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LncRNA XIXT promotes osteogenic differentiation of bone mesenchymal stem cells and alleviates osteoporosis progression by targeting miRNA-30a-5p

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Abstract. – OBJECTIVE: To uncover the role of XIXT in influencing the osteogenesis of hBMSCs by adsorbing microRNA-30a-5p (miRNA-30a-5p) to upregulate RUNX2.

PATIENTS AND METHODS: The serum samples were collected from osteoporosis patients and normal people. hBMSCs were isolated from femoral head tissues. The serum levels of XIXT and miRNA-30a-5p were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The expression levels and activities of the osteogenic differentiation-related genes in hBMSCs after transfection of sh-XIXT, sh-RUNX2, miRNA-30a-5p mimic, and inhibitor were detected by qRT-PCR, Western blot, ALP activity assay, and alizarin red staining. The Dual-Luciferase Reporter Gene Assay was performed to confirm the binding of XIXT to miRNA-30a-5p, as well as the binding of miRNA-30a-5p to RUNX2.

RESULTS: LncRNA XIXT was significantly downregulated, and miRNA-30a-5p was upregulated in the serum of osteoporosis patients. The osteogenic differentiation-related genes (ALP, RUNX2) and XIXT were markedly upregulated in a time-dependent manner, while miRNA-30a-5p level gradually decreased in hBMSCs with the prolongation of osteogenesis. The knockdown of XIXT inhibited the osteogenic differentiation of hBMSCs. In hBMSCs, XIXT regulated RUNX2 expression by targeting miRNA-30a-5p. The knockdown of miRNA-30a-5p partially reversed the inhibitory effect of XIXT on the osteogenesis of hBMSCs. However, the downregulated RUNX2 reversed the promotive effect of miRNA-30a-5p on the osteogenesis of hBMSCs.

CONCLUSIONS: LncRNA XIXT upregulated RUNX2 by absorbing miRNA-30a-5p, and thus induced hBMSCs osteogenesis to alleviate osteoporosis.

Key Words:
HBMSCs, Osteoporosis, LncRNA XIXT, MiRNA-30a-5p, RUNX2, Osteogenic differentiation.

Introduction

Osteoporosis is a group of osteopathy caused by various factors. It is a kind of metabolic bone disease featured with reduced bone tissue volume per unit volume[1]. Osteoporosis, featured with bone pain and susceptibility to fracture, can occur in different genders and ages, especially in postmenopausal women and elderly men[1,2]. Osteoporosis patients are more prone to suffer fractures, bringing a heavy burden on affected people[3]. The imbalanced osteoblast-induced bone formation and osteoclast-induced bone resorption is the major reason for osteoporosis[4].

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs with over 200 nt long. LncRNAs could regulate gene expressions and biological processes[5,6]. They are involved in multiple cellular behaviors, including epigenetic regulation and cell differentiation[7]. LncRNAs have also been reported to influence the osteoporosis progression[8]. For example, lncRNA MEG3 inhibits the osteogenic differentiation of hBMSCs in postmenopausal osteoporosis by targeting miR-133a-3p[9]. LncRNA LINC00311 promotes osteoclast differentiation in osteoporosis rats by targeting DLL3 to activate the Notch signaling pathway by targeting DLL3[10].

MicroRNAs (miRNAs) are functional non-coding RNAs identified in eukaryotic organisms with about 20-25 nt in length[11]. Mature miRNAs

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could recognize their specific target genes by base pairing and further degrade target genes or block their translation. Researchers[13-15] have shown that miRNAs participate in a variety of regulatory pathways.

In this paper, we identified the expression pattern and biological functions of XIXT in osteogenesis of hBMSCs and its underlying mechanism. Our results provide novel ideas for the clinical treatment of osteoporosis.

Patients and Methods

Sample Collection and Patient Data

The serum samples were collected from osteoporosis patients and normal people. Patients with cancer, rheumatoid arthritis, and other metabolic diseases were excluded from this study. All serum samples were frozen in liquid nitrogen and stored in the refrigerator at -80°C. The study was approved by the local Medical Ethics Committee.

Isolation and Culture of hBMSCs

The bone marrow samples were obtained from discarded femoral head tissues of people either with osteoporosis or not during total hip arthroplasty (THA). HBMSCs were cultured in α-Modified Eagle Medium (α-MEM; HyClone, Logan, UT, USA) containing 10% FBS-HI (fetal bovine serum-heat inactivation; Gibco, Grand Island, NY, USA), 100 U/mL penicillin (HyClone, Logan, UT, USA), and 100 μg/mL streptomycin (HyClone, Logan, UT, USA) in a 5% incubator at 37°C. The medium was replaced every day. For osteogenesis induction, the medium containing 1% FBS-HI (in avoidance of possible proliferation and differentiation of hBMSCs), 50 μg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 10 mM glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), and 0.1 μg/mL dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) was prepared. The osteogenic medium was replaced every 3 days.

Knockdown of XIXT and RUNX2

To silence XIXT and RUNX2 in hBMSCs, we constructed the pGLVH1 vectors containing sh-XIXT, sh-RUNX2, and sh-NC (GenePharma, Shanghai, China), which were then transfected into 293T cell lines to produce lentivirus prototype with suitable titer. After packaging, the above lentiviruses were transfected into hBMSCs, and 3 μg/mL puromycin (Invitrogen, Carlsbad, CA, USA) was added to select the cells with stable knockdown of XIXT and RUNX2. QRT-PCR was performed to verify the transfection efficiency. Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) was used for cell transfection.

Cell Transfection

HBMSCs with good growth condition were selected and uniformly inoculated into the 6-well plates with 1×10^4 cells in each well. After 75-85% confluence, the cells were transfected with miRNA-30a-5p mimic, negative control of mimic, miRNA-30a-5p inhibitor or inhibitor negative control (RiboBio Co., Guangzhou, China) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Briefly, the mimic/inhibitor was mixed with 500 mL Lipofectamine™ 2000, and then the mixture was added into each well with 1.5 mL of serum-free and antibiotic-free medium. After incubation at 37°C for 4-6 hours, the complete medium was replaced.

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

TRIzol (Invitrogen, Carlsbad, CA, USA), chloroform, and isopropanol were used to extract the total RNAs. After the concentration was determined by a micro nucleic acid quantifier, the RNA samples were stored at -80°C. The complementary deoxyribose nucleic acid (cDNA) was detected by the SYBR Green method. The PCR amplification was conducted as follows: Pre-denaturation at 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 s, 55°C or 30 s, and 72°C for 90 s.

Western Blot

The transfected cells were inoculated into 6-well plate containing 2.5 mL of medium and cultured for 72 hours before the collection. The cells were lysed on ice and centrifuged. The protein concentration was determined according to the operating instructions of the bicinchoninic acid (BCA) protein quantitative kit (Pierce, Rockford, IL, USA). After heating at 100°C to denature the protein, the samples were separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated in 5% skimmed milk to block the nonspecific antigen. After incubation with the primary and second antibodies, in turn, the protein bands were obtained by exposure.

Dual-Luciferase Reporter Gene Assay

To determine whether miRNA-30a-5p could directly target to XIXT, the 3′-Untranslated region (3′-UTR) and RUNX2 we constructed the pmirGLO luciferase vectors containing the wild-type XIXT (XIXT wt), mutant-type (XIXT mut), wild-type 3'-UTR RUNX2 (RUNX2 3'-UTR wt), and RUNX23'-UTR mut (Promega, Madison, WI, USA). HEK-293T cells were co-transfected with the above plasmids and miRNA-30a-5p mimic or mimic-NC using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). The Luciferase activity was continuously measured by the Dual-Luciferase Assay System (Promega, Madison, WI, USA).

Detection of ALP Activity

After Phosphate-Buffered Saline (PBS) washing, the ALP activity was determined by the release of p-nitrophenol from the ALP colorimetric assay kit (Abcam, Cambridge, UK). The absorbance at 405 nm was recorded.

Alizarin Red Staining (ARS)

The cells were immobilized in pre-cold 70% ethanol for 1 hour, washed with ddH_2O for 5 times, and stained with 40 mM alizarin red (Sigma-Aldrich, St. Louis, MO, USA) for 10-15 minutes. After staining, the orange-red spots indicated that the formation of calcified nodules was calculated.

Statistical Analysis

All data were presented as mean±SD (standard deviation) and plotted with GraphPad Prism 6.0 (La Jolla, CA, USA). The Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) was used to evaluate the differences between the groups by an independent sample *t*-test or Chi-square test. $p < 0.05$ was considered statistically significant.

Results

Expressions of XIXT and MiRNA-30a-5p in Osteoporosis Patients and hBMSCs

The serum levels of XIXT and miRNA-30a-5p in osteoporosis patients and normal controls were detected. The serum level of XIXT was significantly downregulated in osteoporosis patients (Figure 1A). On the contrary, miRNA-30a-5p was significantly upregulated in the serum of osteoporosis patients (Figure 1B). Extracted hBMSCs from femoral head tissue were used in this study. The expression levels of ALP, RUNX2, and lncRNA XIXT showed a remarkable increase with the prolongation of osteogenic differentiation (Figures 1C and 1E), while the level of miRNA-30a-5p gradually decreased (Figure 1F). In conclusion, XIXT and miRNA-30a-5p might be involved in the progression of osteoporosis.

Knockdown of XIXT Inhibits the Osteogenesis of hBMSCs

To explore the effect of XIXT on the osteogenesis of hBMSCs, sh-XIXT was transfected into hBMSCs to effectively downregulate XIXT (Figure 2A). The knockdown of XIXT inhibited the expressions of osteogenesis-related gene ALP (Figures 2B and 2C). Likewise, the silence of XIXT reduced the mRNA and protein expressions of RUNX2 (Figures 2D and 2E). The results of alizarin red staining revealed that the knockdown of XIXT attenuated the mineralization and nodulation in hBMSCs. These results suggested that the downregulation of XIXT inhibited the osteogenic differentiation of hBMSCs.

XIXT Targets to Regulate MiRNA-30a-5p

By online bioinformatics prediction, we identified the binding sites of miRNA-30a-5p in the promoter region of XIXT (Figure 3A). The Dual-Luciferase Reporter Gene Assay verified the binding relationship between XIXT and miRNA-30a-5p (Figure 3B). The knockdown of XIXT upregulated miRNA-30a-5p, while the over-expressed XIXT downregulated miRNA-30a-5p (Figure 3C). In conclusion, XIXT was targeted to miRNA-30a-5p in hBMSCs.

RUNX2 is the Direct Target of MiRNA-30a-5p

To determine the function of miRNA-30a-5p in the osteogenic differentiation of hBMSCs, we focused on RUNX2 gene, a well-known regulator of the osteogenic differentiation hBMSCs. TargetScan website predicted that RUNX2 might be a potential target of miRNA-30a-5p (Figure 4A). The Dual-Luciferase Reporter Gene Assay showed that miRNA-30a-5p directly bound to the 3'UTR of RUNX2 (Figure 4B). The knockdown of miRNA-30a-5p markedly enhanced the expression of RUNX2, while the overexpression of miRNA-30a-5p reduced the expression of RUNX2 (Figures 4C, 4D). In summary, our data indicated that RUNX2 was the downstream target of miRNA-30a-5p in hBMSCs.

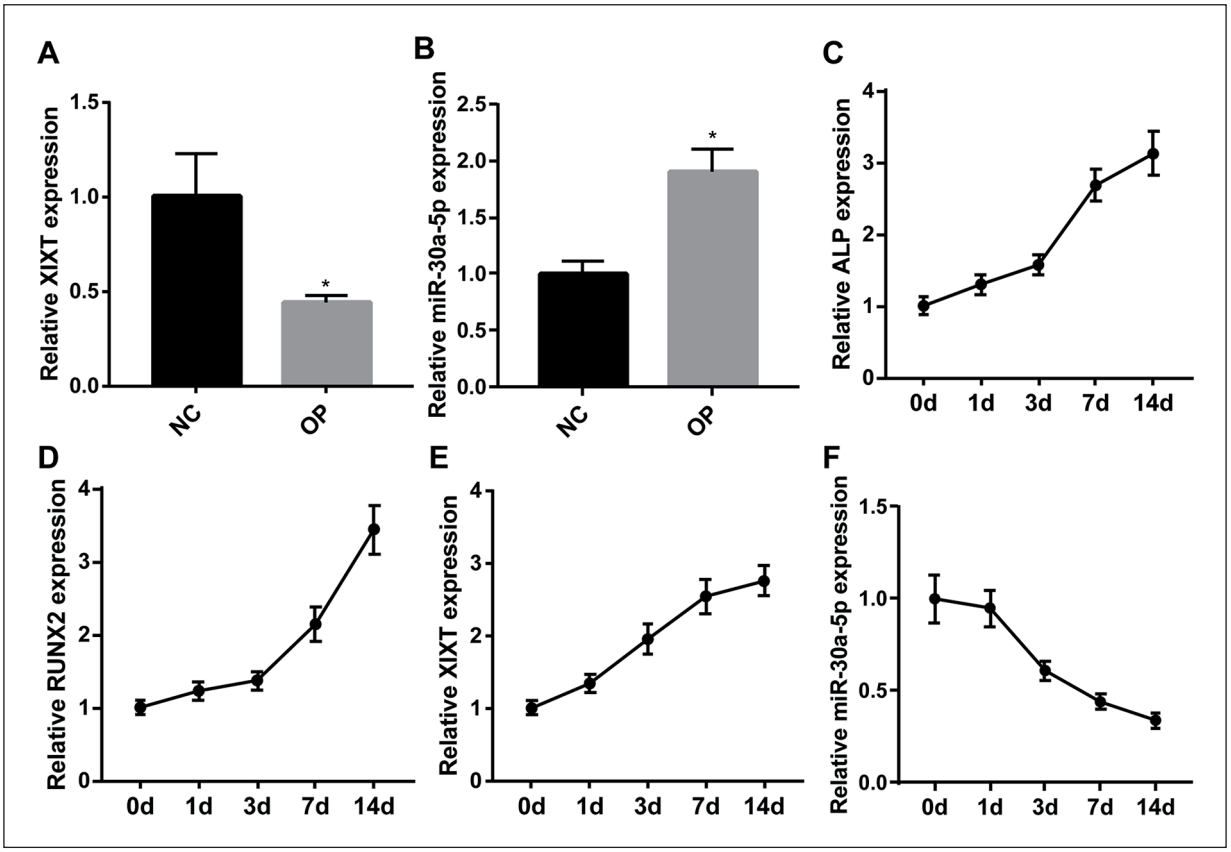

Figure 1. Expression of XIXT and miR-30a-5p in hBMSCs. **A,** qRT-PCR was used to detect the expression of XIXT in the serum of patients with osteoporosis. **B,** qRT-PCR was used to detect the expression of miR-30a-5p in the serum of the osteoporosis patients. ***C,*** qRT-PCR was used to measure the expression of ALP in hBMSCs during osteogenic differentiation. **D,** qRT-PCR was used to measure the expression of RUNX2 in hBMSCs during osteogenic differentiation. **E,** qRT-PCR was used to measure the expression of XIXT in hBMSCs during osteogenic differentiation. **F,** qRT-PCR was used for the expression of miR-30a-5p in hBMSCs during osteogenic differentiation.

Reduced MiRNA-30a-5p Partially Reverses the Inhibitory Effect of XIXT on the Osteogenesis of hBMSCs

Since XIXT inhibited miRNA-30a-5p level and miRNA-30a-5p directly targeted RUNX2 in hBMSCs, we further explored whether XIXT could upregulate the expression of RUNX2 by adsorbing miRNA-30a-5p. The results showed that downregulated XIXT significantly reduced the expression levels of ALP and RUNX2 in hBMSCs, while the co-transfection of miRNA-30a-5p inhibitor partially reversed the decrease in ALP and RUNX2 level (Figures 5A and 5B). ALP activity assay and alizarin red staining further showed that the inhibition of miRNA-30a-5p rescued the reduction of ALP activity and mineralization nodule formation in hBMSCs (Figures 5C and 5D).

Knockdown of RUNX2 Partially Reverses the Osteogenesis of hMSCs Reduced by MiRNA-30a-5p

The knockdown of miRNA-30a-5p significantly upregulated ALP and RUNX2, while the silence of RUNX2 reversed the elevation on ALP and RUNX2 (Figures 6A, 6B). Alizarin red staining and ALP activity assay showed that the inhibition of miRNA-30a-5p markedly attenuated ALP activity and decreased the mineralization formation ability in hBMSCs, while downregulated RUNX2 partially reversed the decrease in ALP activity and mineralization formation ability (Figures 6C and 6D). The above data indicated that the knockdown of RUNX2 could partially reverse the promotion of the osteogenic differentiation of hMSCs caused by miRNA-30a-5p inhibition.

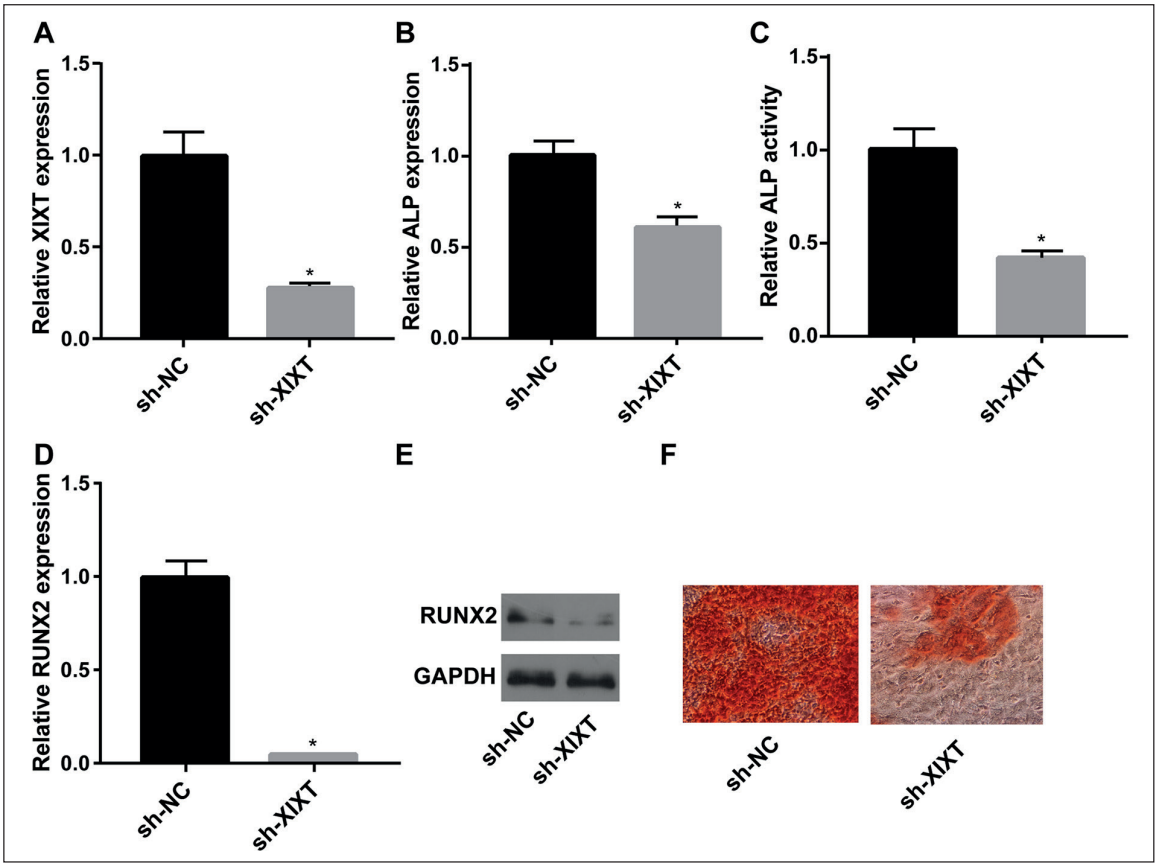

Figure 2. Decreased XIXT inhibits the osteogenic differentiation of hBMSCs. After XIXT was knocked down in hBMSCs, **A,** qRT-PCR was performed to detect the knockout efficiency of XIXT. **B,** qRT-PCR was performed to detect the expression of ALP. **C,** The activity of ALP was detected by ALP kit. **D,** qRT-PCR was performed to detect the expression of RUNX2. **E,** Western blot was performed to detect the expression of RUNX2 protein. **F,** The mineralization ability was measured by alizarin red S staining (× 200).

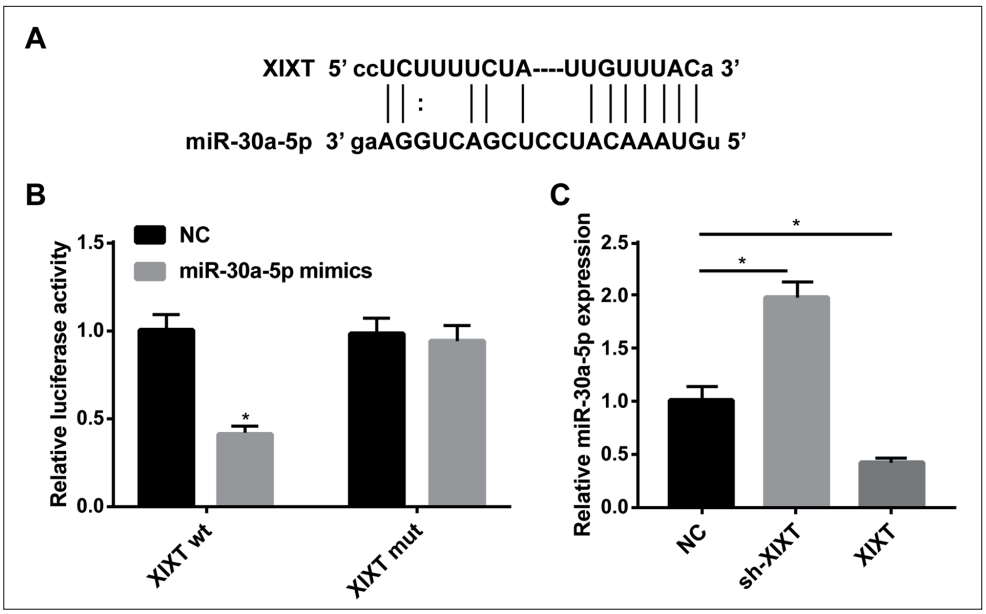

Figure 3. XIXT is targeted to miR-30a-5p. **A,** Bioinformatics analysis showed the binding relationship between XIXT and miR-30a-5p. **B,** Double luciferase reporter gene assays exhibited that miR-30a-5p could bind to the wild type XIXT. **C,** qRT-PCR detection showed that the expression of miR-30a-5p was significantly increased after XIXT knockout, while it decreased after XIXT overexpression.

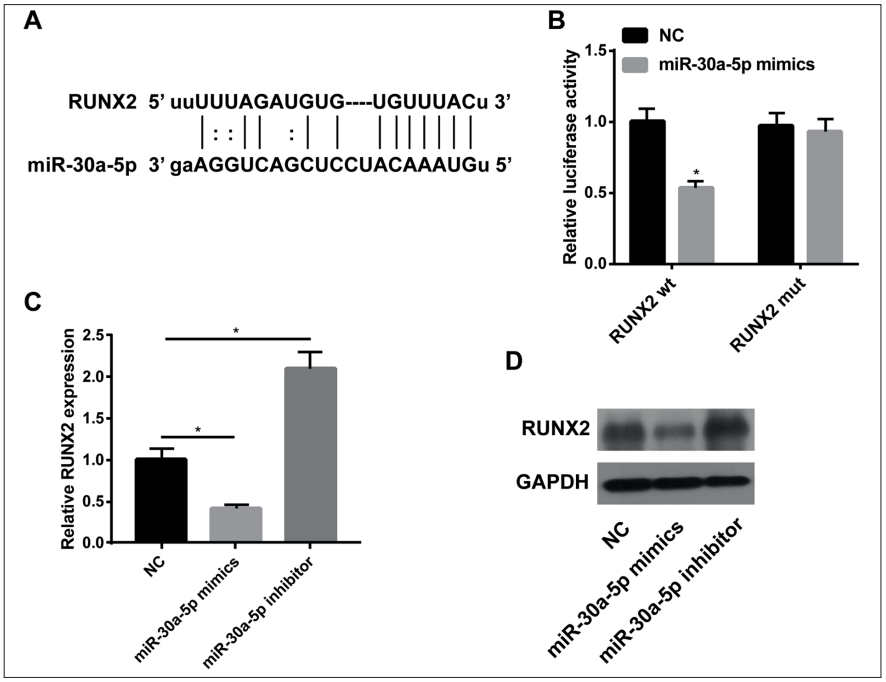

Figure 4. MiR-30a-5p is targeted to RUNX2. **A,** Bioinformatics analysis showed binding relationship between miR-30a-5p and RUNX2. **B,** Double Luciferase reporter gene assays exhibited that miR-30a-5p could bind to the wild type RUNX2. **C,** qRT-PCR detection showed that the expression of RUNX2 was significantly increased after miR-30a-5p knockout, while it decreased after miR-30a-5p overexpression. **D,** Western blot results also showed that the expression of RUNX2 was significantly increased after miR-30a-5p knockout, while it decreased after miR-30a-5p overexpression.

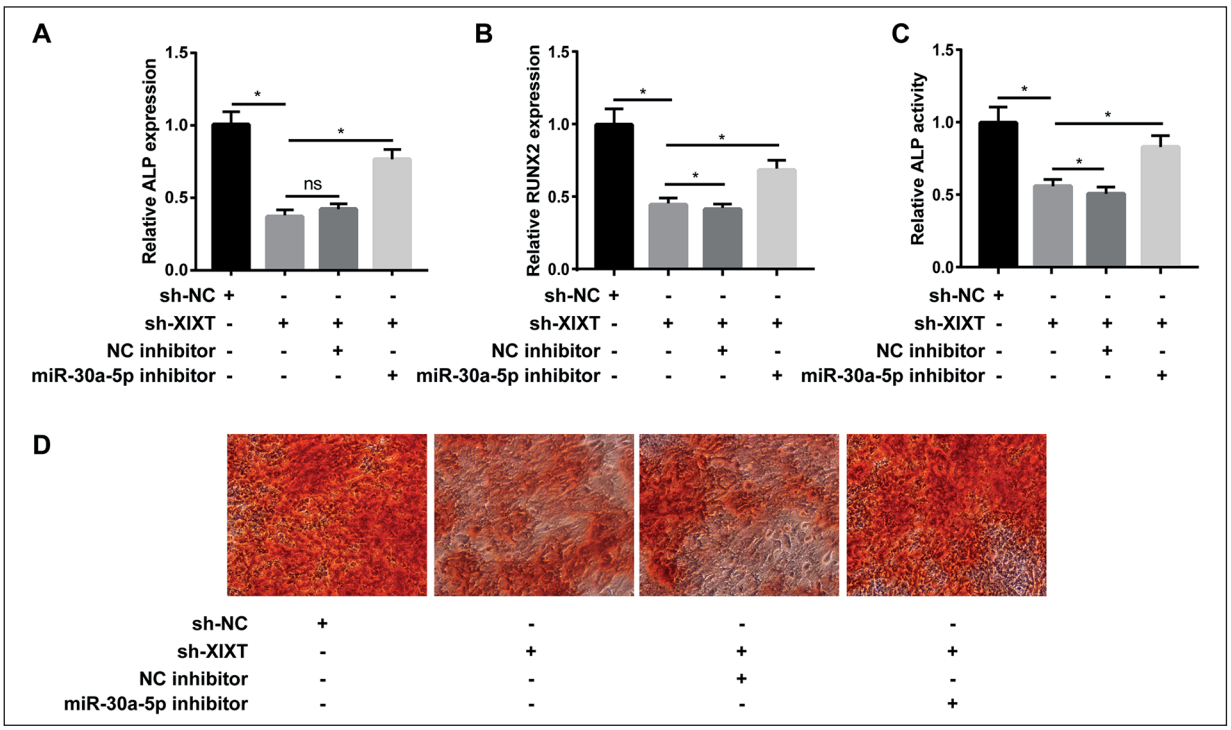

Figure 5. XIXT regulates the osteogenic differentiation of hBMSCs by inhibiting the expression of miR-30a-5p. **A,** qRT-PCR was used to detect the expression of ALP in hBMSCs with different treatments. **B,** qRT-PCR was used to detect the expression of RUNX2 in hBMSCs with different treatments. **C,** ALP assay kit was used to detect the expression of ALP activity in hBMSCs under different treatment conditions. **D,** Alizarin red S staining was used to detect the mineralization ability of hBMSCs under different treatment conditions (×200).

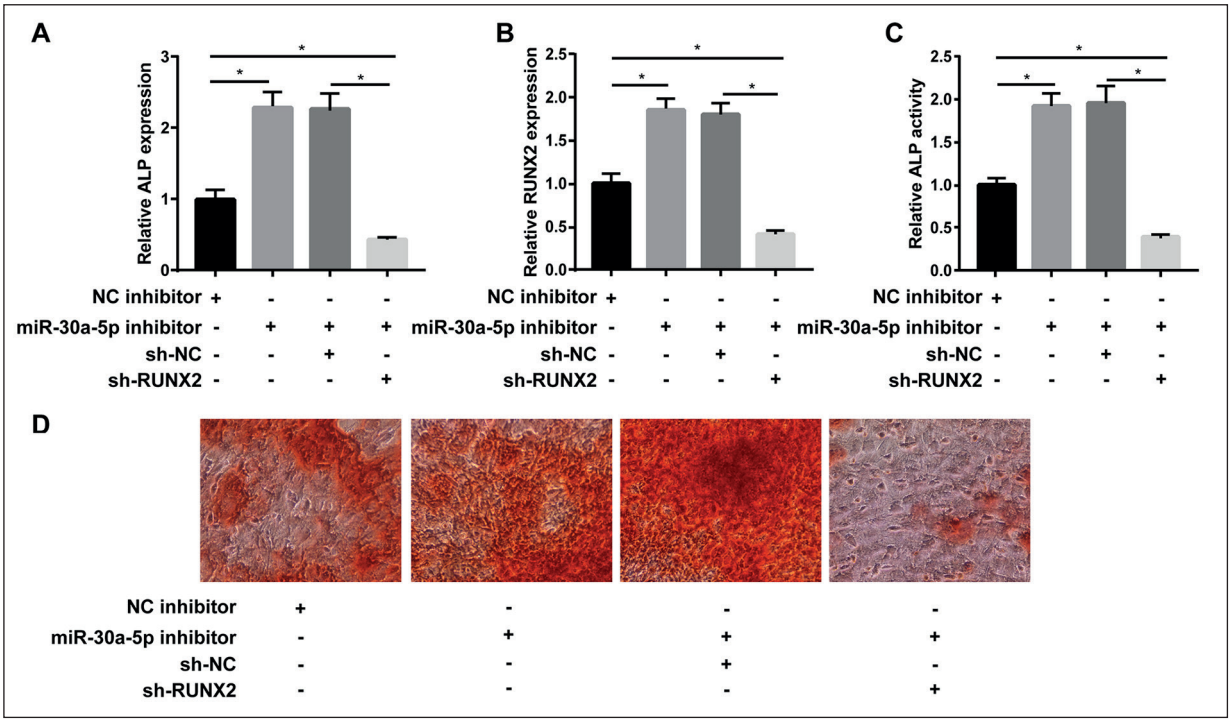

Figure 6. Knockdown of RUNX2 reversed the promotion of the osteogenic differentiation of hBMSCs after miR-30a-5p inhibition. **A,** Knockdown of miR-30a-5p reversed the ALP expression after RUNX2 inhibition. **B,** The expression of RUNX2P was reversed by knocking down RUNX2 and knocking down miR-30a-5p. **C,** Decreased RUNX2 reversed the ALP activity by knocking down miR-30a-5p. **D,** Decreased RUNX2 reversed the mineralization ability by knocking down miR-30a-5p (× 200).

Discussion

Osteoporosis is a systemic osteopathy characterized by bone density and quality decline, bone microstructure destruction, bone fragility, and fracture susceptibility[16]. Osteoporosis includes the primary and secondary types, and the former one is classified into postmenopausal (within post-menopause 5-10 years), senile (in the elderly over 70 years), and idiopathic osteoporosis (adolescents mainly involved)[17-20].

LncRNAs serve as miRNA sponges in regulating the skeletal diseases, and they are considered to provide new clinical therapeutic targets[21,22]. For example, the lncRNA NEAT1/miR-29b-3p/BMP1 axis is reported to promote the osteogenic differentiation in hBMSCs[23]. Although there are investigations supporting that lncRNAs are vital in osteoporosis, little is known about XIXT in hBMSCs. In this paper, we showed that the serum level of XIXT in osteoporosis patients was significantly lower than that of the population with normal BMD. Our results demonstrated that XIXT could target miRNA-30a-5p. The downregulation of XIXT exhibited a remarkable inhibition on the osteogenic differentiation of hBMSCs.

MiRNAs regulate the post-transcriptional gene expressions in plants and animals[24]. Each miRNA can have multiple target genes, and several miRNAs can also regulate the same gene[25]. It is presumed that one third of human genes are regulated by miRNAs[26,27]. Previous studies have been indicated that miRNA-30a-5p is involved in many types of malignant tumors. The downregulated miRNA-30a-5p significantly induces the invasiveness of oral cancer by upregulating FAP[28]. In addition, miRNA-30a-5p enhances the Paclitaxel-sensitivity of NSCLC by targeting Bcl-2[29]. MiRNA-30a-5p also inhibits the growth and metastasis of breast cancer by inhibiting LDHA-mediated Warburg effect. However, the function of miRNA-30a-5p in the osteogenic differentiation and osteoporosis of hBMSCs remains unclear[30]. Our study illustrated that the serum level of miRNA-30a-5p was upregulated in osteoporosis patients, which gradually decreased with the prolongation of osteogenesis induction. Besides, miRNA-30a-5p directly targeted RUNX2 and knockdown of miRNA-30a-5p and partially reversed the regulatory effect of XIXT on osteogenesis of hBMSCs.

RUNX2, located on chromatin 6p21, is the most critical transcription factor for regulating

the differentiation and maturation of hBMSCs into osteoblasts during bone development. RUNX2 expression is usually considered as a marker of osteoblast differentiation, and RUNX2 deficiency would result in the bone dysplasia or termination[31,32]. RUNX2 participates in the formation of many bone-related diseases[33,34]. MiR-221 is involved in the regulation of osteoporosis by regulating the RUNX2 and osteoblast differentiation[35]. Circ-VANGL1 promotes the progression of osteoporosis by regulating miR-217/RUNX2 axis[36]. MiR-338-3p regulates the osteogenesis of mouse BMSCs by targeting RUNX2 and FGFR2[37]. The study of the RUNX2 gene polymorphism also provides new ideas and therapeutic targets for early detection and treatment of diseases. Our data showed that RUNX2 was significantly upregulated with the prolongation of the osteogenic induction days, and downregulated XIXT attenuated the expression of RUNX2. Additionally, the knockdown of RUNX2 partially inhibited the promotion of miRNA-30a-5p on osteogenesis of hBMSCs.

Conclusions

We identified for the first time the effect of XIXT on the osteogenesis of hBMSCs. XIXT inhibited the expression of miRNA-30a-5p by competitive binding with miRNA-30a-5p and downregulated miRNA-30a-5p enhanced the expression of RUNX2 to induce the osteogenic differentiation of hBMSCs.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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