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MiRNA-365a-3p promotes the progression of osteoporosis by inhibiting osteogenic differentiation *via* targeting RUNX2

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Abstract. – OBJECTIVE: The aim of this study was to explore the exact role of miRNA-365a-3p in the progression of osteoporosis, as well as its function in regulating osteogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs).

PATIENTS AND METHODS: The serum level of miRNA-365a-3p in osteoporosis patients and normal controls was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). After transfection of miRNA-365a-3p mimics, miRNA-365a-3p inhibitor or si-RUNX2 in hBMSCs, the relative expression levels of miRNA-365a-3p, osteocalcin (OCN), osteopontin (OPN) and collagen I were determined by qRT-PCR. Western blot was conducted to examine the protein expression of RUNX2 influenced by miRNA-365a-3p. Subsequently, the regulatory effects of miRNA-365a-3p and RUNX2 on osteogenic differentiation and capability of mineralization were evaluated by alkaline phosphatase (ALP) determination and alizarin red staining, respectively. Furthermore, the binding relationship between miRNA-365a-3p and RUNX2 was predicted and verified by miRanda and Dual-Luciferase reporter gene assay, respectively.

RESULTS: MiRNA-365a-3p was highly expressed in osteoporosis patients. The expression of miRNA-365a-3p in hBMSCs decreased gradually with the prolongation of osteogenic differentiation. The subsequent results showed that RUNX2 could bind to miRNA-365a-3p, which was negatively regulated by miRNA-365a-3p in hBMSCs. Down-regulation of miRNA-365a-3p significantly decreased the expression levels of OCN, OPN and collagen I. Furthermore, overexpression of miRNA-365a-3p markedly weakened the capability of mineralization of hBMSCs, whereas was further reversed by transfection of si-RUNX2.

CONCLUSIONS: MiRNA-365a-3p negatively regulates osteogenic differentiation of hBMSCs by targeting RUNX2, thus promoting the progression of osteoporosis.

Key Words:
Osteoporosis, HBMSCs, MiRNA-365a-3p, RUNX2.

Introduction

Osteoporosis is a type of endocrine and metabolic disease, with reduction of bone mineral density (BMD) and bone quality[1-4]. Pathologically, the bone microstructure has severely degenerated in osteoporosis patients. This may result in increased bone fragility, as well as decreased transduction and stress abilities of degenerated bone trabecula. Osteoporosis patients are prone to suffer fragility fracture. Meanwhile, these patients are usually accompanied by symptoms such as shortened length, generalized pain, cramps and difficulty breathing[5]. In addition, statistics have shown that osteoporosis imposes a huge economic burden to society.

Differentiation of human bone marrow mesenchymal stem cells (hBMSCs) is strictly regulated by mechanical and molecular pathways, showing promising potential in stem cell therapy for osteogenic disorders[6]. However, the key regulatory sites and mechanisms of osteogenic differentiation of hBMSCs have not been fully elucidated yet.

Current experimental studies have verified that miRNAs are processed and matured by two RNases, including RNase III Drosha and RNase III Dicer. They are involved in the regulation

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of target gene expression as well[7-9]. In addition, Zhou et al[10] have shown the biological functions of miRNAs in cell proliferation, apoptosis, survival and differentiation. Meanwhile, the role of miRNAs in the pathogenesis of skeletal development and bone diseases has been pronounced. These miRNAs can be utilized as novel potential targets for disease treatment. For example[11], miR-153 inhibits osteogenic differentiation of human mesenchymal stem cells (hMSCs) by targeting to type II bone morphogenetic protein receptors. During osteoblast differentiation, up-regulated miR-214 enhances the number of mesenchymal stem cells (MSCs) in osteoporotic mice. Moreover, miR-214 overexpression[12] inhibits osteoblast differentiation of MSCs. The above findings all suggest that miRNA exerts an important role in osteoporosis. However, the possible underlying mechanism of miRNAs remains unclear.

RUNX2 is a member of the RUNX family, which is encoded by the RUNX gene. As a key regulator of osteoblast differentiation, RUNX2 is essential for normal skeletal development and ossification[13-17]. Enomoto et al[18] have found that periosteum and endochondral ossification are undeveloped in RUNX2$^{-/-}$ mice. This indicates the necessity of RUNX2 for osteoblast differentiation. Gersbach et al[19] have indicated that RUNX2 can induce the transformation of myoblasts into osteoblasts *in vitro*. Meanwhile, it has been found that miRNA regulates osteoblast differentiation through RUNX2. Zhang et al[20] have identified that during osteogenic differentiation of mesenchymal tissues, the expression levels of miRNA-23a, miRNA-30c, miRNA-34c, miRNA-133 a, miRNA-135a, miRNA-137, miRNA-204, miRNA-205, miRNA-217, miRNA-218 and miRNA-338 are negatively correlated with RUNX2. Furthermore, these miRNAs, except for miRNA-218, are capable of inhibiting osteogenic differentiation of mesenchymal tissues.

In this work, we elucidated the function of miRNA-365a-3p in osteogenic differentiation of hBMSCs. Meanwhile, the interaction of miRNA-365a-3p and RUNX2 in osteogenic differentiation was also explored. Our findings aimed to provide novel hallmarks for the treatment of osteoporosis.

Patients and Methods

Sample Collection

Clinically diagnosed osteoporosis patients (n=30) and healthy controls (n=30) were enrolled in this study. Their pathological data were then collected. 8 mL of venous blood was harvested from each subject in the morning under the fasting stage. After standing for 30 min, blood samples were centrifuged at 4°C and 3000 g/min for 10 min. The supernatant serum (non-hemolytic state) was centrifuged again at 4°C and 135,000 g/min for 15 min. Subsequently, the serum samples were sub-packaged in 200 µL/tube and preserved at -80°C for use. This study was approved by the Ethics Committee of People's Hospital of Rizhao. Signed informed consents were obtained from all participants before the study.

Isolation and Culture of hBMSCs

First, hBMSCs were harvested from osteoporosis patients and healthy controls using bone marrow adhesion method. Isolated hBMSCs were cultured in phenol red-free α-minimum Eagle's medium (α-MEM; HyClone, Logan, UT, USA) containing 10% fetal bovine serum-HI (FBS-HI) (Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Grand Island, NY, USA). The culture medium was replaced every two days. Cell passage was performed until 90% of confluence.

First, hBMSCs were cultured in phenol red-free α-MEM with 1% FBS-HI, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL ascorbic acid (Gibco, Grand Island, NY, USA), 10 mM glycerophosphate and 0.1 µg/mL dexamethasone (Sigma-Aldrich, St. Louis, MO, USA). Osteogenic induction was then performed. Meanwhile, osteogenic induction medium was replaced every 3 days.

Cell Transfection

MiRNA-365a-3p mimics, miRNA-365a-3p inhibitor, si-RUNX2 or negative control (GenePharma, Shanghai, China) was transfected into hBMSCs according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in hBMSCs was extracted in strict accordance with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The purity and concentration of extracted RNA were determined using Nanodrop spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, RNA was reverse transcribed into complementary deoxyri-

bose nucleic acid (cDNA) using the cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to amplify obtained cDNA using SYBR Select Master Mix (Applied Biosystems, Foster City, CA, USA) on the ABI7900 Sequence Detection System. The relative expression level of the genes was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: OCN, F: 5'-CGAACCAACTACCGACTC-3', R: 5'-CTAGATCCGAAGCCCGATA-3'; miR-365a-3p, F: 5'-GCGTTTGGACATCCTCGACTG-3', R: 5'-ATGTCGTACAGTGAGTCG-3'; OPN, F: 5'-ATGCGGTGTAAGCGGCAACAA-3', R: 5'-CGTGTGCTTCAGTTAGCGTCT-3'; collagen I, F: 5'-AGAGCTTCGGCAGCAGGA-3', R: 5'-CTTATAGCAGTTCTGCCTGC-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

Total protein in cells was extracted using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China). Subsequently, extracted total protein was separated by electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk for 2 hours, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were incubated with the corresponding secondary antibodies at room temperature for 2 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by Image J Software (NIH, Bethesda, MD, USA).

Alkaline Phosphatase (ALP) Activity Determination

First, hBMSCs were washed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) and lysed in 1% Triton X-100 for 15 min, followed by centrifugation at 10,000 g for 5 min. The supernatant was collected and ALP activity was determined according to the instructions of ALP determination kit (Abcam, Cambridge, MA, USA). OD values at 450 nm were detected by a microplate reader (Promega, Madison, WI, USA).

Alizarin Red Staining

HBMSCs were fixed in pre-cold 70% ethanol and washed with ddH_2O five times. Subsequently, they were dyed with 40 mM of alizarin red for 10-15 min and washed with ddH_2O five times. Calcified nodules (orange) were finally observed and captured using an inverted microscope (Olympus, Tokyo, Japan).

Dual-Luciferase Reporter Gene Assay

Wild-type and mutant-type RUNX2 3'-UTR sequences were inserted into the pmirGLO Luciferase vector. After that, hBMSCs were co-transfected with RUNX2 3'-UTR wt/RUNX2 3'-UTR mut and miRNA-365a-3p mimics/miR-NC using Lipofectamine 2000. 24 h after transfection, the cells were harvested. The Luciferase activity was finally measured using a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for all statistical analysis. Data were expressed as mean ± SD (Standard Deviation). The *t*-test was used to compare the differences between the two groups. One-way analysis of variance (ANOVA) was applied to compare the differences among different groups, followed by the post-hoc test. $p<0.05$ was considered statistically significant.

Results

MiRNA-365a-3p Was Highly Expressed in Serum of Osteoporosis Patients

The serum level of miRNA-365a-3p in osteoporosis patients and healthy controls was first examined by qRT-PCR. The results showed that the level of miRNA-365a-3p in osteoporosis patients was significantly higher than healthy controls (Figure 1A). Subsequently, miRNA-365a-3p expression was determined in hBMSCs undergoing osteogenic differentiation at 0, 5, 10 and 21 day, respectively. The data revealed that miRNA-365a-3p expression decreased gradually with the prolongation of osteogenic induction (Figure 1B). These results suggested that miRNA-365a-3p might be involved in osteogenic differentiation.

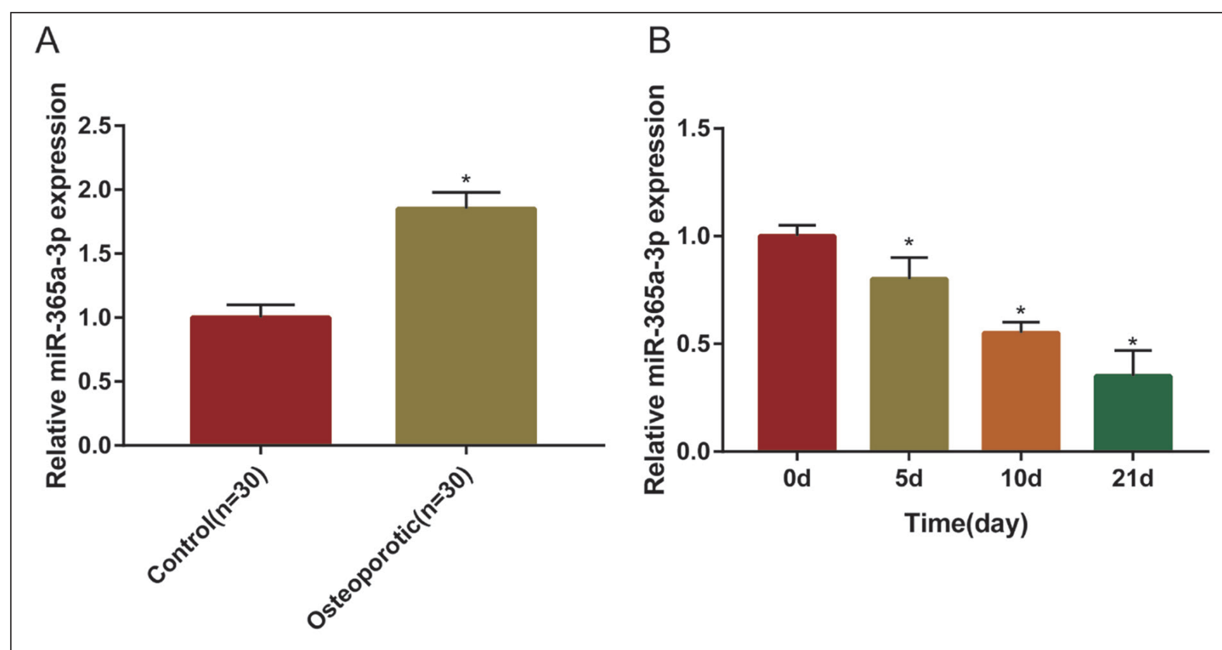

Figure 1. MiR-365a-3p was highly expressed in the serum of osteoporosis patients. **A,** QRT-PCR data showed significantly higher serum level of miR-365a-3p in osteoporosis patients relative to controls. **B,** QRT-PCR data showed that miR-365a-3p expression decreased gradually with the prolongation of osteogenic induction.

MiRNA-365a-3p Overexpression Downregulated the Expressions of Relative Markers for Osteogenic Differentiation

To elucidate the potential function of miRNA-365a-3p in osteogenic differentiation, we first constructed miRNA-365a-3p mimics and inhibitor. Transfection efficacy in hBMSCs was verified by qRT-PCR (Figure 2A). The overexpression of miRNA-365a-3p remarkably down-regulated the expressions of OCN, OPN and collagen I. Conversely, miRNA-365a-3p knockdown significantly up-regulated the levels of the above genes in hBMSCs (Figure 2B-2D). Compared with controls, the capability of mineralization was markedly weakened by miRNA-365a-3p overexpression (Figure 2E). ALP activity of hBMSCs overexpressing miRNA-365a-3p decreased (Figure 2F). It was indicated that miRNA-365a-3p negatively regulated the osteogenic differentiation of hBMSCs.

RUNX2 Was the Target of MiRNA-365a-3p

Subsequently, the potential binding sequences between miRNA-365a-3p and RUNX2 were predicted by miRanda (Figure 3A). Dual-Luciferase reporter gene assay showed that co-transfection of miRNA-365a-3p mimics and RUNX2 3'-UTR WT could remarkably decrease the Luciferase activity (Figure 3B). Hence, the binding relationship of RUNX2 to miRNA-365a-3p was verified. The overexpression of miRNA-365a-3p in hBMSCs significantly down-regulated the mRNA and protein levels of RUNX2. However, miRNA-365a-3p knockdown could up-regulate its expression level (Figure 3C, 3D). Therefore, we believed that miRNA-365a-3p negatively regulated RUNX2 level in hBMSCs.

MiRNA-365a-3p Inhibited Osteogenic Differentiation Via Inhibiting RUNX2 Expression

To further explore the mechanism of miRNA-365a-3p in regulating osteogenic differentiation, hBMSCs were transfected with miR-NC, miRNA-365a-3p mimics or miRNA-365a-3p mimic+si-RUNX2, respectively. The results demonstrated that ALP activity was markedly reduced by overexpression of miRNA-365a-3p, which could be reversed by RUNX2 knockdown (Figure 4A). Identically, alizarin red staining showed remarkably inhibited capacity of mineralization in hBMSCs overexpressing miRNA-

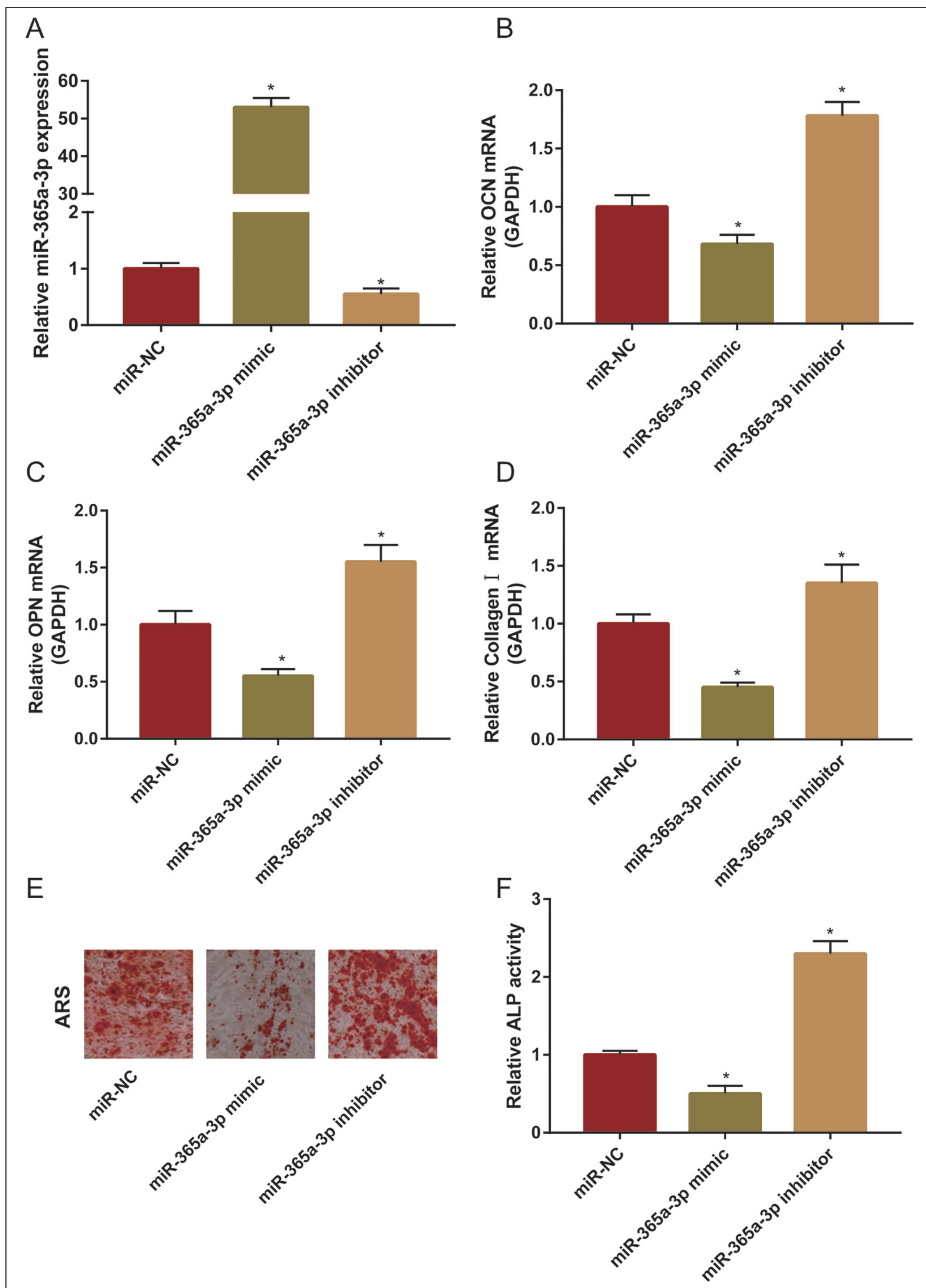

Figure 2. MiR-365a-3p overexpression down-regulated the expression of relative markers for osteogenic differentiation. **A,** Transfection efficacy of miR-365a-3p mimics and inhibitor in hBMSCs. **B-D,** Overexpression of miR-365a-3p markedly down-regulated the levels of OCN, OPN and collagen I. Conversely, miR-365a-3p knockdown up-regulated their expression levels. **E,** Overexpression of miR-365a-3p significantly weakened capability of mineralization in hBMSCs (Magnification × 40). **F,** Overexpression of miR-365a-3p decreased ALP activity in hBMSCs.

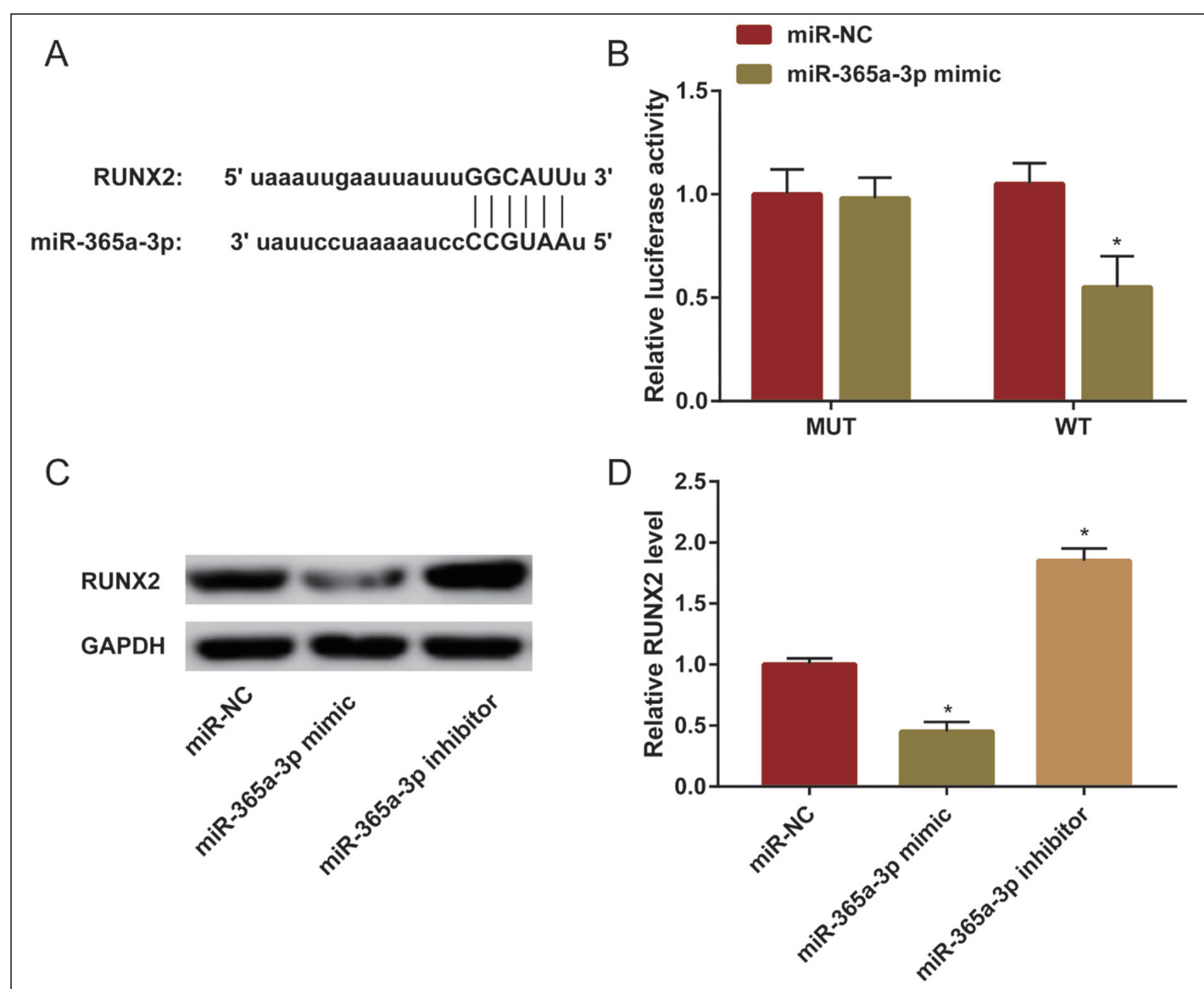

Figure 3. RUNX2 was the target of miR-365a-3p. **A,** The potential binding sequences between miR-365a-3p and RUNX2 were predicted by miRanda. **B,** Dual-Luciferase reporter gene assay confirmed that co-transfection of miR-365a-3p mimics and RUNX2 3'-UTR WT remarkably decreased the Luciferase activity. **C-D,** Overexpression of miR-365a-3p in hBMSCs down-regulated the mRNA and protein levels of RUNX2, whereas miR-365a-3p knockdown up-regulated its expression level.

365a-3p. Meanwhile, mineralization was further elevated by co-transfection of miRNA-365a-3p mimics and si-RUNX2 (Figure 4B). The relative expression levels of OCN, OPN and collagen I were significantly down-regulated after miRNA-365a-3p overexpression. However, the levels of these above genes were markedly elevated by RUNX2 knockdown (Figure 4C-E). The above data demonstrated that miRNA-365a-3p inhibited osteogenic differentiation of hBMSCs *via* targeting RUNX2.

Discussion

MiRNA is closely related to cell proliferation and osteogenic differentiation. It can regulate expressions of key regulatory factors and receptors in osteogenic differentiation pathways by mediating target mRNAs. MiR-365 is located on chromosome 16p13.12. The mature hsa-miR-365 sequence is cleaved from two precursors, including hsa-miR-365-1 and hsa-miR-365-2[21]. Abnormal expression of miR-365 has been observed in various tumors. Meanwhile, the expression patterns and functions of miR-365 vary greatly in different types of tumors. MiRNA-365a-3p is highly expressed in breast cancer and pancreatic cancer, serving as an oncogene[22,23]. However, the role of miRNA-365a-3p in osteoporosis has not been fully elucidated. In this work, we found that the serum level of miRNA-365a-3p in osteoporosis patients was significantly higher than that of healthy controls. Meanwhile, miRNA-365a-3p

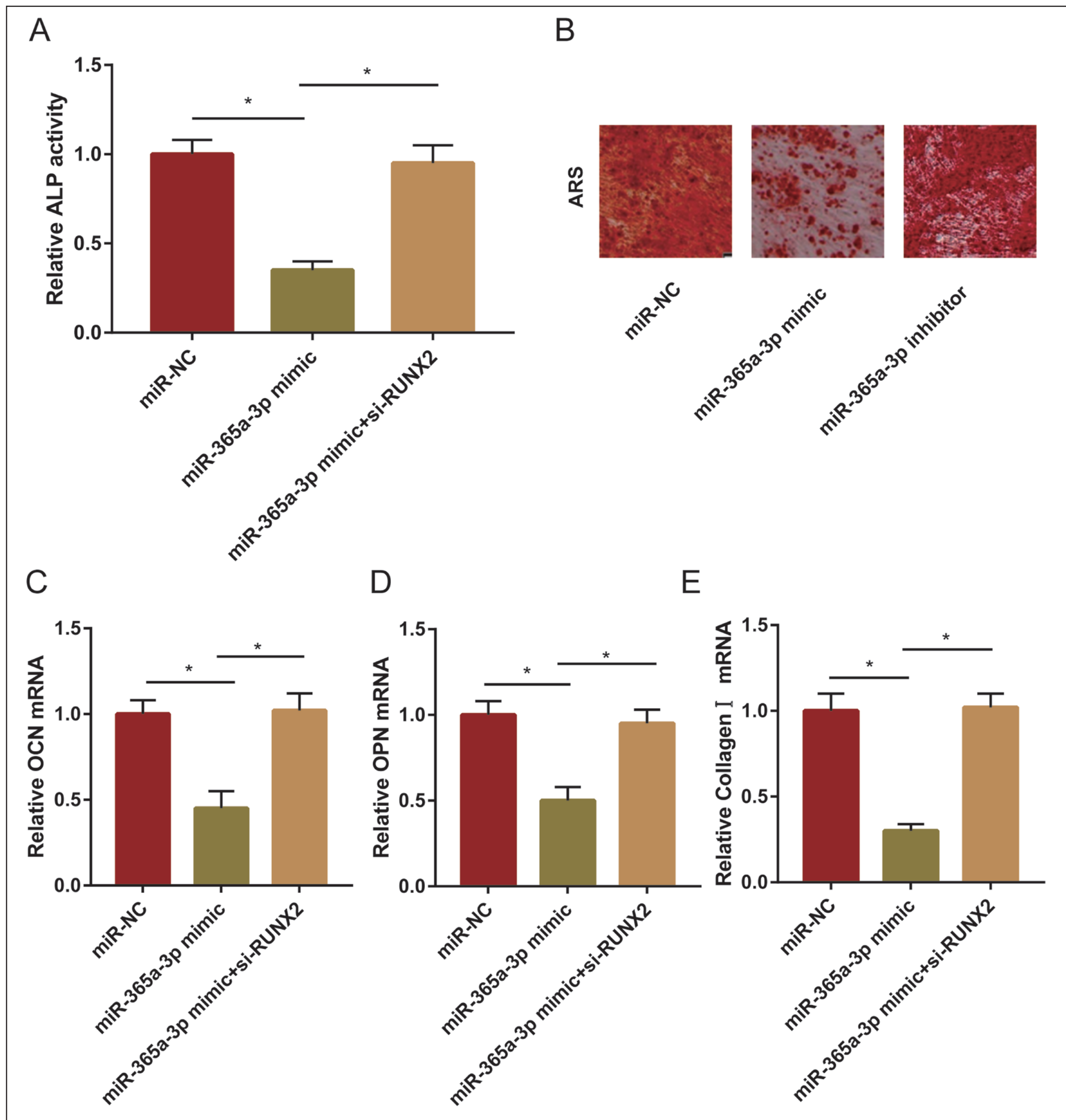

Figure 4. MiR-365a-3p inhibited osteogenic differentiation via inhibiting RUNX2 expression. HBMSCs were transfected with miR-NC, miR-365a-3p mimics or miR-365a-3p mimic+si-RUNX2, respectively. **A,** ALP activity was significantly reduced by overexpression of miR-365a-3p, which was reversed by RUNX2 knockdown. **B,** Alizarin red staining showed markedly inhibited capacity of mineralization in hBMSCs overexpressing miR-365a-3p, which was further elevated by co-transfection of miR-365a-3p mimics and si-RUNX2 (Magnification × 40). **C-E,** The relative expression levels of OCN, OPN and collagen I were significantly down-regulated after miR-365a-3p overexpression. However, the expressions of these molecules were remarkably elevated by RUNX2 knockdown.

expression in hBMSCs decreased gradually with the prolongation of osteogenic induction.

Osteoblast differentiation is divided into three stages, namely proliferative phase, extracellular matrix maturation and extracellular matrix mineralization. Each stage has its differentiation markers. This is an important indicator for studying the differentiation process of osteoblasts. ALP, BSP and Col I are markers for early-stage osteogenic differentiation. However, OCN and OPN are markers for late-stage differentiation[24-27]. By examining the expression levels

of OCN, OPN and Collagen I, we found that the overexpression of miRNA-365a-3p significantly inhibited their expression levels. ALP activity is also a crucial marker of osteogenic differentiation. Our results showed that overexpression of miRNA-365a-3p markedly reduced ALP activity in hBMSCs. These results detected that miRNA-365a-3p played an inhibitory role in osteogenic differentiation.

A variety of transcription factors are involved in the regulation of osteogenic differentiation of hBMSCs, such as Runx2/Cbfa1, Osx and others. Runx2/Cbfa1 and Osx further regulate bone-specific matrix protein products including ALP, COL-I, BSP, OCN and OPN[28]. In recent years, studies have found that miRNA affects osteogenic differentiation by regulating the expression of RUNX2. For example, miR-133 inhibits the differentiation of mouse-derived C2C12 cells into osteoblasts by targeting RUNX2[29]. Kim et al[30] have found that ERRγ inhibits osteoblast differentiation by down-regulating RUNX2 in pre-osteoblasts. Subsequent experiments have also shown that ERRγ directly inhibits the post-transcriptional levels of RUNX2 and BMP-2 by up-regulating miR-433 in mouse-derived C3H10T1/2 cells. Vimalraj et al[31] have demonstrated that Smurf1 degrades RUNX2 through the proteasome pathway. However, miR-15b up-regulates RUNX2 level by down-regulating Smurf1 in BMSCs. Therefore, RUNX2 can interact with upstream and downstream molecules to further influence bone growth and development as crosstalk in osteoporosis. In this work, we further explored the interaction between miRNA-365a-3p and RUNX2 in osteogenic differentiation of hBMSCs. Our findings indicated that miRNA-365a-3p could bind to RUNX2 and negatively regulate its expression.

Conclusions

MiRNA-365a-3p is highly expressed in osteoporosis, whose expression decreases gradually with the prolongation of osteogenic differentiation. In addition, miRNA-365a-3p negatively regulates osteogenic differentiation of hBMSCs by targeting RUNX2, thus promoting the progression of osteoporosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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