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microRNA-1297 promotes the progression of osteoporosis through regulation of osteogenesis of bone marrow mesenchymal stem cells by targeting WNT5A

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Abstract. – OBJECTIVE: This study was designed to investigate whether microRNA-1297 can regulate the osteogenesis of bone marrow mesenchymal stem cells (BMSCs) through WNT5A, thus influencing the progression of osteoporosis.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) assay was performed to analyze microRNA-1297 level and osteogenesis-related markers in osteoporosis patients and controls. The protein levels of the above markers and WNT5A were detected by Western blot. Alkaline phosphatase (ALP) activity assay and ALP staining were used to measure the degree of osteogenic differentiation under the control of microRNA-1297 and WNT5A, and ARS staining was used to detect the mineralization ability of hBMSC after overexpression of microRNA-1297. The binding sites of microRNA-1297 and WNT5A were determined by the dual luciferase-reporting assay. Besides, the activity of Wnt signal transduction pathway in different treatment groups was detected by TOP/FOP report.

RESULTS: MicroRNA-1297 was highly expressed in osteoporotic patients, and its level decreased significantly with the increasing of osteogenic induction. Bioinformatics prediction suggested that microRNA-1297 can target WNT5A. *In vitro* experiments showed that overexpression of microRNA-1297 in hBMSC can reduce the level of WNT5A, while interference with microRNA-1297 can increase the level of WNT5A. Overexpression of microRNA-1297 and transfection of si-WNT5A significantly reduced the mRNA levels of RUNX2, OSX, ALP, OCN, OPN and COL1A1, thereby inhibiting osteogenic differentiation. Overexpression of microRNA-1297 could interfere with WNT signaling pathway regulation and regulate the osteogenic differentiation of hBMSCs.

CONCLUSIONS: microRNA-1297 could regulate the osteogenesis of BMSCs by combining with WNT5A so as to accelerate the progression of osteoporosis.

Key Words:
Osteoporosis, BMSCs, microRNA-1297, WNT5A.

Introduction

Osteoporosis (OP) is the most common bone disease characterized by low bone mass and destruction of bone structure, which could eventually lead to increased bone fragility, decreased bone strength and increased risk of fracture[1]. As the trend of aging in China's society increases, the age-related changes in the systemic system make the problem of osteoporosis more and more significant[2]. Marrow mesenchymal stem cells (MSCs) are a kind of adult stem cells with high self-renewal ability and multi-directional differentiation potential. It exists in almost all tissues and can be separated from bone marrow, adipose tissue and umbilical cord[3-5]. In addition to hematopoietic stem cells, bone marrow tissue contains bone marrow mesenchymal stem cells (BMSCs), which support and nourish hematopoietic cells. The significant biological characteristics of BMSCs are self-renewal, proliferative ability and multi-differentiation potential, which can differentiate into osteoblasts, chondrocytes, fat cells, etc. under certain conditions, and is an ideal bone tissue seed cell[6]. Therefore, human bone marrow mesenchymal stem cells were used in this study for subsequent experiments. In recent years, research on gene level of miRNA regulation has become a hot spot[7]. miRNA is a group of non-coding small RNAs ranging in length from 19 to 25 bases[8]. miRNA mainly inhibits the level of target genes by inhibiting the translation of target gene mRNA by complementary binding to the 3' end of the target gene, thereby affecting cell proliferation, differentiation, apoptosis and invasion

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and metastasis[9]. miRNAs can regulate the level of tens to hundreds of target genes, so it would be more effective to target miRNAs in cell growth, differentiation, proliferation, and anti-cancer process than targeting encoding genes[10-12]. Many current studies[13,14] have confirmed that many miRNAs can play a regulatory role in osteogenic differentiation of BMSCs, such as miR-196a, miR-29b, miR-15b, miR-24 and miR-335-5p, which can promote osteogenic differentiation of BMSCs. Li et al[15] found that miR-29b binds to the 3'-UTR region of target gene, activates the osteogenic differentiation signaling pathway, inhibits the level of HDAC4, TGFβ3, ACR2, CTNNBIP1 and DUSP2 proteins, and promotes osteoblast differentiation. Since miRNAs play such important role in osteoporosis, it is necessary to better understand the effects of more miRNAs on osteoporosis. The Wnt family consists of 19 members, which can combine with their respective Frizzled (FZD) receptors and activate the intracellular and non-canonical Wnt pathways, respectively. Studies have found that Wnt5a can promote the osteogenesis from mesenchymal stem cells (MSC), while Wnt3a inhibits MSC differentiation into osteoblasts, and its effect is inhibited by Wnt5a[16]. Guo et al[17] found that Wnt5a was up-regulated during MSC osteogenic differentiation, while RUNX2, Osterix and alkaline phosphatase (ALP) level were significantly down-regulated in Wnt5a-/-deficient bone cells. Peng et al[18] found that Wnt5a is highly expressed in odontoblasts and dental papilla tissues, especially in differentiated odontoblasts. It is also found that overexpression of Wnt5a could promote the formation of mineralized nodules in human teeth and up-regulate the level of mineralization-related genes, indicating that Wnt5a can promote osteogenic/dentate differentiation of human dental papilla cells. However, the underlying mechanism of microRNA-1297 and WNT5a in osteoporosis is not fully understood. Therefore, this study explored the level of microRNA-1297 in osteoporosis patients and determined the interaction between microRNA-1297 and WNT5A to assess whether they play a role in osteogenic differentiation, aiming at providing theoretical basis for prevention and treatment of osteoporosis.

Patients and Methods

Patients

30 patients clinically diagnosed with osteoporosis and 30 normal controls were enrolled in this experiment. All subjects underwent fasting blood collection in the morning. 8 mL of venous blood was taken from each person, and the serum was collected by centrifugation at 3000 g/min for 10 min at 4°C (non-hemolytic state) and 13500 g/min for 15 min at 4°C. The serum was at -80°C for further experiment. Signed written informed consents were obtained from all participants before the study. This study was approved by the Ethics Committee of Liaocheng People's Hospital.

Cell Extraction and Culture

Human bone marrow mesenchymal stem cells were isolated from osteoporosis patients and normal human bone marrow. After bone marrow collection, it was washed twice with phosphate-buffered saline (PBS) and resuspended in α-Modified Eagle's Medium (α-MEM) cell culture medium containing fetal calf serum, glutamine, penicillin and streptomycin. A certain density of hBMSCs was placed in a cell culture flask and cultured in a 37°C, 5% CO_2 incubator. After 24 h, the culture solution in the culture flask was aspirated, and then fresh culture solution was added. The culture medium was changed every 2 days until the cell density reached 90%, then cells were digested with 0.05% trypsin-EDTA (ethylenediaminetetraacetic acid) and passaged. Cells that passed to the third generation were used for differentiation.

Osteogenic Induction of BMSCs

The well-grown 3rd generation bone marrow mesenchymal stem cells were seeded into a six-well plate at a density of 30×10^{4} /mL for osteogenic differentiation. The induction medium contained 10 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate, 50 μg/mL ascorbic acid and 1% HEPES. The induction time lasted for 7 to 14 days.

Cell Transfection

hBMS cells were transfected with microRNA-1297 mimic, microRNA-1297 inhibitor and si-WNT5A as well as their relative negative controls with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 hours after cell transfection, osteogenesis-related gene level in hBMSCs was analyzed by quantitative Real-time polymerase chain reaction (qRT-PCR).

Quantitative RT-PCR (RT-qPCR)

The total RNA of cells was extracted using TRIzol kit (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into complementary deoxyribose nucleic acid (cDNA). Then relative gene

expression was detected by Real-time PCR. The primer sequences were as follows: RUNX2 (F: 5'-CTTCACAAATCCTCCCCAAG-3', R: 5'-GAATGCGCCTAAATCACTG-3'), ALP (F: 5'-GCGCAAGAGACACTGAAATAT-3', R: 5'-TGGTGGAGCTGACCCTTGAG-3'), OSX (F: 5'-CACCAGGTCCAGGCAACA-3', R: 5'-GAGCAAAGTCAGATGGGTAAGT-3'), OCN (F: 5'-GAAGCCCAGCGGTGCA-3', R: 5'-CACTACCTCGCTGCCCTCC-3'), OPN (F: 5'-CTCCATTGACTCGAACGACTC-3', R: 5'-CAGGTCTGCGAAACTTCTTAGAT-3'), COL1A (F: 5'-GAGGGCCAAGACGAAGACATC-3', R: 5'-CAGATCACGTCATCGCACAAC-3'), WNT5A (F: 5'-CAAATAGGCAGCCGAGAGAC-3', R: 5'-CTCTAGCGTCCACGAACTCC-3'), GAPDH (F: 5'-GGAGCGAGATCCCTCCAAAA-3', R: 5'-GGCTGTTGTCATACTTCTCATGG-3'), microRNA-1297 (F: 5'-ACACTCCAGCTGGGTTCAAGTAATTC-3', R: 5'- GTGCAGGGTCCGAGGT-3').

Western Blot

Total protein was extracted by cell lysate lysis. Protein concentration was determined by the bicinchoninic acid (BCA) protein quantification kit (Pierce, Rockford, IL, USA). Next, 50 ug of total protein was separated by electrophoresis on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The antigen was blocked in 5% skim milk powder blocking solution and incubated with the specific primary antibody overnight. In the next day, the immunoblots were incubated with the secondary antibody.

ALP Vitality Test

ALP activity was analyzed according to the manufacturer's protocol. The cells were incubated in ALP solution for 20 minutes at 37°C while gently shaking in the dark. ALP activity was measured spectrophotometrically by the p-nitrophenyl phosphate liquid substrate system (Sigma-Aldrich, St. Louis, MO, USA).

Alkaline Phosphatase (ALP) Staining

After osteogenic induction for 7 days, cells were washed with PBS for 3 times and placed in a BCIP/NBT alkaline phosphatase color developing mixture, and incubated at 37°C for 30 min. Then cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed once to stop reaction, and photographed.

Alizarin Red Staining

After 14 days of osteogenic induction, the culture medium was discarded, and the cells were washed with PBS for 3 min×3 times, fixed with 60% isopropanol for 60 s, and stained by 10% alizarin red for 3 min. Finally, the liquid was discarded and the mineralized nodules were observed under microscope.

Luciferase Reporter Gene Assay

Cell were transfected with corresponding plasmids by lipo2000. Luciferase activity in the samples was determined using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) 24 hours after transfection.

TOP/FOP Experiment

The Wnt signaling pathway activity was detected using the TOPglow/FOPglow TCF reporter kit (Millipore, Billerica, MA, USA). Cells were seeded in 6-well plates and transfected with TOPglow and FOPglow according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA). The *t*-test was used for comparison between the two groups. $p<0.05$ was considered statistically significant.

Results

microRNA-1297 is Highly Expressed in Osteoporosis

We used qRT-PCR to analyze the level of microRNA-1297 in serum of patients with osteoporosis. The results showed that the level of microRNA-1297 was up-regulated in the serum of patients with osteoporosis compared with that in the normal control group (Figure 1A). qRT-PCR also revealed that during the osteoblast differentiation of hBMSCs, the level of microRNA-1297 was significantly decreased with the increase of osteogenic induction days (Figure 1B). Thus, microRNA-1297 was highly expressed in the serum of osteoporosis patients and may play a role in osteogenic differentiation.

microRNA-1297 Overexpression Inhibits Osteogenic Differentiation of hBMSC

To assess the effect of microRNA-1297 level on osteogenic differentiation, microRNA-1297 mim-

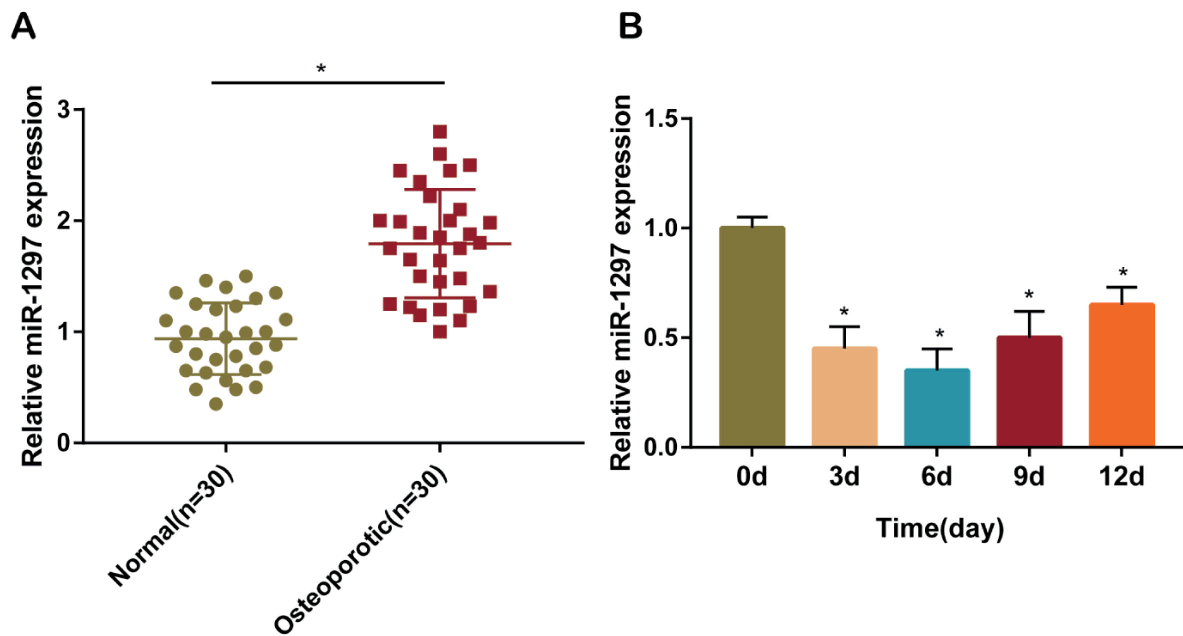

Figure 1. microRNA-1297 is highly expressed in osteoporosis. ***A,*** qRT-PCR detection of microRNA-1297 in 30 osteoporosis patients serum level was significantly higher than the control group. ***B,*** qRT-PCR was used to detect the dynamic level of microRNA-1297 during osteoblast differentiation of hBMSCs. With the increase of osteogenic induction days, the level of microRNA-1297 was significantly decreased.

ic was further transfected into hBMSC isolated from osteoporosis patients and cultured in osteogenic medium. QRT-PCR analysis revealed that microRNA-1297 level was increased in hBMSCs after overexpression of microRNA-1297 (Figure 2A). In addition, we further analyzed the level of key transcription factors and related genes in osteogenic induction, and found that overexpression of microRNA-1297 inhibited the level of RUNX2, OSX, ALP, and OCN, OPN and COL1A1 in hBMSCs (Figure 2B). Similarly, Western blot analysis showed that the level of RUNX2, OSX, ALP, and OPN in the microRNA-1297 mimic group was significantly lower than that in the control group (Figure 2C). ALP activity assay showed that ALP was lower in the microRNA-1297 mimic group than in the control group (Figure 2D). Besides, ALP and ARS staining results showed that overexpression of microRNA-1297 reduced ALP staining and reduced cell mineralization capacity compared to the control group (Figure 2E-F). The above results indicated that overexpression of microRNA-1297 can inhibit osteogenic differentiation of hBMSCs.

WNT5A is the Direct Target of microRNA-1297

We further predicted the binding site of microRNA-1297 to WNT5A using TargetScan7.1 (Figure 3A). Further, it was confirmed by a dual luciferase reporter gene assay that microRNA-1297 could bind to the 3'UTR of wild-type WNT5A (Figure 3B). Furthermore, we transfected microRNA-1297 mimic and inhibitor into hBMSCs isolated from bone marrow of osteoporosis patients, and detected protein expression of WNT5A by Western blot. The results showed that overexpression of microRNA-1297 reduced WNT5A protein level, while knockdown of microRNA-1297 increased its protein expression (Figure 3C). Further, it was found that transfection of si-WNT5A significantly reduced the mRNA level of osteoblast markers compared with that in the negative control group (Figure 3D). These results indicated that microRNA-1297 could bind to WNT5A and that WNT5A could affect osteogenic differentiation of hBMSCs.

microRNA-1297 Regulated Osteogenic Differentiation by Affecting WNT Signaling Pathway

Different concentrations of DKK1 were added to the osteogenic medium. ALP staining was performed in the induced cells, and the results showed that ALP staining gradually decreased as the concentration of DKK1 increased (Figure 4A and 4B). QRT-PCR detection of osteoblast markers showed

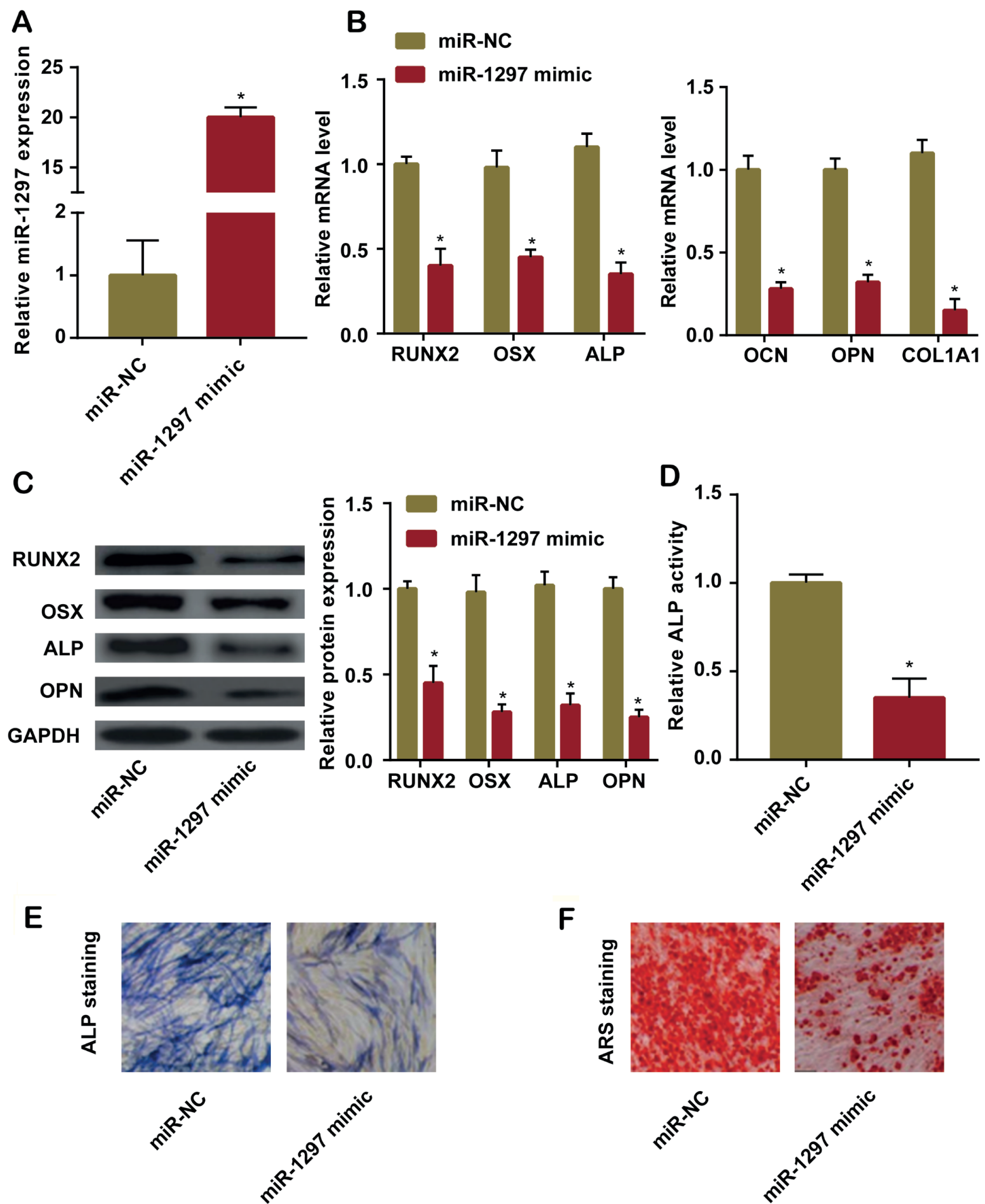

Figure 2. Overexpression of microRNA-1297 inhibits osteogenic differentiation of hBMSCs. ***A,*** qRT-PCR assay detects the level level of microRNA-1297 in hBMSC after overexpression of microRNA-1297. ***B,*** qRT-PCR analysis showed that the level of key transcription factors and osteoblast markers was induced after day 6 of cells. Overexpression of microRNA-1297 group was significantly lower than that of the control group, RUNX2, OSX, ALP, OCN, OPN and COL1A1. ***C,*** Western blot analysis was used to analyze the protein levels of key osteoblast markers on day 6 of induced cells. Compared with the control group, the level of RUNX2, OSX, ALP and OPN in microRNA-1297 mimic group was significantly decreased. ***D,*** ALP activity assay, ALP level in the microRNA-1297 mimic group was lower than the control group. ***E,*** ALP staining, ALP content in the microRNA-1297 mimic group was significantly lower than the control group. ***F,*** ARS was used to indicate the mineral deposition on the 12th day of induction of cells, and the mineralization formation ability was significantly lower in the microRNA-1297 mimic group than in the control group.

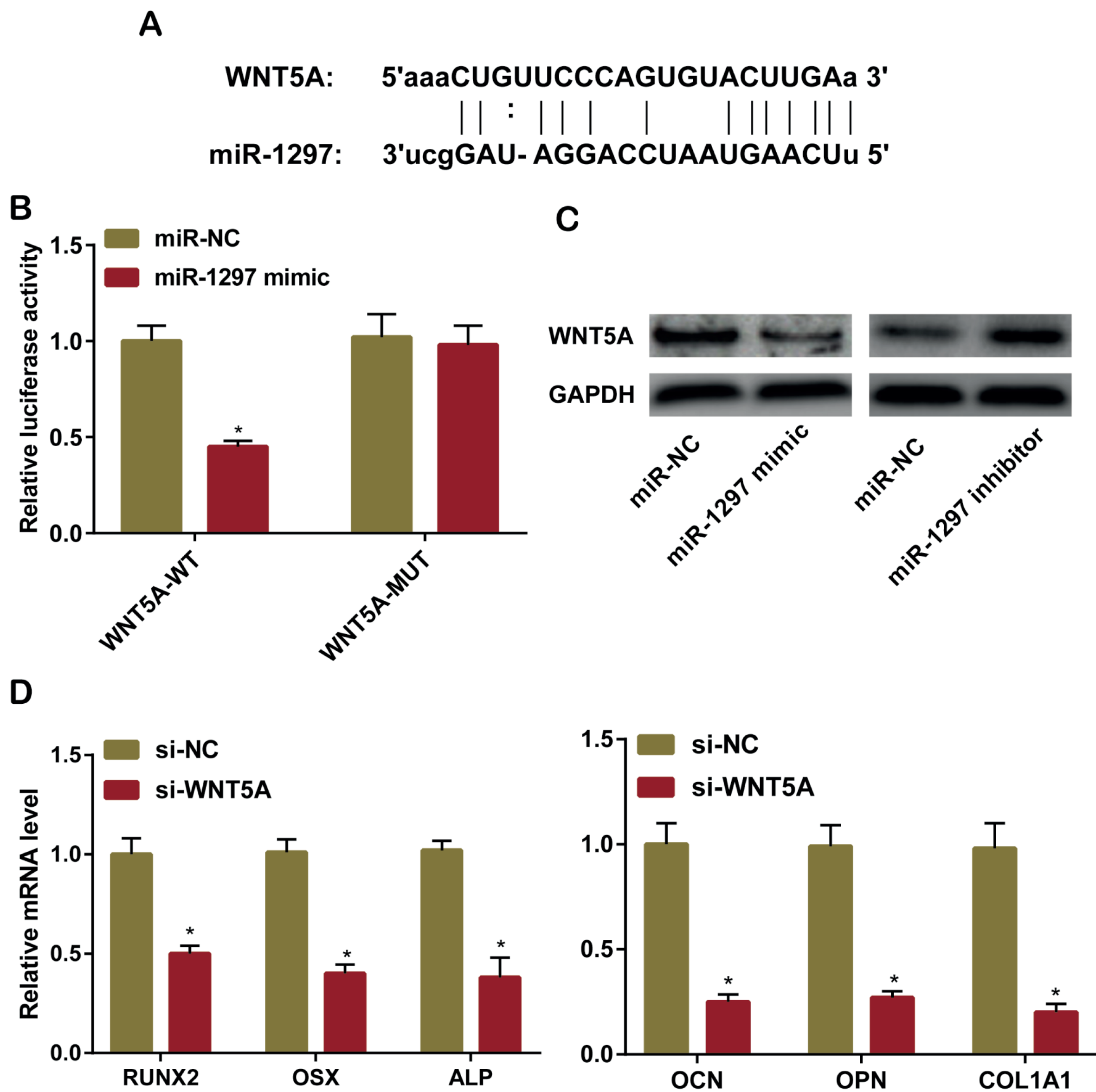

Figure 3. WNT5A is a direct target of microRNA-1297. ***A,*** The binding site of microRNA-1297 to WNT5A was predicted in TargetScan7.1. ***B,*** Dual luciferase reporter assay to confirm the match of microRNA-1297 to the 3'UTR of wild-type WNT5A. ***C,*** Western blot analysis of WNT5A protein level, overexpression of microRNA-1297 in hBMSC can reduce the level of WNT5A, and interference with microRNA-1297 can increase the level of WNT5A. ***D,*** QRT-PCR assay, compared with transfected siRNA negative control (siNC), transfection of siWNT5A significantly reduced the mRNA level levels of RUNX2, OSX, ALP, OCN, OPN and COL1A1.

that 1nM DKK1 blocked the level of marker genes induced by microRNA-1297 inhibitor (Figure 4C and 4D). The above results indicated that microRNA-1297 can interfere with WNT signaling and affect the osteogenic differentiation of hBMSC.

Discussion

Osteoporosis is a common metabolic bone disease in the elderly. It is characterized by decreased bone mass, decreased bone density, and increased risk of fragility fractures. It has become a global public health problem. In recent years, studies have found that a variety of miRNAs play a vital regulatory role in the occurrence and development of abnormal bone metabolism and osteoporosis. Related studies have reported that microRNA-1297 promotes the development of non-small cell lung cancer by targeted down-regulating PTEN level[19]. For example, it

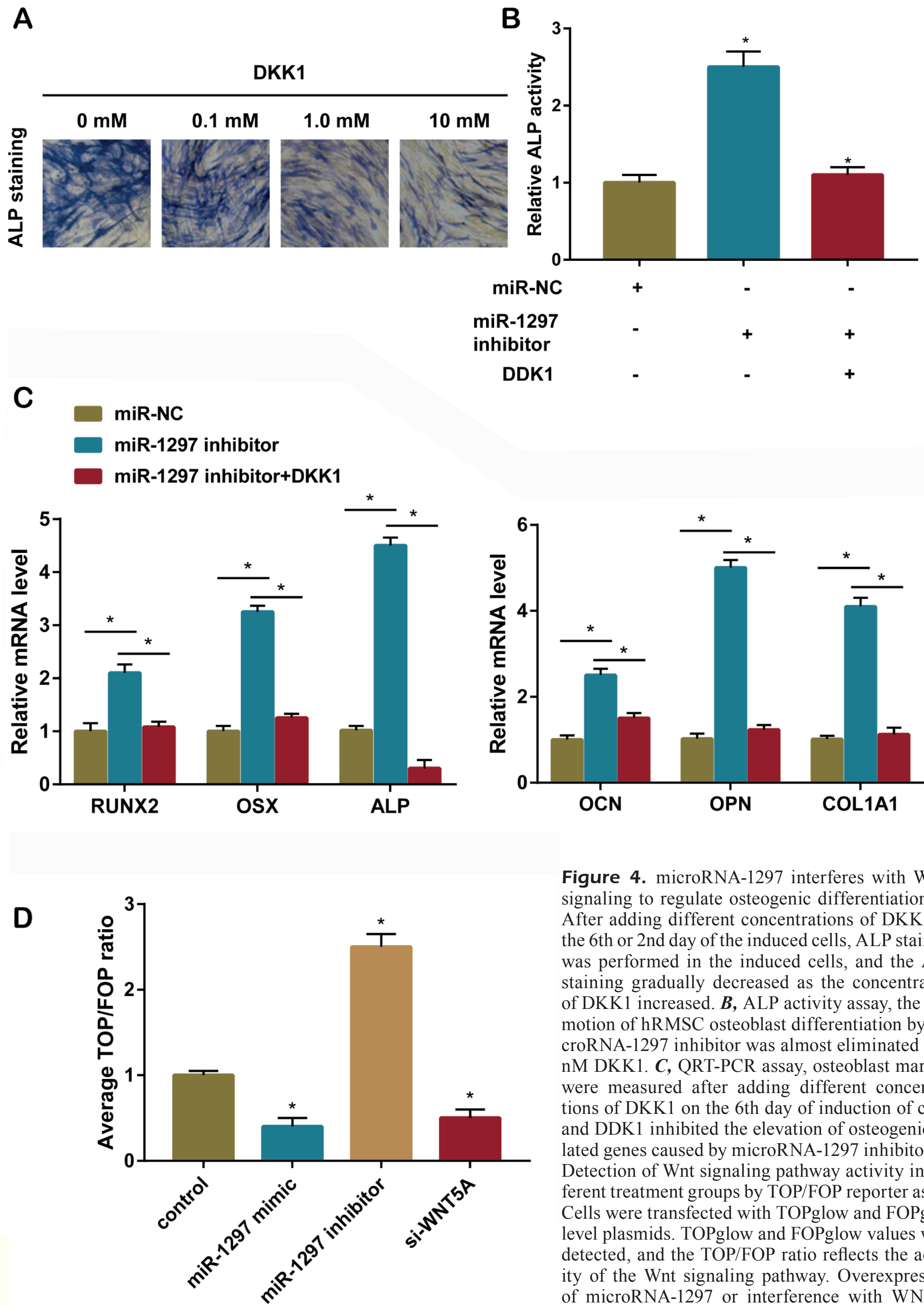

Figure 4. microRNA-1297 interferes with WNT signaling to regulate osteogenic differentiation. ***A,*** After adding different concentrations of DKK1 on the 6th or 2nd day of the induced cells, ALP staining was performed in the induced cells, and the ALP staining gradually decreased as the concentration of DKK1 increased. ***B,*** ALP activity assay, the promotion of hRMSC osteoblast differentiation by microRNA-1297 inhibitor was almost eliminated by 1 nM DKK1. ***C,*** QRT-PCR assay, osteoblast markers were measured after adding different concentrations of DKK1 on the 6th day of induction of cells, and DDK1 inhibited the elevation of osteogenic related genes caused by microRNA-1297 inhibitor. ***D,*** Detection of Wnt signaling pathway activity in different treatment groups by TOP/FOP reporter assay. Cells were transfected with TOPglow and FOPglow level plasmids. TOPglow and FOPglow values were detected, and the TOP/FOP ratio reflects the activity of the Wnt signaling pathway. Overexpression of microRNA-1297 or interference with WNT5A reduces the activity of the Wnt signaling pathway, while microRNA-1297 inhibitors are enhanced.

promotes breast cancer cell proliferation and invasion by targeting down-regulation of PTEN and activating the PI3K/Akt pathway[20], and can bind to PTEN in laryngeal squamous cell carcinoma[21] and testicular germ cell tumors[22]. However, its specific mechanism in osteoporosis has not been reported. Therefore, this study focused on the role of microRNA-1297 in the osteogenic differentiation of hBMSC. We found that microRNA-1297 was highly expressed in the serum of patients with osteoporosis, and its level decreased significantly with the increase of osteogenic induction days, suggesting that microRNA-1297 may be associated with the process of osteogenic differentiation. Wnt signaling pathways can generally be divided into two categories: the canonical Wnt pathway (non-canonical Wnt pathway) and the non-canonical Wnt pathway. The canonical Wnt signaling pathway can be involved in the regulation of BMSCs, osteoblast differentiation, promotion of osteoblast proliferation and differentiation, and inhibition of programmed cell death of osteoblasts, etc., and thus are thought to play an important role in bone development and metabolism[23]. Nonclassical Wnt signals pathway and the classical one have a considerable overlap in function[24]. WNT5A is an important component of the non-canonical Wnt signaling pathway, and Okamoto *et al*[25] found that when the WNT5A gene is deficient, the ability of mouse osteoblast cell lines to differentiate into osteoblasts is diminished. Furthermore, without blocking the Wnt/β-catenin pathway, WNT5A gene silencing can cause down-regulation of Lrp5 and Lrp6, which are two vital transcription factors in the Wnt/β-catenin pathway. This study suggests that the non-canonical Wnt signaling pathway and the canonical Wnt signaling pathway may be transmitted through WNT5A. With the continuous exploration of the osteogenic differentiation process of BMSCs, it is confirmed that many miRNAs are closely related to multiple osteogenesis-related signaling pathways. The differential expression of miRNAs plays a crucial part in the regulation of upstream signals and downstream markers of osteogenic differentiation[26-28]. During osteogenic differentiation, miRNAs are involved in the regulation of the levels of key factors in the Wnt signaling pathway[29]. Zhang *et al*[30] found that miR-335-5p could significantly down-regulate the level of DDK1 and enhance the Wnt signaling pathway through binding to the 3'UTR of DKK1, thus promoting the phosphorylation of glycogen synthase kinase-3β (GSK-3β) and increasing the transcriptional activity of β-catenin. As a result, the level of the osteogenic-associated transcription factor Runx2 (Runt related transcription factor 2), bone sialoprotein (BSP) and osteocalcin (OC) downstream of the Wnt signaling pathway are significantly increased to promote osteogenic differentiation of BMSCs. Su *et al*[31] found that overexpression of miR-26a can activate β-catenin in BMSCs and promote their osteogenic differentiation through Wnt/β-catenin signaling pathway. Further studies confirmed that miR-26a can regulate Wnt signaling pathway through targeting glycogen synthase kinase-3β (GSK-3β). Therefore, this study explored the mutual relationship between microRNA-1297 and WNT5A and found that overexpression of microRNA-1297 or down-regulation of WNT5A can inhibit osteogenic differentiation, and microRNA-1297 can target WNT5A to interfere with WNT signaling and inhibit osteogenic differentiation to promote progression of osteoporosis.

Conclusions

microRNA-1297 is highly expressed in the serum of patients with osteoporosis. *In vitro* studies showed that the level of microRNA-1297 decreased significantly with the increase of osteogenic induction days. At the same time, microRNA-1297 can target WNT5A to interfere with the transmission of WNT signaling and inhibit osteogenic differentiation to promote the progression of osteoporosis.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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