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Teriparatide alleviates osteoporosis by promoting osteogenic differentiation of hMSCs *via* miR-375/RUNX2 axis

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Abstract. – OBJECTIVE: To investigate whether Teriparatide (TP) contributed to the osteogenic differentiation of human marrow mesenchymal cells (hMSCs) through the regulation of miR-375, thereby alleviating osteoporosis (OP).

PATIENTS AND METHODS: The expression levels of miR-375 in the serum and hMSCs of patients with OP were determined by quantitative real time-polymerase chain reaction (qRT-PCR). hMSCs were extracted from bone marrows of OP patients and underwent osteogenic differentiation for 1 day, 3 days, 7 days and 10 days, respectively. The mRNA levels of alkaline phosphatase (ALP), osteocalcin (OCN) and runt-related transcription factor 2 (RUNX2) in TP-treated hMSCs transfected with miR-375 mimics or negative control were detected by qRT-PCR. Western blot was conducted to determine the protein expression of RUNX2 in TP-treated hMSCs transfected with miR-375 mimics or negative control. Besides, the osteogenic capacity and mineralization capacity of hMSCs were evaluated by the detection of ALP activity, ALP staining and Alizarin red staining, respectively. Dual-Luciferase reporter gene assay was performed to verify the binding between RUNX2 and miR-375. Subsequently, RUNX2 expression was detected in hMSCs transfected with miR-375 mimics or inhibitor. Rescue experiments were finally performed to determine whether miR-375 was involved in TP-induced osteogenic differentiation by targeting RUNX2.

RESULTS: MiR-375 remained at high level in serum of OP patients, while gradually decreased with the prolongation of osteogenic differentiation in isolated hMSCs. TP induction increased the osteogenic and mineralization capacities of hMSCs, which were inhibited after miR-375 overexpression. Through Dual-Luciferase reporter gene assay, we confirmed the binding relationship between miR-375 and RUNX2. Besides, both mRNA and protein levels of RUNX2 were negatively regulated by miR-375. Finally, we verified that co-overexpression of miR-375 and RUNX2 in TP-induced hMSCs significantly enhanced the mineralization capacity compared to overexpression of miR-375 alone.

CONCLUSIONS: Teriparatide promoted the osteogenic differentiation of hMSCs through miR-375/RUNX2 axis.

Key Words:
Teriparatide, RUNX2, hMSCs, Osteogenic differentiation.

Introduction

Osteoporosis (OP) is a systemic metabolic bone disease. It is characterized by degeneration of bone micro-structure (thinning and fracture of trabecular bones in cancellous bones, and cortical bone thinning), bone mass reduction and bone fragility enhancement. OP patients are prone to experience fracture due to the imbalance between the bone formation and bone resorption of osteoclasts[1,2]. OP has become the frequent disease in the elderly and severely influences their life qualities. Runt-related transcription factors[3] (RUNX) included RUNX1, RUNX2 and RUNX3 and contain a common structure in the DNA binding region with 128 amino acids, also known as Runt domain[4]. Among them, RUNX2 activated the differentiation of osteoblasts and initiated the development of bone marrow stromal stem cells to osteoblasts or chondrocytes[5]. It is generally considered that RUNX2 is the typical marker in the process of bone formation, which is of great significance in the prevention and treatment of OP[6]. MicroRNAs (miRNAs) are a class of regulatory non-coding RNAs of approximately 18-23 nucleotides in length and widely expressed in eukaryotes[7]. MiRNAs participate in a variety of physiological and pathological processes, such as

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cell differentiation and apoptosis, growth and development, glycolipid metabolism, inflammation, immune response, and tumorigenesis. MiR-375 is highly conserved and located between the CRYBA2 and CCDC108 genes on chromosome 2[8]. MiR-375 was first discovered in islet β cells and involved in islet formation and insulin secretion[9]. With the in-depth study of miRNA expression profiles, miR-375 was found to be widely presented in various tissues and organs and involved in the development of multiple malignant tumors[10-13]. However, the role of miR-375 in OP has not been reported. Teriparatide (TP) is a polypeptide drug developed by Eli Lilly and Company (Indianapolis, IN, USA). The amino acid sequence of TP is as follows: H-Ser1-Val2-Ser3-Glu4-Ile5-Gln6-Leu7-Met8-His9-Asn10-Leu11-Gly12-Lys13-His14-Leu15-Asn16-Ser17-Met18-Glu19-Arg20-Val21-Glu22-Trp23-Leu24-Arg25-Lys26-Lys27-Leu28-Gln29-Asp30-Val31-His32-Asn33-Phe34-OH. TP is a derivative of parathyroid hormone (PTH)[14]. In 2002, TP was approved by the FDA for the treatment of OP in men and postmenopausal women[15]. TP is capable of stimulating osteoblast activity and inhibiting osteoblast apoptosis, thereby promoting bone formation[16]. Currently, it is unclear whether RUNX2 and miRNAs are related to the pathogenic function of TP in alleviating OP. This study investigated the effects of TP on cellular alkaline phosphatase (ALP) activity and RUNX2 expression in hMSCs. A series of functional experiments were conducted to reveal the molecular mechanism of TP and microRNAs in alleviating OP. Our study provided new references for clinical prevention and treatment of OP.

Patients and Methods

Sample Collection

We enrolled 30 OP patients and 30 healthy subjects in this experiment and their clinical data were collected. Under a fasting state, 5 mL of venous blood was harvested from each subject in the morning and stood still for 30 min. Blood samples were then centrifuged at 4°C, 3000 g for 10 min. The supernatant was centrifuged again at 4°C, 13500 g for 15 min. Finally, the supernatant was preserved at -80°C. This study was approved by the Ethics Committee of Gansu Provincial Hospital of TCM. Signed written informed consents were obtained from all participants before the study.

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

Total RNA was extracted from the serum using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the reverse transcription kit (TaKaRa, Otsu, Shiga, Japan) at 37°C for 15 min, 85°C for 5 s and 4°C for 1 min. The reverse transcription system included 500 ng RNA, 2 μL of 5×Primerscrip RT Master Mix and was finally made up to 10 μL with ddH_2O. QRT-PCR was performed to determine the relative expression of ALP, osteocalcin (OCN) and RUNX2. Primer sequences used in this study were as follows: ALP, F: 5'-CCAGGAACCCCTCCTTACTC-3', R: 5'-GCTAGGATGTGTCCGAAGGA-3'; miR-375, F: 5'-TTCGGTGTAAACATCCTCGACTG-3', R: 5'-ATCCGGTGTCGTGGAGTCG-3'; OCN, F: 5'-CCAATGCGGCATTTGGACAA-3', R: 5'-TCGACGAATAGCGACAGTTCT-3'; RUNX2, F: 5'-ACATCGGTTGGACAA-3', R: 5'-CGTGCGAGCAGATAGCGACAGTTCT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Isolation and Culture of hMSCs

Bone marrows underwent Ficoll-Hapague density gradient centrifugation (specific gravity 1.077), washed twice with phosphate-buffered saline (PBS), and resuspended in α-MEM (minimum Eagle's medium) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 2% glutamine and 1% penicillin-streptomycin. hMSCs were seeded into a culture bottle, and fresh medium was replaced every other day. Cells were passaged using 0.05% trypsin-EDTA (Ethylene Diamine Tetraacetic Acid) (Thermo Fisher Scientific, Waltham, MA, USA) until the cell confluence reached 90%.

Osteogenic Differentiation of hMSCs

Sixth-passage hMSCs underwent osteogenic differentiation in α-MEM containing 10% HIFBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 300 ng/mL BMP-2. Medium was replaced twice a week. Cell passage was performed using 0.25% trypsin.

Transfection

hMSCs were seeded into a 24-well plate with 5×10^4 cells per well and cultured with antibiotic-free α-MEM. Cells were transfected with miR-375 mimics, miR-375 inhibitor or pcDNA-RUNX2 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). In brief, 0.8 μg transfection plasmid and 1 μL of Lipofectamine 2000 were respectively diluted in 50 μL α-MEM. After maintenance for 20 min, they were gently mixed and added in each well.

Dual-Luciferase Reporter Gene Assay

Cells were co-transfected with 20 nmol/L miR-375 mimics or negative control and 600 ng RUNX2 WT or RUNX2 MUT. After 24 hours transfection, cells were lysed and centrifuged at 10,000 g for 5 min. A total of 100 μL of suspension was taken for the determination of luciferase activity. Relative light units of Firefly (RLU-1) and Renilla (RLU-2) of target gene were recorded.

Western Blot

Total protein was extracted using the cell lysate for determining protein expression. Protein sample was quantified by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA), separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis, and blocked with 5% skim milk. Membranes were then incubated with the primary antibody and corresponding secondary antibody, successively. Band exposure was developed by chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA).

ALP Activity Determination

hMSCs at 7 days of osteogenic differentiation were incubated with 150 μL of 0.05% Triton-X (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for repeated freeze-thawing. Cells were then centrifuged at 4°C, 15000 rpm for 15 min. The supernatant was collected for the detection of ALP activity by the commercial kit.

Alizarin Red Staining

Alizarin red staining was performed in hMSCs at 21 days of osteogenic differentiation with additional incubation of 10^{-8} mmol/L Vitamin C and 10 ng/mL β-glycerophosphate. Cells were washed with PBS twice, fixed with 4% paraformaldehyde for 15 min and stained with 1% alizarin red staining for 15 min. Calcified nodules were observed and captured using an inverted microscope.

ALP Staining

hMSCs at 7 days of osteogenic differentiation were harvested for ALP staining. Briefly, incubation solution was added on the slides in the 6-well plate and incubated for 15 min at 37°C. ALP staining was performed for 5 min, followed by washing with running water. Images were observed and captured using an inverted microscope.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (IBM, Armonk, NY, USA) was utilized for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x} \pm s$). The *t*-test was used for comparing differences between the two groups. $p<0.05$ was considered statistically significant.

Results

Serum Level of MiR-375 Was Higher in OS Patients Than Controls

Through qRT-PCR determination, we found a higher serum level of miR-375 in OS patients than controls (n=30, Figure 1A). Expression level of miR-375 in hMSCs extracted from bone marrows of OP patients undergoing osteogenic differentiation for 1 d, 3 d 7 d and 10 d, respectively, was determined as well. MiR-375 expression in isolated hMSCs gradually decreased with the prolongation of osteogenic differentiation (Figure 1B). These results indicated a potential role of miR-375 in the occurrence and development of OP.

MiR-375 Was Involved in the TP-Induced Osteogenic Differentiation of hMSCs

Expression levels of osteogenic genes in TP-treated hMSCs with or without miR-375 overexpression were determined by qRT-PCR. TP treatment markedly upregulated the expression levels of ALP, OCN and RUNX2, which were then downregulated after miR-375 overexpression (Figure 2A). Identically, protein level of RUNX2 was also upregulated by TP treatment, but was inhibited after miR-375 was increased (Figure 2C). Furthermore, TP treatment enhanced ALP activity in hMSCs, which was further inhibited by miR-375 mimic transfection (Figure 2B). ALP and Alizarin red staining were carried out to evaluate the osteogenic and mineralization capacities of hMSCs. As the results indicated, ALP staining and Alizarin red staining

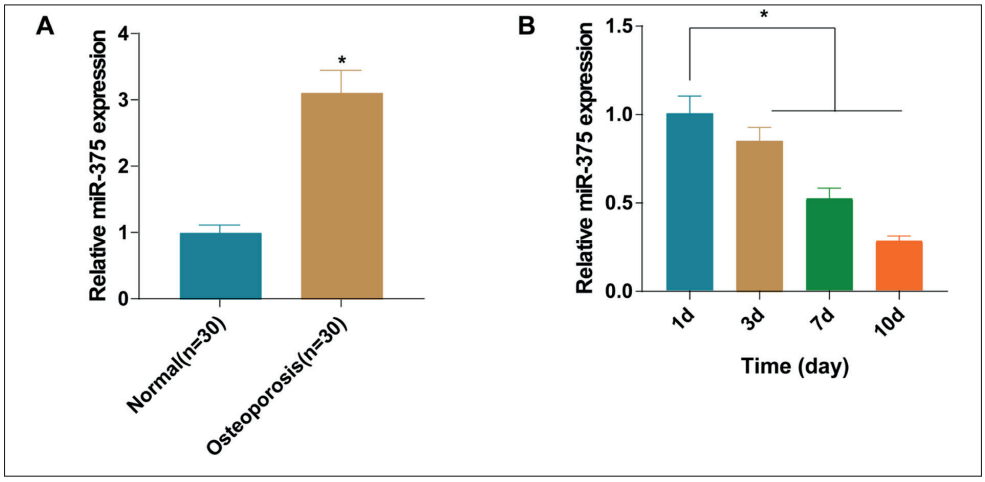

Figure 1. Expression level of miR-375 was higher in OS patients than in controls. **A,** Serum level of miR-375 was higher in OS patients than in controls (n=30). **B,** MiR-375 expression in hMSCs gradually decreased with the prolongation of osteogenic differentiation. *p<0.05.

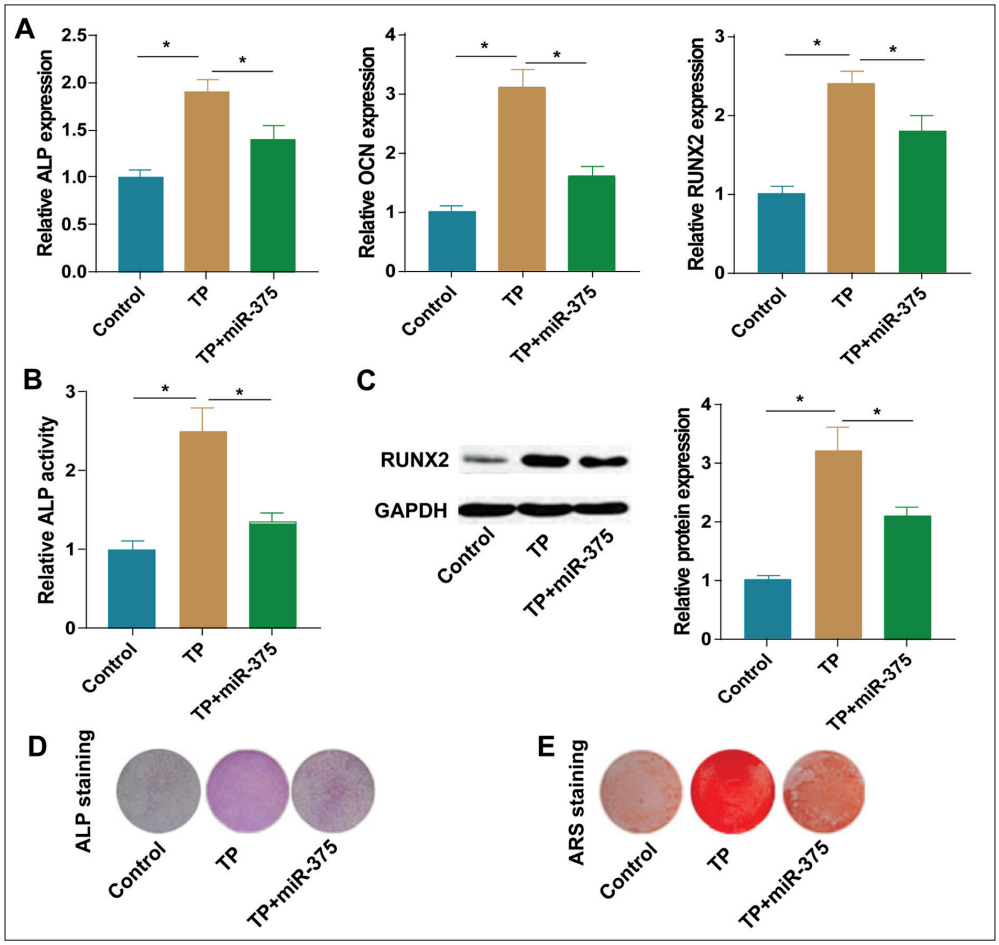

Figure 2. MiR-375 was involved in the TP-induced osteogenic differentiation of hMSCs. **A,** TP treatment markedly upregulated expression levels of ALP, OCN and RUNX2, which were downregulated after miR-375 overexpression. **B,** TP treatment enhanced ALP activity in hMSCs, whereas miR-375 overexpression inhibited ALP activity. **C,** Protein level of RUNX2 was upregulated by TP treatment, but was inhibited after miR-375 overexpression. **D,** ALP staining showed stronger ALP expression in TP-treated hMSCs, but was inhibited after miR-375 overexpression (magnification: 40×). **E,** Alizarin red staining showed stronger mineralization capacity of TP-treated hMSCs, but was inhibited after miR-375 overexpression. *p<0.05 (magnification: 40×).

were pronounced in TP-treated hMSCs. However, enhanced miR-375 significantly attenuated the osteogenic and mineralization capacities of hMSCs (Figure 2D and 2E).

MiR-375 Bound to RUNX2

To further verify the mechanism underlying miR-375 in the osteogenic differentiation of hMSCs, we predicted the targets of miR-375 using TargetScan. Potential binding site in 3'UTR of RUNX2 was observed. (Figure 3A). Subsquently, vectors containing RUNX2 WT and RUNX2 MUT sequences were constructed for detecting the luciferase activity. As Dual-Luciferase reporter gene assay elucidated, relative luciferase activity in hMSCs co-transfected with miR-375 mimics and RUNX2 WT remarkably decreased, whereas no obvious change in RUNX2 MUT group was observed (Figure 3B). We may conclude the binding condition between miR-375 and RUNX2. Moreover, both mRNA and protein levels of RUNX2 were negatively regulated by miR-375 (Figure 3C and 3D). All above results suggested that RUNX2 was the target gene of miR-375, and its expression was negatively regulated by miR-375.

TP Promoted Osteogenic Differentiation of hMSCs Through MiR-375 Targeting RUNX2

Rescue experiments were then performed to determine whether miR-375 was involved in TP-induced osteogenic differentiation by targeting RUNX2. Western blot results indicated that the protein level of RUNX2 in hMSCs co-overexpressing miR-375 and RUNX2 was higher than those in cells overexpressing miR-375 (Figure 4A). More importantly, compared to the hMSCs overexpressing miR-375 only, cells co-overexpressing miR-375 and RUNX2 presented stronger

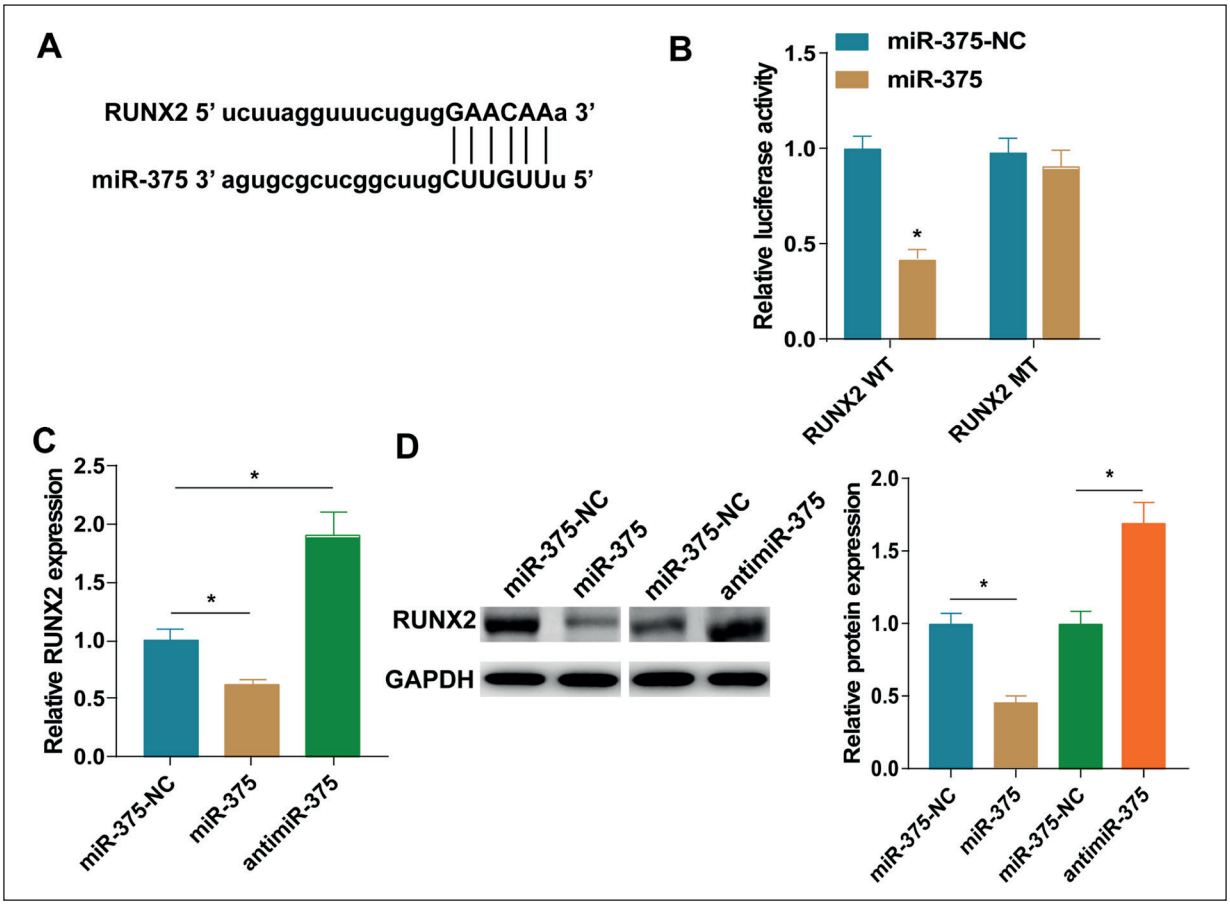

Figure 3. MiR-375 bound to RUNX2. **A,** The binding sites of miR-375 in RUNX2. **B,** Dual-Luciferase reporter gene assay showed that miR-375 overexpression quenched fluoresce of RUNX2 WT. **C,** The mRNA level of RUNX2 decreased by miR-375 overexpression, but increased by miR-375 knockdown. **D,** The protein level of RUNX2 decreased by miR-375 overexpression, but increased by miR-375 knockdown. *$p<0.05$.

ALP and Alizarin red staining, indicating higher osteogenic and mineralization capacities (Figure 4B). Therefore, we detected the involvement of miR-375 in the osteogenic differentiation of hMSCs through targeting RUNX2.

Discussion

OP is a common and frequently-occurring disease that seriously endangers the physical and mental health of the elderly. The incidence of OP increases due to the aging of population and ranks second only to cardiovascular and cerebrovascular diseases[17]. Fracture is the main complication of OP, mainly occurs in the vertebral body, bone marrow, and distal end of bones, and poses great inconvenience to daily life of affected patients. Effective anti-osteoporosis treatment can significantly reduce the occurrence of fractures and recurrent fracture in those who have already suffered from OP-induced fractures. Therefore, early prevention and treatment of OP are of important clinical and social significances. PTH is a major regulator of calcium balance in the body and currently utilized as a representative drug for

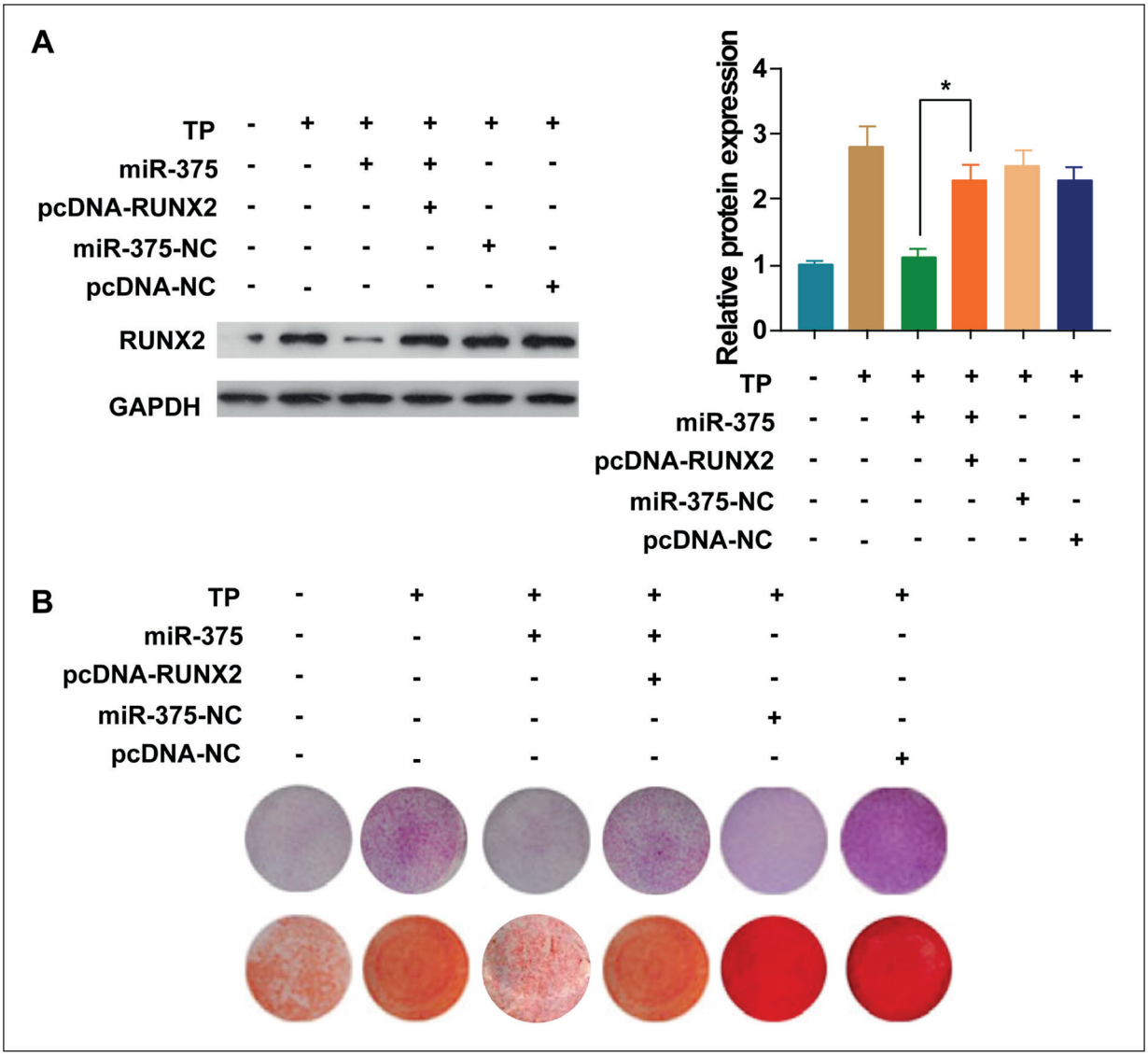

Figure 4. TP promoted osteogenic differentiation of hMSCs through miR-375 targeting RUNX2. **A,** Western blot results indicated a higher protein level of RUNX2 in hMSCs co-overexpressing miR-375 and RUNX2 than those only overexpressing miR-375. **B,** hMSCs co-overexpressing miR-375 and RUNX2 presented stronger ALP expression (upper panel) and mineralization capacity (bottom panel) than those only overexpressing miR-375. *$p<0.05$ (magnification: 40×).

promoting bone formation. TP is a recombinant protein form of PTH consisting of the first (N-terminus) 34 amino acids, which retained the biological activity of PTH. TP exerts the physiology of PTH on bone through binding to PTH1R. Importantly, no adverse effect of C-terminal peptide of TP on bone metabolism was reported before. TP has been approved by the FDA for the treatment of postmenopausal women, hypogonadism, and hormonal osteoporosis. Giannotti et al[18] reported a postmenopausal female patient with femoral fractures of nonunion after 7 months of operation. X-ray showed the appearance of bone bridge after 2 months of TP treatment, and the fracture completely healed at 3 months. However, the pathogenic mechanism of TP in the osteogenic differentiation of hMSCs remains unclear, which is specifically elucidated in this study.

MicroRNAs are a class of small RNAs that post-transcriptionally regulate gene expressions and affecting a variety of biological processes, including cell proliferation[19], differentiation[20], apoptosis[21] and development[22]. The discovery of microRNAs are valuable that provide novel therapeutic targets for OP[23]. Researches on the role of microRNAs in osteogenic differentiation have been advanced. Kahai et al[24] found that miR-378 upregulated nephronectin expression and accelerated the osteogenic differentiation through enhancing the activity of GalNT-7. Eskildsen et al[25] reported that miR-138 attenuated the osteogenic differentiation of hMSCs partially through inhibiting the focal adhesion kinase signaling pathway. Mizuno et al[26] pointed out that miR-125b participated in the directed differentiation of osteoblasts by regulating cell proliferation.

RUNX2 is also an important transcription factor that activates and initiates the differentiation from bone marrow stromal cells into osteoblasts. RUNX2 is mainly considered to have a role in the maturation of osteoblasts during bone development through enhancing various osteogenic genes. It is reported that RUNX2 exerted a crucial significance in the prevention and treatment of OP[27, 28]. Besides, a series of microRNAs have been identified to regulate the expression of RUNX2. Wang et al[29] showed that miR-204 inhibited the *in vitro* osteogenic differentiation by negatively regulating RUNX2 expression, thus further inhibiting expressions of ALP and osteocalcin produced by BMP-2. Huang et al[0] also found that miR-204 was an important endogenous attenuator, which inhibited osteogenesis and promoted the lipogenesis in mesenchymal progenitor cells and MSCs through negatively regulating RUNX2. In this study, miR-375 could bind to RUNX2 and affect the osteogenic differentiation of hMSCs. RUNX2 expression was negatively regulated by miR-375. Furthermore, we observed the involvement of miR-375 in the osteogenic differentiation of hMSCs through targeting RUNX2.

Conclusions

Teriparatide promoted osteogenic differentiation of hMSCs through miR-375 targeting RUNX2.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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