

## The expression and function of microRNAs in bone homeostasis

Caixia Pi<sup>1,2</sup>, Yi-Ping Li<sup>3</sup>, Xuedong Zhou<sup>1,2</sup>, Bo Gao<sup>1,2</sup>

<sup>1</sup>State Key Laboratory of Oral Diseases, Sichuan University, Chengdu, China, 610041, <sup>2</sup>West China School of Stomatology, Sichuan University, Chengdu, China, 610041, <sup>3</sup>Department of Pathology, University of Alabama at Birmingham, SHEL 810, 1825 University Blvd, Birmingham AL 35294-2182

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### 1. ABSTRACT

microRNAs (miRNAs) are a class of small, endogenous, non-coding single strand RNAs. miRNAs are involve in multiple developmental events during embryogenesis and adult tissue homeostasis. miRNAs regulate epigenetic regulating genes through post-transcriptional regulation and that epigenetic factors mediate the regulation of miRNA. Bone tissue homeostasis is maintained through the dynamic balance between osteoclastic bone resorption and osteoblastic bone formation. And miRNAs are important regulators of bone resorbing activity mediated by osteoclasts, as well as osteoblast proliferation and differentiation. This review summarizes recent studies bone-regulating miRNAs, which are divided into two major branches: the osteogenesis regulator and the osteoclastogenesis regulator. miRNAs can positively or negatively regulate osteogenesis and osteoclastogenesis. This review also discusses how miRNAs, target genes, intracellular effectors and transcription factors affect both the bone

homeostasis and bone homeostasis processes. Disrupted the function of miRNAs is related to some bone diseases, such as osteoporosis. Studying the mechanisms underlying the role of miRNAs in the bone and mineral field may reveal potential therapeutic targets for treating metabolic bone disorders, bone loss and bone diseases.

### 2. INTRODUCTION

Bone is a mineralized mesenchymal tissue, and it plays two important biological roles, one is for regulating mineral homeostasis and energy metabolism, the other is for supporting movement and protecting key organs (1). The key point for patients suffering bone metabolic diseases like osteoporosis, osteoarthritis and other diseases is that the balance between bone formation and resorption was disturbed, which lead to bone increase or bone loss (2). This balance is maintained through the dynamic balance between

osteoclastic bone resorption and osteoblastic bone formation. Osteoblasts and osteoclasts are two major cells that affect the homeostasis. In addition, osteocytes, the descendant of the matrix producing osteoblasts, play an important role in activation of bone resorption. Therefore, study of the proliferation and differentiation of osteoblasts, osteoclasts and osteocytes can help us to understand these diseases and develop better treatments (3).

A class of regulatory factors termed microRNAs (miRNAs) has been found to play a crucial role in cell cycle control, apoptosis and other cellular processes including metabolism and differentiation. miRNAs are a class of small, endogenous and non-coding single strand RNAs of 20-25 nucleotides (nts) in length. They play an important negative regulatory in animal by binding specific the 3'-untranslated region (3'-UTR) of their target messenger RNA (mRNA), and leading to mRNA degradation or translation repression (4-6). Besides, miRNAs also can specifically bind to 5'-untranslated region (5'-UTR) (7), or encoding sequence of mRNAs transcripts in order to mediate their effects (8). Published data has shown that many miRNAs regulate bone homeostasis, including bone formation, resorption, remodeling, repair and bone-related disease, by regulating the expression of certain cytokines, transcription factors and signaling intermediates (4). Bone tissue homeostasis is regulated mainly by the activity of osteoblasts and osteoclasts. Many miRNAs can regulate bone resorbing activity mediated by osteoclasts, as well as osteoblast proliferation and differentiation. This review is followed by a summary of recent studies in our understanding of bone-regulating miRNAs and their functions in bone homeostasis. Understanding the cellular and molecular mechanisms is important for the development of better therapeutic options for clinical conditions.

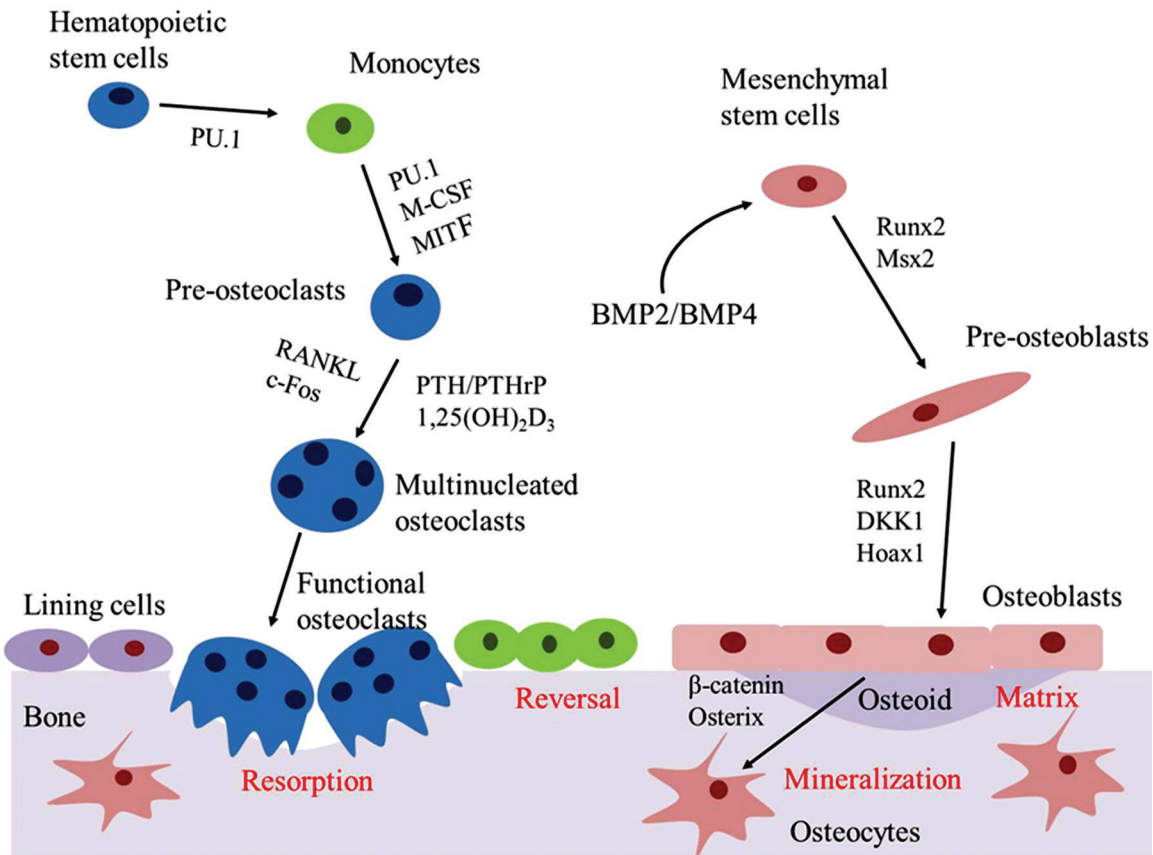
### 3. THE HOMESTASIS OF BONE TISSUE

Bone tissue is continuously remodeled throughout the lifetime of an individual, including four specific cell types: osteocytes, osteoblasts, osteoclasts in bone and chondrocytes in cartilage. And this bone remodeling maintains a dynamic balance between osteoclastic bone resorption and osteoblastic bone formation (9). A bone remodeling cycle begins with the bone resorption, which osteoclasts degrade the bone mineral and matrix. Then monocytes clean the resorbed surface for which osteoblasts generate osteoid matrix. Finally, the remodeling cycle is completed with the matrix

mineralization which replaces damaged bone or maintains the bone metabolism (Figure 1).

Osteoblasts originate from the mesenchymal stem cells (MSCs), which are responsible for bone formation during skeletal development, remodeling and regeneration. Mature osteoblasts can produce characteristic extracellular matrix (ECM) proteins and regulate the matrix mineralization by deposition of hydroxyapatite crystals (10). MSCs differentiated into mature osteoblasts and regulation of osteoblasts functional activities involve multiple layers of regulation mediated by morphogens and regulatory factors (11). Previous data has shown that Runx2/Cbfa1, Sp7/Osterix and  $\beta$ -catenin are the essential transcription factor for osteocytic differentiation (10). Meanwhile, several major signaling pathways such as TGF- $\beta$ /BMP, Wnt/ $\beta$ -catenin and Notch are important for regulating osteoblastic differentiation (12, 13), as well as the CCN family proteins are crucial growth factors for bone formation (14). In addition, endochondral ossification is a complex process involving chondrogenesis and osteogenesis. Intermittent parathyroid hormone (PTH), a key hormone regulating bone metabolism administration presented, also can enhance effects on condylar chondrocyte differentiation and bone formation (15). Other transcription factors including Sox9, Gli2/3, Atf4 and Runx2 are necessary for chondrogenic differentiation (16, 17).

Osteoclasts, bone-resorbing cells, are also important for skeletal development, homeostasis, and regeneration. They originate from monocyte/macrophage precursors differentiated from hematopoietic stem cells. Osteoclastogenesis is regulated by many cytokines, such as tumor necrosis factor (TNF) family cytokine, macrophage colony-stimulating factor (M-CSF), and receptor activator of nuclear factor NF- $\kappa$ B ligand (RANKL) (18). M-CSF and RANKL are important in osteoclast formation and differentiation (19). M-CSF mainly promotes osteoclast precursor cells proliferation and RANK expression. RANKL combines with RANK (a receptor of RANKL) expressed on the cell surface of mononuclear hemopoietic precursors osteoclast to initiate mature osteoclasts. Many transcription factors, such as nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), c-fos, microphthalmia-associated transcription factor and NF- $\kappa$ B, participate in osteoclastogenesis and differentiation. And the PU.1 was proven that involve in early osteoclastogenesis (20, 21). Simultaneously, exogenous hormones are another important



**Figure 1.** Bone remodeling cycle and its regulation. A bone remodeling cycle begins with the bone resorption which osteoclasts degrade the bone mineral and matrix (bone resorption phase). And the formation of osteoclasts is mainly regulated by M-CSF and RANKL. Then mononuclear cells, including monocytes, macrophages remove the debris in the resorbed surface for new osteoblasts and provide signals to recruit them (reversal phase). Next, osteoblasts produce osteoid matrix (bone formation phase). BMP2/BMP4 positively regulates osteoblasts formation. Finally, the remodeling cycle is completed with the matrix mineralization.

regulatory for osteoclast differentiation. For example, parathyroid hormone (PTH) regulates the RANK–RANKL interaction to affect osteoclastogenesis. In addition, osteoblasts are also related to the regulation of osteoclasts differentiation. The osteoblast lineage cells express RANKL, which is coupled to the RANK receptor presented on the osteoclast mononuclear precursors, and also produce the decoy receptor osteoprotegerin (OPG). The RANK–RANKL interaction can promote osteoclastogenesis. But the OPG, a soluble form of RANKL, blocks the interaction of RANKL and RANK, and inhibit osteoclastogenesis. The ratio of RANKL to OPG is a marker of bone-resorbing activity (1, 9). The ligand-receptor interactions of crucial signaling pathways sustain crosstalk between osteoblast and osteoclast lineage cells to regulate the balance between bone resorption and bone formation (1). Osteocytes,

embedded within the bone matrix, are the descendant of the matrix-producing osteoblasts which originate from mesenchymal stem cells (22). During this development of osteocytes, the osteocyte network, which comprises a communication system and canaliculi throughout bone, is forming. This network can convert mechanical signals into biochemical signals (22, 23). Osteocytes are the third cell type playing an indispensable role in bone turnover, because they regulate activation of bone resorption of bone remodeling. Activation is mediated by death of osteocytes in microcracks, and osteocytes actively excrete pro-osteoclastic signals or remove inhibitory signals, such as RANKL, OPG and TGF- $\beta$  (19, 24). In other hand, osteocytes secrete some growth factors that stimulate bone formation. For example, sclerostin stimulates osteocytes promoting osteoclast activity and inhibiting bone formation by

a RANKL-dependent pathway (19). Thus, osteocyte clearly plays an essential role in the maintenance of bone homeostasis and integrity.

### 4. miRNA BIOGENESIS AND OVERALL FUNCTION

Biogenesis of mature miRNAs stems from miRNA genes, which is transcribed into primary miRNA (pri-miRNA) by RNA polymerase II. Then the specific long pri-miRNA stem-loop structures is cleaved and spliced into approximately 60–70 nucleotides miRNA (pre-miRNA) by the ribonuclease III enzyme Drosha in the nucleus (25). By binding with the 3' 2-4 nucleotide overhang motif of pre-miRNA, exportin-5 exports the pre-miRNA into the cytoplasm (26). In the cytoplasm, the pre-miRNA is released from the export complex and processed by another RNase III enzyme (Dicer) to generate an approximately 20-25 nucleotides RNA double-stranded structure (miRNA-miRNA\*). The miRNA duplex incorporates into the RNA-induced silencing complex (RISC) which includes Dicer, TRBP, Ago2 protein and protein activator of PKR (PACT). Then the miRNA duplex is disintegrated by various helicases into a mature miRNA and a miRNA\*. Subsequently, the mature miRNA loaded by Ago2 repress gene expression by recognizing and binding to its specific target site in the complementary 3'-untranslated region (3'-UTR) of mRNAs by complementary base pairing (27). The miRNA\* is degraded by the RISC as its poor stability, and usually considered non-functional (28). However, latest studies found miRNA\* negatively regulate gene expression by influencing on mRNA and 3'-UTR (29). For example, miR-31\* negatively regulates RhoA expression by targeting the 3'-UTR of RhoA, and influences on the function of miR-31 during oral squamous cell carcinoma tumorigenesis (30).

The degree of base pairing between the miRNA and its target mRNA determines the fate of the transcription (27). When miRNAs seed region (2-8 nucleotides) recognizes mRNA sequences for binding perfectly with the 3' end of the transcript's miRNA binding site, the targeted mRNA is degraded. Furthermore, if imperfect base pairing of miRNAs seed region bind with their targets, they can induce translational repression. Most of highly conserved miRNAs are expressed with temporal and spatial specificity in tissues or cells. They mainly mediate the cell proliferation, cell differentiation and apoptosis (2). And distinct miRNA as a stabilized and specific small molecular marker also can be used

to detect various disease, such as osteosarcoma, cancer and congenital diseases (31-33).

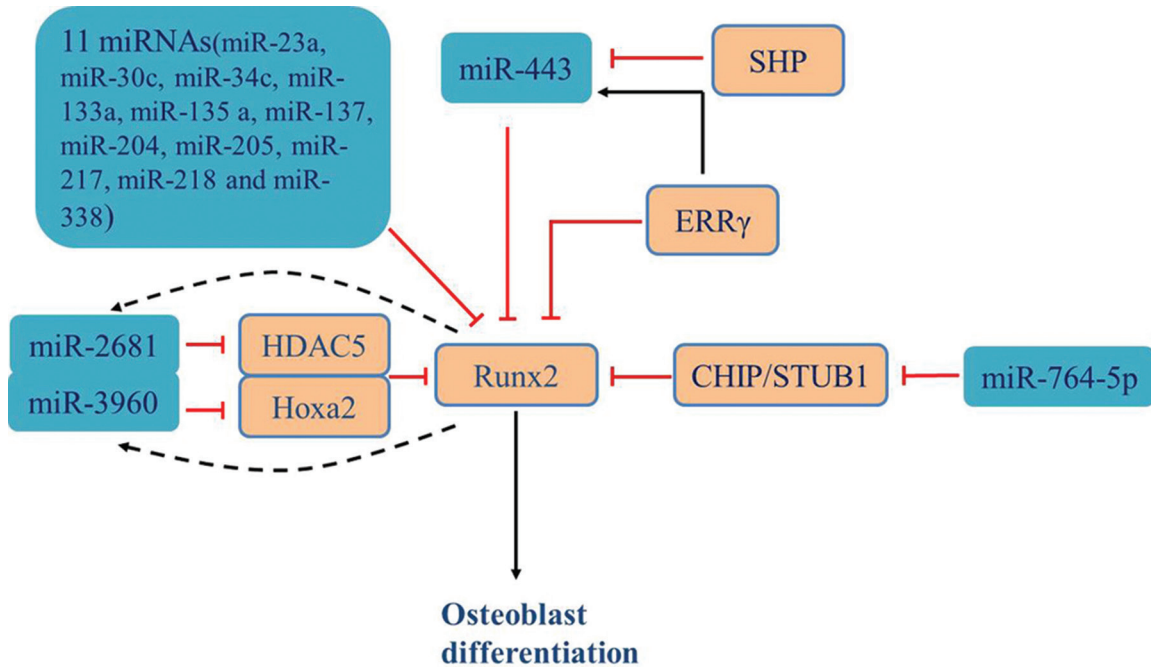
### 5. miRNAs IN OSTEOGENESIS

#### 5.1. Inhibition of osteogenesis by microRNAs

More and more miRNAs have been confirmed to regulate prenatal bone development, postnatal bone formation and to maintain bone homeostasis in adult skeleton. OsteomiRs, bone-regulation miRNAs of expression in osteoblast lineage cells, are able to regulate osteogenesis positively by either direct targeting negative regulators of osteoblast differentiation or negatively targeting osteogenic signal molecules (11). The dysfunction of osteomiRs is an important pathological factor in bone degeneration, bone resorption and other bone-related diseases (34), such as osteoporosis.

##### 5.1.1. The expression of miRNAs downstream of BMP2-signaling

It is found that BMPs play an important role in promoting osteoblast differentiation and bone formation by activating transcription programs. For example, BMP2 can activate transcription factors Runt related transcription factor 2 (*Runx2*) and Osterix (*Osx*) during osteogenesis. And latest studying shows that BMP2 controls tooth root development in coordination with formation of the periodontium (35). Meanwhile, microRNA-34 family including miR-34b and miR-34c, is significantly induced by BMP2 during osteoblast differentiation (36). They are up-regulated during BMP2-induced osteoblastic differentiation of C2C12 pre-myoblast mesenchymal cells by directly targeting Notch1. *In vivo*, overexpression of miR-34c in osteoblasts of mice leads to aged-related osteoporotic phenotype, resulting in both the defective mineralization and the increase of bone resorption. Further studies demonstrated that miR-34c can target directly multiple members of the Notch signaling pathway, including Notch1, Notch2 and Jag1. Notch signaling is an important mechanism to maintain the balance between cell proliferation and differentiation in mammals. Notch differentially regulates trabecular and cortical bone homeostasis in osteocytes (37). In addition, notch signaling influences osteoclast differentiation in non-cell-autonomous fashion. miR-34c mediated post-transcriptional regulation of Notch signaling in osteoblasts is critical in bone homeostasis and the proliferative effect of Notch in the committed osteoblast progenitors.



**Figure 2.** miRNAs regulate osteoblast differentiation by mediating Runx2. 1.1 miRNAs (miR-23a, miR-30c, miR-34c, miR-133a, miR-135a, miR-137, miR-204, miR-205, miR-217, miR-218 and miR-338) and miR-433 inhibit osteoblast differentiation by directly repressing Runx2 activity. miR-443 expression is inhibited by SHP and induced by ERR $\gamma$  which also represses Runx2 expression. miR-2681/miR-3960 and miR-764-5p promote osteoblast differentiation by indirectly increasing Runx2 expression. Runx2 coupled with the promoter of the miR-3960/miR-2861 cluster. Abbreviations: ERR $\gamma$ , Estrogen receptor-related receptors; SHP, a small heterodimer partner; HDAC5, histone deacetylase 5; CHIP/STUB1, C terminus of Hsc70-interacting protein/STIP1 homology and U-Box containing protein 1.

Runx2 is necessary regulator of osteoblastogenesis by involving in the proliferation and differentiation of osteoblasts (Figure 2). And many signaling pathways and transcription factors related to osteoblastogenesis are affected by the production or activity of Runx2 (10). There are 11 Runx2-targeting miRNAs, including miR-23a, miR-30c, miR-34c, miR-133a, miR-135a, miR-137, miR-204, miR-205, miR-217, miR-218 and miR-338, express in mesenchymal cell types (38). And all of them (except miR-218) can control the osteogenic activity of Runx2 and regulate osteoblast differentiation. Besides, 7 Runx2-targeting miRNAs (miR-23a, miR-30c, miR-34c, miR-133a, miR-135a, miR-205, and miR-217) also regulate the chondrogenic GATA transcription factor Trps1 (tricho-rhino-phalangeal syndrome I) (39). Runx2 can down-regulate the expression of miR cluster 23a~27a~24-2, and each of the miRNAs inhibits osteoblast differentiation by targeting the 3'-UTR of SATB2, which is necessary to synergize with Runx2 (40).

Latest studies have shown that miR-433 inhibited BMP2-induced osteoblast differentiation by

reducing the transcription of Runx2 (41). miR-433 and Estrogen receptor-related receptors (ERR $\gamma$ ) are down-regulated during the BMP2-induced osteoblastic differentiation of mesenchymal stem cell lineage C3H10T1/2. ERR $\gamma$ , closely related to estrogen receptor (ER), induces the expression of miR-433. Overexpression of ERR $\gamma$  or miR-433 suppressed the expression of BMP-mediated induction of osteogenic marker genes, such as alkaline phosphatase (ALP) and Runx2. miR-433 was identified that directly binding to the site of the 3'-UTR of Runx2 mRNA and inhibiting the level of Runx2 transcript. Whereas, the down-regulation of miR-433 rescued ERR $\gamma$ -suppressed Runx2 expression and ALP activity. In addition, the expression of miR-433 was inhibited by a small heterodimer partner (SHP) in Hepa-1 cells (42). And SHP can increase the transcriptional activity of Runx2 to promote osteoblast differentiation and bone formation (43).

A number of microRNAs regulate osteogenesis and osteoblast differentiation by their co-expression and/or co-regulated (27). The case of



miR-30 family members that key regulators in bone biomineralization is presented (44). The miR-30 family members, including miR-30a, miR-30b, miR-30c and miR-30d, are down-regulated in the pre-osteoblast differentiation of MC3T3-E1 cells induced by BMP2, and similarly inhibitory effects in mouse bone marrow mesenchymal stem cells. Overexpression of miR-30 family members can result in a decrease of ALP. Evidentially, miR-30 family members restrain osteogenesis by targeting Runx2 and Smad1, which positively mediate osteogenesis through the early down-regulation of miR-30 expression. Besides, miR-30c plays an important role in radiation-induced cell damage by regulating REDD1 expression in CD34+ cells and human fetal osteoblasts (hFOB) (45), and miR-30c targets both TGIF2 and HDAC4, which involve in negative regulation of osteoblast differentiation in nano-bioglass ceramic particles (nBGG)- treatment in human osteoblastic cells (MG63) (46). miR-30d also can target for regulators of bone formation, such as osteopontin /spp1, ccn2/ctgf, ccn1/cyr61 and sox9 in human mesenchymal stem cell (hMSC) (47).

Similarly, miR-133 and miR-135 can express and regulate together, but their effects of regulating cell differentiation are difference in different cell types. miR-133 and miR-135 are down-regulated in the BMP2 induced osteogenesis of C2C12 cells, but are up-regulated in the MEF-2-dependent myogenesis in the same cell (48). Further studies indicated that miR-133 negatively control the transcription of Runx2 by directly binding to the 3'UTR of Runx2 mRNA, as well as miR-135 represses osteogenesis by targeting Smad5 which a BMP intracellular receptor. miR-206 is also expressed in osteoblastic lineage and its expression decreases in BMP2-induced osteoblast differentiation of C2C12 cells (49). Overexpression of miR-206 can repress osteoblast differentiation. Otherwise, the knockdown of miR-206 can promote osteogenic differentiation. Connexin 43 (Cx43), a gap junction protein essential for osteoblast differentiation, is a target gene for miR-206. *In vivo*, transgenic mice overexpressing miR-206 in osteoblasts led to a low bone mass due to osteoblast differentiation. Therefore, miR-206 plays an important role in negative regulation of osteogenesis.

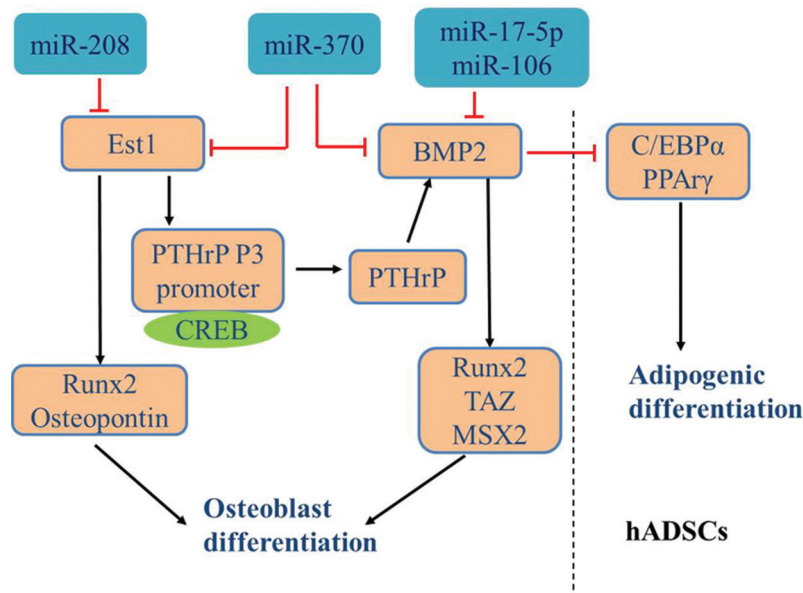
Comparing with non-BMP2-treated MC3T3-E1 cells, miR-370 expression level is reduced significantly in BMP2-treated MC3T3-E1 cells. miR-370 inhibits osteogenic differentiation

of BMP2-stimulated MC3T3-E1 cells by negative regulating the expression of BMP2 and Ets1 (V-ets Erythroblastosis Virus E26 Oncogene Homolog1 stimulated the transcription of osteopontin and Runx2) (50) (Figure 3). Overexpression of mature miR-370 can weaken obviously BMP2-stimulated pre-osteoblast differentiation in primary osteoblasts or MC3T3-E1 cells. Further studies of mechanisms indicated that the regulation of osteogenic differentiation by miR-370 might depend on a BMP2-Ets1-PTHrP feed-forward loop regulation and the regulation of miR-370 can lead to osteogenic disorders. Ets1 activates PTHrP expression by the binding of CREB to the PTHrP P3 promoter region, and PTHrP increases BMP2 mRNA expression. In addition, miR-208, miR-141 and miR-200a are also down-regulated in BMP2-stimulated MC3T3-E1 cells. miR-208 represses the differentiation of pre-osteoblast by targeting Est1 (51) (Figure 3), while miR-141 and miR-200a inhibit osteogenic differentiation by targeting Distal-less homeobox 5 (Dlx5) which a master transcription factor related to osteogenesis (52).

### 5.1.2. The function of miRNAs in human adipose-derived mesenchymal stem cells (hADSCs)

Many studies have proven that human adipose-derived mesenchymal stem cells (hADSCs) have a potential for differentiating into different lineages, such as hADSCs osteogenesis in conjunction with regeneration of bone (53, 54). The effect of miRNAs has been confirmed in the osteogenesis of hADSCs. For example, miR-100 that expressed in hADSCs mediated osteogenic differentiation of hADSCs (55). The enhanced expression of miR-100 inhibited BMPR2 (bone morphogenetic protein receptor type II) mRNA transcription and hADSCs osteogenic differentiation. BMPR2, a kinase receptor of BMPs, was shown to be a target gene of miR-100, and knockdown of BMPR2 by RNAi suppressed osteogenic differentiation. These data indicate that miR-100 plays a negative role in human osteogenesis by targeting BMPR2 in hASCs. Therefore, miR-100 and BMPR2 might be potential therapeutic targets in bone diseases.

Latest research demonstrated that miR-17-5p and miR-106a regulate dually osteogenic differentiation of hADSCs by gain- and loss-of function assays (56) (Figure 3). Both of them can promote adipogenesis and inhibit osteogenesis by



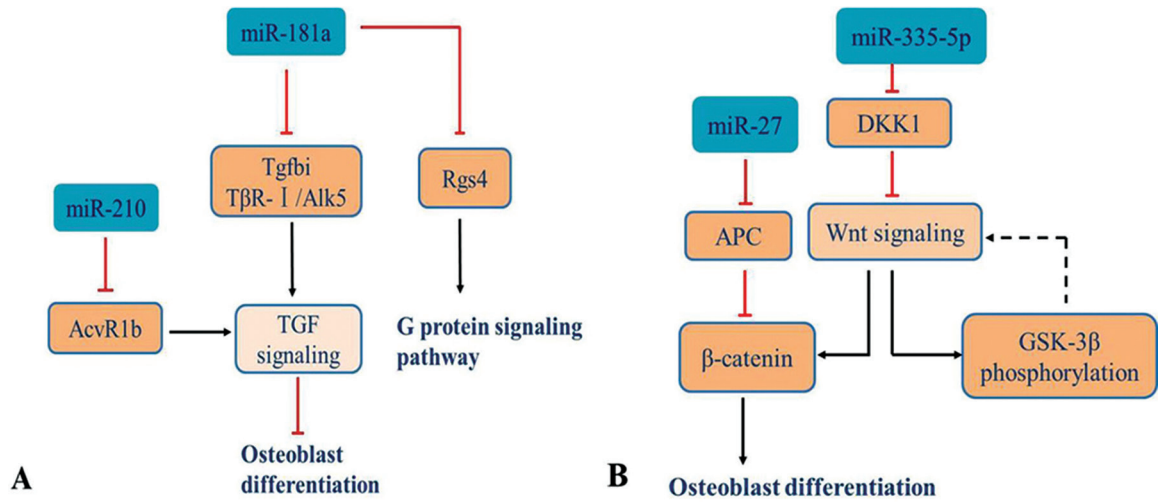
**Figure 3.** miRNAs regulate osteoblast differentiation and adipogenic differentiation. miR-370 negatively regulate osteoblast differentiation via the BMP2-Ets1-PTHrP feed-forward loop. miR-208 also inhibits osteoblast differentiation by targeting Ets1 which stimulates the transcription of osteopontin and Runx2. miR-17-5p and miR-106a regulate dually osteogenic differentiation of hADSCs. Both of them can promote adipogenesis and inhibit osteogenesis by direct targeting BMP2, and consequently increased adipogenic C/EBPα and PPARγ, and reduced osteogenic MSX2 and Runx2. Abbreviations: Est1, V-ets Erythroblastosis Virus E26 Oncogene Homolog1; PTHrP, parathyroid hormone-related protein; hADSCs, human adipose-derived mesenchymal stem cells; C/EBP α, CCAAT/enhancer binding proteins alpha; PPARγ, peroxisome proliferator-activated receptor gamma; TAZ, transcriptional co-activator with PDZ-binding motif; MSX2, mshhomeobox 2.

direct targeting BMP2, and consequently increase adipogenic C/EBPα and PPARγ, and reduce osteogenic MSX2 and Runx2. Knockdown of BMP2 by RNAi facilitated adipogenic differentiation and inhibited osteogenic differentiation, while miR-17-5p and miR-106a up-regulated in the similar stimulation. miR-17-5p and miR-106a may maintain the balance between adipogenesis and osteogenesis of hADSCs. A primate-specific miR-637 also maintains the balance between adipocytes and osteoblasts in hMSCs (57). Expression of miR-637 is decreased during osteoblast differentiation. And miR-637 obviously promotes adipocyte differentiation and inhibits osteoblast differentiation by targeting directly Osterix (Osx) which a transcription factor related osteoblasts. Disruption the expression of miR-17-5p, miR-106a and miR-637, likely result in a disorder for the balance of differentiation in hADSCs or hMSCs. Furthermore, miR-26a represses the expression of Smad1 protein through binding to its 3'UTR of mRNA during the late stage of osteoblastic differentiation of hADSCs (58). Down-regulation of miR-26 increased Smad1 expression, and subsequently up-regulating bone marker genes to promote osteoblastic differentiation. Menin-miR-26a

may be a target site for RNA-based therapy of bone disease (59). Menin is a key factor for multiple endocrine neoplasia type1 syndromes.

### 5.1.3. miRNAs regulation of osteoblast differentiation

Activation of FoxO1, protecting cells in bone from reactive oxygen species (ROS), stimulates proliferation and differentiation of osteoblasts. As a miRNA targeting for FoxO1, miR-182 negatively regulates osteoblastogenesis through suppressing FoxO1 in C3H10T1/2 cells and MC3T3-E1 cells. Overexpression of miR-182 can inhibit osteoblast differentiation and increase cell apoptosis, and miR-182 plays an important role in treatment bone disease related age (60). Both of miR-93 and miR-214 regulate negatively osteoblast mineralization. miR-93 is down-regulated during osteoblast mineralization, and overexpression of it can repress Sp7 protein expression osteoblast mineralization in primary mouse osteoblasts. Sp7/Osterix, a zinc finger transcription factor, is a key regulator of osteoblast mineralization, and Sp7 is a target gene of miR-93. Overexpression of Sp7 decreased miR-93 transcription. Therefore, miR-93



**Figure 4.** miRNAs regulate osteoblast differentiation by TGF signaling pathway and Wnt signaling pathway. a. miR-210 and miR-181a promote osteoblast differentiation through suppressing TGF signaling molecules. b. miR-335-5p and miR-27 promote osteogenic differentiation by specifically regulating Wnt signaling molecular which is activated by associating with phosphorylation-mediated inhibition of GSK-3β. Abbreviations: AcvR1b, activin A receptor type 1B; Tgfb1, Tgf-beta induced; TβR-I/Alk5, TGF-β type I receptor; APC, adenomatous polyposis coli; GSK-3β, a kinase-designated glycogen synthase kinase 3β; DKK1, Dickkopf-related protein 1.

mediates osteoblast mineralization through a novel miR-93/Sp7 regulatory feedback loop (61). Further, miR-214 inhibits osteoblast activity and matrix mineralization by directly targets ATF4, which a gene encoding transcription factors required for osteogenesis (62).

## 5.2. Promotion of osteogenesis by microRNAs

### 5.2.1. The expression of miRNAs in BMP2-induced osteoblast differentiation

miR-181a is up-regulated during BMP induced osteoblastic differentiation of C2C12 and MC3T3 cells (63). Overexpression of miR-181a enhanced ALP levels and induced the expression of osteoblastic maker genes, such as Sp7, Alp1 and Spp. miR-181a promotes osteoblastic differentiation through directly targeting Tgfb1 (Tgf-beta induced) and TβR-I/Alk5 (TGF-β type I receptor), which are TGF-β signaling molecules. TGF-β signaling pathway is indispensable for inhibiting osteoblastic maturation and differentiation, and TGF-β signaling molecules including Tgfb1 and TβR-I/Alk5 are negative regulatory of osteoblastic differentiation. In addition, Rgs4 (regulator of G protein signaling) and Gata6 are potential target genes of miR-181a. Similarly to miR-181a, miR-210 also promotes osteoblastic differentiation via inhibiting the TGF-β/activein signaling molecules by targeting AcvR1b

(activin A receptor type 1B) in BMP4-induced osteoblastic differentiation of bone marrow-derived ST2 stromal cells (64) (Figure 4A). On the contrary of miR-210, miR-125b is increased during BMP4-induced osteoblastic differentiation of ST2 cells, and miR-125b negatively regulated osteoblastic differentiation (65).

Similarly, miR-2861 is transcribed in BMP2-induced osteoblastic differentiation ST cells. miR-2861 promotes osteoblast differentiation by repressing histone deacetylase 5 (HDAC5) expression, leading to the increase of acetylated Runx2 protein (66). Runx2 is a key positive regulator in osteoblast differentiation. The expression of miR-2861 is highly conserved in human, and the homology mutations of pre-miR-2861 might cause primary osteoporosis. Recently, a new microRNA, miR-3960, was demonstrated that play an important role in promotion osteoblast differentiation by a regulatory feedback loop with miR-2861 (67). miR-3960 also was expressed in BMP2-induced osteogenesis ST cells, and overexpression of it promoted BMP2-induced osteoblastogenesis. Homeobox (Hoxa2) that a receptor and negative regulatory of Runx2 is the target gene of miR-3960. Further, Runx2 coupled with the promoter of the miR-3960/miR-2861 cluster. Both miR-3960 and miR-2861 regulate osteoblast differentiation through a



novel positive Runx2/miR-3960/miR-2861 feedback regulation system (Figure 2).

miR-332 is shown to down-regulation during BMP2-induced osteoblast differentiation of C2C12 and MC3T3-E1 cells and primary cultures of murine BM-MSCs (68). Overexpression of miR-332 promotes the BMP2-mediated osteoblastic differentiation by enhancing BMP2 response. Tob2, a negative regulator of Osterix expression, is a target gene of miR-322. Thus, miR-322 can promotes osteoblastic differentiation via increasing Osx expression by inhibition of Tob2 mRNA. In addition, Tob might partly regulate bone development and homeostasis through specific degradation and decay of Osx mRNA. Recently, a research demonstrated that miR764-5p positively promotes osteoblast differentiation in osteoblast progenitor cells by the inhibition of CHIP/STUB1 protein levels, because CHIP/STUB1 negatively regulates osteoblast differentiation by positively regulated the degradation of Runx2 (69).

### 5.2.2. miRNAs mediate osteogenic differentiation via regulating the Wnt signaling pathway

The Wnt family consists of many highly conserved genes that can regulate gene expression, cell adhesion, and cell behavior (3). The Wnt pathway is divided into the canonical pathway and the noncanonical pathway. The canonical pathway is also called the Wnt/ $\beta$ -catenin pathway. It is activated by associating with phosphorylation-mediated inhibition of GSK-3 $\beta$  which a kinase-designated glycogen synthase kinase 3 $\beta$ . Both of miR-335-5p and miR-27 mediate osteogenic differentiation by specifically regulating Wnt signaling molecular (70, 71). Dickkopf-related protein 1 (DKK1) maintains the homeostasis of skeletal as an inhibitor of Wnt signaling. miR-335-5p represses DKK1 expression through binding to 3'-UTR of DKK1 mRNA, sequentially increases the Wnt signaling leading to the increase of GSK-3 $\beta$  phosphorylation and  $\beta$ -catenin transcriptional activity in osteoblast-lineage cells. The accumulation of  $\beta$ -catenin promotes osteoblastic differentiation. Further, miR-335-5p regulates bone development *in vivo* proven by high expression of miR-335-5p in osteoblasts and hypertrophic chondrocytes of mouse embryos. During hFoB1.1.9 cells differentiation, miR-27 expression levels is increase. miR-27 specifically targets adenomatous polyposis coli (APC) 3' UTR and controls APC expression, canonical Wnt signaling activity, and osteogenic differentiation (Figure 4B).

Besides, miR-218 and miR-29 family also mediate osteoblast differentiation through regulating Wnt signaling pathway. miR-218 promotes normal osteoblast differentiation in bone marrow stromal cells (BMSCs) by activating a positive feedback loop between miR-218 and Wnt signaling. miR-218 regulates the Wnt signaling pathway by targeting three Wnt signaling inhibitors, Dickkopf2 (DKK2), Sclerostin (SOST) and secreted frizzled-related protein2 (SFRP2). In reverse, the expression of miR-218 is up-regulated in response to stimulate Wnt signaling molecular and in osteoblasts (72). In addition, a signal- amplification circuit between miR-218 and Wnt signaling can enhance osteomimicry-related tumor activity and promote osteogenic differentiation of human adipose-derived stem cells (hASCs) (73). The latest data demonstrates that Hsa-mir-218 regulates the differentiation of human-derived dental stem cells (DSCs) via targeting Runx2. And hsa-mir-218 expression is down-regulated in DSC mineralized tissue type differentiation (74).

miR-29 family, including miR-29a, miR-29b and miR-29c, target many collagens and extracellular matrix proteins. miR-29a promotes osteoblast differentiation by targeting Wnt signaling inhibitors, Dkk1, Kremen2, and sFRP2 (75). miR-29b promotes osteoblast differentiation and bone extracellular matrix proteins generation through repressed the inhibitors of osteoblast differentiation, HDAC4, TGF  $\beta$  3, CTNNBIP1, ACVR2A (76). Recent data show that miR-29 negatively regulates human osteoclastic cell differentiation (77). In summary, miRNAs play a crucial role in osteogenesis and maintain bone tissue homeostasis by involving in several major signaling pathways including Wnt, TGF- $\beta$ , and Notch. As the essential transcription factors for osteoblast differentiation, ALP, Runx2, Osx and  $\beta$ -catenin also are the markers of osteoblast differentiation (Table 1).

## 6. miRNAs IN OSTEOCLASTOGENESIS

Some regulatory factors of osteoclast differentiation, as well as the markers of bone resorbing activity, are regulated by miRNAs expression. For instance, miR-155 negatively regulates MITF, which is an essential transcription factor in osteoclast differentiation (78). Dicer is a key protein in the formation of mature miRNAs. It can modify the miRNAs through cleaving the pre-miRNAs. Upon removal of Dicer, miRNAs production is down regulated. Deficiency of osteoclast-specific

**Table 1.** Summary of microRNA involvement in osteogenesis

miRNA	Target gene (s)	Cell source	Description	References
Promotion of Osteogenesis by miRNAs				
miR-27	APC	hFoB1.1.9	miRNA promotes osteogenic differentiation	(71)
miR-29a	Dkk1,Kremen2, sFRP2	hFoB1.1.9	miRNA promotes osteoblast differentiation miR-29a inhibits Wnt signaling pathway	(75)
miR-29b	HDAC4, TGFβ3	MC3T3-E1		(76)
miR-181a	Tgfb1, TβR- I /Alk5	C2C12, MC3T3-E1	Overexpression of miR-181a enhances ALP activity miR-181 inhibits TGF-βsignaling	(63)
miR-210	AcvR1b	ST2 cells	miRNA inhibits TGF-β/activin signaling molecules	(64)
miR-218	DKK2,SOST, SFRP2	BMSCs, hASCs	DKK2, SOST and SFRP2 are Wnt signaling inhibitors	(72, 73)
miR-322	Tob2	C2C12, MC3T3-E1, BM-MSCs	Tob2 inhibits Osx expression <i>Tob</i> might partly regulate bone development by degradation and decay of <i>Osx</i> mRNA	(68)
miR-335-5p	DKK1	C3H10T1/2, MC3T3-E1	DKK1 suppresses Wnt signaling pathway	(70)
miR-764-5p	CHIP/STUB1	MC3T3-E1	CHIP/STUB1 promotes the degradation of Runx2	(69)
miR-2681 miR-3960	HDAC5, Hoxa2	ST2 cells	HDAC5 and Hoxa2 repress Runx2 activity, Runx2 increases miRNAs expression	(66, 67)
Inhibition of Osteogenesis by miRNAs				
miR-17-5p miR-106	BMP2	hADSCs	miRNAs maintain the balance between adipogenesis and osteogenesis	(56)
miR cluster 23a~27a~24-2	SATB2	MC3T3-E1	SATB2 is necessary to synthetize Runx2	(40)
miR-26a	Smad1	hADSCs	miRNA inhibits late stage of osteoblastic differentiation	(58, 59)
miR-30	Runx2, Smad1	MC3T3-E1, M-BMSCs	Overexpression of miR-30 family members decreases ALP activity	(44)
miR-34c	Notch1, Notch2, Jag1	C2C12	miRNA increases in BMP2-induced osteoblastic differentiation of C2C12	(36)
miR-93	SP7	primary mouse osteoblasts	miRNA inhibits osteoblast mineralization	(61)
miR-100	BMPR2	hADSCs	Overexpression of miR-100 inhibits BMPR2 mRNA transcription and hADSCs osteogenic differentiation	(55)
miR-125b	Osterix	ST2 cells	miRNA inhibits osteoblastic differentiation	(65)
miR-133	Runx2	C2C12	miR-133 inhibits osteoblast differentiation	(48)
miR-135	Smad5	C2C12	Smad5 is a BMP intracellular receptor	(48)
miR-182	FoxO1	C3H10T1/2, MC3T3-E1	miRNA inhibits osteogenesis FoxO1 simulates proliferation and differentiation of osteoblasts	(60)

(Contd...)

Table 1. Contd...

miRNA	Target gene (s)	Cell source	Description	References
miR-141 miR-200a	Dlx5	MC3T3-E1	miRNAs inhibit osteogenic differentiation	(52)
miR-206	Cx43	C2C12	Cx43 is a gap junction protein essential for osteoblast differentiation	(49)
miR-208	Est1	MC3T3-E1	miRNA represses the differentiation of pre-osteoblast	(51)
miR-214	ATF4		miRNA inhibits osteoblast activity	(62)
miR-370	BMP2/Ets1	MC3T3-E1	miRNA inhibits osteogenic differentiation	(50)
miR-433	Runx2	C3H10T1/2	Overexpression of miRNA suppresses ALP and Runx2 mRNA	(41)
miR-637	Osterix	hMSCs	miRNA promotes adipocyte differentiation and inhibits osteoblast differentiation	(57)

Dicer suppresses the activity of bone-resorbing in vivo (79). In addition, silencing of Dicer, DGCR8 and Ago2 could inhibit osteoclastogenesis and reduce bone resorbing by repressing the expression of miR-223 (80). Therefore, the expression of miRNAs is important for osteoclastogenesis.

In recent years, the function of miRNAs in osteoclast differentiation is a hotpot in research fields, but it remains incompletely understood in present. Many studies demonstrated that miR-223, miR-21 and miR-155 play crucial role in osteoclast differentiation in vivo (81, 82). miR-223 is expressed in mouse osteoclast precursor cell lines (RAW264.7 cells). Overexpression of pre-miR-223 in RAW264.7 cells completely obstructed the formation of TRAP-positive multinucleated cell (82). miR-223 regulates osteoclastogenesis through the feedback loop of PU.1/miR-223/NF1-A/M-CSFR. In this pathway, PU.1 induced by M-CSF promotes the expression levels of miR-223 and RANK in bone marrow osteoclast precursors, then miR-223 down-regulated the NF1-A (a suppressor of osteoclastogenesis) levels by targeting its mRNA to promote osteoclastogenesis. And the down-regulation of NF1-A gene levels can increase M-CSF receptor expression levels in cells, which is important for osteoclastogenesis. As a result, PU.1, miR-223, NF1-A and M-CSFR are linked closely through positive feedback loop (80).

The expression of miR-21 was proven that mediates RANKL-induced osteoclastogenesis in mouse bone marrow macrophage (BMM) (83). Programmed cell death 4 (PDCD4) protein is increased and miR-21 is decreased in BMM lacking Dicer or DGCR8. miR-21 decreases PDCD4 protein

levels by targeting PDCD4 mRNA, subsequently, c-Fos, a key transcription factor and downstream target gene of osteoclastogenesis, is freed from the repression of PDCD4. Further, c-Fos can increase miR-21 gene expression. Therefore, miR-21 positively regulates osteoclastogenesis through the feedback loop of c-Fos/miR-21/PDCD4.

As a regulator of macrophage differentiation, miR-155 plays a unique role in monocyte RAW264.7 progenitors (78). The up-regulation of miR-155 can promote the formation of macrophages, and repress RANKL-induced osteoclastogenesis by inhibiting the expression of MITF. MITF, an essential transcription factor in osteoclast differentiation, is a target gene of miR-155. In addition, miR-155 also targets SOCS1, another positive regulator of osteoclastogenesis (84). Further, the deficiency of miR-155 in mice can reduce generation of osteoclasts and local bone destruction (85). Therefore, the inhibition of miR-155 expression might provide a novel target for the treatment of osteoclast-mediated diseases.

The RANK–RANKL interaction can promote osteoclastogenesis. M-CSF, as a cytokine, promotes osteoclast precursor cells proliferation and RANK expression. Then RANK expressed on the cell surface of mononuclear hemopoietic precursor osteoclast combines with RANKL presented on osteoblast lineage cells to initiate mature osteoclasts. Latest studies demonstrated that miR-31, miR-503 and miR-124 also involve in the osteoclast differentiation. Cytoskeletal organization is dynamically regulated by some small GTPase during osteoclast maturation and bone resorption. And miR-31 was identified that controls cytoskeleton organization in osteoclasts for optimal bone resorption activity by regulating the

**Table 2.** microRNAs in osteoclast differentiation

miRNA	Target gene (s)	Cell source	Description	References
miR-21	PDCD4	BMMs	miR-21 positively regulates osteoclastogenesis through the feedback loop of c-Fos/miR-21/PDCD4	(83)
miR-31	RhoA1	BMMs	miR-31 promotes osteoclast differentiation	(86)
miR-124	NFATc1	BMMs	miR-124 represses osteoclastogenesis	(87)
miR-155	MITF, SOCS1	RAW264.7.	miRNA inhibits osteoclast differentiation	(84)
miR-223	NFI-A	RAW264.7.	NFI-A is a suppressor of osteoclastogenesis, miR-223 promotes osteoclast differentiation through a positive feedback loop	(82)
miR-503	RANK	PBMCs	Over-expression of miRNA inhibits RANKL-induced osteoclastogenesis	(89)

expression of RhoA, one of the small GTPase and miR-31 target genes (86). miR-31 is up-regulated during osteoclast formation and differentiation under RANKL stimulation. Inhibition of miR-31 by specific antagonists promotes the expression of RhoA and represses the RANKL-induced osteoclast formation and bone resorption. Furthermore, exoenzyme C3, a RhoA inhibitor, might save the osteoclastogenesis damaged by miR-31 inhibition. Thus, miR-31 regulates the osteoclastogenesis and bone resorption by targeting the RhoA.

In contrast, the expression of miR-124 rapidly decreased in RANKL-induced BMMs, and miR-124 negatively regulates osteoclastogenesis by suppressing NFATc1 expression (87). NFATc1, nuclear factor of activated T cell cytoplasmic 1, is a master transcription factor of osteoclast differentiation and function. NFATc1 expresses in early RANKL-inducible gene-by-gene expression profiling, and act as cofactors with activator protein-1 (AP-1) (88). Inhibition of miR-124 increases NFATc1 expression and enhances the osteoclast differentiation. Further, the inhibitory effect of miR-124 on osteoclastogenesis might be removed by overexpressing of NFATc1. In addition, the expression of RhoA and Rac1 are decreased in pre-miR-124-treated cells, miR-124 might be involved in the proliferation and migration of osteoclast precursors by affecting the expression of these small G proteins.

miR-503 inhibits osteoclastogenesis via directly targeting RANK, receptor activator of nuclear factor- $\kappa$  B ligand, which is activated by the binding of RANKL (RANK ligand) (89). miR-503 is reduced in CD14<sup>+</sup> peripheral blood mononuclear cells (PBMCs) from postmenopausal osteoporosis patients. Overexpression of miR-503 represses RANKL-induced osteoclastogenesis in CD14<sup>+</sup> PBMCs.

Oppositely, silencing of miR-503 in ovariectomy (OVX) mice can promote RANK expression, increase bone resorption and reduce bone mass. Therefore, miR-503 plays a key role in the pathogenesis of postmenopausal osteoporosis.

Although a few miRNAs have been reported to regulate osteoclastogenesis (Table 2), and the regulation mechanism of most of them is remain unclear, miRNAs directly or indirectly involve in the osteoclast differentiation. Thus, the research of miRNAs in osteoclastogenesis is important for preventing and curing skeletal diseases, such as osteoarthritis (OA) and osteoporosis.

## 7. SUMMARY AND FUTURE DIRECTIONS

Bone tissue homeostasis is maintained through the dynamic balance between osteoclastic bone resorption and osteoblastic bone formation. The activity and numbers of osteoblasts and osteoclasts are important in the homeostasis. There has a crosstalk between osteoblasts and osteoclasts through the RANKL/RANK/OPG system. And osteocytes, as the activator of bone resorption, also involve the regulation of bone tissue homeostasis and play an essential role in the bone remodeling. Osteocytes can promote osteoclastogenesis by the pro- osteoclastic signaling (eg. RANKL), as well as inhibit the bone formation via secreting some factors (eg. Sclerostin) (19). The relationship between osteocytes, osteoblasts and osteoclasts may be complex and significant in bone homeostasis.

miRNAs, post-transcriptional regulatory factors, powerfully involve in each stage of the bone formation and maintain the homeostasis of bone tissue. As specific osteogenesis and osteoclastogenesis regulators, many miRNAs negatively or positively regulate bone formation



and bone loss through targeting key transcription factors. miR-335-5p, miR-27, miR-218 and miR-29 family promote osteoblast differentiation by targeting inhibitors (DKK1, APC, DKK2/SOST/SFRP2, DKK1/HDAC4) of Wnt signaling pathway (70-73, 75, 76). miR-322 promote osteoblastic differentiation via increasing Osterix expression by inhibition of *Tob2* mRNA (68). miR-181a, miR-210 promotes osteoblastic differentiation through targeting promoters (Tgfb1/TβR- I /Alk5, AcvR1b) of TGF-β signaling pathway (63, 64). And miR-764-5p, miR-2681/3960 also promote osteoblast differentiation by suppressing the inhibitor of Runx2 (CHIP/STUB1, HDAC4/Hoxa2) (67, 69). Furthermore, miR-155, miR-124 and miR-503 inhibit osteoclasts differentiation through repressing osteoclastic related factors, including MITF/ SOCS1, NFATc1 and RANK (85, 87, 89). The number of osteoclasts decreased, oppositely, the bone mass is increased. Compared with osteogenesis promoting miRNAs, more miRNAs inhibiting osteogenesis have been introduced in this review. Most of them (e.g. miR-133a, miR-135a, miR-30 family and miR-433) suppress osteogenesis by targeting *Runx2*, which a necessary regulator of osteogenesis (41, 44, 48). miR cluster 23a~27a~24-2 inhibits osteoblast differentiation by suppressing the SATB2, which is necessary to synthesize Runx2 (40). miR-125b and miR-637 inhibit osteoblast differentiation by targeting *Osx* that one of the Wnt signaling molecular (57, 65). In addition, miR-21, miR-223 and miR-31 promote the differentiation of osteoclasts (82, 83, 86). miR-21 regulates osteoclastogenesis through the feedback loop of c-Fos/miR-21/PDCD4, and miR-223 involves in PU.1/miR-223/NFI-A/M-CSFR loop. Above all, many miRNAs maintain bone tissue homeostasis through several bone development signaling pathway such as the Wnt signaling, the TGF-β/BMP pathway, and the PTH pathway (eg. miR-370). The regulator of osteogenesis or osteoclastogenesis could play an important role in the balance between bone resorption and bone formation, which is the key point to maintain bone tissue homeostasis. The disturbance of bone homeostasis is related to some bone diseases, such as osteoporosis.

Despite recent advances that provide abundant evidence that bone phenotypes are regulated by specific miRNAs, the related mechanisms still remain poorly understood. An important and effective approach that deletion Dicer for the research of specific miRNAs expression and function in cells or tissue. And in the research of

miRNAs mechanisms, the identification of miRNA target sites is one of key step. Understanding the mechanisms of osteoblast and osteoclast differentiation regulated by specific miRNAs will be important for developing new therapeutics to the treatment of metabolic bone disorders and bone loss. For example, the inhibition of miR-155 expression might provide a novel target for the treatment of osteoclast-mediated diseases (85). In addition, miRNAs might play an important role in regeneration of bone tissue. Therefore, much more work needs to be conducted in order to identify the importance of miRNAs in bone and to define their roles in bone formation, bone homeostasis, remodeling, fracture repair, and skeletal diseases.

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**Abbreviations:** miRNAs: microRNAs; MSCs: mesenchymal stem cells; hFOB: human fetal osteoblasts; hADSCs: human adipose-derived mesenchymal stem cells; DSCs: human-derived dental stem cells; ECM: extracellular matrix; RISC: RNA-induced silencing complex; PTH: parathyroid hormone; PTHrP: parathyroid hormone-related protein; TNF: tumor necrosis factor; TGF- $\beta$ : transforming growth factor beta; M-CSF: macrophage colony-stimulating factor; RANKL: receptor activator of nuclear factor NF- $\kappa$ B ligand; RANK: a receptor of RANKL; BMP: bone morphogenetic proteins; BMP2: bone morphogenetic protein type II; BMPR2: bone morphogenetic protein receptor type II; PACT: protein activator of PKR; Runx2: Runt related transcription factor 2; Osx: Osterix; ER: estrogen receptor; ERR $\gamma$ : estrogen receptor-related receptors; SHP: small heterodimer partner; ALP: alkaline phosphatase; Cx43: Connexin 43; Ets1: V-ets Erythroblastosis Virus E26 Oncogene Homolog1; Dlx5: Distal-less homeobox 5; ROS: reactive oxygen species; Tgfb1: Tgf-beta induced; T $\beta$ R- I: TGF- $\beta$  type I receptor; Rgs4: regulator of G protein signaling; AcvR1b: activin A receptor type 1B; Hoxa2: Homeobox; DKK1: Dickkopf-related protein 1; APC: adenomatous polyposis coli; SFRP2: secreted frizzled-related protein2; PDCD4: programmed cell death 4; GSK: glycogen synthase kinase-3 $\beta$ ; SOST: sclerostin; Cbfa1: core binding factor 1; ATF4: activating transcription factor 4; NFATc1: Nuclear factor of activated T-cells, cytoplasmic 1; TRBP: Tar Rna Binding Protein; RhoA: ras homolog gene family, member A; SATB2: special AT-rich sequence-binding protein 2; HDAC4: Histone deacetylase 4; CREB: N cyclic amp-response element binding protein; C/EBP $\alpha$ : CCAAT enhancer binding

protein $\alpha$ ; PPAR $\gamma$ : peroxisome proliferator-activated receptor  $\gamma$ ; Gata6: GATA-binding factor 6; MITF: microphthalmia-associated Transcription Factor; SOCS1: suppressor of cytokine signaling 1; CHIP/STUB1: C terminus of Hsc70-interacting protein/STIP1 homology and U-Box containing protein 1; DGCR8: DiGeorge syndrome critical region gene 8; TAZ: transcriptional co-activator with PDZ-binding motif; MSX2: mshhomeobox 2

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**Send correspondence to:** Bo Gao, State Key Laboratory of Oral Diseases, Sichuan University, Chengdu, China, 610041, Tel: 86-28-85503469, Fax: 008602885445625, E-mail: hero\_gb@sina.com