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REVIEW ARTICLE

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Interplay between microRNAs and Wnt, transforming growth factor-β, and bone morphogenic protein signaling pathways promote osteoblastic differentiation of mesenchymal stem cells

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Abstract

Osteoblasts are terminally differentiated cells with mesenchymal origins, known to possess pivotal roles in sustaining bone microstructure and homeostasis. These cells are implicated in the pathophysiology of various bone disorders, especially osteoporosis. Over the last few decades, strategies to impede bone resorption, principally by bisphosphonates, have been mainstay of treatment of osteoporosis; however, in recent years more attention has been drawn on bone-forming approaches for managing osteoporosis. MicroRNAs (miRNAs) are a broad category of noncoding short sequence RNA fragments that posttranscriptionally regulate the expression of diverse functional and structural genes in a negative manner. An accumulating body of evidence signifies that miRNAs direct mesenchymal stem cells toward osteoblast differentiation and bone formation through bone morphogenic protein, transforming growth factor-β, and Wnt signaling pathways. MiRNAs are regarded as excellent future therapeutic candidates because of their small size and ease of delivery into the cells. Considering their novel therapeutic significance, this review discusses the main miRNAs contributing to the anabolic aspects of bone formation and illustrates their interactions with corresponding signaling pathways involved in osteoblastic differentiation.

KEYWORDS
mesenchymal stem cells, microRNAs, osteoblast, osteoblastogenesis, signaling pathways

1 | INTRODUCTION

Mesenchymal stem cells (MSCs) are nonhematopoietic multipotent cells, obtained from several sources including adipose tissue, amniotic fluid, bone morrow, dental pulp, fetal liver, lung, peripheral blood, placenta, skin, tonsils, and umbilical cords. These cells could be cultured in vitro, and depending on the conditions of culture media can differentiate into multiple cell linages such as adipocytes, cardiomyocytes, chondrocytes, dermal-like fibroblasts, hepatocytes, insulin-producing cells, myoblasts, neurons, osteoblasts, Schwann cell-like cells, and vascular endothelial cells (Davies, Cooper, Shelton, Smith, & Scheven, 2015; de Kroon et al., 2017; El-Kehdy et al., 2016; Ingenito et al., 2012; M. Pan et al., 2017; Park et al., 2016; Pop et al., 2015; Sivan, Jayakumar, & Krishnan, 2016; Tancharoen et al., 2017; Teng, Lo, Liu, Hsuan, & Lin 2017; Yu, Zou, Fan, Li, & Ma, 2016). Osteoblasts are bone-forming cells that synthesize and secrete bone matrix proteins (Blair et al., 2017). The potential of MSCs to differentiate into osteoblasts places them in a key position in terms

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of regeneration and repair of bone tissues (Sui et al., 2016; Ye et al 2014). Differentiation of MSCs are carefully controlled by multiple factors including transcription factors (Runx2), signaling pathways (wingless-type [Wnt]), and also microRNAs (miRs). MiRs are short (22 nucleotides) noncoding RNAs, acting as posttranscriptional regulators. Primary transcripts of miRs (pri-miR) are first produced by RNA polymerase II, and then are processed by Drosha to form precursor miR (pre-miR); pre-miRs are transported to cytoplasm and after cleavage by Dicer, are converted into mature miRs. MiRs interact with a particular sequence in the 3′-untranslated region (3′-UTR) of specific target messenger RNA (mRNAs). This interaction may target mRNA to be degraded or otherwise may inhibit the translation of the specific mRNA (C. Huang, Geng, & Jiang, 2017). The importance of miRs in the regulation of osteoblastic differentiation was noticed when deletion of Drosha or Dicer enzymes resulted in the inhibition of this process (Oskowitz et al., 2008). Some miRs such as miR-145, miR-10a, and miR-100 that target core-binding factor subunit β (CBFB), β-catenin, and small mothers against decapentaplegic 1 (SMAD1) have negative impact on osteoblast differentiation (Fu et al., 2016; Fukuda et al., 2015; Li, Zhang, Zhao, Wang, & He, 2015). Others like miR-142-3p, miR-335-5p, and miR-181a improve osteoblast differentiation by downregulating the osteoblastogenesis negative factors like adenomatous polyposis coli (APC), Dickkopf-related protein 1 (DKK1), and transforming growth factor receptor 1 (TGFBR1; Bhushan et al., 2013; W. Hu et al., 2013; J. Zhang, Tu, et al., 2011). Furthermore, transfecting miRs-21, -29a, and -199a into MSCs by nanoparticles efficiently stimulates osteoblast differentiation (Chen et al., 2015; T. Pan et al., 2016; Z. Wang et al., 2015), suggesting the potential therapeutic values of miRs in the management of chronic bone disorders like osteoporosis. In the present review, we aim to discuss the most relevant miRs capable of promoting osteoblast differentiation from a mechanistic viewpoint.

2 | Wnt SIGNALING PATHWAY

Wingless-type (Wnt) family is formed of 19 secreted glycoproteins that involve in various biological processes such as regulation of MSCs fate and includes two distinct canonical (β-catenin dependent) and non-canonical branches (Chang et al., 2007). The effects of Wnt signaling pathway on osteoblastogenesis was shown via overexpression and knockdown of Wnt pathway ligands and components (Chang et al., 2007; Friedman, Oyserman, & Hankenson, 2009; Kugimiya et al., 2007; Kulkarni et al., 2006; Miclea et al., 2009). Wnt5a activated noncanonical Wnt signaling in hASCs and induced osteoblast differentiation by rho-associated kinase upregulation (Santos, Bakker, de Blieck-Hogervorst, & Klein-Nulend, 2010). Wnt4 like Wnt5a initiates noncanonical Wnt signaling and positively affects osteoblastic differentiation of MSCs both in vivo and in vitro (Chang et al., 2007). Wnt6, Wnt10a, and Wnt10b act as MSCs fate regulators; these proteins stimulate differentiation of bone-marrow-derived stromal cells (ST2 cells) to osteoblast in a β-catenin-dependent manner (Cawthorn et al., 2012). Overexpression of Wnt10b is associated with bone mass raise in transgenic mice, which is the result of osteoblast differentiation amplification (Bennett et al., 2007). Wnt11 is upregulated during osteoblastic differentiation; overexpression of Wnt11 in MC3T3E1 cells is associated with the enhancement of osteoblastic maturation and mineralization; in Wnt$^+$ cells, cytoplasmic and nuclear levels of β-catenin are elevated (Friedman et al., 2009). Wnt16 is the other member of this family that inhibits osteoblastic differentiation of MC3T3-E1 cells via Wnt/β-catenin signaling pathway activation (Jiang, Von den Hoff, Torensma, Meng, & Bian, 2014). Wnt3a encourages the proliferation of human MSCs and simultaneously suppresses their osteoblast differentiation that are demonstrated by reduced expression and activities of alkaline phosphatase (ALP; Boland, Perkins, Hall, & Tuan, 2004). β-Catenin plays significant roles both as the effector of canonical Wnt signaling pathway and in cell adhesion independently (Cawthorn et al., 2012). The level of β-catenin is regulated by destruction complex. This complex is composed of Axin as a scaffold, APC and two serine-threonine kinases: casein kinase 1α (CK1α) and glycogen synthase kinase 3β (GSK3β). When Wnt signaling pathway is off, destruction complex phosphorylates β-catenin, first by CK1α and then GSK3β; therefore, phosphorylated β-catenin is instantly ubiquitinated and degraded by the proteasome. By binding Wnt ligand to heterodimer receptor complex in cell membrane that is composed of frizzled (Frz) receptor and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) coreceptors Wnt/β-catenin pathway (canonical pathway) is initiated. In this condition, intracellular domain of LRP5 after phosphorylation recruits Axin and also Disheveled (DVL) that bind collectively to the intracellular part of Frz and accordingly help to the interaction of Axin and LRP5 (Nusse & Clevers, 2017). The resultant complex consists of Wnt ligand, Frz, and LRP5/6; this complex promotes the phosphorylation of GSK3β phosphorylation and its inactivation (Yavropoulou & Yovos, 2007). Consequently, with reduced phosphorylation and destruction of β-catenin, prestored cytoplasmic β-catenins translocate into the nucleus and bind to their related transcription factors, that is, T-cell factor (TCF) and lymphoid enhancer factor (LEF). These transcription factors initiate the transcription of target genes by organizing the DNA binding complex (Yavropoulou & Yovos, 2007). TCF, the transcription factor of the canonical Wnt pathway with a binding site on runt-related transcription factor 2 (Runx2) promoter region; and therefore is able to enhance Runx2 expression (Gaur et al., 2005). Runx2 is a renowned regulator of osteoblast differentiation, activating the expression of principal genes involved in osteoblastic differentiation like osteocalcin (Osc), ALP, type 1 collagen, osteopontin, and bone sialoprotein (Bsp; Vimalraj, Arumugam, Miranda, & Selvamurugan, 2015).

3 | miRs ENHANCING OSTEOBLAST DIFFERENTIATION THROUGH Wnt SIGNALING PATHWAY

3.1 | miR-142-3p

MiR-142-3p has been shown to improve osteoblastic differentiation in human fetal osteoblasts 1.19 cells (hFOB1.19) by targeting APC (W. Hu et al., 2013). The major role of APC by modulating β-catenin

levels has been shown in the regulation of skeletal precursor cells. Moreover, homozygous transgenic mice depleted of functional APC have demonstrated increased perinatal lethality with disturbed osteogenic and chondrogenic differentiation (Miclea et al., 2009). Western blot analysis of cells transfected with miR-142-3p has revealed high nuclear levels of β-catenin and therefore it is postulated that miR-143-3P could be a potential repressor of APC (W. Hu et al., 2013).

Contrarily, Hu et al. have reported that miR-142-3p suppresses Wnt signaling pathway in human embryonic kidney cells 293 (HEK293) by targeting CTNNB1 mRNA posttranscriptionally; CTNNB1 is a gene that codes for β-catenin. Thus the contradictory effects of miR-142-3p on Wnt signaling pathway could possibly be related to the different target mRNAs in different cells and tissues (T. Hu et al., 2016).

3.2 | miR-26a, miR-199b-5p, and miR-346

As mentioned previously, GSK3β participates in silencing of Wnt signaling pathway by phosphorylating cytoplasmic β-catenin, and thus GSK3β is known as an osteogenesis suppressor. GSK3β inhibitors such as AR28 and lithium chloride have promoted osteoblast differentiation in vitro (Galli et al., 2013; Gambardella et al., 2011); GSK3β inhibition has also significantly increased bone mass in vivo (Kulkarni et al., 2006). In addition, GSK3β is able to phosphorylate Runx2, the chief regulator of osteoblastic differentiation; in this manner, the transcriptional activity and DNA binding capacity of Runx2 are suppressed which culminates in the inhibition of osteoblastic differentiation (Kugimiya et al., 2007). MiR-26a, miR-199b-5p, and miR-346 directly target 3′-UTR of GSK3β mRNA and prevent its translation. In addition, the expression of these miRs has been shown to be elevated during osteoblastic differentiation of mouse and human bone marrow stromal cells (BMSCs). MiR-26a, miR-199b-5p, and miR-346 overexpression have increased nuclear β-catenin levels, leading to the activation of Wnt signaling pathway (Su et al., 2015; Q. Wang, Cai, Cai, & Chen, 2013; R. Zhao et al., 2016). It is worthy of note that glucocorticoids inhibit osteoblastogenesis by modulating GSK3β activity. Phosphorylation inactivates GSK3β and glucocorticoids induce GSK3β activities by preventing its phosphorylation, and by this mechanism inhibit osteoblastic differentiation. However, when GSK3β activities are repressed by lithium chloride, the inhibitory effects of glucocorticoids on osteoblastic differentiation fade away (Smith & Frenkel, 2005; Smith, Coetzee, & Frenkel, 2002). Therefore, these miRs could have potential therapeutic applications against glucocorticoid-induced osteoporosis.

3.3 | miR-9, miR-335-5p, and miR-433-3p

These miRs target the inhibitors of Wnt signaling pathway and consequently lead to its activation with resultant enhanced osteoblastic differentiation. MiR-9, miR335-5p, and miR433-3p all have binding sites on 3′-UTR region of DKK1 mRNA, hence blocking its translation (X. Liu et al., 2016; X. Tang, Lin, Wang, & Lu, 2017; J. Zhang, Tu, et al., 2011). DKK1 is a secreted extracellular antagonist of Wnt signaling pathway (Qiang, Barlogie, Rudikoff, & Shaughnessy, 2008). This protein binds to the extracellular regions of LRP5/6 receptors and prevent Frz and LRP5/6 complex formation, that is, the initial step required for Wnt signaling pathway activation (Murrills et al., 2009). DKK1-deficient mice have demonstrated expanding numbers of osteoblasts and increased bone formation (Morvan et al., 2006). Conversely, DKK1-overexpressing transgenic mice have had declined numbers of osteoblasts with osteopenia (J. Li et al., 2006). In line with these findings, DKK1 repression has been observed to abolish long-term dexamethasone-induced osteoporosis (Butler et al., 2010).

MiR-9 could also stimulate osteoblastogenesis by a distinct mechanism, that is, through AMP-activated protein kinase signaling pathway. It has been shown that miR-9 improves osteoblastic differentiation in MC3T3-E1 cells via this signaling pathway (Qu et al., 2016).

It should be emphasized that inhibitory effects of miR-433-3p on osteoblastic differentiation in C3H10T1/2 cells has also been reported via directly targeting 3′-UTR region of principal osteogenic transcription factor, Runx2 (Kim, Kang, Lee, Jang, & Koh, 2013).

3.4 | miR-27a

Secreted frizzled-related protein 1 (sFRP1) is another extracellular antagonist of Wnt signaling pathway. sFRP1 possess a cysteine-rich domain similar to the one found in ligand binding site of Frz receptor, though without intracellular domain of Frz receptor; therefore, it binds to the triggering ligands of Wnt signaling pathways and prevent it to be activated.

MiR-27a is upregulated during osteoblastic differentiation of hFOB cells and targets sFRP1 (Guo et al., 2014). It is known that in sFRP1 deficient mice, osteoblastic differentiation, and bone formation are promoted (Bodine et al., 2004); in contrast, the overexpression of sFRP1 in transgenic mice results in the suppression of osteoblastic differentiation, decreased bone formation, and osteopenia (Yao et al., 2010). Moreover, sFRP1 plays a pivotal role in glucocorticoid-induced suppression of osteoblastic differentiation. Glucocorticoids induce the expression of sFRP1 and when sFRP1 expression is knocked down, the inhibitory effects of glucocorticoids also disappear (F.-S. Wang et al., 2005). From this perspective, miR-27a could be regarded as another therapeutic modality in glucocorticoid-induced osteoporosis. Equally important, the overexpression of miR-27a is associated with β-catenin accumulation in the nuclear regions, accompanied by an upraise in the activity of Wnt signaling pathway which culminates in the induction of osteoblastic differentiation (Guo et al., 2014). MiR-27a has dual beneficial effects on Wnt signaling pathway by regulating it positively through sFRP1 repression, and further promoting its activity by targeting APC. MiR-27a downregulates APC and therefore activates Wnt signaling pathway, and in this way amplifies osteoblastic differentiation in hFOB1.19 cells (T. Wang & Xu, 2010).

Extensive use of steroids could possibly give rise to nontraumatic osteonecrosis of the femoral head (ONFH), a prevalent

orthopedic disorder. In a rat model of steroid-induced ONFH, it was observed that extracted BMSCs strongly differentiated into adipocytes and differentiation into osteoblasts occurred less commonly. It was recognized that as levels of miR-27a decrease, the expression of its target genes including peroxisome proliferator-activated receptor γ (PPARγ) and gremlin1 (GREM1) genes are increased, leading to decreased levels of osteoblastogenesis. On the contrary, the overexpression of miR-27a enhances osteoblast differentiation and suppresses adipocyte differentiation by suppressing of PPARγ and GREM1 (Gu et al., 2016).

A number of investigations have reported an unfavorable role for miR-27a on osteoblastic differentiation. it has been found that miR-27a represses osteoblastic differentiation via downregulating special AT-rich sequence-binding protein 2 (SATB2) in MC3T3-E1 cells (Hassan et al., 2010). SATB2 is a nuclear matrix protein that binds to AT-rich DNA elements and alters the structure of chromatin (Gyorgy, Szemes, De Juan Romero, Tarabykin, & Agoston, 2008). SATB2 regulates expression of Bsp and osteocalcin genes. SATB2 cooperates with Runx2 and activating transcription factor 4 (ATF4), the two transcription factors that strongly induce osteoblastogenesis. This cooperation leads to an overt improvement in the activities of Runx2 and ATF4 (Dobreva et al., 2006). It is noteworthy that Runx2 directly binds to the promoter region of miR-27a to downregulate it. Therefore, there exist a regulatory loop for osteoblastic differentiation composed of miR-27a, Runx2, and SATB2 elements (Hassan et al., 2010). Elsewhere, it has been shown that SATB2 overexpression leads to enhancement of osteoblastic differentiation in mice BMSCs with associated decreasing levels of miR-27a; in these circumstances other targets of miR-27a, that is, BMP2, bone morphogenetic protein receptor I (BMPR1), and SMAD9 are all upregulated. Conversely, miR-27a overexpression mitigates BMP2, BMPR1, and SMAD9 levels; therefore, it is evident that miR-27a plays a central role in osteoblastic differentiation via regulating BMP signaling pathway (Gong et al., 2014). This has been verified by other studies conducted on BMSCs, demonstrating that miR-27a downregulation results in the elevated activities of SATB2; and in this manner, osteoblastic differentiation has been augmented. It should be noted that osterix (Osx), a transcription factor for osteoblastic differentiation, has been reported as a novel target for miR-27a (Gong, Lu, Yu, & Yu, 2016). Mice lacking Osx show disturbed bone formation (Nakashima et al., 2002); in contrast, Osx overexpression accentuates osteoblastic differentiation in mice BMSC cells (Tu, Valverde, & Chen, 2006). Osx is downstream to Runx2 because mice lacking Runx2 are also devoid of Osx (Nakashima et al., 2002). It is worth noting that mice with blocked Osx do not express SATB2; a finding that signifies Osx is upstream to SATB2 and controls its expression (W. Tang, Li, Osimiri, & Zhang, 2011). Finally, miR-27a could also suppress osteoblast differentiation in human BMSCs via downregulating ALP, grancalcin, and peroxisomal biogenesis factor 7 genes (Schoolmeesters et al., 2009).

3.5 | miR-29

MiR-29 is another miR capable of enhancing osteoblastic differentiation. Expression of miR-29 is increased during osteoblastic differentiation in hFOB and MC3T3-E1 cells. The positive effect of miR-29 on osteoblastic differentiation is associated with the modulation of Wnt signaling pathway. MiR-29 targets antagonists of Wnt signaling pathway, that is, DKK1, kremen2 (Krm2), and sFRP2 in hFOB cells. In addition, Wnt signaling pathway inhibitor, inhibitor of β-catenin and TCF-4 (ICAT), is blunted by miR-29 in MC3T3-E1 cells which results in the activation of this signaling pathway (Kapinas, Kessler, Ricks, Gronowicz, & Delany, 2010; Z. Li et al., 2009). The role of DKK1 has already been explained; Krm2 is a transmembrane protein serving as a receptor for DKKs. Krm2 interacts with DKK1, 2, and 4, but not DKK3, and enhances the inhibitory effects of DKKs on Wnt signaling pathway (Mao & Niehrs, 2003). Krm2 together with DKKs form a triple complex with LRP6 that are removed from cell membrane altogether by endocytosis; accordingly, the activation of Wnt signaling pathway is prevented (Davidson, Mao, Barrantes, & Niehrs, 2002). Transgenic mice overexpressing Krm2 especially in their osteoblasts demonstrate severe osteoporosis. In these mice, the osteoblasts numbers are similar to the numbers of these cells in wild-type mice, however, the maturation of osteoblasts and bone matrix mineralization are defective. Conversely, in mice lacking Krm2 gene, osteoblasts numbers are increased with spuriously elevated bone mass. These observations underline the prime role of Krm2 in bone formation (Davidson et al., 2002). sFRP2, the other target of miR-29, represses osteoblastic differentiation induced by BMP2 (Oshima et al., 2005). sFRP2 is expressed in multiple myeloma and ameloblastoma cells and contributes to the inhibition of osteoblastic differentiation in mouse osteoblastic cells (MC3T3-E1 and KUSA/A1; Oshima et al., 2005; Sathi et al., 2009). ICAT is a negative regulator of Wnt signaling pathway, the suppressive functions of which are appeared when interacts with β-catenin and prevents it to form a complex with TCF (Tago et al., 2000).

As described previously, miR-29 stimulates Wnt signaling pathway; however, the stimulatory effects of Wnt signaling pathway on miR-29 expression has also been documented; when Wnt signaling pathway is induced by lithium chloride, the expression of miR-29 is dramatically increased. DKK1 treatment, on the other hand, leads to the inhibition of Wnt signaling pathway which is accompanied by a decrease in miR-29 expression (Kapinas et al., 2010; Kapinas, Kessler, & Delany, 2009); thus, miR-29 and Wnt signaling pathway regulate osteoblastic differentiation with a positive feedback mechanism (Kapinas et al., 2010).

MiR-29 possesses other targets which act independently of Wnt signaling pathway. Histone deacetylase 4 (HDAC4), TGF-β3, and activin A receptor type 2A are negative regulators of osteoblast, the expression of all three genes are downregulated by miR-29 in osteoblastic differentiation of MC3T3-E1 cells (Z. Li et al., 2009). HDAC4 is a member of HDAC protein family that deacetylate lysine residues of histone and nonhistone proteins and is associated with alterations in chromatin structure, affecting genes expression (Huynh, Everts, Pavasant, & Ampornaramveth, 2016). HDAC4 has a central role in the inhibitory effects of TGF-β on osteoblastic differentiation in mouse calvarial osteoblast caIB 2T3 cells. TGF-β

efficiently upregulates the expression of HDAC4, which leads to the deacetylation of SMAD3; after that, Runx2/SMAD3 complex is formed, leading to substantial reductions in Runx2 activities (Kang, Alliston, Delston, & Derynck, 2005). When osteoblastic differentiation of unrestricted human somatic stem cells is induced, a drastic upraise in the expression of miR-29 happens, which promotes osteoblastic differentiation via targeting ICAT and HDAC4 (Trompeter et al., 2013).

The protective effects of miR-29 against glucocorticoid-induced bone loss and suppression of osteoblastic differentiation has been verified. Glucocorticoids decrease miR-29 expression and accordingly increase the HDAC4 levels in MC3T3-E1 cells. β-Catenin is ubiquitinated and degraded after deacetylation, leading to decreased nuclear levels of β-catenin. In contrast, induced miR-29 expression results in the downregulation of DKK1 and HDAC4 proteins; therefore nuclear levels of β-catenin are restored, even with the presence of supraphysiological levels of glucocorticoids (Ko et al., 2013). Likewise, lentivirus-mediated miR-29a precursor increases bone mass, which is associated with a nuclear accumulation of β-catenin and increased activities of osteogenic factor Runx2, in methylprednisolone treated rats (F.-S. Wang et al., 2013). In agreement with these findings, transgenic mice overexpressing miR-29a had less glucocorticoid-mediated bone disorders as compared with wild-type mice. In parallel with in vivo observations, in vitro findings on primary BMSCs driven from transgenic mice and exposed to glucocorticoids showed promoted levels of osteoblast differentiation with decreased levels of HDAC4, and elevated nuclear β-catenin and Runx2 activities (Ko et al., 2015).

3.6 | miR-218

MiR-218 is implicated in the osteoblastogenesis and stimulates this process by positively modulating Wnt signaling pathway in mice BMSCs, MC3T3, and hASCs. Stimulatory effects of miR-218 on Wnt signaling pathway are manifested by downregulating the inhibitors of Wnt signaling pathway, that is, DKK2, sFRP2, and sclerostin (SOST; Hassan et al., 2012; W.-B. Zhang, Zhong, & Wang, 2014). DKK1 has been recognized as an extracellular antagonist of the pathway; DKK2, however, could either act as activator or inhibitor of Wnt signaling pathway with Krm2 controlling these two distinct but salient functions of DKK2 (Mao & Niehrs, 2003). DKK2 is a crucial factor in the later stages of osteoblast differentiation and mineralization processes, with mice not expressing DKK2, osteopenia almost always develops (X. Li, Liu, et al., 2005). SOST is another antagonist of Wnt signaling pathway, with a mechanism of action similar to DKKs, inhibiting the pathway by binding to LRP5/6 receptors (X. Li, Zhang, et al., 2005). Once again, in mice not expressing SOST, high bone mass could be observed (X. Li et al., 2008). It has been shown that BMP2 mediated promotion in Wnt signaling pathway results in increased expression of miR-218; rather, negative regulator of osteoblastic differentiation, that is, TGF-β downregulates miR-218 expressions (Hassan et al., 2012). Wnt-mediated increase in miR-218 further augments the activity of Wnt signaling pathway by suppressing the expression of pathway's inhibitors; therefore a similar positive feedback loop explained for miR-29, exists for the Wnt signaling pathway and miR-218 interaction, upregulating osteoblastic differentiation (Hassan et al., 2012; W.-B. Zhang, et al., 2014).

Supporting aforementioned mechanistic studies, recent findings demonstrate that exosomes derived from human BMSCs during osteoblast differentiation have higher levels of miR-218 (J.-F. Xu et al., 2014). Exosomes are small vesicles present in various body fluids and transport miRs, mRNAs, and protein. Exosomes' content can be altered in relation to the physiological behavior of the cells; for instance, myostatin-treated osteocytes release exosomes with decreased miR-218 content, whereas the levels of DKK1 and SOST are elevated in the same exosomes. When these exosomes are applied to MC3T3 cells, the osteoblastic differentiation process is prohibited (Qin et al., 2017).

3.7 | TGF-β signaling pathway

TGF-β is a big family of cytokines and TGF-βs as well as bone morphogenetic proteins (BMPs) are the salient members of this family. TGF-β is involved in the regulation of many physiological processes including osteoblastogenesis. Effects of TGF-β, however, are contradictory as it can induce dual effects regarding osteoblastic differentiation (de Gorter, van Dinther, Korchynskyi, & ten Dijke, 2011; X. Sun et al., 2017). TGF-β encourages proliferation of osteoblastic precursors and triggers early osteoblastic differentiation, whereas impedes osteoblastic differentiation at the later stages, especially during mineralization (Alliston, Choy, Ducy, Karsenty, & Derynck, 2001; Maeda, Hayashi, Komiya, Imamura, & Miyazono, 2004; Matsunobu et al., 2009; X. Sun et al., 2017). The stimulatory or inhibitory effects of TGF-β on osteoblastic differentiation differs according to the surrounding conditions (de Gorter et al., 2011). Three TGF-βs exist in mammals: TGF-β1, TGF-β2, and TGF-β3 (Maeda et al., 2004). Experiments have indicated that TGF-β2 plays an essential role in the embryonic skeletal development as TGF-β2 deficient mice have shown more severe skeletal abnormalities than TGF-β1 and TGF-β3 deficient mice (Wu, Chen, & Li, 2016). TGF-β ligands can initiates signaling cascades in two SMADs dependent and independent pathways, which the latter is done by mitogen-activated protein kinase (MAPK) cascade activation (Wu et al., 2016). In SMADs dependent pathway, message is transferred to the nucleus by SMADs. There are three types of SMADs in mammalians including receptor-regulated SMADs (R-SMADs), common-partner SMADs (Co-SMADs), and inhibitory SMADs (I-SMADs). R-SMADs are implicated in the process of signal transduction in BMP signaling pathway (i.e. SMADs 1, 5, and 8) and TGF-β signaling pathway (i.e., SMADs 2 and 3). SMAD4 is a renowned Co-SMAD that acts in conjunction with R-SMADs in both already mentioned signaling pathways. Contrarily, SMADs 6 and 7 are known as I-SMADs with negative regulatory effects on BMP and TGF-β signaling pathways by suppressing R-SMADs (Maeda et al., 2004). TGF-β ligand binds to a tetrameric complex receptor that is located in cell membrane; this complex comprises TGFBRI or ALK and TGFBR II. Activin receptor-like

kinase (ALK) is a type I transmembrane serine/threonine kinase receptor that after binding of TGF-β is phosphorylated and activated by type II receptor. Activated ALK phosphorylates SMAD2/3; the phosphorylated form is translocated into the nucleus after cooperation with SMAD4, and together with other cofactors, that is, CREB-binding protein or p300 applies regulatory effects on the gene expression (Wu et al., 2016). Earlier, it was mentioned that SMAD3 is essential for HDAC4-dependent suppression of Runx2 (Kang et al., 2005).

4 | miRs ENHANCING OSTEOBLAST DIFFERENTIATION THROUGH TGF-β SIGNALING PATHWAY

4.1 | miR-181a and miR-210

MiR-181a in mice osteoblastic cells (C2C12 and MC3T3 cells) and miR-210 in ST2 cells promote BMP-induced osteoblastic differentiation through targeting components of TGF-β signaling pathway (Bhushan et al., 2013; Mizuno et al., 2009). ALK is one of these targets. There are seven types of ALK in mammals and only ALK-4 and -5 belong to TGF family (Maeda et al., 2004); ALK-4 is targeted by miR-210 and ALK-5 by miR-181a (Bhushan et al., 2013; Mizuno et al., 2009). Several lines of evidence indicate that the inhibition of ALK with its specific inhibitors including SB431542 and Ki26894 is associated with an improvement in osteoblastic differentiation (Maeda et al., 2004; Mizuno et al., 2009; Takeuchi et al., 2010). Regulatory effects of miR-181a on TGF-β signaling pathway via targeting ALK has been demonstrated in human MSCs and thymic epithelial cells (Guo et al., 2016; L. Liu et al., 2012).

Transforming growth factor β-induced (TGFBI) is a different target for miR-181a; this protein is induced by TGF-β and is an element of extracellular matrix (ECM) like type I collagen, osteocalcin, and fibronectin. The expression of TGFBI is reduced in osteoblastic differentiation of murine preosteoblastic (KS483) cells. TGFBI negatively modulates osteoblastic differentiation and associated matrix mineralization; inhibitory actions of which are mediated by Integrins (Thapa, Kang, & Kim, 2005). Integrins are expressed at the surfaces of osteoblasts which interact with ECM proteins. Integrin αvβ3 is specifically expressed in bones, and when expressed, suppresses osteoblastic differentiation and bone mineralization (Cheng, Lai, Blystone, & Avioli, 2001; Gronthos, Simmons, Graves, & Robey, 2001). Integrin αvβ3 blockade using monoclonal antibodies obviates the negative regulatory effects of TGFBI on osteoblast differentiation (Thapa, Kang, & Kim, 2005).

It has been found that a binding site for TCF4 exists in the promoter region of miR-181a, and therefore Wnt signaling pathway activation is able to enhance the expression of miR-181a in hepatocellular carcinomas. As it has already been mentioned that activation of Wnt signaling pathway has positive regulatory effects on osteoblastic differentiation, it could be inferred that miR-181a is an important link between the two signaling pathways of Wnt and TGF-β, regarding osteoblastic differentiation (Ji, Yamashita, & Wang, 2011).

4.2 | miR-21

MiR-21 modulates TGF-β signaling pathway by downregulating SMAD7 and as a consequence osteoblastic differentiation is improved in MC3T3-E1 cells (H. Li, Yang, Wang, Fu, & Liang, 2015). Repressive effects of SMAD7 on osteoblastic differentiation and mineralization has been demonstrated in MC3T3-E1 cells (Yano et al., 2012). The upraise in miR-21 levels is associated with a reduction in SMAD7 protein expression during osteoblastic differentiation (H. Li et al., 2015). Elevated levels of miR-21 have also been reported in BMP9-induced osteoblastic differentiation in C2C12 cells that targets SMAD7. It should, however, be noted that exogenous miR-21 alone is not enough to induce osteoblastic differentiation (Song et al., 2015). In addition, miR-21 stimulates osteoblastic differentiation through phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling pathway in human umbilical mesenchymal stem cells (Meng et al., 2015), activated PI3K produces phosphatidylinositol 3,4,5-triphosphate (PIP3), which in turn is the activator of Akt, downstream to PI3K (Schindeler & Little, 2006). PI3K/Akt signaling pathway activation augments BMP2-induced osteoblastic differentiation; on the other hand, PI3K/Akt signaling pathway inhibition leads to the suppression of BMP2-induced osteoblastic differentiation (Mukherjee & Rotwein, 2009). Phosphatase and tensin homolog (PTEN) is the inhibitor of PI3K that is targeted by MiR-21; the net effect of which being increased activation of PI3K/Akt signaling pathway. It should be pointed out that activated Akt phosphorylates GSK3β, leading to its inactivation; accordingly β-catenin is preserved, translocate into the nucleus and promotes Runx2 activity. Taken as a whole, the increased levels of miR-21 is associated with elevated levels of phosphorylated PI3K, phosphorylated Akt, and raised activities of nuclear β-catenin and Runx2 (Meng et al., 2015).

Yet, miR-21 could stimulate osteoblastic differentiation through other cellular pathways. Increased expression of miR-21 is associated with increased activation of extracellular-signal-regulated kinase (ERK)/MAPK signaling cascade. The mechanism is as follows: Sprouty2 (SPRY2), the antagonist of ERK, is targeted by miR-21 and accordingly ERK/MAPK signaling pathway is highly activated during osteoblastic differentiation of ADSCs (Mei et al., 2013). Some evidence indicate that suppressing miR-21 has only slight effects on PI3K/Akt and ERK–MAPK signaling pathways activities, rather enhancement of miR-21 expressions deeply affects the activities of these pathways (Mei et al., 2013; Meng et al., 2015).

Serum levels of tumor necrosis factor-α (TNF-α) in postmenopausal osteoporosis patients and also ovariectomized (OVX) mice are elevated; and it has been recognized that high levels of TNF-α is associated with impaired osteoblastic differentiation and also a reduction in miR-21 expression (N. Yang et al., 2013). Moreover, miR-21 targets reversion-inducing cysteine-rich protein with Kazal motifs (RECK), which is a matrix metalloproteinase suppressor. Overexpression of miR-21 obviates TNF-α-induced impaired osteoblastic differentiation in osteoporosis (W. Zhao et al., 2015). In addition to the effects of miR-21 in osteoporosis, its effects on bone fracture healing has also been evaluated in animal models; as overexpression of miR-21 has considerably improved osteoblastic differentiation and fracture healing (Y. Sun et al., 2015).

4.3 | BMP signaling pathway

BMPs, other member of TGF-β superfamily play essential roles in bone formation and osteoblastic differentiation (R. L. Huang, Yuan, Tu, Zou, & Li, 2014). There are 14 BMPs in mammals among which BMPs 2, 4, 5, 6, 7, and 9 have high potencies for the induction of osteogenesis (Wu et al., 2016). Osteoblastic induction effects of BMP2 and BMP7 are investigated in fracture healing of animal models; moreover, a clinical trial have been conducted on the effects of recombinant human BMP2 and BMP7 in fracture healing and the results showed that recombinant BMPs are comparable with autologous bone graft (Gautschi, Frey, & Zellweger, 2007). Runx2 is a downstream target of BMP signaling pathway. BMP2 and BMP7 both up-regulate Runx2 mRNA expression during osteoblastic differentiation; worthy of note that BMP2 and Runx2 play distinct, but at the same time, complimentary roles (S. Yang et al., 2003). Similar to TGF-β signaling pathway, BMP signaling pathway acts through a heteromeric complex receptor. This complex comprises BMPRI and BMPRII. In SMAD dependent pathway, conjunctions of phosphorylated SMADs 1, 5, and 8 with SMAD4 translocate into the nucleus and with cofactor cooperation induce the expression of the target genes (Wu et al., 2016).

5 | miR ENHANCING OSTEOBLAST DIFFERENTIATION THROUGH BMP SIGNALING PATHWAY

5.1 | miR-20a

During osteoblastic differentiation of hADSCs and hMSCs, miR-20a levels are elevated (J. Zhang, Fu, et al., 2011; Z. Zhang et al., 2012). The antagonists of BMP signaling pathway, that is, BMP and activin membrane-bound inhibitor (Bambi) and cysteine-rich motor neuron 1 (Crim1) are the targets of miR-20a, both being cell surface antagonists of BMP signaling pathway (J. Zhang, Fu,

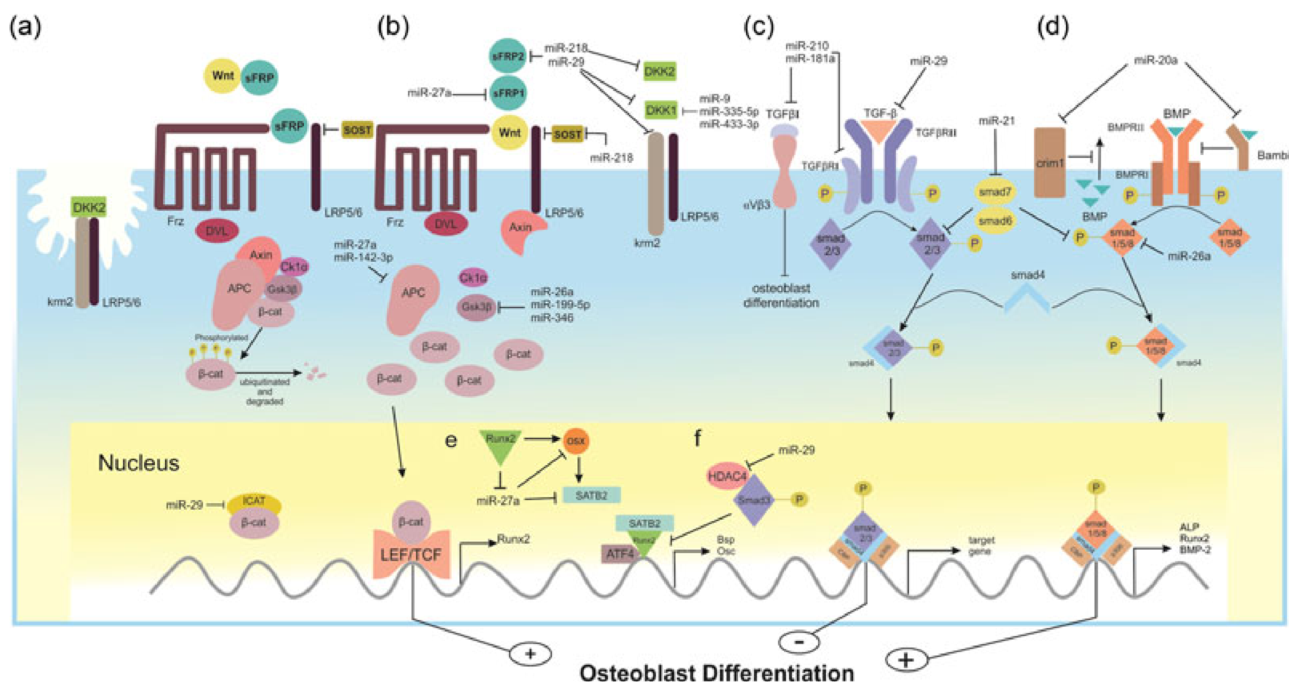

FIGURE 1 Schematic representation of Wnt, TGF-β, and BMP signaling pathways regulated by different miRs during the osteoblastic differentiation of MSCs. (a) On the left side, the antagonists of Wnt signaling pathway, that is, DKK1, DKK2, krm2, sFRP1, sFRP2, SOST, and also ICAT are shown. Wnt signaling pathway is Off, β-catenin is phosphorylated and degregated. (b) The antagonists of Wnt signaling are inhibited by miRs. Signaling pathway is On, β-catenin is accumulated in the cytoplasm and translocates into the nucleus, β-catenin associates with TCF and LEF transcription factors to induce the expression of target genes such as Runx2. (c) TGF-β signaling pathway; SMAD2/3 after phosphorylation joins to SMAD4 and the conjugated form translocates into the nucleus, this complex regulates the expression of target genes together with CBP and p300. MiRs suppress SMAD7, TGF-β ligand, TGFβR I, and TGFβI. (d) On the right side, the antagonists of BMP signaling pathway, that is, Bambi and Crim1 are shown that are suppressed by miRs. BMP signaling pathway is active; the conjugated form of phosphorylated SMAD1/5/8 with SMAD4 translocate into the nucleus, recruit cofactors and induce ALP, BMP2, and Runx2 genes. (e) Runx2 along with Osx and ATF4 induce the osteoblastic genes, that is, Bsp and Osc in the nucleus. A regulatory loop exists between transcription factors Runx2, Osx, SATB2 and miR-27a. MiR-27a inhibits the expression of Osx, downstream gene of Runx2 and also SATB2, while miR-27a is suppressed by Runx2. (f) SMAD3 represses Runx2 after activation via HDAC43 during osteoblast differentiation, HDAC4 is inhibited by miR-29. ALP: alkaline phosphatase; APC: adenomatous polyposis coli; BMP: bone morphogenic protein; CBP: CREB-binding protein; CK1α: casein kinase 1α; DKK1: Dickkopf-related protein 1; DVL: Disheveled; Frz: frizzled; GSK3β: glycogen synthase kinase 3β; Krm2: kremen2; LEF: lymphoid enhancer factor; LRP5/6: lipoprotein receptor-related protein 5/6; miR, microRNA; MSCs: mesenchymal stem cells; TCF: T-cell factor; TGF-β, transforming growth factor β; TGF-βRI: transforming growth factor receptor 1; sFRP: secreted frizzled-related protein; SMAD2: small mothers against decapentaplegic 2; SOST: sclerostin; Wnt: wingless-type [Color figure can be viewed at wileyonlinelibrary.com]

et al., 2011). Bambi is a pseudoreceptor, structure of which is similar to type I transmembrane receptors and binds to extracellular BMP; as it lacks intracellular kinase domain, the message induction is abrogated leading to BMP signaling pathway inhibition (Gazzerro & Canalis, 2006). Crim1 is another transmembrane protein, often present in the cell membranes. Crim1 interacts with BMP and therefore impairs its secretion (Wilkinson et al., 2003). PPARγ is an alternative target of miR-20a (J. Zhang, Fu, et al., 2011), belonging to nuclear receptor families. Despite being as a key factor for adipocyte differentiation, PPARγ has negative regulatory effects in osteoblastic differentiation. PPARγ activation is accompanied by a reduction in mRNA and protein levels of Runx2. Furthermore, PPARγ robustly interacts with Runx2, preventing its transcriptional activity (Jeon et al., 2003). One further action of PPARγ that suppresses osteoblastic differentiation is to inhibit BMP2 expression (Lin, Yang, Tang, Lin, & Fu, 2007). MiR-20a increases Runx2 levels and upregulates BMP signaling pathway via inhibition of PPARγ (J. Zhang, Fu, et al., 2011). It has been shown that during aging, PPARγ is augmented, leading to increased commitment of MSCs into adipocytes, therefore the contributory role of PPARγ to osteoporosis in aging is a topic of investigation (Moerman, Teng, Lipschitz, & Lecka-Czernik, 2004). Additionally, miR-20a levels in MSCs of young donors are higher compared with adults (J. Zhang, Fu, et al., 2011), and miR-20a is decreased during human aging (Hackl et al., 2010); collectively, these findings highlight the presumed contributory role of miR-20a in osteoporosis (Figure 1).

It has been found that Naringin, a flavonoid compound, improves osteoblastic differentiation of rat BMSCs (Z. Xu, Li, Wooley, Yang, & Jiang, 2013), and Fan, Li, and Fan (2015) demonstrated that Naringin does this through elevating the miR-20a levels in rabbit BMSCs that is accompanied by reductions in PPARγ.

6 | CONCLUSION

This paper reviews miRs that promote osteoblastic differentiation chiefly through three principal signaling pathways, that is, Wnt, TGF-β, and BMP. Increasingly recognized roles for these noncoding RNAs in osteoblastogenesis has been warranted. Recently, efforts have been made to develop new MSCs-based therapies for bone diseases, and thus miRs can be helpful in this respect. To achieve this goal, it is imperative to recognize and validate the effects of miRs on osteoblastic differentiation: positive or negative, in addition to identifying their correspondent diverse targets in intracellular signaling pathways. Reciprocally, the impact of signaling pathways and transcriptional factors on the expression of miRs must be identified, and these data can mostly be obtained from preliminary in vitro studies followed by detailed explorations in animal models.

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