



Down-regulation of miR-19b-3p enhances IGF-1 expression to induce osteoblast differentiation and improve osteoporosis

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ABSTRACT

This research was conducted in order to investigate the role of miR-19b-3p in the development of osteoporosis (OP) in rats and the associated mechanisms. This study measured the expression levels of miR-19b-3p and IGF-1 in clinical OP patients and ovariectomy-induced OP rats by qRT-PCR. The osteoprotegerin levels in OP patients were measured by enzyme-linked immunosorbent assay (ELISA). The binding site of miR-19b-3p to IGF-1 was predicted by three prediction sites: Target Scan, miRDB and starbase. Experiments were conducted *in vitro* and *in vivo* using bone marrow mesenchymal stem cells (BMSCs) and OP rats, respectively, to verify the regulatory relationship between miR-19b-3p and IGF-1 and explore the role of miR-19b-3p in the development of OP. Results showed that the expression of miR-19b-3p was elevated in OP patients and rats, while IGF-1 expression was decreased (***) $p < 0.001$. The ELISA assay found that osteoprotegerin levels were inversely correlated with miR-19b-3p and positively correlated with IGF-1. The predictive analysis identified binding sites for miR-19b-3p to IGF-1. The potential regulatory relationship between miR-19b-3p and IGF-1 was validated by *in vitro* and *in vivo* experiments. Moreover, the important role of miR-19b-3p in the regulation of OP was further demonstrated. It was concluded the inhibition of miR-19b-3p has a suppressive effect on the development of OP and the function of miR-19b-3p in OP is likely to be achieved by regulating the expression of the IGF-1 gene.

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Introduction

Osteoporosis (OP) is a systemic disorder of bone metabolism that occurs mainly in the elderly with reduced bone density and degeneration of bone microarchitecture, thereby increasing bone fragility and fracture risk (1-3). As the global population ages, the number of patients suffering from OP increases every year, which places a heavy burden on the country's healthcare system. Therefore, it has become imperative to find effective treatment options to reduce the financial burden on the land and improve the quality of life of the elderly.

The differentiation of BMSCs plays an important role in maintaining normal bone homeostasis (4,5). The increasing evidence suggests that induction of adipocyte differentiation impairs bone formation, while increased osteogenic differentiation is essential for increased bone mass (6-8). Hence, a clear understanding of the pathogenesis of OP requires a complete dissection and testing of the differentiation process of BMSCs. The differentiation of BMSCs

into osteoblasts is regulated by several transcription factors, such as IGF-1, Osterix, Runx2 and others (9-12). It is essential to understand how these factors regulate the differentiation of BMSCs towards osteoblasts and their function in the development of drugs for the treatment of OP and inflammatory bone diseases.

MicroRNAs (miRNAs, miRs) are a group of small non-coding RNAs of approximately 18-25 nucleotides (13). miRNAs induce degradation or reduce translation of target mRNAs by binding to the 3'UTR (12,13). miRNAs are associated with various cellular processes, including cell proliferation, differentiation and apoptosis (13). Meanwhile, miRNAs are able to regulate the differentiation of osteoblasts and osteoclasts. For example, miR-140-3p expression is increased in older people with OP. It affects the development of OP by regulating the expression of PTEN. The high expression of miR-140-3p hinders the differentiation of osteoblasts, resulting in reduced bone mass in patients (14).

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miR-19b-3p is a relatively common microRNA. miR-19b-3p has been found to function in a variety of disease species. It is a potential molecular marker for a number of diseases, including colon cancer, gastric cancer, acute myocardial infarction and Alzheimer's disease (15,16). In the present study, we demonstrated that miR-19b-3p inhibited the differentiation of BMSCs towards osteoblasts and improved the disease sign in OP rats. In addition, the role and function of miR-19b-3p in regulating the development of OP was also explored. miR-19b-3p may play an important role in developing OP by regulating IGF-1 expression.

Materials and methods

Subjects

A total of 63 subjects were recruited for the study, including 32 healthy subjects (Control group) and 31 OP patients. The diagnostic criteria for patients with OP were based on dual-energy X-ray measurements of the bone mineral density of the lumbar spine and hip bones, which were below 2.5% of the standard value. All clinical procedures were approved by the Affiliated Huaian No.1 People's Hospital of Nanjing Medical University Clinical Ethics Committee. All samples were obtained with signed informed consent. The Sprague-Dawley (SD) rats were purchased from the Institute of Model Animals, Nanjing University. All animal protocols were in accordance with the Nanjing Medical University Institutional Animal Care and Use Ethics Committee.

qRT-PCR assay

miRNA was reverse transcribed into complementary DNA (cDNA) by the PrimeScript one-step cDNA synthesis kit (Takara, Tokyo, Japan). mRNA was reverse transcribed into cDNA by the first-strand cDNA synthesis kit (Takara). The qRT-PCR analysis was performed using Power SYBR Green QRT-PCR (Life Technologies, Shanghai, China) as the fluorescent moiety during amplification and TaqMan Master Mix (Applied Biosystems, CA, USA) for the PCR reaction. The relative levels of each miRNA or mRNA were quantified by the $2^{-\Delta\Delta CT}$ method, using U6 and GAPDH as endogenous controls for miRNA and mRNA, respectively.

Enzyme-linked immunosorbent assay (ELISA)

The levels of osteoprotegerin in the patients were assessed using their plasma and according to the instructions for the use of the Human Osteoprotegerin MultiSciences kit (Hangzhou, China).

Isolation and culture of BMSCs

After pentobarbital sodium anesthesia, thirty healthy SD rats, 12 weeks old, were selected and executed. The lower limbs of the rats were separated bilaterally, the epiphyses were removed and the bone marrow was poured out of the bone marrow cavity. BMSCs were gently blown loose in phosphate buffer (PBS) to make a single cell suspension and centrifuged at 1200 r/min for 10 minutes. After discarding the supernatant, the cells were resuspended in a DMEM medium, inoculated into a 25 cm² culture medium and then incubated at 5% CO₂ and 37°C, changing the solution every two days. Cell morphology and growth status were observed under an inverted microscope. The cells were fused, digested with 0.25% trypsin and then passaged at a ratio of 1:2.

Cell transfection and processing

The resulting BMSCs were plated in 24-well plates and incubated for 24 hours. The synthetic pEGFP-N1-IGF-1 and miR-19b-3p mimics were then added and transfected into BMSCs cells to analyse the biological function of miR-19b-3p. Three experimental groups were then established to investigate the potential correlation between miR-19b-3p and osteogenic differentiation: the miR-NC group (negative control, Control), the miR-19b-3p mimics transfection group and the miR-19b-3p mimics+ pEGFP-N1-IGF-1 co-transfection group (miR-19b-3p mimics+p- IGF-1). According to the manufacturer's instructions, all materials were purchased from RiboBio (Guangzhou, China) and transfected using lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA). Osteogenic differentiation of BMSCs to chondrocytes was induced 36 h post-transfection.

Induction of differentiation of BMSCs into osteoblasts

BMSCs were inoculated into the medium at a density of $2 \times 10^4 / \text{cm}^2$ after transfection of the above plasmids. The osteogenic induction medium (α -MEM induction solution containing 10 $\mu\text{mol/L}$ dexamethasone, 50 $\mu\text{mol/L}$ Vitamin C, 10 mmol/L β -phosphoglycerate and 100 mL/L fetal bovine serum FBS) was used when the cells had grown and covered 70%-80% of the bottom of the wells. The freshly differentiated medium was changed every three days and the cells were cultured for 21 days.

Dual-luciferase report analysis

The binding site between miR-19b-3p and IGF-1 was predicted by Target Scan, miRDB and starbase. To validate miR-19b-3p targeting of IGF-1, BMSCs (2×10^4 cells/ml, 200 μl) were inoculated into 48-well plates and transfected with 400 ng of wild-type IGF-1 3'-UTR or mutated IGF-1 3'-UTR along with 50 nmol/l miR-19b-3p NC as a negative control or miR-19b-3p cloned into the psiCHECK2 plasmid (Promega Corporation, Madison, WI, USA). After 6 hours, BMSCs cells were incubated in DMEM for an additional 48 hours. Luciferase activity was measured by a multifunctional enzyme marker (Promega, Madison, WI, USA), and the results were normalised to Renilla fluorophore activity.

MTT for cell proliferation

The cells were collected 21 days after induction of osteogenic differentiation and inoculated into 96-well plates at a density of 2×10^3 cells for 48 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/mL , MultiSciences, Hangzhou, China) was subsequently added to each well. Then, 150 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well to dissolve the formed methanamine. The absorbance was measured at 490 nm by an enzymatic calibrator after 24 h, 48 h, 72 h and 96 h (Bio-Rad, Hercules, CA, USA).

Cell viability assay

The cell viability was measured using the WST 1 assay (Roche Diagnostics). The cells (5×10^3) were inoculated in 96-well plates. WST 1 was added to the cell supernatant according to the instructions for the

instrument and incubated at 37°C for 3 hours in the dark. The absorbance at 450-630 nm was measured using an iD3 ELISA (Molecular Devices, USA).

Apoptosis analysis

The effect of miR-19b-3p on the apoptosis of BMSCs was detected by flow cytometry. 1×10^6 cells were inoculated in 6-well plates and cultured at 37°C , with 95% humidity and 5% CO_2 . After collection, the cells were stained using Annexin V FITC/PI Apoptosis Detection Kit (Sigma Aldrich, Merck KGaA) for 10 mins at room temperature in the dark and then counted by BeamCyte flow cytometry (BITC Bio, Changzhou, China) and analysed by CytoSYS 1.1 software.

Animal experiments

Twenty-four healthy male SD rats (license number: Scxk 2019-0035), 6 to 7 weeks old, weighing 200 ± 20 g, were provided by the Guangdong Medical Laboratory Animal Centre, China. The rats were cultured in SPF conditions. The rats were randomly divided into three groups: sham-operated group (Sham), OP (OP) group, and antago-miR-19b-3p treated group (antago-miR-19b-3p). Adipose tissue was excised near the ovaries in the Sham group, while bilateral ovariectomy was used to construct OP model rats in the OP and antago-miR-19b-3p groups ($n=8$ per group). Four weeks later, when the OP rat model was established, the OP group received 500 μl PBS twice a month for three months by injecting the periosteum into the marrow cavity of the femur. antago-miR-19b-3p group received antago-miR-19b-3p treatment twice a month by injecting the periosteum into the marrow cavity of the femur (80 μmo in 100 μl PBS / L) for three months. After three months, all rats were executed, and femurs were collected for further analysis.

Analysis of alkaline phosphatase (ALP) activity

The activity of ALP, a marker of early osteoblast differentiation, was measured on day 14 after differentiation towards osteogenesis in each group of rats using a phosphatase assay kit (Beyotime Institute of Biotechnology) with an absorbance of 405 nm.

Data processing and analysis

Statistical analysis is performed using GraphPad Prism and indicated inside each figure legend. Data presented in this study were first verified for normal distribution by the D Agostino Pearson normality test. If normally distributed, Student s t-test is used for pairwise comparisons, and one-way ANOVA is used for comparisons among multiple groups, followed by Tukey s multiple comparisons. If not normally distributed, the Mann Whitney U test is used for pairwise comparisons. The Kruskal Wallis test is used for comparisons among multiple groups, followed by Dunn s multiple comparisons.

Results and discussion

miR-19b-3p was significantly increased in OP patients and OP rats, while IGF-1 expression was down-regulated

To investigate the role of miR-19b-3p and IGF-1 in OP disorders, we examined the expression levels of miR-19b-3p and IGF-1 in OP patients and rats. We collected blood samples from a total of 31 OP patients as well as 32 healthy subjects as controls. As shown in Figure-1A and B, plasma levels of miR-19b-3p were significantly higher in patients with OP compared to healthy subjects, while IGF-1 expression was suppressed. Meanwhile, a strong negative correlation between serum miR-19b-3p levels and osteoprotegerin (OPG) concentrations was observed in patients with OP, while IGF-1 expression was positively correlated with OPG (Figure-1C and D). Subsequently, we assessed miR-19b-3p levels in sham-operated and OP rats. The results showed that miR-19b-3p levels were significantly increased in OP rats compared to sham mice, whereas IGF-1 expression was downregulated (Figure 1E and F, ***p<0.001). Overall, these data suggest that miR-19b-3p and IGF-1 expression are closely associated with OP disorders.

IGF-1 was a direct target of miR-19b-3p, and its expression was inhibited by miR-19b-3p

To investigate the potential relationship between miR-19b-3p and IGF-1, we predicted the relationship between miR-19b-3p and IGF-1 using three prediction sites, Target Scan, miRDB and starbase, to elucidate whether IGF-1 was a possible target of miR-19b-3p. Finally, we found that IGF-1 was a putative target of miR-19b-3p (Figure 2-A). To verify whether miR-19b-3p had a regulatory effect on IGF-1, we established a luciferase reporter gene vector containing the binding

region of wild-type or mutant IGF-1 3'UTR to miR-19b-3p. The luciferase activity of the wild-type IGF-1 3' UTR with miR-19b-3p reporter gene was significantly lower than that of the normal control. In contrast, the luciferase activity of the mutant IGF-1 3' UTR with miR-19b-3p reporter gene was not significantly different from that of the normal control (Figure-2B, ***p< 0.001). These results suggest that miR-19b-3p may regulate the expression of IGF-1.

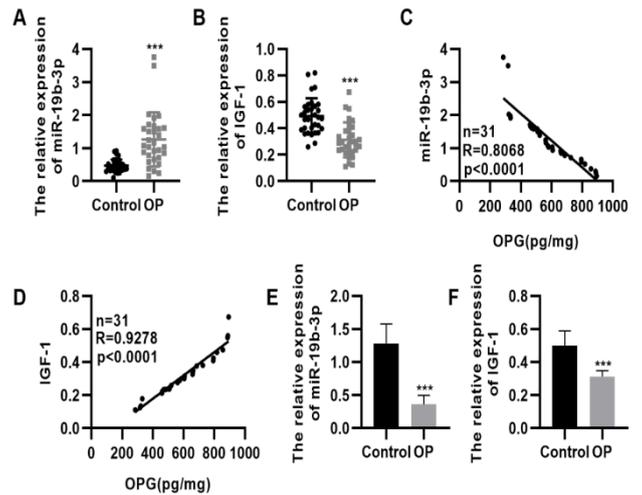


Figure 1. miR-19b-3p expression was elevated in OP patients and OP rats, whereas IGF-1 expression was down-regulated (A and B); the expression levels of miR-19b-3p and IGF-1 in OP patients were detected by qRT-PCR (C and D); the correlation between OPG and miR-19b-3p and IGF-1 expression (E and F); the expression levels of miR-19b-3p and IGF-1 in OP rats were detected by qRT-PCR. ***p<0.0001, Mann-Whitney U test (A), unpaired Student's test (B, E and F).

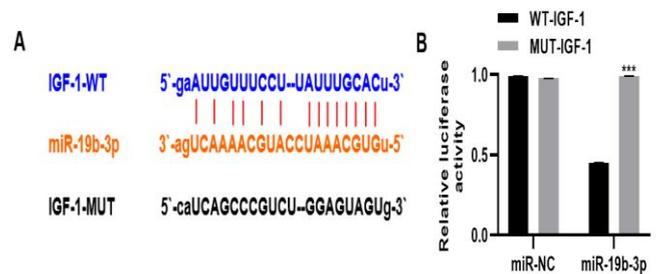


Figure 2. IGF-1 gene was a potential target of miR-19b-3p. (A) The binding site of IGF-1 gene to miR-19b-3p as predicted by Target Scan; (B) The relevant activityluciferase assay. ***p<0.0001, unpaired Student's test.

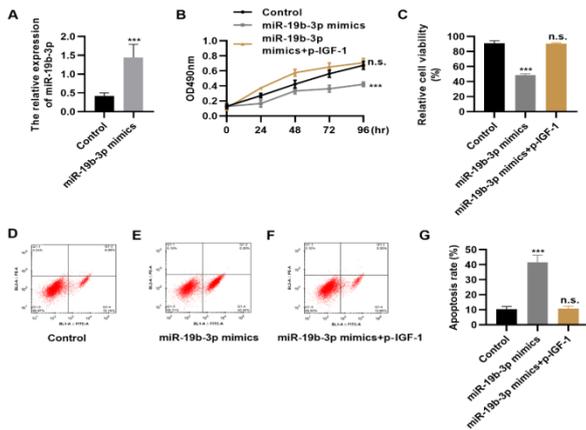


Figure 3. The overexpression of miR-19b-3p inhibited the proliferation and activity of BMSCs and promoted apoptosis. (A) miR-19b-3p mimics promoted miR-19b-3p expression in BMSCs; (B) miR-19b-3p mimics inhibited the proliferation of BMSCs and p-IGF-1 redeemed the inhibitory effect of miR-19b-3p mimics; (C) miR-19b-3p mimics inhibited the activity and p-IGF-1 redeemed the inhibitory effect of miR-19b-3p mimics; (D-G) Apoptosis detection by flow cytometry. n.s., not significant, *** $p < 0.0001$, unpaired Student's test (A, Kruskal-Wallis test followed by Dunn's multiple comparisons test (C and G).

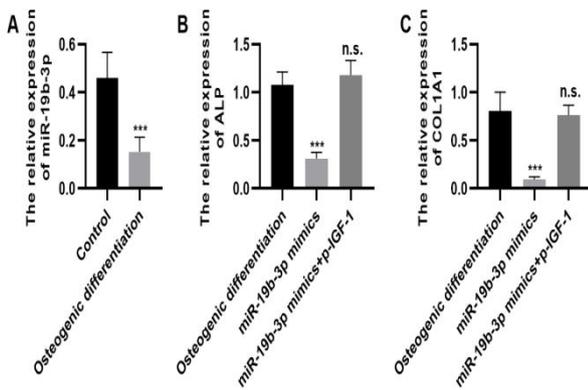


Figure 4. The overexpression of miR-19b-3p inhibited the differentiation of BMSCs towards osteoblasts. (A) The expression of miR-19b-3p was lower during differentiation of BMSCs to osteoblasts; (B and C) miR-19b-3p mimics inhibited the expression of ALP and COL1A1 during osteoblast differentiation. n.s., not significant, *** $p < 0.0001$, unpaired Student's test (A), Kruskal-Wallis test followed by Dunn's multiple comparisons test (B and C).

The overexpression of miR-19b-3p inhibited BMSCs activity

To test the function of miR-19b-3p on the proliferation of BMSCs, we used miR-19b-3p mimics to enhance miR-19b-3p expression in BMSCs (Figure-3A, *** $p < 0.001$). The cell viability and proliferation were subsequently examined 21 days after osteogenic

differentiation. The results showed that miR-19b-3p significantly inhibited the proliferation and viability of BMSCs (Figure-3B-C, *** $p < 0.001$). Next, to verify the potential regulatory relationship existing between miR-19b-3p and IGF-1, we proceeded to transfect p-IGF-1 expression plasmids in the above miR-19b-3p mimics group of BMSCs. It was found that promoting IGF-1 expression enhanced the proliferation of BMSCs and redeemed the reduced viability of BMSCs caused by miR-19b-3p mimics (Figure-3 B-C, *** $p < 0.001$). Similarly, the effect of the different treatments on the apoptosis of BMSCs was again examined using flow cytometry, which revealed that miR-19b-3p mimics promoted the apoptosis of BMSCs, while the p-IGF-1 plasmid inhibited this effect (Figure-3 D-G, *** $p < 0.001$). These results suggest that the activity inhibition and pro-apoptosis of BMSCs by miR-19b-3p are likely to be mediated through the regulation of IGF-1 expression.

The overexpression of miR-19b-3p inhibited the differentiation of BMSCs into osteoblasts

To verify whether miR-19b-3p played a regulatory role in the process of osteogenic differentiation, we induced targeted differentiation of BMSCs using osteogenic induction medium. We detected miR-19b-3p expression after inducing osteogenic differentiation of BMSCs using quantitative qRT-PCR assay (Figure-4A, *** $p < 0.001$). As expected, the expression level of miR-19b-3p was lower during osteogenic differentiation of BMSCs than control (Figure-4A, *** $p < 0.001$). The expression of ALP started to increase early in the differentiation of BMSCs towards osteogenesis and remained high throughout the differentiation process of osteoblasts. Therefore, ALP can be used as an early molecular marker of osteogenic differentiation. We conducted experiments using miR-19b-3p mimics and p-IGF-1. The results revealed that miR-19b-3p significantly inhibited the expression of osteoblast marker genes ALP and COL1A1 mimics, while the inhibition of ALP and COL1A1 expression by miR-19b-3p mimics was improved considerably by the transfer of p-IGF-1 (Figure-4B-C, *** $p < 0.001$). The results suggest that miR-19b-3p may have a regulatory effect on osteogenic differentiation of BMSCs via IGF-1.

The inhibition of miR-19b-3p improved bone loss in OP rats

To further confirm the effect of miR-19b-3p on OP disorders, we treated induced OP rats with antago-miR-19b-3p. The rats in the OP group significantly reduced bone trabecular volume/tissue volume (BV / TV), bone trabecular number (Tb.N.), and bone trabecular thickness (Tb. Th), while bone mineral density (BMD) levels in the rats were also significantly decreased (Figure 5A-D, *** $p < 0.001$). In contrast, antago-miR-19b-3p-treated rats redeemed the pathology of bone loss (Figure 5A-D, *** $p < 0.001$). Overall, these data suggest that miR-19b-3p may play a key role in regulating the development of OP rats.

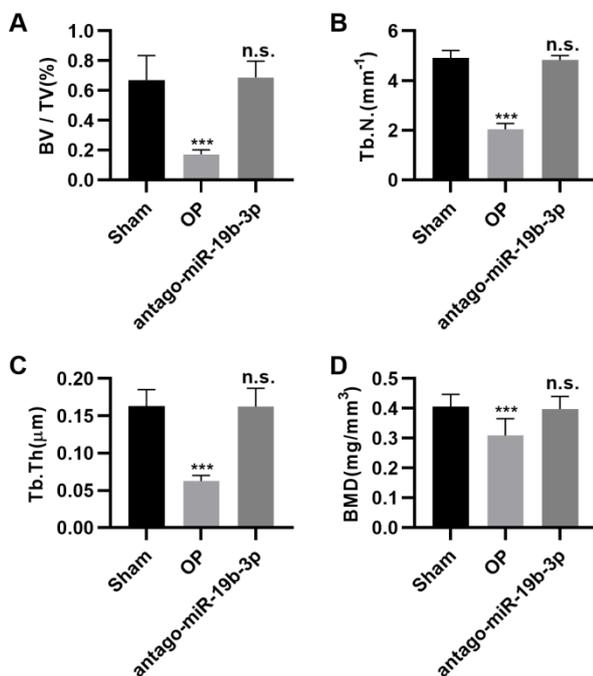


Figure 5. The inhibition of miR-19b-3p expression improved the skeletal pathology of OP rats. (A-D) The inhibition of miR-19b-3p expression increased the BV/TV ratio, Tb.N., Tb. Th, and BMD levels in OP rats. n.s., not significant, *** $p < 0.0001$, Kruskal-Wallis test followed by Dunn's multiple comparisons test.

The inhibition of miR-19b-3p promoted the expression of osteogenic markers in OP rats

To further verify that miR-19b-3p could promote osteogenesis and differentiation in OP rats, we examined the activity of ALP and the expression of osteogenic markers ALP and COL1A1 in OP rats treated with antago-miR-19b-3p. The results showed that antago-miR-19b-3p enhanced the activity of ALP and promoted the expression of ALP and COL1A1

(Figure 6A-D). This result reaffirms the role of miR-19b-3p in regulating the development of OP rats.

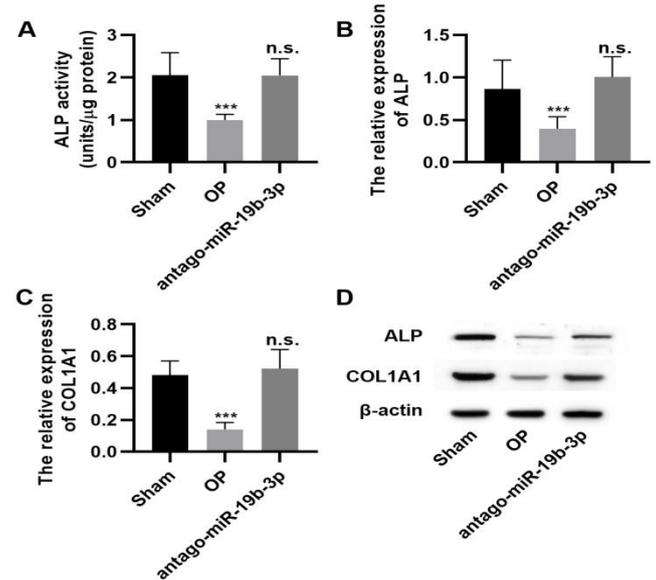


Figure 6. The inhibition of miR-19b-3p promoted the expression of ALP and COL1A1 in OP rats. (A) The inhibition of miR-19b-3p expression enhanced the activity of ALP in OP rats; (B) The inhibition of miR-19b-3p promoted the gene expression of ALP in OP rats; (C) The inhibition of miR-19b-3p promoted the gene expression of COL1A1 in OP rats; (D) Western blot showed that the inhibition of miR-19b-3p promoted the OP rat protein expression of ALP and COL1A1. n.s., not significant, *** $p < 0.0001$, Kruskal-Wallis test followed by Dunn's multiple comparisons test.

In bone metabolism, related miRNAs can affect bone regeneration and remodelling. Moreover, the aberrant expression of miRNAs is closely associated with OP disorders (13,17-19). Further study of these miRNAs will help us to better understand bone metabolism and the pathogenesis of OP disorders.

miR-19b-3p is a type of miRNA, and its relevance to OP has not been demonstrated. However, miR-19b-3p has a potential relationship with a variety of diseases (20,21). Among others, miR-19b-3p promotes proliferation and migration of colon cancer cells by targeting SMAD4 and enhances chemoresistance to the anticancer drug oxaliplatin in colon cancer (15). In Alzheimer's patients, miR-19b-3p plays another active role in preventing amyloid damage by targeting BACE1 in SH-SY5Y cells (22). In the present study, we investigated the role of miR-19b-3p in BMSC differentiation and OP progression. A higher level of miR-19b-3p and a lower level of IGF-1 were found in OP patients compared to healthy volunteers. miR-19b-3p in serum of OP patients was negatively correlated

with osteoprotegerin. Therefore, we suggest that miR-19b-3p may be a potential biomarker for OP disorder and that promoting miR-19b-3p expression levels may contribute to the development of OP disorder.

OP is a complex pathophysiological process that depends on the amount of differentiation of BMSCs towards osteoblasts and the ability of different cells such as osteoblasts and osteoclasts to survive (23-27). miR-19b-3p had been linked to IGF-1, as well as to BMSCs based on increased levels of miR-19b-3p and decreased levels of IGF-1 in BMSCs of OP rats. The functions of miR-19b-3p were more clearly described under *in vitro* BMSCs culture experiments, particularly the ability of miR-19b-3p to inhibit osteogenic differentiation. In addition, an increase in osteogenic markers was clearly found in OP rats following inhibition of miR-19b-3p. These results underline the negative effects of miR-19b-3p.

It is well known that IGF-1 gene expression has an important role in processes such as cell proliferation, migration and differentiation, and its importance in osteogenic differentiation in particular has been well documented (28-30). In the present study, we identified the IGF-1 gene to be associated with miR-19b-3p-mediated osteogenic differentiation. We found evidence that miR-19b-3p inhibited IGF-1 expression and thus the differentiation of BMSCs towards osteogenesis through experimental validation. Similar to previous studies, we found that the inhibition of osteoblast differentiation by miR-19b-3p mimics was rescued by treatment with a p-IGF-1 plasmid (31). miR-19b-3p may regulate multiple targets, and IGF-1 was one of the main targets affecting BMSC differentiation. Therefore, further studies are necessary to investigate the potential mechanisms of miR-19b-3p on BMSC differentiation. Although multiple miRNAs have been reported to be associated with OP disorders, the origin of such dysregulated miRNAs remains unclear. A better understanding of the mechanisms of BMSCs differentiation may provide new ideas for OP disorders and other bone-reduction-related diseases. The current study demonstrated that miR-19b-3p regulated BMSCs differentiation by inhibiting IGF-1 (Figure-7). However, the pathogenesis of OP disorders is complex and the role of miR-19b-3p on other cell types, including osteoblasts and osteoclasts, requires further investigation.

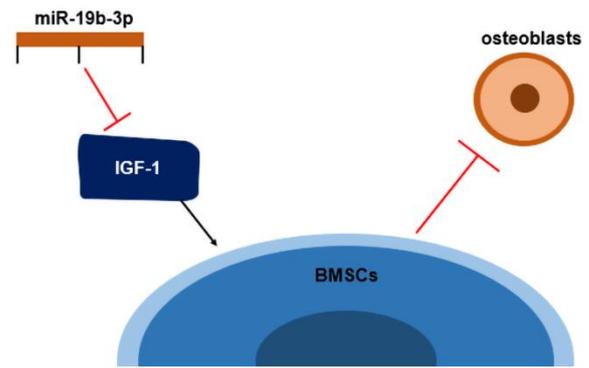


Figure 7. The functional model of the molecular mechanism by which miR-19b-3p regulates OP development. IGF-1 acted as a regulatory target of miR-19b-3p and regulated osteogenic differentiation of BMSCs.

Our findings identify a novel mechanism for miR-19b-3p in BMSC differentiation. miR-19b-3p inhibits IGF-1 expression, thereby suppressing the differentiation of BMSCs towards osteoblasts, which in turn promotes and induces the development of OP disorders. Thus, therapeutic interventions targeting miR-19b-3p hold great promise in treating OP disorders.

Acknowledgments

None.

Conflict interest

The authors declare no conflict of interest.

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