transmission electron microscopy; DLS: Dynamic light scattering; WB: Western blot; ALP: Alkaline phosphatase; OCN: Osteocalcin; Runx2: Runt-related transcription factor 2

array of proteins and mRNAs, as confirmed by proteomic and transcriptomic studies (50–53).

In both healthy and diseased states, RBCEVs containing proteins, lipids, and miRNAs may be essential for interacting with the endothelium to control immunomodulation, redox balance, and NO and O₂ homeostasis. RBCEVs also show pertinent procoagulant effects in a number of disease states and are essential to the dysregulation of hemostasis (54). RBC-derived vesicles demonstrated high biosafety for autologous MSCs, evidenced by cell viability, differentiation, and gene microarray assays.

Their utility extends to imaging, where RBCEVs have served as effective delivery vehicles for iron oxide, specifically targeting human bone marrow mesenchymal stem cells (hBMSCs) in magnetic resonance imaging (55). However, the limitations associated with mass-producing natural exosomes were circumvented by generating exosome mimetics (EMs) from RBCs. In recent years, the approach of producing EMs from RBCs has attracted attention in order to overcome the inherent limitations of natural exosomes, especially low yield, difficulty in extraction, and inability to mass-produce them. Instead of relying on the natural and limited secretion of exosomes by cells, this approach uses a

mechanical and engineered process to create particles with exosome-like properties.

In the study by Gangadaran *et al.*, RBCs were first subjected to a single-step extrusion through a 1-μm pore filter, which applied mechanical stress to the cell membrane and transformed it into smaller, more uniform vesicles. The resulting particles were then purified to remove unwanted components using density gradient centrifugation. This strategy resulted in particles with suitable physical and biological properties, very high yields, and applicability for radiolabeling with technetium-99m (^{99m}Tc). Compared with natural exosomes, this method provided approximately 130-fold higher production efficiency and, at the same time, showed more favorable biodistribution in organs rich in resident phagocytes, such as the liver and spleen (23).

Complementing these advancements in mimetic production, targeted delivery strategies utilizing RBCEVs are also emerging. Xu *et al.* explored a novel peptide-guided delivery strategy using RBCEVs to deliver anti-miR-214 to osteoclasts, a potential treatment for Osteoporosis. The bi-functional peptide, TBP-CP05, was designed to bind both to CD63 on RBCEVs and to receptors on osteoclasts, facilitating targeted delivery. By attaching the TRAP-binding peptide (TBP) to the surface of RBCEVs, the modified extracellular vesicles (OT-RBCEVs) demonstrated enhanced targeting capabilities both *in vitro* and *in vivo*. Following intravenous injection of OT-RBCEVs in osteoporotic mice, there was a notable accumulation of EVs in the bone, leading to significant inhibition of osteoclast activity, increased osteoblast activity, and improved bone density (56).

While exosomes have been extensively studied as key mediators of intercellular communication from viable cells, a growing body of evidence highlights the distinct and significant roles of apoptotic vesicles (apoVs). Studies indicate apoVs possess distinct cargo, physical characteristics, functions, and formation mechanisms compared to EVs from healthy cells. It is these unique properties that establish apoVs as a promising new class of agents for cell-free therapeutic applications (57, 58).

A pivotal study provided key evidence for this therapeutic potential, showing that apoVs from RBCs could selectively enhance osteogenesis in hBMSCs. This activity, demonstrated in a model of calvarial bone defects, was mechanistically linked to the delivery of carbonic anhydrase 1 (CA1) by the RBC-apoVs, which directly orchestrated the osteogenic differentiation of hBMSCs (53).

Platelet-derived exosomes

Platelets, small, anucleate cell fragments produced by bone marrow megakaryocytes, are critical mediators of numerous pathophysiological processes. These include thrombosis, hemostasis, inflammation, and wound healing. They carry out their functions using secretory organelles, such as alpha-granules, which store a diverse array of proteins and GFs (59).

Upon activation, platelets degranulate, releasing a diverse mixture of cytokines, chemokines, GFs, and other inflammatory substances from their alpha-granules (60). The use of platelet-rich plasma (PRP) as an autologous biological therapy in orthopedics has increased drastically in recent years. PRP is an autologous concentrate prepared from a patient's own blood, characterized by a high platelet density within a plasma base, which actively facilitates tissue repair and regenerative processes (61). The therapeutic potential

of this substance is driven by its complex composition, which encompasses over 300 bioactive compounds such as transforming growth factor (TGF), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), along with various adhesion molecules, cytokines, and inflammatory mediators (62). Furthermore, initial investigations into PRP indicate a potential regulatory role for platelets in maintaining bone metabolism and health (63). To prevent the formation of osteoclasts, PRP increases the secretion of osteoprotegerin, an inhibitor of osteoclast production (64). Animals given PRP showed marked improvements in torsional stiffness, three-point load-bearing capacity, overall bone strength, tissue differentiation rates, and bone restoration (65). Bone morphogenetic protein 2-modified MSCs and PRP's prolonged release can also greatly accelerate bone regeneration (66).

However, it is also known that platelets release proteins encapsulated in EVs called exosomes, in addition to free proteins released into the impacted environment. Exosomes have restorative effects, just like PRP (67-69).

However, the effectiveness of PRP is limited by the short-term release of GFs, variations in preparation methods, and poor targeting at the site of injury; more advanced approaches are needed (70). PRP and PRP-derived exosomes (PRP-Exos) possess distinct biological benefits for bone tissue engineering and regenerative medicine. PRP-Exos, a key active component of PRP, contain bioactive molecules that can effectively modulate cellular behavior and signaling pathways, thereby playing crucial roles in bone tissue regeneration (70, 71).

PRP-Exos have emerged as a potent cell-free therapeutic agent across various musculoskeletal injuries, demonstrating efficacy in repairing tendons, treating osteonecrosis, and accelerating bone defect healing.

Li *et al.* addressed the challenge of rapid exosomal clearance by developing a sustained-release system. By

encapsulating highly purified PRP-Exos (isolated via polymerization precipitation and ultracentrifugation) within a biocompatible fibrin gel (FG), they administered a total dose of 500 µg of PRP-Exo via *in situ* gelation at the bone-tendon interface during surgery in rabbit rotator cuff tear models. This approach promoted well-ordered tendon regeneration and complete bone healing, as evidenced by 3D Micro-CT reconstructions that showed morphology closely resembling normal bone (Figure 2) (72).

The protective potential of PRP-Exos extends to more complex pathological environments, such as glucocorticoid-induced osteonecrosis of the femoral head (ONFH). Tao *et al.* demonstrated that PRP-Exos can modulate glucocorticoid-induced damage, which typically leads to cell apoptosis through the induction of endoplasmic reticulum (ER) stress and activation of the PERK/CHOP pathway. Mechanistically, PRP-Exos enhance cell survival and proliferation by activating the Akt and Erk pathways, thereby promoting angiogenesis. Furthermore, they preserve osteogenic differentiation by modulating the Wnt/β-catenin pathway and regulating the Akt/Bad/Bcl-2/Caspase-3 axis to inhibit apoptosis under ER stress conditions (73).

PRP-Exos also act as active drivers of regeneration in skeletal stem cell populations. In the study by Zhang *et al.*, PRP-Exos isolated from rat PRP were shown to significantly enhance the functional capacity of skeletal stem cell-like (SSC-like) cells. The results of *in vitro* experiments showed that treatment of cells with concentrations of 5, 10, and 20 µg/ml of PRP-Exos dose-dependently increased the survival, proliferation, migration, and osteogenic differentiation of these cells. Also, in a rat model of femoral bone defect, local injection of PRP-Exos (5 µg) significantly increased new bone formation, tissue mineralization, and collagen synthesis (70).

Platelet lysates (PLs) are frequently utilized derivatives of PRP. These PLs serve as a cost-effective blend of GFs, primarily comprising PDGF, transforming growth factor-β

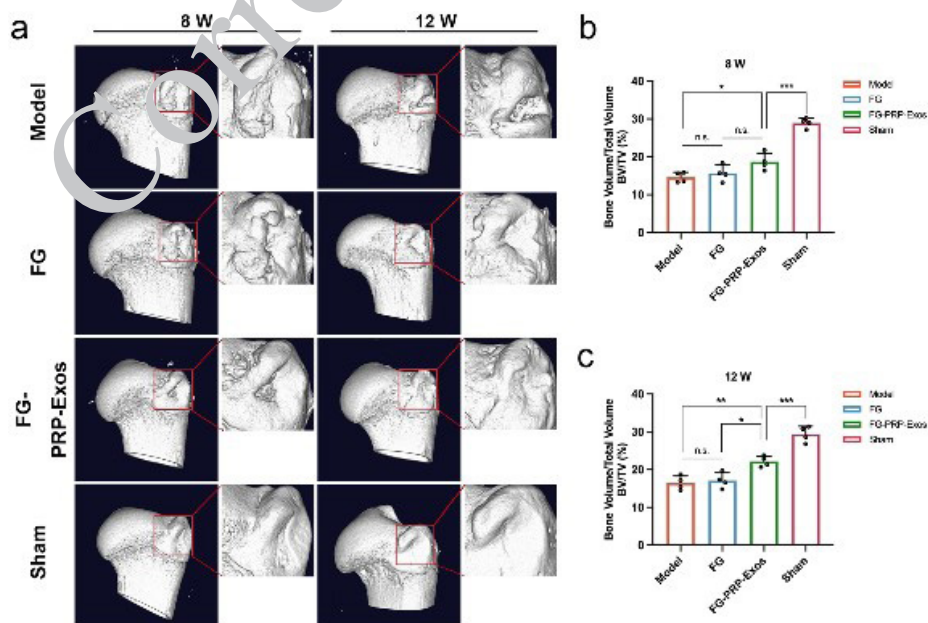


Figure 2. Micro-CT evaluation of bone regeneration

(a) 3D reconstruction of the treated humeral head, with the red area indicating the defined region of interest (ROI). (b, c) Quantitative analysis of the bone volume/total volume (BV/TV) ratio within the ROI. (Reproduced from [72] under CC BY 4.0 license).

Micro-CT: Micro-computed tomography

(TGF- β), and basic fibroblast growth factor (bFGF), and can be conveniently produced through repeated freeze-thawing of PRP. Notably, the removal of platelet debris lowers the immunogenicity of PLs compared to PRP, making them a promising candidate for use in allografts or xenografts (74). PL, as the second generation of PRP, offers a more economical option than commercial recombinant GFs and holds significant promise for broader clinical applications (75).

Torreggiani *et al.* reported for the first time the isolation and purification of exosomes from human PL (PL-Exos). The findings reveal that PL-Exos enhance MSC proliferation and migration more effectively than PL itself, and that the concentration of exosomes influences the osteogenic differentiation of MSCs (76).

In one study, using PL-Exos, BMSCs and endothelial progenitor cells (EPCs) were treated with 50 μ g/mL of the exosomes, resulting in the reversal of the glucocorticoid (GC) inhibition of osteogenesis and angiogenesis in GC-induced osteoporosis rats. GCs can inhibit osteogenic differentiation and impair osseous vasculature. PL-Exos were extracted with ultracentrifugation and were 30–200 nm in size, expressed CD9 and TSG101, and lacked the endoplasmic reticulum protein calnexin. These exosomes contained higher levels of the GFs PDGF-BB, VEGF, TGF- β , and bFGF than the initial lysates. The purified exosomes were chemically conjugated with alendronate-linked PEGylated phospholipid (PL-exo-ALN) to develop a bone-targeting exosome system. The ALN modification dramatically improved the exosome's binding affinity to hydroxyapatite *in vitro*. This effect occurred through the activation of the AKT/ β -catenin pathway in EPCs (enhancing osteogenesis) and the PDGF-BB/PDGF- β /HIF-1 α pathway in endothelial cells (enhancing angiogenesis and migration) (Figure 3) (75).

A summary of the key results is presented in Table 1.

Immune cell-derived exosomes: Mechanisms and applications in bone regeneration

Significant progress has been made in elucidating the immune microenvironment processes that regulate bone, cartilage, and soft tissue regeneration. However, translating these insights into effective clinical applications remains a formidable challenge. The modulation of the immune microenvironment has emerged as a promising strategy for enhancing tissue regeneration. Therefore, a comprehensive understanding of the interactions between immune cells and functional tissue cells is crucial for advancing regenerative therapies (77, 78). Besides platelets and erythrocytes, various blood cells, like immune cells, also produce EVs. Studies have extensively reported EV release by mast cells (79), neutrophils (80), eosinophils (81), basophils (82), macrophages (83), dendritic cells (84), NK cells (85), and B and T lymphocytes (86, 87).

Bone repair and regeneration need a well-timed and managed inflammatory response. A weak pro-inflammatory reaction can hinder bone growth, whereas excessive inflammation results in more bone damage. In order to resolve inflammation during bone repair, immune cells must communicate with multipotent MSCs and other cell populations in the bone microenvironment. Cellular communication can happen through direct interactions or be facilitated by factors secreted by cells, many of which are potentially carried by EVs (88).

Macrophages can differentiate into either pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes, influencing cell metabolism and tissue regeneration through the release of cytokines and other signaling molecules. Similarly, T cells play distinct roles, with helper T1 (Th1) and Th17 cells driving inflammation and

Table 1. Role of blood-derived exosomes in bone regeneration

Source	Type of animal model	Effects on osteogenesis	The key regulatory pathway/ molecule	Ref.
Rabbit blood serum	Rabbit radius	Elevation in levels of PICP ¹ and BALP ² , as a marker of enhanced bone formation	Activation of p38/STAT1 Pathway (Upregulating p38 and suppressing STAT1 signaling)	44
		Reduced osteoclast activity		
		Higher VEGF ³ and FGF2 ⁴ levels, indicating vascularization		
		Restored calcium deposition		45
Young rats' blood serum	Osteoporotic rats	Upregulated osteogenic gene and protein expression	Suppression of PTEN expression (a known inhibitor of osteogenesis) by miR-19b-3p	
		Enhanced ALP ⁵ activity and mineralization		
Young rats' blood plasma	Osteoporotic OVX mice	Significant alleviation of osteoporosis in aged rats	Osteoprotective effects of miR-217-5p	10
Mouse RBCs	Osteoporotic mice	Promoting bone regeneration, likely by enhancing the osteogenic environment.	Suppression of miR-214 level in osteoclasts by the anti-miRNA oligonucleotide	
		Reversing excessive bone absorption by inhibition of osteoclast activity, via osteoclast targeting-RBCEVs ⁶ (containing anti-miR-214 ⁷)		56
Apoptotic human RBCs	Calvarial defect models	Bone-like matrix deposition, activation of osteogenic transcription factors (e.g., RUNX2, Osterix), modulation of signaling pathways such as Wnt/ β -catenin, BMP ⁸ , or MAPK	Delivery of CA1 ⁹ of apoptotic vesicles promoted BMSC ¹⁰ proliferation via RUNX2	
				53
PRP	Glucocorticoid-induced ONFH ¹¹ of rats	Restoring osteogenic protein expression.	Wnt/ β -catenin pathway	
		Suppressing ER ¹² stress-induced apoptosis.		73
Platelet lysate (PL)	Glucocorticoid-induced ONFH of rats	BMSC osteogenesis, EPC ¹³ migration and vascularization, and effectively reversed bone loss	Akt/Bad/Bcl-2/Caspase-3 signaling cascade	
				75

1. Procollagen carboxy-terminal peptide; 2. Bone-specific alkaline phosphatase; 3. Vascular endothelial growth factor; 4. Fibroblast growth factor 2; 5. Alkaline phosphatase; 6. Red blood cell extracellular vesicle; 7. Anti-miR-214 is supposed to reverse bone resorption and provide a potential treatment; 8. Bone morphogenetic protein; 9. Carbonic anhydrase 1; 10. Bone mesenchymal stem cell; 11. Osteonecrosis of the femoral head; 12. Endoplasmic reticulum; 13. Endothelial progenitor cell; 14. Alendronate.

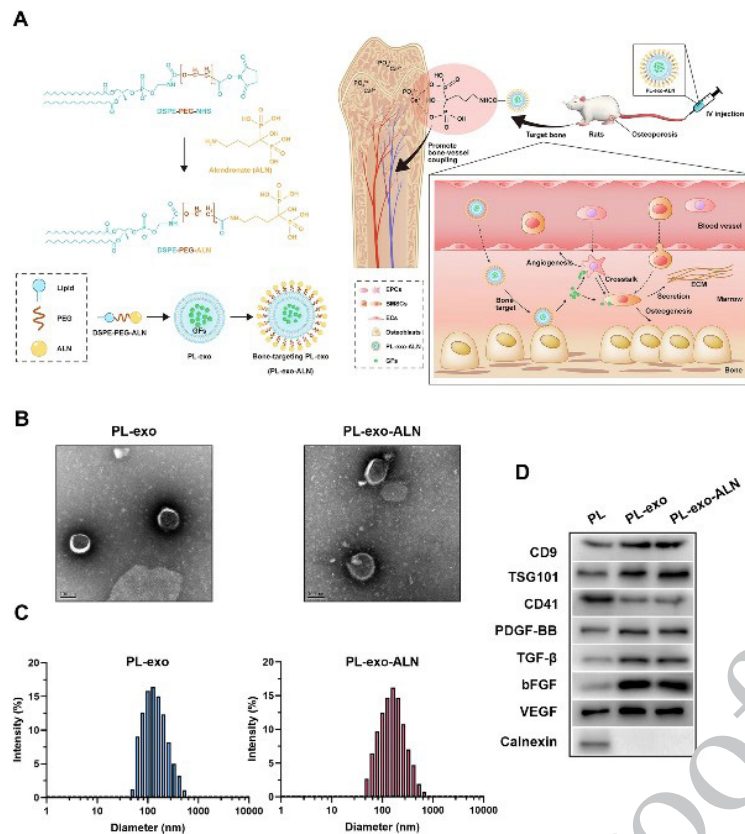


Figure 3. Key characteristics of the isolated rat PL-Exos

(A) Schematic of PL-exo-ALN preparation and bone targeting. (B) TEM images of PL-exo and PL-exo-ALN. (C) DLS size distribution analysis. (D) WB confirming exosomal markers (CD9, TSG101), reduced platelet marker (CD41), enriched growth factors (PDGF-BB, TGF-β, bFGF, VEGF), and the absence of the non-exosomal contaminant calnexin. (Reproduced from [75] under CC BY 4.0 license).

TEM: Transmission electron microscopy; DLS: Dynamic light scattering; WB: Western blot; PL-exo-ALN: Alendronate-conjugated platelet lysate-derived exosomes

osteoclast formation, while Th2 and regulatory T (Treg) cells exhibit anti-inflammatory properties and support tissue reconstruction, effectively promoting osteogenesis. The interactions between macrophages, T cells, and MSCs deeply impact the bone microenvironment and modulate the localized immune response (89). One study revealed that dendritic cells (DCs) can enhance the migration of MSCs *in vitro* via the release of paracrine factors (90). Moreover, additional findings indicate that the paracrine-driven MSC migration stimulated by DCs occurs predominantly through functional exosome-enriched EVs secreted by DCs (88). Other studies have shown similar potential in immune cells like macrophages, NK cells, and T cells (91). Moreover, monocytes/macrophages and their EVs are capable of promoting MSC osteogenic differentiation (92).

Macrophage-derived exosomes

Both inflammatory and resident macrophages coexist in the environment of infected or injured bone and contribute to the progression of bone tissue repair. M1 and M2 phenotypes represent the two ends of a broad spectrum of inflammatory macrophages, derived from a distinct population of blood monocytes that rapidly infiltrate bone tissue following injury, inappropriate functioning, and/or infection damage (93).

Different macrophage depletion strategies have demonstrated the vital role of macrophages in bone healing. Macrophage ablation leads to the deposition of undifferentiated mesenchymal cells, resulting in a fibrotic response within the fracture callus rather than the formation

of cartilage and bone. This fibrotic response persists even when ablation is initiated three or seven days after injury, suggesting that macrophages play roles beyond the initial inflammatory phase of repair (94). A study showed that a diminished population of monocyte/macrophage, as revealed by down-regulated expression of the precursor marker CD14 and the general macrophage marker CD68, along with disruption in the M1-to-M2 switch in aged rats, could lead to non-union when no additional treatment was applied (95).

M2-derived exosomes (M2-Exos) play a substantial role in the M1-to-M2 phenotype switch in cutaneous wound healing (96). M2-Exos have gained recognition in bone tissue engineering, particularly for improving fracture healing in diabetic conditions. In one approach, M2-Exos were recruited to functionalize a 3D-printed nanofibrous scaffold, which resulted in enhanced biocompatibility and regulation of the foreign body response (97). One compelling mechanism involves their potential to modulate type H bone vessels, a type of specialized vascular structure that synchronizes osteogenesis and angiogenesis. These vessels are regulated by PDGF-BB secreted from osteoclast precursor cells originating from monocyte/macrophage fusion, positioning M2-Exos as key mediators in a triad coupling of vascularization, bone formation, and resorption (98). In diabetic db/db mice, the M1/M2 imbalance was demonstrated as a contributing factor to delayed bone repair. Targeting M1 macrophages by M2-Exos showed the activation of the anti-inflammatory PI3K/AKT pathway in M1, resulting in reprogramming of M1 into M2 macrophages

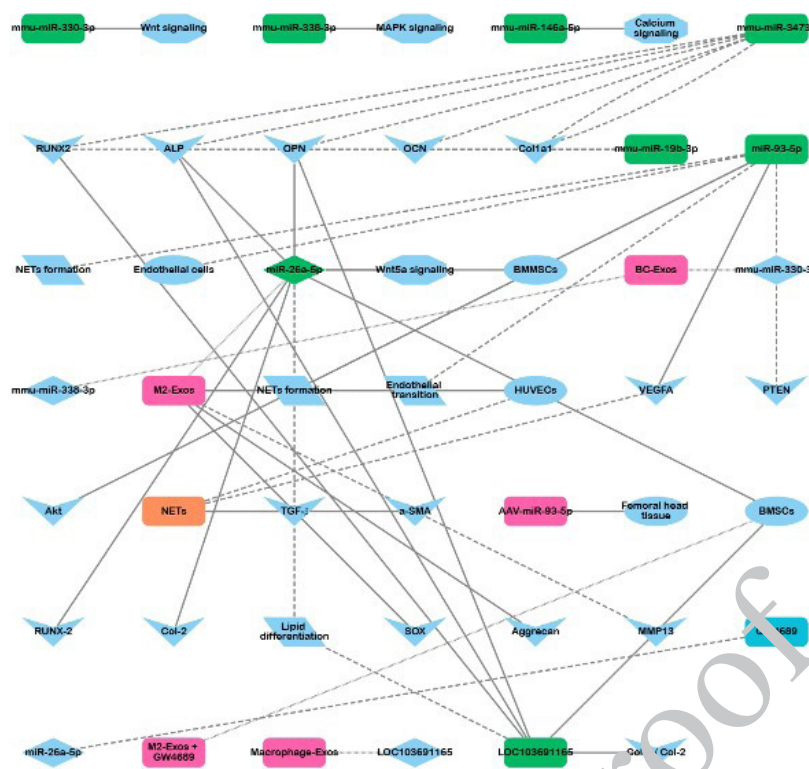


Figure 4. Exosome-mediated regulatory network in osteogenesis, angiogenesis, and endothelial phenotype transition

Figure 2: Exosome-mediated regulatory network in osteogenesis, angiogenesis, and cancer phenotypic transition. Nodes are color-coded by type: Green diamonds for RNA species (miRNA, lncRNA), pink ellipses for extracellular carriers, orange parallelograms for biological processes, and blue ellipses/V-shaped nodes for inhibitor molecules and genes. Shapes indicate entity class: pathways (octagons), KOs (diamonds), cells/tissues (ellipses), processes (parallelograms), and genes (vee). Arrows represent interactions: solid lines for activation, inhibition, differentiation, or expression changes; dashed lines for modulation; dotted lines for cargo delivery. BMSCs: Bone marrow mesenchymal stem cells; NET: Neutrophil extracellular trap. (Created with Cytoscape web application)

in vitro. Additionally, M2-Exos can restore the interrupted osteoimmune microenvironment and improve the delayed healing of diabetic fractures *in vivo*. On day 7 post-surgery, immunofluorescence revealed a marked increase in M2 polarization in the femur of the group treated with M2-Exos, compared to the diabetic control (no Exo treatment) group, confirmed by histological analysis (99).

To further enhance the innate regenerative functions of M2-Exos, their osteogenic potency can be augmented through biochemical pre-treatment strategies. M2-Exos that were pre-treated with exogenous hydrogen sulfide (H₂S) outperformed untreated ones in promoting MSCs' osteogenic differentiation, as evidenced by higher expression of markers ALP and Runx2. H₂S as a gaseous signaling molecule, can regulate the self-renewal and osteogenic differentiation of MSCs and DPSCs through Wnt/ β -catenin signaling (100, 101). H₂S promoted the enrichment of the exosomal protein moesin both in macrophages and their exosomes. The knockdown of moesin with siRNA, markedly reduced the level of moesin protein, which significantly decreased the endocytosis of exosomes to MSCs. Therefore, H₂S promoted the osteogenic differentiation of MSCs with the enrichment of moesin protein in M2-Exos, which facilitated the endocytosis of exosomes by MSCs (102).

Ion-releasing biomaterials such as bioceramics (BCs) represent another broad class of materials that can be used to enhance the osteogenic potency of M2-Exos (103). BCs, through ion products such as SiO_4^{4-} , Ca^{2+} , and Mg^{2+} , have been proven to be important regulators of the immunomodulation of macrophages (104). β -tricalcium phosphate (β -TCP), as a type of BC, stimulates macrophages to release exosomes (BC-Exos) in a dose-dependent

nanoparticles (BC-Exos) may be induced by activating neutral sphingomyelinases (nSMases), possibly triggered by shifts in ionic concentrations in the cell microenvironment. When incorporated into 3D-bioprinted hyaluronic acid/alginate scaffolds, these BC-Exos promote an anti-inflammatory (M2) macrophage phenotype, up-regulating reparative cytokines (IL-10, TGF- β 1) and chemokines (MCP-1, SDF-1 α) (105). Biphasic calcium phosphate (BCP), another BC, regulated macrophage transformation from M1 to M2 phenotype in its submicron surface structure. In this study, two different surface BCPs, one with submicron scale (BCP1) and the other with larger micron-scale (BCP2) particles, were used as bone implants *in vivo*. Exosomes induced by BCP1 were rich in miR-142-5p compared to exosomes in the other group. The overexpression study demonstrated that miR-142a-5p could promote the osteogenic differentiation of MSCs, as evidenced by higher ALP activity, up-regulated gene expression of osteogenic markers like *Runx2*, *Alp*, *Bmp2*, *Opn*, and *Ocn*, and enhanced protein expression of RUNX2 and OCN (106).

A 3D-printed calcium silicate/polycaprolactone scaffold doped with plant extract of *Dictamnii Radicis Cortex* (DICS/PCL), long known for its anti-inflammatory effects in Chinese medicine, stimulated the transition of the osteoimmune microenvironment from an inflammatory M1 state toward a pro-regenerative M2 phenotype. These M2 macrophages released EVs enriched with miR-21-5p, which is known as a key regulatory microRNA through transcriptomic profiling. After uptake by MSCs, these exosomal miR-21 cargos suppressed inhibitory genes such as *Spry1* and *Pten*, thereby activating downstream osteogenic pathways and enhancing the expression of *Runx2*, osteocalcin,

and mineralized matrix formation (107).

Furthermore, titanium nanotube arrays (TNAs) affected the composition of macrophage-derived exosomes, enabling them to be effectively involved in osteoimmunomodulation, angiogenesis, and osteogenesis after stimulation with TNAs. Titanium and titanium-based alloys are among the most widely used scaffolds for clinical implantation, due to their distinguished biocompatibility and mechanical properties (108). Exosomes derived from TNA-stimulated macrophages have been shown to promote angiogenesis and enhance the expression of osteogenesis-related genes and ALP activity through the interaction between miR-3473e and its target gene Akt1 (109).

These biomaterial-induced changes in exosome function suggest underlying molecular mechanisms, among which miRNAs appear to be particularly influential. High-throughput sequencing studies have demonstrated that the therapeutic effects of M2-Exos are closely tied to the rich content of their miRNAs (105, 110, 111). Figure 4 shows the complex and multidirectional connection between miRNAs and osteogenesis-related signaling pathways. Based on miRNA cargo profiling, the expression profile of the previously discussed BC-Exos (105) changed in a direction that promoted pro-osteogenic miRNAs (e.g., mmu-miR-330-3p, mmu-miR-338-3p, mmu-miR-146a-5p) and down-regulated anti-osteogenic ones (e.g., mmu-miR-3473e, mmu-miR-19b-3p). KEGG pathway analysis revealed that the top 20 up-regulated miRNAs in the BC-Exos target key signaling pathways involved in regulating stem cell pluripotency, including MAPK, Wnt, and calcium signaling (105).

M2-Exos have been associated with preventing the development of ONFH by regulating neutrophil extracellular traps, a key driver of ONFH pathogenesis. One of the main molecular mechanisms was miR-93-5p, a potential key factor in the modulation of NET formation and endothelial phenotype transition activation (Figure 5) (110). MicroRNA-26a-5p, another exosomal miR-26a-5p secreted by M2S, showed osteogenic effects on rat BMMSCs (112). In mouse ADSCs and human periodontal ligament stem cells, miRNA-26a-5p promotes osteogenic differentiation by targeting Wnt Ca^{2+} and Wnt5a signaling pathways, respectively (Figure 5, red highlight) (113, 114). In this study, the efficacy of MicroRNA-26a-5p was found to be comparable to that of the osteogenesis induction medium on ALP, RUNX-2, OPN, and Col-2; however, the inability to identify its target gene during osteogenic

differentiation remains a key limitation (112). Long non-coding RNAs (lncRNAs) are key components of exosomes and play important roles in exosomal function. An exosomal lncRNA, named LOC103691165, secreted from either M1 or M2 phenotypes promoted osteogenic differentiation of BMMSCs. When up-regulating the LOC103691165 in macrophage exosomes, BMMSC osteogenesis was promoted, indicating that LOC103691165 was strongly associated with this process. Further research is required to explore the mechanism by which lncRNA LOC103691165 promotes osteogenesis and consider its activity in the context of clinical practice (115).

The delivery of exosomes remains a challenge due to the rapid clearance of exosomes after systemic administration. A 3-way junction (3WJ) RNA nanoparticles-aptamer functionalized exosome system was developed, based on an BMMSC-specific aptamer and 3WJ RNA nanoparticles were applied to conjugate the BMMSC-specific aptamer and M2-Exos ((3WJ-BMSC_{apt}/M2-Exos)). While enabling targeted delivery, the 3WJ-BMSC_{apt} preserved the original physical and biochemical properties of the exosomes. The modified exosomes showed a slight increase in particle size; however, their membrane integrity and characteristic protein markers remained intact. Furthermore, the miRNA profiling in 3WJ-BMSC_{apt}/M2-Exos showed that miR-29a-3p, miR-25-3p, and miR-690, which are responsible for osteogenic differentiation, were enriched in 3WJ-BMSC_{apt}/M2-Exos (116).

While the research has mostly focused on M2-Exos, the role of M1 macrophages is irreplaceable in the initiation and progression of healing, as described earlier in this section. M1 macrophages initiate the ossification process, and their depletion leads to impaired healing (117). This suggests that their role in early bone formation is not only functional but indispensable. Their derived exosomes, rich in miR-21a-5p, further reinforce their osteogenic potential, both *in vitro* and *in vivo* (118, 119). One study identified the potential of M1-Exos to promote the osteogenic differentiation of MC3T3-E1 osteoblasts by delivering miR-21a-5p, which suppresses GATA2, a negative regulator of bone formation (Figure 5, yellow highlight) (118). In another study, to establish the cross-talk among polarized macrophages and BMMSCs, *in vitro* pro-inflammatory M1 and anti-inflammatory M2 macrophages were co-cultured with primary murine BMMSCs, and their effects on osteogenesis were assessed. Results showed that both M1- and M2-Exos can stimulate osteogenesis, especially the M1-Exos

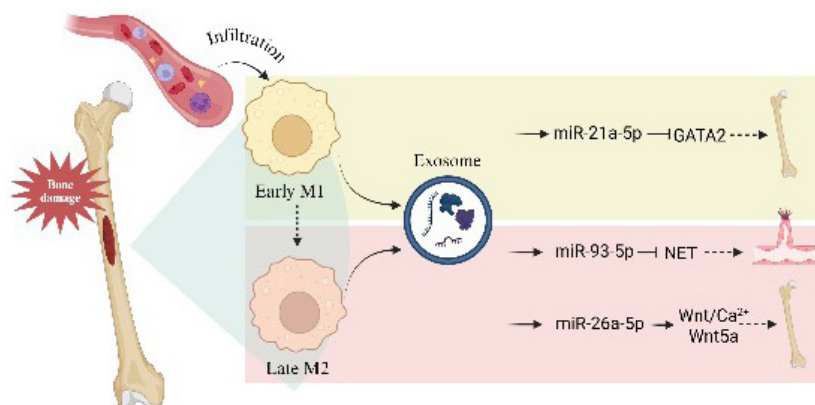


Figure 5. Macrophage-derived exosomes orchestrate bone repair by delivering specific miRNAs that modulate immune and signaling pathways. Early M1 exosomes suppress GATA2 via miR-21a-5p, while late M2 exosomes promote regeneration through NET regulation and Wnt/ Ca^{2+} signaling. (Created with BioRender)

containing microRNA-21a-5p. The results of qRT-PCR revealed that miR-21a-5p was significantly up-regulated in M1-Exos compared with M2-Exos (119).

In conclusion, exosomes secreted from either pro-inflammatory M1 or anti-inflammatory M2 macrophages can enhance osteogenesis. A main target of macrophage exosome delivery is to support the microenvironment for inflammation control and facilitate the timely switch from M1 to M2 phenotype. However, this process is gradual and regulated by a coordinated network of secreted GFs, cytokines, and exosomes. MiRNAs play an important role in BMSC osteogenesis and are abundantly present in macrophage-derived exosomes.

Dendritic cell-derived exosomes

DCs are antigen-presenting cells whose role in immunity is defined by their maturation. Immature DCs (imDCs) are regulatory and suppress immune responses to maintain tolerance by inducing T cell anergy and promoting regulatory T cell (Treg) responses to terminate inflammation, while mature DCs (mDCs) are immunostimulatory. The mDCs' ability to activate strong Th17 responses, though effective against bacteria, can have the adverse effect of promoting bone loss (120-122).

DCs are engaged in every stage of tissue recovery, ranging from the onset of inflammation to the process of tissue remodeling, and they also help maintain skeletal homeostasis and support bone healing (123). DCs have the ability to release different chemical signals and proteins to attract other immune cells, which are necessary for complete bone healing. For example, dendritic cell-derived interferon- λ 1 has been shown to reduce osteoclast formation and bone resorption activity in both *in vitro* and *in vivo* settings (124). Earlier research has established that DCs being new contributors to bone immune responses, interact with biomaterials to enhance their biological effects (125). Recently, the potential crucial role of DCs in biomaterial-induced osteogenesis has been highlighted. Zhang *et al.* demonstrated the role of DCs in modulating immune responses and osteogenesis around β -TCP biomaterials. DCs cultured on β -TCP exhibited an anti-inflammatory phenotype (reduced CD86, CD40/IFN- γ , elevated IL-10/TGF- β 1), and their conditioned media enhanced osteoblast differentiation (RUNX2, ALP, OCN) and mineralization

(126). Additionally, another study revealed that, following implantation of Biphasic calcium phosphate (BCP), danger signals such as HMGB1 accumulate on the material surface, activating DCs via the TLR4/MYD88/NF- κ B pathway, which subsequently recruit MSCs to the implant site to initiate osteoinduction (Figure 6) (127).

Emerging evidence reveals that studies have demonstrated the ability of DCs to promote tissue regeneration by facilitating the recruitment of MSCs via EVs (88). Exosomes derived from DCs (DC-Exos) possess a molecular composition closely mirroring that of DCs. In addition, DC-Exos carry genetic material, soluble proteins, and lipids originating from DCs. As a result, DC-Exos serve not only as an efficient vehicle for drug delivery but also as key mediators of various immune responses, reflecting the functional roles of DCs (128).

In one study, Exos were isolated from DCs to investigate their immunobiological properties in mice and their ability to reprogram immune cells responsible for experimental alveolar bone loss *in vivo* (122). Three subtypes of dendritic cell-derived exosomes were extracted, including immune immature (iDC-EXO), immune stimulatory (stimDC-EXO), and immune-regulatory (regDC-EXO). Exosomes were purified by sequential centrifugation and ultracentrifugation, and regDC-EXO was functionally loaded with TGF- β 1 and IL-10 and was administered either intravenously or via local injection into the soft tissues overlying the alveolar bone. Locally delivered regDC-EXOs exhibited a high tropism for inflamed tissues and were internalized by local DCs and T cells *in situ*. The encapsulated immunoregulatory cargo (TGF- β 1, IL-10) within regDC-EXOs was protected from proteolytic degradation. Furthermore, regDC-EXO treatment suppressed the maturation of recipient DCs and the induction of Th17 effector cells, while simultaneously promoting the recruitment of T-regulatory cells. This immunoregulatory shift ultimately led to the inhibition of bone-resorptive cytokines and a significant reduction in osteoclastic bone loss (122).

Cao *et al.*'s study showed that mature dendritic cell-derived exosomes (mDC-Exo) extracted by sequential centrifugation and ultracentrifugation at a concentration of 10 μ g/ml promoted bone regeneration by BM-MSCs. This process was mediated by miR-335 transfer and targeting of the LATS1 gene, which activates the expression of osteogenic

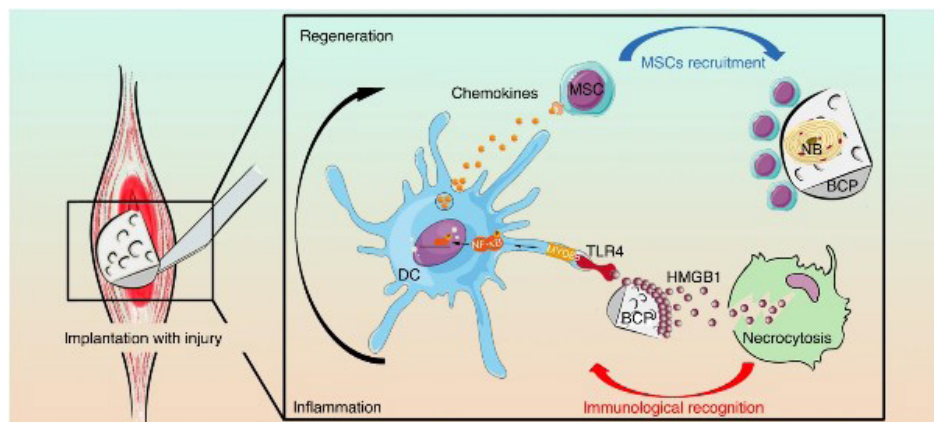


Figure 6. The adsorption of HMGB1 on the BCP surface post-implantation activated DCs through the TLR4/MYD88/NF- κ B signaling pathway, triggering subsequent immune and bone regeneration responses (Reproduced from [127] under CC BY 4.0 license).

HMGB1: High mobility group box 1; BCP: Biphasic calcium phosphate; DCs: Dendritic cells; TLR4: Toll-like receptor 4; MYD88: Myeloid differentiation primary response 88; NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells

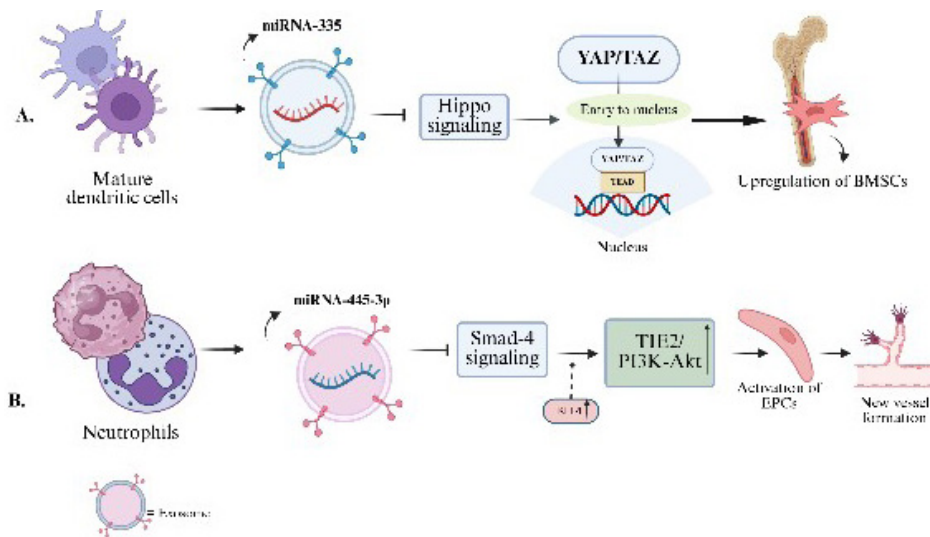


Figure 7. A) Exosomal miR-335, which exists in mature dendritic cell-derived exosomes, inhibits Hippo signaling by targeting LATS1, leading to activation and nuclear translocation of YAP/TAZ. These transcriptional co-activators interact with TEAD (TEA Domain Transcription Factor, nuclear partner of YAP/TAZ) in the nucleus, up-regulating osteogenic gene expression and enhancing BMSC proliferation and differentiation. B) Neutrophil-derived exosomal miR-445-3p promotes endothelial progenitor cell (EPC) activation and vasculogenesis by down-regulating SMAD4, up-regulating KLF4, and enhancing TIE2/PI3K-Akt signaling. (Created with BioRender)

genes by inhibiting the Hippo pathway and reducing YAP/TAZ phosphorylation (121) (Figure 7A).

Neutrophil-derived exosomes

Neutrophils, also called polymorphonuclear leukocytes (PMNs), are the most abundant type of immune cell in the human body and are typically the first to arrive at an injury site (129). Their important function in healing and restoring various types of tissues, such as bone tissue, is becoming increasingly well-recognized (130-133). The importance of PMNs in bone healing is well-established. Evidence shows that depleting PMNs directly impairs fracture repair. Furthermore, these cells are vital for initiating the clotting process and hematoma formation, which creates an essential scaffold for immune and progenitor cells to facilitate bone regeneration (129). Moreover, it has been recently noted that PMNs play a crucial role in recruiting BMSCs during the early phases of bone repair and restoration (134). One of the complex mechanisms underlying this regenerative capacity is the secretion of neutrophil-derived extracellular vesicles (PMN-EVs). For instance, treatment of BMSCs with 80 µg/ml of PMN-EVs has been shown to accelerate cell proliferation, increase ALP activity, and promote mineralization. In the *in vivo* model, BMSC sheets containing PMN-EVs were used to evaluate bone repair. These sheets were formed over 7–10 days in the continuous presence of 80 µg/ml PMN-EVs in the sheet induction medium. Then, the sheets were implanted into a rat calvarial defect. Micro-CT results showed that the BS@PMN-EVs group had a significant increase in new bone regeneration (~39.5%) and increased trabecular thickness compared to the control. Proteomics analysis showed that PMN-EVs increased the expression of two key proteins, SOD2 and GJA1, in BMSCs; These two proteins are associated with osteogenic differentiation and protection against oxidative stress. PMN-EVs also reduced ROS levels in cells (129).

Furthermore, the regenerative influence of PMNs extends beyond direct osteogenesis to the critical process of angiogenesis, which is indispensable for bone graft

survival. Wang *et al.* designed an exosome-capturing scaffold (ECS) that utilizes electrostatic adherence and lipophilic interactions to attract and anchor exosomes. The study demonstrated that the localized concentration of exosomes within the ECS, following implantation into critical mandibular defects, markedly accelerated natural bone healing by encouraging vascularization at the defect site. Among the exosomes identified, neutrophil-derived exosomes (PMN-Exos) emerged as the most prevalent subtype. During the process of intrinsic bone regeneration, PMN-Exos aided in blood vessel formation mainly by helping the growth of endothelial progenitor cells (EPCs), which are essential for creating new blood vessels. On a mechanistic level, this vascularization effect was linked to a specific microRNA (miR455-3p) found in PMN-Exos, which boosts EPC growth by influencing the Smad4 signaling pathway (135) (Figure 7B).

Clinical translation, challenges, and future perspectives

Although preclinical studies have demonstrated the remarkable potential of blood-derived exosomes to enhance osteogenesis, stimulate angiogenesis, modulate inflammatory responses, and accelerate bone tissue repair, translation of these findings into clinical applications is still in its early stages. A phase I, randomized, double-blind clinical trial (NCT04849429) investigated intradiscal injection of exosome-enriched platelet-rich plasma in patients with chronic low back pain. Although the results of this study have not yet been published and do not focus on bone regeneration, it is one of the attempts to assess the safety and feasibility of the clinical application of blood-derived exosomes.

Despite promising results from laboratory studies, several obstacles still prevent this technology from being widely used in clinical treatments. One of the most important challenges is the lack of standardized methods for isolation, purification, identification, and storage of exosomes.

The variability among EV extraction methodologies across the literature is a challenge since not all studies adhere

to standardized extraction guidelines in the field, such as the Minimum Information for Studies of Extracellular Vesicles (MISEV), first established in 2014 and last updated in 2024. The MISEV2023 guideline emphasizes strict control of pre-analytical variables in blood-derived EV research, including reporting characteristic features of the donor source and collection standards such as using large-gauge needles, discarding the first portion of blood, and selecting anticoagulants compatible with downstream EV analyses. The guideline requires early removal of cells and platelets, documentation of all storage conditions, and assessment of major blood co-isolates such as lipoproteins and soluble proteins.

Various methods have been suggested by MISEV to concentrate EVs from the source, including differential ultracentrifugation, density gradient/cushion, size exclusion chromatography, fluid flow-based separation, and charge and molecular recognition-based separation (136). Regarding MISEV, different approaches to separate EVs result in varied levels of yield and purity. For example, differential ultracentrifugation, used frequently for exosome extraction throughout the literature, with sequential increases in spins provides moderate purity, which could be further reinforced by nanoparticle tracking analyzer (NTA) and immunoblot assay to recognize exosomes by the anti-CD9, anti-CD63, and anti-TSG101 antibodies (137). High-resolution transmission electron microscopy (TEM) for visualization of exosome morphology and western blot for a more precise detection of surface markers (e.g., CD9, CD63, CD81, HSP70 and TSG101), along with negative reticulum endothelin canalexin, are complementary assays which help verification of EV presence and the lack of contamination with cell lysate in samples more accurately (9, 73, 99, 107). However, the MISEV 2023 recommends reporting of track EV marker protein levels relative to total protein to estimate fold enrichment, which has often been overlooked in the literature (136). Some studies use commercial kits (115), which theoretically can increase the risk of contamination as a result of co-precipitation of soluble proteins, lipoproteins, nucleoprotein complexes, and non-vesicular extracellular particles with the polymer ingredient of the kit. In such cases, MISEV recommends justifying the reason why a separation/concentration has not been performed.

Taken together, EV isolation strategies should adopt stricter standards according to the MISEV2023 guideline to ensure methodological transparency and reproducibility. Inconsistent methodology between studies limits the comparability and increases the risk of biological interpretations being confounded by non-vesicular contaminants. Future research in the field of EVs and osteogenesis has to move toward clinical translation, while adhering to standardized, MISEV-guided workflows.

Conclusion

Blood-derived exosomes, as a new generation of cell-free therapeutics, have attracted considerable attention in the field of regenerative medicine in recent years. Evidence from preclinical studies suggests that these exosomes can simultaneously enhance key bone remodeling processes, including osteogenesis, angiogenesis, modulation of inflammatory responses, and regulation of tissue regeneration, through the delivery of a variety of miRNAs, proteins, and other bioactive molecules. Compared to other exosomal sources, blood-derived exosomes have

advantages such as easy access, minimal invasiveness in sampling, possibility of autologous preparation, reduced risk of immune responses, and potential for large-scale production, which make them a promising option for clinical applications. However, the lack of standardized protocols for extraction, purification, dosage determination, quality control, and performance evaluation, along with the lack of dedicated clinical studies, remain the most important obstacles to translating this technology into therapy. With the rapid advancement of technologies related to exosome engineering, the development of targeted delivery systems, the design of smart biomaterials and scaffolds, and the expansion of international standards, it is expected that many of the current limitations will be overcome in the coming years. Conducting well-designed and high-quality clinical trials will also play a decisive role in evaluating the safety, efficacy, and clinical application of these therapies.

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Ethical Approval and Consent to Participate

Not applicable.

Authors' Contributions

B N was responsible for Conceptualization, the original draft, and review & editing. N E, A M, and D Y contributed to review & editing. E A handled conceptualization, the original draft, supervision, validation, and project administration.

Conflicts of Interest

The authors declare no competing interests.

Declaration

The authors acknowledge the use of ChatGPT, SciSpace, and Copilot for language editing, paraphrasing, and enhancing the English proficiency of this manuscript. Following the AI-assisted refinement, the authors critically reviewed and revised the text and assume full responsibility for the integrity and accuracy of the final content.

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