REVIEW

Cellular and Molecular Connections Between Bone Fracture Healing and Exosomes

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Summary

Fracture healing is a multifaceted process that requires various phases and intercellular interactions. In recent years, investigations have been conducted to assess the feasibility of utilizing exosomes, small extracellular vesicles (EVs), to enhance and accelerate the healing process. Exosomes serve as a cargo transport platform, facilitating intercellular communication, promoting the presentation of antigens to dendritic cells, and stimulating angiogenesis. Exosomes have a special structure that gives them a special function, especially in the healing process of bone injuries. This article provides an overview of cellular and molecular processes associated with bone fracture healing, as well as a survey of existing exosome research in this context. We also discuss the potential use of exosomes in fracture healing, as well as the obstacles that must be overcome to make this a viable clinical practice.

Key words

Bone fracture healing • Exosomes • Connection • Cellular • Molecular

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Introduction

Bone plays vital roles in the body, including providing structural support for muscles, ligaments, and tendons and protecting internal organs from injury [1]. The process of bone healing when a bone injury has occurred is an issue that is constantly being studied in depth. Osteogenic cells, the key cell in the bone fracture process, mainly comprise three types of cells: the osteoblasts, osteoclasts, and osteocytes. Different from traditional therapies, the characterization of genes and proteins involved in the bone fracture process may lead to the identification of novel therapies that can be used as adjuncts or alternatives. The main research interests in promoting bone healing are vascular and stem cells [2,3]. Wei *et al.* reported that bone marrow mesenchymal stem cells (BMSC)-derived exosomal lncTUG1 enhanced the osteoblastic activity that contributes to bone fracture healing [4]. Saxer *et al.* demonstrated that stromal vascular fraction cells are capable of spontaneously forming bone tissue and vessel structures within a fracture microenvironment, which is also involved in the formation of ossicles at the repair site [5].

Exosomes are cellular membrane-like structures with a diameter of 30-150 nm that can be secreted by most cells of the body and can be present in body fluids to function [6]. Because of the specific proteins and RNA contained on or inside the membrane, it allows more efficient and precise communication between cells [7,8]. Moreover, since exosomes are different from other EV subtypes in terms of origin, production process and site of action, there are more specific roles in exosomes [9-11]. Exosomes have been shown to have the ability of cell-to-cell communication systems and mediator

between cells in osteoarthritis, fracture healing, and cartilage repair [12,13].

Some research has shown that exosomes play important roles in bone extracellular matrix production [14] and mediating signaling stimulation, which causes the osteoblastic activation [15]. Exosome research has gradually expanded in recent years from the diagnostic role of exosomes to their therapeutic role, indicating that exosomes will continue to be studied in depth as a hot spot [16,17]. In this article, we aim to illustrate the connection between exosomes function and bone healing by summarizing recent studies. We also provide some clinical strategy references for the intervention of bone healing using exosomes.

Exosomes in fracture healing

Definition and classification of exosomes

The first study to report EVs was published in 1967. However, limited by the development of technology, exosomes were not found until 1983 [18]. Exosome (30-100 nm), one of the subtypes of EVs, are currently the most widely studied subunits. Exosomes are composed of a lipid bilayer that carries a shell of multiple proteins and contains special molecules such as proteins or RNA, and these special intra-membrane molecules give exosomes their enriched functions [8,19,20] (Fig. 1).

Because exosomes exist widely in body circulation and tissue fluid, they act as a communication bridge between different cells and play a complex role. In the study of bone healing, exosomes are related with BMSCs, macrophages, endothelial cells, myoblasts, human umbilical vein endothelial cells (HUVECs), etc. The classical exosome classification distinguishes the different subspecies to which they belong in EVs according to their diameter size. It is a more effective method of distinguishing exosomes according to their different cell origins (Table 1).

Structural properties and characteristic of exosomes

Exosomes are secreted by plasma membrane fusion with multi-vesicular bodies (MVBs) that are formed by invagination of late endosomal membranes into intraluminal vesicles (ILVs). Exosomes have more specific functions due to the differentiation from other EVs subunits at the production stage.

Exosomes have the ability to transfer contents from cells to cells, which is based on membrane fusion mechanisms [22]. Due to the similar molecular structure of exosomes and cell membranes, this continuous

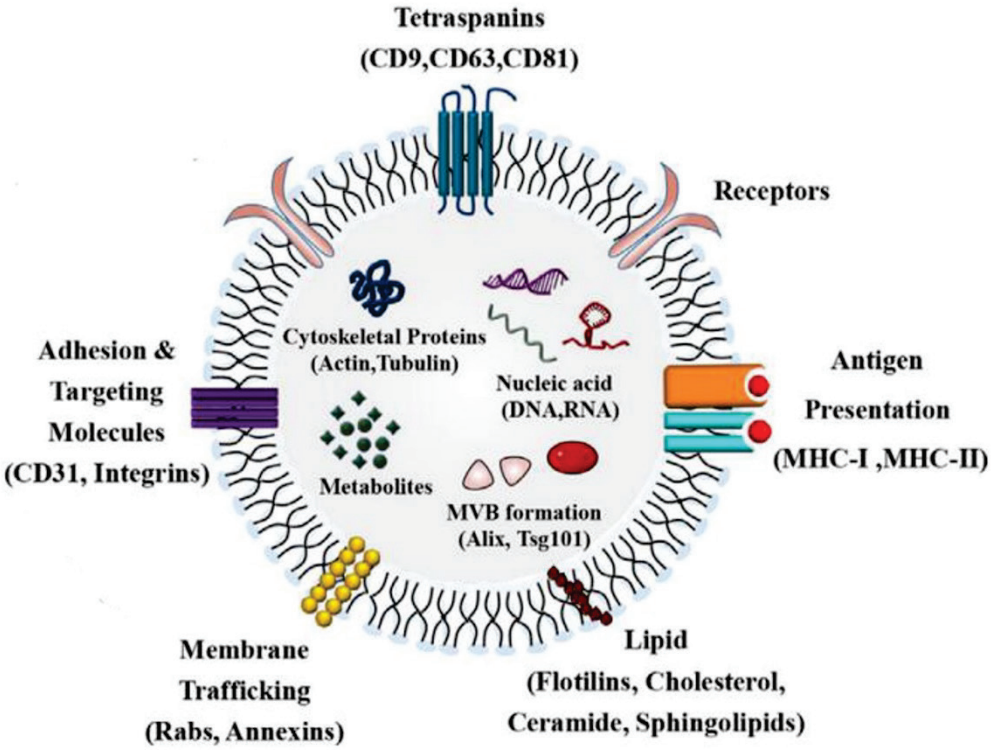

Fig. 1. Compositions of EVs. The mechanisms of EV biogenesis are significantly related to their compositions and functions [21]. They act as endogenous delivery systems for intracellular communication, transporting proteins, lipids, metabolites, and nucleic acids while influencing various aspects of cell life.

Table 1. Different parent cells and mechanisms of exosomes in bone fracture healing.

Parent Cells	Species Information	Key Molecular	Mechanisms	Citation (PMID)
BMSCs	Rat, male, 12 weeks old	BMP-2/ Smad1/RUNX2	Pro-osteogenic differentiation	31992369
BMSCs	Beagle dogs, male, adult	FBXL14	Pro-osteogenic differentiation	35643547
BMSCs	Mouse, male, age NA	Not referred	Pro-osteogenic differentiation	31660556
Mesenchymal stromal cells	Human	Tβ-Fb	Anti-fibrotic	34202139
BMSCs	Mouse, female, 16 weeks old	miR-214-3p	Inhibit angiogenesis	33161580
Umbilical cord mesenchymal stem cells	Human, obtained after healthy neonatal deliveries	HIF-1α	Promotion of angiogenesis	30663158
Adipose stem cells	Rat, male, 15 weeks old	Wnt3a/ β-catenin	Pro-osteogenic differentiation	36902283
Macrophages	Mouse, male, 6 weeks old	miR-5106	Pro-osteogenic differentiation	32345321
Macrophages	Rat, male, 15 weeks old	miR-144-5p/ Smad1	Pro-osteogenic differentiation	34340698
Macrophages	Mouse, male, 6 weeks old	SDF-1α	Pro-osteogenic differentiation/ promotion of angiogenesis	37056272
Endothelial cell	Mouse, gender/age NA	miR-155	Inhibit osteoclast activity and osteoporosis	30968694
Endothelial progenitor cells	Mouse, male, 6 weeks old	miR-124	Pro-recruitment and differentiation of osteoclast precursors	31025509
Endothelial cell	Mouse, gender/age NA	miR-26a-5p	Pro-osteogenic differentiation/ M2 macrophage polarization	34979087
Myoblasts	Mouse, gender/age NA	miR-27a-3p	Pro-osteogenic differentiation	29476741
HUVECs	Human, obtained from healthy donor's marrow and Mouse, gender/age NA	PD-L1	Inhibited T cells activation	35224310

membrane fusion process can be achieved under the action of some molecules such as VAMP7 [23]. In addition, pkh26 serves as a specific molecular marker, and is widely used to expose lipid bilayer in detection [24]. Molecules originally in the mother cell are delivered to the recipient cell by exosomes through this process, where they play a series of roles. Mechanisms of exosomes that effect bone healing include pro-osteogenic differentiation, inhibition of osteoclast activity and osteoporosis, etc. In view of this, we have summarized different exosomes from different parent cells and mechanisms from several representative studies (Table 1).

Exosomes have direct effects on cells, which are sometimes stronger than the parent cells. Rozier *et al*. found that mesenchymal stromal cells (MSC)-derived exosomes were more effective than parent cells in improving myofibroblastic phenotype [25]. Different from classical molecular mechanisms and signal transduction pathways, this enhanced functionality can be achieved by selective encapsulation of exosomes, which provides a functional basis for their use as therapeutics. In addition, there are typically unique molecular arrangements on the surface of exosomes derived from parent cells and obtained through electrostatic interactions with extracellular molecules, which is called "homing ability" [26]. Nevertheless, exosomes can penetrate some barriers such as joint fluid that cannot be reached by ordinary drugs, and provide a better treatment effect for bone injury.

Cellular and molecular mechanisms of fracture healing and exosomes

Applications of exosomes in fracture healing include enhancing bone regeneration, modulating the immune response, improving vascularization, and advantages of exosomes over traditional therapies. We have summarized the role of exosomes in the inflammatory phase, repairing phase, remodeling phase and other phases.

Inflammatory phase

Inflammatory immunity is an important initiating process of bone healing that a variety of immune cells are involved such as Bone cells (BMSCs, osteoblasts (OBs), osteoclasts (OCs), and osteocytes), immune cells (T cells, B cells, neutrophils, and macrophages (Mφ)) and hematopoietic stem cells (HSCs) [27,28]. The inflammatory phase occurs immediately after fracture and cells release a series of molecules for recruitment and activation of other inflammatory cells. A moderate inflammatory response in the early stages is necessary and can be damaged when overactive. Macrophages regulate fracture healing and early angiogenesis to promote additional cell recruitment [29,30]. Cui *et al.* suggested that endothelial progenitor cells (EPCs)-derived exosomes can promote bone repair by enhancing recruitment and differentiation of osteoclast precursors through LncRNA-MALAT1 [31]. Pioneeringly, Chen *et al.* combined Stromal Cell-Derived Factor-1α (SDF-1α) and M2 macrophage derived exosomes (M2D-Exos) with a hyaluronic acid (HA)-based hydrogel precursor solution, which is able to enhanced proliferation and migration of human bone marrow mesenchymal stem cell (HMSCs) and HUVECs, promoting osteogenesis and angiogenesis both *in vivo* and *in vitro* [32]. Another study on HUVECs showed that HUVECs-exosomes overexpressing PD-L1 specifically bind to PD-1 on the T cell surface and inhibit its activation to inhibit overactive inflammation [33].

Numerous factors are involved in the regulation of macrophages by BMSC. Among them, BMSC-derived exosomes are one of the most valuable molecules and are involved in a variety of immune activities. BMSCs are multipotent stem cells found in bone marrow and are attractive sources of regenerative medicine [34,35]. Zhao *et al.* studied that BMSC-EVs attenuated myocardial ischemia-reperfusion by shuttling miR-182 and modified Mφ polarization [36]. In addition, miR-25 secreted by BMSC-Exo can accelerate osteogenic differentiation, proliferation, and migration of osteoblasts *via* the SMURF1/Runx2 axis [37]. In addition, several studies have demonstrated that exosomal miRNAs derived from BMSCs are involved in regulating the pathological process of osteoarthritis (OA), an immune disease that significantly affects the course of bone damage [38,39].

EVs contain two main classes of secreted vesicles: microvesicles and nanometer vesicles, depending on their size, content, origin and biogenesis [6]. Some research has shown that other types of EVs rather than exosomes also play roles in the inflammatory process of bone fracture healing. Zheng *et al.* found that BMSC-derived apoptosis vesicles (apo-Vs) could induce Mφ reprogramming in the treatment of type 2 diabetes liver which may contribute to Mφ polarization towards the anti-inflammation phenotype. Although more experimental evidence is needed, many existing studies suggest that inflammatory immunity in the healing process of bone injury dominated by BMSC-derived exosomes is a promising research direction.

Repairing phase

Osteoblast is a specialized bone-forming cell derived from pluripotent BMSCs that is induced by specific transcription factors [40,41]. Osteogenic cells play a key role in the repair phase, which induces granulation tissue formation by filling the fracture gap and provides early bone scab for sustained recovery. Exosomes from or not from BMSCs may regulate the osteogenic differentiation of BMSC and thus promote bone healing (Fig. 2). Li *et al.* studied that mutant HIF-1α-modified BMSCs exosomes promote the osteoblastic differentiation of BMSCs [42].

Other types of stem cells can also release exosomes and play their vital roles. Zhang *et al.* shown that adipose stem cells (ASCs)-exosomes enhance the osteogenic potential of BMSCs by activating the Wnt/β-catenin signaling pathway, and also facilitate the ability for bone repair and regeneration *in vivo* [43]. Another example is Zhang *et al.* indicated that HIF-1α played an important role in umbilical cord mesenchymal stem cells (uMSCs) exosomes which induced VEGF expression, pro-angiogenesis and enhanced fracture repair to accelerate fracture healing [44].

Nevertheless, exosomes of BMSCs could regulate bone fracture healing process by modulating the expression level of some key molecular [45-47]. Qi *et al.* studied that exosomes secreted by mesenchymal stem

cells derived from human induced pluripotent stem cells (hiPSC-MSCExos) enhanced cell proliferation and alkaline phosphatase (ALP) activity, up-regulated mRNA and protein expression of osteoblast-related genes in bone marrow MSCs derived from ovariectomized rats [48]. Also, evidence showed that BMSC-derived exosomes promote osteoblast proliferation by inhibiting cell apoptosis [49,50].

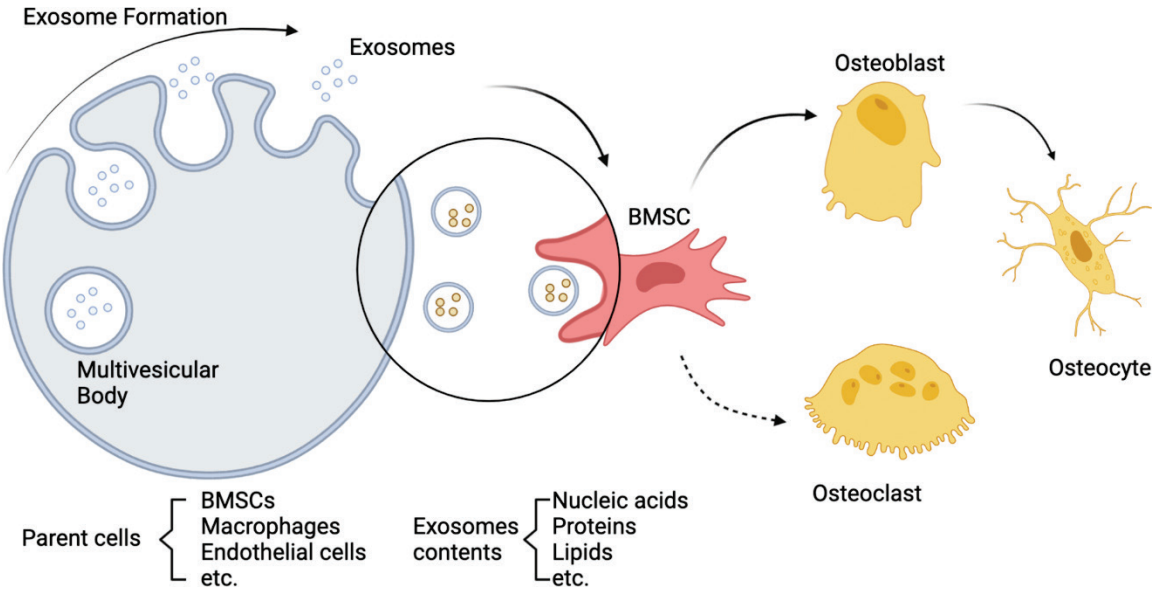

Fig. 2. Exosomes regulate the differentiation process of osteoblasts and bone fracture healing.

Remodeling phase

In the final remodeling phase, osteoclasts resorb the woven bone and osteoblasts replace it with lamellar bone, both of which contribute to restoring the bone to its original structural shape and mechanical properties. There are many connections between osteoclasts and osteoblasts to establish a stable state. However, this imbalance in the steady state is responsible for many bone healing diseases such as osteoporosis. Li *et al.* showed that miR-214-3p in exosomes can be transferred from osteoclasts to osteoblasts and inhibit bone formation by osteoclasts [51]. Exosomes are involved in the differentiation tendency of osteoblasts and osteoclasts, thus promoting or inhibiting bone formation [39]. In view of this, the treatment of osteoporosis can be achieved through exosomes to interfere with the molecules involved.

There is also a strong association between osteoclasts and BMSCs by exosomes. Rong *et al.* reported that miR-31a-5p could promote osteoclasto-genesis and bone resorption, which is increased in BMSC-exosomes in older rats than in younger rats [52]. In addition, exosomal miR-221 expression was decreased in the early stages of osteogenic differentiation of BMSCs, which are considered regulators of osteoblast differentiation [53].

Other phases

In addition to the functions of exosomes described above, we select several other aspects to summarize.

Muscles are an important part of the fracture healing process [54]. Exosomes that are actively secreted by satellite cells, differentiated myoblasts, and mature myotubes can enhance muscle regeneration and muscle-derived circulating [55,56]. Moreover, muscle-derived exosomes appear to play a vital role in muscle-bone communication. Exosomes from C2C12 myoblasts have been shown to promote differentiation of pre-osteoblastic MC3T3-E1 cells into mature osteoblasts [54]. Due to the limited number of studies conducted to assess the impact of exosomes derived from muscle cells on osteoclasts, more studies are needed.

Fracture combined with traumatic brain injury (TBI) is one of the most serious types of compound trauma in the clinic. Research has shown that TBI-Exosomes can be internalized by osteoblasts, inhibiting SMAD7 and promoting osteogenic differentiation, whereas knockdown of miR-21-5p in TBI-Exosomes strongly inhibited this bone-beneficial effect [57]. This shows that exosomes will provide new therapeutic options for difficult diseases combined with bone injuries in some cases.

Blood vessels provide nutrition during bone healing and are an important part of the process [58,59]. Studies have shown that vascular endothelial cell-secreted exosomes (EC-Exos) could also affect bone fracture healing by inhibiting bone marrow macrophages and RAW264.7 cell differentiation into osteoclasts [58]. Also, Hu *et al.* reported that extracellular vesicles from human umbilical cord blood (UCB-EVs) inhibited osteoclastogenesis-related genes and osteoclastic differentiation of RAW264.7 cells induced by RANKL [60].

Prospects and challenges for clinical application

Exosomes have many excellent properties, such as being simultaneously hydrophilic and lipophilic to carry rich types of carriers, having fixed directional delivery properties, and crossing sites such as the blood-brain barrier, which can be used as some outstanding advantages to solve existing challenges in the healing process of bone injuries. Lin *et al.* obtained enriched PD-L1 concentrations from exosomes that are capable of inhibiting T cell activation in peripheral lymphatic tissues for bone fracture therapy [33].

Although exosomes have been well studied, there are still some challenges. Studies have shown that the role of exosomes in inflammation is dual, with most studies suggesting that exosomes promote inflammatory responses and inhibit chondrogenesis, although some studies also suggest that exosomes have positive effects on joints [12,61]. In addition, in most animal experiments, exosomal drugs *in vivo* are injected through animal blood circulation, which tends to be concentrated in the lungs and liver rather than other organs. To solve these kinds of problems, Luo *et al.* made efforts and experienced a stromal cell exosome (STExos). The exosome surface is conjugated with a BMSC-specific aptamer, which delivers STExos into BMSCs within bone marrow with high efficiency [41].

Exosome extraction technology is still not mature enough, especially when a large amount of extraction is required. According to most studies, only 1 μg of exosomes can be extracted from 1 ml of medium for cell culture [62,63]. Although scholars envisioned methods to stimulate cells to produce more exosomes [64], they were unable to increase exosome production, which limited the progress of basic research and clinical therapy research on exosomes.

Conclusions

In this paper, we review the role and molecular mechanisms of exosomes in the three major bone healing processes. Due to these special functions of exosomes, there are clear prospects for clinical applications. In summary, exosomes play an important role in the bone healing process and may be used as potential molecular carriers to promote bone fracture healing. Despite some challenges, exosomes are expected to become an effective therapeutic agent for bone fracture healing as more meaningful studies are conducted and corresponding technologies are optimized.

Conflict of Interest

There is no conflict of interest.

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