MiR-26b-5p Promotes Osteogenesis of Bone Mesenchymal Stem Cells via Suppressing FGF21

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Research Article

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Abstract

Introduction: It has been established that miR-26b-5p actively participate in the osteogenic differentiation of bone mesenchymal stem cells (BMSCs), which is of great value in osteoporosis treatment. Database showed that Fibroblast growth factor(FGF)21 is a potential binding site of miR-26b-5p. This study aimed to investigate the molecular osteogenic mechanisms of miR-26b-5p targeting FGF21 in postmenopausal osteoporosis (PMOP).

Methods: 5ml of bone marrow was aspirated from the anterior superior iliac spine in 10 PMOP women during bone marrow puncture. BMSCs were used to establish an in vitro cell model, and BMSCs markers were analyzed by flow cytometry. miR-26b-5p and FGF21 were overexpressed for 48h, and then placed in an osteogenic induction medium for osteogenic induction culture, the expression of RNA was detect using RT-qPCR. Cells from miR-26b-5p group were collected on days 7, 14 and 21 of induction for ALP and alizarin red S staining. On day 7 of induction, RT-qPCR was used to measure Runx2, Osterix (Osx), and target gene FGF21 expression levels in each group. The dual-luciferase reporter gene system was used to verify that FGF21 was a direct target of miR-26b-5p. FGF21 was measured by western blotting in the miR-26b-5p overexpression group and in the miR-26b-5p inhibition group.

Results: BMSCs were identified according with the antigenic characteristics. miR-26b-5p expression was significantly upregulated after the expression of miR-26b-5p mimics; however, FGF21 expression was downregulated after FGF21 mimics. After overexpression of miR-26b-5p, the alkaline phosphatase activity and nodules of alizarin red S in the culture medium gradually increased as the induction time increased. RT-qPCR showed that the expressions of master osteogenic factors Runx2 and Osx in the BMSC+ osteogenic differentiation medium group was significantly higher than in the BMSC group, the expressions of the factors in the BMSC+ miR-26b-5p overexpression group was significantly higher than in the control group. Target gene FGF21 expression was significantly lower in the BMSC+ osteogenic differentiation medium group than in the BMSC group, and was significantly lower in the BMSC+ miR-26b-5p overexpression group than in the control group. Luciferase reporter assays demonstrated that FGF21 was a direct target of miR-26b-5p. Finally, western blotting analysis showed that FGF21 expression was significantly downregulated in the miR-26b-5p overexpressed group and upregulated in the miR-26b-5p inhibition group.

Conclusion: miR-26b-5p can regulate the osteogenic differentiation of BMSCs and participate in PMOP pathogenesis via suppressing FGF21. The present study provides the basis for further studies on PMOP.

Introduction

Osteoporosis (OP) is a common metabolic bone disease characterized by decreased bone mass and strength [1]. More than 70 million people in China reportedly have osteoporosis, and the prevalence rate in women over 50 years old is 20.7% [2]. It has been shown that the decrease of estrogen levels after menopause leads to endocrine disorders, which further affect calcium absorption and metabolism,

leading to bone resorption, bone destruction and low back pain. The incidence of OP in postmenopausal women is reportedly two to three times that of non-menopausal women [3]. Most importantly, osteoporosis affects patient quality of life and is a major socio-economic health problem [4].

Bone homeostasis is maintained by a balance between bone resorption by osteoclasts and bone formation by osteoblasts to maintain normal structure and function [5]. Abnormal osteogenesis occurs when this balance is disrupted, leading to osteoporosis or metabolic bone diseases [6]. microRNAs (miRNAs) are small non-coding single-stranded RNA molecules that have been extensively studied in tumors, bone metabolism and other research areas [7]. miRNAs mainly bind to the 3' non-coding region of target genes and negatively regulate expression of target factors, leading to degradation or cessation of the translation process, thus affecting cell proliferation, migration, and apoptosis [8]. In recent years, the biological role of miRNA in BMSCs, osteoblasts and osteoclasts has attracted mounting attention[9]. miR-26b promoted BMSC osteogenesis by directly targeting GSK3β and activating canonical Wnt signal pathway, suggesting miR-26b might be serve as potential therapeutic candidate of osteoporosis [9]. Studies have documented that miR-26b-5p is a positive regulator of goat intramuscular preadipocyte via targeting FGF21 [10]. So we hypothesized that FGF21 could be involved in the regulation of osteogenesis.

Fibroblast growth factor (FGF)21, a special member of FGF superfamily, has been proven to have pleiotropic metabolic effects and many potential therapeutic action in various metabolic disorders [11]. Study showed that FGF-21 in high glucose environment could inhibit the osteogenic differentiation of hBMSCs [12]. FGF21 might be an efficient endogenous vasoprotective factor for calcification [13].

It remains unclear whether FGF21 can be leveraged as a target of miR-26b-5p to regulate BMSCs differentiation, warranting further studies. This study aimed to investigate the regulatory mechanisms of miR-26b-5p on BMSCs in humans.

Experiment Instruments And Reagents

Ultraviolet spectrophotometer, Real-time PCR detection system, flow cytometer, electrophoresis apparatus, and transfer membrane apparatus were purchased from Bio-rad Corporation, USA. The Luciferase reporter assay was purchased from Progmega Corporation, USA.

The RNA Extraction Kit, reverse transcription Kit, and primers were purchased from Takara Corporation, China. The BCA Protein Quantitation Kit was purchased from Doyang Corporation, China. Primary and secondary antibodies used during western blotting were purchased from abcam Corporation, USA. ALP kit, alizarin red S staining kit, BCIP®/NBT solution, and carriers were purchased from Bio-Rad, USA. DMEM high sugar medium and osteogenic differentiation medium were purchased from Sigma Corporation, USA. Fetal bovine serum, pMIR-REPORT plasmid, lipidosomes, Lipofectamine 2000, and 293T cells were purchased from Invitrogen Corporation, USA. A plasmid extraction kit was purchased from QIAGEN Corporation, Germany. Mimics and controls and inhibitors, Lipofectamine RNAiMAX Transfection Reagent, and Trizol reagent were purchased from Jima Corporation, China.

Methods

This study was approved by the Ethics Committee of the Foshan Sanshui District People's Hospital in accordance with the Helsinki Declaration of 1975 (revised in 2000). Informed consent was obtained from all individual participants included in the study. Ten postmenopausal women were recruited from July 2021 to October 2021. 5ml of bone marrow was aspirated from the anterior superior iliac spine in all patients during bone marrow puncture. Prior to the study, the study participants exhibited no mental, cognitive or mobility-related impairment. A diagnosis of PMOP was established with bone mineral density (BMD) T value ≤-2.5 measured by dual-energy X-ray absorptiometry. Patients with severe chronic diseases that cause metabolic abnormalities such as secondary PMOP and patients that received drug or hormone therapy within the past year were excluded.

BMSCs culture

5 mL bone marrow was collected in lithium heparin tubes. To establish an in vitro culture system for osteogenic differentiation of BMSCs, the bone marrow was evenly mixed with an equal volume of DMEM culture medium containing 1% double-antibody. The mixture was centrifuged at room temperature, and the supernatant was removed. The cells were re-suspended in a DMEM culture medium. The cell suspension was slowly added to an equal volume Percoll separation solution and centrifuged at room temperature. The middle layer of mononuclear cells was re-suspended with culture medium. After full mixing, a suspension containing BMSCs was obtained and cultured in a 5% CO2 incubator at 37℃. The fluid was changed every 24 hours; the cell morphology, size, and distribution were observed under an inverted microscope. After trypsin digestion, the DMEM medium was used to adjust the cell concentration to 2×10^5 and inoculated in cell culture plates and cultured. When the degree of cell fusion reached 70-80%, trypsin was digested, the digested cell suspension was collected and inoculated into a culture flask for subculture. Third-generation BMSCs in a logarithmic growth stage were added to an osteogenic induction solution (α-MEM culture medium containing fetal bovine serum, dexamethasone, ascorbic acid and β-sodium glycerophosphate) for three weeks. Cells were then collected, and the total RNA was extracted.

Identification of BMSCs

A BMSC suspension of 1×10^6 cells/ml was prepared. The cells were washed twice with cold PBS, centrifuged at 1,000 x g for 5 min at 4˚C, and re-suspended in 100 ml stain buffer. The re-suspended cells were incubated with phycoerythrin-labeled primary antibodies against surface markers CD34, CD45, CD73, CD90, CD105, and a corresponding isotype control antibody at room temperature, according to the manufacturer's protocol. The positively stained cells were analyzed by flow cytometry using FlowJo software 8.7.1. BMSCs from passages 3-6 were used in the experiments.

RT-qPCR

Total RNA was extracted from cultured cells or bone tissues using RNAiso Plus, and cDNA was synthesized using PrimeScript RT Master Mix. Real-time PCR was performed using primers synthesized by Thermo Fisher Scientific and SYBR Premix kit. The sequences of primers used are as follows: 5’ to 3’: Runx2-F CTCCTACCTGAGCCAGATGACG, Runx2-R GTGTAAGTAAAGGTGGCTGGATAGT; Osx-F CCAAGTGGGTGGTATAGAG, Osx-R GGGATGGTGGGTGTAAGA; FGF21-F GCCTCTAGGTTTCTTTGCC, FGF21-R GACTCCTGGTTGCTCTTGG; β-actin-F TGGCACCCAGCACAATGAA, β-actin-R CTAAGTCATAGTCCGCCTAGAAGCA. The PCR procedure consisted of denaturation at 94℃ for 5 minutes; 30 cycles of denaturation at 94℃ for 30 seconds; annealing at 58℃ for 30 seconds and extension at 72℃ for 40 seconds; extension at 72℃ for 10 minutes. β-actin was selected as the internal reference. Data were expressed using the comparative CT (2-ΔΔCT) method and normalized to β-actin.

Protein extraction and western blotting (WB) analysis

Cell samples were rinsed twice with cold PBS and harvested in the lysis buffer. The lysate was centrifuged at 4˚C/16,000g for 30 min, and the suspension was collected. Then, the protein content was examined using a BCA kit. Total proteins (20 μg) were separated by 12% (w/v) SDS-PAGE. The proteins were then transferred onto a polyvinylidene fluoride membrane. The membrane was washed and blocked with freshly prepared TBST containing 5% (w/v) non-fat dry milk for 90 mins at room temperature. The membrane was incubated with antibodies targeting FGF21 and β-actin overnight at 4˚C. After washing three times, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody for 1 h at room temperature. The membrane was again washed three times; then, the protein-antibody complexes were examined using an enhanced chemiluminescent detection reagent. Antibody signals were developed using a Bio-Rad XRS chemiluminescence detection system. Protein band densities were analyzed using Quantity One Software. The mean expression levels of the proteins relative to β-actin were presented [14].

Osteogenic differentiation and ALP/Alizarin Red S staining

ALP staining and Alizarin Red S staining were conducted on days 7, 14 and 21 of induction to assess ALP activity and calcium deposit formation. For ALP staining, cells were fixed with 10% formaldehyde for 15 mins, rinsed three times with deionized water, and treated with the BCIP®/NBT solution for 20 mins. After washing, the stained cultures were photographed. To measure ALP activity, cell lysates were tested using a commercial ALP assay kit. For Alizarin red S staining, BMSCs were stained with pH 4.2, 0.1% Alizarin red S for 5 mins, and the images were captured using a scanner. The calcium deposition was dissolved in 10 cetylpyridinium chloride, and the absorbance of the extracts was determined at 570 nm [14].

Cell transfection

We Purchased miR-26b-5p mimics, FGF21 mimics and the corresponding negative (NC), miR-26b-5p inhibitor and the control. Cells were seeded in six-well plates at a density of 5×10^5; when the cell fusion degree reached 90%, the culture medium was discarded, and cells were washed twice with PBS buffer and placed in 3ml of Opti-MEM culture medium in each well. The plasmids were diluted to 20uM with an Opti-MEM culture medium and transfection reagent. The corresponding groups of RNA were added to the transfection reagent, incubated for 20mins, and then added to the culture medium. After 6h of culture, it was replaced with an ordinary culture medium, and the expression level was verified after culture for 48h. miR-26b-5p and FGF21 were overexpressed for 48h, and then placed in an osteogenic induction medium for osteogenic induction culture, the expression of RNA was detect using RT-qPCR. Cells from miR-26b-5p group were collected on days 7, 14 and 21 of induction for ALP and alizarin red S staining. They were divided into four groups: BMSC group, BMSC+ osteogenic differentiation medium group, BMSC+ miR-26b-5p overexpression control group (NC), and BMSC+ miR-26b-5p overexpression group (mimics). The expressions of osteogenic specific genes Runx2 and Osx and target factor FGF21 were measured by RT-qPCR on day 7 of osteogenic induction. FGF21 was measured by western blotting in the miR-26b-5p overexpression group and in the miR-26b-5p inhibition group.

Dual-luciferase reporter gene assay

Using TargetScanFish software, we found miR-26b-5p may combine FGF21. We performed a dual-luciferase binding assay to verify the binding site and cloned FGF21 3'-UTR containing the predicted miR-26b-5p binding site in the pMIR-REPORTTM miRNA Expression Reporter vector. The plasmid was named pMIR-FGF21-WT (wild type). A mutated FGF21 reporter gene was created from the mutation-binding site region using a site-directed mutagenesis kit and named PMIR-FGF21-MT (mutant type). For transfection, cells were inoculated in 24-well plates, and 100ng pMIR-FGF21-WT or pMIR-FGF21-MT fluorescence reporter vector and 50ng miR-26b-5p mimics were co-transfected into 293T cells using Lipofectamine 2000. After 48h, luciferase activity was measured with the luciferase assay system according to the manufacturer's instructions.

Statistical analysis

All measurements were repeated three times and analyzed using SPSS (v.22.0). Differences between two groups were determined by the Student's t-test, while multigroup comparison was performed by one-way analysis of variance followed by Bonferroni's post hoc test. All data were expressed as mean ± standard deviation. A P-value less than 0.05 was statistically significant.

Results

Culture and identification of BMSCs

The surface markers of BMSCs were analyzed by flow cytometry (Fig 1). We found that the BMSCs were positive for CD73, CD90, and CD105 and negative for CD34 and CD45. These mesenchymal stem cells were confirmed to be BMSCs, which accords with the antigenic characteristics of mesenchymal stem cells.

Overexpression of miR-26b-5p promoted osteogenic diffrentiation and FGF21 inhibited osteogenic diffrentiation

miR-26b-5p expression was significantly increased from 2.33±0.31 to 6.76±1.21 after the expression of miR-26b-5p mimics; however, FGF21 expression was decreased from 5.24±0.23 to 1.43±0.74 after FGF21 mimics, the difference is stark ($P<0.05$).

Overexpression of miR-26b-5p promoted osteogenic diffrentiation

In the meantime, cells from passages 3-6 of each group were transfected for overexpression of miR-26b-5p. After 48h, the culture medium was replaced with an osteogenic induction medium, and ALP and alizarin red S staining experiments were performed on days 7, 14, and 21. The results showed that after miR-26b-5p was overexpressed, the level of ALP in the cell culture medium gradually increased with an increase in induction time, and alizarin red S staining showed progressively increased calcium nodule deposition (Figures 2 and 3).

On day 7 of induction, cells were collected, and RNAs were extracted. RT-qPCR showed that the expressions of master osteogenic factors Runx2 and Osx in the BMSC+ osteogenic differentiation medium group was significantly higher than in the BMSC group, and the expressions of the factors in the BMSC+ miR-26b-5p overexpression group was significantly higher than in the control group. Moreover, target gene FGF21 expression was significantly lower in the BMSC+ osteogenic differentiation medium group than in the BMSC group, and was significantly lower in the BMSC+ miR-26b-5p overexpression group than in the control group (Figure 4).

Binding site verification of miR-26b-5p and FGF21

A microRNA Target Prediction Database showed that FGF21 is a potential binding site of miR-26b-5p according to the sequence information of the miRNA. The identified binding sites are shown in Figure 5. The luciferase reporter assay showed that miR-26b-5p significantly inhibited the luciferase activity of wild-type pMIR-FGF21-WT and did not affect the mutant luciferase reporter gene activity (Figure 6). Furthermore, western blotting analysis showed that FGF21 expression in 293T cells was significantly

decreased in the miR-26b-5p overexpression group (P<0.05) and was significantly increased in the miR-26b-5p inhibition group (P <0.05) (Figure 7).

Discussion

It is widely acknowledged that PMOP results from the rapid decrease in estrogen levels in women after menopause, and an increase in osteoclasts leads to bone resorption. Interestingly, the osteogenic differentiation ability of BMSCs is weakened, which induces relatively greater bone resorption than bone formation and results in metabolic bone disease [13]. As non-coding single-stranded RNAs with regulatory functions, miRNAs are abundant in BMSCs and affect the proliferation, migration, differentiation, and other biological functions of osteogenic and osteoclast cells [9, 10]. In recent years, the effect of miRNA on BMSCs differentiation, osteoclast and osteoblast differentiation has attracted much attention, and miR-26 has been documented to play a certain role in the regulation of osteogenesis and osteoclast differentiation in PMOP [9].

Study found that FGF-21 directly promoted RANKL-induced osteoclastogenesis from bone marrow macrophages (BMMs), as well as promoted adipogenesis while concomitantly inhibiting osteogenesis of bone marrow mesenchymal stem cells (BMMSCs) [11]. A Study reveal that skeletal fragility may be an undesirable consequence of chronic FGF21 administration [15].

Conversely, augmentation of miR-100-5p using a specific agomir in OVX-operated mice decreased the levels of FGF21 in the serum and liver, and prevented osteoclastogenesis and bone loss. The present study revealed that FGF21 may be a signal molecule associated with the mechanism of liver-bone endocrine metabolism and may be targeted by miR-100-5p. In addition, miR-100-5p may serve an important role in protecting against OVX-induced osteoporosis [16]. Studies have documented that miR-26b-5p is a positive regulator of goat intramuscular preadipocyte via targeting FGF21 [10].

However, few studies explored whether the regulatory role of miR-26b-5p in human BMSCs was mediated by FGF21. In the present study, we first identified BMSCs and found they accord with the antigenic characteristics. Then RT-qPCR results showed that the osteogenic differentiation ability of BMSCs was significantly increased after overexpression of miR-26b-5p and was decreased after overexpression of FGF21. This suggests that miR-26b-5p promotes osteogenesis and FGF21 inhibits osteogenesis. Using a target prediction tool, target gene FGF21 was predicted to be the potential binding site of miR-26b-5p. The binding sequences of the wild and mutant FGF21 gene were synthesized. The miR-26b-5p mimics were able to bind to 3 '-UTR of wild-type FGF21 mRNA, and the fluorescence intensity of the pMIR-FGF21-WT+ miR-26b-5p mimics group was significantly reduced, indicating that FGF21 is the target gene of miR-26b-5p. On day 7 of osteogenic induction, Runx2 and Osx expressions were significantly higher in the BMSC+ osteogenic differentiation medium group than in the BMSC group, and were higher in the miR-26b-5p overexpression group than in the control group. FGF21 expression was significantly lower in the BMSC+ osteogenic differentiation medium group than in the BMSC group, and was significantly lower in the BMSC+ miR-26b-5p overexpression group than in the control group. It was concluded that FGF21 might

be a potential binding target of miR-26b-5p. Furthermore, WB analysis showed that FGF21 expression in the miR-26b-5p overexpression group was significantly decreased, and increased FGF21 was found in the miR-26b-5p inhibition group. Accordingly, FGF21 expression negatively correlated with the miR-26b-5p expression level. These results provide evidence that miR-26b-5p influences BMSC's osteogenic differentiation via suppressing FGF21.

A major limitation of the present study is our inability to further validate the regulatory effect of miR-26b-5p on osteocyte function after overexpression of the target gene FGF21. In conclusion, we substantiated that miR-26b-5p regulates BMSCs osteogenic differentiation and participates in the pathogenesis of PMOP via targeting FGF21, This study provided compelling evidence that could be used in the study and treatment of PMOP.

Abbreviations

BMSCs: bone marrow stromal cells; PMOP: Postmenopausal osteoporosis; FGF21: Fibroblast growth factor 21; ALP: alkaline phosphatase; OP: Osteoporosis; BMD: Bone Mineral Density

Declarations

Ethics approval and consent to participate:

Ethics committee approval was received for this study from the Ethics Committee of Foshan Sanshui District People's Hospital (Medical Research in Guangdong Province 2019003 and in Foshan city 2018AB000595).

Consent for publication:

Not applicable.

Availability of data and materials:

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

Bin Wang, Zhenhui Li, Caiyuan Mai, Penglin Mou and Lei Pan declared there were no conflicts of interest.

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Authors' contributions

Bin Wang and Caiyuan Mai designed the study and were responsible for writing and review. Zhenhui Li collected, analyzed the samples, Penglin Mou and Lei Pan interpreted the data for the work, Bin Wang performed statistical analysis. All authors wrote and approved the manuscript.

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Figures

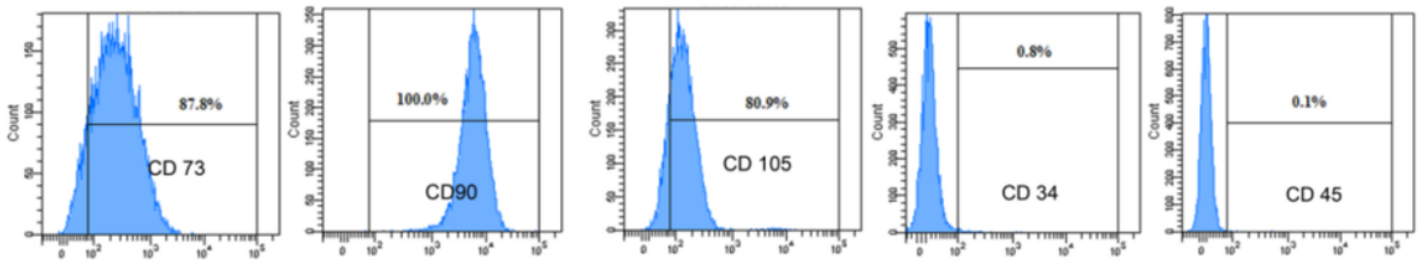

Figure 1

Cell surface marker molecular evidence for identifying circulating BMSCs by flow cytometry.

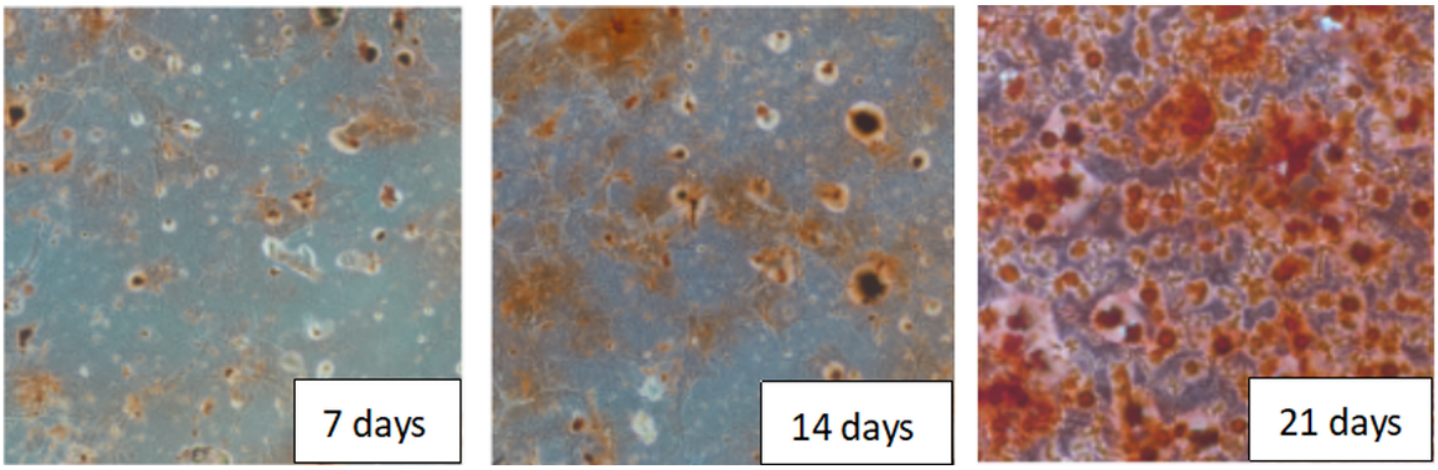

Figure 2

Alizarin red S staining results showed that the osteogenic differentiation ability of BMSCs was significantly weakened after overexpression of miR-26b-5p.

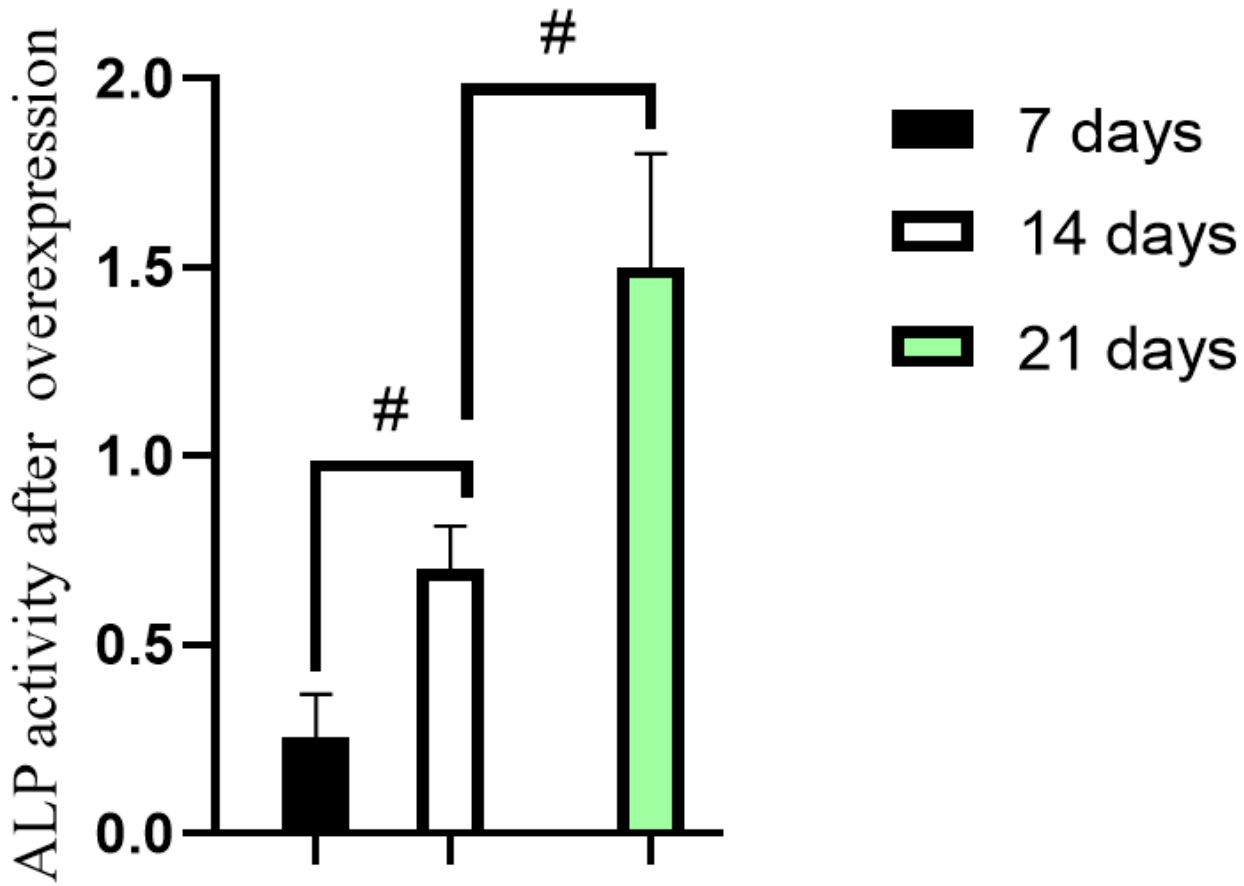

Figure 3

ALP showed that the osteogenic differentiation ability of BMSCs was significantly weakened after overexpression of miR-26b-5p (n=16). #P<0.05.

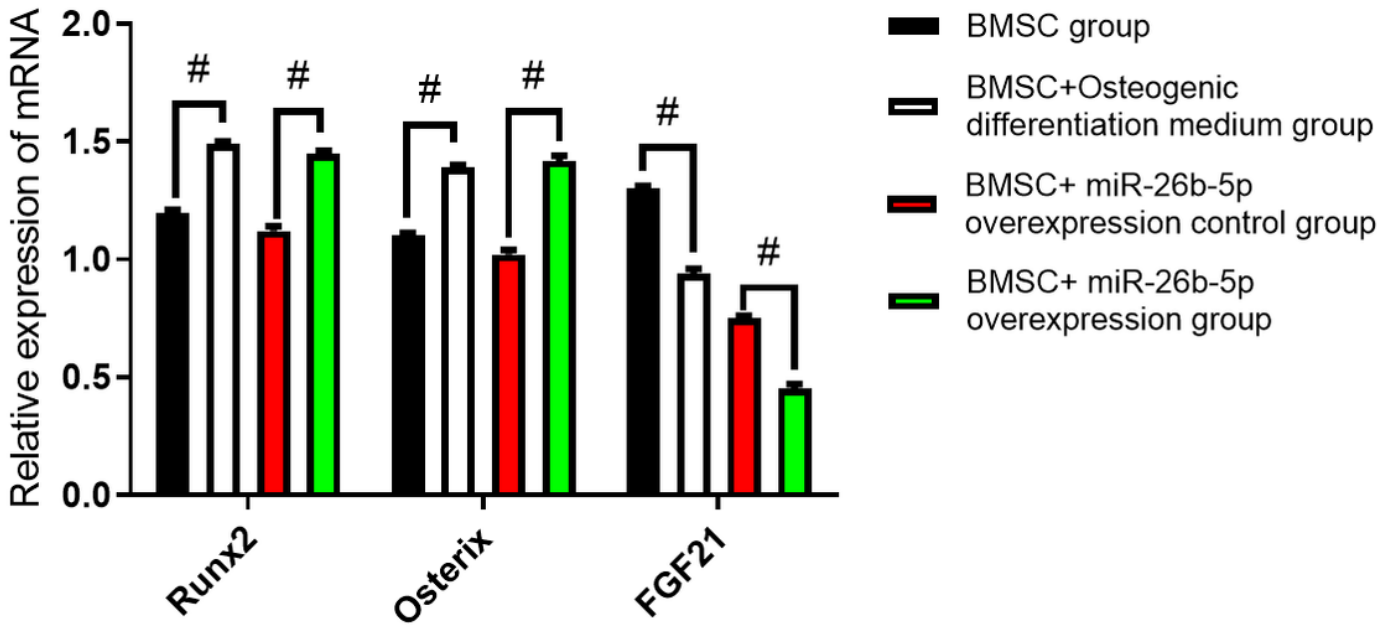

Figure 4

on the 7th day of osteogenesis induction, the expressions of Runx2, Osterix(Qsx) and FGF21 in BMSCs of each group were detected by RT-qPCR (n=16). #P<0.05.

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}	Predicted relative K_D
Position 76-82 of FGF21 3' UTR hsa-miR-26b-5p	5' ...UUUUUUUAUUUUUCUUACUUGAG... 3' UGGAUAGGACUUAAUGAACUU	7mer-m8	-0.33	98	-0.33	0.657	< 0.1	-3.902

Figure 5

The binding site of mRNA of FGF21 and miR-26b-5p.

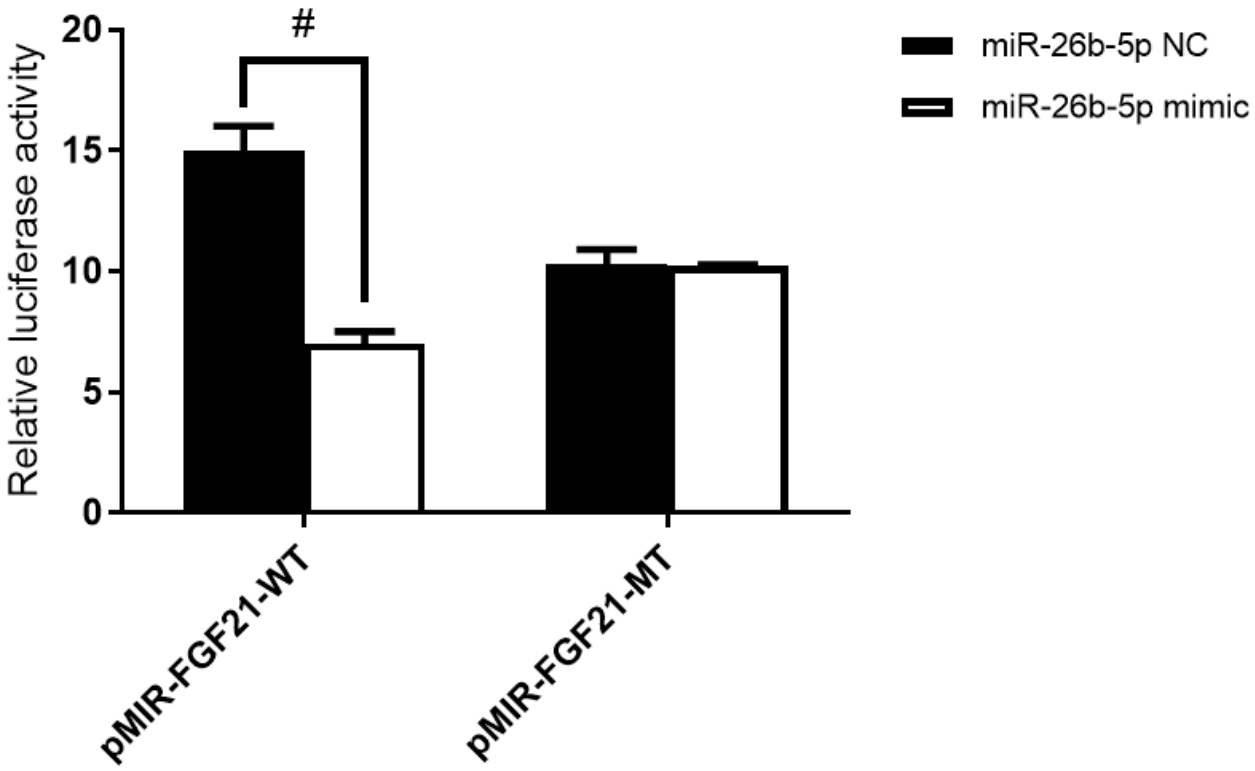

Figure 6

Luciferase reporting experiment (n=16). $^{\#}P<0.05$.

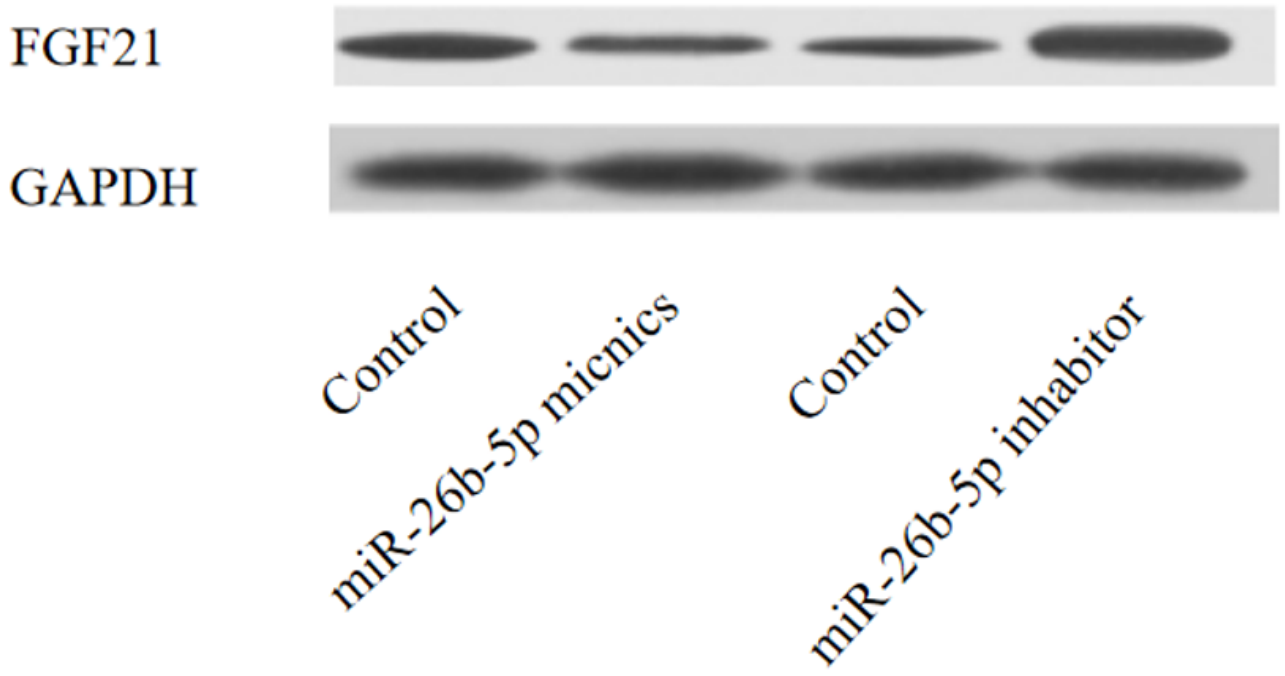

Figure 7

WB detection in BMSCs: expression of FGF21 was negatively correlated with miR-26b-5p expression level.