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**Application of exosome-derived noncoding RNAs in bone regeneration:
Opportunities and challenges**

Exosome-derived ncRNAs in bone regeneration

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Abstract

With advances in the fields of regenerative medicine, cell-free therapy has received increased attention. Exosomes have a variety of endogenous properties that provide stability for molecular transport across biological barriers to cells, as a form of cell-to-cell communication that regulates function and phenotype. In addition, exosomes are an important component of paracrine signaling in stem-cell-based therapy and can be used as a stand-alone therapy or as a drug delivery system. The remarkable potential of exosomes has paved the pathway for cell-free treatment in bone regeneration. Exosomes are enriched in distinct noncoding RNAs (ncRNAs), including μ -microRNAs (miRNAs), long noncoding RNAs (lncRNAs) and circular RNAs (circRNAs). Different ncRNAs have multiple functions. Altered expression of ncRNA in exosomes is associated with the regenerative potential and development of various diseases, such as femoral head osteonecrosis, myocardial infarction, and cancer. Although there is increasing evidence that exosome-derived ncRNAs (exo-ncRNAs) have the potential for bone regeneration, the detailed mechanisms are not fully understood. Here, we review the biogenesis of exo-ncRNA and the effects of ncRNAs on angiogenesis and osteoblast- and osteoclast-related pathways in different diseases. However, there are still many unsolved problems and challenges in the clinical application of ncRNA; for instance, production, storage, targeted delivery and therapeutic potency assessment. Advancements in exosome-derived ncRNA methods and design will promote the development of therapeutics, revolutionizing the present landscape.

Key Words: Exosomes; Non-coding RNA; Bone; Osteogenesis; Angiogenesis; Osteoclasts

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Core Tip: The key to bone regeneration is the mutual balance between osteoblasts, osteoclasts and angiogenesis. As a critical factor in bone regeneration, exosome-derived noncoding RNA (exo-ncRNA) has been extensively studied. However, the detailed mechanism of exo-ncRNA in bone regeneration is still unclear, and further research is necessary. This article summarizes the research on exo-ncRNA in bone regeneration.

INTRODUCTION

Exosomes

Although extracellular vesicles (EVs) were first mentioned in the late 1960s^[1], it was not until the last decade that they were named ^[2]. According to characteristics and cell sources, EVs can be divided into three types: apoptotic bodies, microvesicles, and exosomes^[3]. Exosomes are small EVs^[4] that were first found in reticulocytes^[5]. Johnston named these structures as exosomes in 1987^[6]. The formation and secretion of exosomes is a complex biological process. Exosomes express biological effects through the paracrine pathway and transport bioactive substances to regulate intercellular communication^[7]. Therefore, stable and efficient separation and extraction methods are the prerequisites for their clinical application. Although diverse exosome isolation techniques have been developed based on their biophysical and biochemical properties, there is still a lack of standardized and large-scale clinical isolation and purification methods.

Biogenesis and isolation of exosomes

Exosomes are spherical endocytic vesicles with a diameter of 40–150 nm. They are formed in intracellular multivesicular bodies and removed from various cell types^[8, 9]. Although the biogenesis mechanism of exosomes has not been fully elucidated^[10], recent studies have implicated that exosomes originate from the endocytotic–exogenous pathway^[11]. The formation process of exosomes mainly includes the following three phases: (1) the constitution of endocytic vesicles by invagination of the plasma membrane; (2) multivesicular bodies (MVBs) with intracavitary vesicles are produced in

the Golgi complex; and (3) mature MVBs are fused with the plasma membrane and then released into the extracellular space as exosomes (Figure 1) [2, 12-14].

Exosomes can be identified from the extracellular matrix (ECM) in almost all types of eukaryotic cells^[15]. Based on the different physiochemical properties of exosomes, various separation and purification techniques have been developed^[16, 17]. Ultracentrifugation is the most widely used and most basic isolation method^[18, 19]. However, exosomes obtained by ultracentrifugation are time-consuming and low-yield and contain other vesicles, proteins, or aggregates of proteins and RNAs. Martinez-Greene *et al*^[20] enhanced the production and purity of exosome preparations by combining polymer-based precipitation and size exclusion chromatography (Pre-SEC). Recently, the application of microfluidics in exosome isolation has received more attention. Jie *et al*^[21] used a three-dimensional nanostructured microfluidic chip to capture exosomes. Ultrafiltration with size exclusion indicated higher yields with satisfactory purity^[22]. Unfortunately, so far, no extraction/separation method is perfect.

Identification of exosomes

As a subclass of EVs, exosomes can be obtained from various cell types and extracellular media^[23]. Although there is no consensus on the specific markers of EV subtypes^[24], exosomes are mostly 30–200 nm in diameter and round or oval in shape^[25]. Exosomes are composed of diverse molecules such as RNA, proteins and carbohydrates^[26]. The proteins are composed of the transmembrane family and endosomal proteins. Various proteins in exosomes can be used as potential biomarkers, such as annexin, MVB-producing proteins such as ALIX, and tumor susceptibility gene 101 protein (TSG101)^[27]. The tetraspanins CD9, CD81 and CD63 are well-established markers of exosomes^[28].

Exosomes and noncoding RNA

Exosomes have been demonstrated to contain proteins and nucleic acids, and exosomes can regulate the functional activity of proteins and nucleic acids *via* transcriptional and

translational regulation^[29]. Currently, the genome-wide analysis has demonstrated that a significant portion (> 66%) is actively transcribed into noncoding RNAs (ncRNAs) that have functional roles in regulating the expression of protein-coding genes^[30]. NcRNAs include microRNAs (miRNAs), long noncoding RNAs (lncRNAs) and circular RNAs (circRNAs)^[31]. Length is an essential criterion for defining ncRNA. ncRNA with more than 200nt is called lncRNA and miRNA is about 20 nt and is the best-known group of small ncRNA^[32]. Mature miRNA sequences are located in introns or exons of ncRNA, many of which are produced by introns (mirtron) of Pri-miRNAs (pre-mRNAs)^[33]. CircRNAs range from 100 nt to over 4 kb in length and have remarkable stability due to their lack of exposed ends that are susceptible to nuclear degradation and contain single or multiple exons^[34-36]. Numerous publications have indicated that exosomes are closely related to bone regeneration^[37-40]. In particular, exosome-derived ncRNAs (exoncRNAs) have obtained extensive attention as an essential component of exosomes^[41].

Bone regeneration

Bone is a dynamic tissue that remodels and regenerates itself throughout life activities^[42]. Bone regeneration is a complex procedure that demands coordinating multiple cell types and biogenesis, such as osteoblasts, osteoclasts, endothelial cells, chondrocytes and mesenchymal stem cells (MSCs) (Figure 2)^[43-46].

Mechanistically, intramembranous or endochondral ossification is a pathway to bone regeneration^[47]. Intramembranous ossification is primarily the differentiation of stem cells into osteoblasts, which in turn deposit a mineralized ECM. The sources of stem cells mainly comprise bone marrow, fat, peripheral blood, and the umbilical cord^[48]. In endochondral ossification, osteoblast progenitor cells, osteoclasts, and vascular endothelial cells enter the hypertrophic cartilage. The next step is to differentiate osteoblast progenitor cells into osteoblasts to form trabecular bone, hematopoietic cells, and endothelial cells to form bone marrow so that hypertrophic cartilage is absorbed^[49]. Finally, the balance and coordination among various cells complete bone regeneration. During these processes, several signaling pathways are

involved in osteogenesis, including bone morphogenetic proteins (BMPs), Notch, Hedgehog, and Wnt/ β -catenin [50]. The BMP group is one of three subfamilies of the transforming growth factor (TGF) family [51]. Smad1/5 is regulated by BMP receptor complex [52]. Multiple miRNAs regulate osteogenesis by balancing bone morphogenetic protein receptor 2 (BMPR2)/Activin receptor type 2b (ACVR2b) competition for BMPR-triggered phosphorylation of Smads [53].

ROLES OF EXO-ncRNAs IN BONE REGENERATION

Roles of exo-ncRNAs in osteogenic differentiation

Osteocytes are fully mature and differentiated osteoblasts usually derived from mesenchymal cells, including bone-marrow- and adipose-derived MSCs [54]. Growing evidence indicates that ncRNAs influence MSC differentiation [55]. For example, miR-214 impedes the osteogenic differentiation of MSCs by reducing the expression of bone morphogenetic protein (BMP2) [56]. Furthermore, ncRNAs promote osteogenic differentiation in addition to their inhibitory effect. Dai *et al* remarked that miR-217 improves the expression of Runt-related transcription factor (RUNX2) to promote proliferation and osteogenic differentiation of Bone mesenchymal stem cells (BMSCs) significantly by targeting Dickkopf-1 (DKK1) [57]. Nevertheless, little is understood about the regulatory functions of exo-ncRNAs in these procedures, and we summarize their role in osteogenic differentiation (Table 1).

Regulatory mechanisms of exosome-derived miRNAs in osteogenic differentiation

MiRNAs are 19–24 nucleotide ncRNA molecules [58] that can regulate mRNA transcription and inhibit protein translation [59]. Exo-miRNAs are important components of exosomes and largely determine the impact of exosomes on target cells [60]. Recent studies implicate that BMPR2 is the target gene of exo-miR-100-5p. Further research has confirmed that miR-100-5p inhibits osteogenesis of human BMSCs *via* targeting BMPR2 and inhibiting the BMPR2/smads1/5 pathway [61]. Exo-miRNA-128-3p promotes osteogenic differentiation *via* targeting Smad5 [62]. Jiang *et al* [63] observed that exo-

miRNA-21 inhibits osteogenesis through regulating MSC-derived exosomes pulled from osteoporosis patients *via* targeting Smad7. Additionally, exo-miR-424-5p attenuates osteogenesis *via* regulating the WIF1-mediated Wnt/ β -catenin axis^[64]. The CXCL12/CXCR4 axis regulates osteogenic differentiation by regulating the BMP2/Smad/Osterix axis^[65, 66]. Exo-miR-23a released from fibroblasts inhibits osteogenic differentiation *via* silencing CXCL12^[25]. As a highly conserved signaling pathway, Wnt signaling positively affects osteogenic differentiation^[67]. Previous studies have confirmed that many sirtuin family members are closely related to the Wnt signaling pathway^[68, 69]. Yang *et al* showed that exo-miR-130a-3p promotes the osteogenic differentiation of Adipose- derived stem cells (ADSCs) *via* inhibiting sirtuin 7^[70]. Wnt activates Yes-associated protein (YAP)/transcriptional co-activator with PDZ-binding motif (TAZ), and the Hippo pathway regulates YAP^[71, 72].

As an essential gene in the Hippo signaling pathway, Mps One binder 1 (MOB1) regulates the expression of downstream genes, including YAP/TAZ ^[73]. Exo-miR-186 promotes osteogenesis by targeting Mob1 in postmenopausal osteoporosis^[74]. A study has indicated that FZD4 explicitly activates the Wnt signaling pathway^[75]. In contrast, exo-miR-129-5p from the jaw of diabetic rats targets FZD4 to inhibit the β -catenin signaling pathway ^[76]. As an E3 ubiquitin ligase, FBXW7 can inhibit osteogenic differentiation by regulating the degradation of substrates^[77]. Exo-miR-101 increases osteogenic differentiation *via* inhibiting FBXW7 to control the HIF1 α /FOXO3 axis^[78]. As a tumor suppressor gene, PDCD4 wields antitumor activity *via* facilitating apoptosis^[79]. Zhang *et al*^[80] demonstrated that exo-miR- 135b relieves the harshness of Osteonecrosis of femoral head (ONFH) *via* diminishing the level of PDCD4- induced apoptosis of osteoblasts. insulin-like-growth-factor-binding proteins (IGFBPs) are regulators of the functions of IGF^[81]. A previous study reported that IGF-2 enhanced BMP9-induced osteogenic differentiation^[82]. Recently, it has been reported that exo-miR-375 restricts the expression of IGFBP3 from plying osteogenic effects^[83]. Previous studies have shown that SPRY2 inhibits the Ras/MAPK signaling pathway^[84, 85]. Liao *et al*^[86] demonstrated that miR-122-5p promotes osteoblast differentiation by suppressing the

SPRY2 declaration and creating receptor tyrosine kinase (RTK) activity through RTK/Ras/MAPK signaling. As noted by Zhang *et al*, exo-miR-935 deters signal transducer and activator of transcription 1 expression and stimulates osteoblast expansion and differentiation potential. Also, upregulated exo-miR-935 alleviates osteoporosis presentation^[87]. RUNX2 is concerned with the regulation of bone metabolism *via* multiple pathways^[88]. Yang *et al*^[89] found that osteoclast-derived exosomes including exo-miR-23a-5p inhibit osteogenic differentiation *via* abating RUNX2. There is an interaction between exo-lncRNAs and exo-miRNAs. According to one study, exo-lncRNA-KCNQ1OT1 inhibits apoptosis of primary osteoblasts by sponging miR-141-5p^[90].

Regulatory mechanisms of exosome-derived lncRNAs and circRNA in osteogenic differentiation

LncRNAs are abundant in the genome, and > 27 000 have already been recognized in the human genome^[91]. LncRNA can serve not only as a critical molecule in regulating bone and cartilage degeneration, promoting bone metastasis, and repairing spinal cord injury, but also as a new class of potential biomarkers and therapeutic targets for the treatment of cancer^[92, 93].

More importantly, lncRNAs can action as microRNAs (miRNAs) sponges *via* binding miRNAs^[94]. Furthermore, lncRNAs and miRNAs exert their biological functions by forming a large and complex regulatory network interacting with each other, leading to regulation of gene expression. LncRNA/miRNA interactions allow proper function of the musculoskeletal system, control of bone homeostasis and regeneration, and osteogenic differentiation of stem cells ^[95,96].

It was reported that lncRNA MEG3 could inhibit the adipogenic and osteogenic differentiation of hASCs by regulating the expression of miR-140-5p^[97]. Previous studies indicated that lncRNA Rmst was induced by BMP9 *via* the Smad signaling pathway. Further studies found that the lncRNA Rmst-miRNA-Notch regulatory axis could be a key mediator of BMP9-induced osteogenic differentiation of MSCs^[98]. Moreover,

lncPCAT1 promotes the osteogenic differentiation of PDLSCs by sponging miR-106a-5p and upregulating the expression of the miR-106a-5p-targeted gene BMP2^[99]. Jia *et al.* have found that LINC00707 is involved in the osteogenic differentiation of BMSCs. Mechanistically, LINC00707 can sponge miR-370-3p and upregulate Wnt2B to promote the osteogenic differentiation of HBMSCs^[100]. All these data demonstrate that lncRNAs can promote osteogenesis through multiple pathways. Numerous publications have indicated that the RUNX2 gene plays a vital role in the osteogenic differentiation process^[101]. Li *et al* identified that myeloma-cell-derived exo-lncRNA RUNX2-AS1 could be loaded into exosomes and delivered to MSCs, thereby inhibiting the osteogenesis of MSCs^[102]. Mechanistically, RUNX2-AS1 can form RNA duplexes with RUNX2 pre-mRNA, and this duplex transcriptionally suppresses RUNX2 expression *via* decreasing splicing efficiency^[101, 103]. Previous studies have confirmed that Hoxa10 can participate in regulating osteogenic differentiation^[104]. More recently, Wang *et al* demonstrated that exo-H19 can regulate the expression of Hoxa10 through competitive binding to miR-467 and promote osteogenic differentiation^[105]. Moreover, exo-lncRNA-H19 stimulates osteogenesis *via* mediating Angpt1/Tie2-NO signaling in mice^[106]. Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1) may act as a prognostic biomarker for lung cancer metastasis^[107]. exo-lncRNA MALAT1 improves osteoblast action *via* moderating the miR-34c/SATB2 axis, which may enhance the osteogenic activity *via* functioning as a miR-34c sponge to upregulate SATB2 expression^[108]. In addition, exo-lncRNA NEAT1 boosts osteogenic differentiation of human BMSCs. Mechanistically, NEAT1 upregulates RUNX2 expression through competitively binding to miR- 205- 5p^[109].

Exosome-derived circRNAs play essential roles in osteogenic differentiation. circRNAs are endogenous covalently linked RNA molecules that do not have 5'-3'polarity or poly A tails^[110]. CircRNAs have diverse roles, such as modulating translation and functioning as miRNA sponges^[111]. CircRNAs are enriched in exosomes, implying the potential of circRNAs as biomarkers for complicated diseases^[112]. For example, hsa-miR-31 is considered a miRNA inhibitor of osteogenic differentiation^[113],

¹¹⁴]. Xie *et al* demonstrated that exo-circLPAR1 has an osteogenic effect *via* competitively binding to hsa-miR-31^[115]. Also, previous studies reported Hsa_circ_0006859 expression in different diseases but did not discuss the related cell signaling^[116, 117]. Zhi *et al* reported that exo-Hsa_circ_0006859 inhibits osteogenesis by sponging miR-431-5p to upregulate Rho-associated kinase 1 (ROCK1)^[118].

Roles of exo-ncRNAs in angiogenic differentiation

Blood vessels serve as channels for transporting nutrients and oxygen^[119]. There is a firm connection between the growth of blood vessels in bones and osteogenesis^[120]. Bone tissue is a highly vascularized tissue, and the development of the vascular system requires a synergistic interaction between osteoblasts and angioblasts^[121]. Endothelial progenitor cells (EPCs) are acknowledged to stimulate bone restoration *via* facilitating neovascularization and osteogenesis^[122, 123]. In line with this, endothelial cells are also implicated in the vascularization of the bone tissue^[54].

Regulatory mechanisms of exosome-derived miRNAs and lncRNAs in angiogenic differentiation

Exosomal miRNAs regulate the progression of angiogenic differentiation through diverse mechanisms. ONFH is naturally known to develop at a cellular level, inferring the value of adopting cytototherapy^[124]. Liao *et al*^[86] demonstrated that BMSC-derived exosomes modified through miR-122-5p promote angiogenesis and healing.

However, there is a lack of specifically related mechanisms. According to one study, lower levels miR-224-3p promote angiogenesis of ONFH by upregulating FIP200^[125]. In contrast, according to another study, exo-miR-100-5p inhibits angiogenesis of human umbilical vein endothelial cells (HUVECs) *via* targeting BMPR2^[61]. VEGF is a critical factor in blood vessel growth and is also involved in bone development and regeneration^[126]. For example, miR-126 promotes angiogenesis *via* inhibiting negative regulators of the VEGF pathway^[127]. Huang *et al* demonstrated that exo-miR-126 promoted angiogenesis of HUVECs by suppressing SPRED1 and PIK3R2^[128]. As a

member of the miRNA-26 family, miR-26a has a vital role in bone regeneration by promoting angiogenesis–osteogenesis coupling^[129, 130]. Wu *et al* reported that exo-miR-26a stimulates angiogenesis by upregulation of the TGF- β /Smad2/3 pathway^[131]. Exo-miR-126 downregulated PIK3R2 to trigger the PI3K/Akt signaling pathway in HUVECs. Further analyses in mice confirmed that exo-miR-126 improved angiogenesis in the wound site^[132]. In addition, MSC exo-miRNAs promotes diabetic foot repair. exo-miRNA-210-3p can stimulate angiogenesis *via* promoting VEGF gene expression and triggering proangiogenic essential proteins^[133]. However, exo-miRNA-100 inhibits angiogenesis by regulating the mTOR/HIF-1 α /VEGF pathway^[134]. Exosome-derived miR-let-7c promotes angiogenesis in multiple myeloma (MM)^[135]. Huang *et al* demonstrated that exo-miRNA-21-5p stimulates angiogenesis *via* VEGFR and AKT and MAPK pathway upregulation^[136]. However, exo-miR150 inhibits HUVEC tube formation by downregulation of VEGF^[137]. miR-21-5p is highly expressed in mag-BMSC-Exos and acts as a key mediator of mag-BMSC-Exo-induced regulation; mechanistically, exo-miR-21-5p boosts angiogenesis in HUVECs *via* regulating SPRY^[138]. ASCs-Exos can stimulate angiogenesis and neovascularization in ischemic disease^[139]. Mechanistically, miR-378-ASCs-exos not only promote osteogenic differentiation, but also increase cell migration and angiogenic capacity. miR-378-ASCs-Exos upregulate the Shh signaling pathway by targeting Sufu to enhance osteogenesis and angiogenesis^[140].

Behera *et al* demonstrated that exo-lnc-H19 acts as a sponge to absorb miR-106 and control the expression of Angpt1. In line with this, exosomes promotion of angiogenesis *via* Angpt1 triggers lnc-H19/Tie2-NO signaling in endothelial cells^[106]. XBP1, as a primary transcription factor, has been shown to regulate protein homeostasis in cells under endoplasmic reticulum stress^[141]. Cao *et al* reported that exo-lncRNA SNHG7 inhibited tube formation of Human retinal microvascular endothelial cells (HRMECs) *via* regulating the miR-34a-5p/XBP1 signal pathway^[142].

Roles of exosome-derived ncRNAs in osteoclast differentiation

Osteoclasts are generated by monocyte-macrophage precursors of the hematopoietic lineage in the bone marrow^[143]. The role of osteoclasts is to remove the organic and inorganic parts of bone, and is crucial to healthy bone function^[144]. miR-124 reduces the proliferation of osteoclast precursors and negatively regulates osteoclastogenesis^[145]. miR-214 and miR-21 promote osteoclastogenesis *via* targeting the PTEN/PI3K/AKT pathway^[146]. Although the regulation of osteoclasts by ncRNA is currently comprehended, there have been few studies on the role of exo-ncRNA in osteoclast differentiation.

Regulatory mechanisms of exosome-derived ncRNAs in osteoclast differentiation

Signaling between osteoblasts and osteoclasts is vital for osteoclast maturation^[147]. miRNAs play paramount functions in the post-transcriptional control of gene expression^[148]. miRNAs induce the translational repression or degradation of their target genes *via* binding to the complementary sequences in the 3'-UTRs of their target mRNAs^[149]. Xu *et al*^[150] investigated the effects of osteoclast-secreted exo-lncRNAs on osteogenesis in the process of particle-induced osteolysis. The results showed that **miR-214 Levels in exosomes were significantly up-regulated in osteoclast-specific miR-214 transgenic mice**. Further studies have found that in ovariectomized (OVX) mice, preventing exosome formation by downregulating Rab27a increased osteoblast activity. Taken together, osteoclast-derived exosomes transferred miR-214 into osteoblasts to suppress their activity^[151]. Coculture systems of osteoblasts and osteoclasts to simulate bone regeneration have been reviewed by Borciani *et al*^[152]. Cui *et al*^[153] reported that EPC-derived exosomes stimulated osteoclastogenesis *via* the lncRNA-MALAT1/miR124 pathway. Exo-lncRNA-MALAT1 can negatively control miR-124 activity. Furthermore, there was a negative correlation between miR-124 mRNA and ITGB1. They also indicated that EPC-derived exosomes increased neovascularization in a mouse femoral fracture model. CircRNAs were discovered as ncRNAs with covalently closed structures, and they regulate disease occurrence and development^[154]. m6A methylation is an ordinary state of RNA methylation, and it

participates in and regulates many vital functions of RNA^[155]. circ_0008542 in osteoblast exosomes enables osteoclast-induced bone resorption *via* m6A methylation^[156].

NcRNA includes not only several types that have been introduced above but also other types. For example, tRNA-derived small RNAs (tsRNAs) are a recently discovered form of ncRNA^[157, 158]. TsRNAs participate in translation inhibition and exert control in various physiological phenomena^[159]. Fang *et al* reported that tsRNA-10277-loaded BMSC exosomes improved osteogenic differentiation capacity of dexamethasone-induced BMSCs^[160]. In addition, the ECM plays a significant role in bone repair and regeneration^[161]. Hyaluronic acid (HA) exists naturally as a critical component of the ECM. Zhai *et al*^[162] focused on the recent applications of HA in bone regeneration. Recently, the emergence of decellularized ECM scaffolds have been studied in bone regeneration^[163]. Decellularized ECM scaffolds promote osteogenic differentiation of stem cells and maintain cytokines that regulate bone regeneration^[164, 165].

CONCLUSION

Opportunities

Over the past few decades, due to the development of genetic engineering, the surface of exosomes has been packed with inhibitors of ncRNA and marker molecules by modifying the isolation and purification of exosomes. Researchers have delivered targeted ncRNAs to designated tissues or organs through exosome carriers.

Different research methods can have multiple effects. The most common delivery of miRNAs to target cells is *via* exosomes or liposomes^[166]. Tahmasebi *et al*^[167] **proposed novel tissue-engineering methods premised upon miRNA-incorporated polycaprolactone nanofibers in treating bone lesions and defects**. It is well established that ECs and MSCs are critical performers in orthopedic tissue regeneration and vascularization. Coculture studies have demonstrated that ECs and MSCs have synergistic effects on tissue regeneration^[46, 168].

Hypoxic preconditioning of MSCs can enhance their biological functions^[169]. In fact, hypoxic MSCs are close to the *in vivo* environment^[170]. Liu *et al* showed that hypoxia enhanced the production of exo-miR-126, which further promoted fracture healing^[171]. Liu *et al* explored biomaterial-mediated chemical signaling through a model lithium-binding bioactive glass-ceramic (Li-BGC). Mechanistically, ² Li-BGC-exo transfers proangiogenic miR-130a and in turn, promotes the angiogenesis of ECs *via* activating the AKT pathway^[172]. Liu *et al* previously reported that ¹⁰ knee loading protects against osteonecrosis of the femoral head *via* enhancing vessel remodeling^[173]. Knee loading stimulates type H vessel formation and promotes angiogenesis *via* downregulating exo-miR-214-3p^[174].

In diagnostics, numerous studies have found differential expression of exo-ncRNAs in various diseases^[175, 176]. It implies that exo-ncRNAs have advantages as biomarkers over non-exo-ncRNAs. Meanwhile, exo-ncRNAs are concerned with bone regeneration processes like cell proliferation, migration, and angiogenesis. Exo-ncRNAs promote intercellular and intertissue crosstalk in a paracrine and autocrine manner, leading to multiple applications in diseases like femoral head necrosis, bone defects, and osteoporosis.

In therapeutics, upregulation or downregulation of exo-ncRNAs may have different clinical consequences. For instance, ¹ Lv *et al* utilized electroporation for packaging miR-21-5p mimic into exosomes. The study indicated that the miR-21-5p promotes angiogenesis and vessel maturation^[177]. Additionally, overexpressed exo-miR-122-5p weakens ONFH aggravation^[87]. There may also be therapeutic effects by decreasing the number of harmful ncRNAs in exosomes. In addition, the use of exo-ncRNAs has multiple potential benefits. Exosomes holding a characteristic cargo can function as a drug delivery system. The use of exosomes as endogenous vehicles can evade the immune response. Despite the great potential of exo-ncRNAs as biomarkers and therapeutics for bone regeneration, there are still many obstacles before their clinical application.

Challenges

The investigations and clinical transformation of exo-ncRNAs in bone regeneration have exposed several challenges. First, we need to explore more efficient exosome purification methods to exclude exogenous exosomes and RNA interference. Exosomes, microvesicles, and smaller vesicles are different but still disorganized^[178, 179]. In addition, according to the existing technology, there is still a lack of efficient and fast methods for extracting and isolating exosomes. Second, further study is needed on the pharmacokinetics and toxicity of potential exo-ncRNAs. Expansion of exosome production by increasing intracellular calcium concentration and serum starvation or transfer of the oncogene c-myc may alter exosome content (including ncRNA) and increase tumorigenic potential^[180, 181]. Third, an extensive study is needed to comprehend fully the mechanism by which exo-ncRNAs exert their physiological roles. Fourth, further investigations are demanded in the future to characterize whether miRNAs, circRNAs, lncRNAs, and other ncRNAs may form competing endogenous RNA networks. Hence, if we better understand the bioactive molecules' exact mechanism, we can improve bone tissue regeneration. Fifth, the expression profile of miRNAs is altered with age. For example, ⁵miR-183-5p increases with aging, suppresses osteogenic differentiation in BMSCs, and reduces Hmox1 Levels^[182]. Sixth, most of the current research focuses on cellular experiments and a small number of animal models. It is urgent to use large-scale animal and clinical models to conduct investigations to determine whether exo-ncRNAs can play a role in regulating homeostasis. Overall, ¹the transition of exosomal ncRNAs from basic laboratory studies to clinical application remains challenging. Nonetheless, ¹these studies provide renewed approaches for potential clinical diagnosis and therapeutic direction for exosome-mediated human diseases.

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