MicroRNAs regulate signaling pathways in osteogenic differentiation of mesenchymal stem cells (Review)

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Abstract. Osteogenesis is a complex multi-step process involving the differentiation of mesenchymal stem cells (MSCs) into osteoblast progenitor cells, preosteoblasts, osteoblasts and osteocytes, and the crosstalk between multiple cell types for the formation and remodeling of bone. The signaling regulatory networks during osteogenesis include various components, including growth factors, transcription factors, micro (mi)RNAs and effectors, a number of which form feedback loops controlling the balance of osteogenic differentiation by positive or negative regulation. miRNAs have been found to be important regulators of osteogenic signaling pathways in multiple aspects and multiple signaling pathways. The present review focusses on the progress in elucidating the role of miRNA in the osteogenesis signaling networks of MSCs as a substitute for bone implantation the the field of bone tissue engineering. In particular, the review classifies which miRNAs promote or suppress the osteogenic process, and summarizes which signaling pathway these miRNAs are involved in. Improvements in knowledge of the characteristics of miRNAs in osteogenesis provide an important step for their application in translational investigations of bone tissue engineering and bone disease.

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Abbreviations: OCN, osteocalcin; OPN, osteopotin; ALP, alkaline phosphatase; hBMSCs, human bone marrow mesenchymal stem cells; SCAPs, apical papilla stem cells; BMP2, bone morphogenetic protein 2; hADMSCs, human adipose tissue-derived mesenchymal stem cells; hADSCs, human adipose-derived stem cells; hAT-MSC, human adipose tissue-derived MSC; PDLSCs, periodontal ligament stem cells; OSX, osteoblast-specific transcription factor; FABP4, fatty acid binding protein 4; KLF4, kruppel-like factor 4; TNFα, tumor necrosis factor α; HDAC6, histone deacetylase 6; APC, adenomatous polyposis coli; TCF3, transcription factor 3; SIRT1 silent, information regulator 1

Key words: osteogenic differentiation, microRNAs, mesenchymal stem cells, signaling

Contents

1. Introduction

MicroRNAs (miRNAs) are an extensive family of small (18-24 nucleotides), single-stranded non-coding RNAs, which regulate gene expression in mammalian cells through binding to the seed sequences of the 3'-untranslated region (UTR) of target mRNA sequences and mediate the degradation of mRNA in the RNA-induced silencing complex (1,2). miRNAs repress translation by decreasing stability through targeting specific mRNA targets. Each miRNA regulates numerous mRNAs, and miRNAs are involved in various cellular processes, including proliferation, differentiation, cell cycle, invasion and apoptosis (3-6). The alterations in their expression levels may lead to human diseases, including cardiovascular disease and cancer (7-10). The formation of bone by osteoblast cells and their primary functional activities involve a series of multiple signals, including bone morphogenic protein (BMP), Wnt ligands, Notch ligands, hormones and growth factors, including transforming growth factor (TGF) and tumor necrosis factor (TNF) and cytokines. In addition to these factors, tissue-specific transcription factors and co-factors mediate the expression of genes for the biosynthesis and mineralization of bone matrix, and the remodeling and formation of bone (11-15). Mesenchymal stem cells (MSCs) are induced to differentiate into preosteoblasts, which are regulated by the

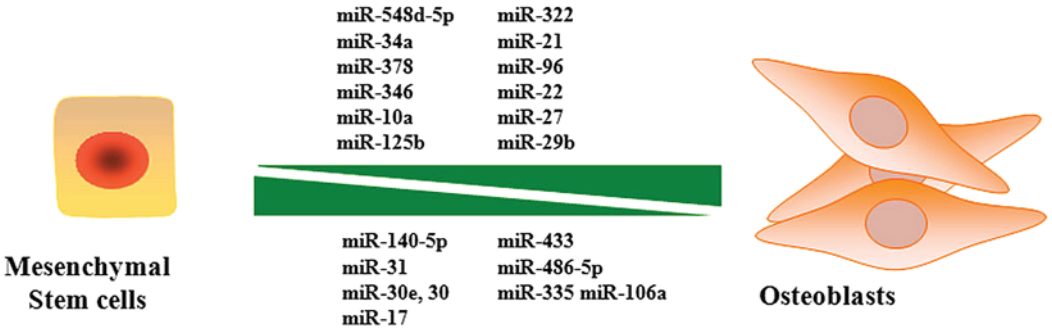

Figure 1. miRNAs control the osteogenic differentiation of mesenchymal stem cells into osteoblasts. The microRNA above the green box indicates that they promote the osteogenic differentiation of mesenchymal stem cells into osteoblasts; the microRNAs listed below the green box indicates that they inhibit the osteogenic differentiation of mesenchymal stem cell into osteoblasts. miRNA/miR, microRNA.

signaling cascades initiated by the various signals, including cytokine stimuli. Pre-osteoblasts differentiate into osteoblasts, which is controlled by gene expression affected by chromatin modifiers, transcription factors or miRNA alterations (16). Osteoblasts produce bone matrix, which mineralizes into bone tissue, and osteoblasts are engulfed in the matrix during bone formation. The entire process is complex and well organized by the signaling network.

Experimental evidence shows that miRNAs are critical for stem cell activities, particularly in the stemness maintenance or direct differentiation into lineage cells (7,8). To date, several studies have examined the functions of miRNAs in MSCs (17,18). Certain miRNAs have been shown to regulate the process of differentiation of MSCs into different cell lineages using high-through gene expression microprofiling assays. This has led to the specific targets of these miRNAs being determined, and the roles of miRNAs are gradually being elucidated (Fig. 1).

2. miRNAs promote the osteogenic differentiation of mesenchymal stem cells

2.1. BMP signaling. BMP-induced osteogenic differentiation is well known (19,20). Hupkes M *et al* reported that the expression of miRNA regulated the terminal differentiation of C2C12 myoblasts via lineage-specific changes. It was found that the overexpression of miR-378 enhances alkaline phosphatase (ALP) activity, calcium deposition and the mRNA expression levels of osteogenic marker genes in the presence of BMP2 (21). miR-322, a BMP-2-downregulated miRNA, is a regulator of osteoblast differentiation. Using gain-and loss-of-function experiments of miR-322 in the C2C12 mouse myoblast cell line, MC3T3-E1 mouse embryonic osteoblasts and primary murine bone marrow-derived MSCs, the overexpression of miR-322 has been demonstrated to enhance the BMP-2 response, increasing the expression of osteoblast-specific transcription factor (Osx) and other osteogenic genes. Transducer of erbB2 (Tob2) has been identified as a target of miR-322, as reporter assays revealed that miR-322 binds to the specific sequence in the 3'-UTR of Tob2. Tob2 is a negative regulator of osteogenesis, which binds to and mediates the degradation of Osx mRNA. This demonstrates a novel molecular mechanism controlling osteogenesis through the specific miR-322/Tob2 regulation of specific target mRNAs (22). miR-196a is involved in the proliferation and osteogenic differentiation of human adipose-derived stem cells (hADSCs). The overexpression of miR-196a inhibits hASC proliferation and enhances osteogenic differentiation by targeting the 3'UTR sequence of homeobox (HOX)C8 mRNA. The expression of Hoxc8 in C2C12 cells decreases the alkaline phosphatase activity induced by BMP-2. The expression of HOXC8 is decreased during the osteogenic differentiation of hADSCs, and concomitant with an increase in the level of miR-196a. Thus, inhibition of miR-196a decreases the protein levels of HOXC8 in hADSCs, and is accompanied by increased osteogenic differentiation. This indicates that miR-196a has a positive effect in osteogenic differentiation in hADSCs by repressing HOXC8 (23).

All the miRNAs promoting osteogenic differentiation are listed in Fig. 2 and Table I.

2.2. Wnt/β-catenin signaling. Wnt/β-catenin signaling has been well defined in the osteogenic differentiation of MSCs (24-27). miR-346 promotes the osteogenic differentiation of human bone marrow MSCs (hBMSCs) by targeting glycogen synthase kinase-3β (GSK-3β) through binding to the 3'-UTR of its mRNA. The decreased GSK-3β results in an increase of β-catenin, which is translocated into the nucleus and activates the downstream genes of the Wnt/β-catenin pathway. β-catenin knockdown almost completely inhibits the positive effect of miR-346 on osteogenic differentiation. Therefore, miR-346 positively regulates the osteogenic differentiation of hBMSCs through the Wnt/β-catenin pathway (28-31). The expression of miR-27 is increased during hFOB1.19 cell (human SV40 transfected osteoblast) differentiation. The ectopic expression of miR-27 promotes the differentiation of hFOB1.19 cells by directly targeting and inhibiting the gene expression of adenomatous polyposis coli (APC). This inhibition of the expression of APC leads to the accumulation of β-catenin, which is a key protein activating Wnt signaling. This suggests that miR-27 is an important promoter of osteogenic differentiation (32). miR-218 positively regulates the osteogenesis of hADSC, directly targeting secreted frizzled-related protein 2 and dickkopf WNT signaling pathway inhibitor 2, thus enhancing Wnt/β-catenin signaling progression. Mimics of the Wnt/β-catenin signal increase the expression of miR-218, which forms a positive feedback loop to promote osteogenesis (33).

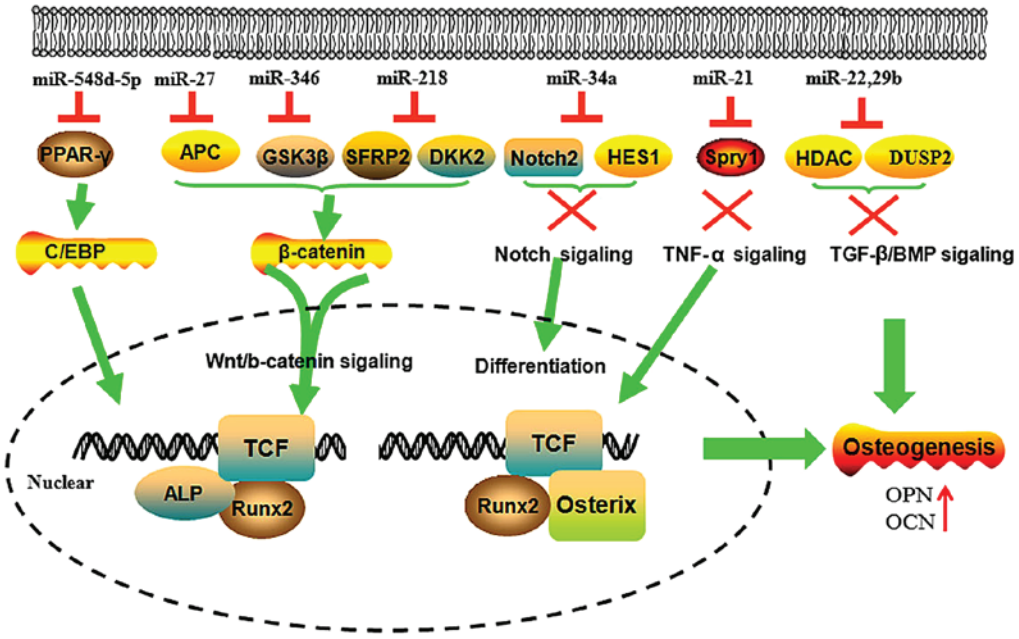

Figure 2. MiR-548, miR-27, miR-346, miR-218, miR-34a, miR-21, miR-22 and miR-29b promote the osteogenic differentiation of mesenchymal stem cells through Wnt/catenin, Notch, TNF-α, TGF-β/BMP signaling pathways. miR, microRNA; PPAR-γ, peroxisome proliferator-activated receptor-γ; GSK-3β, glycogen synthase kinase-3β; Spry1, sprouty homolog 1; HDAC, histone deacetylase; DUSP2, dual specificity phosphatase 2; APC, adenomatous polyposis coli; SFRP2, secreted frizzled-related protein 2; DKK2, dickkopf WNT signaling pathway inhibitor 2; TCF, transcription factor; Runx2, runt-related transcription factor 2; OPN, osteopontin; OCN, osteocalcin; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein.

2.3. Notch signaling. Notch Signaling is important in maintaining the undifferentiated state of MSCs (34-38). The activation of Notch signaling in apical papilla stem cells (SCAPs) inhibits cell differentiation. High expression levels of miR-34a inhibits Notch signaling by directly targeting the 3'UTR of Notch2 and HES1 mRNA then suppressing the binding of Notch2, and HES1, then the inhibition of Notch signaling pathway, resulting in the expression of runt-related transcription factor 2 (RUNX2) and OSX, and enhancing the expression of osteocalcin (OCN). miR-34a promotes osteogenesis in SCAPs through Notch signaling by targeting Notch 2 and HES1 (39).

The upregulation of miR-10a results in increased osteogenic differentiation. miR-10a mimics significantly repress luciferase activity by the direct binding to the 3'-UTR of Kruppel-like factor 4 (KLF4). The inhibition of KLF4 in hBMSCs increases cell differentiation. Therefore, miR-10a enhances the differentiation capability of hBMSCs through the repression of KLF4 (40).

2.4. TNF-α signaling. MiR-21 has been confirmed to promote the osteogenic differentiation of mouse bone marrow cells by targeting Sprouty homolog 1 (Spry1), negatively regulating the osteogenic differentiation of MSCs. The expression of miR-21 partially rescues TNF-α-impaired osteogenesis of MSCs. Inhibition of the TNF-α signaling pathway evidently improves bone formation and down-regulates Spry1 expression, which suggests that miR-21 contributes towards bone formation through targeting Spry1 in MSCs (41).

miR-96 and miR-199a were both up-regulated during osteogenic induction of human bone marrow derived MSCs. They may function through transcription factor SRY-box 9, and fatty acid binding protein 4, however, the detailed molecular mechanism remains poorly understood (42).

The expression of miR-22 is increased during the process of osteogenic differentiation of hADSCs. miR-22 inhibits the expression of histone deacetylase 6 (HDAC6) by binding to the similar sequence of the 3'-UTR (43). HDAC6 deficiency results in a minor increase in trabecular bone density. Thus, miR-22 increases osteogenesis through targeting HDAC6.

miR-29b is understood to promote osteogenesis by supressing the negative regulators, including histone deacetylase 4, TGF-β3, activin A receptor (Type IIA), catenin beta interacting protein 1 and dual specificity phosphatase 2, during the induction of osteogenic differentiation. These anti-osteogenic factors negatively modulate extracellular matrix excretion by differentiated osteoblasts and bone formation (44,45).

3. miRNAs suppress the osteogenesis of mesenchymal stem cells

Several miRNAs directly target transcription factors, including osteopotin (OPN), OCN and RUNX2, or signaling molecules, which promote the osteogenesis of MSCs and inhibit osteogenic differentiation (Fig. 3 and Table I). miR-140-5p inhibits the osteogenic differentiation in hMSCs by directly blocking BMP2, and consequently blocking BMP signaling components and critical regulators (46).

The overexpression of miR-31 represses the osteogenesis of hMSCs by directly targeting special AT-rich sequence-binding protein 2 (SATB2), the knockdown of SATB2 by specific siRNA against SATB2 inhibits osteogenic differentiation (47). Baglio *et al* found an inverse trend in miRNA-target expression during osteogenic differentiation between the levels of miR-31 and OSX. The inhibition of miR-31 leads to an increase in the endogenous expression of OSX (48). Deng *et al* showed that

Table I. miRNAs regulate osteogenesis during the transition of mesenchymal stem cells into osteoblasts.

Function	miRNA	Target gene	Cell	Author	Refs
Promotor	miR-34a	NOTCH2 and HES1	human SCAPs	Sun *et al* (2014)	(37)
	miR-378	None validated	C2C12, myoblasts	Hupkes *et al* (2014)	(21)
	miR-346	GSK-3β	hBMSCs	Wang *et al* (2013)	(26)
				Westendorf *et al* (2004)	(27)
				Logan *et al* (2004)	(28)
				Gaur *et al* (2005)	(29)
	miR-10a	KLF4	hMSCs	Li *et al* (2013)	(38)
	miR-322	Tob2	mBMSCs	Gamez *et al* (2013)	(39)
	miR-21	Spry1	MSCs	Yang *et al* (2013)	(40)
	miR-96	SOX9, aggrecan and FABP4	hMSCs	Laine *et al* (2012)	(41)
	miR-22	HDAC6	hADMSCs	Huang *et al* (2012)	(42)
	miR-27	APC	hFOB1.19 cells	Wang and Xu (2010)	(30)
	miR-218	SFRP2 and DKK2	hASCs	Zhang *et al* (2014)	(31)
	miR-29b	COL1A1, COL5A3 and COL4A2	osteoblasts	Huang *et al* (2012)	(42)
				Crane and Cao (2014)	(44)
	miR-196a	HOXC8	hASCs	Kim *et al* (2009)	(45)
	miR-140-5p	BMP2	hMSCs	Hwang *et al* (2014)	(46)
Suppressor	miR-31	SATB2 and OSX	hMSCs, BMSCs	Xie *et al* (2014)	(47)
				Baglio *et al* (2013)	(48)
				Deng *et al* (2013)	(49)
	miR-30e	TCF	C3H10T1/2 pre-adipocyte 3T3-L1	Wang *et al* (2013)	(50)
	miR-30	Smad1 and Runx2		Wu *et al* (2012)	(51)
	miR-17	TCF3 and Smurf1	PDLSCs	Liu *et al* (2011)	(52)
	miR-17-5p, miR-106a	BMP2	hADSCs	Li *et al* (2013)	(53)
	miR-433	Runx2	C3H10T1/2	Kim *et al* (2013)	(54)
	miR-486-5p	SIRT1	hAT-MSCs	Kim *et al* (2012)	(55)
	miR-335	RUNX2	hMSCs	Tome *et al* (2011)	(56)
	miR-135b	IBSP and OSX	USSCs	Schaap-Oziemlak *et al* (2010)	(57)

miR/miRNA, microRNA; MSCs, mesenchymal stem cells; hBMSCs, human bone marrow MSCs; SCAPs, apical papilla stem cells; hADMSCs, human adipose tissue-derived MSCs; hADSCs, human adipose-derived stem cells; hAT-MSCs, human adipose tissue-derived MSCs; PDLSCs, periodontal ligament stem cells; USSCs, unrestricted somatic stem cells; GSK-3β, glycogen synthase kinase-3β; KLF4, kruppel-like factor 4; Tob2, transducer of erbB2; Spry1, sprouty homolog 1; FABP4, fatty acid binding protein 4; SOX9, SRY-box 9; HDAC6, histone deacetylase 6; APC, adenomatous polyposis coli; SFRP2, secreted frizzled-related protein 2; DKK2, dickkopf WNT signaling pathway inhibitor 2; COL, collagen; HOX, homeobox; BMP2, bone morphogenetic protein 2; SATB2, special AT-rich sequence-binding protein 2; TCF, transcription factor; Smad1, small mothers against decapentaplegic 1; Runx2, runt-related transcription factor 2; Smurf1, Smad ubiquitin regulatory factor 1; SIRT1, silent information regulator 1; IBSP, integrin-binding sialoprotein; OSX, osteoblast-specific transcription factor.

the expression of miR-31 decreased progressively in BMSC cultures during differentiation. The upregulation of miR-31 significantly reduces the expression levels of osteogenic transcription factors, OPN, bone sialoprotein, OSX and OCN. The inhibition of miR-31 markedly decreases the activity of ALP and inhibits osteogenesis. These results suggest that an miR-31/SATB2 axis is involved in the osteogenic differentiation of BMSCs (49).

miR-30 family members are also important regulators during the biomineralization process. The overexpression of miR-30e stimulates adipocyte formation and inhibits osteoblast differentiation from marrow stromal cells. Low-density lipoprotein receptor-related protein 6 (LRP6) is one of the critical co-receptors for Wnts. Blocking LRP6 in 3T3-L1 cells downregulates β-catenin/T-cell factor transcriptional activity and enhances osteogenic differentiation. miR-30 has been reported to target the LRP6 directly and inhibit the expression of LRP6 expression. Thus, miR-30e also controls osteogenesis in periodontal ligament stem cell (PDLSCs) by targeting LRP6 and affecting canonical Wnt/β-catenin signaling (50). There is also evidence that miR-30 family members negatively regulate BMP-2-induced osteoblast differentiation by targeting Small

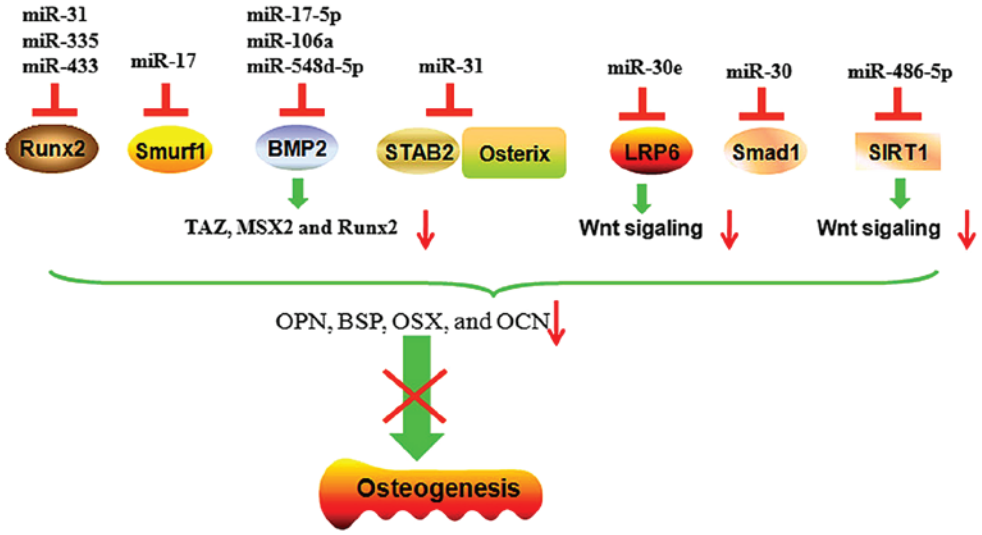

Figure 3. MicroRNAs suppress the osteogenic differentiation of mesenchymal stem cells through targeting transcription factors RUNX2, Smuf1, Osterix or BMP protein. miR, microRNA; Runx-2, runt-related transcription factor 2; BMP, bone morphogenetic protein; STAB2, stabilin 2; LRP6, lipoprotein receptor-related protein 6; Smad1, small mothers against decapentaplegic 1; SIRT1, silent information regulator 1; Smurf1, Smad ubiquitin regulatory factor 1; TAZ, transcriptional coactivator with PDZ-binding motif; MSX2, Msh homeobox 2; OPN, osteopontin; BSP, bone sialoprotein; OSX, osteoblast-specific transcription factor; OCN, osteocalcin.

mothers against decapentaplegic (Smad)1 and RUNX2. As Smad1 and RUNX2 are key positive transcription factors in osteogenic differentiation, miR-30 family members negatively regulate the osteogenic differentiation through Smad1 and RUNX2 (51).

Liu *et al* (52) elucidated that transcription factor 3 (TCF3) enhances osteogenesis in PDLSCs. miR-17 can inhibit this effect by suppressing the target gene, TCF3. Furthermore, the overexpression of TCF3 blocks the effect of miR-17 on the modulation of Wnt signaling (52). Inflammation promotes the downregulation of miR-17 and the subsequent increased expression of Smad ubiquitin regulatory factor 1 (Smurf1), which is an important negative regulator of osteogenic differentiation in MSCs by promoting the degradation of Smurf1-mediated osteoblast-specific factors. Smurf1 has been confirmed as a direct target of miR-17 in PDLSCs using a luciferase reporter assay (52). Li H *et al* revealed that miR-17-5p and miR-106a have dual functions in hADSC fate, which can promote adipogenesis and inhibit osteogenesis. The inhibitory effects of miR-17-5p and miR-106a are due to directly targeting BMP2, and subsequently decreased osteogenic TAZ, MSX2 and RUNX2 protein levels (53).

During osteoblastic differentiation, the overexpression of Estrogen-related receptor γ (ERRγ) or miR-433 inhibits the expression levels of osteogenic marker genes including RUNX2 and ALP. miR-433 directly targets three binding sites on the 3'-UTR of RUNX2 mRNA, and decreases the levels of the RUNX2 transcript in C3H10T1/2 cells. Anti-miR-433 recovers the ERRγ-suppressed expression of RUNX2 and activity of ALP. ERRγ is able to upregulate the levels of miR-433 expression and further enhance the inhibitory role in osteogenic differentiation. This evidence demonstrates that miR-433 suppresses BMP2-induced osteogenic differentiation by targeting RUNX2 C3H10T1/2 cells (54).

The overexpression of miR-486-5p inhibits osteogenic differentiation of hADSCs. miR-486-5p regulates the expression of silent information regulator 1 (SIRT1), a major regulator of longevity and metabolic disorders. SIRT1 also has an important role in the osteogenic process by targeting FOXO3A and then upregulating the levels of RUNX2 promoter activity. miR-486-5p inhibits the expression of SIRT1 through binding to the 3'-UTR region of SIRT1 mRNA (55). The overexpression of miR-335 in hMSCs derived from bone marrow, adipose tissue and articular cartilage inhibits their osteogenic and adipogenic potential. The expression of miR-335 in hMSCs is upregulated by the activated canonical Wnt signaling pathway. It has also been confirmed that RUNX2 is a direct target of miR-335. These results suggest that the downregulation of miR-335 is critical for the acquisition of MSC phenotype differentiation into osteoblasts (56). The overexpression of miR-135b downregulates the osteogenic differentiation of unrestricted somatic stem cells through targeting the key osteogenic factors, integrin-binding sialoprotein and OSX, inhibiting the process of osteogenesis (57).

4. Conclusion

There is substantial evidence and experimental data confirming that miRNAs have multi-dimensional roles in the induction of MSCs into osteoblasts. miRNAs function at all stages of osteoblast differentiation by inhibiting the negative regulators of signaling pathways operating in these cells (16). They also have direct and indirect effects on phenotype development through the promotion or inhibition of positive or negative transcription factors in signaling pathways, which are involved in complex regulatory networks. This indicates an important technique, by which the network of osteogenic differentiation can be regulated through miRNAs. Nicotine can alter the expression of miRNA and reduce human adult stem cell regenerative potential, demonstrating that miRNAs function in the network (58,59).

Currently, only a limited number of miRNAs, identified through *in vitro* expression profiling of bone cells undergoing

differentiation programs, have been characterized for their functional activity and known targets. Numerous miRNAs are associated with bone development, which can be utilized in new bone formation and the therapy of osteoporosis or bone malignant diseases. However, the crosstalk of multiple types of molecules in the regulatory network of the osteogenic differentiation process is not well-defined. LncRNA and circular RNAs which are emerging as novel regulators in the cellular processes, including osteogenesis, require further investigation (60).

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