

miR-144 promotes the proliferation and differentiation of bone mesenchymal stem cells by downregulating the expression of SFRP1

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Abstract. Osteoporosis (OP) seriously affects the health and quality of life of elderly individuals and postmenopausal women, and the need to identify drugs that can prevent or treat OP remains urgent. Recently, several miRNAs have been reported to be involved in the differentiation of mesenchymal stem cells and osteoblasts; however, the role of miRNA (miR)-144 in regulating OP remains to be elucidated. In the present study, the expression levels of miR-144, secreted frizzled-related protein 1 (Sfrp1) and TNF- α in clinical samples were detected by the reverse transcription-quantitative polymerase chain reaction analysis and ELISA, respectively. 5-Ethynyl-2'-deoxyuridine staining, Hoechst 33258 staining, flow cytometry, a clone formation assay and Alizarin red staining were used to assess the effects of miR-144 combined with or without Sfrp1 small interfering RNA on the proliferation, apoptosis and osteoblastic differentiation of primary mesenchymal stem cells isolated from rats. Western blot assays were performed to assess the relevant mechanisms, and a dual luciferase reporter assay was used to detect the interaction between miR-144 and Sfrp1. The results showed that the levels of miR-144, Sfrp1 and TNF- α in clinical serum samples obtained from patients with postmenopausal OP were higher than those in serum samples obtained from postmenopausal women with normal bone density. There was a significant positive correlation between miR-144 and Sfrp1. Functional experiments demonstrated that miR-144 promoted proliferation, inhibited apoptosis and induced the osteoblastic

differentiation of bone marrow-derived mesenchymal stem cells by targeting *Sfrp1*. It was also shown that miR-144 may help regulate OP by activating the Wnt/ β -catenin pathway. These data suggest miR-144 as a novel target for preventing and treating OP.

Introduction

Osteoporosis (OP) is a systemic bone metabolic disorder characterized by reduced bone density and the degeneration of bone microstructure, which results in increased bone brittleness and fracture risk (1,2). As one of the most common bone metabolic disorders, OP adversely affects the health and quality of life of elderly individuals and postmenopausal women (3,4). Numerous studies have sought to identify genes associated with OP and sensitive genetic markers, and then use the information to predict the occurrence and development of OP.

Several hormones, cell types and humoral factors regulate the bone reconstruction process by promoting or inhibiting the development of osteoblasts and osteoclasts during the remodeling/modeling process (5-7). Bone marrow-derived mesenchymal stem cells (BMSCs) are the source osteoblasts in bone tissue, and affect the numbers and functional activities of osteoblasts (8). BMSCs also serve a major role in bone reconstruction and help maintain a balance between bone formation and bone resorption (9,10). A previous study reported that BMSCs can regulate the occurrence and development of postmenopausal OP (11). The most fundamental reason for abnormal bone reconstruction in cases of postmenopausal OP is functional defects of BMSCs, including decreased proliferative activity and adipogenic differentiation ability, and increased lipid differentiation ability (12,13). Therefore, BMSCs were selected as target cells in which to investigate the occurrence and development of OP.

MicroRNAs (miRNAs) are endogenous, non-coding small RNAs composed of 21-23 nucleotides (14), which have been reported to be involved in numerous complex cellular processes, including cell proliferation, differentiation, division, apoptosis and gene regulation (15,16). In recent

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years, miRNAs have been shown to be involved in the occurrence, development and prognosis of various diseases, and have also been used to diagnose and treat diseases (17,18). miRNAs also regulate the differentiation of mesenchymal stem cells and osteoblasts (19,20). During the processes of bone differentiation and regeneration, abnormal miRNA expression is closely associated with OP (21,22). For example, the miR-34c-induced silencing of *MC3T3-E1* reduces the inhibitory effect of vaspin on osteoblast differentiation (23); furthermore, miRNA (miR)-433-3p can regulate osteoblastic differentiation by targeting Dickkopf-1 (*DKK1*) (24). As an important miRNA, miR-144 is known to be involved in the occurrence and development of various human diseases, including ovarian cancer (25), cervical cancer (26) and anaplastic thyroid carcinoma (27). For example, miR-144 acts as a tumor suppressor in osteosarcoma by inhibiting cell proliferation and inducing apoptosis (28). miR-144 also aggravates intestinal hyperpermeability and destroys the epithelial barrier (29). Reports have demonstrated that mammalian target of rapamycin, occludin and zonula occludens 1 are all target genes of miR-144 (29,30). However, the effect of miR-144 on the occurrence and development of OP and the associated molecular mechanisms have not been reported.

The regulation of osteoblast differentiation is a complex process involving multiple genes and pathways, including the Notch signaling pathway, bone morphogenetic protein (BMP)/Smad signaling pathway and Wnt signaling pathway (31-33). The Wnt/ β -catenin signaling pathway serves a vital role in regulating osteoblastic differentiation and osteogenic matrix formation (34,35); therefore, the use of Wnt/ β -catenin pathway antagonists represents a novel approach for treating OP. As a family of secretory glycoproteins, secreted frizzled-related proteins (SFRPs) are a class of extracellular antagonists of the Wnt signaling pathway (36). A previous study demonstrated that SFRPs can antagonize the Wnt signaling pathway by competitively binding to the Frizzled receptor in a cysteine-rich domain (37). SFRP1 is one of the most important proteins in the SFRPs family and a negative regulator of human osteoblast and osteocyte survival (37). However, whether SFRP1 is involved in the process by which miR-144 regulates OP remains to be elucidated.

In the present study, the expression of miR-144 and its target gene *SFRP1* were detected in clinical serum samples obtained from patients with postmenopausal OP and in postmenopausal women with normal bone density. Subsequently, primary bone mesenchymal stem cells (BMSCs) were isolated from rats, and flow cytometry and EdU and Hoechst 33258 staining methods were used to detect cell apoptosis. Following completion of an osteoblast induction culture, a clone formation assay was used to assess changes in the ability of cells to form clones, and Alizarin red staining was used to determine the extent to which the BMSCs had differentiated into osteoblasts. The mechanism by which miR-144 regulates OP was also investigated. The results not only provide a basis for investigating the mechanism by which miR-144 is involved in the occurrence and development of OP, but also suggest a novel molecular target for preventing and treating OP.

Materials and methods

Collection of clinical samples. The collection and analysis of all clinical samples was approved by the Ethics Committee of the Affiliated Hospital of Guilin Medical University (Guilin, China; GLMUIA2016002). In total, 15 pairs of serum samples were collected from patients with postmenopausal OP and postmenopausal women with a normal bone mineral density. The samples were collected from January 2016 to May 2018 at the Affiliated Hospital of Guilin Medical University. The donors, including the postmenopausal women with OP and women with normal bone mineral density had no other diseases, and ranged in age between 54 and 64 years.

Isolation and culture of BMSCs. The gradient centrifugation method was used to isolate MSCs from the bone marrow, which had been collected from Sprague-Dawley (SD) rats. A total of 4 male SD rats (8 weeks old; 200 \pm 20 g) were specific pathogen-free (certificate no. SCXK2011-0015), purchased from the Animal Center of Southern Medical University (Guangzhou, China) and housed in an animal experimental center. The rats were housed under standard conditions (22 \pm 2°C; 40-60% relative humidity; 12:12-h light/dark cycle). Throughout the rearing period, the rats were provided access to food and water *ad libitum*. The protocols for all animal experiments were approved by the Animal Ethics Committee of the Affiliated Hospital of Guilin Medical University. The BM aspirates were cultured in MEM-a medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured in a humidity incubator at 37°C with 5% CO₂. Following culture for 24 h, the suspended cells were removed and the adherent cells were cleaned with phosphate-buffered saline (PBS) solution. The adherent cells were then cultured for ~10 days with two changes of medium (PBS). When the cells reached ~75% confluence, they were harvested for use in experiments.

Transfection. To achieve changes in miR-144 expression, BMSCs (2 \times 10⁴ cells/ml, 200 μ l) were seeded in 48-well plates and transfected with miR-144 mimic (50 nmol/l) or inhibitor (50 nmol/l; Guangzhou Ribobio Co., Ltd., Guangzhou, China) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as a transfection reagent, according to the manufacturer's instructions. Following incubation for 6 h, cells were cultured in MEM-a complete medium for 48 h. The miR-144 mimic and miR-144 inhibitor were used to achieve the overexpression and knockdown of miR-144, respectively. BMSCs treated with Lipofectamine 2000 reagent alone (Mock) were used as control cells. The sequences of the miR-144 mimic, miR nonsense strand negative control (NC) and miR-144 inhibitor were as follows: Mimic, sense 5'-UAC AGUAGAUGAUGUACU-3'; miR NC strand, sense 5'-UUCUCCGAACGUGUCACGUTT-3'; inhibitor, sense 5'-AGUACAUCUAUACUGUA-3'. To achieve changes in the expression of Sfrp1, the BMSCs were treated with Sfrp1 small interfering (si)RNA (5 pmol/l) (three Sfrp1 siRNAs purchased from Sangon Biotech Co., Ltd., Shanghai, China) or a nonsense strand NC. Following incubation for 6 h, cells were cultured in MEM-a complete medium for 48 h. The sequences of the SFRP1 siRNAs and control siRNA were

as follows: NC of siRNA, sense 5'-UUCUCCGAACGUGUC ACGUTT-3'; siRNA-1 (789-811) sense 5'-GCCACAACU UCCUCAUCAUUU-3'; siRNA-2 (561-583) sense 5'-CGU GUGACAACGAGUUGAAUU-3'; siRNA-3 (836-858) sense 5'-CUCACAGCCAUCACAAGUUU-3'. The *sfrp1* siRNAs were used to knock down the expression of *Sfrp1*. siRNA-2 (siSfrp1-2) was selected for use in subsequent knockdown experiments following initial characterization of the three siRNAs. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot methods were used to detect transfection efficiency.

Dual luciferase reporter assay. The binding site for miR-144 in *Sfrp1* mRNA was predicted by using TargetScan release 7.2 (http://www.targetscan.org/vert_72/), and the results indicated that *Sfrp1* was a target gene for miR-144. To confirm that *Sfrp1* is a target gene for miR-144, 293T cells (2×10^4 cells/ml, 200 μ l; American Type Culture Collection, Manassas, VA, USA) were seeded in 48-well plates and transfected with 400 ng wild-type *Sfrp1* 3'-UTR (WT-*Sfrp1*) or mutant *Sfrp1* 3'-UTR (MUT-*Sfrp1*) cloned into the psiCHECK2 plasmid (Promega Corporation, Madison, WI, USA) together with 50 nmol/l miR-144 nonsense strand or mimic using Lipofectamine 2000. Following incubation for 6 h, 293T cells were cultured in DMEM complete medium for 48 h, and then luciferase activity was detected with a dual-luciferase reporter assay (Promega Corporation). The relative luciferase activity was calculated using firefly luciferase activity as an internal control.

RT-qPCR analysis. The expression levels of miR-144, *Sfrp1* and Runt-related transcription factor 2 (*Runx2*) were detected by RT-qPCR analysis. Total RNA was extracted from serum with a GenElute™ Plasma/Serum RNA Purification Mini kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions, and then transcribed into cDNA with the use of a Bestar qPCR RT kit (DBI Bioscience, Ludwigshafen, Germany). RT was conducted as follows: 37°C for 15 min and 98°C for 5 min. qPCR was performed using a Bestar™ qPCR Master Mix (DBI Bioscience) under the following conditions: 95°C for 2 min, followed by 40 cycles of 94°C for 20 sec, 58°C for 20 sec and 72°C for 20 sec, and finally extension at 72°C for 4 min. The RT-qPCR analysis was performed on an Agilent Stratagene Mx3000P Sequence Detection system (Agilent Technologies, Inc., Santa Clara, CA, USA) using the following primer sequences: GAPDH, forward 5'-CCTCGTCTCATAGAC AAGATGGT-3' and reverse 5'-GGGTAGAGTCATACTGGA ACATG-3'; miR-144, forward 5'-ACACTCCAGCTGGGT ACAGTATAGATGATG-3' and reverse 5'-CTCAACTGG TGTCGTGGAGTCGGCAATTCAGTTGAGAGTACATC-3'; U6, forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'; *Sfrp1*, forward 5'-TCA TGCAGTTCTTCGGCTTC-3' and reverse 5'-TTCAACTCG TTGTACACGG-3'; *Runx2*, forward 5'-CATTCGCCTCAC AAACAACC-3' and reverse 5'-AGAAGTTTTGCTGACACG GT-3'. GAPDH and U6 were used as the control for the relative quantification of mRNA or miRNA, respectively. The relative levels of gene expression were calculated using the $2^{-\Delta\Delta C_q}$ method (38).

ELISA. Blood samples from patients with postmenopausal OP and postmenopausal women with normal bone mineral density were collected in serum separator tubes (BD Biosciences, San Jose, CA, USA), clotted for 15 min and then centrifuged at $3,000 \times g$ for 5 min at 4°C. Serum were collected and stored at -80°C until detected. The serum levels of *Sfrp1* and TNF- α in patients with postmenopausal OP and postmenopausal women with normal bone mineral density were determined by ELISA, according to the manufacturer's instructions. The testing kits for *Sfrp1* (cat. no. CSB-E15074h) and TNF- α (cat. no. CSB-E04740h) were purchased from Cusabio Biotech Co., Ltd. (Wuhan, China). The absorbance was measured at 450 nm using the SpectraMax M5 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

5-Ethynyl-2'-deoxyuridine (EdU) staining. Thymidine analog EdU staining was used to detect cell proliferation. The MSCs (1×10^4 /well) were seeded in 96-well plates (Corning Inc., Corning, NY, USA) and cultured in a humidity incubator at 37°C with 5% CO₂ in atmosphere. The MSCs were transfected as previously mentioned and then treated with 50 μ M EdU (cat. no. C10310-2, Guangzhou Ribobio Co., Ltd.) for 2 h, following which, they were collected and stained with reagents in the Apollo® 643 EdU labeling kit (cat. no. C10310-2, Guangzhou Ribobio Co. Ltd.). The cell nuclei were stained with Hoechst 33342 (cat. no. C1022, Beyotime Institute of Biotechnology; Haimen, China). The stained cells were then observed and images were captured with a fluorescence confocal microscope (Olympus FV1000, Olympus Corporation, Tokyo, Japan).

Apoptosis assay. Flow cytometry and a Hoechst staining assay were used to determine the effect of miR-144 on apoptosis. The BMSCs (1×10^6) were seeded into 6-well plates (Corning Inc.), and then transfected with miR-144 mock, miR-144 mimic or miR-144 inhibitor plasmids, respectively. After 48 h, the cells were collected and stained with reagents in an Annexin V-FITC/PI apoptosis detection kit (Sigma-Aldrich; Merck KGaA) at room temperature for 10 min in the dark, following which, they were immediately counted with a BD FACSCalibur flow cytometry (BD Biosciences). CellQuest software (version 3.3; BD Biosciences) was used to assess the resultant data. Apoptosis-induced chromatin pycnosis in the nucleus was detected by Hoechst staining. The transfected cells were seeded into 24-well plates and cultured for 48 h, following which, they were fixed in 4% (w/v) paraformaldehyde at 4°C for 20 min. The cells were then stained with reagents in a Hoechst 33258 staining kit (cat. no. C1011, Beyotime Institute of Biotechnology) at room temperature for 30 min in the dark, and images were captured with a laser scanning confocal microscope (LSM700; Zeiss AG, Oberkochen, Germany).

Colony formation assay. Briefly, the BMSCs transfected with plasmids were seeded into 6-well plates (Corning) and maintained in a medium containing 10% FBS; the medium was refreshed every 2 days. Following 7 days of incubation, the cells were stained with 0.1% crystal violet solution (Sigma-Aldrich; Merck KGaA) at room temperature for 10 min, and the numbers of colonies containing >50 cells were

counted. Colonies were observed using a microscope imaging system (Nikon Corporation, Tokyo, Japan).

Western blot analysis. RIPA buffer (Sigma-Aldrich; Merck KGaA) reconstituted with a proteasome inhibitor (Roche Diagnostics, Indianapolis, IN, USA) and phosphatase inhibitors (Roche Diagnostics) was used to lyse cells. The concentration of samples were determined using a Pierce™ BCA Protein Assay Kit (Pierce; Thermo Fisher Scientific, Inc.). Samples of total cellular protein were loaded onto 5-10% SDS-polyacrylamide gels (20 µg of protein per lane) and then separated by electrophoresis. The separated protein bands were transferred onto PVDF membranes (EMD Millipore, Billerica, MA, USA), then the PVDF membranes were blocked with 5% bovine serum albumin solution (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, and then incubated with antibodies against Runx2 (1:1,000; cat. no. 8486, CST Biological Reagents Co., Ltd.), cyclin-dependent kinase (CDK)4 (1:3,000; cat. no. 12790, CST Biological Reagents Co., Ltd.), Sfrp1 (1:4,000; cat. no. 3534, CST Biological Reagents Co., Ltd.), Wnt1 (1:1,000; cat. no. ab85060, Abcam), β-catenin (1:5,000; cat. no. 8480, CST Biological Reagents Co., Ltd.) or GAPDH (1:10,000; cat. no. ab8245, Abcam) overnight at 4°C. HRP-conjugated goat anti-rabbit (cat. no. BA1054) or anti-mouse (cat. no. BA1050) antibodies (1:20,000; Boster Biological Technology, Pleasanton, CA, USA) were used as secondary antibodies; membranes were incubated with secondary antibodies for 2 h at room temperature. An enhanced chemiluminescence kit (GE Healthcare, Chicago, IL, USA) was used to visualize protein bands.

Immunofluorescence assay. The effects of miR-144 on the expression of Sfrp1 and Runx2 were investigated by immunofluorescence. Briefly, the BMSCs were fixed and permeabilized with 4% (w/v) paraformaldehyde at 4°C for 20 min. The cells were then incubated overnight at 4°C with primary antibodies (anti-Sfrp1 or anti-Runx2, 1:100 dilution), following which they were washed and stained with either FITC-conjugated goat anti-mouse IgG (1:100; cat. no. sc-2010, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or phycoerythrin-conjugated IgG (1:1,000; cat. no. sc-3738, Santa Cruz Biotechnology, Inc.) at 4°C for 30 min in the dark. The cell nuclei were stained with DAPI (10 µg/ml) at room temperature for 5 min in the dark. A laser scanning confocal microscope (LSM700; Zeiss AG) was used to capture the images.

Alizarin red staining. To induce osteoblast differentiation, the BMSCs were cultured in complete medium for the osteogenic differentiation of rat BMSCs (Cyagen Biosciences, Inc., Santa Clara, CA, USA), according to the manufacturer's guidelines. After 3 weeks of culture, calcium nodi, which are indicative of osteogenic differentiation, were detected by Alizarin red staining. The BMSCs were then transferred onto slides and fixed in 10% ethanol, following which the slides were incubated in 0.1% Alizarin red staining solution (cat. no. G8550, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at room temperature for 30 min and then rinsed with distilled water. Following a final wash, images of the stained cells were captured using a microscope imaging system (Nikon Corporation).

Alkaline phosphatase (ALP) assay. The activity of ALP, a marker of early osteoblastic differentiation, was detected and used to demonstrate osteogenic differentiation. ALP activity assays were performed following 14 days of osteogenic differentiation. Briefly, the cells were collected and lysed with lysis buffer, and the total protein concentration was analyzed with a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). ALP activity was determined using a phosphatase assay kit (Beyotime Institute of Biotechnology), with the absorbance measured at 405 nm.

Reactive oxygen species (ROS) assay. Flow cytometric assays were performed to detect the levels of ROS in BMSCs. The BMSCs (1×10⁶) were seeded into 6-well plates and cultured for 24 h. The BMSCs were collected and stained with the reagents of a Reactive Oxygen Species Assay kit (cat. no. MAK144, Sigma-Aldrich; Merck KGaA) at 37°C for 1 h. Following staining, the cells were immediately counted with a BD FACS Calibur flow cytometry (BD Biosciences). CellQuest Pro software was used to analyze the data.

Statistical analysis. Each experiment was repeated at least three times, and results are expressed as the mean ± SEM. Student's t-test and one-way ANOVA with the Newman-Keuls post hoc test were used to analyze the data. All statistical analyses were performed using GraphPad Prism Version 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-144, Sfrp1 and TNF-α in clinical samples. In order to investigate the biological roles of miR-144, Sfrp1 and TNF-α in the development of OP, their respective expression levels were compared clinically in patients with postmenopausal OP (n=15) and postmenopausal women with a normal bone density (n=15). The RT-qPCR results showed that the expression of miR-144 was upregulated in the patients with OP compared with its expression in the normal group (Fig. 1A). The serum levels of Sfrp1 and TNF-α were detected by ELISA, which showed trends that were similar to that of miR-144 (Fig. 1B and C). In addition, correlation analysis (n=30) revealed a significant correlation between the serum levels of miR-144 and Sfrp1 (Fig. 1D). These results indicated that miR-144 and Sfrp1 are involved in the development of OP.

Effect of miR-144 on BMSC proliferation and apoptosis. To examine the role of miR-144 in the function of BMSCs, BMSCs were first isolated from rats and then transfected with miR-144 mimic or the miR-144 inhibitor. EdU staining was used to evaluate the effect of miR-144 on BMSC proliferation. The results showed that overexpression of miR-144 efficiently promoted the proliferation of BMSCs when compared with BMSCs in the mock groups (Fig. 2A and B), whereas the opposite effect was observed for BMSCs in the miR-144 inhibitor group (Fig. 2A and B). In addition, Hoechst 33258 staining was used to detect the effect of miR-144 on the nuclear morphological features of BMSCs. As shown in

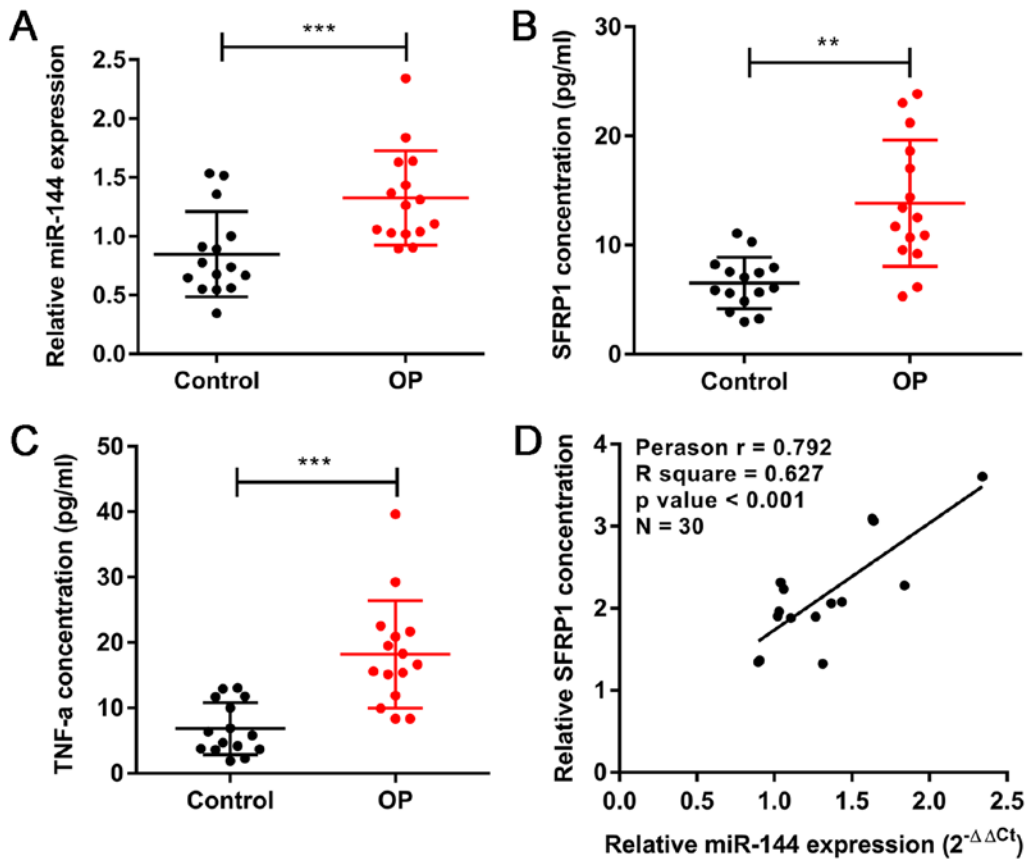


Figure 1. Expression of miR-144, Sfrp1 and TNF- α in clinical samples. (A) Expression of miR-144 was detected by reverse transcription-quantitative polymerase chain reaction analysis. Serum levels of (B) Sfrp1 and (C) TNF- α were detected by ELISA. (D) Correlation analysis was performed to examine the relationship between miR-144 and Sfrp1. Data represent the mean \pm SEM. **P<0.01 and ***P<0.001. OP, osteoporosis; miR, microRNA; Sfrp1, secreted frizzled-related protein 1.

Fig. 2C and D, the number of BMSCs with chromatin condensation was markedly increased following treatment with the miR-144 inhibitor when compared with the BMSCs in the mock group. However, there was no change in the BMSCs treated with the miR-144 mimic (Fig. 2C and D). As shown in Fig. 2B and C, the ratio of positive cells indicates the ratio of positive to total cells. An apoptosis detection kit was then used to detect the role of miR-144 in BMSC apoptosis. The results showed that the miR-144 inhibitor significantly increased the numbers of apoptotic BMSCs when compared with the BMSCs in the mock group, whereas there was no difference between the BMSCs treated with the miR-144 mimic and the mock group (Fig. 2E and F). It was also found that the absence of miR-144 significantly increased the levels of intracellular ROS in BMSCs compared with those in BMSCs in the mock groups (Fig. 2G and H), indicating that the absence of miR-144 may induce apoptosis in BMSCs by increasing their concentrations of reactive oxygen species.

miR-144 promotes the proliferation and differentiation of BMSCs. To further examine the effect of miR-144 on the proliferation of BMSCs, a colony formation assay was used to evaluate the effect of miR-144 on BMSC proliferation. The results indicated that the overexpression of miR-144 efficiently promoted the proliferation of BMSCs, whereas miR-144 knockdown efficiently inhibited BMSC proliferation compared with that in the mock group (Fig. 3A). Subsequently,

Alizarin red staining was performed to detect the numbers of calcium nodi in the BMSCs. The results showed that the overexpression of miR-144 significantly increased the numbers of calcium nodi, whereas miR-144 knockdown efficiently decreased the numbers of calcium nodi in the BMSCs, compared with the numbers in the corresponding mock groups (Fig. 3B), indicating that miR-144 may induce the osteoblastic differentiation of BMSCs *in vitro*. The ALP activity assay was used to further examine the role of miR-144 in the osteogenic differentiation of BMSCs. The results showed that the overexpression of miR-144 significantly increased ALP activity, whereas miR-144 knockdown decreased ALP activity (Fig. 3C). When taken together, these results indicate that miR-144 can induce the osteoblastic differentiation of BMSCs *in vitro*.

Expression of Sfrp1 and Runx2 in BMSCs is regulated by miR-144. It is generally known that miRNA binds to the 3'-UTR of its target mRNAs and thereby regulates gene expression. The TargetScan website (<http://www.targetscan.org/>) was used to predict the binding site for miR-144, and the results indicated that *Sfrp1* was a potential target of miR-144. To investigate the role of miR-144 in the expression of *Sfrp1*, BMSCs were transfected with miR-144 mimic or inhibitor and the expression levels of Sfrp1, miR-144 and Runx2 were detected by RT-qPCR and western blot methods. As shown in Fig. 4, the expression levels of miR-144 in the miR-144 mimic group and

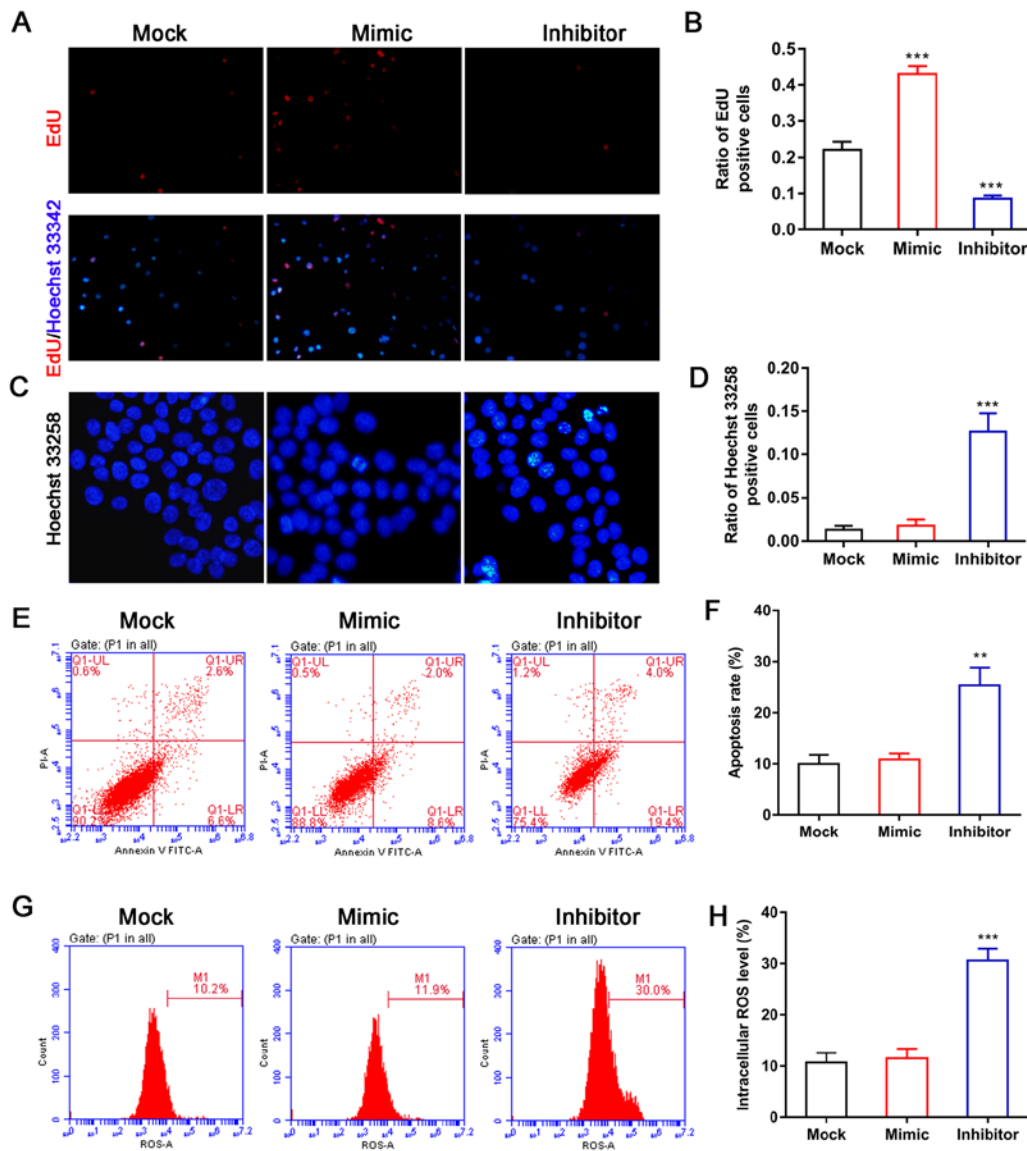


Figure 2. Effect of miR-144 on BMSC proliferation and apoptosis. (A) EdU staining was used to evaluate and the effect of miR-144 on BMSC proliferation (magnification, $\times 100$). (B) Ratio of Edu-positive cells to total cells. (C) Effect of miR-144 on the nuclear morphological features of BMSCs was observed following Hoechst 33258 staining (magnification, $\times 200$). (D) Ratio of Hoechst 33258-positive cells to total cells. (E) Effect of miR-144 on BMSC apoptosis was detected by flow cytometry and (F) quantified. (G) Intracellular ROS in BMSCs were detected by flow cytometry and (H) quantified. The group of BMSCs transfected with miR-144 mimic or the miR-144 inhibitor were compared with BMSCs in the mock groups. Data represent the mean \pm SEM. ** $P < 0.01$ and *** $P < 0.001$. miR, microRNA; BMSCs, bone marrow-derived mesenchymal stem cells; ROS, reactive oxygen species.

miR-144 inhibitor group were significantly upregulated and downregulated, respectively, compared with their levels in the corresponding mock groups (Fig. 4A). Furthermore, the mRNA and protein levels of *Sfrp1* in the BMSCs transfected with miR-144 mimic or miR-144 inhibitor were significantly reduced and increased, respectively (Fig. 4A and B). The immunofluorescence assay showed similar results (Fig. 4C). The present study also investigated how the mRNA and protein levels of Runx2 were altered by miR-144, and found that Runx2 levels in the BMSCs were significantly increased when miR-144 was overexpressed (Fig. 4A and B). By contrast, the expression of Runx2 in BMSCs was significantly reduced when miR-144 was knocked down (Fig. 4A and B), and the immunofluorescence assays showed similar results (Fig. 4C). The protein levels of CDK4, Wnt1 and β -catenin were also markedly increased by miR-144 (Fig. 4B).

miR-144 promotes the proliferation and differentiation of BMSCs by downregulating the expression of Sfrp1. The binding site for miR-144 in *Sfrp1* mRNA was predicted using the TargetScan website, and *Sfrp1* was suggested as a target gene for miR-144 in BMSCs. The luciferase reporter assay was used to examine the direct interaction between miR-144 and *Sfrp1*. The 3'-UTR sequences of the WT and MUT *Sfrp1* were sequenced, and the results revealed that the MUT *Sfrp1* 3'-UTR gene had successfully mutated in the target region (data not shown). The 3'-UTR sequences of the WT and MUT *Sfrp1* were cloned into a psiCHECK2 plasmid in which the firefly luciferase gene is constitutively expressed. The results showed that overexpression of miR-144 significantly decreased the luciferase activity of the WT *Sfrp1* plasmid, compared with that of the other three groups (Fig. 5A). By contrast, there was no effect on the luciferase activity of the MUT *Sfrp1*

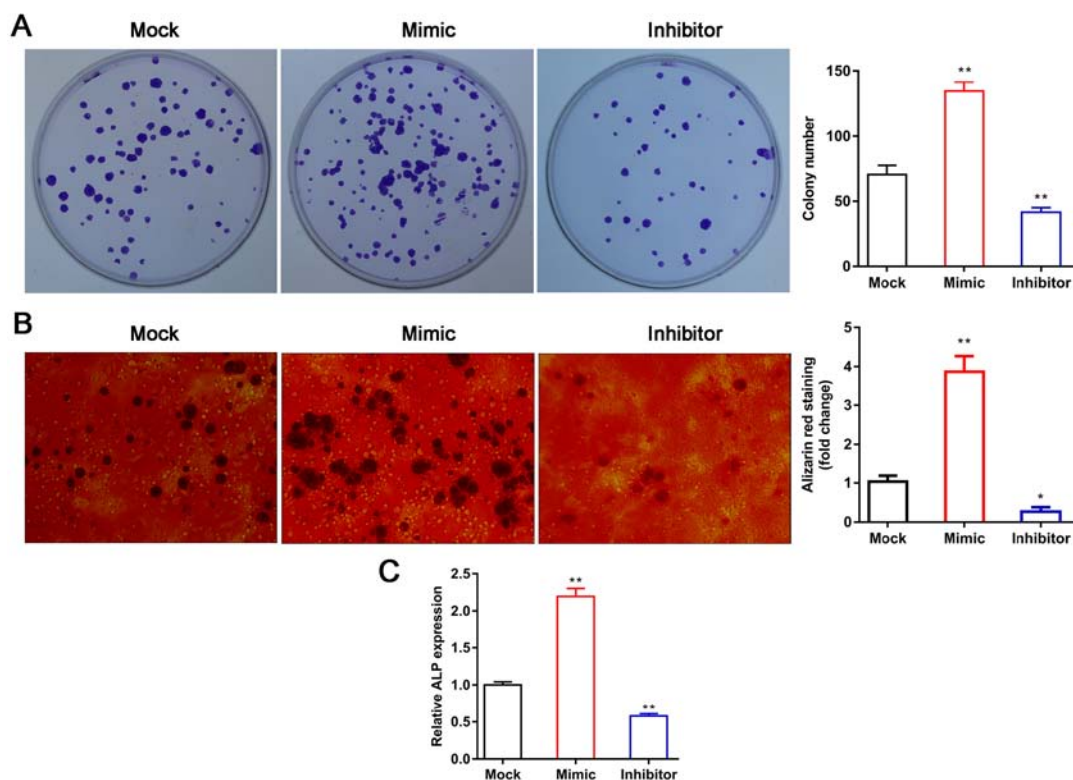


Figure 3. miR-144 promotes the proliferation and differentiation of BMSCs. (A) Effect of miR-144 on BMSC proliferation was evaluated with the colony formation assay. (B) Alizarin red staining was performed following 3 weeks of osteogenic differentiation to detect the calcium nodi in BMSCs (magnification, x200). (C) ALP activity assays were performed following 14 days of osteogenic differentiation. Data represent the mean \pm SEM. $^{\#}P<0.05$ and $^{**}P<0.01$. miR, microRNA; BMSCs, bone marrow-derived mesenchymal stem cells; ALP, alkaline phosphatase.

plasmid compared with that in the NC group (Fig. 5A). Taken together, these results indicate that miR-144 can bind to the 3'-UTR of *Sfrp1* mRNA and thereby regulate the expression of *Sfrp1*. To further examine the effects of *Sfrp1* and miR-144 on the function of BMSCs, BMSCs were transfected with *Sfrp1* siRNA. The mRNA and protein levels of *Sfrp1* were significantly reduced by *Sfrp1* siRNA, particularly in the second *Sfrp1* siRNA group, compared with that in the NC group (Fig. 5B and C). In follow-up experiments, the second *Sfrp1* siRNA was used to interfere with the expression of *Sfrp1*. The colony formation assay, Alizarin red staining and ALP activity assay were used to further examine how *Sfrp1* may influence miR-144 in promoting the proliferation and differentiation of BMSCs. The results showed that *Sfrp1* suppressed the proliferation of BMSCs compared with that of the group of BMSCs co-transfected with the miR-144 inhibitor and NC siRNA (Fig. 5D and F), and also decreased calcium nodus formation (Fig. 5E and G) and ALP activity (Fig. 5H). In addition, the protein levels of Runx2, CDK4, Wnt1 and β -catenin were markedly increased in the BMSCs co-transfected with the miR-144 inhibitor and *Sfrp1* siRNA compared with the those in the group of BMSCs co-transfected with the miR-144 inhibitor and NC siRNA (Fig. 5I). It is well known that the Wnt/ β -catenin pathway serves an important role in cell proliferation and differentiation, and that Wnt signaling can be downregulated by *Sfrp1* (39). It is also known that Runx2 is a key transcription factor associated with osteoblastic differentiation. When combined with our previous studies, these results suggest that miR-144 promotes the proliferation and

differentiation of BMSCs by upregulating the expression of Runx2, CDK4, Wnt1 and β -catenin and downregulating the expression of *Sfrp1*.

Discussion

OP is a common disease among older adults and postmenopausal women (1,3). The degeneration of osteoblasts, in terms of their function and quantity, aggravates the occurrence and development of OP; however, BMSCs can continue to differentiate into osteoblasts following directed induction, which has become an important method for preventing and curing OP (10,40). The numbers and functions of BMSCs are key factors involved in maintaining the normal physiological function of bones, and the osteogenic capacity of BMSCs in bone marrow decreases when OP occurs (8-10). Therefore, investigations on how BMSCs differentiate into osteoblasts have become a primary focus in the search for novel treatments for OP. A recent study showed that miRNAs may be involved in cell proliferation and osteoblastic differentiation (41). The present study confirmed that the expression of miR-144 was markedly increased in clinical samples from patients with OP. Furthermore, miR-144 silencing inhibited the proliferation and promoted the apoptosis of BMSCs, suggesting that miR-144 may have a key regulatory role in the progression of OP. In addition, the numbers of calcium nodi in BMSCs was significantly increased following treatment with miR-144 mimic, indicating that miR-144 induced the osteoblastic differentiation of BMSCs.

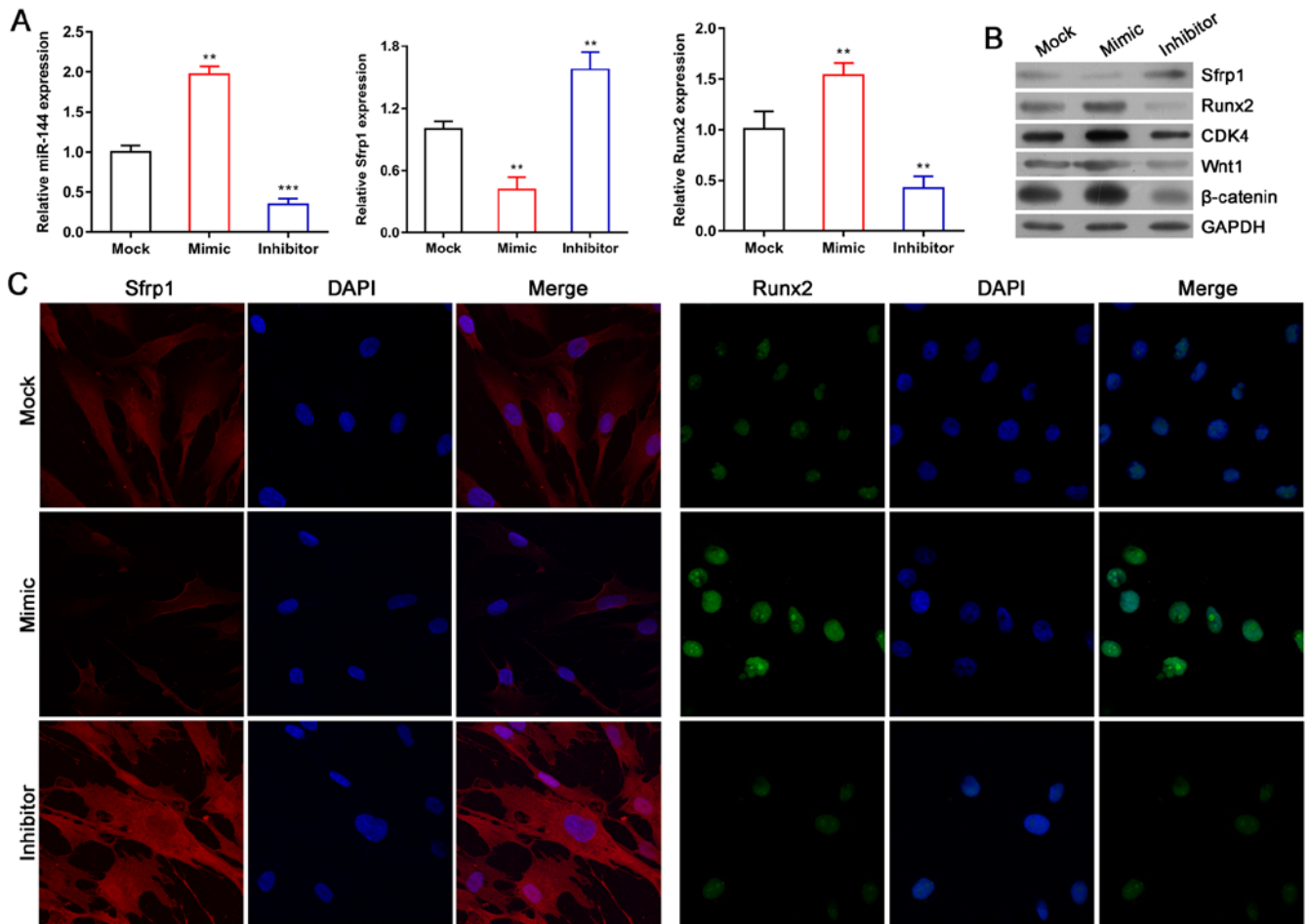


Figure 4. Expression of Sfrp1 and Runx2 in BMSCs is regulated by miR-144. (A) Expression levels of miR-144, Sfrp1 and Runx2 were detected by reverse transcription-quantitative polymerase chain reaction analysis. (B) Protein expression of Sfrp1, Runx2, CDK4, Wnt1 and β -catenin was analyzed by western blot analysis. (C) Distributions of Sfrp1 and Runx2 in BMSCs were observed using an immunofluorescence assay (magnification, $\times 200$). Data represent the mean \pm SEM. ** $P < 0.01$ and *** $P < 0.001$. miR, microRNA; BMSCs, bone marrow-derived mesenchymal stem cells; Sfrp1, secreted frizzled-related protein 1; Runx2, Runt-related transcription factor 2; CDK4, cyclin-dependent kinase 4.

The regulation of osteoblast differentiation is a complex process involving multiple genes and multiple pathways, including the Notch signaling pathway, BMP/Smad signaling pathway and Wnt signaling pathway (31-33). The Wnt/ β -catenin signaling pathway serves a vital role in regulating the proliferation and differentiation of osteoblasts and regeneration of bone tissue. Studies have shown that miRNAs with abnormal expression patterns specifically regulate expression of the Wnt signaling pathway, and are thus closely associated with the occurrence of OP. The expression of miR-218 is regulated by the Wnt pathway. The upregulation of miR-218 inhibits the expression of sclerostin, DKK2 and sFRP2, to create a similar positive feedback regulatory loop leading to osteogenic differentiation (42). Therefore, antagonists of Wnt/ β -catenin may be useful as novel agents for treating OP. In the present study, it was found that the Wnt/ β -catenin signaling pathway was significantly activated by the overexpression of miR-144 and sharply suppressed by miR-144 silencing, indicating that miR-144 partially promotes osteoblast differentiation via the Wnt/ β -catenin signaling pathway. It was further shown that *Sfrp1* was a target gene of miR-144 and negatively regulated by miR-144 in BMSCs. However, it was found that the miR-144 and Sfrp1 were expressed at high levels in the serum of patients

with OP, which may be due to the secretion of miR-144 out of the cell. When miR-144 is secreted out of the cell, such as in the blood, serum detection becomes correspondingly higher. Therefore, a positive regulation was found between miR-144 and Sfrp1 in the serum of patients with OP. Of note, Sfrp1 is an antagonist of the Wnt signaling pathway; it can inhibit the downward transduction of Wnt protein, and thereby inhibit osteoblast activity and osteogenic activity (43,44). In the present study, it was also found that suppression of the Wnt/ β -catenin signaling pathway as a result of miR-144 knockdown was partially reversed by Sfrp1 siRNA, suggesting that miR-144 may regulate osteoblast differentiation by targeting Sfrp1, which is an antagonist of the Wnt signaling pathway.

During the process by which BMSCs differentiate into osteoblasts, numerous transcription factors become activated or deactivated, and a series of osteoblast-related genes, including *Runx2* and *Osterix*, are expressed (43,45). As an important factor affecting osteoblast differentiation and functional activity, *Runx2* is regulated by BMP, TGF- β , Wnt and other signaling pathways (32,46). Altering the expression of *Runx2* affects the osteogenic differentiation of BMSCs and further influences the formation of normal osteoblasts and bone tissue. In the present study, it was shown that the

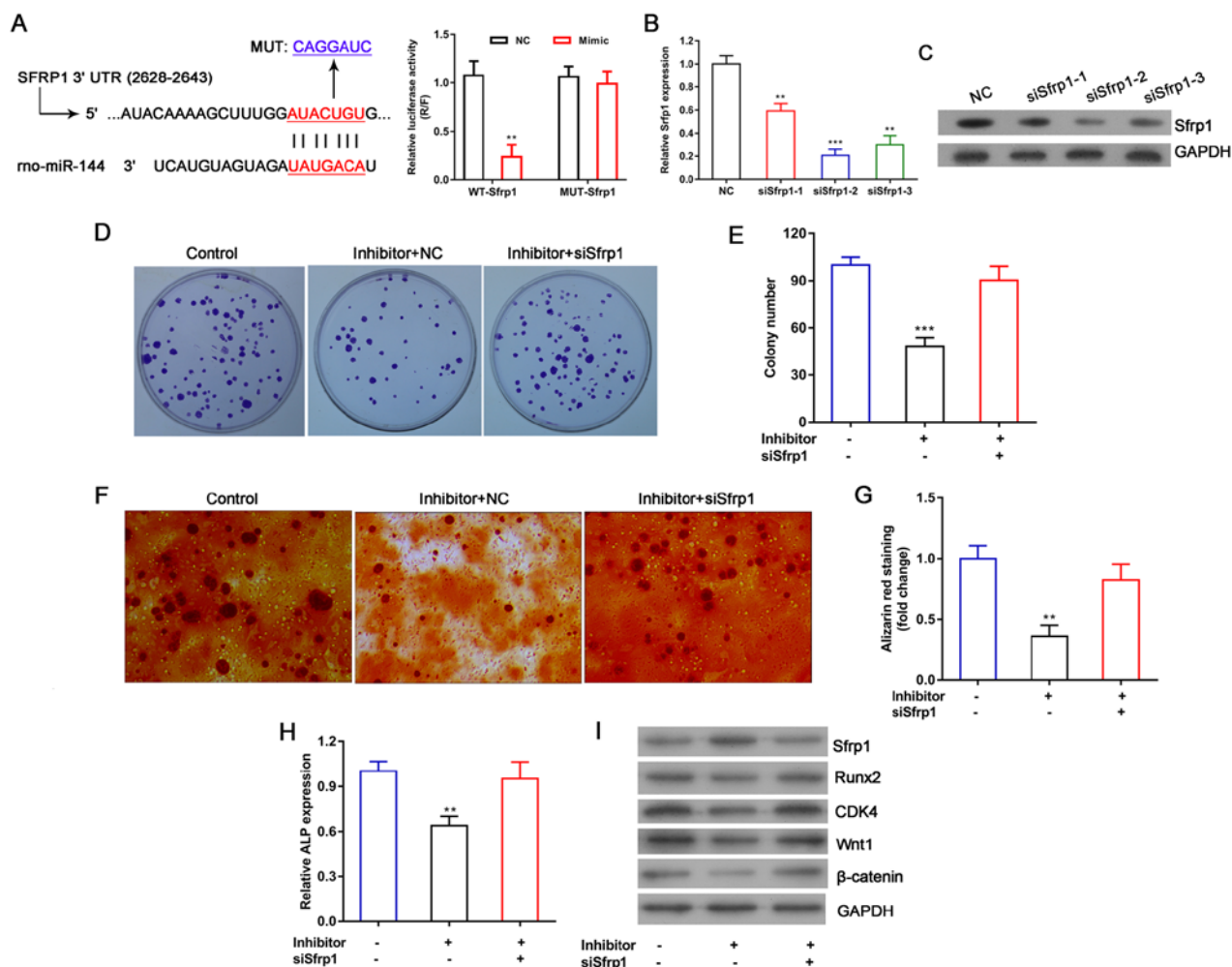


Figure 5. miR-144 promotes the proliferation and differentiation of BMSCs by downregulating the expression of Sfrp1. (A) A luciferase reporter assay was used to examine the direct interaction between miR-144 and Sfrp1. The group of BMSCs were co-transfected with WT Sfrp1 plasmid and miR-144 mimic was compared with the other three groups. BMSCs were transfected with Sfrp1 siRNA, and the interference efficiency was analyzed by (B) reverse transcription-quantitative polymerase chain reaction and (C) western blot analysis, respectively. (D) A colony formation assay was used to determine how Sfrp1 affected the ability of miR-144 to promote BMSC proliferation, and (E) quantified. (F) Alizarin red staining was performed following 3 weeks of osteogenic differentiation to detect the calcium nodi in BMSCs (magnification, x200), with (G) quantification of staining. (H) ALP activity assays were performed following 14 days of osteogenic differentiation. The group of BMSCs co-transfected with the miR-144 inhibitor and NC siRNA was compared with other two groups. (I) Protein expression levels of Sfrp1, Runx2, CDK4, Wnt1 and β -catenin were analyzed by western blot analysis. GAPDH was used as an internal control. Data represent the mean \pm SEM. ** $P < 0.01$ and *** $P < 0.001$. miR, microRNA; BMSCs, bone marrow-derived mesenchymal stem cells; WT, wild-type; MUT, mutant; siRNA, small interfering RNA; NC, negative control; ALP, alkaline phosphatase; Sfrp1, secreted frizzled-related protein 1; Runx2, Runt-related transcription factor 2; CDK4, cyclin-dependent kinase 4.

downregulation of miR-144 suppressed the expression of *Runx2*; however, this effect was partially reversed following treatment with Sfrp1 siRNA, demonstrating that miR-144 promotes the osteoblastic differentiation of BMSCs by targeting *Sfrp1*. CDK4 is reported to regulate the transition from the G1 phase to the S phase, and thus promote the growth of osteoblasts (47). In the present study, it was shown that miR-144 silencing significantly suppressed the expression of CDK4, but that effect was partially reversed when Sfrp1 was simultaneously knocked down. This indicates that miR-144 promotes the proliferation of BMSCs by enhancing the expression of CDK4, and thereby promoting the transition from the G1 phase to the S phase.

In conclusion, the data obtained in the present study showed that the levels miR-144, Sfrp1 and TNF- α were significantly increased in the serum of patients with OP, and there was a significant positive correlation between miR-144 and Sfrp1. In

addition, functional experiments demonstrated that miR-144 promoted the proliferation of BMSCs, inhibited the apoptosis of BMSCs and induced the osteoblastic differentiation of BMSCs, partially by targeting Sfrp1, which is an antagonist of the Wnt signaling pathway. These results provide a basis for investigating the mechanism by which miR-144 is involved in the occurrence and development of OP, and also suggest a novel molecular target for preventing and treating OP.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

LT and SL conceived and supervised the study. LT, WL and JH designed and performed experiments. XT and HZ analyzed the data. LT and SL drafted the manuscript and made revisions. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The collection and analysis of all clinical samples was approved by the Ethics Committee of the Affiliated Hospital of Guilin Medical University (Guilin, China; GLMUIA2016002). The protocols for all animal experiments were approved by the Animal Ethics Committee of the Affiliated Hospital of Guilin Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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