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LncRNA TERC alleviates the progression of osteoporosis by absorbing miRNA-217 to upregulate RUNX2

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Abstract. – OBJECTIVE: To elucidate the role of telomerase RNA elements (TERC) in alleviating osteoporosis (OP) by absorbing microRNA-217 (miRNAs-217) to regulate runt-related transcription factor 2 (RUNX2) level.

MATERIALS AND METHODS: The serum levels of TERC and miRNA-217 in OP patients and healthy controls were determined. During the osteogenic process, the relative levels of alkaline phosphatase (ALP), RUNX2, and Osterix were determined in hMSCs. The regulatory effects of TERC, miRNA-217, and RUNX2 on ALP and RUNX2 levels in hMSCs were examined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot. In addition, the changes in ALP activity and calcification ability in hMSCs influenced by them were assessed through ALP activity determination and alizarin red staining, respectively. The interaction of TERC/miRNA-217/RUNX2 regulatory loop and its role in influencing hMSCs osteogenesis were assessed by Dual-Luciferase Reporter Gene Assay and a series of rescue experiments, respectively.

RESULTS: The downregulated TERC and upregulated miRNA-217 were identified in the serum of the OP patients. Consistently, the downregulated TERC and upregulated miRNA-217 were discovered in in vitro osteogenic process of hMSCs. The silence of TERC, or RUNX2 downregulated ALP and RUNX2 levels, decreased ALP activity and attenuated the calcification ability in hMSCs. The overexpression of miRNA-217 gave similar results. The binding relationship in TERC/miRNA-217/RUNX2 regulatory loop was verified. At last, rescue experiments suggested that TERC accelerated hMSCs osteogenesis by absorbing miRNA-217 to upregulate RUNX2.

CONCLUSIONS: The serum level of TERC is lowly expressed in OP patients. TERC influences hMSCs osteogenesis by absorbing miRNA-217 to upregulate RUNX2, thus alleviating the progression of OP.

Key Words:
Osteoporosis, TERC, MiRNA-217, RUNX2.

Introduction

Osteoporosis (OP) is a common orthopaedic disease mainly affecting the elderly. The bone mass decline and the microstructure changes of bone tissues are the characteristic phenotypes of OP[1]. It is reported that the incidence of OP in fracture females is about 33%, which is 20% in male fracture patients[2]. In the past 30 years, the number of OP patients in China has increased threefold, affecting 90 million people[3]. Currently, basic drugs (Vitamin D and calcium) anti-bone resorption drugs and osteogenic drugs are the three major kinds of drugs applied for the treatment of OP. However, the former two drugs could not induce bone formation and their adverse effects are unavoidable. It is urgent to uncover the mechanism of osteogenesis, thus providing an effective approach to alleviate OP.

Telomerase is a special nuclear protein reverse transcriptase complex responsible for the resynthesis of telomeric DNA repeats, ensuring normal repair of telomeres and maintenance of telomere length (TL). Telomerase is composed of telomerase RNA elements (TERC), telomerase reverse transcriptase (TERT), and telomerase-related proteins[4]. In most tumor cells, telomerase is remarkably upregulated[5-7]. LncRNA TERC is a non-coding RNA that provides a template sequence for telomere synthesis, which is closely related to the

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maintenance of the telomerase activity[8]. It is reported that TERC gene detection can be utilized for the screening of cervical diseases[9]. However, the role of TERC in OP has been rarely reported.

Numerous studies have demonstrated the crucial role of non-coding RNAs in malignant progression and/or carcinogenesis[10]. MicroRNAs (miRNAs) are 22 nt non-coding RNAs, and they are involved in the tumorigenesis[11]. The interaction between lncRNAs and miRNAs has been identified[12]. MiRNA-217 is a vital oncogene involved in many types of malignancies by targeting EZH2, Cox-2, Mcl-1, or Fos[13].

Runt-related transcription factor 2 (RUNX2) acts as a specific transcription factor to regulate the osteogenesis of hMSCs[14]. The overexpression of RUNX2 is able to promote stem cell differentiation to osteoblasts and upregulate ECM proteins, thereafter, influencing bone formation and development[15]. As a result, RUNX2 may be important in influencing the progression of OP by regulating osteogenesis. Yang et al[16] pointed out that miR-203 inhibits trauma-induced ectopic osteogenesis by targeting RUNX2. In prostate cancer, miRNA-217 targets RUNX2 to further affect the disease progression[17]. In this paper, we mainly explored the role of TERC/miRNA-217/RUNX2 regulatory loop in hMSCs differentiation, thus alleviating the progression of OP.

Materials and Methods

Cell Culture and Osteogenesis

MC3T3-E1 cells and hMSCs were cultured in α-Minimum Eagle's Medium (α-MEM) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin. Osteogenesis was induced in α-MEM containing 10% FBS and 1% penicillin-streptomycin, 10 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate, and 50 μg/ml ascorbic acid. The medium was replaced every day. Osteoclast differentiation of RAW264.7 cells was conducted in medium containing 100 ng/mL RANKL for 6 d.

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

The total RNA in cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and subjected to reverse transcription. The extracted complementary deoxyribose nucleic acid (cDNA) was applied for PCR using the SYBR Green method (TaKaRa, Otsu, Shiga, Japan). The primer sequences were as follows: TERC: forward: 5′-CCGCCTTCCACCGTRCATTC-3′ and reverse: 5′-ACAGAGCCCAACTCTTCGC-3′; MiRNA-217: forward: 5′-GGCGAGGTGAGGGTGAGGCAGAGTC-3′ and reverse: 5′-AATGCGGTTTATTTATGGCGAGATTG-3′; UNX2: forward: 5′-GGGTAAGACTGGTCATAGGACC-3′ and reverse: 5′-CCCAGTATGAGAGTAGGTGTCC-3′; ALP: forward: 5′-ACCACCACGAGAGTGAACCA-3′ and reverse: 5′-CGTTGTCTGAGTACCAGTCCC-3′; Osterix: forward: 5′-CAGGCTATGCTAATGATTACC-3′ and reverse: 5′-GGCAGACAGTCAGAAGAG-3′; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward: 5′-TTCTTTTGCGTCGCCAGCCGA-3′ and reverse: 5′-GTCACCACCCGCCCAATACGA-3′.

Cell Transfection

Cell suspension (1×10^6 /mL) was inoculated in a 24-well plate with 100 μL per well. HiperFect solution and 50 nmol/L shRNA were dissolved in 100 μL of serum-free medium. At 6 h, 400 μL of the medium was applied. sh-NC: forward, 5′-CCGGTTTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCGGAGAATTTTTG-3′ and reverse, 5′-AATTCAAAAAGTTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCGGAGAA-3′; sh-TERC forward, 5′-CAACCTGGCAGATGCTATGAAAG-3′ and reverse, 5′-CAGGCTGGATGGAGTCTAGTGG-3′.

Alkaline Phosphatase (ALP) Activity Determination

The ALP activity in the cells was determined by releasing p-nitrophenol using an ALP colorimetric assay kit (Abcam, Cambridge, MA, USA). The absorbance at 405 nm was recorded.

Alizarin Red Staining

The cells were washed with Phosphate-Buffered Saline (PBS) twice, fixed in 4% paraformaldehyde for 10 min and stained with 2% alizarin red staining (pH 4.1) for 15 min. The calcification nodules were observed and captured using an inverted microscope (magnification 200×) (Leica, Wetzlar, Germany).

Dual-Luciferase Reporter Gene Assay

The cells were co-transfected with wild-type/mutant-type vectors and NC/miRNA-217 mimics. After transfection for 48 h, 50 μL of 1× PLB was added in each well to fully lyse the cells. Finally, the luciferase activity was determined.

Western Blot

The total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein sample was loaded for electrophoresis and transferred on a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). The membranes were blocked in 5% skim milk for 2 h and subjected to incubation with primary and secondary antibodies. The bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The intergroup differences were analyzed by the *t*-test. $p<0.05$ was considered as statistically significant.

Results

Serum Levels of TERC and MiRNA-217 in OP Patients

The serum level of TERC was downregulated in OP patients than those of the healthy controls, while the miRNA-217 level was upregulated (Figures 1A, 1B). Osteogenesis was induced in hMSCs for 0, 1, 3, 7, and 14 days, respectively. The mRNA levels of ALP, RUNX2, and Osterix were time-dependently upregulated in hMSCs, indicating the successful construction of *in vitro* osteogenesis model (Figure 1C). During the osteogenic progression of hMSCs, the TERC level was upregulated and the miRNA-217 level was downregulated (Figure 1D).

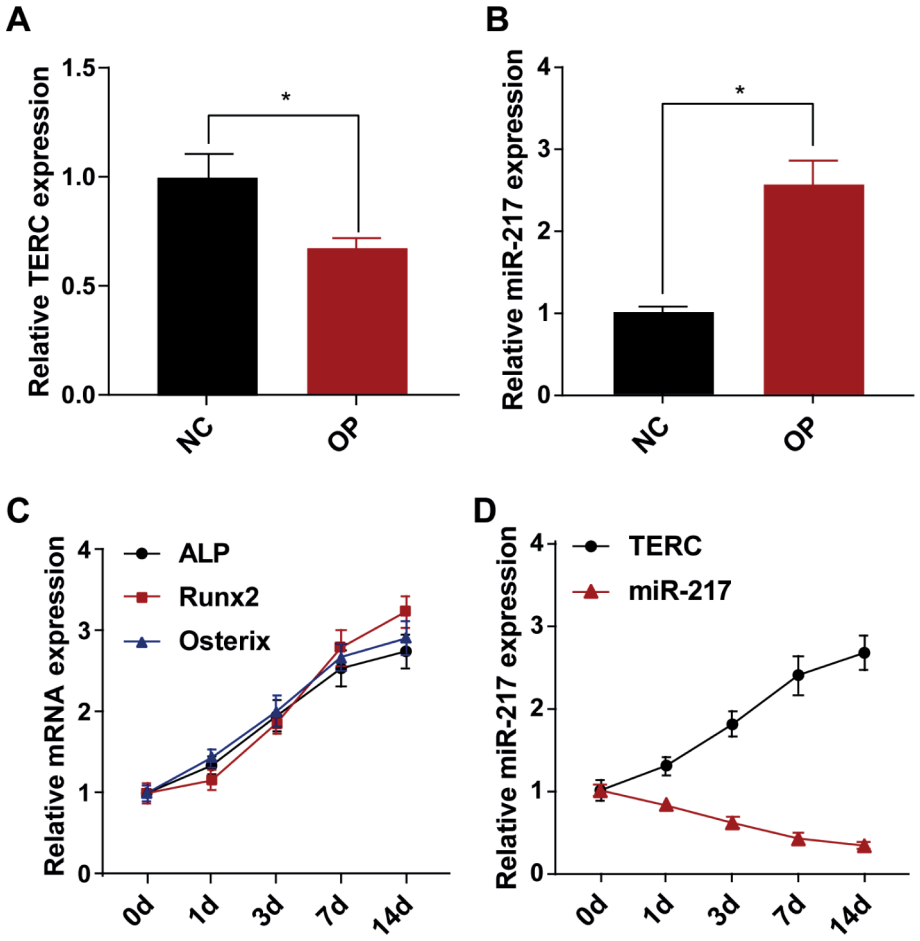

Figure 1. The serum levels of TERC and miR-217 in OP patients. **A,** The serum level of TERC in osteoporosis patients and healthy controls. **B,** The serum level of miR-217 in osteoporosis patients and healthy controls. **C,** The relative levels of ALP, RUNX2, and Osterix in hMSCs undergoing osteogenesis for 0, 1, 3, 7, and 14 days. **D,** The relative levels of TERC and miR-217 in hMSCs undergoing osteogenesis for 0, 1, 3, 7, and 14 days.

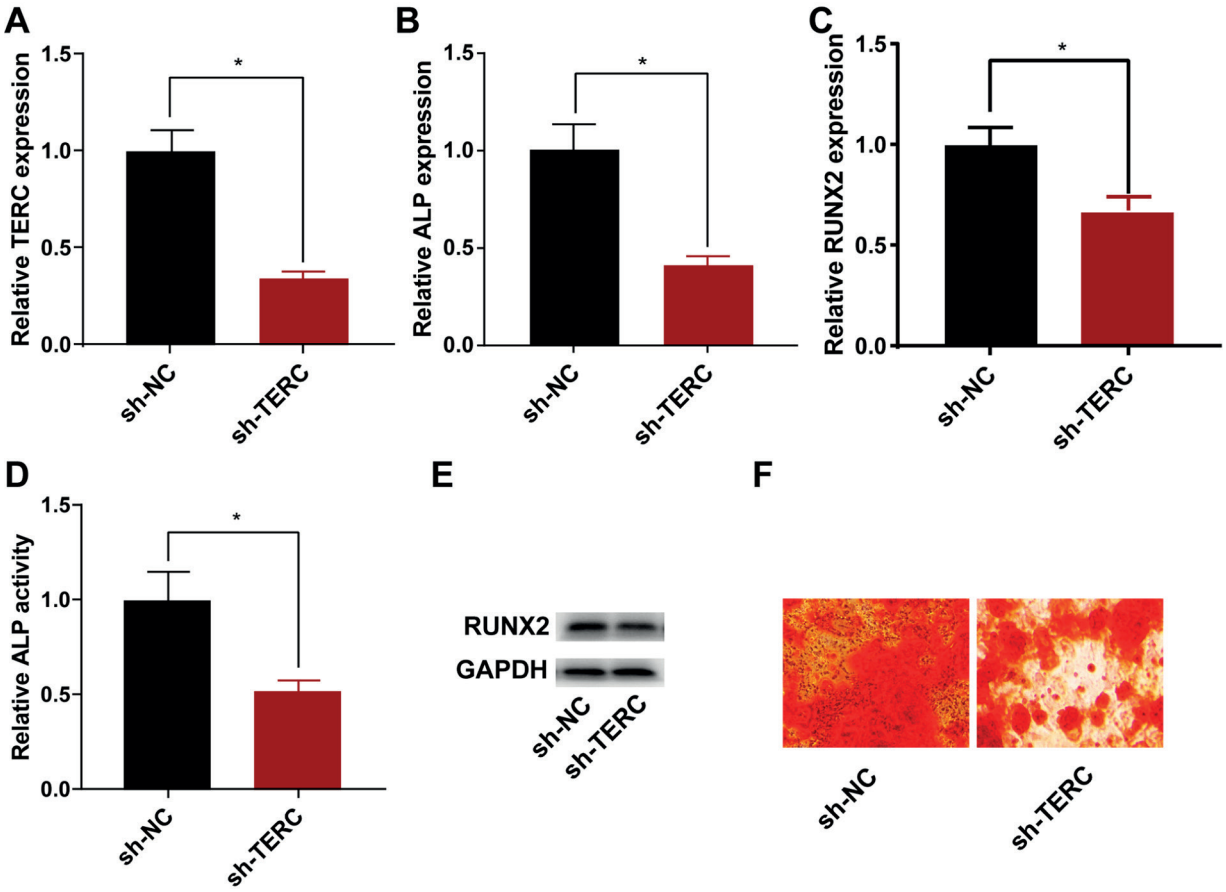

Figure 2. The silence of TERC inhibited hMSCs osteogenesis. **A,** The transfection efficacy of sh-TERC in hMSCs. **B,** The relative level of ALP in hMSCs transfected with sh-NC or sh-TERC. **C,** The relative level of RUNX2 in hMSCs transfected with sh-NC or sh-TERC. **D,** The ALP activity in hMSCs transfected with sh-NC or sh-TERC. **E,** The protein level of RUNX2 in hMSCs transfected with sh-NC or sh-TERC. **F,** The calcification nodules in hMSCs transfected with sh-NC or sh-TERC (magnification: 40×).

Silence of TERC Inhibited hMSCs Osteogenesis

To uncover the role of TERC during hMSCs osteogenesis, sh-TERC was constructed. The transfection of sh-TERC remarkably downregulated TERC level in hMSCs (Figure 2A). The knockdown of TERC decreased the mRNA level of ALP and ALP activity in hMSCs (Figures 2B, 2D). Moreover, both mRNA and the protein levels of RUNX2 were downregulated in hMSCs transfected with sh-TERC (Figures 2C, 2E). Alizarin red staining showed fewer calcification nodules in hMSCs with TERC knockdown compared to the controls, indicating attenuated osteogenesis (Figure 2F).

TERC Directly Bound to MiRNA-217

Through bioinformatics prediction, the binding sites in the promoter regions of TERC and miRNA-217 were discovered (Figure 3A). The luciferase activity was reduced after the co-transfection of TERC wt and miRNA-217 mimics (Figure 3B). In addition, the transfection of sh-TERC remarkably upregulated miRNA-217 level in hMSCs (Figure 3C). Collectively, TERC directly bound to miRNA-217and downregulated its level.

RUNX2 Was the Target of MiRNA-217

Similarly, the binding sites between miRNA-217 and RUNX2 were identified (Figure 4A). The decreased luciferase activity in hMSCs co-transfected with RUNX2 wt and miRNA-217 mimics verified the binding relationship between miRNA-217 and RUNX2 (Figure 4B). The transfection of miRNA-217 mimics downregulated mRNA and the protein levels

of RUNX2, and conversely, the transfection of miRNA-217 inhibitor upregulated RUNX2 (Figures 4C, 4D).

TERC Accelerated hMSCs Osteogenesis by Downregulating MiRNA-217

Both ALP level and ALP activity were reduced by the transfection of miRNA-217 inhibitor. Interestingly, the reduced ALP level and its activity in hMSCs transfected with sh-TERC were partially reversed by the co-transfection of miRNA-217 inhibitor (Figures 5A, 5C). The knockdown of TERC downregulated RUNX2 level, which was partially reversed by the silence of miRNA-217 (Figure 5B). Furthermore, the attenuated calcification ability of hMSCs transfected with sh-TERC was reversed after the co-transfection of miRNA-217 inhibitor (Figure 5D). As a result, it is believed that TERC accelerated hMSCs osteogenesis via downregulating miRNA-217.

Silence of RUNX2 Reversed the Regulatory Effect of MiRNA-217 on hMSCs Osteogenesis

It is shown that the transfection of miRNA-217 inhibitor could enhance ALP level and activity in hMSCs, which were found to be reduced after the co-transfection of sh-RUNX2 (Figures 6A, 6C). Similarly, the upregulated RUNX2 in hMSCs transfected with miRNA-217 inhibitor was greatly downregulated by the silence of RUNX2 (Figure 6B). The calcification ability was stimulated by the knockdown of miRNA-217, which was further attenuated by the co-transfection of sh-RUNX2 (Figure 6D). Therefore, a regulatory loop TERC/miRNA-217/RUNX2 was identified by influencing hMSCs osteogenesis.

Discussion

Bone development is the process of enhanced osteogenic differentiation and attenuated osteoclast differentiation. Once the balance breaks, it would lead to the occurrence of OP[18]. It is very significant to uncover the genes that are capable of regulating osteoblast and osteoclast activities. In recent years, OP-related miRNAs have been discovered and their functions are explored. LncRNAs have been well concerned in the progression of OP in recent years[19].

LncRNAs exert transcriptional and post-transcriptional regulations on the gene expressions. Dysregulated lncRNAs are closely related to various types of diseases[20-23]. During the osteogenic process, MIR31HG directly binds to IκBα and activates NF-κB, and the formed positive feedback loop suppresses osteogenesis[24]. Our findings

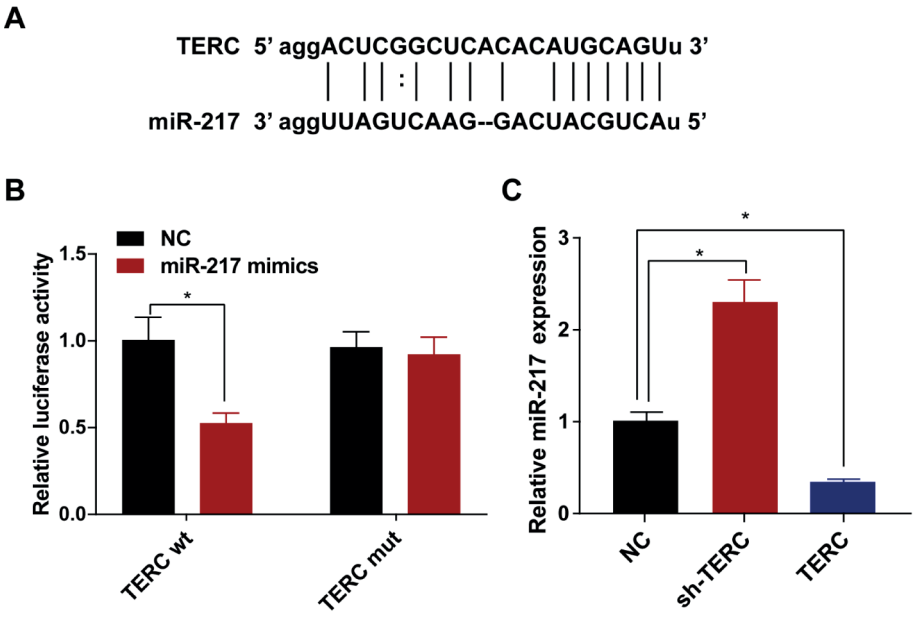

Figure 3. TERC directly bound to miR-217. **A,** The binding sites in the promoter regions of TERC and miR-217. **B,** The relative luciferase activity in hMSCs co-transfected with TERC wt/TERC mut and NC/miR-217 mimics. **C,** MiR-217 level in hMSCs with blank control, or transfected with sh-TERC or sh-NC.

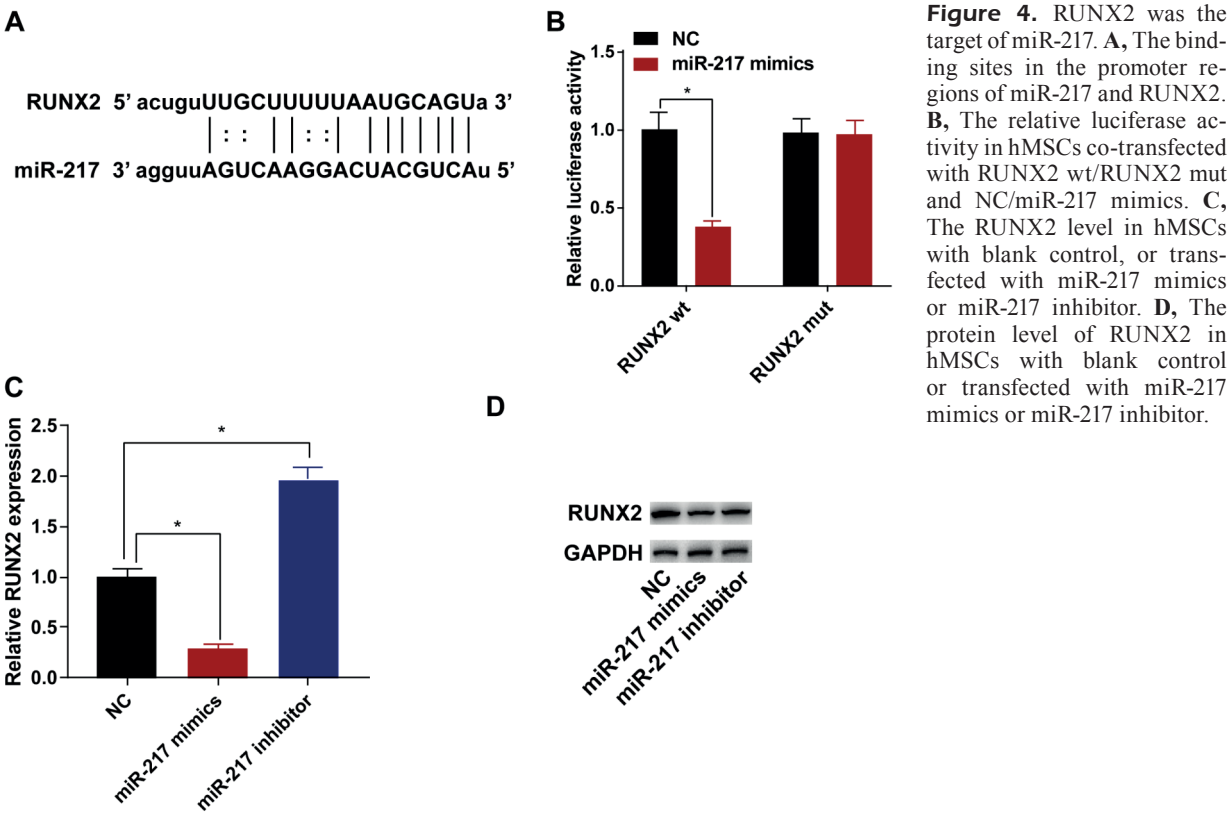

Figure 4. RUNX2 was the target of miR-217. **A,** The binding sites in the promoter regions of miR-217 and RUNX2. **B,** The relative luciferase activity in hMSCs co-transfected with RUNX2 wt/RUNX2 mut and NC/miR-217 mimics. **C,** The RUNX2 level in hMSCs with blank control, or transfected with miR-217 mimics or miR-217 inhibitor. **D,** The protein level of RUNX2 in hMSCs with blank control or transfected with miR-217 mimics or miR-217 inhibitor.

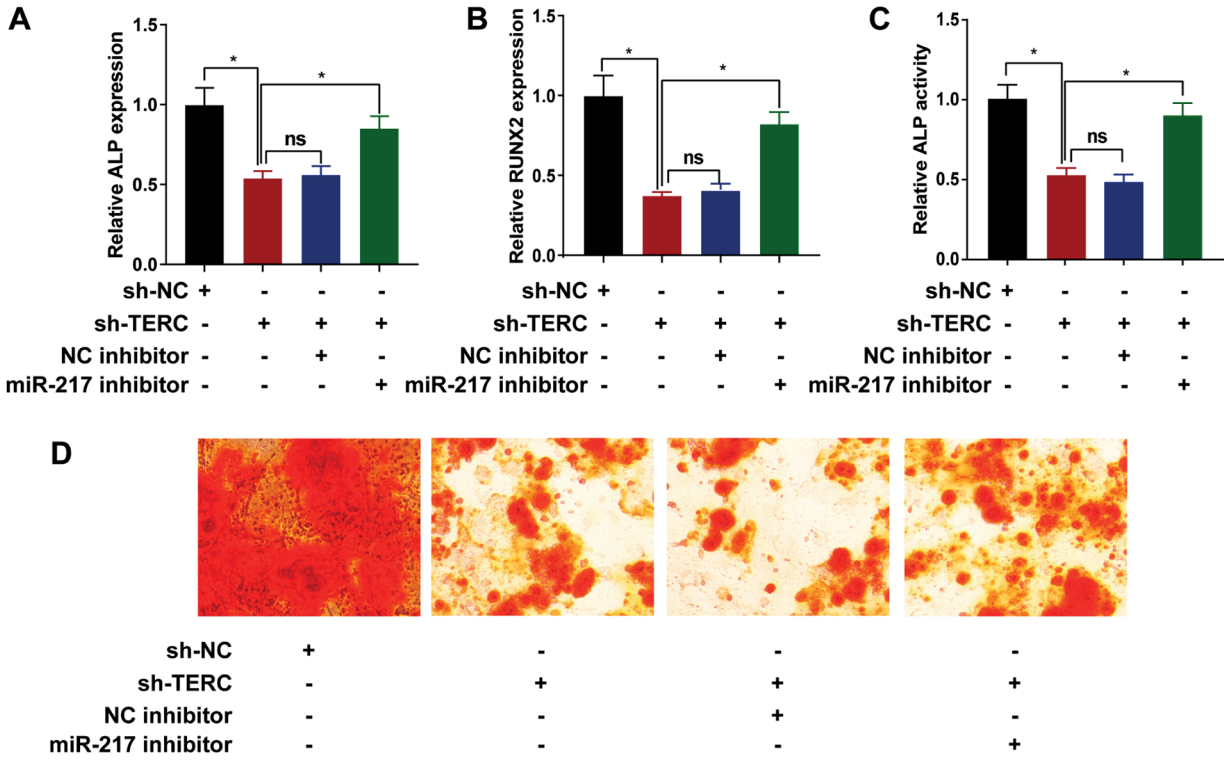

Figure 5. TERC accelerated hMSCs osteogenesis by downregulating miR-217. hMSCs were transfected with sh-NC, sh-TERC, sh-TERC + NC inhibitor or sh-TERC + miR-217 inhibitor, respectively. **A,** ALP level. **B,** RUNX2 level. **C,** ALP activity. **D,** Calcification nodules (magnification: 40×).

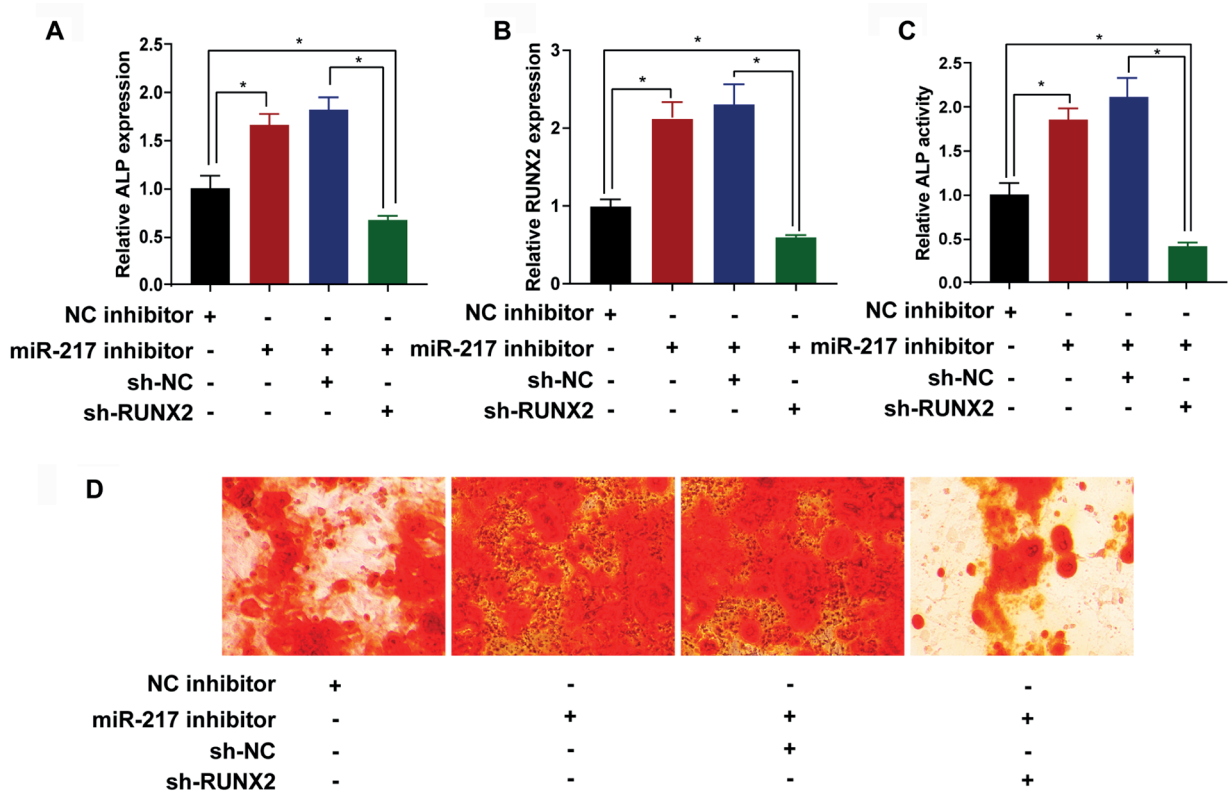

Figure 6. Silence of RUNX2 reversed the regulatory effect of miR-217 on hMSCs osteogenesis. hMSCs were transfected with NC inhibitor, miR-217 inhibitor, miR-217 inhibitor + sh-NC or miR-217 inhibitor + sh-RUNX2, respectively. **A,** ALP level. **B,** RUNX2 level. **C,** ALP activity. **D,** Calcification nodules (magnification: 40×)

uncovered the promotive effect of TERC on the osteogenesis of hMSCs.

MiRNA expressions have the characteristics of the tissue and developmental stage-specificity. Nearly one-third of human protein-coding genes could be regulated by miRNAs[25]. Bone metabolism could be regulated by certain miRNAs[26-28]. Hence, abnormally expressed miRNAs are believed to influence bone metabolism-related diseases, such as OP. The interaction between lncRNAs and miRNAs has been extensively investigated. On the one hand, lncRNA serves as a ceRNA to competitively bind to the target miRNA. On the other hand, miRNA suppresses lncRNA level through Ago2-mediated pathway[12]. For example, in bladder cancer, circRNA 000044 alleviates cancer progression by downregulating miRNA-217 to suppress RUNX2 level[17]. Serving as a ceRNA, lncRNA H19 stimulates osteogenesis by sponging miR-141 and miR-22, thus activating the Wnt pathway[29]. Consistently, our results clarified the TERC/miRNA-217/RUNX2 regulatory loop in alleviating OP, providing novel ideas for the clinical treatment of OP.

Conclusions

Briefly, the serum level of TERC is lowly expressed in OP patients. TERC influences hMSCs osteogenesis by absorbing miRNA-217 to upregulate RUNX2, thus alleviating the progression of OP.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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