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MiR-1301 promotes adipogenic and osteogenic differentiation of BMSCs by targeting Satb2

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Abstract. – OBJECTIVE: Bone marrow mesenchymal stem cells (BMSCs) have the ability to differentiate into several cell lines and are critical for skeletal microenvironment and bone development. MiR-1301 is involved in multiple pathological and physiological processes. However, miR-1301's role in BMSCs adipogenic and osteogenic differentiation remains unclear.

MATERIALS AND METHODS: Rat BMSCs were isolated and randomly divided into control group, miR-1301 group, and miR-1301 siRNA group followed by analysis of the expression of miR-1301, Bax, Bcl-2, UNX2, and OPN, as well as FABP4 and PPARγ2 by Real Time-PCR. Cell proliferation was assessed by MTT assay and the relationship between miR-1301 and Satb2 was evaluated by the Dual-Luciferase reporter assay. Satb2 expression was detected by Western blot.

RESULTS: The pcDNA-miR-1301 plasmid was transfected into BMSCs to upregulate the expression of miR-1301, which promoted cell proliferation, decreased Bax expression, and increased Bcl-2 expression and ALP activity. In addition, it also elevated the expression of RUNX2 and OPN and decreased the expression of FABP4, PPARγ2, and Satb2. Compared with the control group, the difference was statistically significant ($p<0.05$); Satb2 was the target gene of miR-1301. MiR-1301 siRNA transfected into BMSCs down-regulated miR-1301 expression, inhibited cell proliferation, increased Bax expression and decreased Bcl-2 expression and ALP activity. Meanwhile, miR-1301 siRNA also reduced RUNX2 and OPN expression and increased expression of FABP4, PPARγ2 and Satb2. The difference was statistically significant compared with control group ($p<0.05$).

CONCLUSIONS: Regulation of miR-1301 expression in BMSCs can improve BMSCs proliferation and regulate their adipogenic and osteogenic differentiation by regulating Satb2.

Key Words:
MiR-1301, Satb2, BMSCs, Proliferation, Adipogenesis, Osteogenic differentiation.

Introduction

Osteoporosis (OP) is a systemic skeletal disorder that is common in the elderly, especially in older women, characterized by decreased bone mass, deterioration of microstructure of bone tissue, and subsequent increase in bone fragility and fracture susceptibility[1]. Because estrogen is required, menopausal elderly women suffer from osteoporosis up to 50%, and osteoporotic fractures are highly prone to occur[2,3]. However, the current clinical treatment for osteoporosis is difficult to achieve satisfactory outcomes, while the side effects of drug treatment and patient compliance also affect the treatment effect[4]. Therefore, how to promote osteogenic differentiation and then increase bone content has become an important strategy for the treatment of osteoporosis[5]. Bone marrow mesenchymal stem cells (BMSCs) are mesodermal cells that can be obtained from a variety of sources including adipose tissue, periosteum, and bone marrow[6]. BMSCs are similar to hematopoietic stem cells and have the potential for multi-lineage self-differentiation and high self-renewal[7,8]. BMSCs are the main source of cells for the body to increase and maintain bone mass and health[8]; they can provide intrinsic osteogenesis and thus supplement a large amount of bone loss[9]. BMSCs are easy to obtain and separate and widely used in many fields such as tissue engineering, especially in ideal seed cells for bone tissue engineering[10,11]. Due to their unique ability to differentiate into osteoblasts, chondrocytes, and adipocytes, BMSCs are widely used in the treatment of osteoporosis[12].

In recent decades, a large number of studies have shown that non-coding RNA involves in many biological processes. MicroRNAs, also known as miRNAs, small RNAs or microRNAs, are widely found in animals and plants. Small molecule short-chain RNA involves in cell sur-

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vival, proliferation and differentiation[13,14]. Abnormal expression of miRNAs may be involved in the occurrence and development of diseases and may serve as a central regulator of proliferation and differentiation of BMSCs, thus playing a key role in regulating the differentiation of BMSCs into specific lineages[15]. MiR-203 and miR-320 negatively regulate BMP-2-induced osteogenic differentiation, while miR-495 inhibits new bone regeneration by targeting high mobility proteins[16,17]. Therefore, miRNAs can regulate osteogenic transcription and differentiation. MiR-1301 is involved in several pathological and physiological processes[18], but the role of miR-1301 in the transformation of adipogenic and osteogenic differentiation of BMSCs has not been reported.

Materials and Methods

Experimental Animals

Ten healthy female Sprague-Dawley rats, 2 months old, SPF grade, body weight (250±20) g, were purchased from the experimental animal center of the unit and fed by the SPF animal experiment center. Feeding conditions include maintaining the temperature at (21 ± 1)°C and maintaining relative humidity (50-70%) under constant temperature and constant humidity conditions, ensuring a 12/day cycle every 12 hours. Animal experiments were performed in strict accordance with the experimental design and performed by experienced technicians to minimize animal suffering. This investigation was approved by the Ethics Committee of Qilu Hospital (Qingdao, China).

Reagents and Instruments

Sodium pentobarbital was purchased from Shanghai Zhaohui Pharmaceutical Co., Ltd (Shanghai, China). pcDNA-miR-1301 plasmid and miR-1301 siRNA was constructed by Shanghai Jima Gene Company (Shanghai, China). Fetal bovine serum (FBS) and cyan chain double antibody were purchased from HyClone Corporation (San Angelo, TX, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) powder was purchased from Gibco (Grand Island, NY, USA); trypsin-ethylenediaminetetraacetic acid (EDTA) digest was purchased from Sigma-Aldrich (St. Louis, MO, USA). The lipo2000 transfection reagent was purchased from Invitrogen (Carlsbad, CA, USA). Western blot related chemical reagents were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China), enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences, rabbit anti-mouse Stab2 monoclonal antibody, and goat anti-rabbit horseradish peroxidase (HRP)-labeled IgG secondary antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). The RNA extraction kit and the reverse transcription kit were purchased from Axygen (Union City, CA, USA). Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd (Shanghai, China). The alkaline phosphatase (ALP) activity detection kit was purchased from Wuhan Boster Bio Co., Ltd (Wuhan, China). The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI (Foster City, CA, USA). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Corporation (Hercules, CA, USA). The clean workbench was purchased from Suzhou Purification Equipment Factory in Jiangsu Province (Suzhou, China). Surgical microscopy equipment was purchased from Suzhou Medical Instrument Factory (Suzhou, China). The Hera cell CO_2 incubator was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Isolation and Culture of Rat BMSCs

The rats were sacrificed by cervical dislocation and immersed in 70% alcohol for 20-30 min. Cut the skin layer by layer, subcutaneous tissue, expose the femur and tibia, transfer the separated femur and tibia into a sterile plate, extract 3 ml of bone marrow with a syringe containing 2 ml of heparin anticoagulation, add 3 ml of phosphate-buffered saline (PBS), and blow to make a cell suspension. Centrifuge at 1000 rpm for 10 min; discard the supernatant and fat, gently add the suspension to the preset equal volume of lymphocyte separation solution, add PBS, and centrifuge for 25 min at room temperature. A liquid capillary pipette was used to slowly inject 5 ml of a suspension containing bone marrow mesenchymal cells along the wall of the tube onto the mononuclear cell separation solution and centrifuged at room temperature for 20 min at 2000 rpm. Centrifuge at 1000 rpm for 5 min and wash twice. The precipitates, i.e., BMSCs, were placed in a medium containing 5 ml of α-MEM containing 10% FBS, 1% double antibody, 5% CO_2, and cultured in a 37°C incubator. After 48 h, the medium was changed, the non-adherent cells were removed, the culture was continued, and passage was performed until the cell colonies

reached 80% of confluence. The 3-5 generation BMSCs cells were taken for testing.

BMSCs Grouping and Processing

Rat BMSCs were isolated and cultured *in vitro* and randomly divided into control group, miR-1301 group and miR-1301 siRNA group, in which miR-1301 plasmid and miR-1301 siRNA was transfected into BMSCs, respectively. The miR-1301 siRNA sequence was as follows: the upstream sequence 5'-CTGGAGAGCGAATCTTCA-3'; the downstream sequence 5'-GAAGACGGTCACG-GTCA-3. The cell density was fused to 70-80% in a 6-well plate; pcDNA-miR-1301 plasmid or miR-1301 siRNA liposome was added to 200 μl of serum-free medium, mixed well, and incubated at room temperature for 15 min. The mixed Lipofectamine 2000 was separately mixed with the corresponding dilution and incubated for 30 min at room temperature. The serum of the cells was removed, PBS was gently rinsed, 1.6 ml of serum-free medium was added, and each system was added to each system, and cultured in a 5% CO_2 incubator at 37°C for 48 hours for experimental research.

MTT Assay for the Proliferation of BMSCs In Each Group

The BMSCs collected in logarithmic growth phase were inoculated into the 96-well culture plate with 10% fetal bovine serum α- Minimum Essential Medium (MEM) culture medium at a cell number of 5×10^3, and the supernatant was discarded. After 24 hours of culture, the supernatant was discarded and randomly grouped. The treatment method was the same as above. After 48 hours of cell treatment in each dose group, 20 μl of sterile MTT was added to the wells to be tested, and 3 replicate wells were set in each treatment group. After 4 hours of continuous culture, the supernatant was completely removed, 150 μl/well of DMSO was added, and the shaker was shaken 10 min until the purple crystals were fully dissolved, followed by measuring the absorbance (A) value at a wavelength of 570 nm by a microplate reader. The experiment was repeated three more times.

Real Time PCR Detection of Bax, Bcl-2, RUNX2, OPN, FABP4 and PPARγ2 Expression In BMSCs of Rats In Each Group

RNA from each group of rat BMSCs was extracted from ice by TRIzol reagent, and DNA reverse transcription synthesis was performed according to the kit instructions. Primers were designed according to each gene sequence by Premier 6.0 and synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Table I). Real-time PCR reaction conditions: 55°C 1 min, 92°C 30 sec, 58-60°C 45 sec, 72°C 35 sec, a total of 35 cycles. Data was collected using the PCR reactor software and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference. According to the fluorescence quantification, the starting cycle number (CT) of all samples and standards was calculated. Based on the standard CT value, a standard curve was drawn, and then the semi-quantitative analysis was carried out by the $2^{-\Delta Ct}$ method.

Determination of ALP Content

The ALP content was determined according to the instructions of the ALP test kit, centrifuged at 1000 rpm for 10 min, the supernatant was discarded, and the precipitate was collected by adding Triton-X100. After mixing, various OD values were measured at 520 nm, and the ALP content was calculated.

Western Blot Analysis of Satb2 Expression Changes

Extract cell protein: add lysate, lyse tissue on ice for 15-30 min, 5 s × 4 times sonication, centrifugation at 4°C, 10000 × g for 15 min, transfer supernatant to new Eppendorf (EP) tube and

Table I. Primer sequences.

Gene	Forward 5′-3′	Reverse 5′-3′
GAPDH	AGTATGTTGTCACCGCTGG	TAACCTGTCTATACGGAGGGT
RUNX2	CATGGGGACGAATGGA	GCCTCTCAAAGTGGTA
OPN	TCGCTGGCTCAAGGAC	AGTTCGTTACGTAAAGTC
Bax	CTGGCCAGTTCGCTCC GA	ACTGAAATTCGTTACCGGAG
Bcl-2	GGCATTCGCTTATCCTGTC	GACATGGACGAACTGAAAA
FABP4	GTCGCTAATTGAATCCAAT	GTTCGAGTTCCGAACTCT
PPARγ2	GACGTTTGATCGTTCC	ATTCTCTATGTGAGTACT

stored at -20°C for Western blot experiments. The isolated protein was electrophoresed on a 10% sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was transferred to a polyvinylidene difluoride (PVDF) membrane by semi-dry transfer and blocked with 5% skim milk powder for 2 h to remove the non-specific background using room temperature, followed by incubation with 1:1000 dilution of Satb2 monoclonal antibody at 4°C overnight. After washing with phosphate-buffered-saline and Tween (PBST), 1:2000 goat anti-rabbit secondary antibody was added and incubated for 30 min under dark, followed by washing with PBST and development after addition of enhanced chemiluminescence (ECL) for 1 min. X-film and strip density measurements were separately scanned using protein image processing system software and Quantity one software. The procedure was repeated four times (n=4) and statistical analysis too.

Dual-Luciferase Reporter Gene Assay

The predicted miR-1301 sequence was inserted into the pmirGLO vector, and the reporter and Satb2 mimics were co-transfected into BMSCs for 48 h. Luciferase activity was determined by Dual-Luciferase assay and *Renilla* Luciferase activity was corrected and calculated.

Statistical Analysis

Data were processed by Statistical Package for the Social Sciences (SPSS) 16.0 statistical software (SPSS Inc., Chicago, IL, USA) The count data was expressed as a percentage and a Chi-square test was performed. Measurement data were expressed as mean ± standard deviation (SD), and comparison of multiple groups of samples was performed using one-way ANOVA. $p < 0.05$ was considered statistically significant.

Results

Effect of Regulating MiR-1301 on the Expression of MiR-1301 In BMSCs

Real time PCR analysis of the effect of miR-1301 on the expression of miR-1301 in BMSCs showed that pcDNA-miR-1301 plasmid transfection into BMSCs up-regulated the expression of miR-1301. Compared with the control group, the difference was statistically significant ($p < 0.05$). MiR-1301 siRNA transfection into BMSCs down-regulated the expression of miR-1301, and the difference was statistically significant ($p < 0.05$; Figure 1).

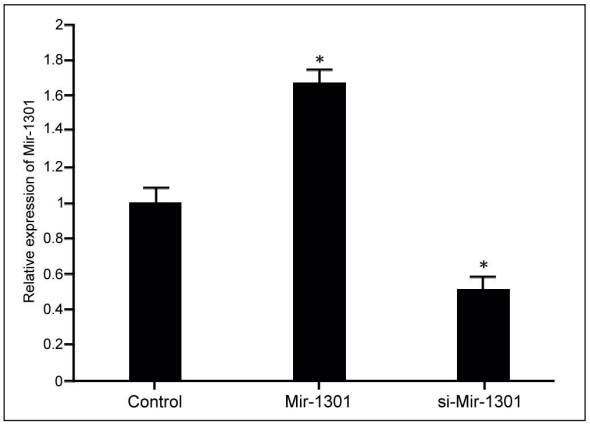

Figure 1. Effect of regulating miR-1301 on the expression of Mir-1301 in BMSCs. Compared with the control group, * $p < 0.05$.

Effect of MiR-1301 on the Proliferation of BMSCs

MTT assay was used to analyze the effect of miR-1301 on the proliferation of BMSCs. The pcDNA-miR-1301 plasmid was transfected into BMSCs to up-regulate the expression of miR-1301, which promoted the proliferation of BMSCs. Compared with the control group, the difference was statistically significant ($p < 0.05$). MiR-1301 siRNA transfection into BMSCs down-regulated the expression of miR-1301, which inhibited the proliferation of BMSCs. Compared with the control group, the difference was statistically significant ($p < 0.05$; Figure 2).

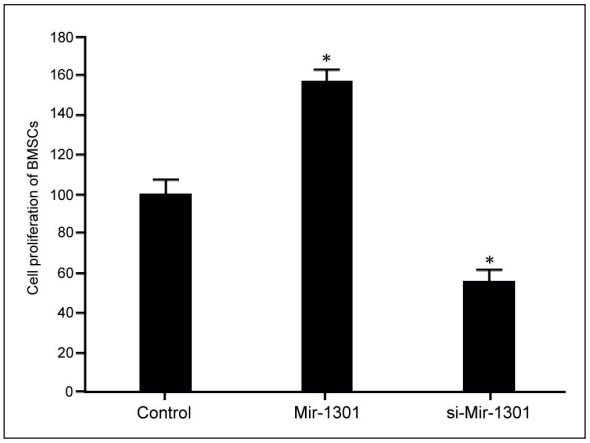

Figure 2. Effect of regulating miR-1301 on the proliferation of BMSCs. Compared with the control group, * $p < 0.05$.

Effects of MiR-1301 on the Expression of Apoptosis and Anti-Apoptosis Genes In BMSCs

Real time PCR analysis of the effect of miR-1301 on the expression of apoptosis and anti-apoptotic genes Bax and Bcl-2 in BMSCs showed that pcDNA-miR-1301 plasmid transfection into BMSCs up-regulated the expression of miR-1301, decreased Bax expression and increased Bcl-2 expression. Compared with the control group, the difference was statistically significant ($p < 0.05$). In BMSCs transfected with miR-1301 siRNA, Bax expression was decreased and Bcl-2 expression was increased, compared with the control group, the difference was statistically significant ($p < 0.05$; Figure 3).

Effects of MiR-1301 on the Expression of Osteogenesis Genes RUNX2 and OPN In BMSCs

pcDNA-miR-1301 plasmid transfection into BMSCs up-regulated the expression of miR-1301 and increased the expression of osteogenic genes RUNX2 and OPN. Compared with the control group, the difference was statistically significant ($p < 0.05$). miR-1301 siRNA transfection into BMSCs decreased RUNX2 and OPN expression, compared with the control group, the difference was statistically significant ($p < 0.05$; Figure 4).

Effects of MiR-1301 on the Expression of Adipogenic Related Genes FABP4 and PPARγ2 In BMSCs

The pcDNA-miR-1301 plasmid transfection into BMSCs upregulated the expression of miR-1301 and decreased the expression of FABP4 and PPARγ2. Compared with the control group, the difference was statistically significant ($p < 0.05$). In BMSCs transfected with miR-1301 siRNA, miR-1301 was down-regulated and the expression of FABP4 and PPARγ2 was increased. Compared with the control group, the difference was statistically significant ($p < 0.05$; Figure 5).

Effect of MiR-1301 on ALP Activity In BMSCs

The pcDNA-miR-1301 plasmid transfection into BMSCs up-regulated the expression of miR-1301 and increased the ALP activity. Compared

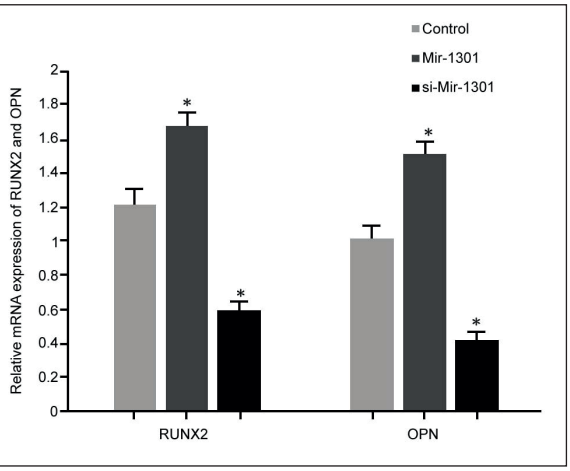

Figure 4. Effects of mirR-1301 on the expression of osteogenesis genes RUNX2 and OPN in BMSCs. Compared with the control group, * $p < 0.05$.

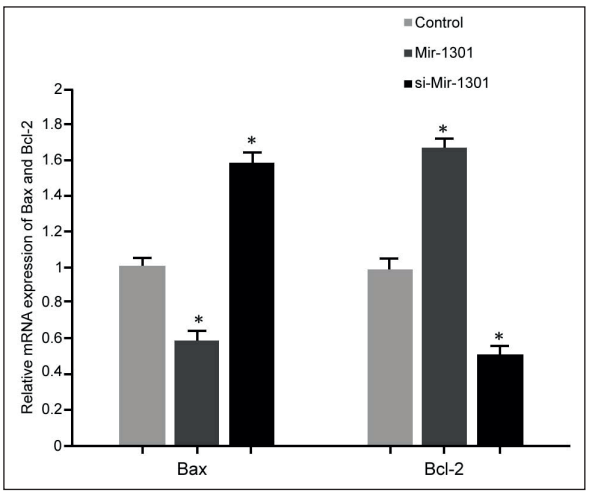

Figure 3. Effects of miR-1301 on Apoptosis and Anti-apoptosis Genes in BMSCs. Compared with the control group, * $p < 0.05$.

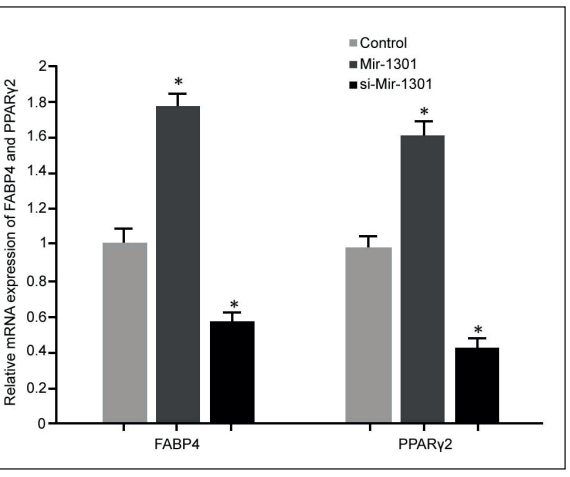

Figure 5. Effects of miR-1301 on the expression of adipogenic related genes FABP4 and PPARγ2 in BMSCs. Compared with the control group, * $p < 0.05$.

with the control group, the difference was statistically significant ($p < 0.05$). In BMSCs transfected with miR-1301 siRNA, ALP activity was reduced, and the difference was statistically significant ($p < 0.05$) compared with the control group (Figure 6).

Targeted Analysis of MiR-1301 In BMSCs

Dual-Luciferase reporter gene assay was performed to analyze the targeting genes of miR-1301 in BMSCs after construction of the miR-1301 wild type (WT) and variant (Mut). Targeted report analysis predicted that Satb2 was the target gene of miR-1301 and Satb2 mimics significantly inhibited the Luciferase activity of cells transfected with miR-1301 WT (Figure 7).

Effect of MiR-1301 on the Expression of Satb2 In BMSCs

The pcDNA-miR-1301 plasmid was transfected into BMSCs to up-regulate the expression of miR-1301 and the expression of Satb2 was decreased. Compared with the control group, the difference was statistically significant ($p < 0.05$). MiR-1301 siRNA was transfected into BMSCs to down-regulate the expression of miR-1301 and increase the expression of Satb2. Compared with control group, the difference was statistically significant ($p < 0.05$; Figure 8).

Discussion

Osteoporosis (OP) is high in the world and is common in middle-aged and elderly people. In

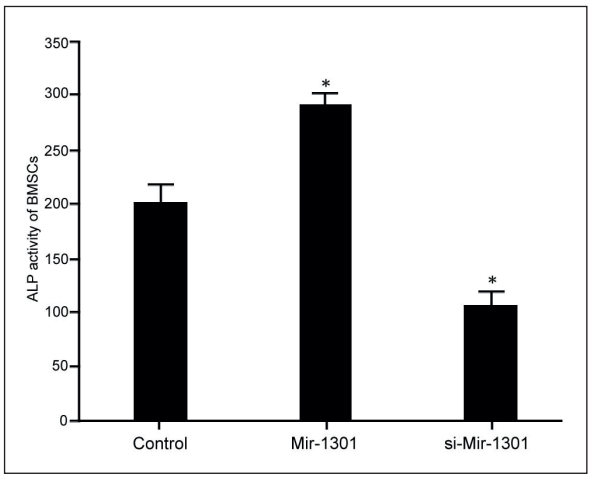

Figure 6. Effect of regulating miR-1301 on ALP activity in BMSCs. Compared with the control group, * $p < 0.05$.

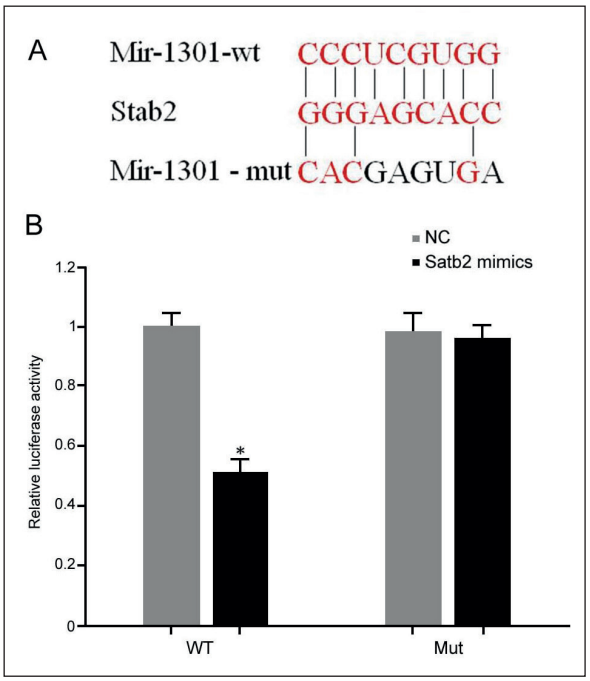

Figure 7. Targeted gene analysis of miR-1301 in BMSCs. **A,** Mir-1301 targets gene analysis in BMSCs. **B,** Luciferase reports statistical analysis, compared with NC group, * $p < 0.05$.

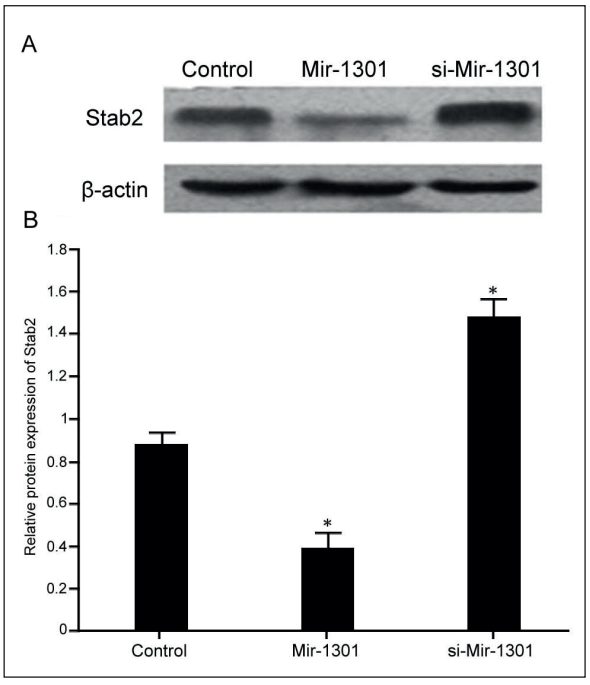

Figure 8. Effect of regulating mirR-1301 on the expression of Satb2 in BMSCs. **A,** Western blot analysis regulates the effect of Mir-1301 on the expression of Satb2 in BMSCs. **B,** Statistical analysis of the effect of Mir-1301 on the expression of Satb2 in BMSCs, compared with the control group, * $p < 0.05$.

particular, postmenopausal estrogen levels decline in women, leading to a large proportion of osteoporosis in women. Osteoporosis can cause fractures and other injuries, and the quality of life of patients is seriously degraded[19]. Although there are many clinical treatments for osteoporosis, including symptomatic methods such as supplementation of hormones, calcium supplementation, and inhibition of bone resorption, OP treatment is not effective due to problems such as poor drug absorption and drug side effects[20]. Osteoporosis severely affects the health and quality of life of older and postmenopausal women, and effective drugs that prevent or treat OP must be identified[21]. BMSCs can differentiate in multiple directions, and the direction of osteogenic differentiation and adipogenic differentiation is a key factor limiting bone growth and plays an important role in bone development, regeneration and repair[22]. Therefore, regulation of osteogenic differentiation and adipogenic differentiation of BMSCs during the prevention and treatment of osteoporosis is a key target and therapeutic direction.

MiRNAs have been shown to be involved in the development, progression and prognosis of various diseases and can be used to diagnose and treat diseases. Some miRNAs are involved in the differentiation of mesenchymal stem cells and osteoblasts during bone differentiation and regeneration. The expression of miRNAs is closely related to bone tissue hyperplasia[23,24]. However, the role of miR-1301 in the regulation of BMSCs remains to be elucidated. This study confirmed that pcDNA-miR-1301 plasmid transfection into BMSCs up-regulated miR-1301 expression, promoted cell proliferation, decreased ALP activity, increased expression of osteogenesis-related genes RUNX2 and OPN, and decreased expression of adipogenic related genes FABP4 and PPARγ2; miR-1301 siRNA transfection into BMSCs down-regulated miR-1301 expression and significantly reversed the above changes. These results suggest that miR-1301 is involved in the regulation of apoptosis and proliferation of BMSCs, and the differentiation of BMSCs into osteoblasts. This study confirmed that Satb2 is a miR-1301 target gene. Specific AT sequence binding protein 2 (Satb2) DNA binding protein, which can specifically bind to the DNA nuclear matrix binding region, plays an important role in chromatin transcription and remodeling, cortical neuron differentiation and osteoblast development, and its increased expression is associated with osteosarcoma formation and osteogenesis impediment[25]. This research confirmed that pcDNA-miR-1301 plasmid transfection BMSCs up-regulated miR-1301 expression, decreased Satb2 expression, and inhibited BMSCs differentiation into adipogenic direction, and down-regulated miR-1301 expression in BMSCs increased expression of Satb2 and reversed the above changes.

Conclusions

In short, the regulation of miR-1301 expression in BMSCs can improve BMSCs proliferation and regulate their adipogenic and osteogenic differentiation by regulating Satb2.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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