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MiRNA-27a-3p promotes osteogenic differentiation of human mesenchymal stem cells through targeting ATF3

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Abstract. – OBJECTIVE: To elucidate whether miRNA-27a-3p can promote osteogenic differentiation of hMSCs by targeting ATF3, thus alleviating osteoporosis symptoms.

PATIENTS AND METHODS: The serum levels of miRNA-27a-3p in osteoporosis patients (n=20) and normal controls (n=20) were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Human bone marrow mesenchymal stem cells (hMSCs) were subjected to osteogenic differentiation for 1, 3 and 7 days. Subsequently, mRNA levels of miRNA-27a-3p, ALP, and Bglap in hMSCs were determined by qRT-PCR. The regulatory effects of miRNA-27a-3p levels and the mRNA levels of ALP, Bglap, and Runx2 were detected. After the overexpression or knockdown of miRNA-27a-3p, we evaluated the changes in the osteogenic differentiation by alizarin red staining and ALP staining. Through Dual-Luciferase Reporter Gene Assay, we verified the binding relationship between miRNA-27a-3p and ATF3. Rescue experiments were finally conducted to prove whether miRNA-27a-3p regulated the osteogenic differentiation by targeting ATF3.

RESULTS: The serum level of miRNA-27a-3p remained lower in osteoporosis patients relative to controls. With the prolongation of osteogenic differentiation, the mRNA levels of miRNA-27a-3p, ALP, and Bglap gradually increased. The overexpression of miRNA-27a-3p upregulated mRNA and the protein levels of osteogenesis-related genes, increased ALP activity, and enhanced mineralization capacity. The knockdown of miRNA-27a-3p obtained the opposite trends. MiRNA-27a-3p could target ATF3, and the overexpression of ATF3 reversed the promotive effects of miRNA-27a-3p on osteogenic differentiation.

CONCLUSIONS: MiRNA-27a-3p promotes the differentiation of hMSCs into osteoblasts by targeting ATF3, thus alleviating osteoporosis symptoms.

Key Words:
hMSCs, Osteogenic differentiation, Osteoporosis, MiRNA, ATF3.

Introduction

Osteoporosis is one of the common diseases in the elderly and menopausal women[1]. Osteoporosis manifests as decreased bone density, bone quality, and bone microstructural destruction. Osteoporosis patients are prone to experience a fracture due to elevated skeletal fragility[2]. Derived from mesenchymal precursors, the osteoblasts secrete the extracellular matrix that is necessary for bone mineralization during embryonic development and postpartum remodeling[3]. Osteoblasts are vital cells for maintaining adult skeletal homeostasis. Imbalanced osteoblasts and osteoclasts lead to bone mass reduction, further resulting in osteoporosis or ossification[4]. Osteoporosis is the main hazard for menopausal women[5]. Estrogen is closely related to bone repair. Estrogen deficiency leads to bone changes[6].

Human bone marrow mesenchymal stem cells (hMSCs) are isolated from the marrow and have diverse differentiation potentials, which are widely concerned in relative researches[7]. hMSCs can differentiate into a variety of mature cells

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under certain conditions, including osteoblasts, adipocytes and chondrocytes[8]. Induction of hMSCs into osteoblasts has been well studied in recent years.

MicroRNA (miRNA) is a small non-coding RNA of 19-25 nucleotides in length. It binds to a non-coding region of messenger RNA (mRNA), thus degrading or inhibiting mRNA[9]. MiRNAs participate in various biological behaviors, including cell proliferation[10], differentiation[11,12], and apoptosis[13]. Many studies[14] have shown that a single miRNA can simultaneously target multiple mRNAs, and conversely, different miRNAs can synergistically target a single mRNA. There are several miRNAs identified in regulating the osteogenic differentiation of hMSCs.

In this study, qRT-PCR was performed to detect the serum level of miRNA-27a-3p in osteoporosis patients and healthy controls. We found that miRNA-27a-3p was lowly expressed in osteoporosis patients. Moreover, the levels of miRNA-27a-3p and osteogenic-specific genes were upregulated with the prolongation of osteogenic differentiation. Our results indicated that miRNA-27a-3p was significantly up-regulated during osteogenic differentiation. The silence of miRNA-27a-3p inhibited osteoblast activity and matrix mineralized nodule formation, whereas the overexpression of miRNA-27a-3p promoted osteogenic differentiation. In addition, ATF3 (activation of transcription factor 3) is a stress-inducing gene[15] and was identified as a direct target of miRNA-27a-3p in this study. The overexpression of miRNA-27a-3p abolished ATF3-mediated and inhibited osteogenic differentiation. However, the specific molecular mechanism of miRNA-27a-3p in promoting the osteogenic differentiation of hMSCs remains unclear. We hypothesized that miRNA-27a-3p exerts an important role in inducing osteogenic differentiation of hMSCs cells, thus protecting osteoporosis.

Patients and Methods

Subjects

Osteoporosis patients (n=20) and healthy controls (n=20) were enrolled from April 2016 to April 2017 in Gong'an County People's Hospital. We collected their serum samples and preserved them at -80°C with approval. This experimental study was approved by the Ethics Committee of Gong'an County People's Hospital.

Construction of LV-ATF3 and shRNA-ATF3

For primary cells that are difficult to transfect, the use of lentiviral vectors can greatly improve transfection efficiency. The oligonucleotide encoding the ATF3 shRNA was inserted into the plasmid, and the plasmid containing the fragment of the H1 promoter and the ATF3 shRNA were inserted into the lentivirus, which was ATF3 shRNA lentiviral vector. A plasmid containing a full-length complementary deoxyribose nucleic acid (cDNA) amplified by Polymerase Chain Reaction (PCR) amplification of ATF3 and a fragment of the H1 promoter was inserted into the lentivirus, which was LV-ATF3.

Culture and Transfection of hMSCs

hMSCs were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) at 37°C, 5% CO_2. The cells with good growth conditions were seeded in a 6-well plate with 1×10^4 cells per well. Transfection was performed until cell confluence reached 75-85%. The complete medium was replaced 4 hours later. The transfected cells were harvested at 24-48 h for subsequent experiments.

The culture medium: high-glucose DMEM containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 1% penicillin and 1% streptomycin.

Growth medium: α-MEM supplemented with 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin and 1% HEPES.

The induction medium: High-glucose DMEM supplemented with 10% FBS, 10 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate, 50 μg/ml ascorbic acid, 1% L-glucose, 1% penicillin-streptomycin and 1% HEPES.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA of hMSCs was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA). RNA purity was measured by an ultraviolet spectrophotometer and RNA samples were stored at -80 °C until use. The cDNA was obtained through reverse transcription of RNA, and the SYBR Green method (TaKaRa, Otsu, Shiga, Japan) was used for PCR detection. The expressions of osteogenic-specific genes (ALP, Bglap,

Runx2) were examined. The primer sequences were: ALP, F: 5'-AAGGCTTCTTCTTGCTG-GTG-3', R: 5'-GCCTTACCCTCATGATGTCC-3'; Bglap, F: 5'-AGCAAAGGTGCAGCCTTTGT-3', R: 5'-GCGCCTGGTCTCTTCACT-3'; Runx2, F: 5'-ACTTCCTGTGCTCCGTGCTG-3', R: 5'-TC-GTTGAACCTGGCTACTTGG-3'; GAPDH, F: 5'-ACCCACTCCTCCACCTTTGA-3', R: 5'-CT-GTTGCTGTAGCCAAATTCGT-3'.

Alizarin Red Staining

After osteogenic differentiation of hMSCs, the cells were washed with Phosphate-Buffered Saline (PBS) twice, fixed with 4% paraformaldehyde for 15 min and stained with 1% alizarin red staining for 5 min. Calcified nodules were observed and captured using an inverted microscope.

ALP Staining

The transfected hMSCs were induced in osteogenic induction medium (5 mL of 3% β-glycerophosphate sodium + 5 mL of 2% barbital sodium + 10 mL of distilled water + 10 mL of 2% calcium chloride + 1 mL of 2% magnesium sulfate). Osteogenic induction medium was added on the slides placed in a 6-well plate, incubated at 37°C for 15 min and washed for 2 min. The cells were re-stained with the hematoxylin counterstaining solution for 5 min, washed with 2 min and air dried. Finally, the cells were captured under an optical microscope.

Western Blot

The total protein from cells was extracted using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) and loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. The bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Dual-Luciferase Reporter Gene Assay

hMSCs were seeded in a 24-well plate with 3×10^5 cells per well. The cells were co-transfected with wild-type or mutant-type psiCHECK-2 ATF3 and miR-130-5p mimic using Lipofectamine 2000. After 24 hours, the cells were harvested and the Luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. The data were expressed as mean ± SD (Standard Deviation). The *t*-test was used to analyze the differences between the two groups. $p<0.05$ indicated the significant difference (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

Serum Level of MiRNA-27a-3p Remained Low in Osteoporosis Patients

We detected miRNA-27a-3p level in the serum samples of 20 osteoporosis patients and 20 normal controls. The lower serum level of miRNA-27a-3p was observed in osteoporosis patients (Figure 1A). hMSCs were incubated in osteogenic induction medium for 1, 3, and 7 days, respectively. The levels of miRNA-27a-3p, and the mRNA levels of ALP, Runx2, and Bglap gradually increased with the prolongation of osteogenic differentiation (Figure 1B). The above results indicated that miRNA-27a-3p remained at a low level in the serum of osteoporosis patients. Moreover, miRNA-27a-3p expression increased with the prolongation of osteogenic differentiation in hMSCs.

Overexpression of MiRNA-27a-3p Promoted Differentiation of hMSCs into Osteoblasts

To further investigate the effect of miRNA-27a-3p on the differentiation of hMSCs into osteoblasts, the transfection efficacy of miRNA-27a-3p mimic and inhibitor was verified at first. The transfection of miRNA-27a-3p mimic greatly increased the miRNA-27a-3p level, and the transfection of miRNA-27a-3p inhibitor reduced miRNA-27a-3p level (Figure 2A). The overexpression of miRNA-27a-3p upregulated mRNA levels of ALP, Runx2, and Bglap in hMSCs. Conversely, miRNA-27a-3p knockdown downregulated their mRNA levels (Figure 2B). The protein levels of ALP and Runx2 were positively regulated by miRNA-27a-3p as well (Figure 2C). Moreover, ALP activity was positively influenced by miRNA-27a-3p expression (Figure 2D). hMSCs overexpressing miRNA-27a-3p showed a higher activity of ALP. The mineralized nodules are important markers of osteoblast maturation. The results of alizarin red staining showed that

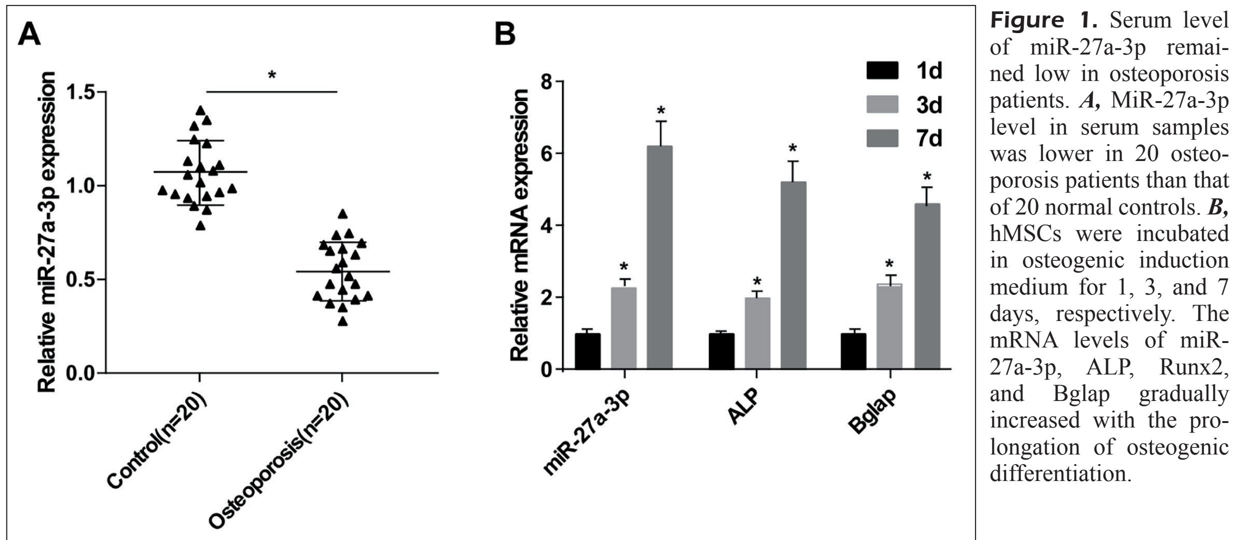

Figure 1. Serum level of miR-27a-3p remained low in osteoporosis patients. ***A,*** MiR-27a-3p level in serum samples was lower in 20 osteoporosis patients than that of 20 normal controls. ***B,*** hMSCs were incubated in osteogenic induction medium for 1, 3, and 7 days, respectively. The mRNA levels of miR-27a-3p, ALP, Runx2, and Bglap gradually increased with the prolongation of osteogenic differentiation.

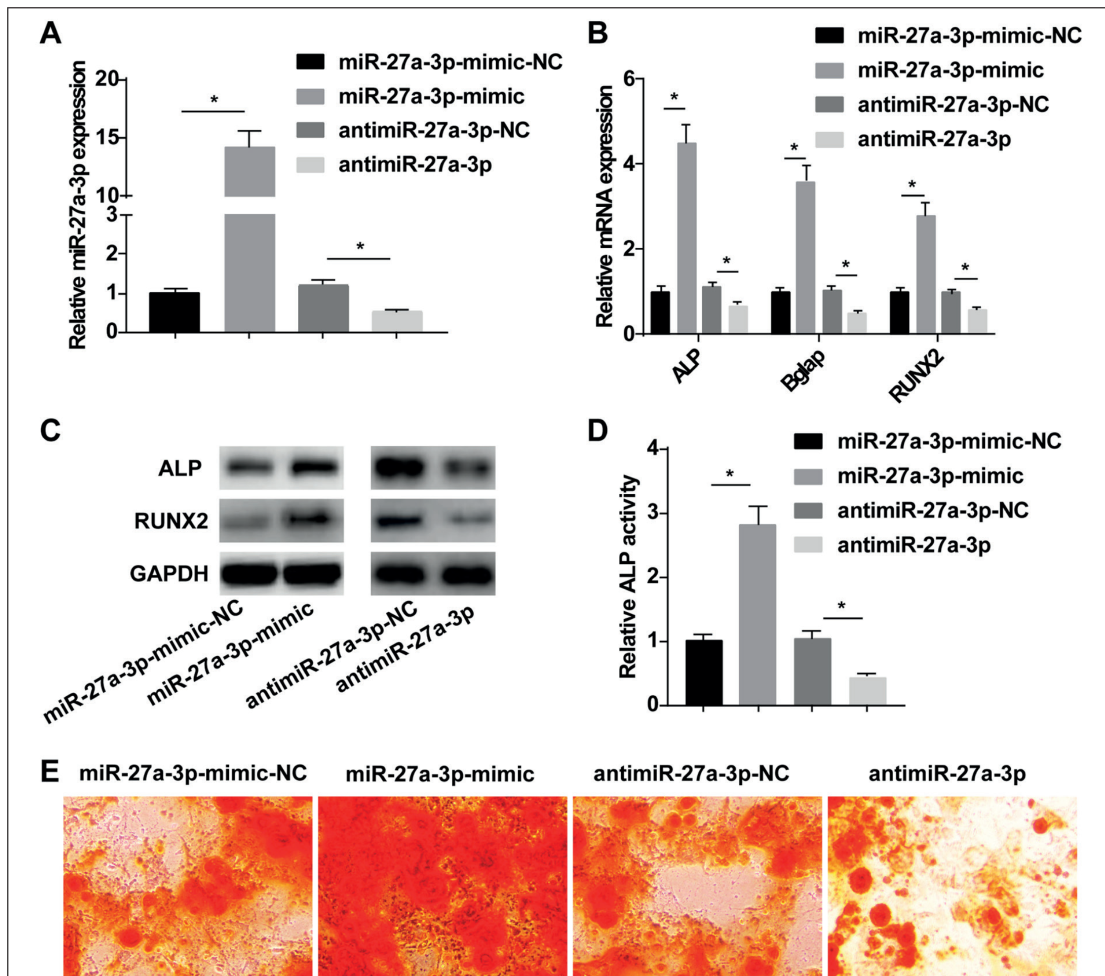

Figure 2. Overexpression of miR-27a-3p promoted the differentiation of hMSCs into osteoblasts. ***A,*** The transfection of miR-27a-3p mimic greatly increased miR-27a-3p level, and the transfection of miR-27a-3p inhibitor reduced miR-27a-3p level. ***B,*** Overexpression of miR-27a-3p upregulated mRNA levels of ALP, Runx2, and Bglap in hMSCs. Conversely, miR-27a-3p knockdown downregulated their mRNA levels. ***C,*** The protein levels of ALP and Runx2 were positively regulated by miR-27a-3p. ***D,*** ALP activity was positively influenced by miR-27a-3p expression. ***E,*** MiR-27a-3p overexpression promoted the formation of mineralized nodules in hMSCs and differentiation into osteoblasts.

miRNA-27a-3p overexpression promoted the formation of the mineralized nodules in hMSCs and differentiation into osteoblasts (Figure 2E). These results indicated that the overexpression of miRNA-27a-3p promoted the differentiation of hMSCs into osteoblasts.

MiRNA-27a-3p Directly Targeted ATF3

The binding sites between miRNA-27a-3p and ATF3 were found by the bioinformatics prediction website TargetScan (Figure 3A). The Dual-Luciferase Reporter Gene Assay confirmed that miRNA-27a-3p could bind to ATF3 (Figure 3B). The transfection of miRNA-27a-3p mimic markedly downregulated mRNA level of ATF3, whereas miRNA-27a-3p knockdown upregulated ATF3 level, showing a negative correlation between them (Figure 3C). Identically, miRNA-27a-3p negatively regulated the protein level of ATF3 (Figure 3D). The above experimental results suggested that miRNA-27a-3p directly targeted ATF3.

Overexpression of ATF3 Inhibited Osteogenic Differentiation of hMSCs

The transfection of LV-ATF3 inhibited mRNA levels of ALP, Bglap, and Runx2, whereas the transfection of shRNA-ATF3 upregulated their levels (Figures 4A, 4B). It is suggested that ATF3 inhibited the osteogenic differentiation of hMSCs cells. Similarly, the protein levels of ALP and Runx2 were negatively regulated by ATF3 (Figure 4C). hMSCs overexpressing ATF3 showed lower ALP activity relative to controls (Figure 4D). Alizarin red staining showed that the overexpression of ATF3 inhibited the formation of mineralized nodules in hMSCs, and the knockdown of ATF3 promoted the capability of mineralization in hMSCs (Figure 4E). The above results demonstrated that overexpression of ATF3 inhibited the osteogenic differentiation of hMSCs.

Overexpression of ATF3 Reversed the Promotive Effect of miRNA-27a-3p on Osteogenic Differentiation of hMSCs

To evaluate whether ATF3 was involved in the regulatory effect of miRNA-27a-3p on osteogenic differentiation, hMSCs were co-transfected with miRNA-27a-3p mimic and LV-ATF3. MiRNA-27a-3p overexpression upregulated mRNA levels of ALP, Bglap, and Runx2, but was further reversed by the co-transfection of LV-ATF3 (Figure 5A). Moreover, the increased ALP activity due to the overexpressed miRNA-27a-3p was inhibited by ATF3 overexpression (Figure

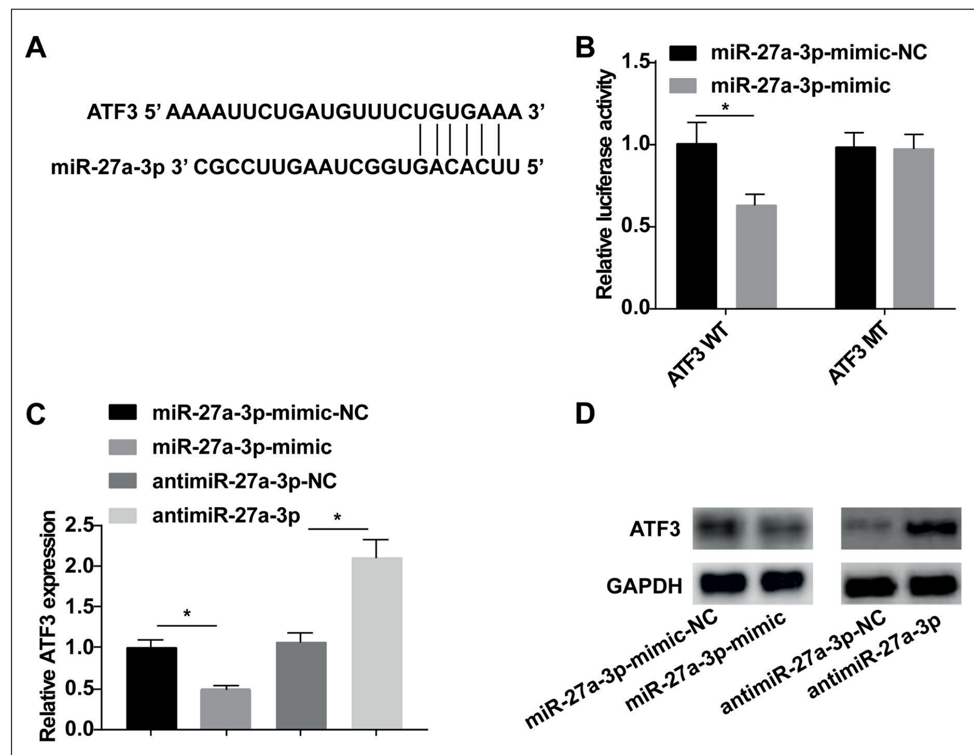

Figure 3. MiR-27a-3p directly targeted ATF3. ***A,*** The binding sites between miR-27a-3p and ATF3. ***B,*** Dual-Luciferase Reporter Gene Assay confirmed that miR-27a-3p could bind to ATF3. ***C,*** The transfection of miR-27a-3p mimic markedly downregulated mRNA level of ATF3, whereas miR-27a-3p knockdown upregulated ATF3 level. ***D,*** MiR-27a-3p negatively regulated the protein level of ATF3.

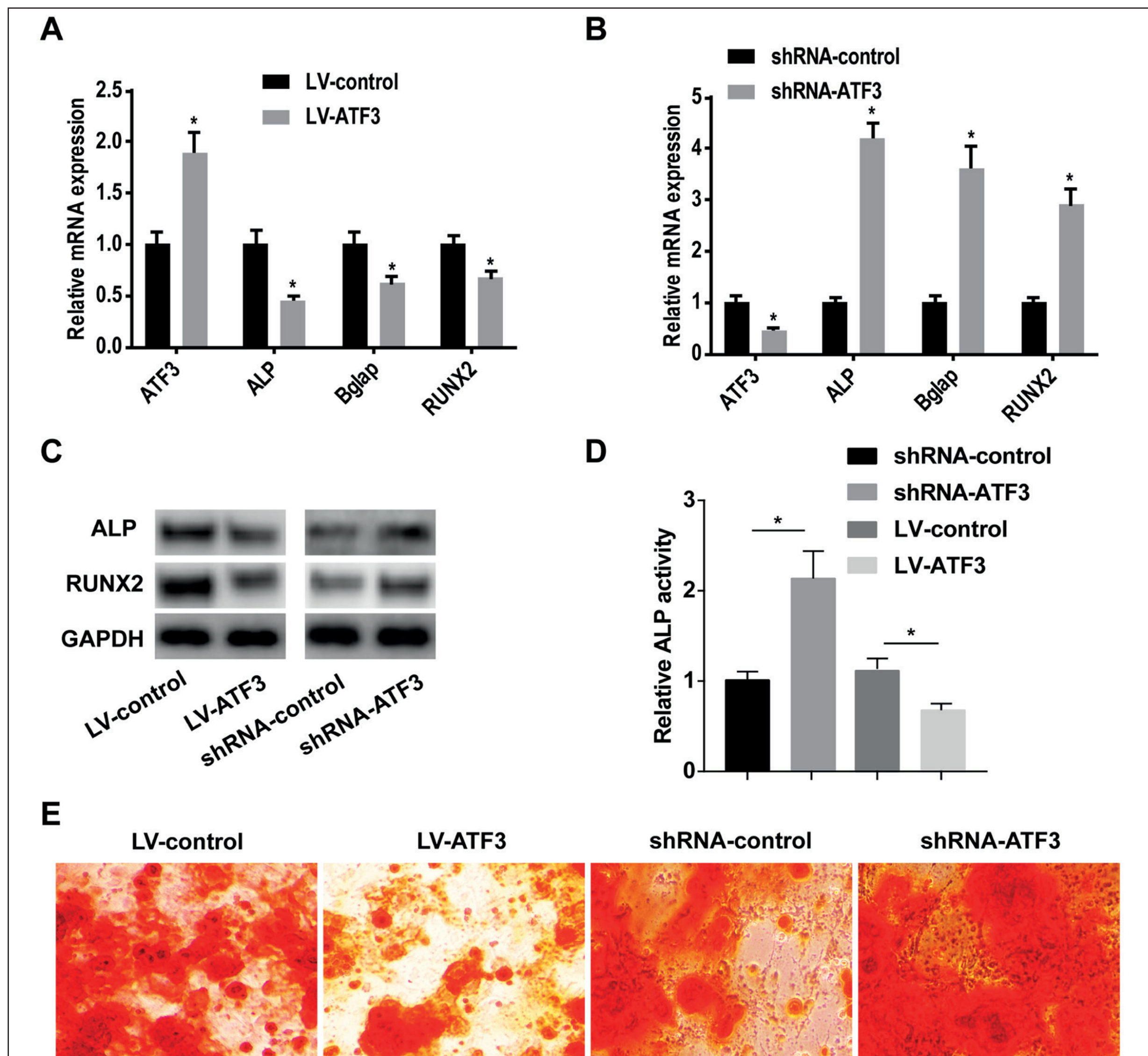

Figure 4. Overexpression of ATF3 inhibited the osteogenic differentiation of hMSCs. ***A,*** Overexpression of ATF3 through transfection with LV-ATF3 inhibited the mRNA levels of ALP, Bglap, and Runx2. ***B,*** Transfection with shRNA-ATF3 upregulated mRNA levels of ALP, Bglap, and Runx2. ***C,*** The protein levels of ALP and Runx2 were negatively regulated by ATF3. ***D,*** ALP activity was negatively influenced by ATF3 expression. ***E,*** Alizarin red staining showed that the overexpression of ATF3 inhibited the formation of mineralized nodules in hMSCs, and the knockdown of ATF3 promoted the capability of mineralization in hMSCs.

5B). The above data proved that the overexpression of ATF3 reversed the promotive effect of miRNA-27a-3p on the osteogenic differentiation of hMSCs.

Discussion

The growth and maintenance of human bones depend on the dynamic balance in the activities and numbers of osteoblasts and osteoclasts. Osteoblasts are cell units responsible for bone formation and bone remodeling[16]. Our study found that miRNA-27a-3p was an important regulator of bone formation. The experimental results showed that miRNA-27a-3p remained low in the serum level of osteoporosis patients, but increased with the prolongation of the osteogenic differentiation, accompanied by mRNA levels of osteogenesis-specific genes. The overexpression of miRNA-27a-3p enhanced the mineralization capacity and ALP activity in

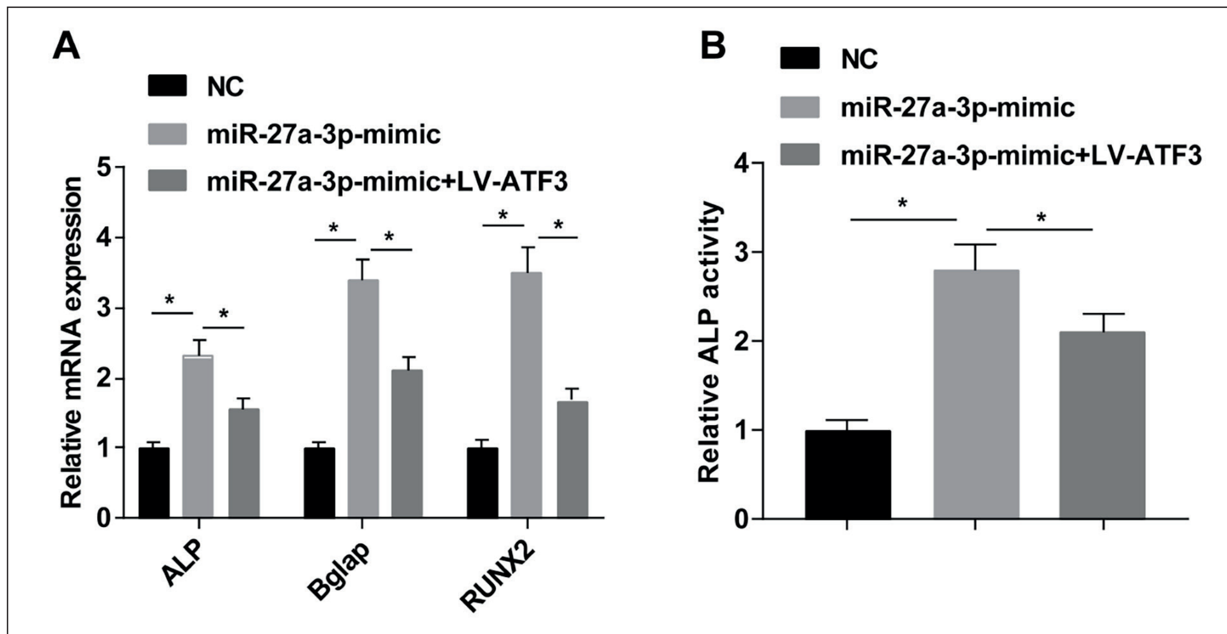

Figure 5. Overexpression of ATF3 reversed the promotive effect of miR-27a-3p on osteogenic differentiation of hMSCs. ***A,*** MiR-27a-3p overexpression upregulated mRNA levels of ALP, Bglap, and Runx2, but were further reversed by the co-transfection of LV-ATF3. ***B,*** The increased ALP activity due to overexpressed miR-27a-3p was inhibited by ATF3 overexpression.

osteoblasts. MiRNAs have been extensively studied in various biological processes, including osteogenic homeostasis[17]. Multiple miRNAs are involved in the post-transcriptional regulation of genes in osteogenic differentiation[18-20]. In this study, we found that miRNA-27a-3p promoted the osteogenic differentiation of hMSCs by targeting ATF3, providing novel directions for revealing the pathogenesis of osteoporosis. MiRNAs are involved in multiple cellular behaviors[10]. A single miRNA simultaneously targets multiple mRNAs, and different miRNAs could cooperatively target a single mRNA to form a complex regulatory network[21]. Recent studies have shown that microRNA-451 blocks YWHAZ-mediated stabilization of Runx2, thus accelerating osteoblast differentiation and bone anabolism[22]. In this study, we identified a novel mechanism that miRNA-27a-3p promoted osteogenic differentiation of hMSCs by targeting ATF3. The regulatory effects of ATF3 on the transcription of target genes are influenced by a variety of factors, including targeted regulation of miRNAs. ATF3 expression is strictly translated in accordance with the biological process of transcription and translation. In this paper, we confirmed that AFT3 was a direct target of miRNA-27a-3p through Dual-Luciferase Reporter Gene Assay and participated in the process of miRNA-27a-3p promoting the osteogenic differentiation of hMSCs.

It is well known that ATF3 binds to the promoter of ALP and regulates its expression and activation, exerting a key role in the osteoblast maturation[23]. High expression of ALP reflects the increased activity of hMSCs cells, which promotes the mineralization capacity and differential potential into osteoblasts. After overexpression of ATF3 by lentivirus transfection, ATF3 directly inhibited the transcription and translation of ALP, Runx2, and Bglap. To sum up, our study demonstrated that miRNA-27a-3p promoted osteogenic differentiation by targeting ATF3 in hMSCs.

Conclusions

We demonstrated that miRNA-27a-3p promotes the differentiation of hMSCs into osteoblasts by targeting ATF3, thus alleviating the symptoms of osteoporosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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