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Exosomes to exosome-functionalized scaffolds: a novel approach to stimulate bone regeneration

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Abstract

Bone regeneration is a complex biological process that relies on the orchestrated interplay of various cellular and molecular events. Bone tissue engineering is currently the most promising method for treating bone regeneration. However, the immunogenicity, stable and cell quantity of seed cells limited their application. Recently, exosomes, which are small extracellular vesicles released by cells, have been found to effectively address these problems and better induce bone regeneration. Meanwhile, a growing line of research has shown the cargos of exosomes may provide effective therapeutic and biomarker tools for bone repair, including miRNA, lncRNA, and proteins. Moreover, engineered scaffolds loaded with exosomes can offer a cell-free bone repair strategy, addressing immunogenicity concerns and providing a more stable functional performance. Herein, we provide a comprehensive summary of the role played by scaffolds loaded with exosomes in bone regeneration, drawing on a systematic analysis of relevant literature available on PubMed, Scopus, and Google Scholar database.

Keywords Exosome, Osteogenesis, Biomaterial, Tissue engineering

Background

With increased life expectancy and an aging global population, bone defects caused by trauma, fractures, osteoporosis, and bone metastases significantly contribute to a decline in people's quality of life and an increase in economic burden [1]. Among, osteoporosis is a common disease in the elderly, and is highly associated with an increased risk of fractures. As the global population is rapidly aging, the economic and health burden of this disease is increasing [2]. It is estimated that there are around 5.5 million men and 22 million women with osteoporosis in the European Union, leading to approximately 3.5 million fractures per year [3–5]. The first-year mortality rate for hip fractures is close to 20% [6]. While, benign bone tumors and tumor-like lesions are common in children and adolescents. In most cases, regular observation is suitable for the patients, but for the lesions that threaten the structural bone stability, curettage is often required to achieve a lower recurrence rate and better limb function, which may lead to bone defects beyond a certain size or located in weight-bearing areas [7–9]. Bone regeneration is a complex, multi-stage physiological process that involves various cellular components, cytokines, chemokines, growth factors, and intercellular signaling pathways [10–12]. Although bones possess a certain degree of regenerative capability, bone defects exceeding a critical size threshold (which typically depends on the anatomical location, usually > 2 cm)

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cannot self-heal. Achieving functional restoration and complete healing in such cases requires clinical intervention and additional treatments to promote bone regeneration [13].

Autologous or allogeneic bone are ideal grafts used to treat such long bone defects. However, there are still shortcomings associated with bone grafting. Among them, autologous graft is limited by availability, requiring an additional surgical site and presenting variable quality. Allogeneic bone transplantation provides an abundant supply and avoids an extra surgical site, but carries a risk of rejection, disease transmission, and reduced osteogenic potential [14, 15]. Therefore, bone tissue engineering, a scaffold material loaded with seed cells and growth factors, has become the most attractive solution to promote bone regeneration [16, 17]. Previous studies have indicated that seed cells, usually mesenchymal stem cells (MSCs), serve as a cellular basis of osteogenic differentiation in the bone regeneration process. However, accumulating evidence indicates that MSC-derived paracrine signaling, such as exosomes, has been proposed to be more responsible for the bone regeneration and not the cell themselves [18, 19].

Exosomes, with diameters ranging from 30 to 150 nm extracellular vesicles (EVs), originate from the endosomal system, specifically as intraluminal vesicles (ILVs) within multivesicular bodies (MVBs). They contain a variety of bioactive molecules such as proteins, lipids, and nucleic acids, which can be transferred to target cells. Exosomes serve as a means of cell communication by delivering their cargo to recipient cells, where they can induce various biological effects [20–22]. Once inside the recipient cell, the exosome cargo can modulate various cellular processes such as gene expression, protein synthesis, and signaling pathways [23, 24]. With the revelation of exosomes' ability to influence the behavior of recipient cells, such as osteoblast and MSCs, their potential as key regulators of bone remodeling and repair has been underscored [25]. Wei et al. reported that bone marrow stem cells (BMSCs)-derived exosomes regulate WIF1-mediated Wnt/β-catenin axis inhibition of osteogenic differentiation via miR-424-5p [26]. Lin et al. found that human umbilical vein endothelial cell derived exosomes with overexpressing PD-1 induces osteogenic differentiation and promotes fracture healing by binding to PD-1 on the T cell surface and suppressing the activation of T cells as an immunosuppressant [27]. Additionally, exosomes exhibit high stability, low tumorigenicity, and possess an inherent homing effect that allows for organ targeting [28, 29]. Therefore, these findings have driven the search for alternate cell-free therapies based on exosomes have become strongly established in the landscape of bone regeneration [30]. However, the application of exosomes in clinical bone regeneration is still limited by issues such as short half-life. Currently, the application of exosomes in bone repair is mainly as carriers of bioactive molecules in the construction of acellular bone tissue engineering scaffolds.

Here, we will provide a comprehensive review of the research progress on the use of exosomes in bone regeneration in recent years, focusing on the classification of active substances in exosomes, their mechanisms of action, and the strategies for constructing engineered exosomes and exosomes functionalized scaffolds.

Biosynthesis and phenotypic characterization of exosomes

Biological synthesis of exosomes

Extracellular vesicles are vesicles that are secreted from parent cells into the extracellular environment and encapsulated with a double-layered phospholipid membrane. Based on their size, EVs can be classified into the following categories: (1) apoptotic bodies (ApoBDs) with a diameter range of 1–5 μm; (2) microvesicles (MVs) with a diameter range of 50–1000 nm; and (3) exosomes with a diameter range of 30–150 nm [26, 31]. ApoBDs are a major subset of membrane-bound apoptotic extracellular vesicles generated during the final stages of cellular programmed death, typically considered to range in diameter from 1 to 5 μm [32]. Their formation involves a series of morphologically regulated processes, including membrane budding, the formation of apoptotic membrane protrusions, and subsequent fragmentation into ApoBDs [33, 34]. ApoBDs play a significant role in the clearance of apoptotic cells and intercellular communication, as they enhance phagocyte engulfment rates and can participate in intercellular communication through their contents, such as DNA [35]. MVs range in diameter from 50 to 1000 nm and are produced through outward budding of the plasma membrane. This process requires several molecular rearrangements within the plasma membrane, including changes in lipidcomponents and protein composition, as well as fluctuations in calcium ion (Ca^{2+}) levels. Ca^{2+}-dependent enzymatic mechanisms lead to physical bending of the membrane and restructuring of the underlying actin cytoskeleton, facilitating membrane budding and the formation of microvesicles. MVs also play a role in intercellular communication among various cell types, including cancer cells [36]. Among them, the role of exosomes in different biological processes has been widely confirmed. Meanwhile, the formation of exosomes has been proved to be a complex and highly regulated process. Firstly, under the influence of different cell types and stimuli factors, the cell membrane forms inward protrusions of varying shapes and sizes through "inverted budding." Subsequently, these

protrusions gradually expand and detach from the parent cell membrane to form early endosomes [37]. Then, the early endosomes undergo inward invagination to generate tubular structures, forming mature endosomes. At the same time, processed within the Golgi complex and rough endoplasmic reticulum, result in the mature endosomes carrying differentially modified proteins and nucleic acids. The mature endosomes evolve into multivesicular bodies, which ultimately fuse with the cell membrane under the action of Rab enzymes and are released into the extracellular space via exocytosis, thus forming exosomes. These modification processes include lipid modification, glycosylation, phosphorylation, etc., imparting exosomes with distinct specificity and biological activity. Therefore, exosomes can carry a large amount of biologically active substances such as proteins, lipids, DNA, mRNA, and miRNA [38, 39]. As shown in Fig. 1.

Phenotypic characterization of exosomes

Exosomes were first discovered in the 1980s, and they were initially thought to be a way for cells to rid themselves of unnecessary proteins. However, it was not

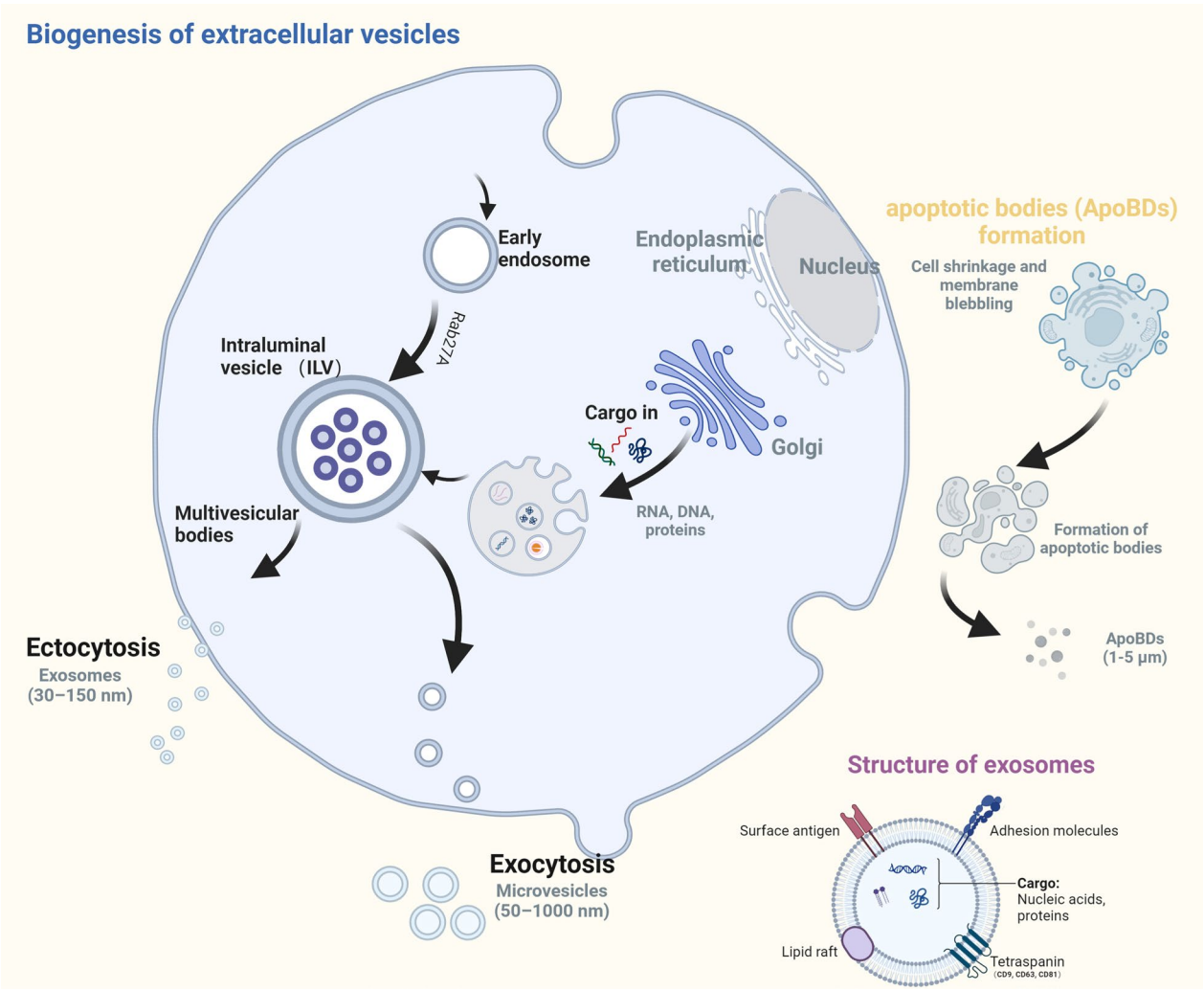

Fig. 1 Biosynthesis and Phenotypic Characterization of Exosomes. Exosomes are small extracellular vesicles that play a role in cell-to-cell communication by transporting proteins, nucleic acids, and lipids between cells. Biosynthesis of exosomes involves sorting cargo molecules into intracavicular vesicles (ILV) in endosomal compartments, which then mature into multivesicular bodies (MVBs). These MVBs can either fuse with lysosomes for degradation or with the plasma membrane to release exosomes with a diameter range of 30–100 nm into the extracellular environment. Exosomes can be characterized based on their size, morphology, surface markers, and cargo molecules such as proteins, lipids, and nucleic acids, including CD63, CD9, CD81, TSG101, Alix, etc. ApoBDs generated during the final stages of cellular programmed death, typically considered to range in diameter from 1 to 5 μm. MVs range in diameter from 50 to 1000 nm and are produced through outward budding of the plasma membrane

until the late 1990s and early 2000s, when researchers at Utrecht University in the Netherlands discovered that exosomes could transfer functional MHC Class II molecules between dendritic cells, thereby activating T cells, that exosomes began to be recognized as important mediators of intercellular communication [40]. In recent years, the field of exosome research has expanded rapidly, with numerous studies exploring the potential applications of exosomes in diagnostics, therapeutics, and regenerative medicine. Choi and Dong et al. have confirmed that the degree of protein phosphorylation on the surface of exosomes isolated from the blood of patients with breast cancer, lung cancer, and pancreatic cancer is significantly higher than that of healthy individuals, suggesting that it plays a key role in the early development of cancer cells [41]. Changes in their surface polysaccharides and lipid molecules can also be used to detect pancreatic cancer, lung cancer, liver cancer, and colorectal related cancers [42, 43]. Exosomes are closely associated with the development and progression of bone and soft tissue sarcomas, and exosome-based liquid biopsies are also utilized in sarcomas [44]. Tao Wan et al. have constructed a new gene editing delivery system based on CRISPR-Cas9 technology and exosomes, named exosomeRNP. Cas9 RNPs are loaded into purified exosomes isolated from hepatic stellate cells through electroporation. The exosomeRNP has been proven to effectively deliver RNPs to the cytoplasm and specifically accumulate in the liver tissue, exerting its gene therapy effect on acute liver injury and chronic liver fibrosis in mice by targeting p53 up-regulated modulator of apoptosis, cyclin E1 and lysine acetyltransferase 5 [45]. Meanwhile, exosomes enriched with human vascular endothelial growth factor A (VEGF-A) and human bone morphogenetic protein 2 (BMP-2) mRNAs have also been attempted for the treatment of critical-size femoral defects in rats. The results show that these therapeutic exosomes can be locally and controllably released at the defect site, achieving highly efficient induction of bone regeneration [46]. Furthermore, technological advances have greatly facilitated the identification and characterization of exosomes.

As proposed by the International Extracellular Vesicle Society (ISEV) in 2014, there are three aspects for exosome identification: ultrastructure (transmission electron microscope, TEM), particle size (nanoparticle tracking analysis, NTA), and protein markers (western blot, WB). In details, NTA indicate that exosomes from different sources have slightly different particle sizes, with a diameter range of 30–150 nm [47]. Under TEM, exosomes exhibit rounded, full-bodied particles with a discoidal bilayer membrane structure. The outer layer of the exosomal phospholipid bilayer contains components such as mannose, polylactosamine, and sphingolipids, which are involved in signal recognition and maintenance of exosome morphology. The interior is rich in proteins, including tetraspanins (CD9, CD63, CD81, etc.) that interact with integrins, signaling proteins (protein kinases, β-catenin, etc.), and heat shock proteins (HSP70, HSP90, etc.) [48–50]. Among these, the high expression of tetraspanins can serve as exosomal markers for their characterization and identification. Meanwhile, tetraspanins play a role in multiple important processes of exosomes, including formation, secretion, and functional transfer. For example, CD9 mediates exosome exocytosis and promotes exosome release, CD63 assists exosomes in transporting carried miRNA to target cells, CD81 can regulate the transfer of integrins and affect exosome formation. As shown in the lower right of Fig. 1.

Exosomes effect on different cells during bone regeneration

Bone regeneration is a complex process involving a variety of cells, including osteoblasts, osteoclasts, chondrocytes, osteocytes, and immune cells. Exosomes functioning as messengers after being released by cells, they are taken up by recipient cells through binding to target cell receptors, endocytosis/phagocytosis, or membrane fusion, thereby transferring various bioactive compounds and playing a role in intercellular communication [51]. As shown in Fig. 2.

Osteoblasts are functional cells that secrete bioactive substances to regulate bone formation and remodeling. The proliferation rate of osteoblasts is found to be positively correlated with different concentrations of exosomes derived from BMSCs [52]. Runx2 and osterix are important transcription factors in osteoblast differentiation that regulate the expression of bone-related genes such as OPN, BSP, and OCN [53]. BMSC-derived exosomes have been shown to upregulate the expression of Runx2, OSX, OCN, and other osteogenic genes [54]. Osteoclasts are closely related to bone resorption, and exosomes act as important regulatory factors in the paracrine signaling of osteoclasts. RANKL plays a key role in osteoclast differentiation, and RANK has been confirmed to be abundantly expressed in exosomes from osteoclasts, where it binds to RANKL and competitively inhibits the RANK pathway, thereby regulating bone resorption [55].

Meanwhile, cartilage is a connective tissue that provides major support in bone tissue. Exosomes derived from BMSCs significantly attenuate the inhibitory effects of IL-1β on chondrocyte proliferation and migration, as well as downregulation of COL2A1 and ACAN, and upregulation of MMP13 and ADAMTS5 in chondrocytes [56]. BMSC-derived exosomes exert anti-inflammatory effects either by inhibiting glycolysis or through the NF-κB signaling pathway and the Nrf2/HO-1 axis,

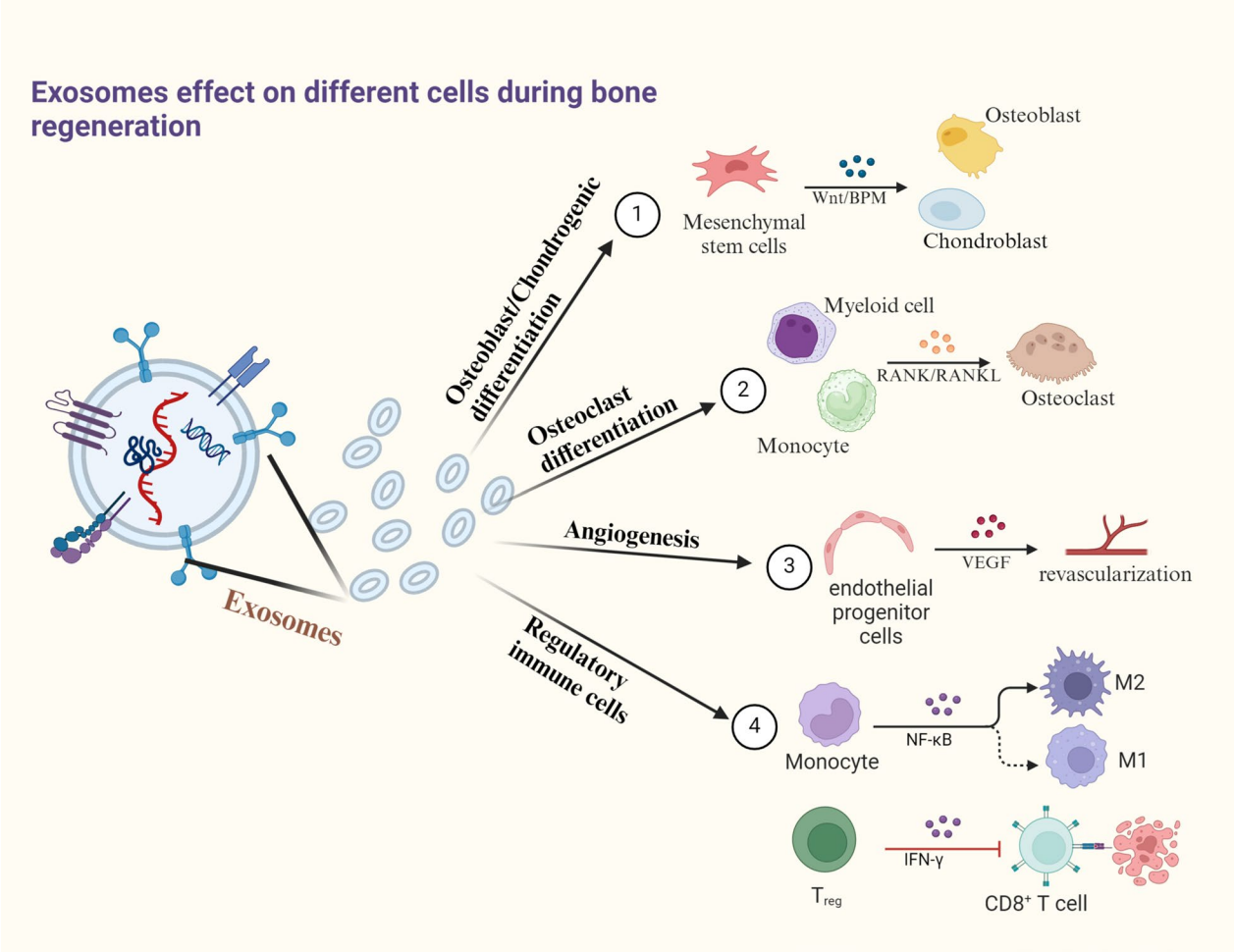

Fig. 2 Exosomes effect on different cells during bone regeneration. Exosomes regulate the differentiation and maturation of bone-related cells, including the regulation of MSC osteogenesis and chondroblast differentiation; monocyte differentiation into osteoclasts; endothelial progenitor cell angiogenesis, and macrophage polarization in the bone immune microenvironment

inhibiting M1 macrophage activity while promoting the generation of M2 macrophages [57, 58]. Exosomes from regulatory dendritic cells inhibit the maturation of dendritic cells and promote the recruitment of regulatory T cells, thereby suppressing the production of osteoclastic cytokines and reducing bone loss [59].

Additionally, the process of bone remodeling requires ongoing metabolic regeneration to maintain the proper morphology and physiological function of bone tissue. Exosomes derived from various cells participate in bone metabolism via regulating bone formation and angiogenesis. For example, endothelial progenitor cells (EPCs) indirectly stimulate new bone formation by promoting the development of new blood vessels in order to complete the repair process. Exosomes produced by EPCs have been shown to enhance the motility of bone marrow-derived macrophages and promote osteoclast differentiation by competitively binding and regulating miR-124 [60]. Similarly, exosomes released by endothelial cells (ECs) are more targeted towards bone than those produced by osteoblasts or BMSCs. EC-derived exosomes are known to promote the differentiation and development of macrophage osteoclasts through miR-155 [61].

Exosomes promote bone regeneration though functional cargo transportation

Exosomes carry a large number of active factors and have been widely confirmed to play a regulatory role in various biological functions. Bone regeneration involves the proliferation and differentiation of various cell types, including MSCs, osteoblasts, osteoclasts, chondrocytes, and vascular endothelial cells, among others. Moreover, exosomes from different sources have been proven to be involved in regulating every stage of bone regeneration. The effects of exosomes from different sources on

osteogenesis-related cells and their active factors are shown in Fig. 3.

Exosomal microRNAs

MiRNAs are double-stranded non-coding RNAs of about 22 nucleotides generated by RNA polymerase II transcription and processing of series complexes. MiRNAs mediate post-transcriptional gene silencing by binding to complementary sequences of target genes to regulate the translational process in a wide range of biological processes [62]. MiRNAs were first observed in exosomes in 2007, and now miRNAs have become the most studied cargos in exosomes. Recently, a large number of studies have shown that exosomal miRNAs from different sources are widely involved in regulating osteogenesis. The effects of exosomal miRNAs on osteogenesis are summarized in Table 1.

MSCs are important osteoprogenitor cells in the bone regeneration process, which can differentiate into osteogenic or chondrogenic progenitor cells and ultimately differentiate into osteoblasts or chondrocytes. Their derived exosomes play an important regulatory role in these processes. Tao Xu and colleagues discovered that the regenerative capacity of exosomes derived from MSCs decreased as the cells aged, as evidenced by reduced efficacy in treating fractures in rats [63]. Subsequent investigations revealed that the levels of miR-128-3p in MSC-derived exosomes increased with cellular aging, and its suppression of Smad5 expression attenuated the therapeutic impact of exosomes on fractures [63]. Consequently, they propose that antagonists targeting miR-128-3p may represent a promising and innovative strategy for treating fractures in elderly patients [63]. The upregulation of WWP1 or Smurf2 in BMSCs leads to the degradation of the target protein KLF5 through ubiquitination, ultimately inhibiting fracture healing. Exosomes derived from BMSCs target WWP1 or Smurf2 through microRNA-19b, activating the KLF5/β-catenin signaling pathway and promoting fracture healing [64].

Bone immune microenvironment is closely related to bone regeneration. Immune cells derived exosomes also play an important regulatory role in the process of bone regeneration. Jincheng et al. found that miR-486-5p was significantly overexpressed in M2 macrophage-derived exosomes [65]. They demonstrated that M2 macrophage-derived exosomal miR-486-5p influences the differentiation potential of BMMSCs through the miR-486-5p/SMAD2/TGF-β signalling pathway and

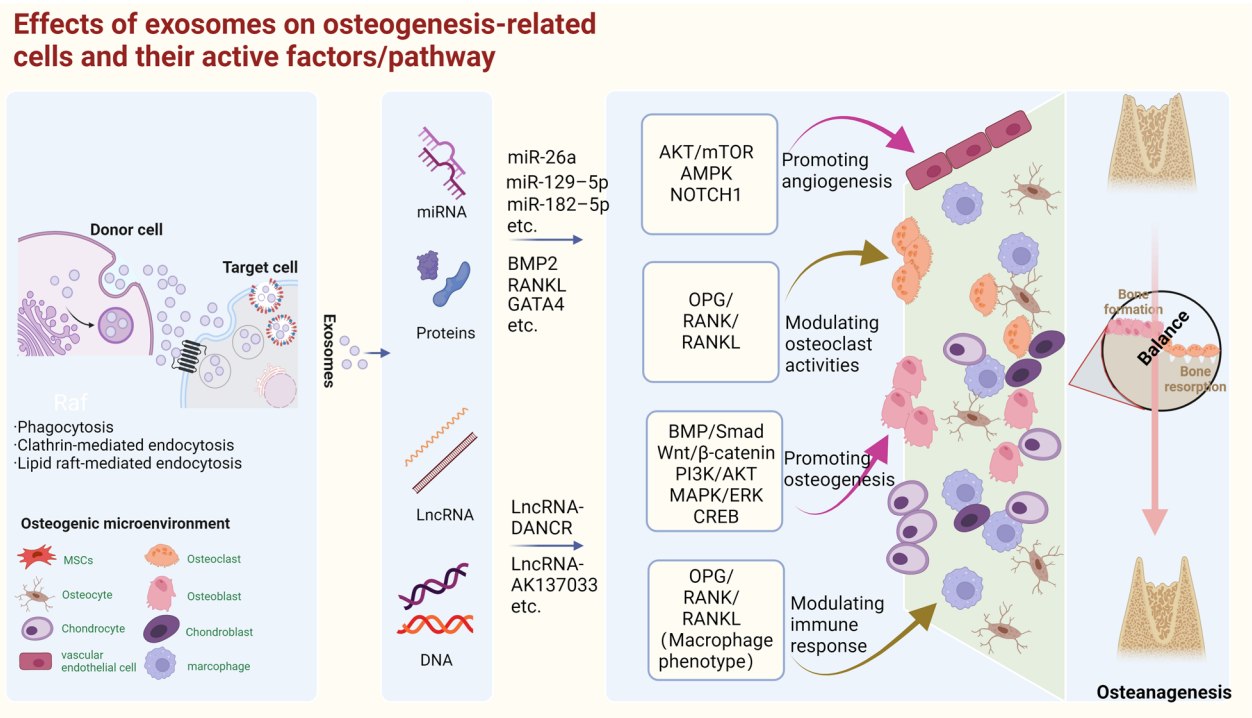

Fig. 3 Effects of exosomal cargos on osteogenesis-related cells and their active factors/pathway. Effects of exosomal cargos on osteogenesis-related cells and their active factors/pathway. Effect of donor cell derived exosomes act on osteogenesis-related cells, including MSCs, osteoblasts, osteoclasts, chondrocytes, osteocytes, chondroblast, vascular endothelial cell, and the local immune microenvironment composed of macrophages, etc. They regulate bone formation by delivering miRNAs, lncRNAs, and proteins to modulate signaling pathways like AKT/mTOR, AMPK, Wnt, and RANKL

Table 1 The Role of Different Exosomal Contents in Bone Regeneration

miRNAs	Effect	Target gene	Source	References
miR-130a-3p	Pro-	SIRT7	ADSCs	[93]
miR-140-3p	Pro-	Plxnb1	BMSC	[101]
miRNA-128-3p	Pro-	Smad5	MSC	[63]
miR-23a-5p	Anti-	Runx2	Osteoclast	[94]
miR-122-5p	Pro-	SPRY2	BMSC	[95]
miR-223	Anti-	cGMP-PKG	Neutrophil-like cells	[96]
miR-181b-5p	Pro-	PTEN	Osteocyte	[97]
miR-375	Pro-	IGFBP3	hADSCs	[98]
miR-17-5p	Pro-	SMAD7	BMSC	[100]
miR-155	Anti-	BMP2/BMP9 /Runx2	M1 macrophage	[68]
miR-486-5p	Pro-	SMAD2	M2 macrophage	[65]
miR-26a	Pro-	angiogenesis	$hCD34^+$ stem cells	[74]
miR-19b	Pro-	WWP1 and Smurf2	BMSC	[64]
miR-100-5p	Anti-	mTOR	Bone tissues	[102]
miR-335	Pro-	LATS1	Mature dendritic cells	[103]
miR-1260a	Pro-	HDAC7 and COL4A2	BMSC	[104]
miR-92a-1-5p	Anti-	COL1A1	Prostate cancer	[70]
miR-140-5p	Anti-	IGF1R	(OPLL)cell	[69]
miR-5134-5p	Anti-	JAK2/STAT3 Axis	Osteoclasts	[105]
miR-424-5p	Anti-	wif1	BMSCs	[106]
miR-151-3p	Anti-	PAFAH1B1	BMSC	[108]
LncRNAs				
Lnc TCONS_00072128	Pro-	caspase 8	PMOP serum	[107]
Lnc NEAT1	Pro-	DDX3X/NLRP3	HUVECs	[71]
Lnc RNA H19	Pro-	miR-467/HoxA10	BMSC	[73]
Lnc NONMMUT000375.2	Anti-	Wnt11	RAW264.7	[110]
Lnc RNA MALAT1	Pro-	microRNA-34c/SATB2	BMSC	[111]
Lnc TUG1	Pro-	miR-22-5p/Anxa8	BMSC	[112]
Lnc RUNX2-AS1	Anti-	RUNX2	multiple myeloma cells	[75]
Lnc NEAT1	Pro-	miR205-5p/SFPQ/PTBP2	prostate cancer cells	[77]
Lnc LIOCE	Pro-	osx	inflammatory osteoclasts	[76]
circRNAs				
circ_HIPK3	Pro-	miR-29a-5p /PINK1	BMSCs	[80]
circ_0006859	Anti-	miR-431-5p	Osteoporosis serum	[79]
circ_LPAR1	Pro-	hsa-miR-31	DPSCs	[114]
circ_FAM63	Anti-	miR-578/HMGA2	PMOP serum	[84]
Protein				
Prrx2	Anti-	lncRNA-MIR22HG	Myoblast	[86]
VEGF	Pro-	VEGF/ VEGFR	ATDC5	[115]
IL-10	Pro-	IL-10/IL-10R	M2	[116]
UCHL3	Pro-	SMAD1	BMDM	[92]
MATN3	Anti-	TGF-β	UDSCs	[117]
URG-4	Pro-	Wnt	BMSCs	[118]

osteoporosis [66]. Furthermore, Miya Kang and colleagues revealed that polarized macrophage derived exosomal miRNAs play a positive or negative in osteogenic differentiation. They discovered that M1 macrophage exosome-enriched miR-155 reduced MSC osteogenic differentiation and M2 macrophage exosome-enriched miR-378a increased the expression of osteogenic genes in MSCs [67]. In addition, miRNAs

in other cell-derived exosomes are also involved in the regulation of osteogenic differentiation. Ossification of posterior longitudinal ligament (OPLL) is a disabling disease with unknown pathogenesis, and there is no effective interventions yet [68]. Yifan Tang et al. found that miR-140-5p was significantly downregulated in OPLL cell-derived exosomes. Mechanistic studies also indicate that miR-140-5p was transferred to MSC, where it targets IGF1R to inhibit osteogenic differentiation [69]. Patients with prostate cancer (PCa) often experience pathological fractures, and histopathologic assessment reveals bone resorption in all metastatic lesions. Lijuan Yu and colleagues made an unexpected discovery that exosomal miR-92a-1-5p derived from PCa plays a critical role in regulating bone homeostasis, leading to osteoclastic lesions and promoting tumor growth in bone [70]. Importantly, these results suggest that exosomal miRNA may be potential therapeutics for different diseases.

Exosomal lncRNAs

Apart from microRNAs, long non-coding RNAs (LncRNAs), a class of non-coding RNAs more than 200 nucleotides in length, are also a significant category of non-coding RNAs, and research has identified exosome-derived lncRNAs as having a crucial role in bone regulation (Table 1) [71, 72].

Fracture is a prevalent traumatic condition in clinical practice, characterized by a high incidence, prolonged healing process, and challenging treatment, often resulting in significant financial burden for patients [73]. BMSCs-derived exosomes have been shown to improve fracture healing caused by obesity, and subsequent investigations have revealed that exosomal LncRNA H19 regulated osteogenic differentiation through miR-467/HoxA10 axis [74]. LncRUNX2-AS1 has also been verified to transferred from multiple myeloma to MSCs via exosomes, targeting RUNX2 to involve in the osteogenesis suppression [75]. Importantly, the equilibrium between the process of bone formation and bone resorption is crucial for bone remodeling. Persistent inflammation in bone infections may result from an imbalance in bone remodeling, marked by excessive activation of osteoclasts ultimately causing bone destruction. Inflammatory osteoclasts-derived exosomal lncRNA LIOCE have been reported to stabilize osteogenic transcription factor Osterix by interacting and reducing the ubiquitination level of Osterix [76]. In addition, Chengqiang Mo et al. demonstrated that prostate cancer cells derived exosomes transferred NEAT1 to hBMSCs, and play a role in inducing osteogenic differentiation of hBMSCs in vitro and in vivo by competitively binding with miR-205-5p and regulating SFPQ/PTBP2, up-regulating RUNX2 [77].

Exosomal circRNAs

Circular RNAs (circRNAs), a novel subclass of lncRNAs with a circular structure, are evolutionarily conserved and are abundant in eukaryotes, suggesting their biological functions (Table 1). As one of the most common chronic diseases in the world, osteoporosis is characterized by bone mass loss and tissue microstructure degeneration, resulting in a high risk of bone fracture, especially in postmenopausal women [78]. Feng Zhi et al. found that the expression level of hsa_circ_0006859 was found to be significantly upregulated in the exosomes derived from the serum of osteoporosis patients [79]. The exosomal circular RNA was observed to regulate the balance between osteogenic and adipogenic differentiation through modulation of the miR-431-5p/ROCK signaling pathway [79]. The circHIPK3 was highly expressed in the exosomes derived from BMSCs, and that it promoted the osteogenic differentiation of MC3T3-E1 cells through the miR-29a-5p/PINK1 axis [80]. Furthermore, osteoporosis in postmenopausal women, which is the most common type in older females, is caused by reduced estrogen levels that are unable to effectively regulate osteoclast activity, leading to accelerated breakdown and absorption of bone tissue [81–83]. Exosomal circFAM63B derived from the serum of postmenopausal osteoporotic patients has been shown to suppress bone regeneration via miR-578/HMGA2 axis and regulate postmenopausal osteoporosis [84].

Exosomal protein

As one of the abundant contents of exosomes, proteins are not only the markers of exosomes but also play a role in regulating multiple processes of bone regeneration by transmitting different information between cells. These proteins include transcription factors, cytokines, and signaling molecules (Table 1).

The rotator cuff tear is the primary type of injury to the shoulder joint, leading to a significant impairment of shoulder joint function. Han L. et al. reported that BMSC-derived exosomal BMP2 promote tendon bone healing via activating Smad/RUNX2 signaling pathway [85]. Additionally, it has been revealed that myoblast-derived exosomal Prrx2 can mitigate the occurrence of "osteosarcopenia," the simultaneous manifestation of sarcopenia and osteoporosis, in the elderly population. In details, exosomal Prrx2 plays a role in the transcriptional activation of miR-22HG, which in turn activates the YAP pathway by sponging miR-128, thereby promoting the osteogenic differentiation of BMSCs [86]. Furthermore,

malocclusion refers to the misalignment or improper contact of teeth, and orthodontic treatment is an effective method for correcting malocclusion by inducing tooth movement through prolonged mechanical forces [87–89]. Orthodontic tooth movement (OTM) is essentially a process of alveolar bone remodeling induced by mechanical forces and regulated by local inflammation, making the exploration of its underlying mechanisms crucial for anatomical studies [90, 91]. Panjun Pu et al. revealed that the mechanical force induced macrophage-derived exosomal UCHL3 enhancing BMSCs osteogenic differentiation via targeting SMAD1 [92].

The strategies of engineering exosomes for bone regeneration

Although an increasing number of exosome cargos have been proven to have a beneficial role in bone regulation, a sufficient amount of functional contents is often required in bone defect applications to meet the needs of injury repair [114]. Engineering exosomes is the process of modifying or manipulating exosomes, which refers to altering the contents of exosomes, such as proteins or nucleic acids, to enhance their therapeutic properties, as well as modifying their surface to improve targeting and delivery to specific cells or tissues [98, 115, 116].

In the past decade, there has been rapid development in the field of engineered exosomes aimed at obtaining exosomes enriched with functional cargo. This involves two main approaches: loading cargo into exosomes before their isolation from cells, and loading cargo into isolated exosomes, as shown in Fig. 4. The pre-isolation loading methods include specific cargo exogenous transfection, transduction, as well as co-culture of cells with small molecules, resulting in modifying the parent cells to produce exosomes with specific cargo. Si Chen et al. obtained engineered exosomes with high expression of miR-375 derived from human adipose mesenchymal stem cells (hADSCs) through lentivirus infection and

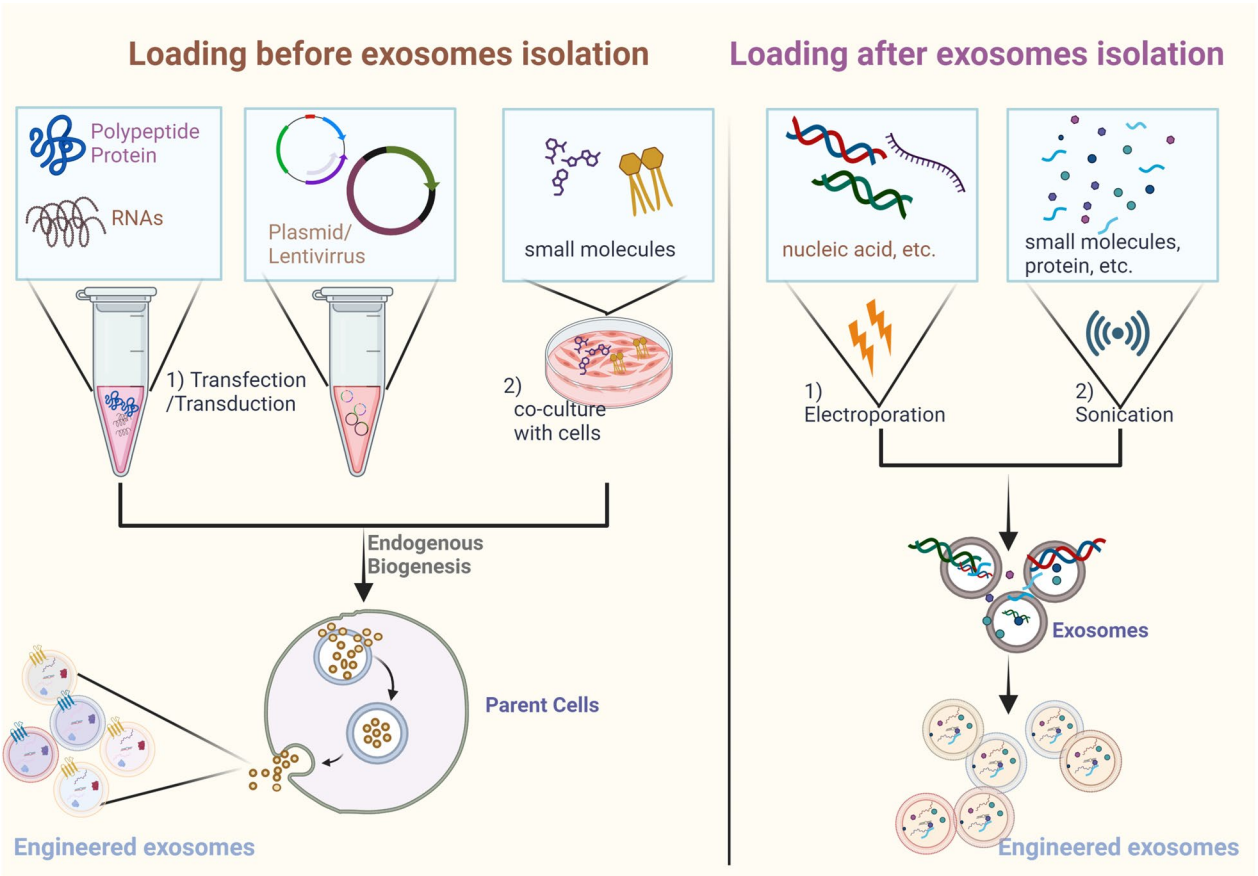

Fig. 4 The strategies of engineering exosomes. The strategy of engineering exosomes typically involves two approaches, including loading target cargos before exosomes isolation and loading after exosomes isolation. Left panel: Pre-isolation loading is usually achieved by transfection, transduction, etc., or by co-culturing small molecules to introduce RNA, proteins, plasmids, etc., into the parent cells and then isolating engineering exosomes. Right panels: Post-isolation loading is typically done by electroporation, ultrasonication, etc., to directly transfer nucleic acids, small molecules, proteins, etc., into the exosomes

demonstrated good osteoinductive properties in vitro and in vivo [117]. Furthermore, Zin and colleagues established a genetically Human Umbilical Vein Endothelial Cell (HUVECs) with overexpression of PD-L1 through plasmid transfection, and subsequently isolated engineered exosomes with high PD-L1 expression using ultracentrifugation [118]. The engineered exosomes induced MSCs osteogenic differentiation by suppressing T cell proliferation [119]. Yang et al. designed engineered exosomes enriched with a higher abundance of Bmp2 mRNA, which were extracted from 293 T cells after co-transfection of non-annotated P-body dissociating polypeptide and Bmp2 artificial plasmid, demonstrating enhanced osteoinductive properties [28].

Post-isolation loading means directly encapsulate cargos into isolated exosomes via electroporation or sonication. Blood vessels are essential for bone regeneration as they supply oxygen, nutrients, immune cells, and growth factors to the bone cells, supporting their growth and repair [120]. The inadequate vascular communication at the site of injury significantly impacts the bone injury repair process, especially the large segmental bone regeneration [121]. Zha and colleagues generated gene-activated engineered exosomes by employing electroporation to encapsulate the VEGF gene within exosomes derived from ATDC5 cells [122]. The engineered exosomes have been shown to have the dual role of osteogenic matrix and the release of VEGF gene vectors, thereby remodel the vascular system and ultimately achieving large bone repair [122]. Moreover, Zhu et al. loaded miRNA into exosomes through co-incubation to obtain engineered exosomes containing miR-182–5p mimic, miR-182–5p inhibitor, and NC [112]. Exosomes loaded with the miR-182–5p inhibitor promote bone regeneration by activating the PI3K/Akt signaling pathway, which confirmed the availability of direct modification of exosomes strategy from the opposite [112]. Similarly, Choi et al. disrupted the function of pre-osteoblast exosomal let-7, a crucial miRNA involved in regulating osteogenesis, by introducing a let-7 inhibitor into exosomes using electroporation. They observed that these modified exosomes no longer possessed the capability to promote osteogenic differentiation [123].

The engineering of exosomes with distinct modifications pre- and post-isolation has led to the development of engineered exosomes enriched with functional bone-related regulatory factors and exhibiting strong osteoinductive properties. Nevertheless, these strategies still encounter challenges. For instance, direct modification of exosomes seems to be a straightforward and effective method for engineering exosomes, but factors such as the length, size, and charge of the active substances can impact the efficiency of exosome internalization [124–126]. Additionally, ultrasonication may cause the most significant disruption to exosome membrane integrity [127]. Meanwhile, overexpressing circular RNA within cells through transfection and similar methods is limited by low circularization efficiency and accuracy, which subsequently restricts its osteogenic effects. Several studies have also revealed the potential influence of genetic modifications on other biologically active molecules within exosomes, such as lncRNAs and circRNAs [128]. Hence, there is a need to explore new and high-efficiency genetic modification technologies to address these issues.

Exosomes functionalized scaffolds in bone regeneration

Although exosomes have shown excellent bone inductivity in both in vivo and in vitro studies. However, using exosomes alone for bone repair in bone tissue engineering is not recommended due to challenges in their retention and sustained release at the target site. Therefore, it is preferable to utilize functional scaffolds loaded with exosomes in combination with scaffolds, as this approach allows for sustained and localized delivery of exosomes, enhancing bone regeneration. In recent years, a large number of studies have applied exosomes in combination with biomaterial scaffolds to tissue defects to improve the bone repair effect of simple scaffold implantation, presenting a novel and promising approach for achieving cell-free bone regeneration in tissue defects (Fig. 5).

Exosomes functionalized metallic scaffolds

Metal scaffolds play a crucial role in bone tissue engineering, providing reliable support for bone regeneration and repair. Typical metal scaffolds utilized in bone tissue engineering consist of titanium alloy,metal–organic frameworks, stainless steel scaffolds, etc. To achieve cell-free tissue regeneration, exosomes functionalized metal-based scaffolds improved cell signaling and communication, enhanced tissue regeneration and repair.

Zhang et al. attempted to load exosomes derived from human dental pulp stem cell (hDPSCs) onto metallic titanium scaffolds for rat models with cranial defects, demonstrating promoted new bone formation which is due to modulate bone repair through the induction of differential miRNA expression, included upregulating osteogenic miRNAs (hsa-miR-29c-5p, hsa-miR-378a-5p, hsa-miR-10b-5p and hsa-miR-9-3p) and downregulating anti-osteogenic miRNAs (hsa-miR-31–3p, hsa-miR-221–3p, hsa-miR-183–5p and hsa-miR-503–5p) [129]. Titanium alloys are commonly utilized as orthopedic implants with a high success rate. However, porous titanium alloys often struggle to integrate effectively with surrounding bone tissue, leading to limited bone ingrowth into

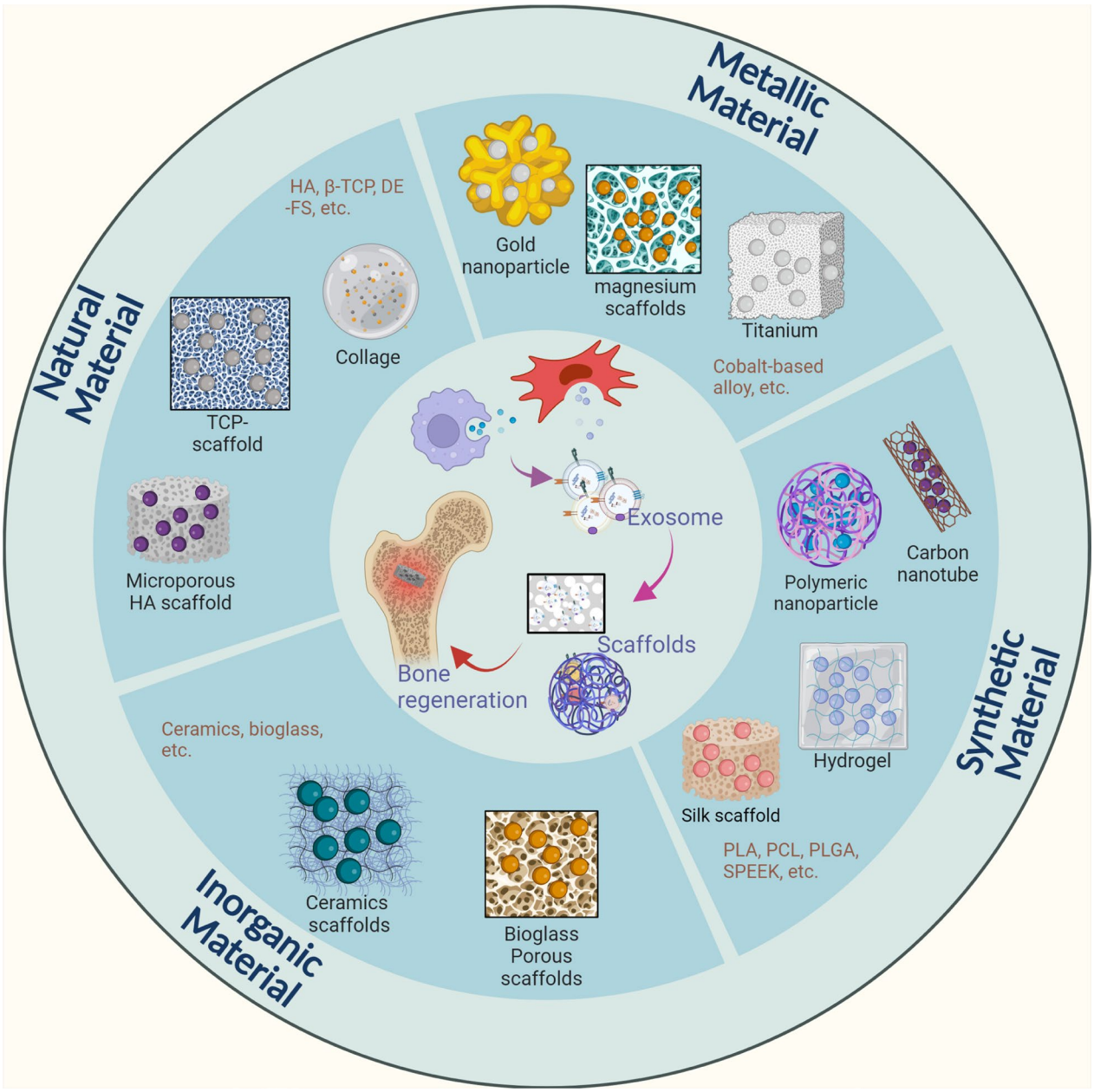

Fig. 5 Exosomes functionalized scaffolds in bone regeneration**.** Exosomes functionalized scaffolds in bone regeneration. Scaffolds combined with different derived-exosomes for bone regeneration can be classified into 4 categories: Natural Material, Metallic Material, Synthetic Material and Inorganic Material

the implants. Zhigang Wu et al. and colleagues loaded Schwann cell-derived exosomes on a titanium alloy scaffold and revealed that the incorporation of exosomes could enhance the migration, proliferation, and differentiation of BMSCs, significantly improving bone repair [130]. Furthermore, in the field of orthopedics, addressing defects in large bones has presented significant challenges. The combination of BMSCs derived exosomes and tantalum metal (pTa) scaffold showed effective bone repairation. Among, pTa acted as a core scaffold for cell adhesion, while the exosomes enhanced the proliferation and differentiation of BMSCs [131]. Moreover, the repair of large segmental bone defects is currently a major challenge in bone regeneration due to the need for extensive bone regeneration and vascular reconstruction in the affected bone region. Hao Liu and colleagues

have developed an innovative 3D-printed titanium scaffold loaded with serum-derived exosomes using a cell-free scaffold strategy, demonstrating clear benefits in the treatment of significant bone defects via osteoconduction, osteoinduction, and revascularization [132].

Exosomes functionalized naturally derived scaffolds

Biomaterials derived naturally from living organisms, such as collagen, silk fibrin, chitosan, and hyaluronic acid, exhibit excellent biocompatibility, minimal adverse immunoreactions, impressive plasticity, and abundant sources. These materials are ultimately degraded into carbon dioxide and water, making them ideal for use in clinical applications of bone defect repair [133].

Periodontal disease is an inflammatory condition that affects the gums in the mouth due to bacterial infection, and it is closely associated with the occurrence of bone loss. DPSC-derived exosomes incorporated chitosan hydrogel (DPSC-Exo/CS) have been shown to accelerate the healing of alveolar bone and the periodontal epithelium caused by periodontitis [103]. In detail, DPSC-Exo/CS converted macrophages from a pro-inflammatory phenotype to an anti-inflammatory phenotype via exosomal miR-1246 [103]. Furthermore, in a mouse calvarial defect model, the combination of hMSC-derived exosomes with injectable chitosan hydrogel led to bone regeneration by downregulating noggin through the inhibition of miR-29a [134]. Hyaluronic acid is a linear nonsulfated glycosaminoglycan composed of alternating repetitions of D-glucuronic acid and N-acetyl-D-glucosamine connected by β-1,3 and β-1,4 glycosidic bonds, which can be synthesized by most cells in the body [135, 136]. Hyaluronic acid-based scaffolds have been widely used in the field of biomedicine due to their biocompatibility, biodegradability, viscoelasticity, and other characteristics [137–139]. However, simply implanting hyaluronic acid scaffolds into damaged areas is insufficient for repairing severe bone defects. Researchers have achieved better bone repair in defect areas by constructing various cell-derived exosome-hyaluronic acid-based scaffolds. Zhang et al. used umbilical cord mesenchymal stem cell (UCMSCs)-derived exosomes carried by hyaluronic-based scaffolds to repair skull defects in rats, and found that exosome-derived miRNA miR-21, as an intercellular messenger, promoted angiogenesis by inhibiting NOTCH1/DLL4 pathway to achieve the purpose of bone defect repair [140].

Exosomes functionalized synthetic scaffolds

In the practical application of bone defect repair, traditional metals and natural materials often fall short of meeting clinical needs. Synthetic biomaterials have emerged as the leading trend in bone tissue engineering, harnessing the combined benefits of diverse materials. This category enables large-scale, precise design and production while minimizing the risk of immune response, encompassing polymers, organic synthetic materials, synthetic inorganic materials, and composite materials. Meanwhile, exosomes functionalized synthetic scaffolds demonstrated better osteoinductive effects in bone repair.

The organic synthetic material polylactic acid (PLA) is widely used in tissue defect repair and has been increasingly utilized to construct 3D-printed bone implants [141]. Zhang et al. modified the surface of PLA by carrying MSC derived exosomes, and developed a 3D PLA/MSC-Exo scafold. Exosomes released from the scafold greatly improved its osteogenic and immunoregulatory potential, the pro-inflammatory markers in macrophages were reduced, and the osteogenic differentiation ability of MSCs were significantly enhanced [142]. Meanwhile, Yue Kang and colleagues designed and synthesized a novel exosomes functionalized scaffold, PLGA/Exo-Mg-GA MOF, with a unique nanostructured interface using hADSCs-Exos, $Mg2+$, and gallic acid (GA) [143]. In vitro experiments demonstrated that the synthesized scaffold promoted osteogenic differentiation of hBMSCs and vascular formation in HUVECs [143]. Furthermore, the slowly released hADSCs-Exos from the synthesized scaffold, were phagocytosed by co-cultured cells, released their bioactive contents, stabilized the osteogenic environment, ensured blood supply, and promoted new bone formation in a rat calvarial defect model [143]. Moreover, bone regeneration usually takes a long time, shuo Yang et al. combined the hUCMSCs derived exosomes with an injectable composite scaffold constructed by alginate, hyaluronic acid and hydroxyapatite, achieved both controlled exosome delivery and physical support of the defects scaffold [144].

Discussion

The significant role of exosomes in bone regeneration has been recognized by many researchers. Yunhao Qin et al. have summarized the characteristics, origins, and biogenesis of exosomes as small endogenous vesicles that deliver functional cargos between cells, thereby regulating the differentiation, function, and proliferation of target cells, highlighting their potential applications in bone regeneration [145]. Additionally, in the construction of scaffold materials for bone regeneration, metallic ions are widely utilized due to their superior ability to promote angiogenesis and osteogenesis. Xuwei Luo et al. focused on the relationship between metallic ions and exosomes, systematically analyzing the effects of metallic ions and their associated biomaterials on the secretion of exosomes from MSCs and macrophages, as well

as the roles of secreted exosomes in inflammation, angiogenesis, and osteogenesis [146]. Here, we takes a broader perspective by examining exosomes from different sources and the functionalized scaffolds that incorporate exosomes with varying material compositions, providing a more comprehensive summary of the research and application prospects of exosomes and their functionalized scaffolds in bone regeneration.

Exosomes, intrinsic membrane-bound vesicles crucial for intercellular communication, play a pivotal role in this process. Because of its low immunogenicity, high stability, low tumorigenicity and the innate homing effect of targeted organs, it is a good bioactive substance. The present review primarily focuses on the synthesis and characterization of exosomes, the molecular basis between exosomal cargos and bone regeneration, strategies for engineering exosomes, and the properties of exosome-functionalized scaffolds necessary for bone regeneration. Natural exosomes from different sources have demonstrated excellent regulatory abilities in bone regeneration by targeting various downstream signals, including MSCs, ADSCs, macrophages, among others [96–98]. Additionally, as crucial factors involved in bone regeneration within exosomal cargos are identified, engineered exosomes produced through transfection methods exhibit improved osteoinductive potential. Meanwhile, leveraging scaffolds in bone tissue engineering for bone repair, exosome-functionalized scaffolds meet a wider range of conditions for bone regeneration.

In the context of clinical bone defect repair, particularly when addressing significant bone injuries, it is essential to carry out large-scale preparation and isolation of exosomes, tailor biological materials to accommodate various defect conditions, and proficiently incorporate exosomes into scaffold materials. Improving the separation and purification of exosomes and the preparation of engineered exosomes is an effective means to improve the consistency of exosomes and stabilize their activity after loading. With the emergence of biological materials with different functions such as immune regulation, vascular regeneration, and bone repair targeting, the self-renewal of exosome functionalized scaffolds has been greatly promoted.

Prospect

Despite the significant potential of exosomes and their functionalized scaffolds in the field of bone regeneration, several challenges remain. Exosome isolation techniques primarily include ultracentrifugation, density gradient centrifugation, precipitation, membrane filtration, chromatography, and microfluidics, each with its own advantages and disadvantages [147, 148]. While ultracentrifugation can yield high purity and concentration of exosomes, it is time-consuming and requires advanced equipment. Conversely, precipitation is favored for its simplicity and low cost, although it typically results in lower purity. Membrane filtration is suitable for large-scale separations, whereas chromatography can enhance purity through specific labeling. Microfluidic technologies offer high-throughput capabilities but involve more complex design and implementation [148–150]. Currently, the exosomes obtained through different isolation methods vary in content and quality, which may influence their subsequent biological functions [151, 152]. Therefore, the development of standardized methods for exosome isolation and ensuring the reproducibility of exosome quality across different batches is crucial. Additionally, achieving scalability in the fabrication of functionalized exosome scaffolds remains a pressing issue. In existing studies, mice are commonly used as experimental models, but the translation of research findings to clinical applications must address practical challenges such as longer treatment durations and increased demand. Future research utilizing 3D printing technology to create functionalized exosome scaffolds for bone defect repair may represent a promising direction.

Furthermore, future studies may focus on uncovering the critical molecules that drive the functionality of exosomes in bone regeneration, as well as the chemokines that attract cells involved in bone repair across different types of bone defect conditions. Developing engineered exosomes rich in diverse factors with multifunctional and multi-target capabilities could facilitate the complex regulatory needs of the bone regeneration microenvironment. Moreover, in light of the challenges posed by complex fractures, trauma, and serious underlying diseases in bone defect repair, there is a pressing need to develop innovative scaffold materials and explore combinatorial therapies involving different exosomes to optimize outcomes. Overall, leveraging exosomes and functionalized scaffolds holds significant potential in advancing regenerative medicine strategies for promoting bone healing and repair.

Abbreviations

ApoBDs	Apoptotic bodies
BMSCs	Bone marrow stem cells
BMP-2	Bone morphogenetic protein 2
Ca^{2+}	Calcium ion
circRNAs	Circular RNAs
ECs	Endothelial cells
EPCs	Endothelial progenitor cells
EVs	Extracellular vesicles
GA	Gallic acid
hADSCs	Human adipose mesenchymal stem cells
HUVECs	Human Umbilical Vein Endothelial Cell
hUCMSCs	Human umbilical cord mesenchymal stem cells
hDPSCs	Human dental pulp stem cell
ISEV	International Extracellular Vesicle Society
ILVs	Intraluminal vesicles

LncRNAs	Long non-coding RNAs
miRNAs	MicroRNAs
MSCs	Mesenchymal stem cells.
MVBs	multivesicular bodies.
MVs	Microvesicles.
NTA	Nanoparticle tracking analysis.
OTM	Orthodontic tooth movement.
OPLL	Ossification of posterior longitudinal ligament.
PCa	Prostate cancer.
PLA	Polylactic acid.
TEM	transmission electron microscope.
pTa	tantalum metal.
VEGF-A	vascular endothelial growth factor A.
WB	Western blot.

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Availability of data and materials
The data presented in this study are available on request from the corresponding author.

Declarations

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Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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