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REVIEW

The role of microRNAs in bone remodeling

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Bone remodeling is balanced by bone formation and bone resorption as well as by alterations in the quantities and functions of seed cells, leading to either the maintenance or deterioration of bone status. The existing evidence indicates that microRNAs (miRNAs), known as a family of short non-coding RNAs, are the key post-transcriptional repressors of gene expression, and growing numbers of novel miRNAs have been verified to play vital roles in the regulation of osteogenesis, osteoclastogenesis, and adipogenesis, revealing how they interact with signaling molecules to control these processes. This review summarizes the current knowledge of the roles of miRNAs in regulating bone remodeling as well as novel applications for miRNAs in biomaterials for therapeutic purposes.

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INTRODUCTION

Long-standing clinical observations indicate that numerous patients suffer from bone loss, bone defects, and bone non-union due to trauma, osteoporosis, osteoarthritis (OA), cancer, and other diseases. The leading edge in the prevention and treatment of these diseases is the study of how to promote osteogenesis and accelerate bone formation.[1] Studies have revealed that bone tissue is continuously remodeled throughout the lifetime to create new bones and to maintain normal bone homeostasis. Mature bone tissue is maintained through the balancing activities of bone-forming osteoblasts, which originate from mesenchymal stem cells (MSCs), and of bone-resorbing osteoclasts, which arise from haematopoietic stem cells (HSCs).[2] In recent years, investigators have unraveled several key signaling mechanisms involved in the regulation of these cells at both the genetic and molecular levels, including major signaling pathways, such as the Notch,[3] bone morphogenetic protein (BMP),[4] canonical WNT,[5] and the receptor activator of nuclear factor κB (RANK)-osteoprotegerin (OPG)-and RANK ligand (RANKL) pathways,[6] which are critical for bone formation and resorption. In addition to these genetic mechanisms, bone functions are also controlled by epigenetic mechanisms, which include the regulation of genes by DNA methylation, microRNA (miRNA) function, and chromatin architecture modification.[7] miRNAs, which are considered the key epigenetic mechanisms for the control of expressed genes, provide a novel dimension to the post-transcriptional control of cells involved in bone remodeling by inhibiting mRNA translation.[8–9] However, the current understanding of how miRNAs mediate the regulatory interplay between and among different cells in bone remodeling is minimal, and the role of miRNAs in the differentiation and function of mature cells derived from MSCs or HSCs remains to be elucidated. Herein, we review the role of miRNAs in the regulation of bone remodeling and discuss their implications as promising therapeutic targets.

BONE REMODELING AND ITS TRANSCRIPTIONAL REGULATION

Bone remodeling is a dynamic process regulated by numerous biological factors and signals.[10] Osteocytes, which act as sensors of chemical signals and mechanical loading, orchestrate bone activity and communicate with osteoblast and osteoclast effectors.[11] Osteoblasts are differentiated from MSCs, and they play a critical role in the production and mineralization of extracellular matrix.[12] In addition, osteoblasts can regulate the differentiation of osteoclasts. Currently, several transcription factors (TFs) in bone development have been identified, such as runt-related transcription factor 2 (Runx2) and Cbf β, which are key factors both in bone formation and osteoblast differentiation.[13]

Osteoclasts are essential bone-resorbing cells during bone homeostasis and regeneration. They originate from HSCs or haematopoietic progenitors in the monocyte/macrophage lineage. The TF PU.1 contributes to the early stage of osteoclastogenesis. Microphthalmia-associated transcription factors (MITF) and transcription factor E3, located downstream of macrophage colony-stimulating factor (M-CSF)/c-fms and RANKL/RANK, are also important in the differentiation of monocytic osteoclast precursors into multinucleated osteoclasts.[14] In addition to these signaling factors, abundant transcriptional factors have been shown to be involved in bone remodeling.[11,15]

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miRNAs: DEFINITION AND BIOLOGICAL FUNCTIONS

miRNAs are short, single-strand, non-coding RNAs approximately 20–25 nucleotides in length that have emerged as a novel mechanism capable of post-transcriptionally regulating the expression of mRNAs and proteins.[16] They can silence their cognate target genes by inhibiting mRNA translation or degrading the mRNA molecules by binding to their 3′-untranslated regions (UTR).[17] The transcription of miRNAs is mostly modified by RNA polymerase II, and it can also be performed by RNA polymerase III.[18] Sequences encoding miRNAs are often found around the genome as separate transcriptional units, located within the introns of coding genes.[19] The 5′ ends of mature miRNAs contain the seed region (nucleotide positions 2–7 or 2–8), which has the ability to identify the complementary bases of the 3′-UTR of the target mRNAs and trigger their degradation.[20]

miRNAs have been found to be involved in multiple biological processes, including cell differentiation, cell proliferation, development timing, apoptosis, transposon silencing, and antiviral defense.[21] Hundreds of miRNAs, which are evolutionarily conserved, have been identified in humans.[22] So far, more than 3% of the genes in humans have been identified as encoding for miRNAs, and approximately 40% to 90% of the human protein-encoding genes are under miRNA-mediated gene regulation.[23] Therefore, numerous miRNAs have been found to be involved in the regulation of bone homeostasis, and they play critical roles in bone remodeling (Table 1).

miRNAs IN OSTEOBLAST LINEAGE AND BONE FORMATION

MSCs are the sources of osteoblasts, chondrocytes, and adipocytes. They differentiate into osteoblast lineages, determined by a variety of osteogenic factors and signals, which are regulated by post-transcriptional control, and miRNAs are among the most crucial, validated members (Figure 1). The inactivation of Dicer, which is a prerequisite for the manufacture of canonical miRNAs, leads to bone defects and impaired mineralization during the embryonic stage and a delayed increase in bone mass during the post-natal stage.[24] In addition, miRNAs can bind to target sequences in the UTR of mRNAs, leading to translational arrest or causing the RNA-induced silencing complex (RISC) to degrade messages.[25] Therefore, miRNAs can display considerable enhancing or inhibitory functions in osteogenesis by binding to important osteogenic TFs.[26]

miRNAs that inhibit osteogenesis

Runt-related transcription factor 2. Runt-related transcription factor 2 (Runx2) – also referred to as core-binding factor α1 (Cbfα1), osteoblast-specific factor 2 (Osf2), polyomavirus enhancer binding protein (PEBP2a2), and acute myeloid leukemia gene 3 (AML3) – acts as a core TF in the regulation of osteogenesis.[27] Runx2 binds to osteoblast-specific cis-acting element (OSE2), which exists in the promoter region of osteogenic-related genes, such as bone gamma-carboxyglutamic acid-containing protein (BGLAP), secreted phosphoprotein 1

Table 1 microRNAs in bone remodeling

Targets	miRNAs	Sample resources	References
Osteogenesis inhibited			
Runx2	miR-34c		
	miR-133a		
	miR-135a		
	miR-137		
	miR-205	MC3T3-E1	[33]
	miR-217		
	miR-338		
	miR-23a	MC3T3-E1	[33,34]
	miR-30c	MC3T3-E1, hADSCs	[33,35,36]
	miR-204/211	MC3T3-E1, C3H10T1/2, ST2, hMSCs	[33,37,38,70]
	miR-103a	hFOB 1.19, C57BL/6J mice	[45]
Cbfβ	miR-125b	BMSCs, C3H10T1/2, ST2	[39-42]
JMJD3	miR-146a	hMSCs	[32]
Smad1	miR-30	MC3T3-E1, BMSCs	[35]
SATB2	miR-23a~27a~24-2	MC3T3-E1	[50]
	miR-31	hMSCs	[55]
	miR-34b/c	Mouse osteoblasts, C2C12, miR-34a/b/c$^{-/-}$ mice	[52,53]
Notch1/2, JAG1	miR-34a/c	hMSCs, miR-34c Tg mice	[53,219]
Osx	miR-31	hMSCs	[62]
	miR-93	MC3T3-E1	[63]
	miR-143	MC3T3-E1	[64,66]
	miR-145	MC3T3-E1, C2C12	[65,66]
	miR-637	hMSCs	[67]
	miR-214	hMSCs	[68]
Smad1	miR-26a/b	hADSCs, USSCs	[75]
Smad5	miR-135b	hMSCs, C2C12, hUSSCs	[76-78]
BMPR2	miR-100	hADSCs	[80]
BMP2	miR-17-5p	hADSCs	[79]
	miR-106a		
	miR-140-5p	hMSCs	[81]
TCF3	miR-17	hPDLSCs, NOD/SCID mice	[87]
CDC25A	miR-141-3p	hMSCs, ST2	[91]
ATF4	miR-214	MC3T3-E1, human osteoporotic bone specimens	[101]
COL1A1, Col5A3	miR-29a/b	Rat osteoblasts, MC3T3-E1	[140,141]

Table 1 (Continued)

Targets	miRNAs	Sample resources	References
SOCS1	miR-155	MC3T3-E1	[210,211]
Fas	miR-23a	MC3T3-E1	[214]
FAK	miR-138	hMSCs, MC3T3-E1	[113]
Fgfr2	miR-233	MC3T3-E1, C3H10T1/2, ST2	[115]
	miR-338	BMSCs, OVX mice	[116]
Cx43	miR-206	C2C12, Cx43$^{-/-}$ mice	[119]
Dlx5 SVCT2	miR-141/200a	MC3T3-E1 BMSCs	[120] [121]
Dlx5/Msx2	miR-124a/181a	iPS cells, hMSCs, BMSCs, MC3T3-E1, C2C12	[122,123]
IGF2	miR-30e	BMSCs, SMCs, ApoE$^{-/-}$ mice	[230]
Osteogenesis promoted			
Tob2	miR-322	BMSCs, MC3T3-E1, C2C12	[69]
Smurf1	miR-15	hMSCs	[159]
PPARγ	miR-20a/b	hMSCs	[133,134]
	miR-548d-5p	hMSCs	[132]
Bambi, Crim1	miR-20b	hMSCs	[133]
DDK1	miR-335-5p	C3H10T-1/2	[139]
	miR-29a/c	hFOB1.19, MC3T3-E1	[19,141]
Kremen2, SFRP2	miR-29a/c	hFOB1.19, MC3T3-E1	[19,141]
DDK2, SFRP2, SOST	miR-218	hADSCs, MC3T3-E1, BMSCs	[137,138]
Hoxa2	miR-3960	Mouse osteoblasts	[151]
Axin2	miR-let-7	hMSCs	[142]
HMGA2		hADSCs	[231]
APC	miR-27	hFOB1.19	[144]
SFRP1			[143]
HDAC1	miR-449a	Human iPS cells	[148]
HDAC4	miR-29b	MC3T3-E1, USSCs	[141,149]
HDAC5	miR-2861	Mouse osteoblasts, ST2, OVX mice	[150,151]
HDAC6	miR-22	hADSCs	[153]
HDAC9	miR-188	BMSCs, 188-Tg mice	[154]
TFG-βi/Alk5	miR-181a	MC3T3-E1, C2C12	[156]
Acvr1b	miR-210	ST2, NRG cells	[157]
Smad2/3	miR-146a	Fetal femur-derived cells	[158]
COUP-TFII	miR-194	BMSCs	[161]
	miR-302a	MC3T3, C2C12	
HB-EGF	miR-96	BMSCs, MC3T3-E1,OVX mice	[163]
	miR-1192	C2C12/Runx2^{Dox} cells	[164]
Spry1	miR-21	hMSCs, OVX mice	[213]
SOX2		SS-AF-hMSCs	[232]
SPARC	miR-29a	ABSa15	[88]
STAT1	miR-194	BMSCs	[233]
Hoxa8	miR-196a	hADSCs	[234]
CHIP/STUB1	miR-764-5p	MC3T3-E1, mouse osteoblasts	[235]
Osteoclastogenesis inhibited			
RANK	miR-503	PBMCs	[174]
TRAF6	miR-125a	PBMCs	[175]
NFATc1	miR-124	BMMs	[176]
FasL	miR-21	BMMs, osteoclast precursors, CAG-Z-miR-21-EGFP transgenic mice	[183]
CTGF	miR-26a	Murine osteoclasts	[186]
NFI-A	miR-233	RA/OA patients	[168]
SOCS1, MITF	miR-155	FNb$^{-/-}$/IFNAR1$^{-/-}$ mice, RAW264.7	[211]
Tgif2	miR-34a	Tie2-cre mice, 34a-KO/Het mice, OVX mice,34a-Tg mice	[220]
DC-STAMP	miR-7b	RAW264.7	[236]
Osteoclastogenesis promoted			
PIAS3	miR-9718	RAW 264.7	[172]
RhoA	miR-31	BMMs	[177]
PDCD4	miR-21	BMMs, c-Fos$^{-/-}$ mice	[181]
MAFB	miR-148a	PBMCs	[184]
Pten	miR-214	BMMs, RAW 264.7, OC-214Tg mice	[200]

hMSC, human mesenchymal stem cell; BMSC, bone marrow stem cell; ADSC, adipose-derived stem cell; PDLSC, periodontal ligament stem cell; USSC, cord blood stem cell; MC3T3-E1, mouse osteoblastic cell line; C2C12, mouse premyogenic cell line; ST2, mouse bone marrow stromal cell line; SMC, aortic smooth muscle cell; hFOB1.19, human osteoblastic cell line; PBMC, peripheral blood mononuclear cell; BMM, bone marrow macrophage; RAW264.7, Murine macrophage cell line; OVX mice, ovariectomized mice; PDCD4, programmed cell death 4; MAFB, V-maf musculoaponeurotic fibrosarcoma oncogene homolog B.

(SPP1), collagen type I alpha 1 (COL1A1), alkaline phosphatase (ALP), and bone sialoprotein (BSP).[28–29] It is primarily required for the induction of osteoblast differentiation rather than for adipogenesis or chondrogenesis, while ossification is suppressed when Runx2 is overexpressed during osteoblast maturation.[30–31] It has also been found that Runx2 expression can be affected by multiple epigenetic regulatory mechanisms, including a methylation regulatory circuit in which jumonji domain-containing 3 (JMJD3) acts as a histone demethylase to increase Runx2 levels via the negative modulation of histone H3 lysine 27 trimethylation (H3K27 me3), and miR-146a targets JMJD3 to reverse osteogenic differentiation.[32]

miRNA microarray is a common method for the screening of miRNAs because it has been discovered that 10 miRNAs (miR-23a, miR-30c, miR-34c, miR-133a, miR-135a, miR-137, miR-204, miR-205, miR-217, and miR-338) inhibit osteoblast differentiation in MC3T3-E1 premature osteoblasts by directly targeting Runx2, showing either high or medium levels.[33] They also target TRPS1 (except for miR-137, miR-204, and miR-338) to redirect MSCs to adipogenic cell fates.[34] Moreover, members of the miR-30 family have been shown to target both Runx2 and Smad1, acting as key regulators in bone mineralization in BMP-2-mediated osteogenesis and stimulating adipogenesis in hADSCs.[35–36] miR-204 and its homolog miR-211 act as endogenous attenuators of Runx2, inhibiting osteogenesis instead of adipogenesis in C3H10T1/2 and ST2 cell mesenchymal progenitor cells and human mesenchymal stem cells (hMSCs), as well as contributing to vascular smooth-muscle cell (VSMC) calcification.[37–38] miR-125b is also involved in the inhibition of osteoblastogenesis, and ErbB2 has previously been verified as a target gene.[39] Recently, another target, Cbf β, has been found, which is a cofactor enhancing Runx2 activity, causing miR-125b to indirectly act on Runx2 during the early stages of osteogenesis.[40] In addition, miR-125b is significantly increased in the hMSCs derived from senile osteoporotic patients, and osterix (Osx) levels change,[41] establishing the potential role of miR-125b in bone disease therapy, although a contradiction occurred in the 'gain and loss' experiments of miR-125b *in vitro*.[42] In this case, miRNAs and their targets vary accordingly across different osteogenesis stages, and *in vivo* research is needed to elucidate the mechanism.

Recent studies have found and affirmed some specific miRNAs functioning in cyclic mechanical stretch-induced osteoblast differentiation,[43] especially in human periodontal ligament cells (PDLCs) and alveolar bone cells (ABCs). For example, miR-29b and miR-103a act as mechanosensitive regulators post-transcription, and they can also target Runx2 to suppress osteoblasts activity and bone mineralization.[44–45] These new findings will broaden our horizons in considering the triangular relationship between miRNA, mechanics and bone remodeling.

Special AT-rich sequence-binding protein 2. Special AT-rich sequence-binding protein 2 (SATB2) binds to nuclear matrix attachment regions and interacts synergistically with Runx2, promoting osteoblast differentiation and positively regulating the expression of osteoblast-specific genes, such as BSP, osteocalcin (OCN), Osx, and vascular endothelial growth factor A.[46–47] Gel shift assays have shown that Osx, as an upstream regulator, binds directly to the SATB2 promoter sequence, while conversely, SATB2 up-regulates Osx dependently or independently of Runx2.[47–48] In addition, SATB2 has also been found to be a novel marker of osteoblast differentiation in some mesenchymal tumors.[49]

Study of the miR-23a~27a~24-2 cluster indicates a 'feed-forward' regulatory circuit in the osteoblast lineage. Each of the components targets SATB2 directly and is suppressed by Runx2 in the mineralization stage, leading to osteoblastogenesis and attenuation by Hoxa10 and Runx2 targeted by miR-27a and miR-23a.[50] However, miR-23a has failed to play such a role in animal research,[51] which should trigger discussion regarding why discrepancies have occurred in *in vitro* and *in vivo* studies. In addition, *in vivo* research has discovered that the miR-34 family inhibits osteoblast differentiation in the terminal stage by targeting SATB2, while it inhibits osteoblast proliferation by targeting cyclin D1, CDK4, and CDK6, thus suppressing bone formation.[52–53] Recently, seeding anti-miR-31-modified bone marrow stem cells (BMSCs) onto poly(glycerol sebacate) (PGS) scaffolds showed significant generation of critical-sized bone defects,[54] and miR-31 was shown to suppress osteogenesis by targeting SATB2 in hMSCs.[55] At the same time, miR-31 is directly suppressed by Runx2, forming a feedback loop in the regulatory circuit of miR-31, Runx2, and SATB2.[56]

Osx. Osx, also called Sp7, is a special zinc-finger-containing TF required for bone formation and bone homeostasis. Deletion or deactivation of Osx in mice led to spinal deformities.[57] Osx has been found downstream of Runx2, and it is involved in several pathways, such as MAKP signaling.[58–59] In addition, the BMP-2-mediated induction of Osx may be inhibited by blocking Runx2.[60–61]

As mentioned above, miR-31 targets primarily SATB2, but it also directly regulates the Osx 3′-UTR during the osteogenic differentiation of BMSCs. At the same time, five miRNAs were found to be modulated during the mineralization process.[62] miR-93 has been found to target Osx in osteoblast mineralization and to be negatively regulated by Sp7, forming a feedback loop.[63] miR-143 and miR-145 directly target Osx and act as crucial regulators of osteogenic differentiation,[64–65] consisting of another feedback loop with Klf4 and Osx in odontoblast differentiation, thus suggesting a complex but relevant application for osteogenesis.[66] miR-637 has been indicated as being indispensable for maintaining the balance of osteogenesis and adipogenesis by targeting Osx in hMSCs.[67] For C2C12 myoblast cells, miR-214 has been verified as a new regulator of Osx to suppress osteogenic differentiation.[68] Researchers have also found that Osx may be positively modulated by miR-322 targeting of Tob2, which binds to Osx and regulates Osx degradation.[69] Controlled by Osx, miR-133a and miR-204/211 are down-regulated, with their Runx2 targets up-regulated.[37] In addition, miR-133a and miR-204/211 can negatively regulate ALP and Sost, which antagonize Wnt and enhance Runx2, to form an intricate loop.[70]

Smads. The Smad factor is a crucial intracellular receptor that participates in canonical Transforming growth factor beta (TGF-β) and BMP superfamily pathways, and it separately activates RSmad2/3 and Rsmad1/5/8 signals.[71] Activated Rsmads form a complex bond with Co-Smad4 and translocate into nuclei to regulate responsive genes.[72] Smad acts as a common stimulator of osteogenesis and bone regeneration in a number of mechanisms, such as its interaction with Runx2.[73] Smad directly induces Msx2 to increase Osx expression, and it is involved in many other pathways.[72] However, a non-Smad pathway exists in BMP-7-induced osteogenesis, which regulates the proliferation and survival of osteoblasts.[74]

miR-26a, which is involved in BMP pathways, can also target Smad1 to guide osteoblast differentiation.[75] MSCs derived from multiple myeloma (MM) patients showed impaired bone formation and higher miR-135b, which targets Smad5 to control bone disease and C2C12 mesenchymal cells and human umbilical cord blood-derived unrestricted somatic stem cell (USSC) differentiation.[76–78] BMP2 and BMPR2 can also be targeted directly by miR-17-5p, miR-106, miR-100, and miR-140-5p and by

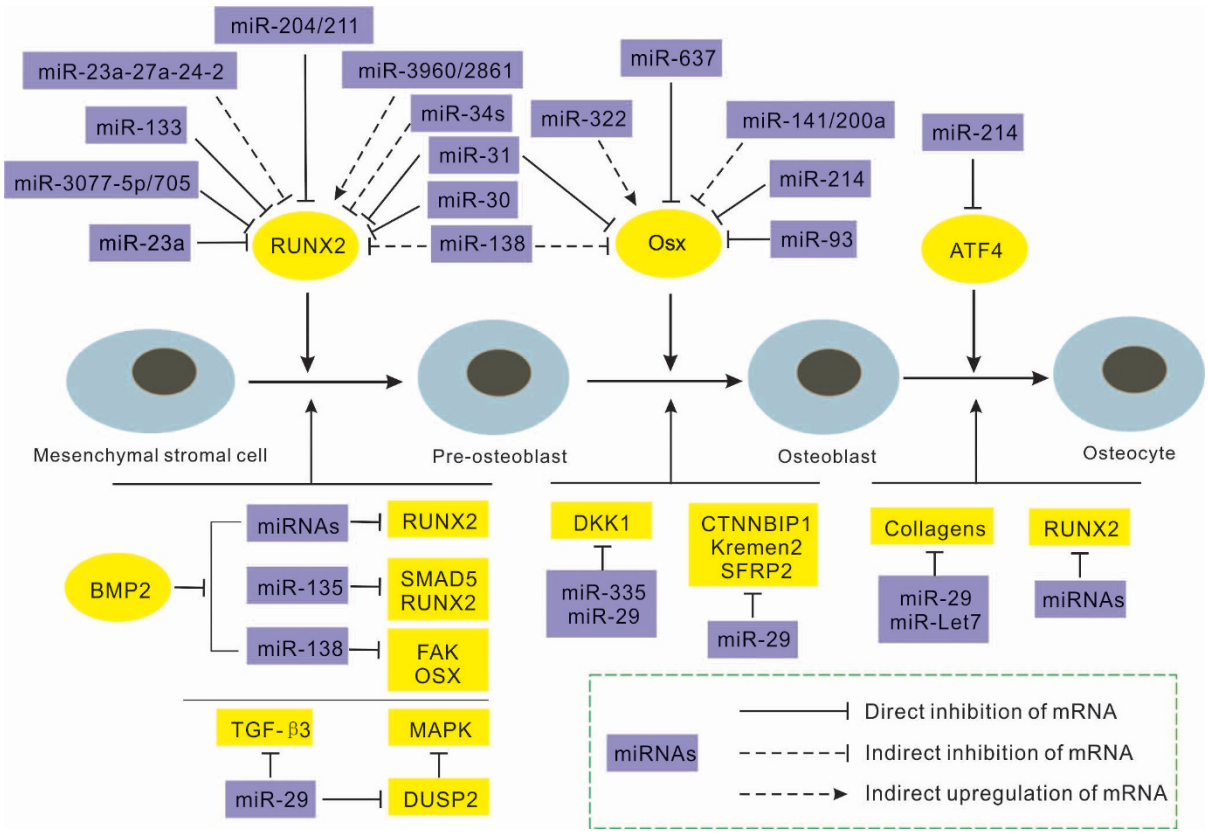

Figure 1 Schematic summary of miRNAs implicated in osteoblast differentiation. This figure indicates the major activities of miRNAs influencing the continuous maturation of cells from mesenchymal stromal cells to osteocytes. Selected miRNAs (purple) relative to their targets (yellow) and influencing each stage to regulate the progression of differentiation are indicated. BMP, bone morphogenetic protein.

decreased osteogenic TAZ, Msx2, and Runx2 through Smad1/5, while inducing adipogenesis.[79–81] Conversely, miR-17-5p plays a key role in this reaction and was recently proven to target Smad7. It can also increase the Runx2 and COL1A1 levels and promote nuclear translocation of β-catenin, preventing non-traumatic osteonecrosis and maintaining bone remodeling to some extent.[82] miR-542-3p can also target BMP-7, inhibiting both Smad-mediated osteoblast differentiation and BMP-7/PI3K-survivin non-Smad-induced proliferation.[74] In contrast, inhibition of miR-203 may rescue suppressed PI3K expression and stem cell activity.[83]

β-catenin. β-catenin is a master downstream multi-functional protein, which is actively involved in the canonical Wnt signaling pathway and displays characteristics essential for determining the differential direction of MSCs,[84–85] forming an intricate network in different stages and microenvironments.[86–87] In this pathway, miR-29a may induce β-catenin protein levels,[88] but conversely, when β-catenin degradation was inhibited, it accumulated and localized in the nuclei of active T-cell factor (TCF)/lymphoid enhancer factor (LEF), which regulates target gene expression.[89] TCF3 has been elucidated as an enhancer of osteogenesis both *in vivo* and *in vitro*, and miR-17 is a direct modulator of TCF3.[87] Additionally, both β-catenin and TCF can be down-regulated if low-density lipoprotein receptor-related protein 6 (LRP6), a co-receptor of Wnts, is blocked by miR-30e in the early stage of stem cell differentiation.[90] Moreover, a major regulator of the cell cycle, called cell division cycle 25A (CDC25A), can be knocked down by miR-141-3p in the initial phase of the osteoblast differentiation of human stromal stem cells.[91]

Activating transcription factor 4. Mice with activating TF (ATF) 4 deficiency ($ATF4^{-/-}$) display severely reduced boss mass and bone toughness, as well as decreased COL1A1 synthesis.[92–93] As a crucial transcriptional factor in bone formation, ATF4 works with Runx2 and Osx. ATF4 regulates OCN expression positively by binding to osteocalcin-specific element (OSE1), and it interacts cooperatively with Runx2 through SATB2.[92,94–95] Factors inhibiting ATF4-mediated transcription (Fiat) have been found to suppress ATF4 activity and, co-expressed in osteoblasts[96–97] and modulated by the Sp family, to exclude Osx.[98] In the matrix mineralization stage, ATF4 is positively regulated, to a large extent, by c-Jun N-terminal kinase (JNK), affecting the expression of OCN and BSP.[99] Moreover, ATF4 can also bind to the promoter of RANKL and can ultimately inhibit bone resorption.[100] Recently, high miR-214 levels were found in samples from older individuals with lower BGLAP and ALP levels, verified by their inhibitory roles in bone formation by targeting ATF4 directly, thus providing a vital potential therapy from ameliorating osteoporosis.[101]

TAZ. Transcription coactivator with binding capacity to PDZ motifs (TAZ) is a 14-3-3-binding transcriptional modulator with WW domains that binds to the motifs in many bone-related transcriptional genes to determine stem cell fate. During osteogenic differentiation, TAZ can be induced by extracellular signal-regulated kinase (ERK), activated by FGFs.[102] It can also be facilitated by PP1A-induced Wnt3a,[103] and some chemical compounds, such as phorbaketal A and TM-25659, have been shown to modulate TAZ.[104–105] In addition, TAZ can coactivate with Runx2-dependent genes (such as OCN) to

activate osteogenesis while suppressing PPARγ-dependent gene transcription (such as of adipocyte protein 2) to block adipogenesis.[106–107] miR-17-5p has been confirmed to regulate BMP2 and to participate in TAZ-regulated Runx2 and PPARγ modulation.[79] Moreover, TNF can activate NF-κB, which induces osteogenesis via subsequent up-regulation of TAZ expression, but miR-146a inhibited all of these processes.[108]

Another nuclear TF, called Yes-associated protein (YAP), coactivates with TAZ, and YAP/TAZ are key downstream effectors of Hippo signaling, which has been defined as necessary for the Dicer-mediated pre-miRNA process.[109–110] YAP/TAZ are key mechanical signals influenced by extracellular matrix rigidity and cell shape, and they have determinable effects on cell fate.[111–112]

Focal adhesion kinase. Focal adhesion kinase (FAK), which is encoded by the PTK2 gene, facilitates contact between ECM proteins and activation of ERK1/2, namely via the ERK-dependent pathway, which is found to be concentrated on the transcriptional regulation of bone formation. Downstream cascade Runx2 phosphorylation and Osx expression are affected accordingly. Previous studies have indicated that FAK was targeted by miR-138, and it blocked such pathway-inhibited bone formation.[113] New research has found that platelet-derived growth factor (PDGF) acts as the upstream regulator of miR-138.[114]

In addition, ERK1/2 phosphorylation and its pathway can also be blocked by fibroblast growth factor receptor 2 (Fgfr2), which is a critical regulator of osteogenesis and bone tissue regeneration. Conversely, CCAAT/enhancer binding protein α (C/EBPα) and PPARγ can be activated, with miR-233 and miR-338-3p targeting Fgfr2, resulting in a feedback loop.[115–116]

Other targets. In addition to these mutual targets preferred by respecle miRNAs, there are still some crucial and special targets mediated by a small number of miRNAs.

Connexin 43(Cx43), belonging to the gap junction proteins family, is mainly expressed in the vasculature, ventricular myocardium, and astrocytes. While Cx43-deficient mice showed decreased bone mass due to osteoblast dysfunction,[117] it was surprising to find that miR-206, a muscle-related miRNA,[118] inhibited osteoblast differentiation by targeting Cx43 in osteoblasts. Transgenic mice with specifically expressed miR-206 in osteoblasts showed this result as well, resulting in a significant decrease in the bone formation rate.[119]

Distal-less homeobox 5 (Dlx5), a member of a homeobox TF gene family, is also regarded as one of the osteogenic TFs. miR-141 and miR-200a are members of the miR-200 family and have been identified as targeting Dlx5 to affect the transcriptional expression of Osx indirectly,[70,120] as well as targeting sodium-dependent vitamin C transporter 2 (SVCT2) to suppress osteogenic differentiation.[121] Furthermore, miR-124a and miR-181a were predicted and verified to regulate the differentiation of iPS cells into osteoblasts by targeting Dlx5 and Msx2, respectively,[122] and *in vivo* ectopic bone formation assay strengthened these findings.[123]

miRNAs in promoting osteogenesis

PPARs. Peroxisome proliferator-activated receptors (PPARs), which belong to the ligand-dependent activated nuclear hormone receptor gene superfamily, include PPARβ, PPARδ, and PPARγ. PPARγ plays a decisive role in adipogenic differentiation and therefore in the regulation of osteogenesis.[124–125] Adipogenesis occurs through the activation of C/EBPβ and C/EBPδ, which activate C/EBPα and PPARγ, respectively.[126] PPARγ$^{-/-}$ ES cells and PPARγ$^{+/-}$ mice show enhanced osteoblastic differentiation, with high bone mass but normal osteoblast functions.[127] In addition, the existence of PPARγ suppresses Runx2 activity and subsequent osteocalcin expression.[128] It can also induce β-catenin degradation.[129] Recent epigenetic research has found that DNA hypermethylation prevented PPARγ from activating C/EBPα and that histone deacetylase (HDAC) 1 occupied the region of and disequilibrated the balance between osteogenesis and adipogenesis.[130] Nevertheless, the stimulatory role of PPARγ has been found not only to promote adipogenic differentiation but also to induce osteogenesis by BMP2 when PPARγ is overexpressed.[131] Recently, miR-548d-5p was found to directly target PPARγ when C/EBPα levels were decreased, which may suppress adipogenesis rather than osteogenesis.[132] When BMP2 is inhibited by miR-17-5p and miR-106a, Runx2, TAZ, and Msx2 decrease, which decreases their ability to inhibit PPARγ, which leads to adipogenesis.[79] miR-20a and miR-20b share the same function in directly repressing PPARγ, with Bambi and Crim1 activating the BMP/Runx2 pathway to enhance osteogenesis.[133–135]

Wnt inhibitors. Wnt inhibitors consist of the Dickkopf family (DKK) and the secreted frizzled-related family (SFRP), which competitively combine with LRP5/6 to inhibit Wnt signaling.[135] Numerous intracellular factors, such as APC, Axin, GSK3, NLK, and NKD, are also negative regulating proteins.[136] miR-218 has been repeatedly proven to be an up-regulator of osteogenic differentiation in hADSCs and osteoprogenitors, and the direct targets of miR-218 are DKK2, SFRP2, and sclerostin (SOST), while Wnt3a signals up-regulate miR-218, forming a 'feed-forward' regulatory circuit.[137–138] DKK1, which is another important DKK family member, is directly targeted by miR-335-5p.[139] Moreover, the miR-29 family plays crucial roles in osteogenesis by suppressing osteonectin expression rather than reducing collagens and extracellular matrix proteins.[19,140–141] Furthermore, Axin2 can be decreased by high levels of miR-let-7 in tissue inhibitor of metalloproteinase 1$^{-/-}$ (TIMP1$^{-/-}$) hMSCs, which is another antagonist of β-catenin and increases osteoblast differentiation.[142] Adenomatous polyposis coli (APC) and sFRP1 inhibition by miR-27 share the same mechanism.[143–144]

HDACs. HDACs are a class of enzymes that modify chromatin structure and transcriptional activity balanced by histone acetyltransferase (HAT). HDACs are Runx2 inhibitors: HDAC1 down-regulates histone acetylation levels, and HDAC4/5 are recruited to interact with Smad3, forming a co-repressor complex to inhibit osteoblast differentiation,[145–147] while miR-449a was detected to target HDAC1 in human iPS cells,[148] and miR-26a/b and miR-29 targeted HDAC4 and CDK6 in USSCs.[141,149] Two other enhancers of Runx2 degradation, HDAC5 and Hoxa2, are inhibited by miR-3960 and miR-2861, respectively, and they can be up-regulated by Runx2 to form an amplification loop.[150–151] Moreover, deficiency in HDAC6, which is targeted by miR-22, leads to increased mouse trabecular bone density.[152–153] HDAC9 also plays a switchable role in deciding the direction of stem cell differentiation, which decreases in age-dependent adipogenesis, and transgenic miR-188 mice targeted for HDAC9 showed increased bone loss and fat accumulation.[154]

BMP/TGF-β/activin receptors. The TFG-β/Smads pathway is also involved in osteogenesis. Anti-mir-221-transfected USSCs showed increased expression of SMAD3 and TGF-βR and up-regulation of osteogenesis.[155] miR-181 suppressed TGF-β signaling by inhibiting TFG-βi (TFG-β-induced) and TGF-βRI, which are both osteoblas-

tic-negative regulators.[156] Activin receptor type-1B (AcvR1b), which belongs to TGF-β superfamily, transmitting signals to activate Rsmad2/3. With miR-210 targeting AcvR1b directs and miR-146a targets Smad2/3,[157–158] TGF-β signaling is inhibited, and osteoblastogenesis is promoted. Additionally, Smad-specific E3 ubiquitin protein ligase 1 (Smurf) has been reported to bind with miR-15 and to degrade Runx2.[159] The miR-497∼195 cluster, a member of the miR-15 family, has similar functions.[160] Interestingly, chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), targeted by miR-194 and miR-302a, plays critical roles in inhibiting BMP-2-induced osteogenesis.[161–162]

Other targets. In situations such as osteoblast inhibition, there are some novel target genes and pathways involved in the process of osteogenesis that cannot be ignored. Heparin-binding EGF-like growth factor (HB-EGF), which is a member of the epidermal growth factor (EGF) family, is likely to bind to epidermal growth factor receptor (EGFR) and can activate the downstream factors ERK1, phosphatidylinositol 3-kinase (PI3K)/Akt, JNK, and c-Jun to proceed to the HBEGF–EGFR signaling pathway, which exerts effects on many physiological processes, including bone formation. miR-96, which has previously been recognized as a crucial mediator in cancer, has also been found to be a positive regulator of osteogenic differentiation by targeting HB-EGF.[163] miR-1192, which can be up-regulated by Runx2, showed the same effects.[164] Human umbilical cord mesenchymal stem cells (hUMSCs) have also been used to test the osteogenesis ability controlled by miRNA. PI3K/Akt signaling is activated, and the downstream β-catenin cascade is increased obviously after miR-21 treatment.[165]

miRNAs IN OSTEOCLAST LINEAGE AND BONE RESORPTION

Osteoclasts, which are derived from immature mononuclear phagocytic cells that originate from HSCs, make a significant contribution to bone resorption in bone remodeling processes. They have been shown to be regulated by numerous cytokines and exogenous hormones.[166–167] miRNAs have also been validated as vital regulators of osteoclasts by knocking out the osteoclast-specific Dicer gene.[168] In addition, researchers have found that DiGeorge syndrome critical region gene 8 (DGCR8)-dependent miRNAs are indispensable for osteoclastogenesis.[169] To date, the mechanism of how miRNAs work in osteoclastogenesis has been thoroughly researched, although to a lesser extent than that in osteogenesis (Figure 2).

RANK. Receptor activator for nuclear factor κB (RANK) is a type I membrane protein expressed on osteoclast precursors to activate osteoclast formation in combination with RANK ligand (RANKL) on the surfaces of osteoblasts, osteocytes, stromal cells, and T-cells by means of intimate cell–cell contact via ligands and receptors.[170–171] In the downstream cascade, transduction factor TNF receptor-associated factors (TRAFs), especially TRAF6, can bind to RANK to activate NF-κB and the JNK/AP-1 pathway.[166] c-Fos, which is another determinant transcriptional factor in osteoclast functions, is also RANKL-induced and can improve osteoclast differentiation,[170] but c-Fos-mediated osteoclast-associated receptor (OSCAR) and nuclear factor of activated T-cell calcineurin-dependent 1 (NFATc1) may be attenuated by protein inhibitor of activated STAT 3 (PIAS3) to suppress osteoclastogenesis.[172] In this system, a natural decoy receptor for RANKL, called osteoprotegerin (OPG), can compete with RANK to prevent osteoclast maturation, while M-CSF can up-regulate RANK expression.[173]

Currently, researchers have reported that peripheral blood mononuclear cells (PBMCs) from post-menopausal osteoporosis patients expressed markedly low levels of miR-503, and RANK has been verified as a direct target of miR-503, the inhibition of which promotes RANKL-induced osteoclastogenesis and bone resorption both *in vitro*

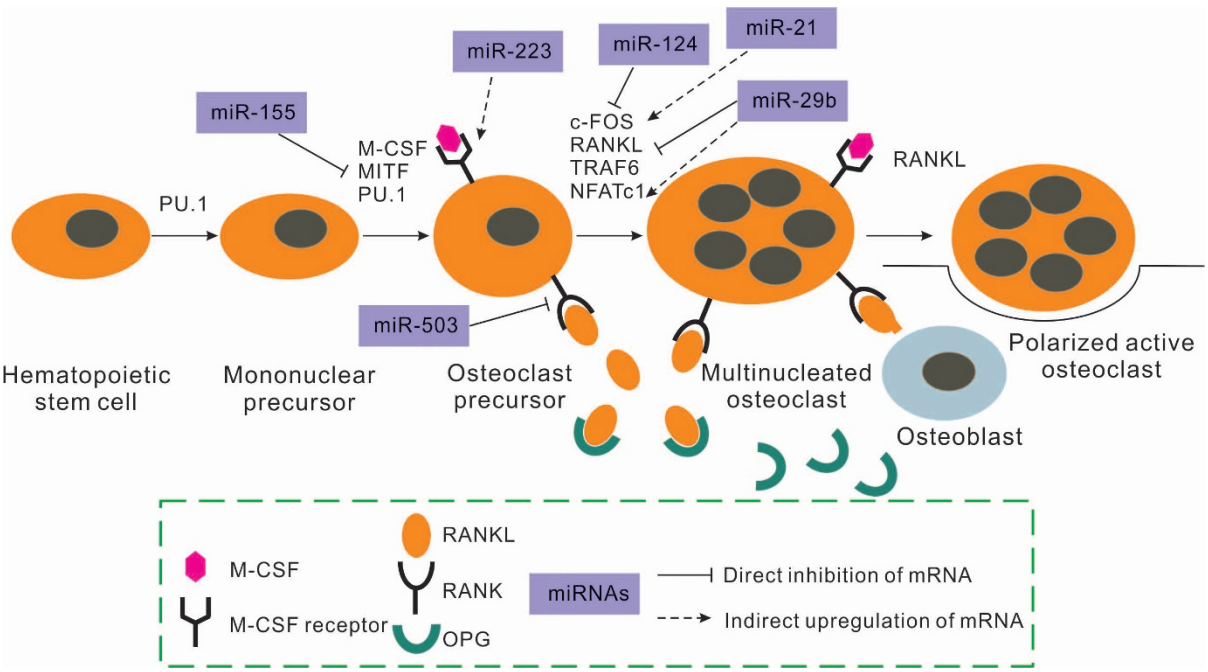

Figure 2 Effects of miRNAs on osteoclast differentiation. This figure indicates the major activities of miRNAs influencing osteoclast commitment. miRNAs affect different molecular mechanisms related to this commitment, such as RANK or M-CSF receptor, among others. miRNA expression leads to alterations in osteoclast activity *in vitro* and changes in bone resorption *in vivo*. M-CSF, macrophage colony-stimulating factor; OPG, osteoprotegerin; MITF, microphthalmiaassociated transcription factor; RANK, receptor activator for nuclear factor κB; RANKL, RANK ligand; NFATc1, nuclear factor of activated T-cell calcineurin-dependent 1.

and *in vivo*.[174] In the downstream signals, TRAF6 is directly targeted by miR-125a, while NFATc1, which is activated by NF-κB, can bind to the promoter of miR-125a and inhibit the suppressing function of TRAF6.[175] miR-124, a specific suppressor of NFATc1, can regulate osteoclast differentiation, proliferation, and migration.[176] The expression of RhoA, which controls the formation of actin stress fibers and focal adhesions, is suppressed and stabilized by miR-31, an important up-regulated miRNA in osteoclast formation, to influence osteoclast motility.[177–178] NFATc1, a cofactor of activator protein-1 (AP-1), consists of Fos/Jun proteins.[179] It can enhance the expression of the osteoclast-specific markers tartrate-resistant acid phosphatase (TRAP) and cathepsin K, and it is therefore an indispensable TF for RANKL-induced osteoclastogenesis.[180] c-Fos triggers the expression of miR-21, which can down-regulate programmed cell death 4 (PDCD4) protein levels to activate c-Fos, forming a positive 'feed-forward' loop.[181] Furthermore, miR-21 has been verified to play a critical role in particle-induced osteolysis (PIO),[182] and estrogen has been found to regulate miR-21 negatively by c-Fos inhibition and Fas ligand (FasL), the target of miR-21, to induce osteoclast apoptosis.[183] Further studies have also found that miR-148a can inhibit V-maf musculo-aponeurotic fibrosarcoma oncogene homolog B (MAFB), which can attenuate the transactivation of NFATc1 and the binding ability of c-Fos DNA, to enhance osteoclastogenesis.[184] In addition, connective tissue growth factor (CTGF) has the dual ability of promoting endochondral ossification and increasing RANKL-mediated osteoclastogenesis.[185–186]

Rheumatoid arthritis (RA) is an autoimmune disease in which osteoclasts make major contributions to bone destruction and in which the cross-talk between RANKL and interferon-γ plays a critical role.[187] Evidence has emerged indicating that miRNAs are also involved in the pathogenesis of RA. In two groups of miR-155$^{-/-}$ mice with collagen-induced arthritis (CIA) and K/BxN, serum-transfer arthritis, the mice showed blocked autoreactive B- and T-cell formation and decreased bone impairment, as in RANKL-induced osteoclastogenesis.[168,188] In addition, miR-223 has been found in high-level RA synovium patients with CIA rather than OA, and overexpression of miR-223 results in reduced osteoclastogenesis.[189–191] In human PBMCs of CIA, miR-146a is up-regulated, but c-Jun, NFATc1, PU.1, and especially TRAP expression is down-regulated in a dose-dependent manner, indicating the osteoclastic inhibition ability of miR-146a.[192–194]

M-CSFR. M-CSF receptor (M-CSFR)/CSF-1R/c-Fms, which exists in osteoclast precursors and is bound by M-CSF, is another essential secreted cytokine in osteoclast differentiation. M-CSFR is also released by osteoblasts, and it connects to receptors to initiate physiological functions through oligomerization and transphosphorylation.[195–196] PU.1, which stimulates osteoclast-specific markers and RANK expression, can positively bind to the promoter of the M-CSFR gene for activation.[197] M-CSF then participates in the latter differentiation process by stimulating the Akt, c-Fos, and ERK pathways.[198] CSF-1R has been shown to be expressed extensively in human RA, and injection of its monoclonal antibody provided effective protection against RA and inhibited osteoclast formation.[199] Moreover, a positive feedback loop of M-SCF/PU.1/miR-233/NFI-A/M-CSFR exists. M-CSF activates PU.1, which then up-regulates miR-233 and gives rise to the reduction of M-CSFR through negative regulation of nuclear factor I-A (NFI-A).[168]

During osteoclastogenesis, the expression level of miRNAs can be regulated by both RANKL and M-CSF induction. Of these two mechanisms, miR-214 plays a significant role in osteogenesis by targeting ATF, which is also a crucial regulator of osteoclast differentiation.[200] However, how miR-214 controls homeostasis and how to target it specifically remain elusive.

miRNAs BETWEEN OSTEOBLAST AND OSTEOCLAST LINEAGES

Beyond osteogenesis and osteoclastogenesis in bone remodeling, numerous signaling communications that affect each other also exist. Osteoclast-specific Dicer gene deficiency analysis has shown that activity of both osteoclasts and osteoblasts is inhibited, while bone mass is increased, indicating simultaneous and unequal epigenetic regulatory mechanisms.[168]

TNF-α/cachexin

TNF-α/cachexin is an inflammatory cytokine in bone homeostasis. It can stimulate osteoclastogenesis, but it also dually inhibits or induces osteoblastogenesis. TNF-α binds to TNFR1/2 and then acts via NF-κB, MAPK, and the AP-1 pathway, in association with the pathogeneses of RA and ankylosing spondylitis (AS).[201]

For osteoblast differentiation, TNF-α plays an inhibitory role via Osx, ALP, and Runx2 by up-regulating Smurf1.[202] Its dual function depends on the concentration of TNF-α, with low concentrations leading to osteogenic differentiation of MSCs,[203] but a low-dose stimulus may inhibit the development of bone in young mice.[204] Additionally, JNK signaling was found to be strongly expressed in the MAPK pathway, both increasing ALP activity and decreasing Runx2 and Smads.[205–206] For osteoclast differentiation, TNF-α indirectly stimulates osteoclastic bone resorption;[207] additionally, TNF-α can synergize with RANKL and M-SCF or directly target osteoclast precursors for the further activation of NF-κB and AP-1 signaling to induce osteoclastogenesis.[201,208] However, pre-osteoclast and osteoclast precursors can induce apoptosis by the interaction of TNF-induced Fas with IFN, thus inhibiting osteoclastogenesis.[208–209]

Knockdown of miR-155 directly increases target SOCS1 expression, which is a negative regulator of TNF-α and JNK signaling, as well as limiting NF-κB signaling. Down-regulation of JNK led to the inhibition of osteogenesis in MC3T3-E1 cells, as well as the apoptosis of TNF-α-related cells.[210] At the same time, by targeting SOCS1 and MITF, miR-155 can suppress the function of IFN-β in osteoclast differentiation, providing new insight into the dimorphic role of one miRNA balanced in both osteogenesis and osteoclastogenesis.[211] In addition to miR-155, miR-21, miR-29b, miR-146, and miR-210 have all been found to be involved in the osteoclast differentiation of TNF-α-regulated RAW264.7 cells.[212] miR-21 has also been found to be down-regulated in estrogen-deficiency-induced osteoporosis samples, and it has been verified to be suppressed by TNF-α; furthermore, miR-21 can promote hMSC osteogenesis by targeting Spry1.[213] The knockdown of miR-23a significantly enhanced the apoptosis of TNF-α-induced osteoblast precursors by reversing the inhibition of target Fas.[214]

Notch

Notch is a receptor that exists in highly conserved cells and is always triggered by direct contact among neighbor cells that expose the Notch ligands (Delta-like1, 3, 4, Jagged1, Jagged2). After silencing, the NICD/CSL complex forms and then activates Hes/Hey/Herp. In addition to numerous functions in nerve/cardiovascular/endocrine development, Notch has also emerged as an important regulator of multiple stages of skeletogenesis.[215] It appears to inhibit the terminal osteoblastic differentiation of mesenchymal precursors and bone formation by dimin-

ishing Runx2 activity via Hes/Hey.[216–217] However, Notch is also required for proper regulation of osteoclast differentiation. The Delta1 ligand acts as an inhibitor of CSF-1R, and physiologic Notch signaling, enabled by presenilin-1/2, balances osteoblast-dependent osteoclastogenesis via the regulation of OPG.[217–218] The Notch signaling-related miRNAs focus on the miR-34 family. miR-34a/b/c has been previously shown to inhibit osteoblast differentiation by targeting multiple components of the Notch signaling pathway, such as Notch1/2, Jagged1, and SATB2.[52–53] However, how miR-34 regulates osteoclastogenesis is non-cell-autonomous, involving an osteoblast–osteoclast coupling mechanism, whereby miR-34c transgenic osteoblasts increase the production and proportion of RANKL/OPG and induce osteoclast differentiation.[219] Simultaneously, evidence has revealed that miR-34a is a novel and critical blocker of transforming growth factor-B-induced factor 2 (Tgif2), which results in suppressed bone resorption.[220] Therefore, different members of the same miRNA family may play distinct roles in osteoporosis and other bone-related diseases.

miRNAs IN BIOMATERIALS AND THERAPEUTICS

Based on the increasing body of research on miRNAs, the concepts regarding biomaterials for the repair of bone defects and the restoration of bone functions have expanded. Previous research focused on the different materials and constructions that induce different miRNAs in the bone formation process, such as inorganic silicate-based PerioGlas, inorganic bovine bone, and organic collagen materials that act to alter osteoblast activity due to the exposure of different cell-binding domains and miRNA translations.[221–222]

Conversely, the use of modified materials to deliver a specific miRNA into a body makes precise regulation achievable. Extensively applied in bone formation, a polymeric scaffold has good biocompatibility and controlled biodegradability. Recently, poly(glycerol sebacate) and porous β-TCP scaffolds seeded with anti-miR-31-modified BMSCs showed significant capacity to repair critical-sized defects (8 mm diameter in the cranium and 10 mm diameter in the canine medial orbital wall).[54,223] In addition, another microgrooved poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) seeded with osteogenic miRNAs triggered the expression of multiple osteogenic markers.[224] To increase the efficiency of miRNA transfection, researchers have used a microporous titanium oxide surface functionalized with miR-29b/anti-miR-138 or 2000-miR-148b/anti-miR-138 lipoplex reverse transfection to enhance osteogenic activity.[225–226]

In addition to transfection, direct delivery is also convenient. The miR-29b mimic combines with a synthetic cell-penetrating peptide, forming a nanoparticle complex and promoting osteogenesis by directly down-regulating osteogenic inhibitors.[227] Another study used silver nanoparticles with localized surface plasmon fields with increased sensory ability, and the miR-148b mimic is linked to such nanoparticles through photocleavable linkers, resulting in PC-miR-148-SNP conjugation in the modulation of osteogenesis in hASCs.[228] The miR-148 family is utilized again in a novel delivery system, but eight repeating sequences of aspartate (D-Asp8)-liposome-antagomir-148a have been created to target osteoclasts specifically to attenuate bone resorption. Therefore, it may be regarded as one promising approach for the treatment of osteoclast-dysfunction-induced skeletal diseases.[229]

CONCLUSIONS AND FUTURE PROSPECTS

In general, bone remodeling is controlled by the balance between bone formation and bone resorption. However, remodeling is a complicated process involving numerous signals and pathway modifications. This review summarized the current knowledge regarding miRNAs and their biological functions related to some specific regulatory molecules in regulating multiple stages of bone remodeling. Numerous *in vitro* and *in vivo* studies have been undertaken to demonstrate the importance and complexity of post-transcriptional regulation by miRNAs, and these studies have provided the foundation for potential therapy. However, further efforts should be undertaken to investigate the transduction and communication pathways between miRNAs and intracellular signal, as well as the communication of different miRNAs in different pathways of both osteogenesis and osteoclastogenesis, the direct and indirect targets of different miRNAs, and even some of the less desirable effects, such as stress loading, oxygen pressure, and microenvironment changes, on miRNA functions in bone remodeling. Therefore, to realize the ultimate goal of maintaining balance in bone remodeling, increased focus should be placed on treatments that cause specific miRNAs to function efficiently in specific locations and during certain stages of bone diseases.

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