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miR-485-5p promotes osteoporosis via targeting Osterix

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Abstract. – OBJECTIVE: To investigate the effect and related mechanisms of miR-485-5p on the osteogenic differentiation of rat bone marrow mesenchymal stem cells (BMSCs).

PATIENTS AND METHODS: The expression level of miR-485-5p was detected in clinical cases and during the osteogenic differentiation. Three group were established to study the potential function between miR-485-5p and osteogenic differentiation: miR-NC group (negative control), miR-485-5p mimics (BMSCs transfected by miR-485-5p mimics), and mimics + si-Osx (BMSCs transfected by miR-485-5p mimics and si-Osx), after the induction of osteogenic differentiation, the cell viability of BMSCs and osteogenic markers were determined.

RESULTS: In our work, miR-485-5p was found up-regulated in patients with osteoporosis by comparing with health cases. Besides, during osteogenic differentiation, miR-485-5p was suppressed. These results suggest miR-485-5p has a negative regulating effect. To research potential target of miR-485-5p, we checked it in three publicly available algorithms, TargetScan, miRDB and microRNA. We found that Osterix (Osx) is a direct target of miR-485-5p, and Luciferase assays confirmed our hypothesis, the subsequent experiments showed that decreased expression of Osx resulting from the up-regulation of miR-485-5p could restrain the cell viability and the expression level of osteogenic markers

CONCLUSIONS: Our research revealed the promote function of miR-485-5p on osteoporosis, indicating that miR-485-5p could be a potential therapeutic strategy for the treatment of osteoporosis.

Key Words:

Osteoporosis, miR-485-5p, Osterix (Osx), Osteogenic differentiation.

Introduction

Osteoporosis is a kind of systematic bone disease characterized by reduced bone mass, destruction of bone microstructure, increased bone fragility and proneness to fracture[1]. With the increasing aggravation of population aging in the world, osteoporosis has become one of the most common and expensive diseases globally. There are more than 9 million cases of fracture caused by osteoporosis every year around the world, about 2 million of which occur in the United States, directly resulting in 17 billion medical expenses. Therefore, it is urgent to find the effective therapeutic regimen to reduce the financial burden on the country and improve the life quality of the aged[2].

Bone is a kind of continuous dynamic balance tissue, which, through continuous remodeling, maintains its mineralization balance and integrity of its own structure. Bone remodeling consists of two processes, the osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Once the equilibrium state of bone remodeling is broken, such bone diseases as osteoporosis will be caused. Osteoblasts are differentiated from mesenchymal stem cells, and such a process is finely regulated by some transcription factors[3-7]. Therefore, understanding how to regulate the function of differentiated or mature osteoblasts after birth is crucial for the development of therapeutic drugs for osteoporosis and inflammatory bone diseases[8].

In recent years, studies have found that micro ribonucleic acid (miRNA) is closely related to the molecular pathogenesis of bone diseases, such as osteoarthritis[9], ankylosing spondylitis (AS)[10], osteomyelitis[11], and osteoporosis[12]. As miRNA plays such an important role in the process of osteogenic differentiation and occurrence and development of disease, it can be used as a potential target in the treatment of osteoporosis.

As a member of the miRNA family, miR-485-5p has demonstrated its unique advantages in the diagnosis and treatment of many diseases,

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such as breast cancer[13], gastric cancer[14], hepatocellular carcinoma[15], and cardiac hypertrophy[16]. However, there are few reports about the role of miR-485-5p in the occurrence and development of osteoporosis and its related molecular mechanism. In this study, the expression of miR-485-5p and the effects of miR-485-5p on biological behaviors in the progression of osteoporosis were analyzed, so as to clarify the role of miR-485-5p in the occurrence and development of osteoporosis and its related molecular mechanisms.

Patients and Methods

Clinical Samples

Human blood samples were collected from 30 aged patients who were diagnosed according to the BMD or BMC, in brief, osteoporosis was diagnosed if the femoral neck and/or lumbar spine T score was less than –2.5 SD, and the other normal people were established for control. Blood samples were centrifuged at 1000 g for 10 min and the serum were stored in aliquots at -20°C until assayed. Serum miR-485-5p in patients with osteoporosis and health samples were determined by qRT-PCR. The Declaration of Helsinki should be mentioned and respected. This study was approved by the Ethics Committee of The Second Hospital of Jilin University. The signed written informed consents were obtained from all participants before the study.

Isolation and Culture of BMSCs

A total of 30 healthy Sprague-Dawley (SD) rats aged 12 weeks old were selected and executed under anesthesia. Bilateral lower extremities of rats were isolated, the epiphyseal ends were cut off, and the bone marrow in marrow cavity was poured out. Bone marrow mesenchymal stem cells (BMSCs) were gently blown and beaten using phosphate-buffered saline (PBS) to be prepared into single-cell suspension, and centrifuged at 1200 r/min for 10 min. After the supernatant was discarded, cells were resuspended in F12/Dulbecco's Modified Eagle Medium (DMEM), inoculated into a 25 cm^2 culture flask, and cultured under 5% CO_2 at 37°C. The solution was replaced once every 2 d. The cell morphology and growth status were observed under an inverted microscope. When cells were fused, they were digested using 0.25% trypsin, followed by passage at a ratio of 1:2.

Cell Transfection and Treatment

BMSCs at passage 3 were pre-cultured in a 24-well plate for 24 h. miR-485-5p mimics and si-Osx were synthesized and transfected to MSCs cell to analyze biological function of miR-485-5p. Then, three groups were established to study the potential relevance between miR-485-5p and osteogenic differentiation: miR-NC group (negative control), miR-485-5p mimics (BMSCs transfected by miR-485-5p mimics), and mimics + si-Osx (BMSCs transfected by miR-485-5p mimics and si-Osx). All the stuff were purchased from RiboBio (Guangzhou, China), and were transfected by using lipofectamin RNAiMAX (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. At 36 h after transfection, the osteogenic differentiation of bone marrow-derived MSCs into chondrocytes was induced.

Induced Osteogenic Differentiation of Bone Mesenchymal Stem Cells (BMSCs)

After transfection, BMSCs were inoculated at $2\times10^4/cm^2$. When cells grew and covered 70%-80% of the bottom of wells, they were cultured using the osteogenic induction medium (α-MEM inducing solution containing 10 μmol/L dexamethasone, 50 μmol/L VitC, 10 mmol/L β-phosphoglycerate, and 100 mL/L fetal bovine serum – FBS), freshly differentiated medium was replaced once every 3 days, and cells were cultured for 21 days.

Luciferase Reporter Assays

In TargetScan, miRDB and microRNA websites, it was found that Osterix (Osx) was the target gene of miR-485-5p. BMSCs cells were co-transfected with pMIR-30UTR-Osx or pMIR-30UTR-Mut Osx and miR-485-5p mimic or negative control (NC), and the pMIR-Renilla plasmid (Promega, Madison, WI, USA) followed being seeded into a 12-well plate. The cells were then lysed post-transfection. The luciferase activity was detected in a multi-function microplate reader (Promega, Madison, WI, USA), and results were normalized to Renilla luciferase activity.

Cell Viability

At 21 days after induction of osteogenic differentiation, cells were harvested and inoculated into 96-well plates at a density of 2 x 10^3 cells for 48 hours, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-di-

phenyl tetrazolium bromide) solution (5 mg/mL, MultiSciences, Hangzhou, China) was appended to each well after 4-hour incubation. Then, 150 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well for solubilizing the formazan formed. After half hour, the absorbance was measured by a microplate reader (Bio-Rad, Hercules, CA, USA) set at 490 nm.

Alkaline Phosphatase (ALP) Activity Analysis

According to instructions of the ALP activity assay kit, the ALP activity was quantified using the ALP yellow liquid substrate system *via* enzyme-linked immunosorbent assay (ELISA) (Beyotime, Shanghai, China). After induced osteogenic differentiation for 21 days, BMSCs were collected to extract the total protein, and the ALP activity was normalized based on the total amount of protein. The absorbance was measured at a wavelength of 405 nm using an ultraviolet spectrophotometer, and the ALP activity was calculated using the following formula: ALP activity (U/g) = [(absorbance $_{\text{measured tube}}$/absorbance $_{\text{standard tube}}$) × nitrophenol amount $_{\text{standard tube}}$]/total protein (g).

Alizarin Red Staining

The operation was performed according to the reagent instructions. After induced osteogenic differentiation of BMSCs for 21 days, cells were washed twice with balanced salt solution, and fixed with 75% ethanol solution for 10 min. Then, the fixing solution was discarded, 500 μL 0.1% alizarin red-Tris-HCL staining solution (pH=8.2) was added into each well for staining for 30 min, followed by rinsing with distilled water, observation and photography (×200) under a light microscope.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was procured by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. SYBR green quantitative polymerase chain reaction (qPCR) assay was used to measure the level of Osx expression and endogenous controlled by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) was used to measure the level of miR-485-5p expression normalized to miRNA U6.

Western Blotting

At 21 days after induction of osteogenic differentiation, cells were collected and lysed using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). The total protein was extracted, and its concentration was determined according to instructions of the bicinchoninic acid (BCA) protein concentration kit (Pierce, Rockford, IL, USA). The same amount of total protein was separated *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a nitrocellulose membrane, sealed with 5% skim milk and incubated with rabbit anti-rat Osx primary antibodies (1:1000, Abcam, Cambridge, MA, USA) at 4°C overnight. After the membrane was fully washed with Tris-buffered saline with Tween-20 (TBS-T), anti-rabbit secondary antibody (coupled by horseradish peroxidase) was added for incubation at room temperature for 2 h, followed by development *via* enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Waltham, MA, USA), exposure in gel imaging system, fixation and observation of results. With β-actin as an internal reference, the relative changes in protein expression were detected.

Statistical analysis

Statistical analysis was performed with a Student's *t*-test or *F*-test. All *p*-values were two-sided, and $p < 0.05$ was considered statistically significant. All data were analyzed by Prism 6.02 software (La Jolla, CA, USA).

Results

miR-485-5p Was Up-Regulated in Patients With Osteoporosis

To examine the role of miR-485-5p in osteoporosis, we detected the level of miR-485-5p expression in clinical samples. The results showed that the expression of miR-485-5p was pretty high in patients with osteoporosis compared with health cases (Figure 1A).

miR-485-5p Was Up-Regulated During Chondrogenic Differentiation of BMSCs

The expression of miR-485-5p was detected after the induction of osteogenic differentiation of BMSCs by quantitative qRT-PCR. As expected, the expression level of miR-485-5p was decreased during the osteogenic differentiation of BMSCs.

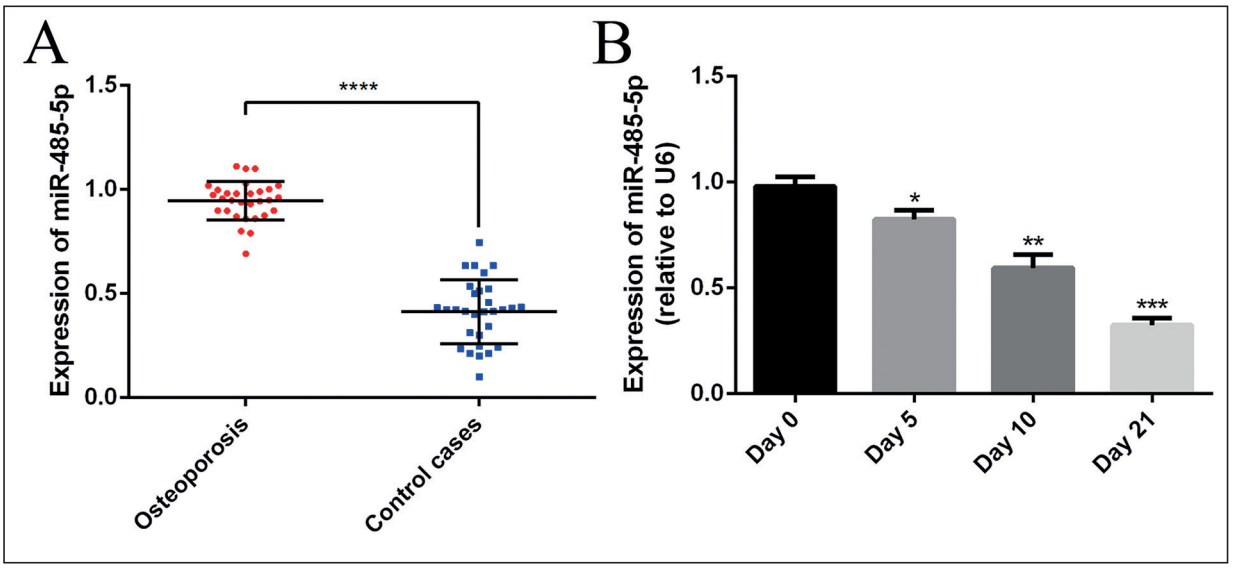

Figure 1. ***A,*** The expressions of miR-485-5p in clinic cases. ***B,*** The expressions of miR-485-5p during the osteogenic differentiation. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$).

The results indicated that miR-485-5p may have a negative regulating effect on the chondrogenic differentiation of MSCs (Figure 1B).

Osterix is a Direct Target of miR-485-5p

To research potential target of miR-485-5p, we checked it in three publicly available algorithms, Target Scan, miRDB and microRNA to elucidate the putative and possible targets of miR-485-5p. Finally, we found the Osterix (Osx) was checked supposed target of miR-485-5p (Figure 2A). Thus, Osx has caught our attention. To confirm whether miR-485-5p has regulation effect on Osx, we established luciferase reporter vectors containing the wild or mutant-type miR-485-5p seed sequences of the Osx 30UTR. The luciferase activity of the wide-type Osx 3`UTR reporter gene in miR-485-5p group was significantly lower than that in normal control, but no significant differences of the luciferase activity were observed in mutant-type Osx 3`UTR reporter gene (Figure 2B). These results suggested that the expression of Osx could be regulated by miRNA-485-5p.

miR-485-5p Decreased the Expression Level of Osx

To further explore the correlate relation of miR-485-5p and Osx, three groups, miR-NC group, miR-485-5p mimics group, and the mimics + si-Osx group, were established in BMSCs cells. The results showed that the expression level of Osx was suppressed by up-regulation of miR-485-5p in BMSCs cell in both PCR analysis and Western blot experiment (Figure 3A, 3B, 3D). These data further confirmed that Osx could be negatively regulated by miR-485-5p.

miR-485-5p Inhibited the Cell Viability of BMSCs

To examine the function of miR-485-5p on proliferation of BMSCs, we used MTT assay to detect the cell viability at 21 days of osteogen-

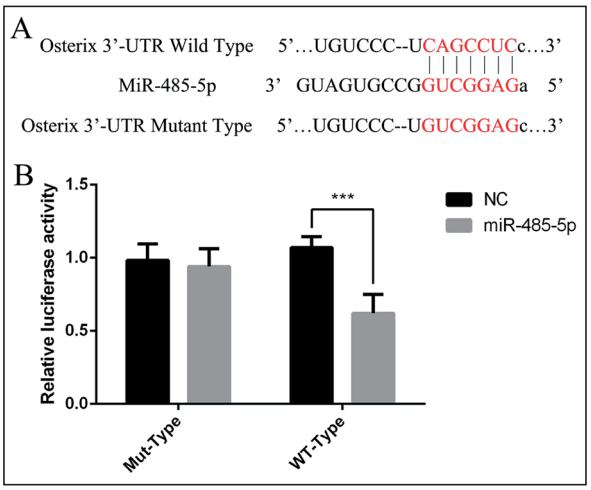

Figure 2. Osterix is a direct and functional target of miR-485-5p. BMSCs were transfection with miR-485-5p mimics and inhibitor. ***A,*** Diagram of putative miR-485-5p binding sites of Osterix. ***B,*** Relative activities of luciferase reporters (***$p < 0.001$).

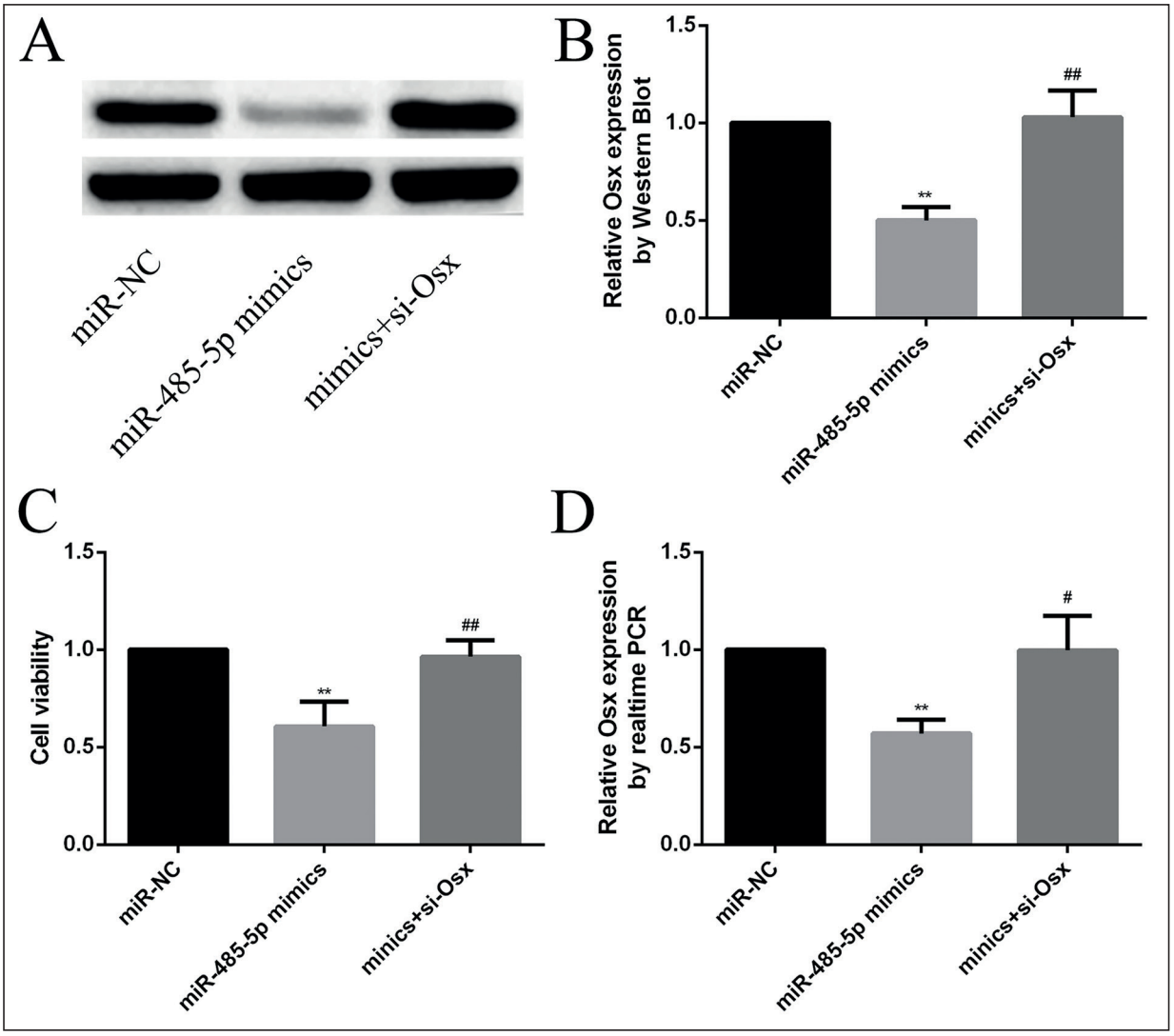

Figure 3. ***A,*** Protein expression of Osterix determined by Western blotting. ***B,*** was statistical analysis of A. Data were presented as means ± standard deviations. ***C,*** Cell viability of BMSCs detected by MTT assay. ***D,*** Expression of Osterix determined by PCR. (**$p < 0.01$ *vs.* miR-NC group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* Mimics group).

ic differentiation. The results from the MTT assay revealed that miR-485-5p significantly suppress the viability of BMSCs, while the viability was restored in mimics + si-Osx group (Figure 3C).

miR-485-5p Decreased the Expression of Osteogenic Markers

Since the expression of ALP began to increase early in osteogenic differentiation of BMSCs. And it maintains a high concentration throughout the whole process of osteogenesis. Therefore, ALP staining can be used as an early marker of osteogenic differentiation.

Alizarin Red staining was a method to detect the calcium compounds deposited in the extracellular matrix (ECM) as a result of bone mineralization in the later stages of osteogenesis.

The results indicated a higher degree of ALP activity and mineral nodule formation compared with the control croup after the transfection of miR-485-5p, while the addition of si-Osx can subside the effect of miR-485-5p (Figure 4).

The results suggest that miR-485-5p has a negative effect during osteogenic differentiation of MSCs.

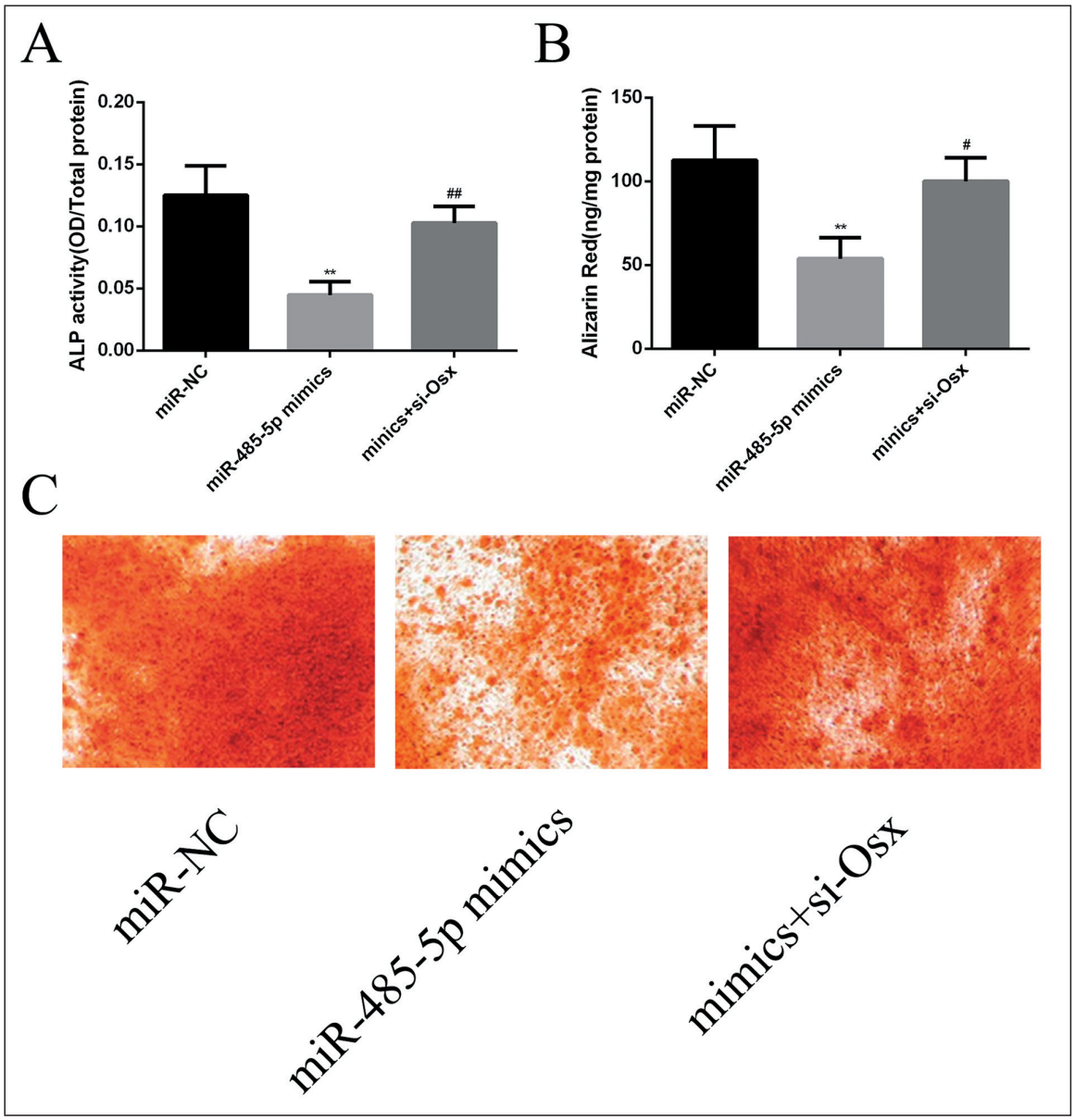

Figure 4. The effect of miR-485-5p on the expression of osteogenic markers. ***A,*** Activity of ALP determined by ELISA. ***B,*** and ***C,*** were Alizarin Red staining and the quantitative analysis of matrix mineralization. Data were presented as means ± standard deviations. (**$p < 0.01$ *vs.* miR-NC group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* Mimics group).

Discussion

Normal bone metabolism in the body is a complex process of bone tissue remodeling, mainly including osteoblast-mediated bone formation and osteoclast-mediated bone resorption. First, many osteoclasts are activated to dissolve the original matrix and form lacunae. Then, osteocytes assemble on the surface of bone resorption and synthesize non-mineralized bone matrix. Finally, calcium and phosphorus crystals are gradually deposited in the bone matrix for calcification, forming new bone. When the bone resorption rate is greater than the bone formation rate, bone loss, osteoporosis, and osteomalacia may occur[4,17-19]. The decline in osteoblast function is a key link in osteoporosis.

As an important member of post-transcriptional regulation, miRNA is involved in the expressions of many transcription factors and the ac-

tivation process of regulatory pathways in cells, which plays an important role in the growth and development of organisms and the occurrence and development of diseases. In view of such importance, miRNA has become a research hotspot in the field of life science in recent years. Bone metabolism-related miRNAs affect the bone regeneration and remodeling, and the abnormal expression of miRNA is closely related to osteoporosis. The in-depth study on these miRNAs will help us to better understand the bone metabolism and pathogenesis of osteoporosis.

As a member of the large family of miRNAs, miR-485-5p is involved in the regulation of a variety of genes in the body[13-16]. In current study, it was found in the blood samples of patients with osteoporosis that the expression of miR-485-5p was significantly increased compared with that in control group.

In 2002, Cell proposed that Osterix (Osx also known as Sp7) is a transcription factor containing novel zinc finger, which is essential for osteoblast differentiation and osteogenesis[20]. Several investigations have verified this point[21-23].

BMSCs, as a kind of important precursor cell of osteoblasts, play important roles in bone formation and osteogenic differentiation. With the constant deepening of research on BMSC osteogenetic mechanism, the pathological processes of various bone diseases will be better understood, seeking new ideas for the treatment of such diseases. In addition, BMSCs have strong proliferative ability and multi-directional differentiation potential, and they are the most important cell sources in bone tissue engineering and treatment.

To investigate the role of miR-485-5p in BMSCs, BMSCs were transfected with miR-485-5p to search the biological effects of miR-485-5p. Firstly, the cell viability of BMSCs was detected *via* methyl thiazolyl tetrazolium (MTT) assay. The results of MTT showed that miR-485-5p plays a negative regulating effect on the osteogenetic differentiation. However, the mechanism of miR-485-5p remains unclear. In this work, targetScan and miRanda software was used to predict the possible target sequences of miR-485-5p and Osx caught our attention. We further confirmed that Osx is a direct target gene of miR-485-5p *via* luciferase reporter system. To describe the osteogenic capability of cells more comprehensively, the classic osteogenetic markers were selected. After osteogenic induction of BMSCs transfected with miR-485-5p, the secretion of osteogenic factor ALP and the formation of calcified nodules were decreased. These findings indicated that miR-485-5p, as an inhibitor, is involved in the whole process of osteogenic differentiation of BMSCs. On the contrary, calcified nodules and expressions of ALP, were significantly up-regulated after transfection with si-Osx, and differences were statistically significant. These results suggested that miR-485-5p regulates osteogenic differentiation *via* targeting the Osx gene.

Conclusions

This study suggested that miR-485-5p regulates osteogenic differentiation of BMSCs through regulating the level of Osterix, thus affecting the osteoporosis symptoms. Our work provides new targets and strategies for the treatment of osteoporosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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