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MicroRNA-449b-5p promotes the progression of osteoporosis by inhibiting osteogenic differentiation of BMSCs via targeting Satb2

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Abstract. – OBJECTIVE: We aimed to explore the role of microRNA-449b-5p in osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) and its mechanism of action.

MATERIALS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay was used to detect the expression levels of microRNA-449b-5p and osteogenic markers including RUNX2, OCN during BMSCs differentiation. The microRNA-449b-5p mimic and microRNA-449b-5p inhibitors were transfected into BMSCs to achieve microRNA-449b-5p overexpression and knockdown, then the expressions of osteogenic markers were detected by qRT-PCR. The ALP activity staining and the alizarin red staining were used to detect the activity of ALP and the mineralization ability of cells after overexpression and knockdown of microRNA-449b-5p. Binding sites for microRNA-449b-5p and Satb2 were predicted by TargetScan, the PicTar and microRNAanda programs, and confirmed by dual-luciferase reporter gene assay. The relationship between microRNA-449b-5p and Satb2 was analyzed by QRT-PCR and Western blot. The microRNA-449b-5p inhibitor and shSATB2 lentivirus were simultaneously transfected in BMSCs, and the expression levels of RUNX2, OCN and ALP were detected by qRT-PCR and ALP activity assays.

RESULTS: microRNA-449b-5p expression gradually decreased during osteogenic differentiation. Overexpression of microRNA-449b-5p inhibited BMSCs differentiation by down-regulating ALP activity, RUNX2, and OCN expression, while the opposite result was observed after knockdown of microRNA-449b-5p. MicroRNA-449b-5p can bind to the 3'UTR end of Satb2, which was involved in the osteogenic differentiation of microRNA-449b-5p-regulated BMSCs, and silencing of Satb2 can abolish the positive effect of the microRNA-449b-5p inhibitor on osteoblasts differentiation.

CONCLUSIONS: microRNA-449b-5p could aggravate osteoporosis by inhibiting osteogenic differentiation of BMSCs through targeting Satb2.

Key Words:
Osteoporosis, BMSCs, MicroRNA-449b-5p, Satb2.

Introduction

Osteoporosis is characterized by reduced bone mass and abnormal microstructure of bone tissue, resulting in increased bone fragility and a multi-gene metabolic bone disease that is prone to fracture[1]. In the study of osteoporosis, the most studied are bone marrow mesenchymal stem cells (BMSCs) and adipose stem cells, each with advantages and disadvantages, but BMSCs are easier to isolate and have more effective osteogenic differentiation[2]. Therefore, BMSCs play a crucial part in studying the pathophysiological role of bone anabolism in osteoporosis.

BMSCs, as common progenitor cells of osteoblasts and adipocytes[3], are important in bone physiology and are also partly involved in the pathophysiological process of osteoporosis[4]. BMSCs have the characteristics of wide source and good proliferation and differentiation ability, and are easy to obtain and culture, besides, they rarely have immune rejection and do not involve ethical issues, all of which makes them ideal carrier cells for gene therapy[5]. It has been shown that the imbalance of osteoblasts differentiation and adipogenic differentiation of BMSCs leads to a decrease in bone mass, which may be one of the

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causes of osteoporosis[6]. Therefore, understanding the differentiation regulation mechanism of BMSCs is very important for clarifying the pathogenesis of osteoporosis and discovering new disease treatment methods.

MicroRNAs are non-coding single-stranded RNA molecules of about 22 nucleotides in length. MicroRNAs are highly tissue-specific, conserved, and can be incompletely complementary to the target genes to perform post-transcriptional regulation[7]. It accounts for about 1% of the human genome, but regulates more than 30% of human gene expression. Choi et al[8] have indicated that, microRNA has important regulatory functions in cell proliferation, differentiation, apoptosis, secretion, migration, malignant tumors and other cellular functions, and are involved in the growth and development, disease occurrence, aging and death. Many studies have showed that microRNAs can play a regulatory role in osteogenic differentiation of BMSCs, such as microRNA-196a, microRNA-29b, microRNA-15b, microRNA-24 and microRNA-335-5p, which can promote osteogenic differentiation of BMSCs[9,10]. Besides, they can promote or inhibit the differentiation of bone precursor cells and osteoblasts by regulating key transcription factors and osteogenic markers on the osteogenic signaling pathway. For example, microRNA-133 and microRNA-135 inhibit stem cell differentiation into osteoblasts by inhibiting Runx2 and Smad-5, key transcription factors in osteogenic differentiation[11-13]. MicroRNA can affect the osteogenic differentiation process of BMSCs, so exploring the mechanism by which microRNA regulates osteoblasts differentiation of BMSCs can be used as one of the targets for the treatment of osteoporosis.

MicroRNA-449b-5p is a miRNA that has been shown to be essential in the development of tumors. Jeon et al[14] showed that microRNA-449a/b expression was down-regulated in lung cancer tissues and could induce growth arrest by targeting HDAC-1. Zhen et al[15] demonstrated that NEAT1 acts as a molecular sponge of microRNA-449b-5p and could lead to up-regulation of c-Met. Zheng et al[16] have also found that microRNA-449b-5p plays a vital part in skeletal muscle aging. However, its role in osteoporosis has not been reported so far.

Therefore, this study focused on the role of microRNA-449b-5p in osteogenic differentiation of BMSCs, and studied the mechanism of action to provide new potential targets for the treatment of osteoporosis.

Materials and Methods

Cell Culture and Osteogenic Differentiation

Primary rat BMSCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in a 5% CO_2 incubator at 37°C. The 3rd generation BMSCs with good growth of rats were plated into a six-well plate at a density of 30×10^4 /mL for osteogenic differentiation. The osteogenic induction group was prepared by adding 10% fetal bovine serum (FBS), 10 mmol/L β-glycerophosphate, 10 nmol/L dexamethasone, 50 μg/mL ascorbic acid, 1% penicillin-streptomycin in high glucose medium (DMEM-HG) containing 1% HEPES, and the cells were induced in the medium for a total of 7 d-14 d.

Cell and Lentivirus Transfection

MicroRNA-449b-5p mimic, microRNA-449b-5p inhibitor as well as their negative controls were obtained from GenePharma (Shanghai, China) and transfected into BMSCs using Lipofectamine 2000 (Sigma-Aldrich, St. Louis, MO, USA). The lentivirus of SATB2 shRNA and its corresponding control was obtained from GenePharma (Shanghai, China). Cells were infected with lentivirus and were harvested 48 hours after transfection.

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

Total RNA was extracted from the differently treated cells using the TRIzol method (Invitrogen, Carlsbad, CA, USA). The reverse transcription reaction was carried out according to the instructions of PrimeScript RT reagent Kit. The complementary deoxyribose nucleic acid (cDNA) obtained by reverse transcription was diluted to an appropriate multiple and then subjected to experiments according to the instructions of the real-time quantitative premix. The appropriate amount of cDNA, primers, premixed liquid and ultrapure water were mixed into a system of 20 tubes. After mixing and centrifuging, the cells were amplified by PCR on the ABI 7500 FAST real-time PCR instrument (Applied Biosystems, Foster City, CA, USA) according to the recommended parameters, and the fluorescence signals were collected and analyzed. The expression level of the gene of interest was calculated by the $2^{-\Delta\Delta CT}$ method based on the gene of interest relative to the reference gene. Primer sequences are detailed in Table I.

Table I. The primer sequences were as follows.

Target gene	Primer sequence
miR-449b-5p	F: 5′-GGGAGGCAGTGTATTGTTA-3′ R: 5′-CAGTGCGTGTCGTGGAGT-3′
U6	F:5’- CGCTTCGGCAGCACATATACTAAAATTGGAAC-3’ R: 5’- GCTTCACGAATTTGCGTGTCATCCTTGC-3’
RUNX2	F:5′-GGACGAGGCAAGAGTTTCA-3′ R:5′-TGGTGCAGAGTTCAGGGAG-3′
OCN	F:5′-GAGGGCAATAAGGTAGTGAA-3′ R:5′-CATAGATGCGTTTGTAGGC-3′
Satb2	F: 5'-CCTGGCCCT GGGGTATTCT-3' R: 5'-GTGCATCTGTCACATAACTGAGG-3'
GAPDH	F: 5′-TGCACCACCAACTGCTTAGC-3′ R: 5′-GGCATGCACTGTGGTCATGAG-3′

Western Blot

Total proteins of cell samples were extracted by radioimmunoprecipitation assay (RIPA) lysis (Beyotime, Shanghai, China). 20 μg of proteins in each group was separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was then blocked with 5% non-fat milk for 1 hour and incubated with primary rabbit monoclonal antibodies against STAB2 and β-actin diluted 1:500 and secondary goat anti-rabbit IgG antibody. Protein bands were detected by enhanced chemiluminescence (ECL) kit while protein levels were calculated relative to β-actin.

Alkaline Phosphatase (ALP) Activity

ALP activity was determined using an ALP kit from Beyotime (Shanghai, China). Cells were collected with lysis buffer from the kit. After centrifugation, the supernatant was collected for ALP activity detection by measuring the absorbance at 450 nm on a microplate reader (Bio-Tek, Biotek Winooski, VE, USA), then the total protein concentration was determined by bicinchoninic acid (BCA) assay protein assay kit (Pierce, Rockford, IL, USA), which was used for ALP activity normalization.

Alizarin Red Staining

BMSCs were inoculated into 24-well plates and applied for osteogenic induction for 14 days. The differentiated cells were washed three times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, and then stained with 2% alizarin red. After the staining, cells were observed and photographed under a microscope. To assess mineralization, the alizarin red dye was eluted with cetylpyridinium chloride for 1 hour, then the optical density (OD) value at 570 nm of the extracted alizarin red dye was measured.

Luciferase Reporting Assay

The luciferase pGL3 reporter gene containing the 3’-Untranslated Region (3’-UTR) region of human SATB2 was cloned to the Renilla Luciferase pRL-TK plasmid. BMSC cells were transfected with microRNA-449b-5p mimic and microRNA-NC as well as SATB2 luciferase plasmid using LiRfectamine® 2000 (Invitrogen, Carlsbad, CA, USA). 24 hours after transfection, cells were harvested and lysed. Luciferase activity was measured using a Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI, USA).

Statistical Analysis

Statistical evaluation was performed using Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA). Comparisons between the two groups were analyzed by Student’s *t*-test. Comparison between three groups was done using One-way ANOVA test followed by Post-Hoc Test LSD (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

MicroRNA-449b-5p was Down-Regulated During Osteoblasts Differentiation

To detect the expression of microRNA-449b-5p during osteoblasts differentiation, we per-

formed osteogenic induction in BMSCs and detected the expressions of osteogenic markers ALP, RUNX2 and OCN on days 1, 7 and 14 of induction, respectively. ALP activity assays showed increased ALP activity during osteoblasts differentiation (Figure 1A). The levels of osteogenesis-related genes RUNX2 and OCN increased with the number of osteogenic induction days (Figure 1B, 1C). At the same time, QRT-PCR showed that the level of microRNA-449b-5p gradually decreased with the increase of osteogenic induction days (Figure 1D). These results suggested that microRNA-449b-5p may be involved in the osteoblasts differentiation of BMSCs.

Effect of microRNA-449b-5p on Osteogenic Differentiation of BMSCs

To investigate the effect of microRNA-449b-5p on osteoblasts differentiation, we overexpressed and knocked down microRNA-449b-5p in BMSCs 14 days after osteoblast induction. QRT-PCR results showed that microRNA-449b-5p was increased in BMSCs after overexpression of microRNA-449b-5p. Conversely, the expression of microRNA-449b-5p in BMSCs was decreased after knockdown of microRNA-449b-5p (Figure 2A). After osteogenic induction, overexpression of microRNA-449b-5p reduced RUNX2 and OCN expression, while knockdown of mi-

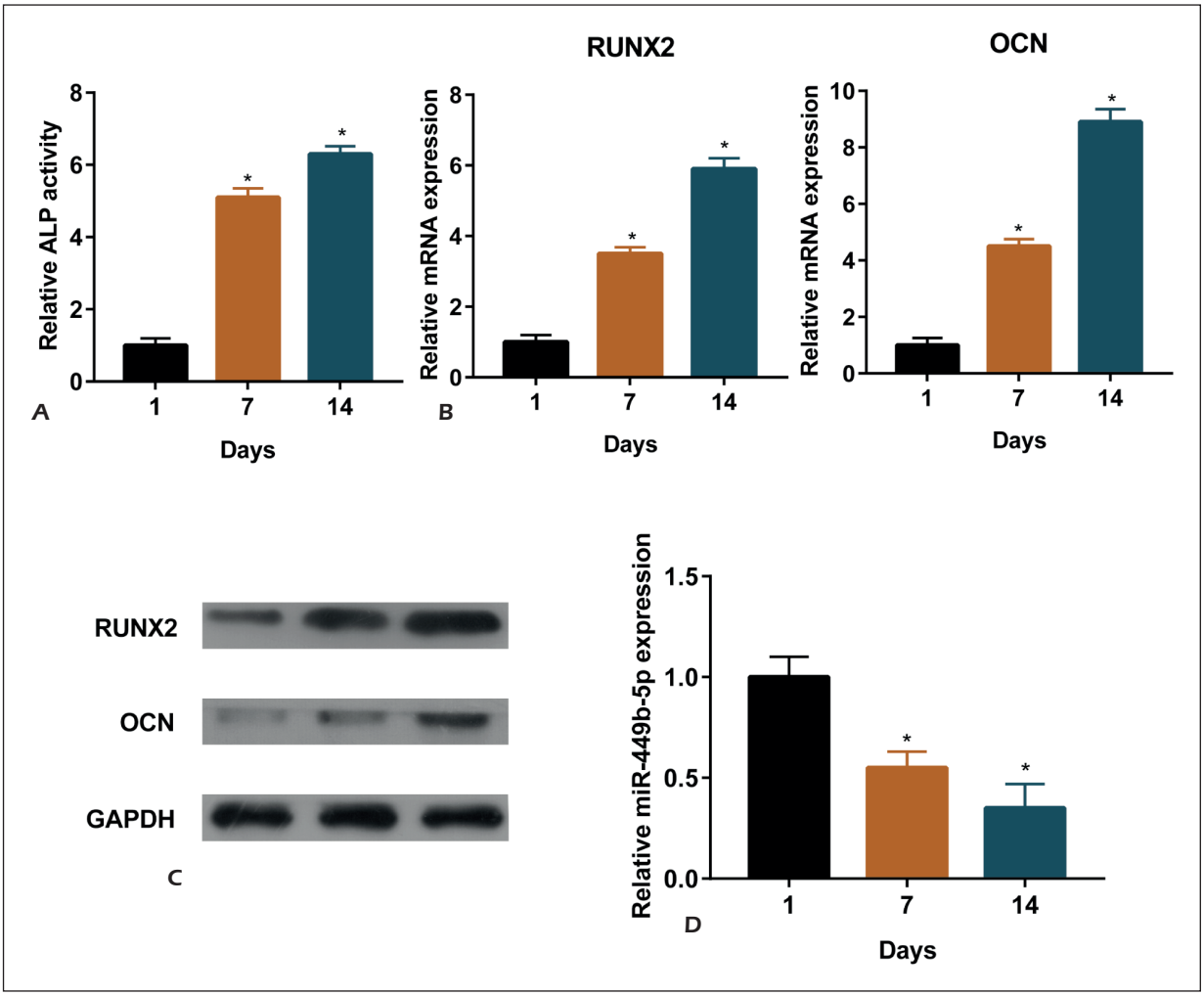

Figure 1. MiR-449b-5p is down-regulated in osteoblasts differentiation. **A**, ALP activity was measured on days 1, 7, and 14 of BMSCs osteogenic induction. **B**, qRT-PCR detected the expression levels of the osteogenic related genes such as RUNX2 and OCN during osteoblasts differentiation of BMSCs. **C**, Western blot analysis showed the expression levels of osteogenesis-related genes (RUNX2, OCN). With the increase of osteogenic induction days, the expression of RUNX2 and OCN increased gradually. **D**, qRT-PCR was used to detect the expression level of miR-449b-5p during osteoblasts differentiation of BMSCs. With the increase of osteogenic induction days, the expression of miR-449b-5p decreased gradually.

croRNA-449b-5p increased expression (Figure 2B). The results of ALP activity assay showed that microRNA-449b-5p overexpression reduced cell viability compared to the normal control group, while the opposite result was observed after knockdown of microRNA-449b-5p (Figure

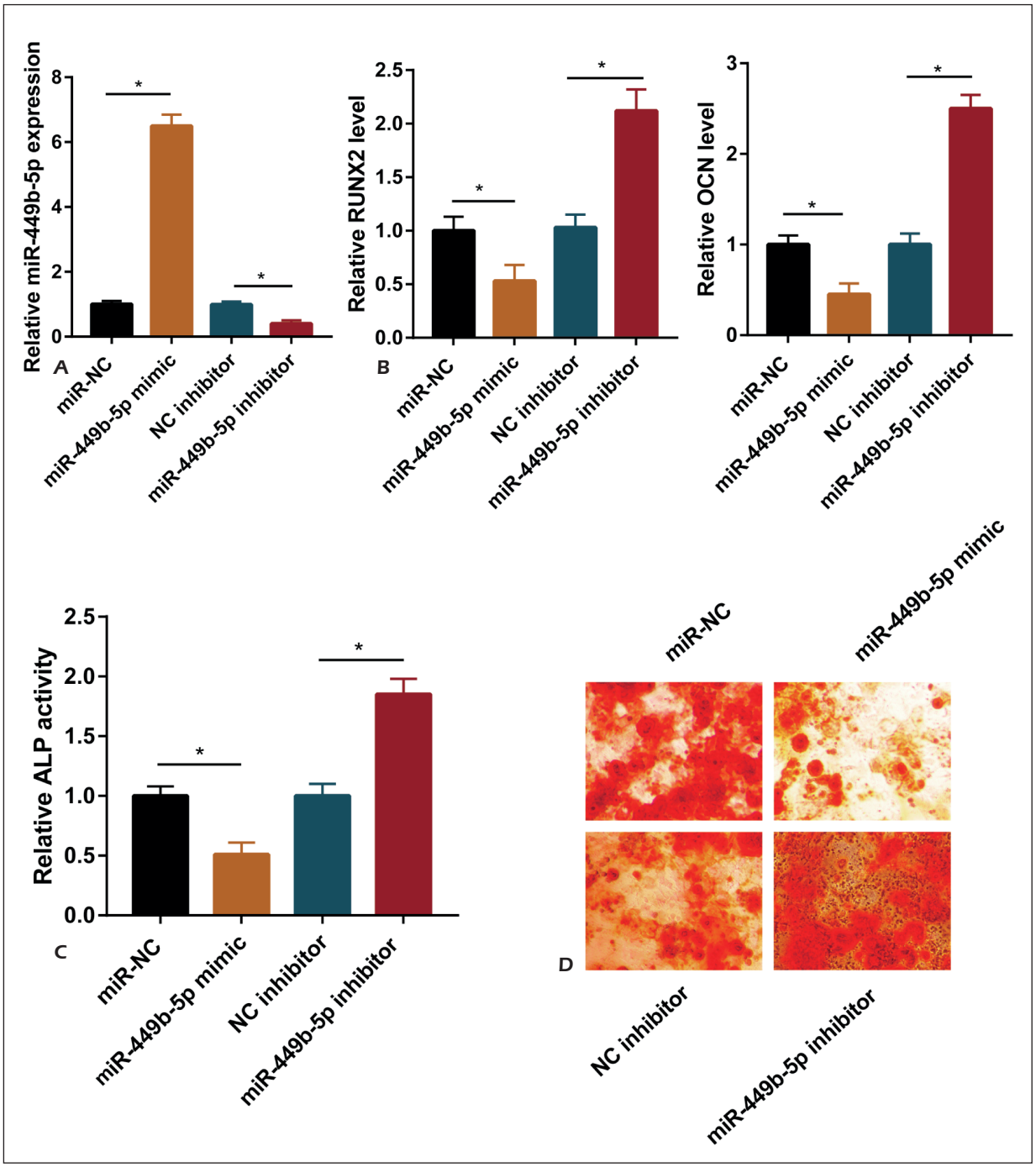

Figure 2. Effect of miR-449b-5p on osteogenic differentiation of bone marrow mesenchymal stem cells was explored. After 14 days of osteogenic induction, miR-449b-5p mimic, miR-449b-5p inhibitor and its negative control were transfected into BMSCs. **A**, qRT-PCR assay detected the expression level of miR-449b-5p in BMSCs after overexpression and knockdown of miR-449b-5p. **B**, ALP activity kit detected the level of ALP in BMSCs after overexpression and knockdown of miR-449b-5p. **C**, QRT-PCR detection revealed that overexpression of miR-449b-5p decreased RUNX2 and OCN mRNA expression, while knocking down miR-449b-5p increased that. **D**, Alizarin red staining indicated that compared with the control group, the mineralization ability of cells in the overexpressing miR-449b-5p group was weakened and was enhanced after knockdown of miR-449b-5p.

2C). In addition, alizarin red staining showed that microRNA-449b-5p overexpression inhibited the mineralization ability of cells compared with the control group, but enhanced the mineralization ability of cells after knocking down microRNA-449b-5p (Figure 2D). These results indicated that overexpression of microRNA-449b-5p could inhibit osteogenic differentiation of BMSCs.

Satb2 is a Direct Target of microRNA-449b-5p

We predicted the binding site of microRNA-449b-5p to Satb2 by TargetScan, PicTar and miRNAanda programs, and found a potential microRNA-449b-5p binding site at the 3'UTR end of Satb2 (Figure 3A). Next, in order to determine whether microRNA-449b-5p can regulate Satb2 expression by binding to the predicted target site in the 3'-UTR of its mRNA, the dual luciferase reporter results show that microRNA-449b-5p can inhibit the luciferase activity of wild-type Satb2, but had no effect on the luciferase activity of mutant Satb2 (Figure 3B). In addition, we determined the expression of Satb2 by over-expressing and knocking down microRNA-449b-5p. Results showed that Satb2 expression decreased after microRNA-449b-5p overexpression, while knocking of microRNA-449b-5p increased the expression of Satb2 (Figure 3C, 3D). The above results indicated that microRNA-449b-5p could bind directly to the 3'UTR terminus of Satb2 to negatively regulate the expression level of Satb2.

Satb2 is Involved in Osteoblasts Differentiation of BMSCs Regulated by microRNA-449b-5p

To verify whether microRNA-449b-5p is dependent on Satb2 during osteoblasts differentiation, we infected shSATB2 lentivirus or shNC lentivirus to stably express BMSCs expressing microRNA-449b-5p inhibitors or NC inhibitors. The level of Satb2 was detected by qRT-PCR, and knockdown of microRNA-449b-5p and Satb2 simultaneously reversed the increase in Satb2 caused by knockdown of microRNA-449b-5p alone (Figure 4A). ALP activity assay showed that transfection of shSATB2 alone inhibited ALP activity, while transfection of microRNA-449b-5p inhibitor and shSATB2 reversed the increase in ALP activity induced by transfection of microRNA-449b-5p inhibitor alone (Figure 4B). The levels of osteogenesis-related genes were further examined. QRT-PCR results showed that silencing of Satb2 expression abolished the increased expression of RUNX2 and OCN caused by transfection of microRNA-449b-5p inhibitor (Figure 4C). These results indicate that Satb2 is involved in the osteogenic differentiation of BMSCs induced by microRNA-449b-5p.

Discussion

With the advent of society, osteoporosis and its related complications have gradually attracted the attention of the society. However, due to the fact that the pathogenesis of osteoporosis is not completely clear, the current treatment for osteoporosis is not ideal. Therefore, further research on the formation mechanism of osteoporosis and the search for better treatment methods, reducing the incidence of fractures and improving the quality of life are the focus of current osteoporosis treatment research.

A large number of studies have showed that BMSCs have the ability to multi-directional differentiation, and can be transformed into osteoblasts after the corresponding induction stimulation[17]. MicroRNAs maintain the metabolic balance of bone by regulating the phenotypic differentiation of MSC and hematopoietic stem cells, ultimately affecting bone metabolic homeostasis and bone formation[18,19]. A large body of evidence indicates that microRNAs play an important regulatory part in osteogenic differentiation and bone growth and development. For example, Qu et al[20] reported that overexpression of microRNA-9 can cause osteoblasts differentiation and promote angiogenesis in MSCs by acting on the adenylate-activated protein kinase (AMPK) signaling pathway. Meng et al[21] observed that microRNA-21 promotes osteoblasts differentiation of hMSCs by acting on the PI3K-AKT-GSK3β signaling pathway. Huang et al[22] found that microRNA-125b also significantly promotes osteoblasts differentiation in hMSCs by targeting Cbf'b. In this study, we explored the role of microRNA-449b-5p in osteoblasts differentiation. We found that microRNA-449b-5p was down-regulated during osteogenic differentiation, furthermore, microRNA-449b-5p overexpression could inhibit the differentiation. Thus, microRNA-449b-5p can inhibit bone formation. Satb 2 is located in a gene deletion region of 2q32-q33 and was originally identified as kaia1034[23]. Is a recently cloned member of the special AT-rich binding protein family that binds to MAR and activates transcription[24,25]. It has been reported that Satb2 is up-regulated in newly formed osteoblasts in the mandibular defect site. Satb2 not only enhances osteoblasts differentiation by actively regulating the expression of multiple osteoblast-spe-

cific genes, but also promotes bone formation and participates in the formation of zygomatic arch by inhibiting several Hox genes including Hoxa2[26]. Satb2 has been reported to play a crucial part in the regulation of skeletal development and osteoblasts differentiation in transcriptional networks[27,28]. Wei et al[29] studied that the microRNA-34 family can inhibit osteogenic differentiation by inhibiting the nuclear matrix protein Satb2 to inhibit the terminal differentiation of osteoblasts. Hu et al[30] showed that microRNA-205 can negatively regulate osteogenic differentiation by affecting ERK phosphorylation and regulating SATB2 expression. Tang et al[31] found that microRNA-383 inhibits osteoblasts differentiation by binding to the 3'-UTR of Satb2 mRNA. In the present study, we also demonstrated that microRNA-449b-5p binds to the 3'-UTR of Satb2 to regulate osteogenic differentiation. Inhibition of Satb2 significantly inhibited the activity of ALP, inhibited the expression of RUNX2 and OCN, and knockdown of Satb2 abolished the role of microRNA-449b-5p inhibitor. These evidences suggest that microRNA-449b-5p regulates osteogenic differentiation of BMSCs by targeted binding to Satb2.

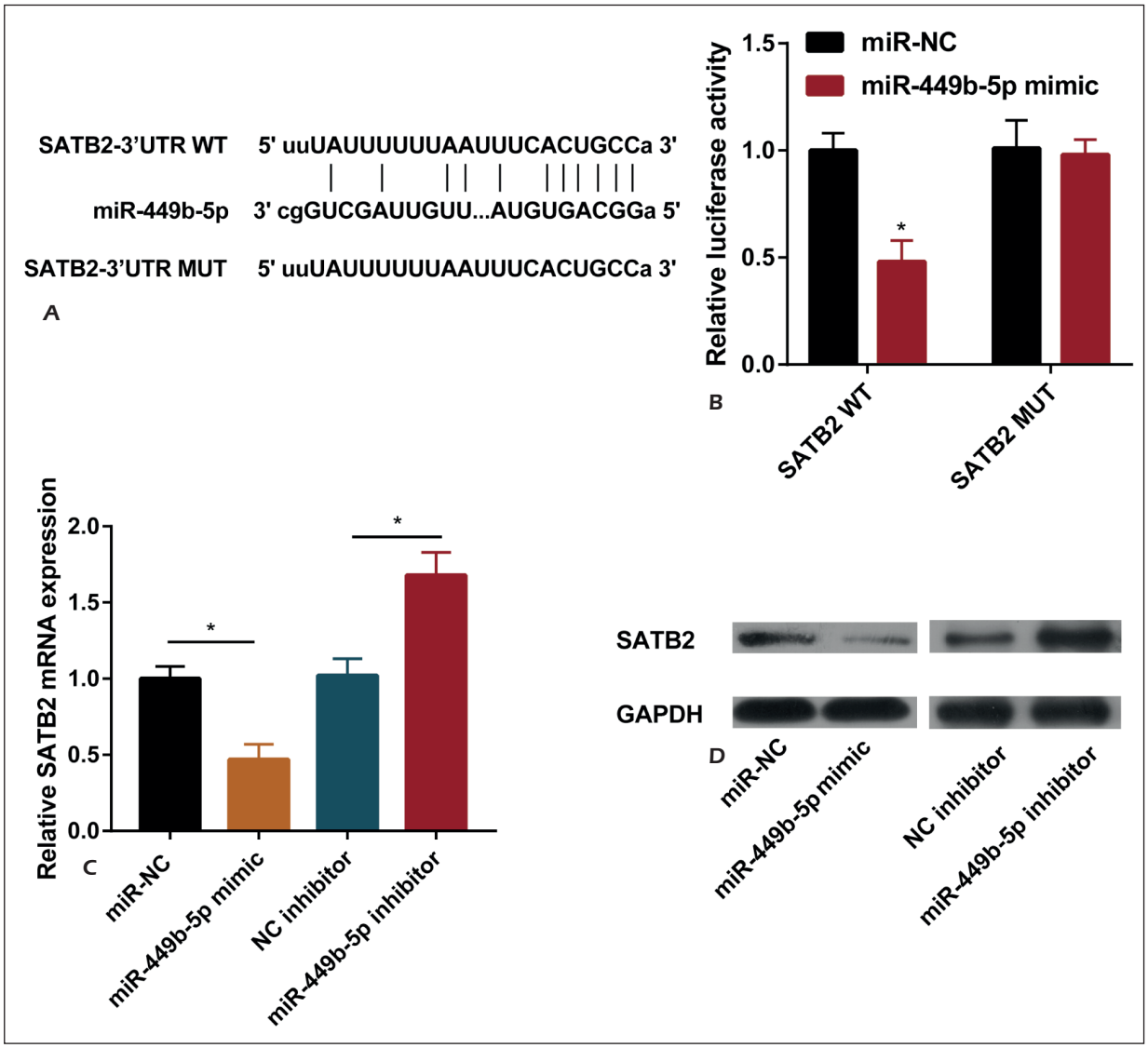

Figure 3. Satb2 is a direct target of miR-449b-5p. **A**, The binding site of miR-449b-5p to Satb2 was predicted by TargetScan, PicTar and miRanda programs, and miR-449b-5p can bind to the 3'UTR end of Satb2. **B**, Dual luciferase reporter assay confirmed that miR-449b-5p inhibited the luciferase activity of the Satb2 wild-type reporter gene. **C**, After transfection of miR-449b-5p mimic and miR-449b-5p inhibitor into BMSCs, qRT-PCR revealed that the expression level of Satb2 was negatively correlated with miR-449b-5p. **D**, Western blot analysis was used to determine the expression level of Satb2 protein in BMSCs that were transfected with miR-449b-5p mimic and miR-449b-5p inhibitor.

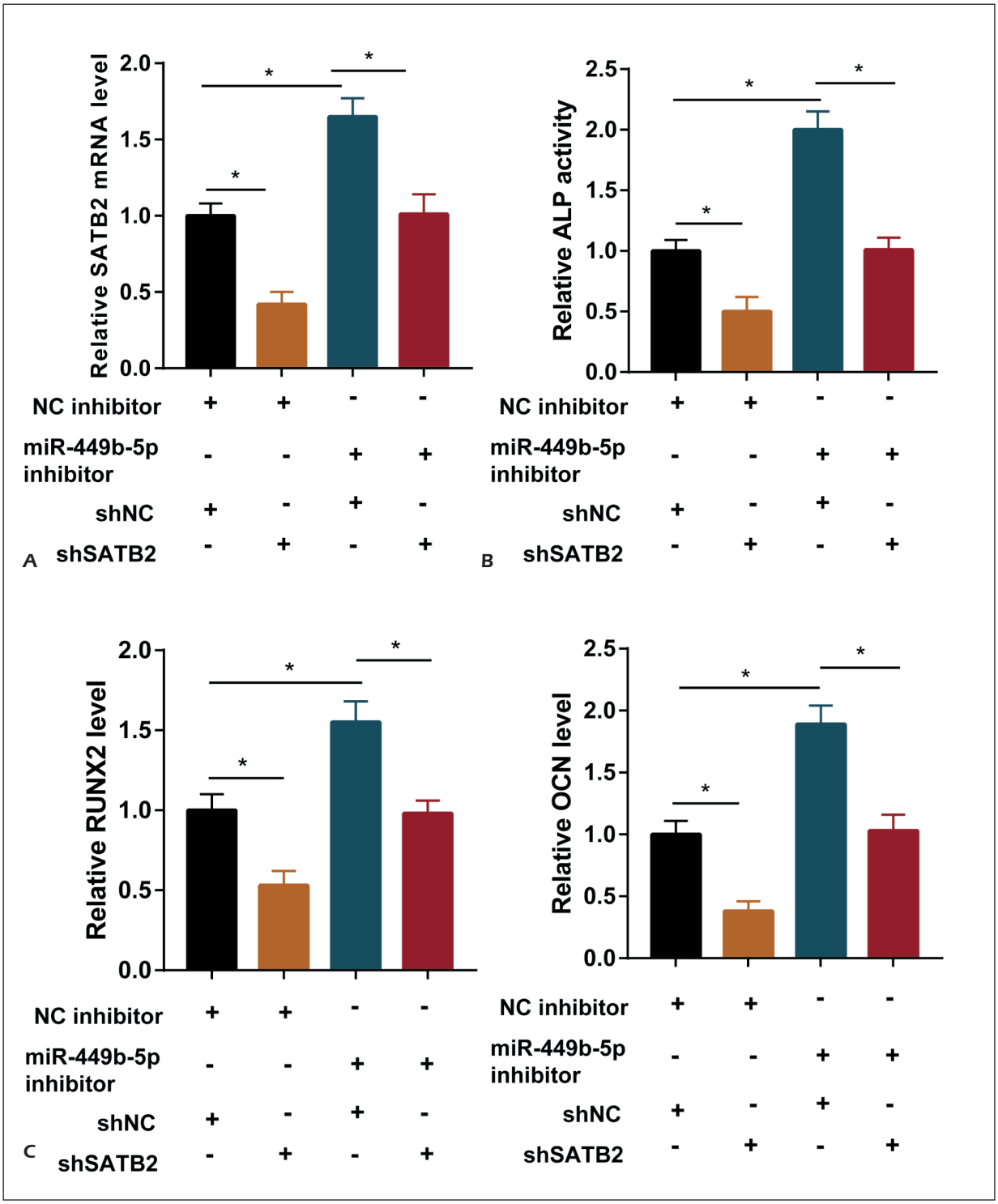

Figure 4. Satb2 is involved in the osteoblasts differentiation of BMSCs regulated by miR-449b-5p. **A**, After the BMSCs stably expressing the miR-449b-5p inhibitor were infected with shSATB2 lentivirus or shNC lentivirus, the expression level of Satb2 was detected by qRT-PCR. **B**, ALP activity assay revealed that knocking down SATB2 alone can inhibit the activity of ALP, while transfection of miR-449b-5p inhibitor and shSATB2 reversed the increase of ALP activity induced by transfection of miR-449b-5p inhibitor alone. **C**, shSATB2 alone can inhibit the expression of RUNX2 and OCN, while simultaneous transfection of miR-449b-5p inhibitor and shSATB2 can reverse the increased expression of RUNX2 and OCN induced by transfection of miR-449b-5p inhibitor alone.

Conclusions

microRNA-449b-5p is decreased during osteogenic differentiation, and microRNA-449b-5p overexpression can inhibit osteogenic differentiation. In addition, microRNA-449b-5p can inhibit the osteogenic differentiation of BMSCs by targeting binding to Satb2 and promote the progression of osteoporosis.

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Conflict of Interests

The authors declared no conflict of interest.

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