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The Regulatory Effect of Mir-181c-5p On the Differentiation Function of Bone Marrow Mesenchymal Stem Cells in Postmenopausal Osteoporotic Mice

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Abstract

Background: *Osteoporosis (OP) is a metabolic bone disease syndrome for which there is no good treatment. In this study, we investigated the expression changes of miR-181c-5p in osteoporosis-derived BMMSCs, and the role and molecular mechanism in the osteogenic-lipogenic differentiation of BMMSCs.*

Methods: *In this study, an OP mouse model was successfully established using the ovariectomy method, and osteoporotic-derived BMMSCs (O-BMMSCs) and sham-operated-derived BMMSCs (S-BMMSCs) were isolated and cultured using the whole bone marrow method. A genetic screen revealed that miR-181c-5p was differentially expressed in O-BMMSCs and S-BMMSCs. The expression levels of miR-181c-5p in BMMSCs were overexpressed or inhibited by cell transfection, and the regulatory effects of miR-181c-5p on the proliferation and osteogenic-adipogenic differentiation of BMMSCs were examined using MTT, multi-directional differentiation induction, alizarin red staining, oil red O staining, qRT-PCR and Western blot. Candidate target genes for miR-181c-5p were screened by target gene prediction software and bioinformatics websites, and target gene validation was performed.*

Results: *The study found that overexpression of miR-181c-5p or inhibition of miR-181c-5p had no significant effect on the proliferation ability of BMMSCs. Upregulation of miR-181c-5p could reduce the osteogenic ability and enhance the adipogenic ability of BMMSCs, while downregulation of miR-181c-5p could increase the osteogenic ability and inhibit the adipogenic ability of BMMSCs. Besides, Foxo1 was confirmed as a direct target gene of miR-181c-5p, and miR-181c-5p negatively regulated Foxo1 expression. Downregulation of miR-181c-5p in O-BMMSCs promoted Foxo1 expression, improved the osteogenic differentiation of O-BMMSCs, and reduced abnormal lipogenic differentiation of O-BMMSCs and eventually partially restored the normal differentiation ability of O-BMMSCs.*

Conclusion: *miR-181c-5p regulated the osteogenic and adipogenic differentiation of BMMSCs by negatively regulating the expression of target gene Foxo1. The overexpression of miR-181c-5p in the process of osteoporosis leads to the disruption of the balance of osteogenic and adipogenic differentiation of BMMSCs, and reduces the bone formation ability of stem cells.*

Keywords: Osteoporosis, Mesenchymal stem cells, microRNA, Ovariectomy, Multidirectional differentiation.

Introduction

Osteoporosis (OP) is a metabolic bone disease syndrome characterized by osteopenia and destruction of bone microarchitecture. Patients have decreased osteogenic strength, increased fragility, and are prone to fractures. Postmenopausal osteoporosis, also known as type I OP, generally occurs about 10 years after menopause, and is most commonly seen clinically in women aged 60-80 [1]. At present, more than 200 million people in the world suffer from osteoporosis, and there are more than 88 million in China, and its incidence has jumped to the 7th place of common diseases and morbidities[2]. The underlying cause is an imbalance between osteoblastic bone resorption and osteoblastic bone formation, resulting in a decrease in bone mass and structural changes in bone tissue, which increases bone fragility and leads to fractures. Epidemiological studies in North America have shown that the risk of osteoporotic fractures in white women over 50 years of age is

17.5% for hip fractures, 15.6% for vertebral fractures and 16.9% for distal forearm fractures; the corresponding risks for men are 6.0%, 5.0% and 3.0% respectively [3]. These fractures (especially hip fractures) have a high incidence, poor prognosis, high disability rate and high medical costs, often causing a large financial burden to the patient, family and society. Therefore, the prevention and treatment of fractures in patients with postmenopausal osteoporosis (OPM) is one of the hot topics of clinical research in internal medicine and orthopaedic clinical research in recent years. However, the current clinical treatment of OPM is mainly based on hormone replacement, calcium supplementation and inhibition of bone resorption, but the treatment is not effective due to drug toxicity, malabsorption, unstable efficacy and poor patient compliance.

Bone marrow mesenchymal stem cells (BMMSCs) are adult stem cells derived from mesoderm with self-renewal and multi-directional differentiation potential [4]. BMMSCs play an important role in bone development, regeneration and repair by differentiating into various cells. BMMSCs are the source cells of both osteoblasts and adipocytes, and the balance of their osteogenic-adipogenic differentiation capacity is closely related to the homeostatic balance of bone remodeling. In the process of osteoporosis, the osteogenic differentiation ability of BMMSCs decreases, while the adipogenic differentiation ability increases. This may be one of the reasons for osteoporosis, but the specific regulatory mechanism of the multi-directional differentiation of BMMSCs is still unclear.

MicroRNAs (miRNAs) are a newly discovered class of small non-coding RNAs that widely exist in animals and plants, and can bind to the 3′-untranslated region of target genes in a base-pairing manner [5]. The miRNA can silence the expression of target genes at the post-transcriptional level, and play important regulatory roles in a variety of physiological and pathological processes. A variety of miRNAs have been identified that are involved in the osteogenic-adipogenic differentiation process of stem cells and play an important role in regulating stem cell fate. However, it is still unclear which miRNAs play key roles in the multidirectional differentiation of BMMSCs, the molecular signaling pathways that specifically regulate the differentiation of BMMSCs, and whether miRNAs play a role in the occurrence and development of OPM.

In this study, BMMSCs were isolated and purified from normal mice and osteoporosis model mice [6]. It has been reported that miR-705 abnormally regulates the differentiation of bone marrow mesenchymal stem cells in postmenopausal osteoporotic mice. Studies have shown that BMMSCs from two sources, post-O-BMMSCs and post-S-BMMSCs, both express normal mesenchymal stem cell surface markers, but do not express hematopoietic system-derived cell surface markers, and have clonogenic ability. The osteogenic differentiation ability of O-BMMSCs was significantly lower than S-BMMSCs after osteogenic induction, while the adipogenic differentiation ability of O-BMMSCs after adipogenic induction was significantly higher than S-BMMSCs. Gene screening revealed that miR-181c-5p was differentially expressed between normal mice and osteoporosis model mice. In this experiment, miR-181c-5p was selected for further functional study.

The miR-181 family is an evolutionarily conserved family of microRNAs with a wide range of functions involved in the regulation of immune system function [7], vascular inflammatory response [8], and hematopoietic development [9]. In addition, the miR-181 family is aberrantly expressed in a variety of tumors, however the role in osteogenic differentiation is not reported. In osteoporosis caused by estrogen deficiency, high expression of miR-181a can inhibit the proliferation of BMMSCs and reduce the osteogenic capacity of bone marrow stromal cells, which may be one of the important factors affecting the pathogenesis of osteoporosis [10]. In addition, miR-181b was found to significantly reduce the osteogenic differentiation ability of BMMSCs in vitro [11].

This study further explored the role and mechanism of miR-181c-5p in the pathogenesis of OPM, both to deepen the understanding of the molecular mechanism of OPM pathogenesis and to clarify the underlying causes of bone loss in osteoporosis, and to provide important guidance for the prevention and treatment of various postmenopausal osteoporosis-induced fracture diseases.

2. Methods and Materials

2.1 Establishment of postmenopausal osteoporosis (OPM) mouse model

All protocols in this study were approved by the Committee on the Ethics of Animal Experiments of Yangzhou University Medical College, Xuzhou Medical University and the Xuzhou Council on Animal Care, Xuzhou, China, in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no.85-23, revised 1996). The study was performed in accordance with ARRIVE guidelines too.

Female healthy C57BL/6 mice (5 weeks old, weighing 15 ± 2 g) were purchased from the animal center. Mice were housed in cages with appropriate water and food at an ambient temperature of 25 °C ± 1 °C, humidity of 50% ± 5%, and light conditions with a 12-h light/dark cycle.

The procedure was performed according to previous reports [12]. Briefly, the mice in the castration group were anesthetized by inhaling isoflurane gas (300ml/min), the skin was disinfected, and an approximately 1.5 cm incision was made in the midline of the abdomen to expose the bilateral fallopian tubes and ovaries. Lateral ligation was performed, the ovaries and ligated fallopian tubes were removed, the wounds were sutured after the operation, and penicillin was injected intraperitoneally for 3 consecutive days after the operation. In the sham-operated group, only equal amounts of adipose tissue around the ovaries were excised, the wounds were sutured after the operation, and penicillin was injected intra-

peritoneally for 3 consecutive days after the operation. All animals at 3 months after surgery were weighed and recorded for data analysis and comparison.

2.2 Hematoxylin-Eosin (HE) staining for femoral bone morphology

The distal femoral stem ends of mice from the ovariectomized group (OVX) and the sham-operated group (SHAM) (5 mice each) were fixed overnight in 10% formalin at 3 months post-operatively. Then, decalcify with 10% EDTA (PH=7.4) at room temperature for 3 weeks, and replace the decalcification solution every 3 days. The decalcified specimens were rinsed overnight in running water, paraffin embedded and cut into paraffin sections of approximately 10 um thickness. The sections were routinely dewaxed and then stained with HE solution. Histomorphological observation and photography were performed under an optical microscope.

2.3 Isolation and culture of bone marrow mesenchymal stem cells (BMMSCs)

BMMSCs were isolated from C57BL/6 mice using a whole bone marrow apposition screening method [13]. briefly, bone marrow was isolated from the femur and tibia of the mice and all bone marrow cells were placed in DMEM culture medium containing 20% FBS and cultured in primary culture at 37 °C in a 5% CO2 incubator. After 48 h of incubation, the first full volume of fluid was changed to remove any cells that were not adherent to the wall, followed by half volume changes every 3 days. When the walled cells covered 80%-90% of the bottom area of the dish, the cells were digested with 0.25% trypsin. When BMMSCs were expanded to generation 3 for subsequent experiments.

2.4 Cell transfection

The miR-181c-5p mimics, miR-181c-5p inhibitor and mus-miR-negative control were synthesized by GenepPharma (Suzhou, China). The miR-181c-5p mimics, miR-181c-5p inhibitor and mus-miR-negative control were transfected into BMMSCs according to Lipofectamine 2000TM instructions (Thermofisher Scientific). Transfection efficiency was examined by qRT-PCR.

2.5 Osteogenic and adipogenic differentiation

To induce osteogenic-adipogenic differentiation, P2-5 BMMSCs were seeded into 6-well plates to about 80% confluence, and the osteogenic group was supplemented with DMEM containing 10% FBS, 10 mmol/L sodium β-glycerophosphate and 0.2 mmol/L ascorbic acid. The adipogenic group was supplemented with 0.01 uM dexamethasone, 0.5 mM IBMX, 60 uM indomethacin, 2 ml insulin.The medium was changed every other day during differentiation.

2.6 MTT assay

To detect the effect of miR-181c-5p on the proliferation of BMMSCs, BMMSCs were transfected with miR-181c-5p mimics or miR-181c-5p inhibitor, and then inoculated in well plate. Replaced with complete medium after 12 h, and continued to incubate for 6 days. Adding 20 ul MTT solution to the culture wells, incubated at 37°C for 4 h and then discarded. 150 ul DMSO was added and shaken for 10 min avoiding light. The OD value was detected with a microplate reader at 490 nm wavelength and the growth curve was drawn.

2.7 Alizarin red staining and quantification

The cells in osteoblast group were washed 3 times with PBS on 21 days, added with 4% paraformaldehyde for 15 min, and washed 3 times with distilled water. Then adding an appropriate amount of alizarin red staining solution at room temperature for 30 min, rinse with distilled water for 2 minutes, observe under a microscope and take pictures. For quantification, 10% cetylpyridinium chloride solution was added to the stained calcified nodule samples for 15 minutes, the lysate was collected, and the OD value of the lysate was detected by a microplate reader (wavelength 570 mm).

2.8 Oil Red O staining and quantification

The cells in the adipogenic group were washed 3 times with PBS on 10 days, then stained with oil red dye solution for 10 min, observed under a microscope and photographed. For quantification, isopropyl alcohol was added to the stained oil droplet samples for 10min, the lysate was collected and the OD of the lysate (wavelength 520mm) was detected by enzyme marker.

2.9 Quantitative Real-time PCR (qRT-PCR) analysis

Total RNA was extracted from BMMSCs after osteogenic or adipogenic differentiation using RNA-easy Isolation Reagent (Vazyme) and cDNA was then obtained using a reverse transcription kit. cDNA was subsequently quantified by real-time PCR using SYBR Green (Takara). primers were synthesized by Sangon Biotech (Shanghai, China). and the sequences are shown in the following table1 [14].

Table 1: Primer sequences for PCR

Gene	Sequence (5'-3')
RUNX2-F	TGTCATGGCGGGTAACGAT
RUNX2-R	AAGACGGTTATGGTCAAGGTGAA
PPAR-γ-F	ACTGCCGGATCCACAAAA
PPAR-γ-R	TCTCCTTCTCGGCCTGTG
Foxo1-F	ACGAGTGGATGGTGAAGAGC
Foxo1-R	TGCTGTGAAGGGACAGATTG
β-actin-F	CTGGAGAACATTCATTGCTGTC
β-actin-R	GTGCAGGGTCCGAGGT

2.10 Western blot assay

After osteogenic or adipogenic differentiation of S-BMMSCs and O-BMMSCs, cell samples were collected and operated according to the previously reported methods. Briefly, cells were lysed with RIPA lysis buffer (Beyotime) containing protease inhibitor cocktail (Roche) to obtain total protein. The supernatant was separated by centrifugation at 12,000g and the total protein content was assayed using a BCA kit (Beyotime). The proteins were separated by 12% SDS-PAGE and transferred onto PVDF membranes (Millipore). The membranes were then sealed with a sealing solution containing 5% skimmed milk powder. After sealing for 1 hour, the protein-containing membrane was incubated overnight at 4°C with primary antibodies, including Anti-mouse RUNX2 and Anti-mouse PPAR-γ (Abcam, USA). The next day the membrane was washed five times with TBS buffer containing 1% Tween. The membranes were then incubated with HRP-labelled secondary antibodies for 2 hours at room temperature and developed with ECL luminescent solution. Anti-mouse β-actin (Cell signaling, USA) was used as a control protein [14].

2.11 Dual luciferase reporting assay

Foxo1 and control plasmids were co-transfected with miR-7-5p mimic or mock control into BMMSCs cells. After 48 hours of transfection, cells were collected and luciferase activity was assessed using a dual luciferase assay kit (Solarbio).

2.12 Statistical analysis

All data were statistically analyzed using SPSS17.0 software, and measurement data were expressed as mean±standard deviation (SD). The t-test was used for comparison between two groups. All experiments were repeated more than 3 times. The datasets used and analysed during the current study available from the corresponding author on reasonable request,we will reply as soon as we receive the emails.

3 Results

3.1 Identification of the mouse OPM model

To identify the OPM mouse model, the weight of the mice was measured and samples were collected after 3 months of surgery. The body weight of the mice in the OVX group was significantly higher than SHAM group, indicating that ovariectomy caused excessive obesity in the mice, which was consistent with previous reports (Figure 1). HE staining was performed on the histological sections of the femur, and the comparison under the light microscope showed that the trabecular bone in the OVX group was slender, few in number and disorderly arranged, and osteoporosis such as enlarged medullary cavity appeared (Figure 2). These results proved that the mice showed significant osteoporosis in the femur and the OPM model was successfully established after the removal of the ovaries.

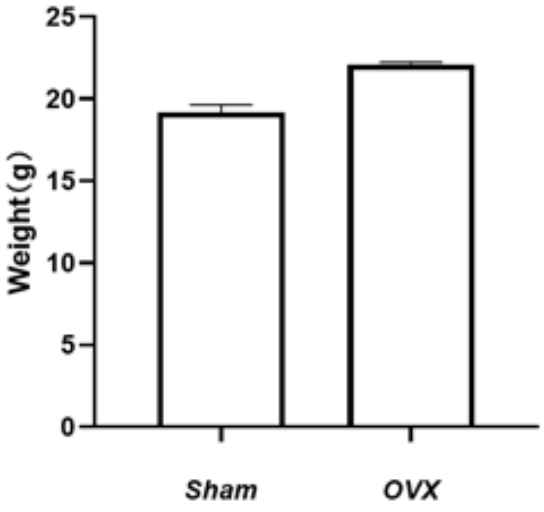

Figure 1: Comparison the body weight of mice in each group after 3 months of OPM surgery

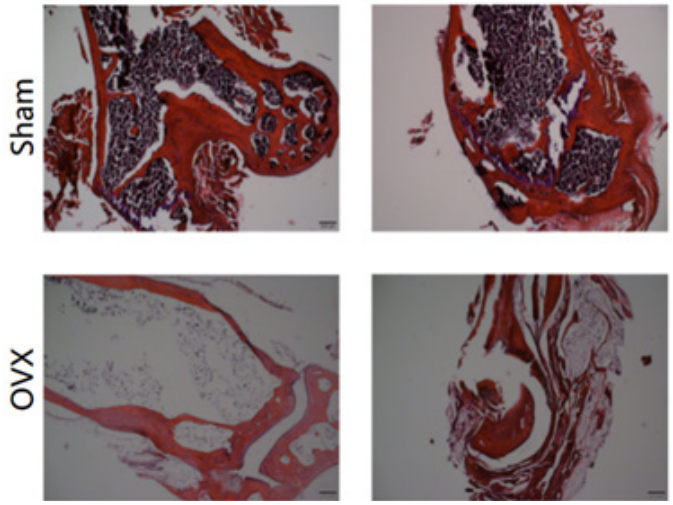

Figure 2: Bone morphology of the distal femur of mice in each group after 3 months of OPM surgery (n=5).

3.2 Compare the cell morphology of BMMSCs

BMMSCs were obtained from the bone marrow of mice and cultured to compare the morphology of cells in the normal and surgical groups. After three days of culture by whole bone marrow apposition method, both groups of BMMSCs were able to grow appositionally with similar cell morphology, both were shuttle-shaped or triangular, with little cytoplasm, large nuclei, strong refractive index and uneven cell protrusions (Figure 3A and C). After 12 days of culture, most of the cell colonies fused with each other and grew to the bottom of the bottle (Figure 3B and D).

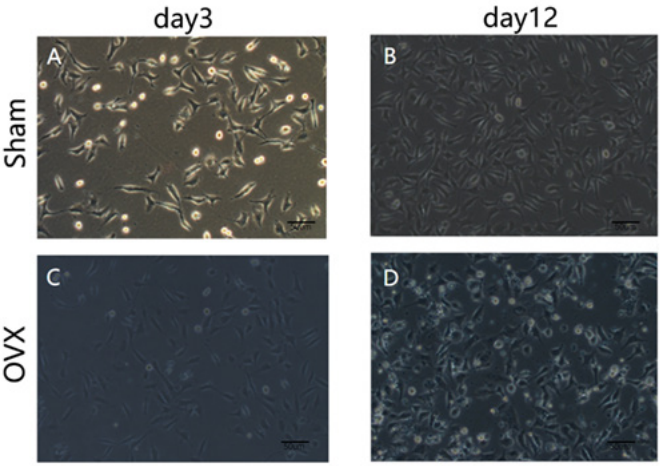

Figure 3: Optical microscope images of primary cultured S-BMMSCs and O-BMMSCs. (A) Growth and cell morphology of primary cultured S-BMMSCs on day 3 (×40); (B) Growth and cell morphology of primary cultured S-BMMSCs on day 12 (×100); (C) Growth and cell morphology of primary cultured O-BMMSCs on day 3 (×40); (D) Growth and cell morphology of primary cultured O-BMMSCs on day 12 (×100).

3.3 The miRNA screening in BMMSCs

Previous studies compared the miRNA expression profiles of O-BMMSCs and S-BMMSCs, and the screening found that the expression of miR-181c-5p was specifically increased in BMMSCs of OPM (Figure 4), suggesting that miR-181c-5p is related to the functional changes in BMMSCs of OPM. It was previously reported that the miR-181 family can reduce the osteogenic differentiation ability of BMMSCs [7] [8]. Based on the above evidence, we hypothesized that the abnormal increase of miR-181c-5p during the occurrence of OPM changes the differentiation phenotype of BMMSCs, causing an imbalance in their osteogenic-adipogenic differentiation balance and thus contributing to the development of OPM. Therefore, we further used synthetic miRNA mimics and inhibitors to specifically upregulate and downregulate the expression of miR-181c-5p in BMMSCs, and observe the changes in the expression level of miR-181c-5p on the biology of BMMSCs. Furthermore, the key target genes regulated by miR-181c-5p were further identified by bioinformatics analysis, and the molecular mechanism of its regulation of BMMSCs differentiation was discussed.

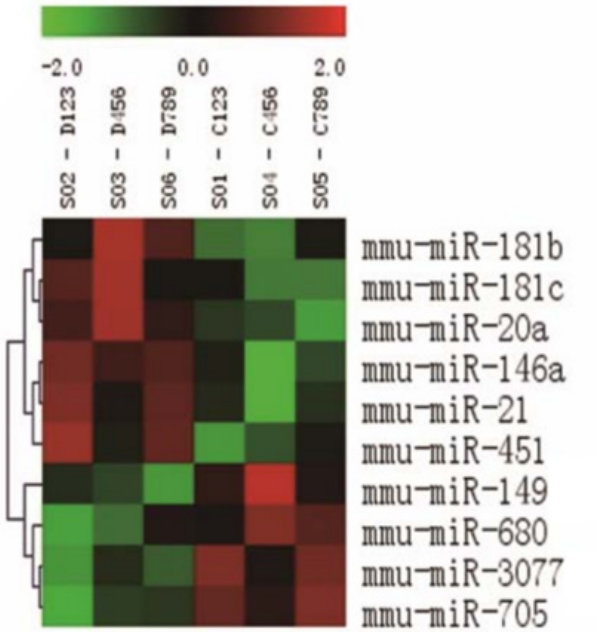

Figure 4: Heatmap of differentially expressed miRNAs in S-BMMSCs and O-BMMSCs miRNA microarray screening.

3.4 Functional characterization of chemically synthesized mimics and inhibitor

To test whether the synthesized miR-181c-5p mimics and miR-181c-5p inhibitor could specifically alter the expression level of miR-181c-5p in cells, we detected the expression levels of miR-181c-5p in BMMSCs transfected with miR-181c-5p mimics, miR-181c-5p inhibitor and negative control by qRT-PCR. The results are shown in Figure 5. After 72h of transfection, the expression

of miR-181c-5p in the mimics transfected group was upregulated approximately 39-fold compared to the control group, while the expression in the inhibitor transfected group was downregulated approximately 5-fold compared to the control group. This indicates that the expression level of miR-181c-5p in BMMSCs could be effectively regulated by transfection with specific mimics or inhibitor.

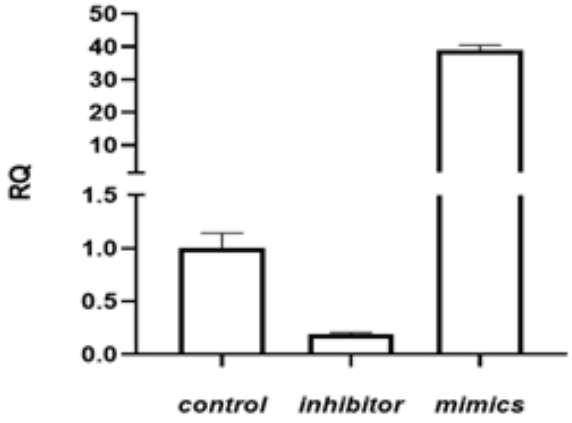

Figure 5. The effect of chemically synthesized miR-181c-5p mimics and miR-181c-5p inhibitor on the expression level of iR-181c-5p detected by qRT-PCR (n=5; *P<0.05 vs control).

3.5 Effect of miR-181c-5p on the proliferative capacity of BMMSCs

The proliferation of BMMSCs transfected with miR-181c-5p mimics or miR-181c-5p inhibitor was detected by MTT assay. The results showed that there was no significant change in cell growth rate and cell proliferation curve after upregulation or downregulation of miR-181c-5p compared with control group (Figure 6, P > 0.05). These results demonstrate that miR-181c-5p does not affect the proliferative capacity of BMMSCs.

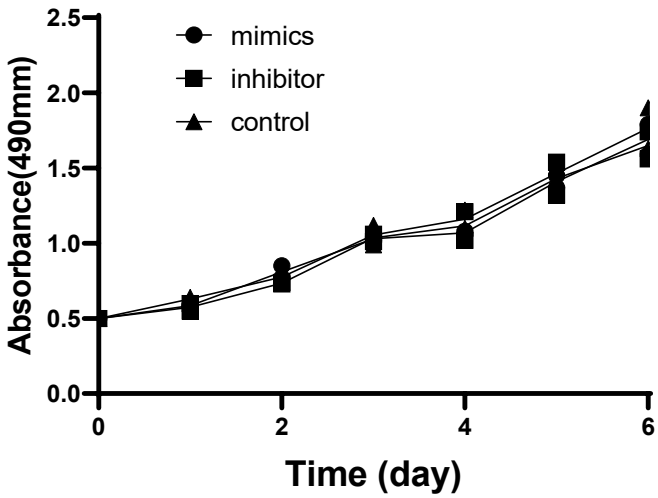

Figure 6: The effect of miR-181c-5p on the proliferation of BMMSCs detected by MTT assay

3.6 Effect of miR-181c-5p on the osteogenic differentiation of BMMSCs

To investigate the effect of miR-181c-5p on the osteogenic differentiation ability of BMMSCs, we performed alizarin red staining analysis on BMMSCs after 14 days with osteogenic induction, and detected the expression of osteogenic-related genes by Western blot. The results showed that the red-positive nodules formed in the miR-181c-5p inhibitor group were larger, more numerous and darker than miR-181c-5p mimics group (Fig. 7A-B). It showed that the expression level of miR-181c-5p was negatively correlated with calcium deposition in BMMSCs. The results of western blot showed that the protein expression of Runx2 was reduced in the mimics group compared to the control group, while Runx2 was significantly higher in the inhibitor group (Figure 7C). These results demonstrated that miR-181c-5p inhibited the osteogenic differentiation of BMMSCs.

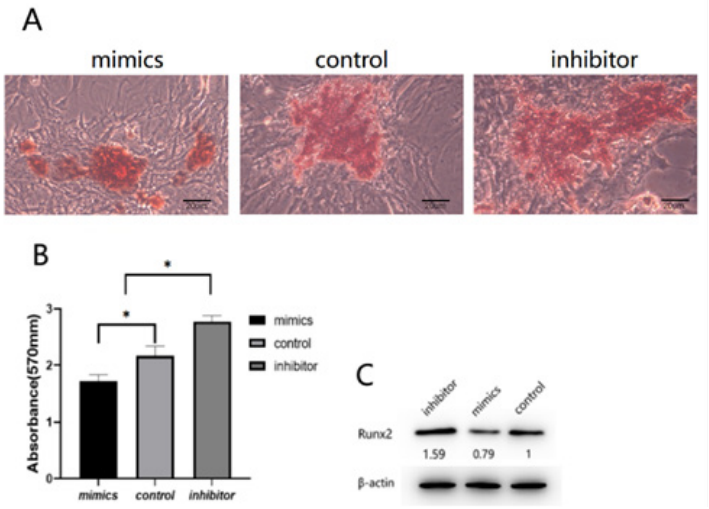

Figure 7: Effect of miR-181c-5p on osteogenic differentiation of BMMSCs. (A) Alizarin red staining (×200); (B) Quantification of alizarin red staining; (C) Western blot detection of Runx2 protein expression. (n = 5; * indicates P<0.05 vs control).

3.7 Effect of miR-181c-5p on the adipogenic differentiation of BMMSCs

Next, we detected the effect of miR-181c-5p on the adipogenic differentiation ability of BMMSCs by Oil Red O Staining and Western blot. The results of Oil Red O staining showed that lipid droplets formed in the miR-181c-5p inhibitor group were smaller in volume and less in number than those in the miR-181c-5p mimics group, indicating that the expression level of miR-181c-5p was positively correlated to the lipid formation of BMMSCs (Figure 8A-B). As shown in Figure 8C, the protein expression of PPAR-γ was lower in mimics group, while PPAR-γ was significantly increased in inhibitor group. These results indicated that miR-181c-5p promoted adipogenic differentiation of BMMSCs.

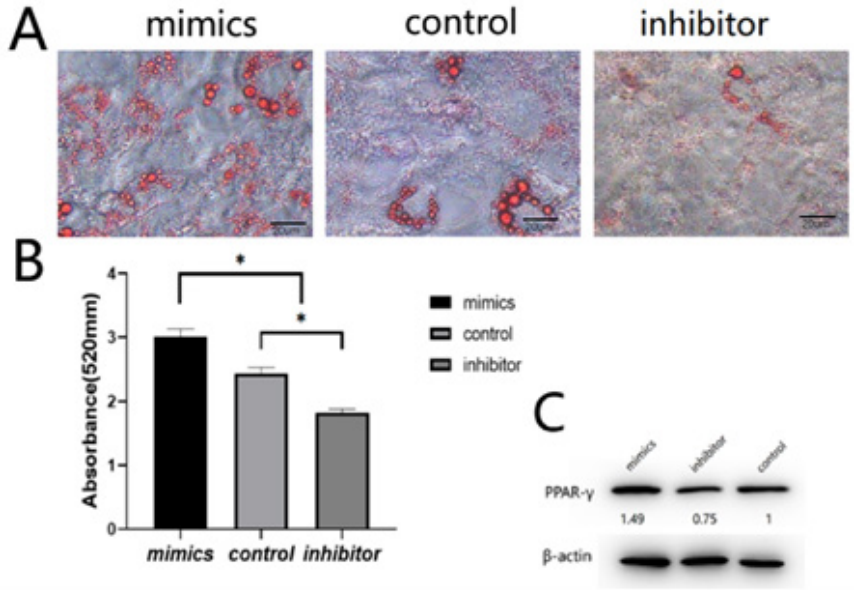

Figure 8: The effect of miR-181c-5p on adipogenic differentiation of BMMSCs. (A) Oil red O staining (×200); (B) Oil red O staining quantification; (C) Western blot detection of PPAR-γ protein expression. (n = 5, * means P<0.05 vs control).

3.8 The miR-181c-5p target gene screening

The above experiments proved that miR-181c-5p is an inhibitor of osteogenic differentiation and a promoter of adipogenic differentiation in BMMSCs. To clarify the mechanism of miR-181c-5p in differentiation of BMMSCs, we performed bioinformatics analysis through target gene prediction software and websites (TargetScan, miRanda, miRwalk, PITA and RNAhybrid), and predicted that miR-181c-5p may interact with more than 700 genes. Among them, miR-181c-5p had binding sites with 3'-UTR region of Foxo1. In addition, Foxo1 has been confirmed to be a transcription factor that promoted the osteogenic differentiation and inhibited the adipogenic differentiation in BMMSCs. Therefore, we hypothesize that Foxo1 is a target gene of miR-181c-5p, and the expression pairing diagram is shown in Figure 9.

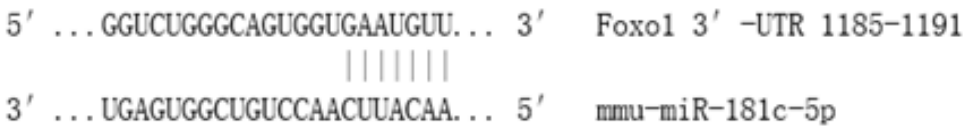

Figure 9: Schematic representation of miR-181c-5p paired with the 3′-UTR region of Foxo1.

3.9 Foxo1 negatively regulates miR-181c-5p

To investigate the relationship between Foxo1 and miR-181c-5p, we detected the expression level of Foxo1 in BMMSCs by Western blot. The results showed that overexpression of miR-181c-5p prevented the expression of endogenous Foxo1 protein in BMMSCs, while the protein level of endogenous Foxo1 was significantly increased in BMMSCs after downregulation of miR-181c-5p (Figure 10A). This indicated that miR-181c-5p prevented Foxo1 protein translation. The receptor tyrosine kinase RTK-MAPK plays an important role in embryonic somite development and osteogenic differentiation [15]. Various cytokines including BMP, bFGF and IGF can regulate osteogenic differentiation by activating RUNX2 through the MAPK/ERK signaling pathway [16]. RUNX2 is a transcription factor that plays a key role in osteogenic differentiation and bone formation, and miR-181c-5p may inhibit the normal osteogenic and adipogenic differentiation of BMMSCs by

inhibiting the MAPK/ERK signaling pathway. Furthermore, in the comparison between Sham and OVX,qRT-PCR and Westernblot showed that the mRNA and protein expression of Foxo1 was significantly lower in O-BMMSCs than S-BMMSCs(Figure 11), in contrast to the expression pattern of miR-181c-5p in O-BMMSCs versus S-BMMSCs [10]. These results suggest that miR-181c-5p causes imbalance in adipogenic-osteogenic differentiation in O-BMMSCs by repressing the expression of target genes.

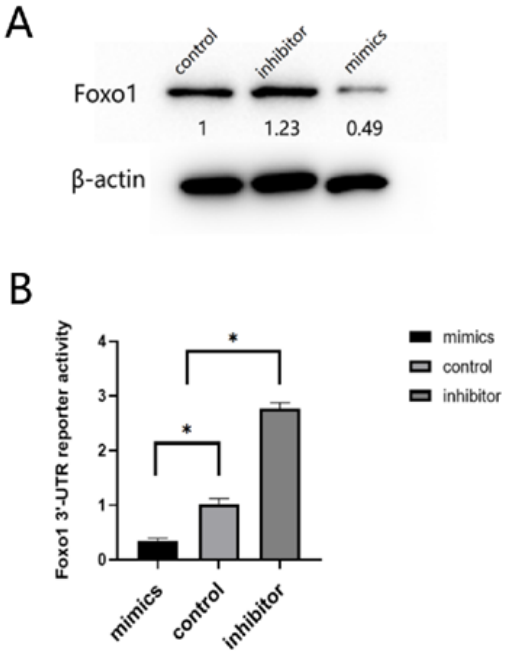

Figure 10: The regulatory effect of miR-181c-5p on Foxo1. (A) The protein expression of Foxo1 after miR-181c-5p mimics, inhibitor and control transfection; (B) Foxo1 3′-UTR luciferase activity after transfection with miR-181c-5p mimics, inhibitor and control. (n = 5, * means P<0.05 vs control).

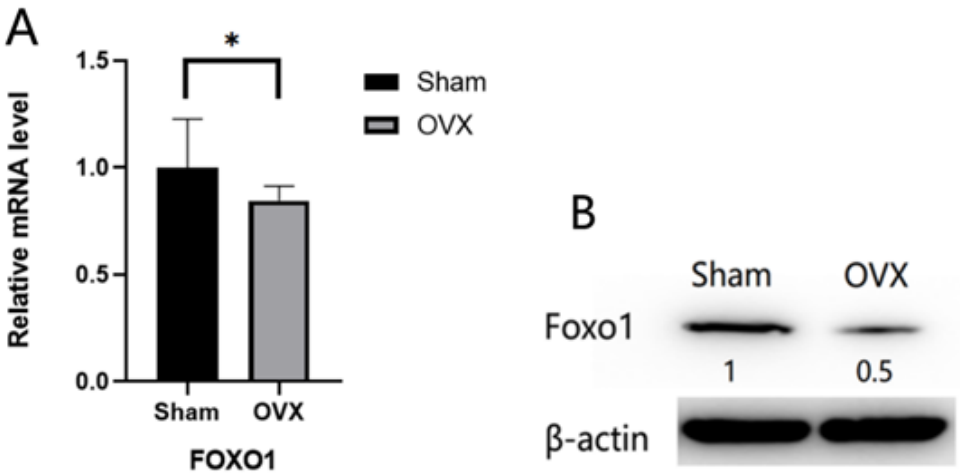

Figure 11: Endogenous expression of Foxo1 in O-BMMSCs and S-BMMSCs. (A) Comparison of Foxo1 mRNA levels in O-BMMSCs and S-BMMSCs; (B) Comparison of Foxo1 protein content in O-BMMSCs and S-BMMSCs. (n=5, * means P<0.05 vs Sham).

3.10 Foxo1 is a direct target of miR-181c-5p

Next, we verified the relationship between miR-181c-5p and Foxo1 by dual luciferase reporter. We constructed a luciferase gene plasmid containing the 3′-UTR sequence of Foxo1, and cotransfected the gene plasmid and the internal control plasmid with miR-181c-5p mimics, inhibitor and negative control to measure Foxo1 luciferase activity. As shown in Figure 10B, the activity of Foxo1 luciferase decreased by 55% in all mimics transfected groups compared to the control group, while Foxo1 luciferase activity increased by approximately 65% in the inhibitor transfected group ($P<0.05$). These results indicate that miR-181c-5p plays a role in inhibiting the protein expression of the target gene by binding directly to the Foxo1 3′-UTR region.

3.11 The reversal effect of miR-181c-5p inhibitor on the osteogenic and adipogenic differentiation of O-BMMSCs

To further investigate the mechanism of miR-181c-5p in BMMSCs, we intervened the role of miR-181c-5p by miR-181c-5p inhibitor. The miR-181c-5p inhibitor or inhibitor-control (NC) were transfected into O-BMMSCs and S-BMMSCs. The transfected cells were subjected to osteogenic induction for 14 days followed by alizarin red staining. The results showed that, compared with the OVX+NC group, the calcified nodules in the transfected miR-181c-5p inhibitor group were larger, more numerous, and darker in color, but not as much as the SHAM+NC group (Figure 12A-B). The OD value of miR-181c-5p inhibitor alizarin red was higher than OVX+NC group, which indicated that miR-181c-5p inhibitor had a certain recovery effect on the osteogenic differentiation defect of O-BMMSC s (Figure 12C). Western blot results showed that the expression of Foxo1 and Runx2 was significantly increased in O-BMMSCs with downregulated miR-181c-5p (Figure 12D-E), indicating that the miR-181c-5p inhibitor was effective in restoring the osteogenic ability of O-BMMSCs by elevating the expression levels of endogenous Foxo1 and RUNX2.

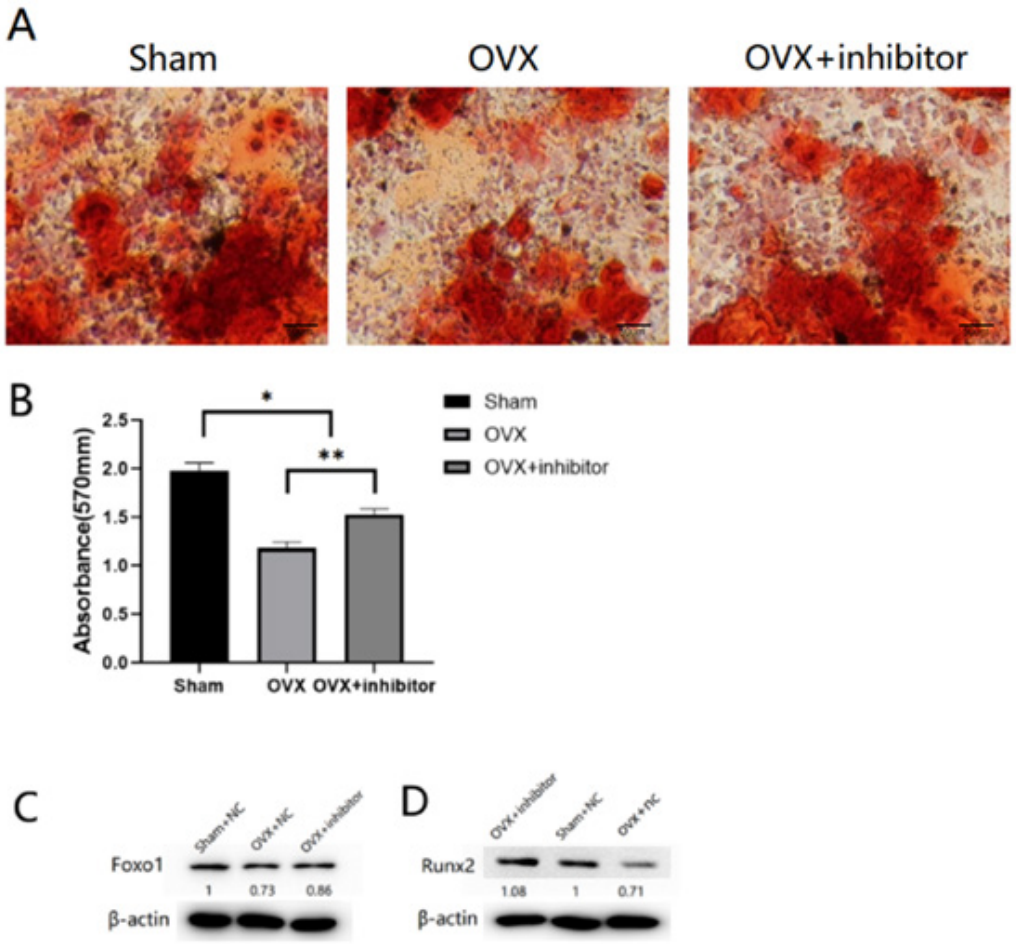

Figure 12: Effect of miR-181c-5p inhibitor on osteogenic differentiation of O-BMMSCs. (A) Alizarin red staining micrograph (x200); (B) Quantification of alizarin red staining; (C) Protein expression of Foxo1; (D) Protein expression of Runx2. (n=5, * indicates $P<0.05$ vs control, ** indicates $P<0.01$ vs control).

In addition, the adipogenic induction group was stained with Oil Red O. The results showed that the miR-181c-5p inhibitor transfected group had smaller and fewer lipid droplets for oil-red O staining compared to the OVX+NC group, but larger and more lipid droplets than the SHAM+NC group (Figure 13A). Quantitative analysis of Oil Red O showed that BMMSCs with downregulated miR-181c-5p had lower Oil Red OD value and lower adipogenic differentiation ability, but higher OD value than SHAM+NC group (Figure 13B). As shown in Figure 13C and 13D, Foxo1 protein levels were significantly increased and PPAR-γ protein levels were significantly decreased compared to the OVX+NC group, but their expression levels were not as high as those of the SHAM+NC group. This indicates that miR-181c-5p inhibitor increases the expression level of endogenous Foxo1, thereby inhibiting the expression of PPAR-γ, but miR-181c-5p inhibitor can only partially counteract the high adipogenic differentiation of O-BMMSCs, and could not completely restore the lipogenic differentiation ability of O-BMMSCs to the similar differentiation characteristics of S-BMMSCs.

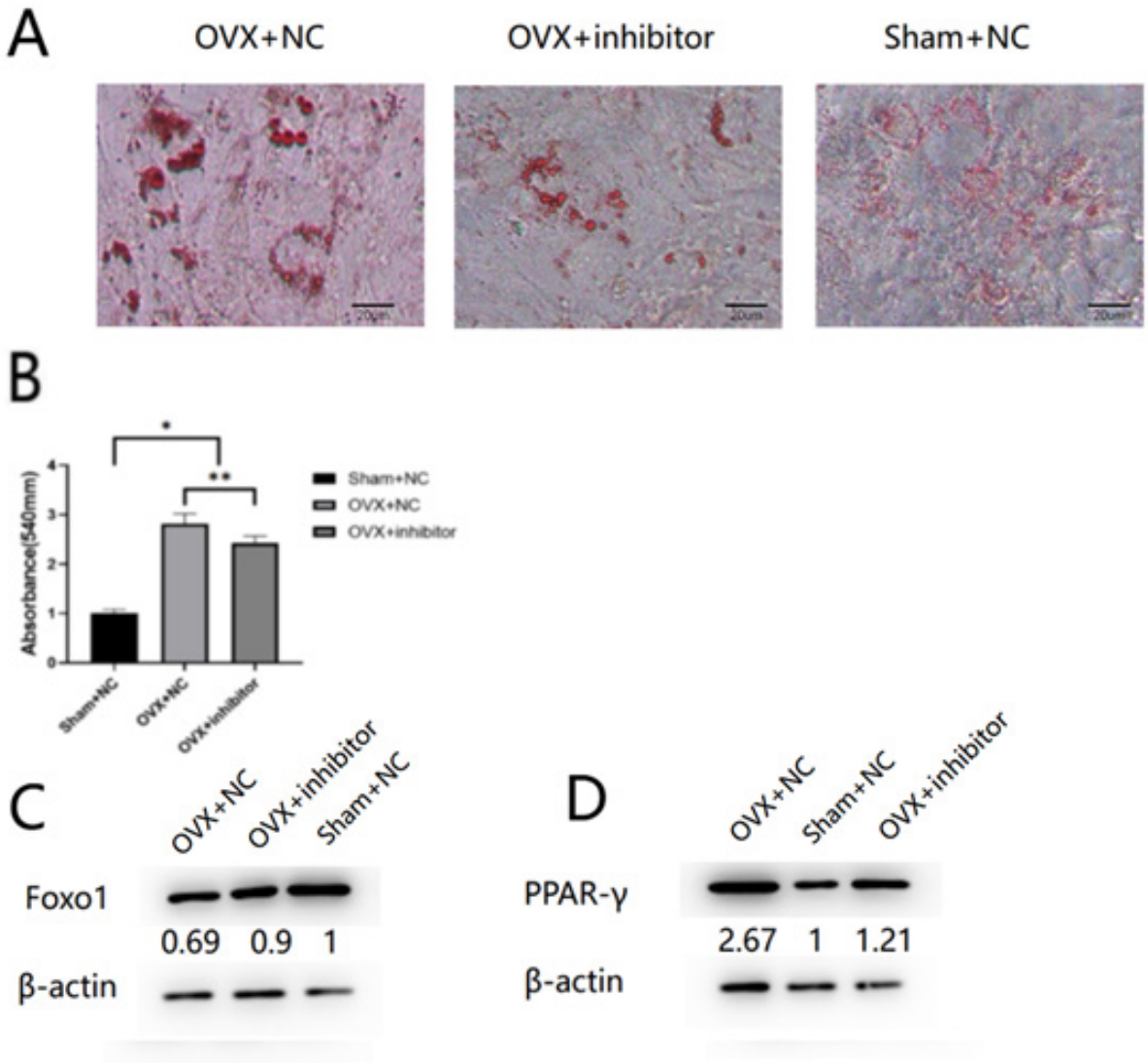

Figure 13: The effect of miR-181c-p inhibitor on the adipogenic differentiation of O-BMMSCS. (A) Oil red O staining; (B) Oil red O staining quantification; (C) Protein expression of Foxo1; (D) Protein expression of PPAR-γ. (n=5, * means P<0.05 vs control, ** means P<0.01 vs control).

4. Discussion

In order to clarify the biological function of miR-181c-5p on BMMSCs, this studay specifically regulated the expression level of miR-181c-5p in BMMSCs by transfecting chemically synthesized miR-181c-5p mimics and inhibitors. The results of cell differentiation showed that miR-181c-5p was involved in the osteogenic- adipogenic differentiation of BMMSCs, inhibiting osteogenic differentiation and promoting adipogenic differentiation of BMMSCs.

To further clarify the multifunctional role of miR-181c-5p in promoting adipogenic and inhibiting osteogenic differentiation in BMMSCs, we first hypothesized that Foxo1, a gene that has been shown to be important in promoting osteogenesis and inhibiting lipogenesis, would most likely be a candidate for miR-181c-5p based on biological information analysis software, common target gene prediction software, databases and the negative regulation mechanism of miRNAs and target genes. Bioinformatics results showed that the predicted target of miR-181c-5p was located at the 3'-UTR sequence of Foxo1. Foxo1 has been reported to be an important transcription factor that promotes bone formation, bone matrix mineralization, and osteoblast differentiation [17, 18, 19]. It was found that the mRNA levels of Col-1, RUNX2, OCN and MMP13 in osteogenic differentiation were significantly decreased after Foxo1 knockout in osteoblasts [20]. RUNX2 is a transcription factor that plays a key role in osteogenic differentiation and bone formation, and miR-181c-5p may inhibit the osteogenic differentiation of BMMSCs by inhibiting the MAPK/ERK signaling pathway. The study found that the promoter of RUNX2 is the direct target of Foxo1, and verified that RUNX2 is an important downstream gene of Foxo1. In addition, Foxo1 has been found to be involved in adipocyte differentiation, negatively regulates adipocyte differentiation and inhibits the formation of lipid droplets in precursor adipocytes [21, 22]. Osteoblasts and adipocytes are derived from a common progenitor cell, and inhibition of adipogenic differentiation will inversely promote osteoblast differentiation [23]. These findings suggest that Foxo1 may first regulate the differentiation of blasts toward osteogenic and away from adipogenic differentiation [20]. To clarify whether miR-181c-5p can regulate the expression of predicted target genes and Foxo1, this study analyzed the protein expression level of Foxo1 by overexpressing and inhibiting the expression level of miR-181c-5p in BMMSCs. The results showed that the protein level of Foxo1 were significantly decreased in the miR-181c-5p overexpression group, while the protein levels of Foxo1 in the miR-181c-5p inhibition group showed an increasing trend. These results indicated that miR-181c-5p was negatively regulated with Foxo1, validating Foxo1 as a direct target gene of miR-181c-5p mediated by translational repression.

Next, we cotransfected miR-181c-5p and a luciferase reporter gene containing the 3'-UTR sequence of Foxo1 into BMMSCs to further verify the relationship between miR-181c-5p and Foxo1. The results showed that the luciferase activity of target genes in miR-181c-5p upregulated group was significantly decreased, while the luciferase activity in the miR-181c-5p downregulated group was significantly increased. This strongly indicates that Foxo1 is a target gene of miR-181c-5p, and miR-181c-5p prevents the expression of Foxo1 through translational inhibition, which can inhibit osteogenic differentiation and promote adipogenic differentiation in BMMSCs, ultimately causing an imbalance in osteogenic- adipogenic differentiation in BMMSCs.

Previous experiments confirmed that miR-181c-5p targets Foxo1, inhibits its synthesis post-transcriptionally, and alters the osteogenic-adipogenic differentiation balance of BMMSCs. To clarify whether miR-181c-5p regulates the imbalance of BMMSCs differentiation by interacting with Foxo1 target genes, we investigated the mRNA and protein levels of endogenous Foxo1 in O-BMMSCs and S-BMMSCs. These results indicated that miR-181c-5p in OPM caused the adipogenic and osteogenic differentiation bias of O-BMMSCs by inhibiting the expression of target gene Foxo1, and also proved that miR-181c-5p is a positive regulator of OPM. Studies have shown that target genes mediate the role of miRNAs. Therefore, modulating the expression levels of endogenous target genes by changing miRNAs is an effective way to regulate various biological processes [24, 25]. In this study, we reduced the expression of miR-181c-5p in O-BMMSCs by inhibiting miR-181c-5p and detected the level of endogenous target genes. The results showed that miR-181c-5p inhibitor partially elevated endogenous Foxo1 expression, and promote RUNX2 expression and calcified nodule formation, reduce PPAR-γ levels and lipid droplet formation, thereby improving Osteogenic-adipogenic differentiation balance of O-BMMSCs. However, miR-181c-5p inhibitor could not fully restore expression levels of Foxo1 to normal levels. This may be due to the presence of other signaling molecules involved in the osteoblastic-lipogenic differentiation of O-BMMSCs [26, 27], which warrants further investigation. In conclusion, miR-181c-5p inhibitor could partially restore the low osteogenic capacity and inhibit the high adipogenic capacity of O-BMMSCs, thereby correcting the osteogenic-adipogenic differentiation balance of O-BMMSCs and improving the bone remodeling microenvironment of OPM.

5. Conclusion

In conclusion, this study reveals a new mechanism for the development of osteoporosis due to reduced estrogen levels, which may provide new targets and new ideas for the prevention and treatment of bone resorption in osteoporosis.

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