

M2 macrophage extracellular vesicles in bone regeneration: Applications and challenges

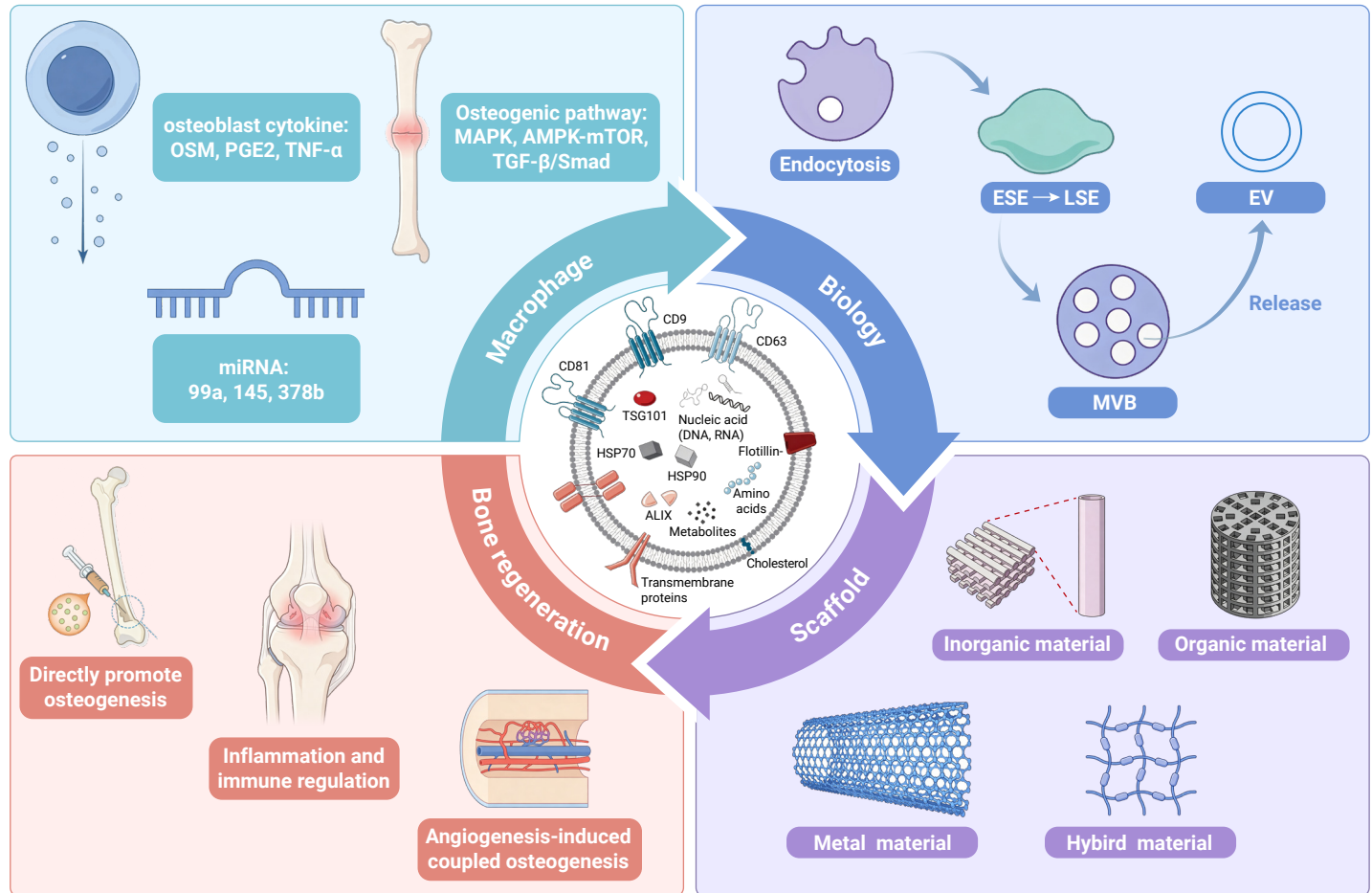
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- M2 macrophage extracellular vesicles promote bone repair by reducing inflammation and stimulating blood vessel growth.
- When combined with functionalized scaffolds, these vesicles significantly improve implant integration and defect healing.
- Challenges remain in targeting, scalable production, and quality control for clinical use.

M2 macrophage extracellular vesicles in bone regeneration: Applications and challenges

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Extracellular vesicles (EVs) can carry bioactive molecules and mediate intercellular communication. Among EVs from various sources, those derived from M2 macrophages (M2-EVs) have attracted widespread attention in the field of bone regeneration due to their inherent anti-inflammatory and osteogenic properties. M2-EVs promote osteogenic differentiation of mesenchymal stem cells by delivering specific microRNAs and proteins that activate key signaling pathways, such as MAPK, TGF- β /Smad, AMPK-mTOR, and PI3K/Akt. They also modulate the osteoimmune microenvironment by suppressing inflammation and inducing M2 polarization, while enhancing angiogenesis through VEGF and miR-21 transfer. In bone tissue engineering, M2-EVs can be integrated with various scaffolds—such as β -TCP, PCL fibrous scaffolds, and hydrogels—to improve osseointegration and bone defect repair, as confirmed in preclinical models. However, clinical translation faces challenges, including insufficient targeting, heterogeneity, isolation standardization, and quality control. Despite these limitations, M2-EVs represent a promising cell-free therapeutic strategy for bone repair. Future research may focus on engineering approaches to enhance their specificity, stability, and large-scale production, ultimately unlocking their full potential in regenerative medicine.

INTRODUCTION

Various cells secrete extracellular vesicles (EVs) under both normal and pathological conditions.¹ EVs originate mainly from intracellular multivesicular bodies (MVBs) and are released into the extracellular matrix upon fusion of the outer membrane of MVBs with the cell membrane. Early studies regarded EVs merely as vehicles for cellular waste disposal.² However, with in-depth exploration of their biogenesis, molecular composition, transport pathways, and functions in intercellular signaling, EVs are now recognized to possess diverse physiological and pathological functions, including involvement in bone and tissue regeneration and repair.³ The function of EVs is highly dependent on their cell type of origin. During inflammation, implant integration, and tissue healing, the regulatory role of the immune system—particularly macrophages—is attracting increasing attention. Macrophages, which can originate from either circulating monocytes or embryonic progenitor cells, are core components of the immune system and play critical roles in host defense and the regulation of inflammation.⁴

Bone regeneration is a highly complex and dynamically coordinated biological process that relies on the precise and orderly interplay of multiple cell types and molecular signaling pathways. Traditional views often simplistically interpret bone regeneration as a balance between bone formation and resorption. However, with the advancement of osteoimmunology, it has become increasingly recognized that bone regeneration is essentially a close interaction among multiple systems, including the skeletal, immune, and even nervous systems. Within the inflammatory microenvironment following bone injury, immune cells such as macrophages secrete cytokines and chemokines that not only clear necrotic tissue but also profoundly influence the processes of osteogenesis and angiogenesis by regulating the recruitment and differentiation of mesenchymal stem cells.⁵ In bone tissue healing, macrophages have been shown to be critical for cytokine regulation and the promotion of regeneration: they phagocytose invading foreign materials, restore tissue homeostasis, and recruit progenitor cells while secreting anti-inflammatory factors and growth factors, thereby promoting cell differentiation and angiogenesis and ultimately completing tissue repair.⁶ During the integration of biomaterials with host tissues, macrophages also maintain

tissue homeostasis and assist in repair, while the physicochemical properties of the implant in turn modulate the phenotype and biological behavior of macrophages. Of particular note, osteal macrophages (osteomacs), a subset of macrophages widely distributed in the endosteal and periosteal tissues, account for approximately one-sixth of all cells in the bone marrow.⁷ Therefore, in-depth understanding of the biological functions of macrophages (particularly their specific subsets) and their interactions with biomaterials is of great significance for achieving optimal bone repair and material integration.

The functional diversity of macrophages arises from their high plasticity. Driven by microenvironmental signals, macrophages can switch from the classical pro-inflammatory M1 phenotype to the alternatively activated M2 phenotype.⁸ M2 macrophages are typically induced by IL-4 or IL-13, highly express molecules such as arginase-1, CD206, and Fizz1, and exhibit properties such as suppressing inflammation, promoting angiogenesis, and initiating tissue repair.⁹ In recent years, EVs have emerged as important mediators for macrophages to exert long-range regulation, fundamentally changing our understanding of the osteoimmune microenvironment. Critically, M2-EVs have been shown to be effective in promoting bone regeneration and angiogenesis.¹⁰

In osteogenesis-oriented research, M2-EVs have been shown to directly or indirectly promote bone formation. On one hand, M2-EVs deliver specific microRNAs or functional proteins to target and regulate the osteogenic differentiation of mesenchymal stem cells (MSCs) and osteoprogenitors.¹⁰ For example, miR-26a enriched in M2-EVs suppresses the expression of glutamate dehydrogenase (GLUD1), thereby activating the activity of Runx2, a key osteogenic transcription factor.¹¹ Other studies have shown that BMP-2 or related signaling molecules carried by M2-EVs can directly induce MSCs to differentiate toward the osteogenic lineage and promote calcium nodule formation and matrix mineralization. On the other hand, M2-EVs provide a favorable immune microenvironment for bone healing by inhibiting local excessive inflammation (e.g., reducing IL-1 β and TNF- α levels) and promoting the polarization of macrophages toward the M2 phenotype. Moreover, M2-EVs possess potent pro-angiogenic capacity (e.g., delivering miR-21, VEGF, etc.), thereby accelerating bone defect repair by coupling angiogenesis with osteogenesis.¹²

In the fields of bone tissue engineering and biomaterial integration, M2-EVs show great potential as a cell-free therapeutic strategy. Loading M2-EVs onto biomaterials (such as hydrogels, coatings, or scaffolds) significantly improves the osseointegration of implants: they guide host macrophage polarization toward the M2 phenotype, reduce foreign body reactions, and continuously recruit endogenous MSCs to differentiate toward the osteogenic lineage.¹³ In vivo studies have confirmed that local delivery of M2-EVs effectively repairs bone defects in rat calvarial and femoral condyle models, with both new bone formation and bone mineral density significantly increased.

There are currently many reviews on EVs, but they mostly focus on applications in inflammatory diseases and regenerative medicine. Moreover, in terms of engineering, they also highlight the roles in controlling inflammation and tissue regeneration, as well as the underlying therapeutic mechanisms, without specifically addressing osteogenesis. This review systematically outlines the biogenesis mechanisms of M2-EVs, their key osteogenic molecules (miRNAs, proteins, etc.), and their specific roles in the regulation of the osteoimmune microenvironment, osteogenic differentiation of mesenchymal stem cells (MSCs), angiogenesis, and the osseointegration of biomaterials. We further discuss the interaction mechanisms between M2-

EVs and the osteomac network, summarize current preclinical research progress on the use of M2-EVs in bone regeneration medicine, and analyze challenges such as standardization of production, heterogeneity resolution, and long-term safety. Although many of the current experiments are primarily based on in vitro and rodent models, they still help us gain a deeper understanding of the regulatory mechanisms of M2-EVs in the bone formation process, and are expected to provide a new theoretical foundation and engineering strategies for designing next-generation osteogenic biomaterials and achieving efficient acellular bone repair.

EXTRACELLULAR VESICLES

Structure and composition

Currently, research on EVs is highly active. As a key subtype of EVs, small EVs (sEVs) are small membrane vesicles containing complex RNA and proteins—specifically disc-shaped vesicles with diameters of 30–200 nm.¹⁴ They are endosome-derived lipid nanoparticles actively secreted via exocytosis by most living cells. Macrophage-derived sEVs, in particular, can regulate the biological processes of bone-related cells by carrying various biomolecules, including proteins and RNAs (especially miRNAs), and they further target and modulate the osteogenic microenvironment through the loading of these miRNAs. Regarding the M2 macrophage-derived sEVs discussed in this article, they contain a variety of active molecules, including miRNAs, lipids, proteins, and others, and express specific markers such as ALIX, CD63, and TSG101. Compared to M1 macrophages, these sEVs exhibit higher expression of anti-inflammatory miR-99a, miR-146b, and miR-378a. Through the transfer of active molecules such as miRNAs, lipids, and proteins, these sEVs facilitate intercellular communication and thereby influence cell behavior and function.

Biogenesis

EVs are generally believed to originate from endosomes, a process that involves a double invagination of the plasma membrane and the formation of intracellular multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs). The ILVs are ultimately released via fusion of MVBs with the plasma membrane and subsequent exocytosis. During the first invagination of the plasma membrane, a cup-shaped structure is formed, which encloses some cell surface proteins and soluble proteins associated with the extracellular environment. This step leads to the de novo formation of early sorting endosomes (ESEs) and, in some cases, may also directly merge with pre-existing ESEs. ESEs can then mature into late sorting endosomes (LSEs) and eventually give rise to MVBs, which are also referred to as multivesicular endosomes. MVBs are formed by inward invagination of the limiting membrane of the endosome—a process that corresponds to the previously mentioned double invagination of the plasma membrane. This process results in MVBs containing multiple ILVs. MVBs can either fuse with lysosomes or autophagosomes for degradation, or fuse with the plasma membrane to release their contained ILVs, which are then known as EVs.¹⁵

Furthermore, studies have also shown that the trans-Golgi network and the endoplasmic reticulum contribute to both the formation and the content of ESEs.¹⁶ EVs, which originate from endosomes, are subsequently generated through interactions with other intracellular vesicles or organelles. Their diverse components, including nucleic acids, proteins, lipids, amino acids, and metabolites, reflect their cellular origin. In various diseases, EVs provide a window into altered cellular or tissue states, and their detection in biological fluids may offer multi-component diagnostic readouts. The efficient exchange of cellular components via EVs informs their application in the design of extracellular vesicle-based therapies. Additionally, engineering approaches can also efficiently extract macrophage-derived EVs, primarily by using physical methods to disrupt and reconstitute the plasma membrane for encapsulating active substances, thereby generating “EV-like vesicles” loaded with target molecules during the membrane extrusion process. This strategy intervenes in the formation process of EVs to achieve their engineering (Figure 1).

Extraction and identification

As mentioned above, increasing attention has been focused on studying the functions of EVs, and a series of studies have been conducted on their

potential use in treating various diseases, such as cancer, cardiovascular, neurological, and musculoskeletal disorders. Recently, scientists have developed many new techniques for extracellular vesicle isolation, including ultracentrifugation, immunomagnetic bead separation, polyethylene glycol precipitation, and size-exclusion chromatography.¹⁷ Next, we will discuss how to isolate, purify, and characterize EVs.

Extraction method. Extracellular vesicle isolation can be achieved through a variety of techniques, each with distinct principles, advantages, and limitations. The choice of method depends on the specific requirements of purity, yield, throughput, and downstream applications. The following section describes commonly used approaches: ultracentrifugation, immunomagnetic bead separation, polyethylene glycol (PEG)-based precipitation, size-exclusion chromatography (SEC), ultrafiltration, and microfluidics.

Ultracentrifugation is the most commonly used method and a classic approach for extracellular vesicle isolation. This technique exploits differences in size and density between EVs and contaminants, using gradient centrifugation to achieve separation by differential sedimentation (Figure 2). Typically, it is combined with sucrose density gradient centrifugation or a sucrose cushion to isolate low-abundance EVs, requiring centrifugation at 100 000–200 000 × *g* for 120 minutes to enrich EVs within a density range of 1.13–1.19 g/mL.¹⁸ While the method offers simple operation and relatively high yield, it has drawbacks including high equipment cost, limited throughput, time-consuming protocols, and possible extracellular vesicle aggregation. To improve purity, some researchers incorporate additional sucrose density gradient centrifugation, though this adds cumbersome preparatory steps and may introduce chemical contaminants that are difficult to remove.¹⁹ Immunomagnetic bead separation utilizes monoclonal antibodies against extracellular vesicle-associated antigens—such as CD9, CD81, CD63, CD82, Hsp70— that specifically bind to surface markers, enabling selective isolation.²⁰ The procedure involves incubating antibody-coated beads with EVs, followed by centrifugation, washing, and resuspension in PBS buffer. Because marker abundance varies with extracellular vesicle heterogeneity, specific antibody combinations can capture distinct subtypes. This approach ensures high specificity and intact morphology while eliminating the need for expensive equipment; however, variations in solvent pH and concentration may compromise bioactivity, and the beads themselves are costly. In addition, polyethylene glycol (PEG)-based precipitation is another widely used method: PEG, a hydrophilic polymer, alters extracellular vesicle solubility and dispersity, causing precipitation. It is simple, requires no specialized equipment, and offers high yield, but may co-precipitate non-exosomal hydrophobic substances and proteins, resulting in lower purity. Pre-treating samples with low-speed centrifugation, incubating with PEG at 4 °C overnight, and then applying SEC or filtration can effectively remove contaminants and improve purity.²¹ Size-exclusion chromatography (SEC) isolates EVs based on size rather than molecular weight (Figure 2). Using a porous gel bead matrix, large molecules (including EVs) elute first while smaller molecules are retained longer, allowing high-purity separation with excellent preservation of structure and function.²² However, loading volume must be minimized to maintain efficiency. In practice, SEC is often combined with other methods to further enhance purity. Ultrafiltration (centrifugal ultrafiltration) employs membranes with different molecular weight cutoffs to selectively retain EVs while allowing smaller molecules to pass through (Figure 2).²³ It is simple and preserves bioactivity, but EVs tend to clog filter pores, shortening membrane lifespan and reducing efficiency. To mitigate this, ultrafiltration is often combined with SEC or centrifugation to remove larger aggregates beforehand, thereby improving throughput and purity. Microfluidic-based technologies represent a rapidly evolving frontier in EVs isolation and engineering. These platforms operate on fundamental principles such as laminar flow and nanoscale manipulation, enabling precise and reproducible control over particle transport and separation. By leveraging these micro-scale forces, microfluidic devices integrate various isolation strategies—including immunoaffinity capture, size-based nanofiltration, acoustic separation, and dielectrophoresis—to achieve higher recovery rates, purity, and throughput compared with traditional ultracentrifugation, all while requiring smaller sample volumes and less processing time. Beyond separation, microfluidics also enable precise loading of therapeutic cargo into EVs and surface modification, with integrated bioreactor platforms combining cell culture, EVs

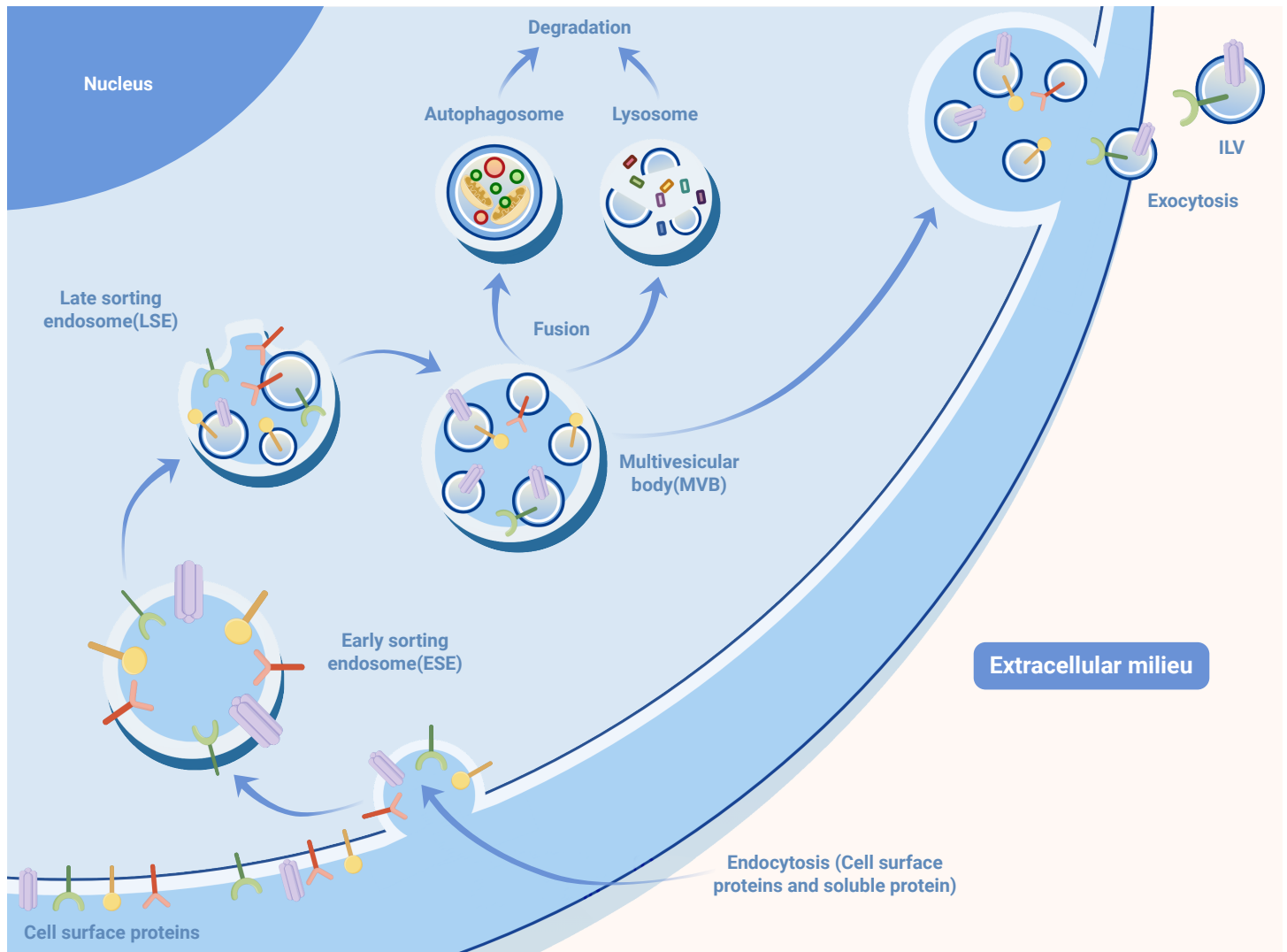


Figure 1. Extracellular vesicle biogenesis: Plasma membrane endocytosis internalizes cell surface and soluble proteins → early sorting endosomes (ESEs) → late sorting endosomes (LSEs) → multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs) MVBs either fuse with lysosomes/autophagosomes for degradation, or fuse with plasma membrane to release ILVs as EVs via exocytosis.

production, capture, loading, and functionalization within closed, automated systems. Despite these advantages, broader adoption remains constrained by high costs, limited scalability, and a lack of standardized protocols. Nevertheless, microfluidic platforms hold particular promise for clinical translation and point-of-care applications, especially when combined with emerging detection technologies such as electrochemical, impedance-based, and optical biosensing modalities for EV-based liquid biopsies (Table 1).^{24,25}

Identification method. Transmission electron microscopy (TEM) is a powerful tool for characterizing nanoparticle morphology. It uses an accelerated and focused electron beam projected onto a very thin sample, causing electrons to collide with atoms and change direction, thereby generating solid-angle scattering. The scattering angle correlates with sample density and thickness, producing images with varying brightness that reveal ultrastructures.²⁶ With a resolution of 0.1–0.2 nm and magnification up to millions of times, TEM can visualize substructures smaller than 0.2 μm—invisible under light microscopy—making it particularly suitable for observing the size and morphology of EVs, which possess a characteristic bilayer envelope. Complementing morphological analysis, nanoparticle tracking analysis (NTA) determines particle size distribution in liquid suspension by exploiting light scattering and Brownian motion. During measurement, an optical microscope directly visualizes nanoparticles, collects scattered light signals, and tracks their Brownian trajectories, providing high resolution even for complex samples. Using NTA, researchers have confirmed that EVs are relatively uniform in size, with diameters ranging from 40 to 180 nm.²⁷

While physical characterization reveals extracellular vesicle size and shape, biochemical identification is essential for confirming their identity. Western blotting (WB) is a standard method that detects hallmark proteins specific to EVs, such as CD9, CD63, CD81, and HSP70. By using specific antibodies to stain proteins separated by gel electrophoresis, WB analyzes staining position and intensity to determine protein expression levels. This technique has confirmed the presence of typical exosomal markers, including CD63 and CD81 (members of the tetraspanin superfamily) as well as TSG101 (a tumor susceptibility gene product).²⁸ Thus, WB not only verifies the exosomal nature of isolated vesicles but also provides quantitative information on their protein cargo, complementing the physical data from TEM and NTA.²⁹

M2 MACROPHAGE-DERIVED EVS

Macrophages

Macrophages (mφ) are white blood cells located within tissues, some originate from monocytes, which in turn originate from hematopoietic progenitor cells in the bone marrow. Both macrophages and monocytes are phagocytes that participate in both innate and adaptive (cellular) immunity in vertebrates. Their primary function is to phagocytose (i.e., engulf and digest) cellular debris and pathogens—either as fixed or free cells—and to activate lymphocytes or other immune cells to mount responses against pathogens. With their diverse functional repertoire, macrophages are key subjects in studies of phagocytosis, cellular immunity, and molecular immunology. These cells arise from monocytes that are released into the peripheral blood after differ-

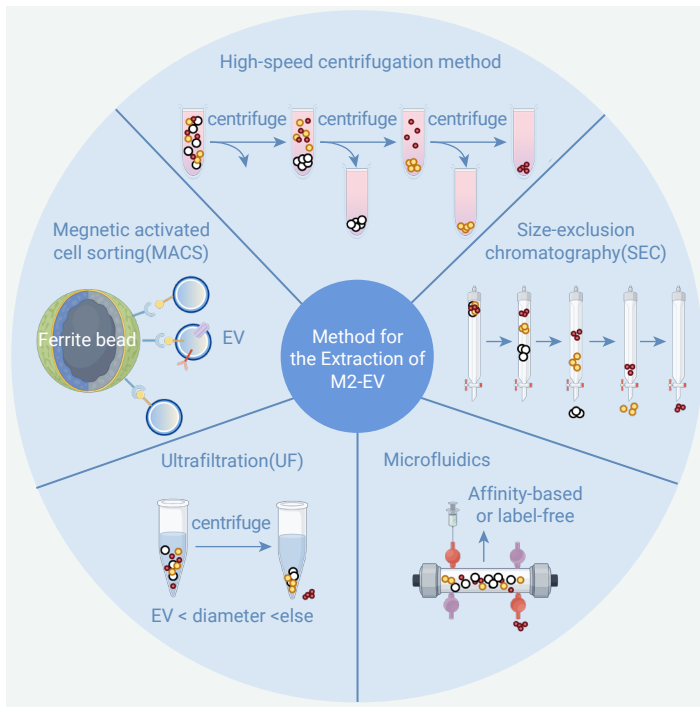


Figure 2. Classic extracellular vesicle isolation methods: high-speed centrifugation method; magnetic-activated cell sorting (MACS); size-exclusion chromatography (SEC); ultrafiltration (UF); microfluidics.

entiation from bone marrow progenitors, and then further differentiate into tissue-resident macrophages, such as dust cells in the lungs, Kupffer cells in the liver, microglial cells in the brain, and osteoclasts in bone tissue.³⁰

Macrophage polarization. Macrophages are highly plastic cells capable of altering their phenotype and function in response to microenvironmental cues in vivo or in vitro, thereby forming a spectrum of functionally distinct macrophage subsets.³¹ The differential expression of surface molecules, secretion of cytokines, and functional characteristics of macrophages under such microenvironmental influences is referred to as macrophage polarization. Based on phenotypic differences and cytokine secretion profiles, macrophages can be classified into classically activated macrophages (M1 type) and alternatively activated macrophages (M2 type). The M2 type can be further divided into three subtypes—M2a, M2b, and M2c—each exerting distinct functions in different biological contexts.

The microbial component lipopolysaccharide (LPS) drives macrophage polarization toward the M1 phenotype, whereas interleukin-4 (IL-4) induces polarization toward the M2 phenotype.³² A key aspect of macrophage polarization is the altered expression of cell surface markers. M1 macrophages overexpress CD80, CD86, and CD16/32, and secrete pro-inflammatory cytokines.³³ In contrast, M2 macrophages show elevated expression of arginase-1 (Arg-1), the mannose receptor (CD206), anti-inflammatory cytokines (e.g., IL-10), and the chemokines CCL17 and CCL22. These M2 cells play important roles in tissue repair, angiogenesis, and metabolism.

For M2 macrophages, they also known as alternatively activated macrophages, primarily secrete anti-inflammatory cytokines such as interleukin-10 (IL-10), transforming growth factor- β (TGF- β), and alternative activation-associated chemokines, thereby inducing a Th2-type immune response, suppressing inflammation, and promoting tissue repair.³⁴ These macrophages are actively recruited to injury sites during the late inflammatory phase, where they release tissue repair signals and exert anti-inflammatory effects, making them crucial for tissue regeneration.^{4,35} The identification of M2 macrophages relies on several surface markers, with CD163 and CD206 being the main ones.³⁶ CD163 is considered highly specific for M2 macrophages, as it is more selective than CD68, another commonly used marker. Additional markers include arginase-1 (Arg1), DECTIN-1, as well as FIZZ1, Ym1, and Ly6C, which are associated with both M1 and M2 subsets.^{37,38} CD206, also known as mannose receptor 1, is a C-type lectin expressed on macrophages and immature dendritic cells; it promotes

macrophage activation, antigen presentation, and immune responses, and is closely linked to tumor cell proliferation and metastasis, with demonstrated associations with breast, ovarian, pancreatic, and prostate cancers. Similarly, CD163, a cysteine-rich scavenger receptor exclusively expressed in the monocyte/macrophage lineage, serves as a marker for M2 tumor-associated macrophages (TAMs) and plays dual roles in counteracting inflammation and promoting tumor progression; its expression is associated with breast, bladder, lung, and colorectal cancers. CD68, a transmembrane glycoprotein highly expressed on monocytes and macrophages, is involved in phagocytosis and may facilitate macrophage homing to specific tissues via binding to selectins; it is also expressed in many tumor cell lines and contributes to metastatic spread, making it a potential marker of tumor metastasis. Recent studies have identified MS4A4A as a myeloid-specific marker that is differentially regulated in M1 versus M2 macrophages, and other novel markers such as CD38, Gpr18, and Fpr2 for M1, and Egr2 and c-MYC for M2, have been proposed. Flow cytometry using CD38 and Egr2 has shown advantages over classical markers like iNOS, Arg1, and CD206 in distinguishing M1 from M2 macrophage subsets.

For M1 macrophages, surface markers such as CD80, CD86, CD64, CD16, and CD32 are commonly used, along with the intracellular marker inducible nitric oxide synthase (iNOS) as a phenotypic indicator. CD80 (also known as B7, B7-1, or BB1) and CD86 (B7-2) are T-lymphocyte activation antigens that synergistically activate T cells, playing key roles in autoimmune surveillance, humoral immunity, and transplant rejection; CD86 also interacts with CD28 and CTLA4 to induce T-cell proliferation and IL-2 production.³⁹ CD64, the high-affinity Fc γ receptor I (Fc γ RI), mediates both innate and adaptive immune responses, binds IgG monomers with high affinity, and has diagnostic value in early infection detection—it is constitutively expressed on monocytes, macrophages, and dendritic cells, but is upregulated on neutrophils upon infection.⁴⁰ CD32, or Fc γ RIIb, is a low-affinity receptor involved in immune complex phagocytosis and regulation of B-cell antibody production, and is expressed on all blood cells except erythrocytes. Macrophage polarization, particularly toward the M2 phenotype, is crucial for immune tolerance but may also promote tumorigenesis; this process is regulated by multiple factors—including cytokines, growth factors, and chemical substances from tumor cells—that affect intracellular signaling pathways. Beyond protein markers, long non-coding RNAs (lncRNAs) also play important roles in monocyte/macrophage differentiation. For instance, lnc-MC, a long non-coding monocyte RNA, is transcriptionally activated by PU.1 and promotes monocyte/macrophage differentiation in THP-1 cells and CD34(+) hematopoietic stem/progenitor cells (HSPCs).⁴¹ Similarly, lncRNA-MM2P was identified as the only lncRNA upregulated during M2 polarization (and down-regulated in M1 macrophages); its knockdown blocks M2 polarization and impairs the angiogenesis-promoting function of M2 macrophages via reduced STAT6 phosphorylation, and its manipulation in macrophages affects tumorigenesis and tumor angiogenesis.⁴² Mechanistically, lnc-MC acts as a competing endogenous RNA that sequesters miR-199a-5p, thereby relieving inhibition of ACVR1B (an important regulator of monocyte/macrophage differentiation); conversely, miR-199a-5p inhibits lnc-MC expression and function, whereas PU.1-mediated downregulation of miR-199a-5p attenuates this inhibition.⁴¹

Macrophage differentiation and recruitment. During bone injury repair, surrounding M1 macrophages are first recruited to the damaged area to initiate the phagocytosis and clearance of injured cells and foreign materials, thereby creating a favorable environment for regeneration. Alternatively, macrophages polarize into the M1 phenotype, which secretes various pro-inflammatory cytokines (including tumor necrosis factor- α , interleukin-1, and interleukin-6) to combat infection, promote osteoclast differentiation, and engulf bone debris.⁴³ As bone tissue healing progresses, the number of M1 macrophages gradually decreases, whereas the number of M2 macrophages increases. M2 macrophages secrete multiple factors such as bone morphogenetic proteins and osteoblastogenesis-related factors, which stimulate the differentiation of bone marrow stromal cells into osteoblasts and promote bone tissue formation. Additionally, M2 macrophages promote osteoblast proliferation and differentiation by secreting various growth factors and cytokines, thereby facilitating bone regeneration.

Effect of M2 macrophages on inflammation. Tissue injury induces an

Table 1. Comparison of six EVs isolation methods in terms of their principles, yield, purity, operational complexity, cost, impact on M2-Exo bioactivity, and suitability for different applications

Method	Principle	Yield	Purity	Operational complexity	Cost	Impact on bioactivity	Suitability for M2-EVs applications
Ultracentrifugation (UC)	Sequential centrifugal forces based on size and density to pellet EVs	Moderate	Moderate	High-requires specialized equipment and careful protocol optimization	Moderate to high	Moderate to high-high shear forces and prolonged processing may affect EVs integrity and surface protein conformation	Large-volume preparations; routine isolation but requires additional purification steps
Immunomagnetic bead separation	Antibody-coated magnetic beads recognizing EVs surface markers (CD9, CD81, CD63)	Low to moderate	High to very high	Moderate-requires antibody selection, incubation, and washing steps	High	Moderate-binding and elution may alter EVs surface properties or reduce recovery	Specific EVs subpopulation isolation; applications requiring high purity and defined subtyping
PEG-based precipitation	Hydrophilic polymer alters EVs solubility, causing precipitation	High	Low	Very low-simple mixing and centrifugation	Low	Low to moderate-but co-precipitated contaminants may interfere with functional assays	Initial screening or large-scale recovery where high purity is not critical; often combined with post-purification steps
Size-exclusion chromatography (SEC)	Porous gel beads separate particles by size; larger EVs elute first	Moderate	High	Low-column equilibration and collection of fractions	Moderate	Low-gentle, preserves EVs structure and bioactivity	Bioactivity-sensitive applications; removal of soluble contaminants
Ultrafiltration (UF)	Membrane with defined molecular weight cutoff retains EVs; small molecules pass through	Moderate	Moderate	Low-membrane selection and centrifugation steps	Moderate	Low-generally preserves bioactivity, but membrane clogging may reduce yield	Concentration, desalting, and buffer exchange; often used as a pre-concentration step
Microfluidics	Laminar flow and micro-scale forces	Moderate to high	High	High-requires specialized chips and equipment; limited standardization	High	Low-mild conditions preserve EVs integrity and cargo activity	Small sample volumes; clinical and point-of-care applications; integrated analysis and engineering; emerging as a versatile platform

inflammatory response, which recruits immune cells such as macrophages and neutrophils, promotes their proliferation and differentiation, and activates them, thereby achieving tissue repair.⁴⁴ In the case of minor tissue injury, the inflammatory response resolves quickly, allowing the body or the affected organ or tissue to recover its normal function. However, when injury is severe or when repeated injury occurs within a short period of time, chronic inflammation develops at the injured site, which may lead to organ fibrosis, progressive loss of normal tissue function, and ultimately organ failure; in severe cases, it may even result in death.⁴⁵ Therefore, inflammatory responses during tissue repair and regeneration must be tightly controlled to avoid the development of chronic inflammation as much as possible. Among the various immune cells involved in the inflammatory response to tissue injury as mentioned above, macrophages have been shown to be key players in both the inflammatory response and organ fibrosis.⁴⁶ Owing to the important roles of macrophages in tissue injury-induced inflammation and fibrosis, researchers in recent years have become increasingly interested in the contribution of different macrophage subsets to these processes.

During the inflammatory response, M1 and M2 macrophages exert distinct functions and can transition from one phenotype to the other. In the early stage of inflammation, M1 macrophages release pro-inflammatory cytokines, recruit and activate adaptive immune cells, induce Th1 and Th17 responses, and promote inflammation to eliminate pathogens, although this may also cause damage to normal host tissues. In the resolution phase of inflammation, these cells convert to the M2 phenotype, which counteracts the inflammatory response, induces Th2 responses, and participates in tissue healing and repair.⁴⁷ Furthermore, macrophage polarization is also widely involved in

the onset and progression of tumors, metabolic diseases, and autoimmune disorders. A deeper understanding of macrophage polarization and its underlying molecular mechanisms in these diseases may provide new targets for their control and prevention.⁴⁸ In parallel, studies have shown that EVs secreted by macrophages ameliorate the inflammatory response of recipient cells by modulating the levels of cytokines and microRNAs (miRNAs).

Meanwhile, studies have shown that EVs secreted by macrophages ameliorate inflammation by modulating cytokines and microRNAs, while M2 macrophages themselves suppress local inflammatory responses, improve the tissue microenvironment, and promote wound repair. Because of the detrimental effects of M1 macrophages in late-stage inflammation, M1 cells are often induced to polarize into the M2 phenotype during this phase, and M2 macrophages secrete anti-inflammatory mediators such as IL-4, IL-10, and TGF- β , thereby counteracting inflammation and inducing Th2 responses that participate in tissue healing. In addition, cytokines from M2 macrophages antagonize M1 macrophages: IL-1 receptor antagonist inhibits IL-1's pro-inflammatory effect; TGF- β induces regulatory T-cell differentiation; and IL-10 interrupts antigen presentation by APCs and inhibits IL-12/23 synthesis, thereby affecting Th1 and Th17 differentiation. Researchers have proposed that the transition of macrophages from a pro-inflammatory to an anti-inflammatory phenotype marks the shift from the inflammatory phase to the proliferative phase during wound healing.⁴⁹ However, M1 macrophages also play a significant role in inflammation: they release pro-inflammatory cytokines such as IL-1, IL-6, IL-12, and TNF- α , recruiting and activating neutrophils, macrophages, and NK cells to the inflammatory site, thereby inducing Th1 and Th17 responses and contributing to wound repair.⁵⁰ At the

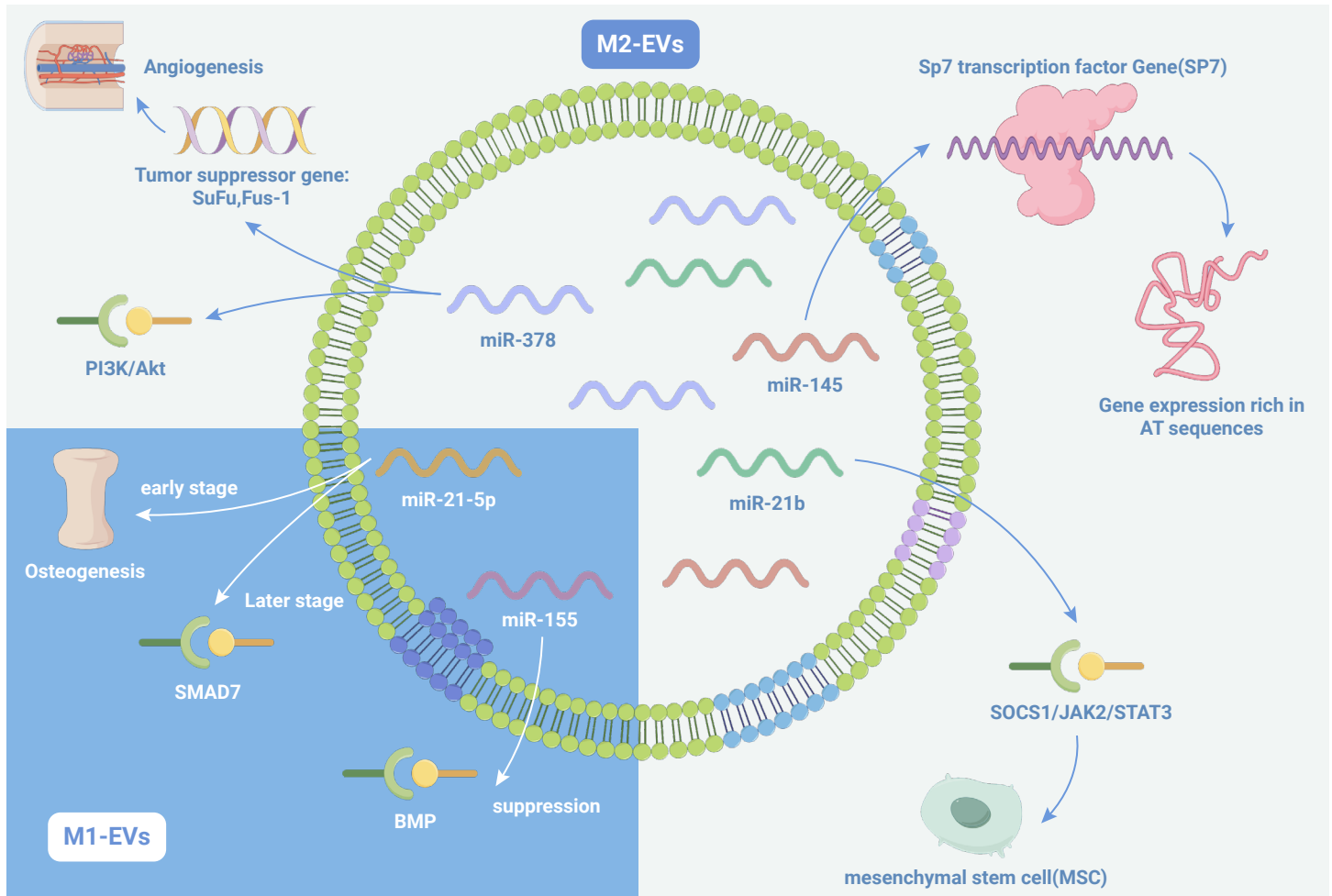


Figure 3. Osteogenic role of miRNAs: miR-378a positively regulates osteogenesis, promotes MSC osteogenic differentiation, and is highly enriched in M2-EVs It also promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1, and attenuates high glucose-suppressed osteogenic differentiation via CASP3 targeting and PI3K/Akt activation. miR-223 directly targets SP7 (osterix) to promote osteoblast differentiation and bone formation. miR-21-5p is released from M1-EVs in the context of fracture combined with traumatic brain injury and promotes early osteogenesis; pre-injection of TBI-EVs enhances osteogenic differentiation of BMSCs by targeting the SOCS1/JAK2/STAT3 signaling axis.

same time, however, macrophages also release large amounts of TNF and IL-1, which can trigger systemic inflammation, acute-phase reactions, and tissue damage; moreover, M1 macrophages promote nitric oxide synthesis through ROS release, exacerbating injury. Thus, M1 macrophages exert diametrically opposite effects in early versus late inflammation: in the early stage, they mount immune responses, phagocytose necrotic tissue, present antigens, and exert anti-infective effects to repair damage, whereas in the late stage, their pro-inflammatory action inhibits wound healing.⁵¹ It should be noted that in the early stage of bone healing, the presence of pro-inflammatory macrophages is indispensable for initiating the recruitment of osteoprogenitors and subsequent angiogenesis, rather than merely serving as a detrimental population to be swiftly eliminated.

M2 macrophage-derived exosomal miRNAs

EVs secreted by both M0 and M2 macrophages enhance the promoter activity of osteoinductive genes in MSCs to varying degrees, and studies have demonstrated that macrophage-derived EVs exert their regulatory effects via the BMP2 signaling pathway.⁵² Among these, M2-EVs highly express anti-inflammatory miR-99a, miR-146b, and miR-378a, which can target the IRS-1/TAZ and SIK2/SIK3 pathways to promote BMSC osteogenic differentiation, while also suppressing inflammation through NF- κ B and TNF- α signaling and promoting M2 polarization of recipient macrophages. Among these, miR-378a acts as a positive regulator of osteogenesis—in contrast to miR-155, which downregulates osteogenic differentiation—and is highly enriched in both M0 and M2-EVs, with M2-EVs showing even higher levels.^{53,54} Mechanistically, miR-378 promotes cell survival, tumor growth, and angiogenesis by

targeting SuFu and Fus-1, and its overexpression attenuates high glucose-suppressed osteogenic differentiation through targeting CASP3 and activating the PI3K/Akt pathway (Figure 3).^{55,56} Another pro-osteogenic miRNA, miR-145, promotes osteoblast differentiation and bone formation by directly targeting SP7 (Figure 3).⁵⁷ Furthermore, miR-26a-5p promotes osteogenesis and inhibits adipogenesis in BMSCs, and miR-21b enhances BMSC osteogenic differentiation via the SOCS1/JAK2/STAT3 axis (Figure 3).

In contrast, M1 macrophages exert opposing effects: co-culture of M1 macrophages with MSCs negatively regulates BMP2, BMP9, and RUNX2, thereby inhibiting MSC osteogenic differentiation. This suppression is mediated by M1-derived extracellular vesicles (M1-EVs), which carry specific miRNAs that directly affect the BMP cascade. For instance, miR-155 is highly expressed in M1-EVs and impairs osteoblast differentiation by selectively inhibiting BMP signaling and by directly targeting the SMAD5 pathway to suppress osteoblast proliferation and differentiation (Figure 3). However, the role of M1-EVs is not exclusively inhibitory: in a mouse fracture model, miR-21-5p released from M1-EVs in the context of trauma combined with traumatic brain injury promoted early osteogenesis, and pre-injection of TBI-EVs enhanced osteogenic differentiation, indicating that certain miRNAs in M1-EVs can also support bone formation under specific conditions (Figure 3).⁵⁸ Beyond osteogenesis, miR-93-5p—highly abundant in therapeutic EVs for osteonecrosis of the femoral head (ONFH)—reduces neutrophil extracellular trap formation and enhances endothelial cell angiogenic capacity, further expanding the functional repertoire of exosomal miRNAs in bone repair.⁵⁹

Other factors affecting extracellular vesicle function

Recent studies have shown that H₂S is an important regulator of macrophage polarization, promoting the shift toward the M2 phenotype and altering the protein profile of M2-EVs, leading to high enrichment of moesin—a protein that controls clathrin-mediated endocytosis of S1PR1 in T cells and facilitates extracellular vesicle uptake into mesenchymal stem cells (MSCs), thereby accelerating osteogenesis.⁶⁰⁻⁶² In parallel, engineering approaches have been developed to enhance extracellular vesicle functionality: genetic modification techniques can load target gene expression vectors into cells, which then secrete EVs displaying targeting peptides or carrying specific genes; alternatively, chemical and mechanical methods—such as electroporation, extrusion, sonication, incubation, bioconjugation, and click chemistry—can be applied directly to isolated EVs, achieving high loading efficiency with relative ease. Despite the broad promise of EVs in bone regeneration, their clinical translation is hindered by inherent limitations, including susceptibility to degradation and clearance. To overcome these barriers, Wu et al. recently developed macrophage-derived calcium phosphate nanoparticle-immobilized small vesicle nanohybrids (M2EV@CaP). In this system, the chemically inert CaP nanoparticle shell provides structural protection for M2-EVs, inhibits aggregation caused by membrane protein denaturation or lipid phase transition, and acts as a physical barrier. Moreover, M2EV@CaP supplies calcium/phosphorus ions and signaling molecules essential for bone regeneration, and subsequent *in vitro* and *in vivo* experiments confirmed that it promotes osteogenesis via the Akt signaling pathway in diabetic rats, demonstrating a promising strategy to address the delivery and stability challenges of extracellular vesicle-based therapies.⁶³

MACROPHAGE-DERIVED EVS IN OSTEOGENESIS

Application of M2-EVs in tissue engineering

Currently, with the growing body of research on EVs, they have been widely recognized as a new direction for the treatment of various diseases. Therefore, a major challenge in their application is how to maintain the retention and stability of EVs in the body over time after transplantation. To address this, numerous studies have combined EVs with different forms of tissue engineering materials, such as hydrogels, nanoparticles, scaffolds, and conduits to promote tissue repair and regeneration, including bone tissue regeneration, muscle regeneration, skin wound healing, angiogenesis, and peripheral nerve regeneration. Moreover, as the focus of this review, M2-EVs have been applied in tissue engineering mainly through their regulatory effects on cell proliferation, apoptosis, and invasion, as well as their promotion of angiogenesis and neointimal repair after scaffold implantation. Furthermore, these EVs exert significant effects on cell behavior, promoting cell proliferation and invasion while inhibiting apoptosis, and these effects are likely related to specific miRNAs carried by the EVs, which play important roles in regulating cell proliferation, apoptosis, and invasion.⁶⁴

In addition, M2-EVs have shown positive effects on angiogenesis and neointimal repair after scaffold implantation. Studies indicate that M2 macrophages influence the dedifferentiation of vascular smooth muscle cells through the secretion of EVs, a process accompanied by cytoskeletal depolymerization and decreased cell stiffness. Exogenous administration of M2-EVs accelerates vascular re-endothelialization and promotes vascular repair following stent injury. These findings suggest that M2-EVs hold potential therapeutic value in the treatment of cardiovascular diseases, providing new therapeutic targets and insights for percutaneous coronary intervention (PCI).⁶⁵

Role of macrophages in bone regeneration

Mata et al. demonstrated that the inflammatory microenvironment is a prerequisite for the differentiation of BMSCs into osteoblasts, and that macrophages play a key role in this early inflammatory microenvironment.⁶⁶ This established that macrophages can regulate the inflammatory microenvironment to control osteogenesis. Meanwhile, Champagne et al. showed that macrophages may promote bone healing by providing osteoinductive and osteogenic cytokines within the bone trauma healing environment.⁶⁷ As early as a decade ago, Pirraco et al., in their study on the effect of macrophages on the early osteogenic differentiation of BMSCs, confirmed that peripheral blood macrophages can influence the metabolism of BMSCs. The underlying mechanism may involve bone morphogenetic proteins

(BMPs) produced by macrophages, which participate in and promote BMSC proliferation, thereby making them more prone to differentiate into osteoblasts and thus enhancing osteogenesis.⁶⁸

The process of bone regeneration. Bone healing is a complex and dynamic process involving the interplay of multiple cell types and molecular signaling pathways. In addition to cells of the immune system, there are cell lineages that directly contribute to bone regeneration: the skeletal cell lineage includes osteoblasts, osteocytes, and chondrocytes, which maintain and repair bone during homeostasis and after injury, while osteoclasts—responsible for bone resorption—originate from the hematopoietic lineage. The body maintains normal bone homeostasis through a balance between osteoblast and osteoclast activities, and mesenchymal stem cells, skeletal stem cells, and others also participate in bone regeneration. With the advancement of osteoimmunology, a growing body of evidence indicates that bone regeneration is not merely a process of bone formation and resorption, but rather a close interaction between multiple systems, including the skeletal and immune systems.⁶⁹

After bone trauma, niche-related cells respond to changes in the microenvironment, leading to precursor cell differentiation into osteoblasts and subsequent tissue repair. Following acute injury, inflammatory cells are the first to be activated, resulting in hematoma formation characterized by hypoxia and low pH, which promotes macrophage and neutrophil infiltration.⁷⁰ Macrophage infiltration leads to secretion of pro-inflammatory chemokines that, through interactions with osteoprogenitors, promote osteogenic differentiation and generation of the osteoblast lineage.⁷¹ Bone regeneration and repair—similar to embryonic bone development—can be divided into intramembranous ossification (direct differentiation of mesenchymal aggregates into bone) and the more complex endochondral ossification, which requires a cartilage intermediate. In endochondral ossification, mesenchymal cells differentiate into chondrocytes that secrete cartilage matrix, while surrounding cells form the perichondrium; boundary cells exit the cell cycle, undergo hypertrophy, and promote osteoblast differentiation.⁷² In parallel, vascular remodeling is essential: newly formed vasculature delivers nutrients, and during endochondral ossification, blood vessels infiltrate hypertrophic cartilage, triggering osteoblast differentiation and marrow cavity formation. Vascularization also recruits cartilage-resorbing cells and osteoprogenitors, and allows perichondrial cells to enter the marrow cavity. Endothelial cells mediate vessel repair and interact with osteoprogenitors to generate a supportive microenvironment; in particular, type H endothelial cells—present in the metaphysis and endosteum of postnatal long bones—express PECAM1 and endomucin and provide Notch signals to osteoprogenitors, and disruption of this signaling reduces bone formation by decreasing Spp1, Runx2, and Osx expression.⁷²

Finally, both sensory and sympathetic nerves play crucial roles in skeletal homeostasis and repair. Nerve growth factor (NGF), a neurotrophin involved in nerve development and regeneration, was shown in a rabbit mandibular distraction osteogenesis model to reduce the consolidation period of bone repair when applied exogenously, indicating that NGF naturally secreted by nerves within the bone niche promotes osteogenesis.⁷³ This model also revealed elevated NGF and VEGF expression during bone repair, with NGF from nerve cells and VEGF from Schwann cells.⁷⁴ Collectively, these findings indicate that nerve cells and Schwann cells in the bone niche support osteoprogenitor differentiation into osteoblasts, thereby promoting new bone formation. In summary, the commitment of the osteoblast lineage depends on an appropriate local environment that provides the necessary signals to induce angiogenesis and ultimately form the bone matrix for new bone formation.

Osteoblast Cytokines. Several macrophage-derived molecules have been identified as key regulators of osteogenesis. Oncostatin M (OSM), a cytokine of the IL-6 family produced by monocytes and macrophages, plays an important role in promoting osteogenic differentiation: in 2012, Guihard et al. demonstrated that macrophage-derived OSM binds to the oncostatin M receptor (OSMR) on mesenchymal stem cells (MSCs), activating signal transducer and activator of transcription 3 (STAT3) and thereby inducing osteogenic differentiation and matrix mineralization of MSCs, with subsequent work by the same team further confirming that OSM promotes osteogenic differentiation of mesenchymal progenitor cells via OSMR and

STAT3 signaling.^{75,76} Similarly, prostaglandin E2 (PGE2), a member of the prostaglandin family also produced by macrophages within bone, is an important regulator of bone metabolism. In 2011, Ninomiya et al. injected a selective agonist of the EP4 receptor (a PGE2 receptor subtype) into rats and observed a marked increase in bone formation, indicating that PGE2 exerts its regulatory effects mainly through EP4.⁷⁷ In 2017, Lu et al. further showed that co-culture of MSCs with macrophages for four weeks regulated PGE2 secretion during early osteogenesis, thereby enhancing bone mineralization.⁷⁸

In addition, BMP-2, which is known to be produced by various cell types, including macrophages, has been shown to activate the intracellular signaling molecule SMAD in osteoblasts and further promote the expression of *Dlx5* and *Runx2*, thereby enhancing the osteogenic differentiation of BMSCs.⁷⁹ In contrast, tumor necrosis factor- α (TNF- α), a pro-inflammatory cytokine mainly secreted by macrophages, exhibits a more complex role. It is an important regulator of bone remodeling: Cho et al. found that TNF- α enhances osteogenesis by activating NF- κ B and increasing transcriptional coactivators with a PDZ-binding sequence, while Briolay et al. reported that TNF- α significantly elevates *Wnt5a* levels in MSCs, promoting osteogenesis, and that blocking *Wnt5a* attenuates osteoblast mineralization.^{80,81} However, contrary to these pro-osteogenic effects, some studies have shown that TNF- α can also inhibit osteogenic differentiation, and subsequent evidence indicates that macrophages influence bone formation through TNF- α in a concentration-dependent manner, with different outcomes depending on its local levels.

Macrophage-mediated bone regeneration. As mentioned above, multiple local and systemic factors, including inflammation, influence bone regeneration, and evidence suggests that macrophages contribute to the immunomodulation of MSC and osteoblast functions during this process, with recent studies indicating that macrophage polarization further modulates this regulation. Specifically, in fracture healing, resident macrophage populations in the periosteum and on its surface play a key role in intramembranous ossification, whereas recruited inflammatory macrophages are important for endochondral ossification; the influx of these inflammatory cells leads to the secretion of chemokines such as IL-6 and CCL2, and together with BMP4 and VEGF, these factors are released into the microenvironment at the injury site, where they promote osteogenic differentiation through interactions with newly recruited osteoprogenitors, which subsequently differentiate into the osteoblast lineage to accomplish bone repair. Furthermore, specialized macrophages termed "osteal macrophages", located in the canopy structure above the osteoblast layer on the bone surface, directly contribute to bone repair, as experimental depletion of these cells has revealed reduced osteoblast coverage and decreased bone formation, and macrophage reduction has also been shown to impair intramembranous bone repair and reduce growth associated with a decrease in mesenchymal progenitor osteoblasts.^{82,83} Moreover, emerging studies indicate that selected miRNAs from M1 and M2 macrophage-derived EVs can differentially regulate osteoinduction *in vitro*; however, further research is needed to determine the impact of these individual miRNAs on bone regeneration in the context of corresponding injured tissue models.⁸⁴

Pathways related to M2 macrophage polarization and osteogenesis

To understand the osteogenic mechanisms of M2-EVs, extensive studies have been conducted in recent years on the osteogenic pathways associated with macrophages, along with investigations into certain pathways involved in macrophage polarization. However, three signaling pathways—AMPK-mTOR, Notch, and MAPK—have received considerable attention in recent years. Other pathways involved include TGF- β /Smad, JAK/STAT, PI3K/AKT, Wnt, AMPK, HIF, and RhoA/ROCK.

The MAPK pathway consists of four branches: ERK1/2, JNK, ERK5, and p38MAPK.⁸⁴ Among these, the JNK pathway plays the most important role in macrophage polarization and subsequent secretion of inflammatory cytokines, primarily by increasing p-JNK and the downstream transcription factor c-Myc to alter the polarization phenotype, and relevant studies have also shown that JNK signaling is key in M2 macrophage-mediated osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) (Figure 4).⁸⁵ Meanwhile, the p38 and ERK branches regulate osteoblast proliferation, differentiation, and apoptosis by modulating cell cycle regulators and

Runx2 activity.⁸⁶ In parallel, the TGF- β /Smad pathway is another critical signaling cascade: TGF- β (existing as three isoforms, TGF- β 1, - β 2, and - β 3) chemoattracts osteoblasts, influences their proliferation, enhances BMSC differentiation into osteoblasts, and accelerates bone healing. Signal transduction is mediated by a transmembrane receptor complex with serine/threonine kinase activity, where T β RII phosphorylates T β RI, which then transmits signals to the nucleus via Smad protein complexes (Figure 4). Furthermore, the TGF- β /Smad pathway regulates macrophage polarization toward the M2 phenotype, leading to increased TGF- β secretion, which in turn activates Smad signaling and upregulates osteogenic marker genes such as *RUNX2*, thereby promoting osteoblast proliferation and enhancing BMSC osteogenic differentiation.^{87,88}

Regarding the AMPK-mTOR pathway, Byles et al. demonstrated that loss of tuberous sclerosis complex 1 (TSC1) leads to mTORC1 activation, which inhibits IL-4-induced M2 polarization of macrophages, whereas Han et al. showed that everolimus (an mTOR inhibitor) suppresses mTOR and increases Akt activity, thereby promoting M2 macrophage polarization.^{89,90} Meanwhile, activation of the JAK/STAT signaling pathway by various cytokines and growth factors *in vivo* triggers immune responses that lead to osteoblast proliferation and differentiation, and the JAK/STAT inhibitor AG490 has been shown to significantly suppress M2 polarization, further confirming the osteogenic role of this pathway.^{91,92} In addition, PI3K/Akt not only regulates bone growth and formation but also influences macrophage survival; external stimuli activate PI3K and differentially regulate Akt1 and Akt2, with Akt1 upregulating miR-155 expression, which activates the RelA/NF- κ B pathway and downregulates *SOCS1* transcription, leading to increased expression of pro-inflammatory macrophage genes.⁹³ Collectively, these three pathways—AMPK-mTOR, JAK/STAT, and PI3K/Akt—interconnect to modulate macrophage polarization, inflammatory responses, and osteogenesis, highlighting their integrated roles in bone metabolism and immune regulation.

In addition, the Wnt signaling pathway—which mainly includes the Wnt/ β -catenin pathway, the intracellular pathway for asymmetric cell division, the Wnt planar cell polarity pathway (regulating spindle orientation), and the Wnt/Ca²⁺ pathway—plays diverse roles in osteogenesis and cell regulation.⁹⁴ Furthermore, the HIF signaling pathway, mediated by an $\alpha\beta$ heterodimeric transcription factor composed of three α subunits and one β subunit, is activated under hypoxic conditions and stimulates the transcription of multiple genes, thereby mediating chemotactic recruitment, differentiation, and angiogenesis of mesenchymal stem cells.⁹⁵ Moreover, the RhoA/ROCK signaling pathway involves RhoA, a guanosine triphosphatase (GTPase) that participates in the composition of RhoA, Rac1, and CDC42; increased levels of these molecules in turn promote the polarization of macrophages toward the M2 phenotype, further linking cytoskeletal dynamics to immune modulation.⁹⁶

M2 MACROPHAGE-DERIVED EXTRACELLULAR VESICLE-FUNCTIONALIZED SCAFFOLDS FOR BONE REGENERATION

Currently, tissue engineering has been applied in various clinical settings, including wound healing, corneal regeneration, bone repair, and even certain visceral diseases such as heart valve repair.⁹⁷ Bone tissue engineering, specifically for bone repair, offers advantages such as rapid bone growth, abundant raw material supply, low immunorejection rate, and low infection rate.⁹⁸ The benefits of bone repair are further enhanced when EVs derived from macrophages or other sources are incorporated into appropriate bone repair materials via various delivery routes.⁹⁹ As scaffold materials in bone tissue engineering, EVs also possess many excellent properties that support their widespread application, including good biocompatibility, biodegradability, and excellent interfacial properties.¹⁰⁰ Moreover, immobilizing EVs onto the biomaterials used in bone tissue engineering not only provides a stable platform for the EVs but also circumvents many of the issues associated with cell-based approaches. The relationship between scaffold materials and EVs in bone tissue engineering extends beyond a simple drug-carrier paradigm. Rather than viewing scaffolds merely as passive delivery platforms, we propose that a bidirectional synergistic interplay exists between the material and the EVs. The following section briefly introduces the current types of scaffolds used in bone tissue engineering.

Inorganic materials

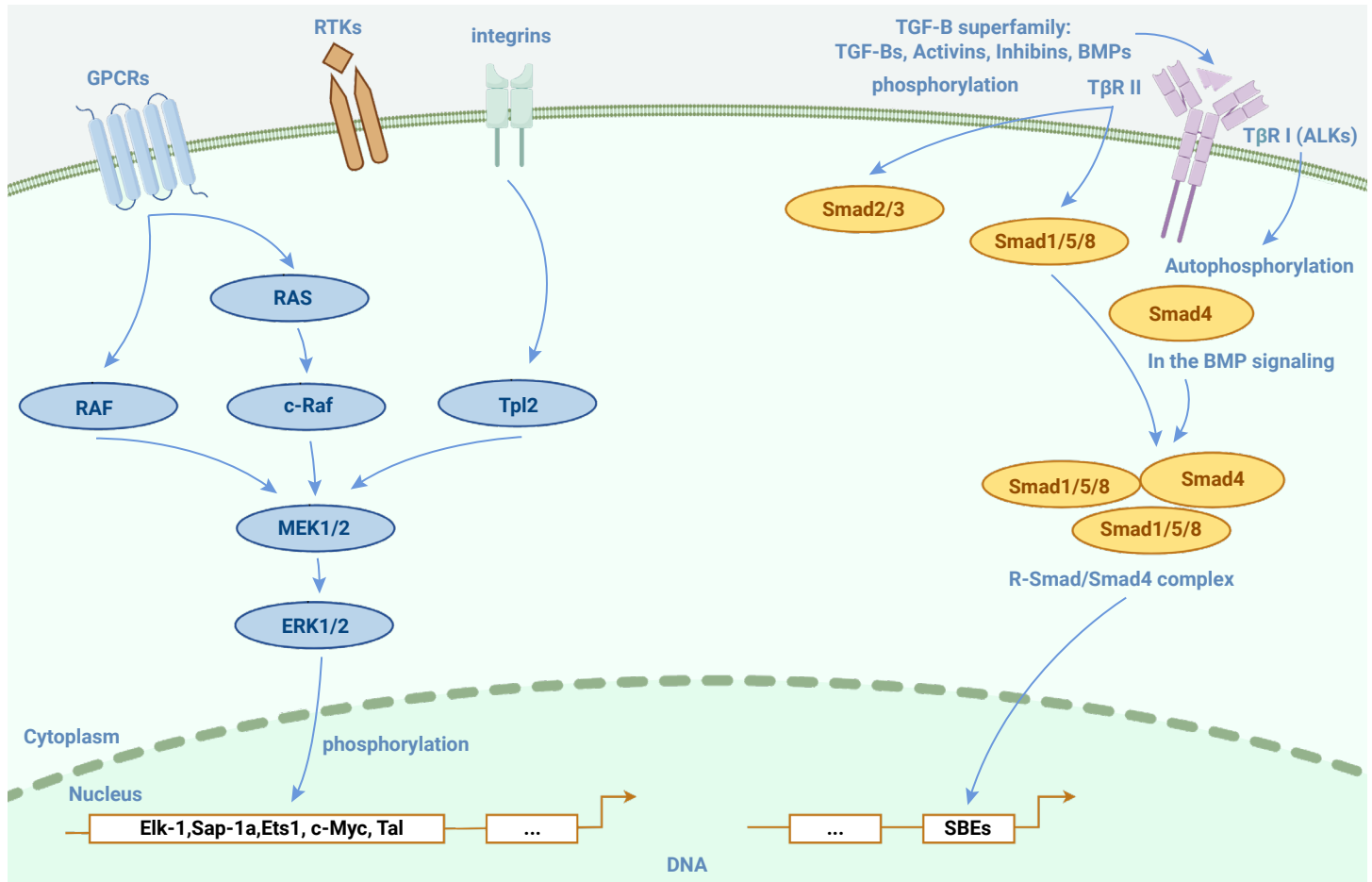


Figure 4. MAPK signaling pathway and TGF-β/Smad signaling pathway involved in osteogenesis

β-Tricalcium phosphate (β-TCP) is compositionally similar to human autologous bone and offers advantages such as abundant sources, high safety, easy storage, sterility, and degradability. It has the ability to release calcium ions in vivo, thereby maintaining a balance between material degradation and osteogenesis and promoting bone regeneration.¹⁰¹ Subsequently, researchers have modified β-TCP scaffolds with EVs derived from human induced pluripotent stem cell mesenchymal stem cells (hiPS-MSC-EVs), and studies have shown that, compared with pure β-TCP scaffolds, the extracellular vesicle-modified β-TCP scaffolds exhibit increased expression of the PI3K/Akt signaling pathway and display higher osteogenic activity (Figure 5A).¹⁰² Although the EVs loaded onto this scaffold are not derived from macrophages, they may still serve as a methodological reference. Similarly, hydroxyapatite (HAP), which is a major mineral component of human bone tissue, is generally regarded as possessing favorable biocompatibility and osteogenic potential. It is typically associated with minimal toxicity, low inflammatory reactions, and negligible foreign body responses in vivo. However, HAP also has some disadvantages: while it exhibits high hardness, it is brittle and has poor fracture toughness; in addition, HAP materials have a long degradation time and poor cell adhesion.^{103,104} To address these limitations, researchers have attached EVs derived from human mesenchymal stem cells (hMSCs) onto classic HAP scaffolds stimulated with low-density dimethyloxylylglycine (DMOG) (Figure 5B). This approach activates the AKT/mTOR pathway, promotes the regeneration of human umbilical vein endothelial cells (HUVECs), and further enhances the osteogenic capacity of HAP scaffolds.¹⁰⁵

Organic materials

Currently, many scaffolds are fabricated by loading EVs onto biomolecules. Such materials generally exhibit good adaptability and targeting properties. An example is a recently developed scaffold based on aptamer loading. Aptamers (Apts) are functional nucleic acids that can serve as recognition

ligands with high specificity and affinity. In this study, an engineered extracellular vesicle-modified biomimetic periosteum termed PEC-Apt-NP-EV was designed. Amino- and cholesterol-modified BMSC aptamers were conjugated with M2-EVs via a three-way junction (3WJ) platform to generate engineered EVs, which were then covalently linked to the ECM shell of the PEC membrane to form a biomimetic periosteum. Subsequent experiments demonstrated that these engineered EVs promoted the osteogenic differentiation of BMSCs, and that the modification process did not alter the original osteogenic properties of the EVs.¹⁰⁶

Polycaprolactone (PCL), a biodegradable polyester polymer, has been extensively explored in various tissue engineering applications. In bone tissue engineering, PCL is often employed as a scaffold material, as it can offer favorable biocompatibility, plasticity, toughness, and tunable degradation rates. However, PCL itself has low mechanical strength, cannot bear weight, and lacks bioactivity or osteoconductive properties. Moreover, the low surface energy and lack of surface signals of pure PCL make cell attachment difficult.¹⁰⁷ Zha et al. encapsulated VEGF plasmids into ATDC5-derived EVs, implanted these EVs into 3D-printed PCL scaffolds, and modified the PCL scaffolds with the extracellular vesicle-anchoring peptide CP05 to improve implantation efficiency. The scaffolds were then implanted into a rat radial defect model. The final experimental results showed that the VEGF plasmids exhibited strong angiogenic capacity, and the PCL scaffolds combined with EVs and VEGF plasmids promoted both osteogenesis and angiogenesis (Figures 5C-E).¹⁰⁸ In addition, PLA and PGA are also frequently used in tissue engineering.

In a study by Jin et al., electrospun scaffolds that combined physical and biological cues were fabricated by anchoring reparative M2-EVs onto topological pore-structured nanofibrous scaffolds (Figure 6A). In vitro cell experiments showed that these EVs could be internalized by target cells, promoting cell migration, tube formation, osteogenic differentiation, and anti-inflammatory macrophage polarization. Through subcutaneous implantation in mice,

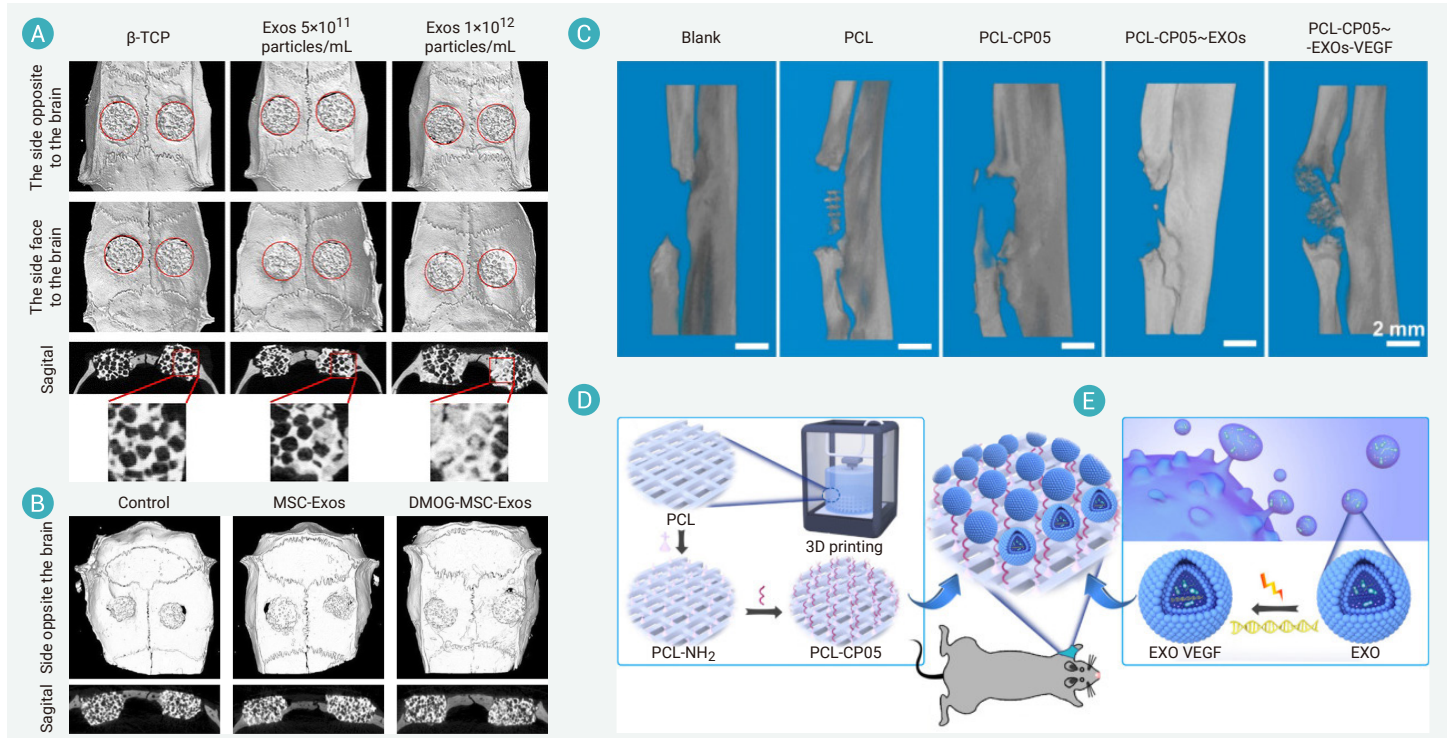


Figure 5. Comparative evaluation of bone defect repair for different biomaterial scaffolds loaded with various engineered extracellular vesicles (A) Repair outcomes when using EVs or tricalcium phosphate (β -TCP) alone. (B) HA, MSC-EVs, and DMOG-MSC-EVs have varying reparative effects. (C) The PCL-CP05-EVs-VEGF experimental group showed the best repair results 12 weeks after transplantation. (D,E) VEGF was encapsulated into EVs derived from ATDC5 cells, and the PCL scaffold was modified using CP05. (A,B) Reproduced under the terms of Creative Commons Attribution 4.0 International License. Copyright 2016 and 2019, The Authors, Published by Springer Nature. (C-E) Reproduced under the terms of Creative Commons Attribution 4.0 International License. Copyright 2021, The Authors, Published by Ivyspring.

the authors elucidated the activation of fibrosis, angiogenesis, and macrophage activity during the extracellular vesicle-functionalized fibrous scaffold-mediated foreign body response (FBR). Furthermore, in a critical-sized rat cranial bone defect model, the extracellular vesicle-functionalized nanofibrous scaffolds enhanced vascularized bone formation. Specifically, histological analysis revealed that the biofunctional scaffolds regulated the coupling effect of angiogenesis, osteoclastogenesis, and osteogenesis by stimulating type H vessel formation. Moreover, in the mouse subcutaneous implantation model, the exosome-functionalized scaffold system mitigated foreign body response, including excessive fibrosis, tumor-like vascularization, and macrophage activation.¹⁰⁹ In addition, Jin et al. also proposed a strategy based on materials biology and immune-guided regeneration that integrates physical and biological cues. They designed and fabricated a Janus electrospun nanofibrous scaffold loaded with immunomodulatory EVs ($\text{Exo}^{\text{TFGRN/IL-4}}$), which possesses distinct surface structures and mechanical properties on its two sides, enabling it to modulate the immune responses required for bone and muscle repair as well as inhibit osteoclastogenesis (Figures 6B-E).¹¹⁰ Furthermore, foreign body reaction assessments indicate that this stent does not cause long-term inflammation or systemic toxicity.

Metal materials

Metal materials offer advantages such as high mechanical strength, good biocompatibility, low extensibility, ease of processing, corrosion resistance, and low modulus; however, they have poor absorbability and are generally not naturally degradable, so in clinical practice they are primarily used as platforms to support the materials employed for repair.¹¹¹ Among them, titanium alloys exhibit particularly prominent advantages: in addition to their excellent biocompatibility, high friction coefficient, high porosity, and corrosion resistance, their micro-/nano-textured layered titanium morphology can also promote extracellular vesicle secretion, thereby enhancing bone regeneration (Figures 7A and 7B). Furthermore, with the development of 3D printing technology, studies have found that the excellent porosity of 3D-printed titanium alloy scaffolds not only facilitates extracellular vesicle attachment but also benefits cell migration and proliferation; 3D-printed titanium alloy scaffolds modified with EVs derived from human mesenchymal stem cells can upregulate

late osteogenic miRNAs via the PI3K/Akt and MAPK pathways and promote osteogenesis (Figure 7C).^{112,113} Moreover, recent studies have also shown that titanium nanotube-loaded EVs can stimulate autophagic activation of BMSCs, thereby regulating the differentiation and self-renewal of MSCs and playing a key role in their osteogenic differentiation.¹¹⁴

Hybrid materials

Hybrid materials are composites composed of two components at the nanoscale or molecular level, typically one inorganic and one organic, and thus differ from conventional composites whose constituents are mixed at the macroscopic (micrometer to millimeter) level; mixing at the microscopic scale results in more homogeneous materials that either exhibit properties intermediate between the two original phases or display entirely new properties. Similarly, hydrogels are hydrophilic gels with crosslinked networks, including natural, synthetic, and biohybrid types; natural hydrogels contain polymers such as collagen, hyaluronic acid, fibrin, alginate, agarose, and chitosan. Hydrogels possess high plasticity, allowing their molecular structure to be modified into temperature- or pH-responsive forms or molecularly imprinted hydrogels for molecular recognition, and their plasticity, porosity, and biocompatibility render them widely used in therapy and diagnostics, particularly in tissue engineering, controlled drug delivery, and bionanotechnology applications. Subsequently, chondroitin sulfate (OCS) was incorporated into a hydrogel together with EVs that promote cartilage regeneration to form GMOCS-EVs hydrogels; experimental results showed that these hydrogels not only sustained extracellular vesicle release for 14 days but also promoted the polarization of RAW cells from M1 to M2 via the NF- κ B pathway, alleviated IL-1-induced damage to chondrocytes, and enhanced cartilage regeneration (Figure 7D).¹¹⁵ Similarly, A novel adhesive dual-sensitive F127/HA-NB hydrogel loaded with M2 macrophage-derived exosomes (M2-Exos@F127/HA-NB) achieved sustained release of exosomes for up to two weeks and exhibited superior bone regeneration-promoting effects in a rat cranial defect model.¹¹⁶ In parallel, nanocomposite scaffolds—composed of polymers and nanomaterials—are expected to better mimic the extracellular matrix (ECM) environment of bone, and their favorable mechanical and

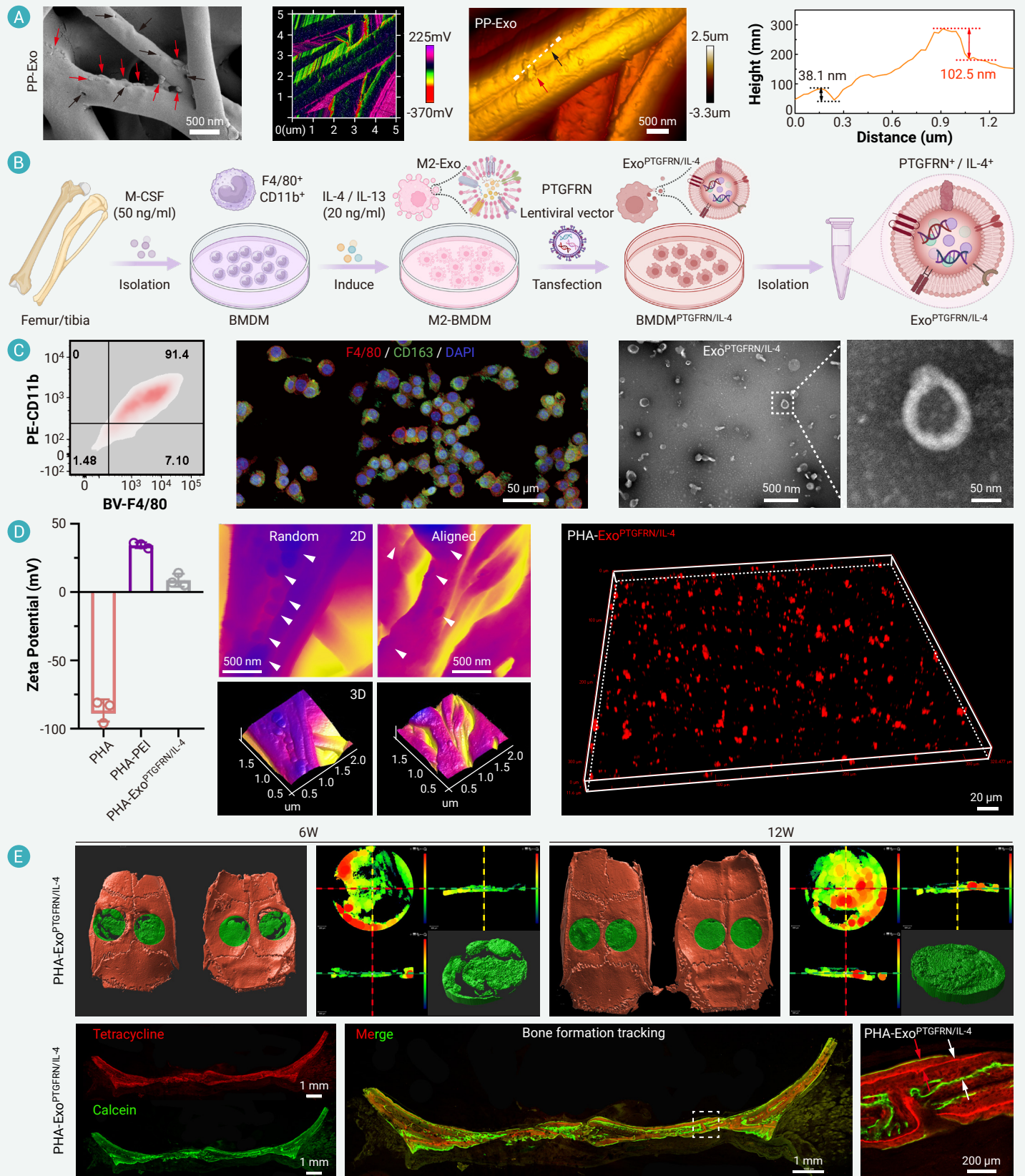


Figure 6. Preparation, characterization and bone regeneration of PTGFRN/IL-4-EV-modified Janus electrospun nanofibrous scaffolds (A) M2-EVs modified topological pore structured nanofibrous scaffolds. (B) The schematic diagram of the preparation of PTGFRN- and IL-4-overexpressing EVs. (C) The identification of BMDM cells and EV^{PTGFRN/IL-4}. (D) The modification of Janus electrospun nanofibrous scaffold with PEI and EV^{PTGFRN/IL-4}. (E) The EV^{PTGFRN/IL-4} functionalized Janus electrospun nanofibrous scaffold mediated bone regeneration. (A) Adapted with permission. Copyright 2024, Acta Materialia Inc. Elsevier Ltd. (B-E) Adapted with permission. Copyright 2026, PERGAMON, Elsevier Ltd.

biomimetic properties make them suitable for enhancing scaffold–cell interactions to repair damaged bone and adjacent tissues. Currently, research on nanofibrous materials has become increasingly mature, with techniques such

as electrospinning, 3D printing, self-assembly, and solvent casting successfully employed to replicate the fibrous structure of the native ECM and design diverse fibrous scaffolds; recent advances in nanotechnology have driven the

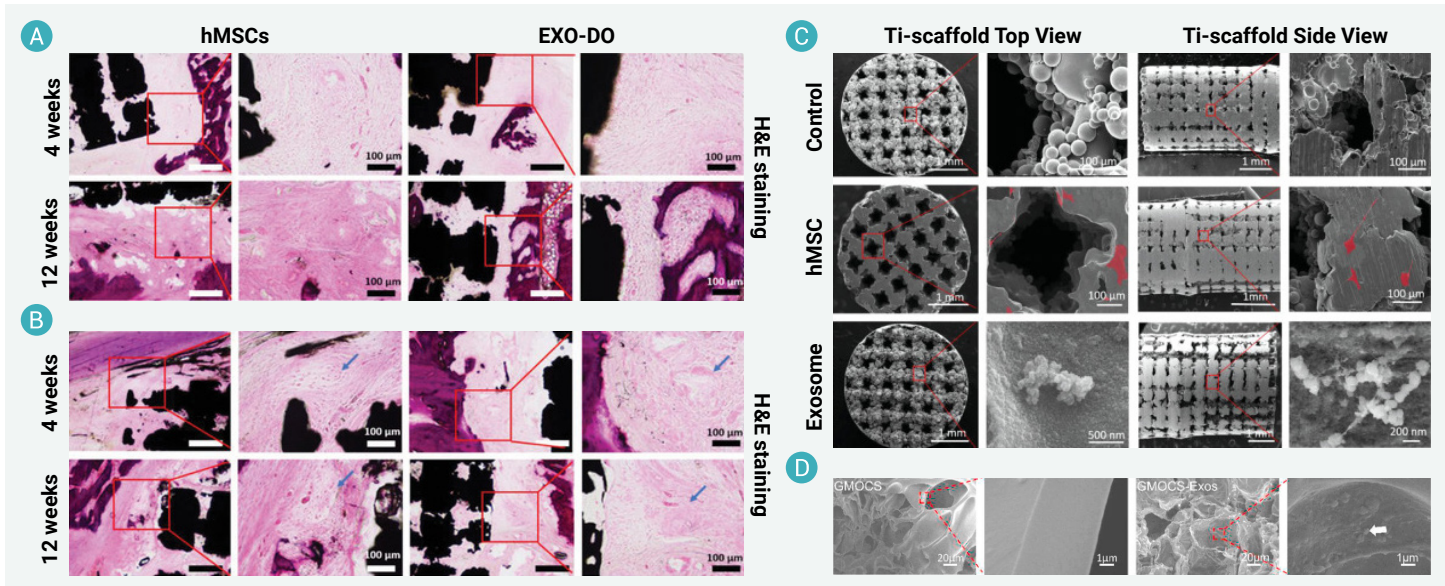


Figure 7. Histological and SEM characterization of EV-modified metal scaffolds and hydrogel biomaterials (A,B) Repair of metal scaffolds at 4 and 12 weeks as observed under H&E staining. (C) Images of the Ti scaffold, hMSCs-Ti scaffold, and extracellular vesicle-Ti scaffold under SEM. (D) Images of GMOCS hydrogels under SEM. (A–C) Reproduced under the terms of Creative Commons Attribution 4.0 International License. Copyright 2020, The Authors. Published by Wiley-VCH GmbH. (D) Reproduced under the CC BY-NC-ND license. Copyright 2022, The Authors. Published by KeAi Communications Co., Ltd.

emergence of novel bone grafts and scaffold systems, advancing bone tissue engineering.¹¹⁷ Because the surface characteristics of nanofibrous scaffolds can be readily modified, and surface topography significantly impacts stem cell morphology and differential differentiation, scaffolds fabricated from nanofibrous materials can markedly enhance the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs).

CHALLENGES AND PROSPECTS

Although EVs derived from various cell types—such as mesenchymal stem cells, osteoblasts, endothelial cells, and tumor cells—have been explored for bone repair, M2-EVs exhibit distinct advantages that make them particularly attractive for immunomodulatory osteogenesis. Compared with MSC-derived EVs, which are widely studied for their pro-osteogenic and pro-angiogenic properties, M2-EVs inherently carry a repertoire of anti-inflammatory miRNAs and proteins that not only promote osteogenic differentiation but also actively suppress pro-inflammatory signaling and foster an M2-dominant microenvironment. This dual function—direct osteostimulation coupled with microenvironmental immunomodulation—distinguishes M2-EVs from EVs derived from osteoblasts or endothelial cells, which primarily exert effects on differentiation or angiogenesis without robust anti-inflammatory activity. Moreover, compared with M1 macrophage-derived EVs, which tend to inhibit osteogenesis through pro-inflammatory mediators, M2-EVs consistently support bone formation and are less likely to induce detrimental inflammatory side effects. Nevertheless, the clinical translation of M2-EVs faces challenges similar to those of other EVs types, including heterogeneity, scalability, and in vivo stability, which require further engineering solutions. Thus, while multiple EVs sources hold promise for bone regeneration, M2-EVs offer a unique combination of immunomodulatory and osteogenic activities that align particularly well with the complex inflammatory and regenerative demands of bone healing.

Beyond their osteogenic role, M2-EVs exhibit a variety of other functions. In terms of inflammation regulation, M2-EVs can target inflammatory sites through specific interactions of surface LFA-1 and VLA-4 with receptors such as ICAM-1, thereby exerting anti-inflammatory effects.^{118,119} Meanwhile, EVs serve as ideal vehicles for drug and RNA delivery. Engineered M2-EVs can encapsulate drugs such as melatonin, avoiding clearance by the mononuclear phagocyte system and enriching in target tissues, thereby achieving periodontal regeneration in periodontitis models.¹²⁰ Similarly, lipid nanoparticle-based delivery of siRNA has been used for the treatment of genetic diseases. Fusion of M2-EVs with bone marrow mesenchymal stem cell-derived EVs (BMSCs-EVs) generates engineered hybrid EVs that combine

targeting and immunomodulatory properties. In a model of periprosthetic osteolysis, such hybrid EVs exhibited a 2.67-fold increase in targeting precision and effectively inhibited osteoclast differentiation.¹²¹ Furthermore, EVs offer advantages in disease diagnosis: they are widely present in body fluids, are structurally stable, and contain high concentrations of biomarkers. For example, miRNAs in liver macrophage-derived EVs can be used for the diagnosis of nonalcoholic fatty liver disease.¹²² In the context of aging regulation, EVs derived from bone marrow macrophages of aged mice promote aging burden in multiple tissues, whereas EVs from young mice downregulate aging markers in the liver, bone, and brain of aged mice, demonstrating anti-aging effects.¹²³ For specific diseases, M2-EVs inhibit macrophage uptake of oxidized low-density lipoprotein (ox-LDL); after platelet modification, their miR-99a-5p targets HOXA1, thereby delaying the progression of atherosclerosis.¹²⁴ In hepatic ischemia-reperfusion injury, the DATS@M2-EVs delivery system releases H₂S in a concentration-dependent manner, exhibiting protective effects against oxidative stress, inflammation, and ferroptosis.¹²⁵

Despite the great potential of M2-EVs in osteogenesis, their clinical application still faces multiple limitations. First, the targeting ability of EVs is insufficient: when delivered in vivo, they may be taken up by non-target tissues and fail to act effectively on the intended cells. One study constructed engineered EVs via Fc modification to enable tumor site targeting without altering the half-life, and similar strategies may enhance the osteogenic specificity of EVs.¹²⁶ Second, the osteogenic effect of EVs is not absolutely beneficial—under certain pathological conditions, they can instead lead to bone degeneration. For example, EVs derived from bone marrow macrophages of high-fat diet-induced obese mice can regulate the Ppara-Abca1 axis via miRNA-378a, thereby causing dysfunction of skeletal stem/progenitor cells and leading to bone degeneration, whereas EVs from healthy mice restore bone vitality.¹²⁷ Moreover, current studies mostly focus on local effects or single cell types, and a systemic understanding of the underlying mechanisms, as well as the mutual promotion or inhibition among EVs from different cellular origins, remains lacking. At the technical level, isolation and characterization methods for EVs still require optimization: ultracentrifugation cannot effectively separate EVs with different physical properties or biological origins, and variations in centrifugation conditions may affect their performance. In addition, the preparation of M2-EVs is limited by the heterogeneity of macrophage origin (most studies use mouse-derived cells, which may introduce experimental bias).¹²⁸ Furthermore, analytical tools for the quality attributes of EVs remain scarce. Compared with biomolecules such as antibodies, EVs—owing to their complex composition (including small molecules, proteins, nucleic acids, etc.)—present greater

challenges in defining their identity, purity, and the isolation of homogeneous subpopulations.¹²⁹ It still remains unclear how cells control the release of EVs, and how the release mechanisms correlate with their physicochemical properties and biological functions. Finally, akin to stem cell therapy, EVs represent a paradigm within the cell-free therapeutic arena, yet they face analogous challenges in safety and regulatory oversight. Extracellular vesicle-based drugs or implants must undergo rigorous approval and regulatory procedures. However, such extracellular vesicle products, often crossing national and regional borders, are subject to divergent regulatory frameworks across governments and agencies, leading to discrepancies in potency, safety profiles, and clinical trial designs. Hence, there is an urgent need to promote the establishment of a globally harmonized regulatory framework.¹³⁰ These limitations collectively point to key issues that must be addressed before engineered M2-EVs can be used in osteogenic therapy.

Having acknowledged the above limitations, it is still important to recognize that despite the serious challenges facing M2-EVs in terms of druggability, targeting uniformity, and quality control, the functional diversity of these vesicles remains undiminished. On the contrary, as researchers work to overcome these obstacles, the broad potential of M2-EVs beyond osteogenesis continues to be uncovered, including their roles in inflammation regulation, drug delivery, extracellular vesicle fusion engineering, disease diagnosis, aging modulation, and the treatment of various specific diseases.

The researchers believe that future directions for M2-EVs in osteogenic applications should focus on two interconnected aspects: the mechanistic deepening of their molecular actions and the rational integration of M2-EVs with biomaterial scaffolds. Mechanistically, current understanding remains largely descriptive, and further studies are needed to dissect the precise molecular logic underlying M2-EVs-mediated osteogenesis—moving beyond bulk cargo correlations toward establishing direct causal links between specific miRNAs or proteins, their direct intracellular targets in recipient cells (including mesenchymal stem cells, endothelial cells, and osteoclast precursors), and the resulting spatiotemporal dynamics of the osteoimmune microenvironment. In addition, we could consider upgrading these brackets. The incorporation of M2-EVs into tissue-engineering scaffolds should evolve from a simple drug-carrier paradigm toward a synergistic framework that exploits bidirectional interactions: scaffolds should enable intelligent, inflammation-responsive release of M2-EVs, actively modulate host macrophage polarization toward the M2 phenotype to create positive feedback loops, and integrate immunomodulatory and pro-osteogenic EVs activities with the material's inherent osteoconductive and angiogenic properties. Attention must also be paid to loading efficiency, retention, release kinetics, bioactivity maintenance, and potential confounding effects of scaffold degradation products. Together, these mechanistic and engineering efforts will advance our fundamental understanding of M2-EVs biology and pave the way for next-generation M2-EVs-based systems with improved specificity, efficacy, and translational feasibility. This requires us to transform EVs from "passive cargo" into "reactors." Current research primarily treats M2-EVs as pre-packaged drug carriers. We propose a paradigm shift: engineering M2-EVs into "responsive devices" capable of sensing and responding to the pathological microenvironment. This goes beyond simply loading therapeutic molecules; it also involves integrating biosensors so that the release of osteogenic factors is triggered only upon encountering specific inflammatory signals, such as changes in pH or elevated levels of inflammatory cytokines. This responsive design can significantly enhance on-demand therapeutic efficacy while minimizing off-target effects. Furthermore, future research needs to overcome the "all-or-nothing" delivery dilemma. Current rapid injection or passive loading strategies result in sudden release and rapid clearance. We envision a "two-stage release system" in which M2-EVs are embedded in the scaffold in a tiered manner. The first stage allows for immediate, low-dose release to initiate an early anti-inflammatory response; the second stage, triggered by the enzymatic degradation of the scaffold, provides sustained, high-dose release during the proliferation phase to drive robust osteogenesis and angiogenesis. This may enhance the osteogenic function of EVs, and it may even excel in other functions as well.

CONCLUSION

This review provides a comprehensive overview of the current understand-

ing of M2-EVs in bone regeneration, spanning from their biogenesis and molecular cargo to their multifaceted roles in osteoimmunomodulation, osteogenic differentiation, angiogenesis, and biomaterial integration. M2-EVs have emerged as a promising cell-free therapeutic platform that not only inherits the pro-regenerative and anti-inflammatory characteristics of their parent cells but also offers distinct advantages over other EVs sources by actively shaping a favorable immune microenvironment for bone healing. Their ability to deliver functional miRNAs and proteins to recipient cells, coupled with their compatibility with various scaffold systems, positions them as versatile tools in bone tissue engineering. However, despite the encouraging preclinical evidence, significant hurdles remain—including issues of targeting specificity, production scalability, quality control, and regulatory standardization—that must be addressed before clinical translation can be realized. Moreover, future efforts should move beyond descriptive observations toward mechanistic dissection and engineering innovation, aiming to transform M2-EVs from passive drug carriers into intelligent, responsive therapeutic systems. In summary, while challenges persist, M2-EVs represent a paradigm shift in bone regenerative medicine, and continued interdisciplinary research will be essential to unlock their full clinical potential.

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AUTHOR CONTRIBUTIONS

P.L. designed the manuscript, prepared and wrote the first draft. S.J. designed the manuscript, wrote the draft and supervised this project. Z.Z. and F.L. supervised and reviewed the project. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.