MiR-17-3p inhibits osteoblast differentiation by downregulating Sox6 expression

Nan Chen, Di Wu, Hua Li, Yi Liu and Hao Yang

Department of Orthopedics, the First Affiliated Hospital of Kunming Medical University, Kunming, China

Keywords
MC3T3-E1; miR-17-3p; osteoblast; Sox6

Correspondence
H. Yang, Department of Orthopedics, the First Affiliated Hospital of Kunming Medical University, No. 295 Xichang Road, Kunming 650032, Yunnan Province, China
Tel: +86 13312542641
E-mail: doctoryanghao@163.com

(Oceived 14 April 2020, revised 15 August 2020, accepted 10 September 2020)
doi:10.1002/2211-5463.12979

Osteoporosis and osteoarthritis are orthopedic disorders that affect millions of elderly people worldwide; stimulation of bone formation is a potential therapeutic strategy for the treatment of these conditions. As the only bone-forming cells, osteoblasts play a key role in bone reconstruction. The microRNA miR-17-3p is downregulated during osteogenic differentiation of human bone marrow mesenchymal stem cells, but its precise role in this process is unknown. Here, we investigated the role of miR-17-3p in osteoblast differentiation. An in vitro model of osteogenesis was established by treating MC3T3-E1 murine preosteoblast cells with bone morphogenetic protein 2 (BMP2). The expression of miR-17-3p in BMP2-induced MC3T3-E1 cells was detected by reverse transcription-quantitative PCR, and its effects on cells transfected with miR-17-3p mimic or inhibitor were evaluated by Alizarin Red staining, alkaline phosphatase (ALP) activity assay, and by detection of osteoblast markers including the ALP, collagen type I α1 chain, and osteopontin genes. Bioinformatics analysis was carried out to identify putative target genes of miR-17-3p, and the luciferase reporter assay was used for functional validation. Rescue experiments were performed to determine whether SRY-box transcription factor 6 (Sox6) plays a role in the regulation of osteoblast differentiation by miR-17-3p. We report that miR-17-3p was downregulated upon BMP2-induced osteoblast differentiation in MC3T3-E1 cells, and this was accompanied by decreased differentiation and mineralization, ALP activity, and expression of osteogenesis-related genes. Sox6 was confirmed to be a target gene of miR-17-3p in osteoblasts, and the inhibitory effect of miR-17-3p on osteoblast differentiation was observed to occur via Sox6. These results suggest the existence of a novel mechanism underlying miRNA-mediated regulation of osteogenesis, which has potential implications for the treatment of orthopedic disorders.

Abbreviations
ALP, alkaline phosphatase; BMP2, bone morphogenetic protein 2; COLIA1, collagen type I α1 chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miR-17-3p, microRNA-17-3p; miRNA, microRNA; MUT, mutation-type; NC, negative control; OPN, osteopontin; RT-qPCR, reverse transcription-quantitative PCR; SD, standard deviation; SiRNA, Small interfering RNA; Sox6, SRY-box transcription factor 6; UTR, untranslated region; WT, wild-type.
Bone formation involves the differentiation of progenitor cells into osteoblasts; inhibiting this process can have pathologic consequences [3]. Osteogenic induction increases the expression of osteogenesis-related genes including osteopontin (OPN), collagen type I α1 chain (COL1A1), and alkaline phosphatase (ALP) [4], which is a key event in osteoblast differentiation.

MicroRNAs (miRNAs) regulate a variety of cellular processes, and their dysregulation has been implicated in several diseases. Their activity mainly involves binding to the 3′ untranslated region (UTR) of target transcripts to alter gene expression [5,6]. The role of miRNAs in osteogenesis and bone development has been widely investigated. Multiple miRNAs including miR-138, miR-2861, and miR-148b have been shown to modulate the development of bone precursor cells [7–9]. MiR-17-3p is downregulated during osteogenic differentiation of human bone marrow mesenchymal stem cells [10], but its precise role in this process is unknown.

Transcription factors such as Runt-related transcription factor 2 (Runx2), Osterix, mothers against DPP homolog 1 (SMAD), T-cell factor (TCF)/Lymphoid enhancer-binding factor (LEF), nuclear factor of activated T-cell cytoplasmic 1 (NFATc1), Twist, activator protein 1 (AP-1), and activating transcription factor 4 (ATF4) are known to play an important role in osteogenic differentiation [11,12]. The transcription factor SRY-box transcription factor 6 (Sox6) is involved in the differentiation of various tissues [13]. It was previously reported that Sox6 is a tumor suppressor gene that is downregulated in osteosarcoma (OS) tissues and cell lines. Osteogenic differentiation defects promote the development of OS; therefore, stimulating this process is a potential treatment strategy [14].

Based on the above findings, the present study investigated the roles of miR-17-3p and Sox6 in osteoblast differentiation in order to assess their potentiality as therapeutic targets in the treatment of bone disorders.

**Materials and methods**

**Cell culture**

MC3T3-E1 murine preosteoblast cells were obtained from ScienCell (Carlsbad, CA, USA) and cultured in α-minimal essential medium supplemented with 10% fetal bovine serum, penicillin (100 U·mL−1), and streptomycin (100 μg·mL−1) at 37 °C and 5% CO2. Cells in passages 2 and 3 were used for experiments. To induce osteoblast differentiation, the cells were cultured in medium containing 200 ng·mL−1 BMP2 for 21 days.

**Transfection of MC3T3-E1 cells**

MiR-17-3p mimic and miR-17-3p inhibitor, along with small interfering RNA (siRNA) targeting Sox6 or a scrambled control siRNA, were synthesized by GenePharma (Shanghai, China). The plasmids were transfected into MC3T3-E1 cells at a concentration of 10 nm using Lipofectamine RNAiMAX transfection reagent (cat. no. 13-778-075; Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

**Real-time quantitative PCR**

After osteoinduction of MC3T3-E1 cells for 0, 1, 3, 7, 14, and 21 days, total RNA was extracted from cells using TRIzol reagent (GenePharma). cDNA was synthesized from 500 ng total RNA using the Bestar qPCR RT kit (DBI Bioscience, Shanghai, China), and 20–100 ng of cDNA served as the template for RT-qPCR using Bestar SybrGreen qPCR Master Mix (DBI Bioscience) according to the manufacturer’s protocol. MiR-17-3p and Sox6 levels and the transfection efficiency of miR-17-3p mimic or inhibitor in MC3T3-E1 cells were determined by RT-qPCR. The expression of osteogenic differentiation-related genes was also detected by RT-qPCR in 3 independent experiments. Primers used for RT-qPCR are shown in Table 1. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as internal controls for miRNA and mRNA, respectively. Relative expression levels of target genes were calculated with the 2−ΔΔCt method, with GAPDH level used for normalization.

**Western blot analysis**

Total protein was extracted using radioimmunoprecipitation assay buffer (pH 7.4) and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% gel. The proteins were transferred over 2 h to a nitrocellulose membrane (Millipore, Billerica, MA, USA) that was then blocked with 5% low-fat milk at room temperature for 2 h and incubated overnight with a primary antibody against Sox6 (1:1000, cat. no. ab66316; Abcam, Cambridge, MA, USA), followed by horseradish peroxidase-conjugated secondary antibody (1 : 5000, goat anti-rabbit IgG H&L, cat. no. ab205718; Abcam) for 2 h at room temperature. Protein bands were detected with enhanced chemiluminescent reagent (Amersham Biosciences, Piscataway, NJ, USA). GAPDH was used as the loading control.

**ALP activity and Alizarin Red staining**

ALP activity was detected to assess the degree of differentiation of MC3T3-E1 cells. After transfecting BMP2-treated MC3T3-E1 cells with miR-17-3p mimic or inhibitor or siRNA, ALP activity was determined using a commercial assay kit (Jiancheng Biotech, Nanjing, China), with
absorbance measured at 405 nm. Calcification in MC3T3-E1 cells was also detected by staining with Alizarin Red for 30 min at room temperature, and the absorbance was measured at 540 nm using a microplate reader. Images of stained cells were acquired on a light microscope.

**Plasmid construction and dual-luciferase activity assay**

Sox6 was predicted as a target gene of miR-17-3p using TargetScan (http://www.targetscan.org/vert_72/). The fragment of Sox6 containing miR-17-3p binding sites was amplified by PCR with specific primers and cloned into the psi-CHECK2 vector (Promega, Madison, WI, USA) to obtain wild-type (WT) Sox6 plasmid. A fragment containing mutated miR-17-3p-binding sites was also amplified and inserted into psi-CHECK2 to generate the mutant (MUT) Sox6 plasmid. WT and MUT Sox6 3' UTR DNA sequences were synthesized by GenePharma. MC3T3-E1 cells incubated overnight in 24-well plates were cotransfected with WT or MUT Sox6 plasmid and miR-17-3p mimic or negative control (NC) using Lipofectamine 2000 (Invitrogen). Luciferase activity was determined with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions, with absorbance measured at 560 nm.

**Statistical analysis**

Statistical analysis was performed using PRISM v5.0 software (GraphPad, La Jolla, CA, USA). All data are presented as mean ± standard deviation (SD). Differences between groups were evaluated with the Wilcoxon test. The experiments were carried out independently three times. P < 0.05 was considered statistically significant.

**Results**

**MiR-17-3p is downregulated during BMP2-induced osteoblast differentiation**

MiR-17-3p expression levels in MC3T3-E1 cells on days 0, 1, 3, 7, 14, and 21 of osteoblast differentiation were determined by RT-qPCR. MiR-17-3p expression decreased over time in BMP2-treated cells compared to the untreated control group (Fig. 1A). In MC3T3-E1 cells transfected with miR-17-3p mimic or inhibitor, miR-17-3p level was higher and lower, respectively, than in cells transfected with NC, which confirmed the efficiency of transfection (Fig. 1B).

**MiR-17-3p attenuates osteoblast differentiation**

To examine the role of miR-17-3p in osteoblast differentiation, miR-17-3p mimic or inhibitor was transfected
into MC3T3-E1 cells following BMP2 induction. Overexpression of miR-17-3p significantly reduced calcification compared to BMP2-treated NC-transfected control cells, whereas inhibition of miR-17-3p had the opposite effect. Similar trends were observed for mineralization (Fig. 2A,B). Furthermore, compared to cells with BMP2 treatment, ALP activity was decreased in BMP2-induced cells transfected with miR-17-3p mimic and increased in those transfected with miR-409-3p inhibitor (Fig. 2C), with corresponding decreases or increases in the expression levels of the osteoblast marker genes *ALP*, *COLIA1*, and *OPN* (Fig. 2D). These results confirm that miR-17-3p inhibits BMP2-induced osteoblast differentiation in MC3T3-E1 cells.

**Sox6 is a target gene of miR-17-3p**

The TargetScan prediction algorithm identified putative miR-17-3p-binding sites in the Sox6 3’ UTR (Fig. 3A). We found that luciferase activity was reduced in MC3T3-E1 cells cotransfected with miR-17-3p mimic and Sox6 WT 3’ UTR compared to those that were cotransfected with NC mimic + Sox6 WT 3’ UTR, whereas an increase in activity was observed in cells cotransfected with miR-17-3p inhibitor + Sox6 MUT 3’ UTR. On the other hand, there was no change in luciferase activity in cells cotransfected with miR-17-3p mimic or inhibitor and Sox6 MUT 3’ UTR (Fig. 3B). Furthermore, in cells transfected with miR-
17-3p mimic, Sox6 mRNA and protein levels were markedly reduced compared to cells transfected with NC mimic, as determined by RT-qPCR and western blotting, respectively (Fig. 3C,D). These results demonstrate that Sox6 expression in osteoblast differentiation is regulated by miR-17-3p.

**Sox6 knockdown reverses the inhibition of osteoblast differentiation by miR-17-3p**

To examine the relationship between miR-17-3p and Sox6 in greater detail, we cotransfected miR-17-3p inhibitor and Sox6 siRNA into MC3T3-E1 cells for 24 h, followed by BMP2 treatment for 14 days to induce osteoblast differentiation. Sox6 knockdown decreased the level of Sox6 in cells transfected with miR-17-3p inhibitor compared to those transfected with NC or miR-17-3p inhibitor + scrambled siRNA (Fig. 4A). Additionally, Sox6 depletion reduced mineralization in the miR-17-3p inhibitor group compared with the miR-17-3p inhibitor + scrambled siRNA group (Fig. 4B). Similar trends were observed for ALP activity and ALP, COLIA1, and OPN mRNA levels (Fig. 4C,D). Thus, miR-17-3p inhibits osteoblast differentiation via negative regulation of Sox6 expression.

**Discussion**

In the present study, we observed that miR-17-3p expression decreased during BMP2-induced osteoblast differentiation in MC3T3-E1 cells. Gain- and loss-of-function experiments using miR-17-3p mimic or inhibitor, respectively, showed that miR-17-3p suppressed mineralization, ALP activity, and the expression of osteogenesis-associated genes (ALP, COLIA1, and OPN). We also confirmed that miR-17-3p directly regulates Sox6 expression in MC3T3-E1 cells during osteoblast differentiation. These data provide evidence for miR-17-3p as a negative regulator of osteogenesis that acts by inhibiting Sox6 and its downstream targets.

Osteoblasts are essential for maintaining the stability of the intraosseous environment. Multiple miRNAs have been identified that participate in osteoblast differentiation. For example, miR-29b, miR-210, miR-335-5p, and miR-2861 were shown to enhance this process [15–18], whereas miR-125a-3p, miR-145-5p, miR-106b-5p, and miR-17-5p exert suppressive effects [19–21]. MiR-17-3p is variably expressed in different cancer types [22–24] but its role in osteogenesis has not been previously reported. The MC3T3-E1 pre-osteoblast cell line has been widely used for in vitro studies of osteogenesis. We used these cells in the present study to investigate the mechanism by which miR-17-3p regulates osteoblast differentiation. Interestingly, the miR-17-92 cluster has been shown to regulate bone growth and development; as a mature miRNA within this cluster, miR-17-3p has been suggested to play an essential role in bone formation [25], which is supported by the current findings.

MiRNAs mainly act by modulating the expression of target transcripts. Numerous miRNAs are known to participate in osteogenesis either as positive or negative regulators [26]. Sox6 encodes a transcription factor that is involved in the differentiation of various cell types including mesenchymal stem cells and neurons [27,28,14]. OS, which is common in children and adolescents, is characterized by impaired bone
formation resulting from abnormal osteogenesis [29]. Sox6 was shown to suppress proliferation, invasion, and epithelial-to-mesenchymal transition in OS cells by targeting TWIST1 [14]. Reduced differentiation and loss of function of osteoblasts are key features of osteoporosis and osteoarthritis. A large-scale meta-analysis identified Sox6 as a candidate gene that increased bone mineral density and thereby improved osteoporosis in women [30]. Sox6 and Sox9 are also important factors in cartilage homeostasis that stimulate cartilage formation, which may promote bone growth and prevent osteoarthritis [31]. In our study, we found that miR-17-3p suppressed osteoblast differentiation by downregulating the expression of Sox6, although the clinical significance of this observation in the context of OS or osteoporosis remains to be determined. Additionally, the detailed mechanism of Sox6 regulation by miR-17-3p in osteogenesis warrants further study, although there is evidence suggesting that Sox6 specifically activates enhancers of target genes in primary osteoblasts [32].

In conclusion, the results of our study demonstrate for the first time that miR-17-3p negatively regulates osteoblast differentiation by suppressing Sox6 expression. Thus, therapeutic strategies that inhibit miR-17-3p could potentially stimulate bone formation and may be an effective treatment for OS and orthopedic disorders such as osteoporosis and osteoarthritis.

Acknowledgements

The present study was supported by the Yunnan Scientific and Technology Committee and Kunming Medical University (Kunming, China) [Grant No.: 2018FE001(-199)]. We acknowledge and appreciate Dr. Ge Zhu (Changhai Hospital of Second Military Medical University) for his valuable efforts and constructive comments on this manuscript.

Conflict of interest

The authors confirm that they have no financial or non-financial conflicts of interest.

Author contribution

HY conceived and designed the entire study; NC and DW analyzed the data, performed literature research,
and drafted the paper. HL and YL were responsible for data analysis and visualization. HY guided the entire study and revised it critically for important intellectual content. All authors have read and agreed with the final version of this manuscript.

Data Availability Statement

Data will be available from the corresponding author with the final version of this manuscript.

References

1 Dong S, Yang B, Guo H and Kang F (2012) miRNAs regulate osteogenesis and chondrogenesis. *Biochemical and Biophysical Research Communications* **418**, 587–591.

2 Corrado A, Sanpaolo ER, Di Bello S and Cantatore FP (2017) Osteoblast as a target of anti-osteoporotic treatment. *Postgrad Med* **129**, 858–865.

3 Raggett LJ and Partridge NC (2010) Cellular and molecular mechanisms of bone remodeling. *J Biol Chem* **285**, 25103–25108.

4 Adamzyk C, Kachel P, Hoss M, Modabber A, Holzle F, Tolba R, Neuss S and Lethaus B (2016) Bone tissue engineering using polyetherketoneketone scaffolds combined with autologous mesenchymal stem cells in a sheep calvarial defect model. *J Cranio-maxillofac Surg* **44**, 985–994.

5 Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215–233.

6 Eric H and Izaurrelde E (2011) Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet* **12**, 99–110.

7 Eskildsen T, Taipaleemmäki H, Stenvang J, Abdallah BM, Ditzel N, Nossent AY, Bak M, Kauppinen S and Kassem M (2011) MicroRNA-138 regulates osteogenic differentiation of human stromal (mesenchymal) stem cells in vivo [Cell Biology]. *Proc Natl Acad Sci U S A* **108**, 6139–6144.

8 Li H, Xie H, Liu W, Hu R, Huang B, Tan YF, Xu K, Sheng ZF, Zhou HD, Wu XP and et al, (2009) A novel microRNA targeting HDAC5 regulates osteoblast differentiation in mice and contributes to primary osteoporosis in humans. *J Clin Invest* **119**, 3666–3677.

9 Schoolmeesters A, Eklund T, Leake D, Vermeulen A, Smith Q, Aldred SF and Fedorov Y (2009) Functional profiling reveals critical role for miRNA in differentiation of human mesenchymal stem cells. *PLoS One* **4**, e5605.

10 Baglio SR, Devescovi V, Granchi D and Baldini N (2013) MicroRNA expression profiling of human bone marrow mesenchymal stem cells during osteogenic differentiation reveals Osterix regulation by miR-31. *Gene* **527**, 321–331.

11 Deng ZL, Sharff KA, Tang N, Song WX, Luo JY, Luo XJ, Chen J, Bennett E, Reid R, Manning D et al, (2008) Regulation of osteogenic differentiation during skeletal development. *Front Biosci* **13**, 2001–2021.

12 Karsenty G (2003) The complexities of skeletal biology. *Nature* **423**, 316–318.

13 Zou L, Zou X, Li H, Mygind T, Zeng Y, Lü N and Bünger C (2006) Molecular mechanism of osteochondrogenesis fate determination during bone formation. *Adv Exp Med Biol* **585**, 431–441.

14 Wang Z, Li J, Li K and Xu J (2018) SOX6 is downregulated in osteosarcoma and suppresses the migration, invasion and epithelial-mesenchymal transition via TWIST1 regulation. *Mol Med Rep* **17**, 6803–6811.

15 Hu R, Liu W, Li H, Yang L, Chen C, Xia ZY, Guo LJ, Xie H, Zhou HD, Wu XP et al, (2011) A Runx2/miR-3960/miR-2861 regulatory feedback loop during mouse osteoblast differentiation. *J Biol Chem* **286**, 12328–12339.

16 Zhang J, Tu QS, Bonewald LF, He X, Stein G, Lian J and Chen J (2011) Effects of miR-335-5p in modulating osteogenic differentiation by specifically downregulating Wnt antagonist DKK1. *J Bone Miner Res* **26**, 1953–1963.

17 Mizuno Y, Tokuzawa Y, Ninomiya Y, Yagi K, Yatsuka-Kanesaki Y, Suda T, Fukuda T, Katagiri T, Kondoh Y, Amemiya T et al, (2009) miR-210 promotes osteoblastic differentiation through inhibition of AvcR1b. *FEBS Lett* **583**, 2263–2268.

18 Li ZY, Hassan MQ, Jafferji M, Aqeilan RI, Garzon R, Croce CM, van Wijnen AJ, Stein JL, Stein GS and Lian JB (2009) Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. *J Biol Chem* **284**, 15676–15684.

19 Tu XM, Gu YL and Ren QG (2016) miR-125a-3p targetedly regulates GIT1 expression to inhibit osteoblastic proliferation and differentiation. *Exp Ther Med* **12**, 4099–4106.

20 Liu S, Gao G, Yan D, Chen X, Yao C, Guo S, Li G and Zhao Y (2017) Effects of miR-145-5p through NRAS on the cell proliferation, apoptosis, migration, and invasion in melanoma by inhibiting MAPK and PI3K/AKT pathways. *Cancer Med* **6**, 819–833.

21 Fang T, Wu Q, Zhou L, Mu S and Fu Q (2016) miR-106b-5p and miR-17-5p suppress osteogenic differentiation by targeting Smad5 and inhibit bone formation. *Exp Cell Res* **347**, 74–82.

22 Yang X, Du WW, Li H, Liu F, Khorshidi A, Rutnam ZZ and Yang BB (2013) Both mature miR-17-5p and passenger strand miR-17-3p target TIMP3 and induce prostate tumor growth and invasion. *Nucleic Acids Res* **41**, 9688–9704.

23 Asakura K, Kadota T, Matsuzaki J, Yoshida Y, Yamamoto Y, Nakagawa K, Takizawa S, Aoki Y,
Nakamura E, Miura J et al, (2020) A miRNA-based diagnostic model predicts resectable lung cancer in humans with high accuracy. Commun Biol 3, 134.

24 Shincy JS, Panagal M, Jereena J, Vengatagiri GY, Vittalrao KR, Sivakumar P, Gopinath V, Kumar KM and Sekar D (2017) Computational identification of microRNA-17-3p in breast cancer cells. MicroRNA (Sharjah, United Arab Emirates) 6, 208–212.

25 Bai X, Hua S, Zhang J and Xu S (2019) The MicroRNA family both in normal development and in different diseases: the miR-17-92 cluster. BioMed Res Int 2019, 9450240.

26 Jia B, Zhang Z, Qiu X, Chu H, Sun X, Zheng X, Zhao J and Li Q (2018) Analysis of the miRNA and mRNA involved in osteogenesis of adipose-derived mesenchymal stem cells. Exp Ther Med 16, 1111–1120.

27 Wang H, Shan XB, Qiao YJ, Wang H, Shan XB and Qiao YJ (2017) PDK2 promotes chondrogenic differentiation of mesenchymal stem cells by upregulation of Sox6 and activation of JNK/MAPK/ERK pathway. Braz J Med Biol Res 50, e5988.

28 Hamadakanazawa M, Ogawa D, Takano M and Miyake M (2016) Sox6 suppression induces RA-dependent apoptosis mediated by BMP-4 expression during neuronal differentiation in P19 cells. Mol Cell Biochem 412, 49–57.

29 Wagner ER, Luther G, Zhu G, Luo Q, Shi Q, Kim SH, Gao JL, Huang E, Gao Y, Yang K et al, (2011) Defective osteogenic differentiation in the development of osteosarcoma. Sarcoma 2011, 325238.

30 Hsu YH, Zillikens MC, Wilson SG, Farber CR, Demissie S, Soranzo N, Bianchi EN, Grundberg E, Liang L, Richards JB et al (2010) An integration of genome-wide association study and gene expression profiling to prioritize the discovery of novel susceptibility Loci for osteoporosis-related traits. PLoS Genet 6, e1000977.

31 Liu CF and Lefebvre V (2015) The transcription factors SOX9 and SOX5/SOX6 cooperate genome-wide through super-enhancers to drive chondrogenesis. Nucleic Acids Res 43, 8183–8203.

32 Kawane T, Komori H, Liu W, Moriishi T, Miyazaki T, Mori M, Matsuo Y, Takada Y, Izumi S, Jiang Q et al (2014) Dlx5 and mef2 regulate a novel runx2 enhancer for osteoblast-specific expression. J Bone Miner Res 29, 1960–1969.