

Original Article

Mechanism of miRNA-31 Regulating Wnt/ β -catenin Signaling Pathway by Targeting Satb2 in the Osteogenic Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells

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Abstract

Objective: To explore the expression of miR-31 and Satb2 gene in the serum of postmenopausal women with osteoporosis (OP). **Methods:** 97 postmenopausal women with OP and 100 healthy women were selected as research subjects. MSCs were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. Bone marrow-derived mesenchymal stem cells (BMSCs) were isolated, identified and transfected, and then quantified by alkaline phosphatase (ALP) levels. The expression levels of miR-31 and Satb2 gene mRNA were determined by qRT-PCR. The proteins of RUNX2, OCN and BMP and Wnt/ β -catenin pathway-related proteins (GSK-3, Frizzled 1, Lrp5, Lrp6 and β -catenin) were tested by Western blotting. **Results:** In the OP group, the relative expression of miR-31 was 3.61 ± 0.54 , significantly higher than that (1.75 ± 0.27) in the healthy control group ($t=9.422$, $P<0.001$). The relative expression of mRNA of Satb2 gene was 0.86 ± 0.12 , significantly lower than that (1.35 ± 0.21) in the healthy control group ($t=5.897$, $P<0.001$). **Conclusions:** The increase in miR-31 expression can down-regulate the Wnt/ β -catenin pathway by targeting the expression of Satb2 gene, thereby inhibiting the osteogenic differentiation of BMSCs. This provides an important reference for further understanding the mechanism of OP and identifying targets for early diagnosis and treatment.

Keywords: miRNA-31, Osteogenic Differentiation, Osteoporosis, Satb2 Gene, Wnt/ β -catenin Signaling Pathway

Introduction

Osteoporosis (OP) is a disorder of bone metabolism in which the bone mass reduces, leading to an increased risk of osteoporotic fractures¹. After the onset of OP, it is easy to develop fractures, especially in the spine and hip, which may eventually lead to disability, or even death. The occurrence of OP is more prevalent in postmenopausal women, which

may be associated with the decrease of estrogen levels². Approximately 50% of postmenopausal women worldwide are affected by OP. In China, the incidence of OP is increasing every year, seriously affecting the health and quality of life of postmenopausal women^{2,3}. OP mainly results from an imbalance in bone formation and resorption during bone metabolism⁴. The pathogenesis of OP has not been fully understood yet, and many studies suggested the important role of Wnt/ β -catenin signaling pathway in bone growth and development. Its activation can promote osteoblast differentiation and suppress osteoclast proliferation, thus participating in the pathogenesis of OP^{5,6}.

MicroRNAs (miRNAs) are endogenous single-stranded RNA consisting of approximately 21-25 nucleotides that play a critical role in the transcriptional and post-transcriptional regulation of genes⁷. The primary functions of miRNA include silencing or down-regulating the expression of target genes with strong specificity, enabling precise regulation of multiple biological processes based on timing

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and tissue specificity⁸. Numerous studies have shown that miRNAs regulate osteoblast differentiation by modulating various signaling pathways or transcription factors. They also play a vital role in regulating bone metabolism and osteogenesis, and alterations in their expression levels can lead to a range of skeletal disorders^{9,10}. The miRNA-31 is located on chromosome 9p21.3 and has been associated with various bone disorders such as ankylosing spondylitis and osteoarthritis^{11,12}. Bone marrow-derived mesenchymal stem cells (BMSCs) have the potential to differentiate in multiple directions. A study¹³ has demonstrated that miR-31 plays a vital role in regulating osteogenic differentiation and bone-related diseases in BMSCs. Bioinformatics analysis revealed a targeted binding site of miR-31 with special AT-rich sequence binding protein 2 (Satb2). The present study aimed to validate this targeting relationship. Dual luciferase reporter assay results demonstrated that Satb2 is a target gene of miR-31. Satb2 is a binding protein in the matrix attachment region for tissue-specific expression, and can participate in the development and maturation of multiple cells. Studies have revealed that Satb2 acts as a positive regulator for the Wnt/ β -catenin signaling pathway and plays a crucial role in the occurrence and development of postmenopausal OP^{14,15}. Therefore, this study aims to explore the expression levels of miR-31 and Satb2 gene in the serum of postmenopausal patients with OP. Furthermore, the molecular mechanism of miR-31 targeting Satb2 in regulating osteogenic differentiation in osteogenic environment was investigated to provide a reference for further understanding of the pathological mechanism of OP and identifying effective therapeutic targets, thus contributing to the prevention and treatment of OP.

Materials and Methods

Research subjects

Postmenopausal women with OP who attended Xuzhou Third People's Hospital from January 2019 to December 2020 were selected as the research subjects. The inclusion criteria were as follows: patients diagnosed with OP according to the World Health Organization (WHO) criteria; natural menopause for 1-20 years; and patients who voluntarily participated in this study. The exclusion criteria were as follows: patients with diseases or malignant tumors related to heart, brain, lung, liver and kidney; and patients with other diseases affecting bone metabolism, such as diabetes, hyperthyroidism, rheumatoid arthritis and ankylosing spondylitis. Ultimately, 97 postmenopausal OP patients were included in this study, with ages ranging from 48 to 75 years old, a mean age of 60.14 ± 7.61 years old and a body mass index (BMI) of 25.47 ± 4.27 kg/m². Additionally, 100 healthy postmenopausal who underwent a physical examination at our hospital during the same period were included as healthy controls. They were aged from 45 to 78 years old, with a mean age of 60.58 ± 8.24 years old and BMI of 24.89 ± 5.87 kg/m². There were no

Table 1. Primer sequences for miR-31, Satb2 and β -actin.

mRNA	PCR primer
miR-31	Forward: 5'-AAAAGTAAGGATTGAGCTCAGC-3'
	Reverse: 5'-TCACTGATTTCTGTCTCCATC-3'
Satb2	Forward: 5'-TGGTGAGTACCATGCATTTTC-3'
	Reverse: 5'-CGTGCGTGGTATGGGGCGTA-3'
β -actin	Forward: 5'-TATCCCCTTGGCTTCGATCTGC-3'
	Reverse: 5'-GGCAATATGTATAGTTTATG-3'

significant differences in age, BMI and estrogen levels between the OP and control groups ($P > 0.05$), and they were comparable. This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of our hospital (Approval No.: 2022-02-009-KO1). All research participants volunteered to participate in this study and provided signed informed consent.

Experimental materials

Primary cells of Human Bone Marrow-Derived Mesenchymal Stem Cells (BMSCs) (Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd.); DMEM medium, FBS (Gibco, USA); ALP detection kit (Beyotime Biotechnology Co., Ltd.); Trizol (Thermo Fisher Scientific Company, USA); Anti-BMP antibody, anti-OPG antibody, RUNX2 antibody, anti-GSK-3 antibody, anti-Frizzled 1 antibody, anti-Lrp5 antibody, anti-Lrp 6 antibody, and anti-actin antibody (Abcam Company, USA); 293T cells (Sciarray Biotech Co., Ltd.); hsa-miR-31 mimics, hsa-miR-31 mimics NC, hsa-miR-31 inhibitor, and hsa-miR-31 inhibitor NC (Shanghai Genepharma Co., Ltd.); Liposome 3000 transfection reagent (Thermo, USA); Serum-free culture medium, Opti-MEM (Invitrogen, USA); Dual luciferase reporter gene detection kit, Cat #: RGO09 (Beyotime Biotechnology Co., Ltd.).

Empirical methods

Isolation, culture and identification of BMSCs

Primary BMSCs were cultured in DMEM medium with 10% FBS and 5% CO₂, at 37°C, under 95% relative humidity. The culture medium was replaced once every 3 to 4 days. Subculture was performed when the cells reached 80-90% confluence at the bottom of the culture flask. To confirm cell growth in the logarithmic phase, adherent cells were stained with immunocytochemistry for the expression of associated surface antigen markers.

Osteogenic differentiation ability of BMSCs *in vitro* detected by alkaline phosphatase

Third-generation BMSCs in logarithmic phase were seeded in 24-well plates, with three wells per group. After 24h of culture, the cells were further cultured with osteogenic induction medium (DMEM medium containing 10% FBS,

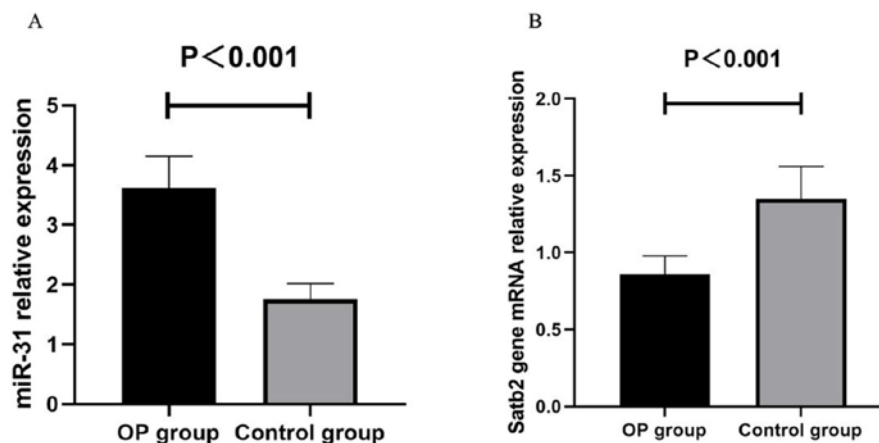


Figure 1. Expression of miR-31 and Satb2 gene mRNA in serum from the OP and healthy control groups. A: miR-31 relative expression; B: Satb2 gene mRNA relative expression.

0.1 $\mu\text{mol/L}$ dexamethasone, 10 mmol/L sodium glycerol phosphate, 50 mg/L vitamin C, 1% penicillin, and 2.5 mg/L streptomycin). The ALP levels were measured using an ALP kit on days 7, 14, and 21 of induction culture. The cell culture medium was collected and the supernatant was removed after a transient centrifugation. The samples were added to 96-well plates, with all the sample and control distributions shown in Table 1. The sample volume was 50 μL , and the standard dosages had six gradients of 4, 8, 16, 24, 32, and 40 μL . The optical density (OD) of samples was measured at 405 nm by a UV spectrophotometer, and the ALP activity value of each group was calculated according to the enzyme activity calculation method.

Cell transfection

The expression levels of miR-31 and Satb2 were regulated by transfecting miR-31 mimics, miR-31 mimics negative control (NC), miR-31 inhibitor, miR-31 inhibitor NC, and short hairpin RNA (shRNA) of Satb2. The following steps were performed: Cells were seeded in a 6-well plate at a density of 2×10^6 cells per well and transfected using the Lipofectamine 3000 transfection reagent according to the manufacturer's instructions when cell confluence reached 70%. After 48 hours of transfection, cells were harvested for analysis.

Relative expression of miR-31 and Satb2 gene mRNA in serum detected by qRT-PCR

Peripheral blood samples of 5 mL were collected from both patients with and healthy control participants of our study and placed in anticoagulant tubes containing EDTA. Total RNA was extracted from the serum using Trizol reagent, and the RNA concentration was measured using a UV spectrophotometer. The RNA was then reverse transcribed into cDNA using the PrimeScriptTM RT kit (Takara, Japan, Art. No.: RRO37A). The expression levels of miR-31 and mRNA in

the Satb2 gene were determined using the SYBR Green kit in the qRT-PCR system (Takara, Japan, Art. No.: BL698A). The internal control used was β -actin, and the primer sequences for miR-31, Satb2, and β -actin are listed in Table I. 20 μL of total reaction system was selected. A total reaction system of 20 μL was used with the following reaction conditions: pre-denaturation at 94 $^{\circ}\text{C}$ for 10 min, denaturation at 95 $^{\circ}\text{C}$ for 15s, annealing at 60 $^{\circ}\text{C}$ for 30s, and extension at 75 $^{\circ}\text{C}$ for 1 min, for a total of 40 cycles. After the reaction, the relative quantitative analysis of miR-31 and Satb2 gene mRNA expression was performed, with $2^{-\Delta\Delta\text{CT}}$ for calculation.

Targeting relationship between miRNA31 and Satb2 gene verified by immunofluorescence reporter assay

After searching the Targetscan database, the regulatory binding site of miR-31 on Satb2 gene was obtained. The targeting relationship was validated by performing a dual luciferase reporter assay in 293T cells, following the instructions of the Bright-GloTM Luciferase Detection System (Promega, USA, Art. No.: E2610/E2620/E2650).

Osteogenic differentiation related proteins and Wnt/ β -catenin pathway related proteins detected by Western blotting

RUNX2, OCN, and BMP proteins as well as Wnt/ β -catenin pathway-related proteins (GSK-3, Frizzled 1, Lrp5, Lrp6, and β -catenin), were detected in BMSCs. After 48 hours of transfection, BMSCs were collected, and washed with PBS. Protein lysate was then added, and centrifuged at 14000 r/min for 30 min. The supernatant was collected and the protein concentration was determined using a BCA kit. Protein loading buffer was added to the sample and heated to boiling for denaturation. The sample was then loaded onto an SDS-PAGE gel and electrophoresed at 80V for 2 hours to separate the proteins. The proteins were then transferred to a PVDF membrane by electrowetting at 350mA for 2 hours. The PVDF

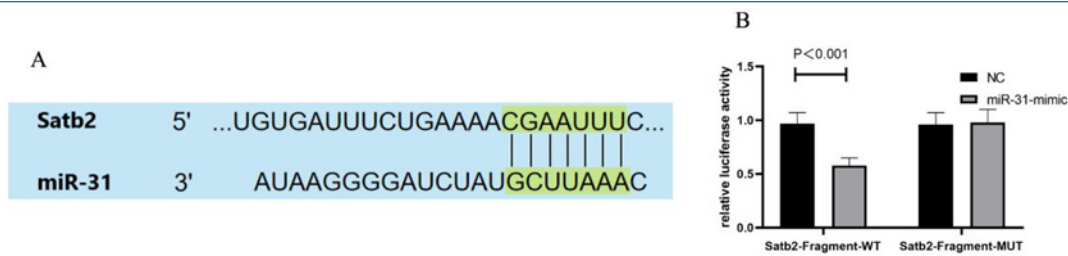


Figure 2. Verification of targeting relationship between miR-31 and Satb2 gene. Note: A: targeted binding site of miR-31 and Satb2; B: Dual luciferase report assay results.

membranes were incubated in 5% BSA at room temperature for 1h, and then incubated with the primary antibody at 4°C overnight. The membranes were then incubated with a sheep anti-rabbit IgG horseradish peroxidase secondary antibody (ab7090, diluted 1:1,000, Abcam, UK) for 2 hours at room temperature. The protein bands were exposed using an ECL chemiluminescence solution, developer solution, and fixing solution, and the protein grayscale values were measured using Image J software. The relative expression of the target protein was calculated as the gray scale of the target protein divided by the gray scale of GAPDH protein, which was used as the internal reference.

Statistical analysis

All statistical analyses were performed with the SPSS 22.0 and GraphPad Prism 8.0.1 software. Quantitative data that followed a normal distribution were described with mean \pm standard deviation (SD). T-test was used to compare data between two groups, and pairwise comparisons of data were performed among multiple groups using the SNK-q test. Pearson correlation analysis was used to examine correlations between quantitative variables that followed a normal distribution. A P-value of less than 0.05 was considered statistically significant.

Results

Expression of miR-31 and Satb2 mRNA in serum from OP and healthy control groups

In the OP group, the relative expression of miR-31 was 3.61 ± 0.54 , which was significantly higher than the expression level in healthy controls (1.75 ± 0.27), with statistical significance ($t=9.422$, $P<0.001$), as shown in Figure 1A. Additionally, the relative expression of Satb2 mRNA in the OP group was 0.86 ± 0.12 , which was significantly lower than that of healthy controls (1.35 ± 0.21), with statistical significance ($t=5.897$, $P<0.001$), as shown in Figure 1B. Correlation analysis of the expression of miR-31 and Satb2 mRNA from patients with OP revealed a significant negative relationship between the two groups ($r=0.754$, $P<0.001$).

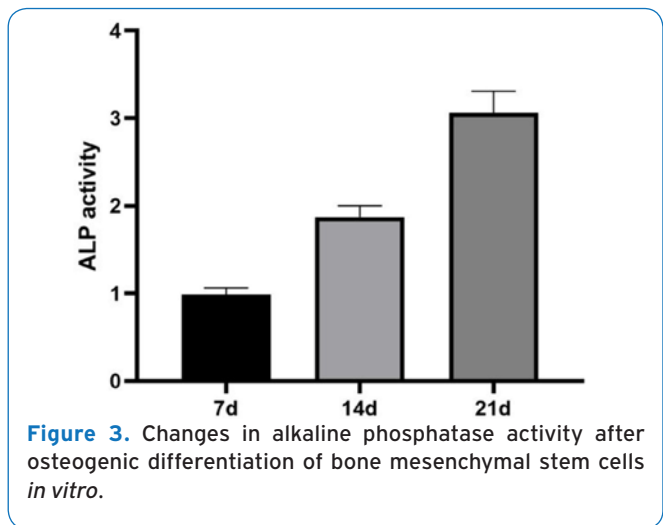


Figure 3. Changes in alkaline phosphatase activity after osteogenic differentiation of bone mesenchymal stem cells *in vitro*.

Verification of targeting relationship between miR-31 and Satb2 gene

Figure 2 illustrates the targeted binding site between miR-31 and the Satb2 gene. The results of the dual luciferase reporter assay confirmed that Satb2 is indeed a target gene for miR-31.

Alkaline phosphatase activity after osteogenic differentiation of BMSCs in vitro

ALP activity was quantitatively measured on Days 7, 14, and 21 of BMSC induction. The results indicated a significant increase in ALP expression in BMSCs as the induction period progressed, with statistically significant differences ($P<0.001$), as depicted in Figure 3.

miR-31 inhibited the osteogenic differentiation of BMSCs

BMSCs were transfected with miR-31mimic, inhibitor, mimic NC, and inhibitor NC. After osteogenic induction, ALP staining was conducted on Day 21. The results indicated a significant decrease in ALP activity in the miR-31 mimic

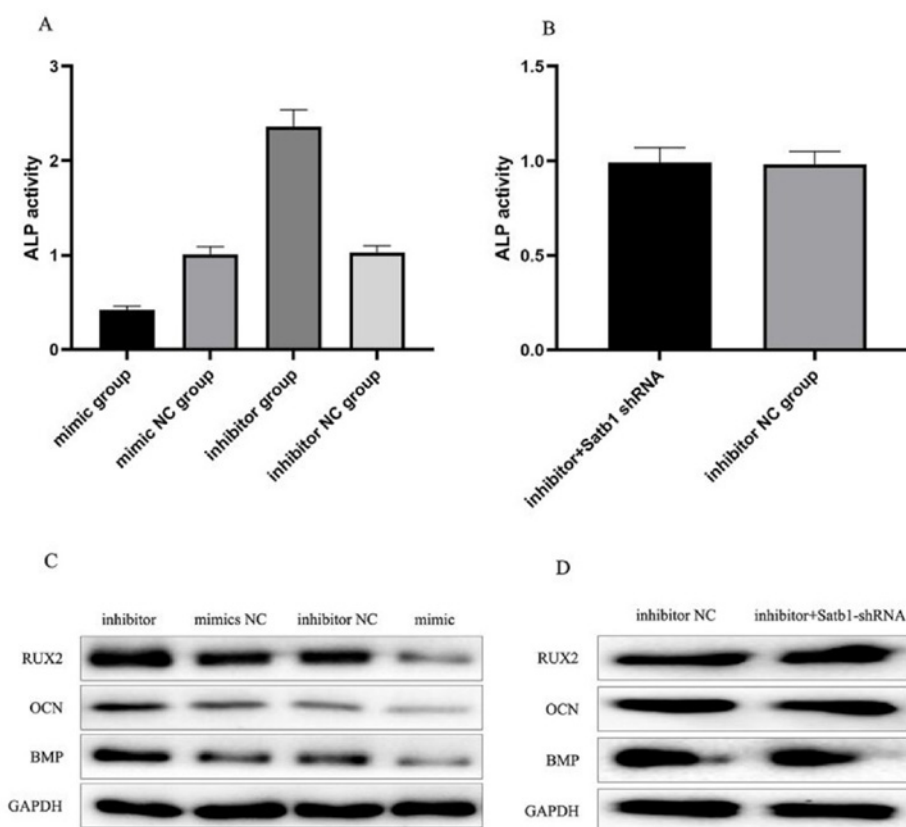


Figure 4. ALP activity and expression levels of osteogenic marker genes in each group after transfection. A: Comparison of ALP activity after transfection with miR-31mimic, inhibitor, mimic NC, and inhibitor NC; B: Comparison of ALP activity after transfection with miR-31 inhibitor + Satb1-shRNA and inhibitor NC; C: Expression levels of osteogenic marker genes after transfection with miR-31mimic, inhibitor, mimic NC, and inhibitor NC; D: Expression levels of osteogenic marker genes after transfection with miR-31 inhibitor + Satb1-shRNA and inhibitor NC.

group compared to the other three groups, and a significant increase in ALP activity in the inhibitor group compared to the other three groups, with statistical significance ($P < 0.001$), as demonstrated in Figure 4A. The expression levels of osteogenic marker genes (RUNX2, OCN, and BMP) were analyzed by Western blotting after transfection. The results showed a significant decrease in RUNX2, OCN, and BMP expression in the miR-31 mimic group, and a significant increase in the inhibitor group, as depicted in Figure 4C. However, co-transfection of miR-31 inhibitor and Satb1 shRNA to BMSCs did not show any significant differences in ALP activity and the expression levels of RUNX2, OCN, and BMP compared to the negative transfection controls, as shown in Figure 4B and 4D.

miR-31 inhibited mRNA expression in Satb2 gene

The mRNA expression of Satb2 gene was determined 48 hours after transfection. The results showed that the expression of Satb2 gene mRNA in the miR-31 mimic group

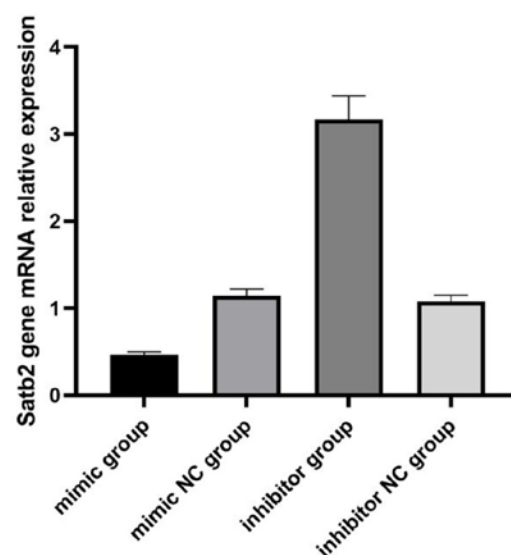


Figure 5. mRNA expression in Satb2 gene in each group after transfection.

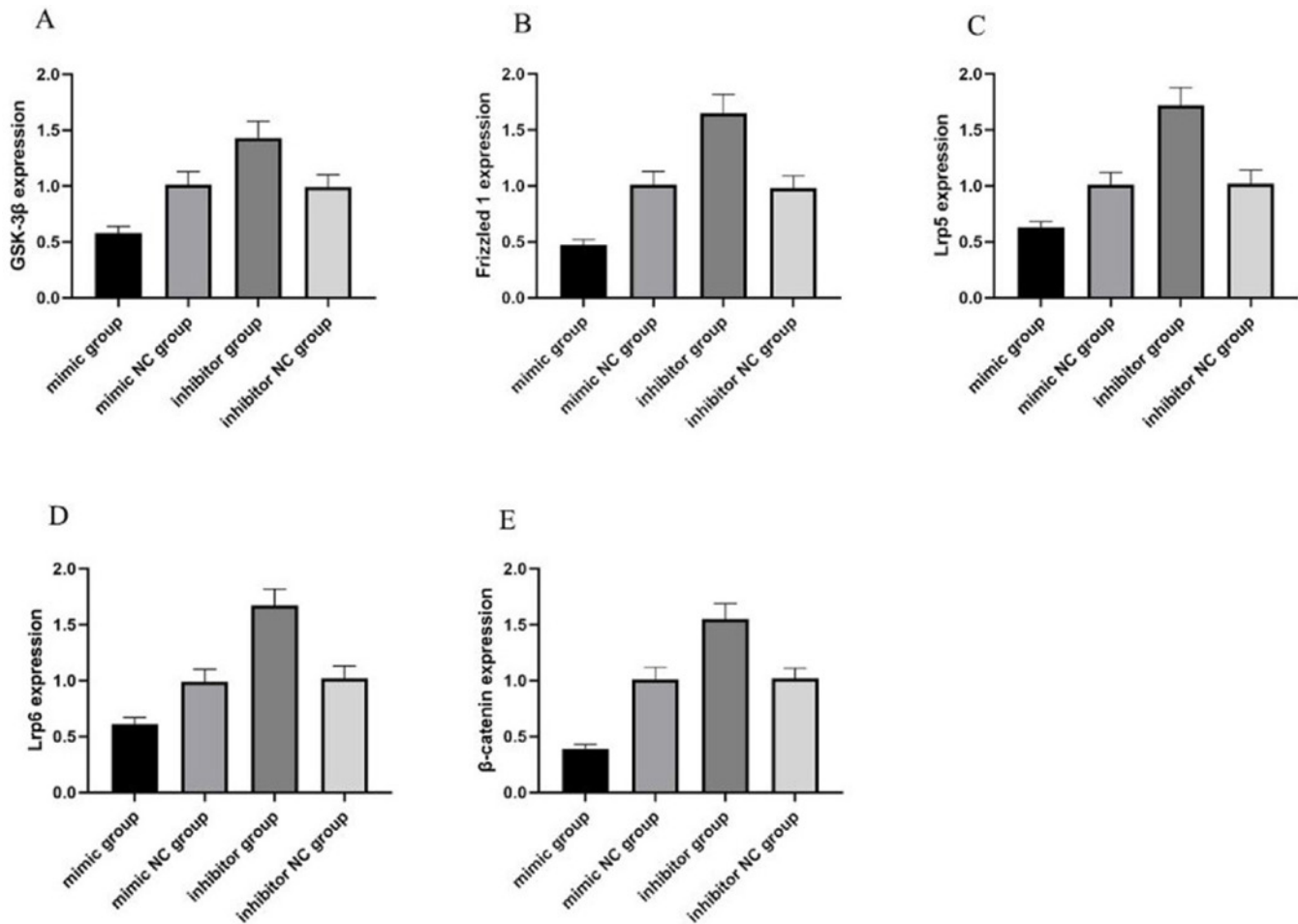


Figure 6. Expression levels of Wnt/ β -catenin pathway related proteins in bone mesenchymal stem cells after 48h of transfection. A: GSK-3 β ; B: Frizzled 1; C: Lrp5; D: Lrp6; E: β -catenin.

(0.46 ± 0.04) was significantly lower than that in the mimic NC group (1.14 ± 0.08), the inhibitor group (3.17 ± 0.27), and the inhibitor NC group (1.08 ± 0.07), with statistical significance ($P < 0.001$). However, the mRNA expression of *Satb2* gene was significantly higher in the inhibitor group than in the other three groups, with statistical significance ($P < 0.001$), as shown in Figure 5.

miR-31 regulated the expression of Wnt/ β -catenin pathway related proteins

The protein expression levels of GSK-3, Frizzled 1, Lrp5, Lrp6, and β -catenin were measured 48 hours after transfection. The results indicated that the expression levels of proteins related to the Wnt/ β -catenin pathway decreased in both the mimic and inhibitor groups, as illustrated in Figure 6. However, when miR-31 inhibitor and *Satb1* shRNA were co-transfected into BMSCs, there was no significant difference observed in the expression levels of proteins related to the Wnt/ β -catenin pathway when

compared to the negative transfection control group, as shown in Figure 7.

Discussion

In recent years, miRNAs have received significant attention due to their broad regulatory function in affecting gene transcription and post-transcriptional protein synthesis. This study demonstrates that miR-31 expression is significantly increased in the serum of patients with OP, and acts as a negative regulator of the osteogenic differentiation of BMSCs. The upregulation of miR-31 expression leads to downregulation of the *Satb2* gene, which negatively regulates the Wnt/ β -catenin signaling pathway, ultimately inhibiting the osteogenic differentiation of BMSCs. This effect is enhanced by miR-31 inhibition. The findings of this study shed light on the molecular mechanisms underlying the weakening of osteogenic differentiation of BMSCs in OP, which could

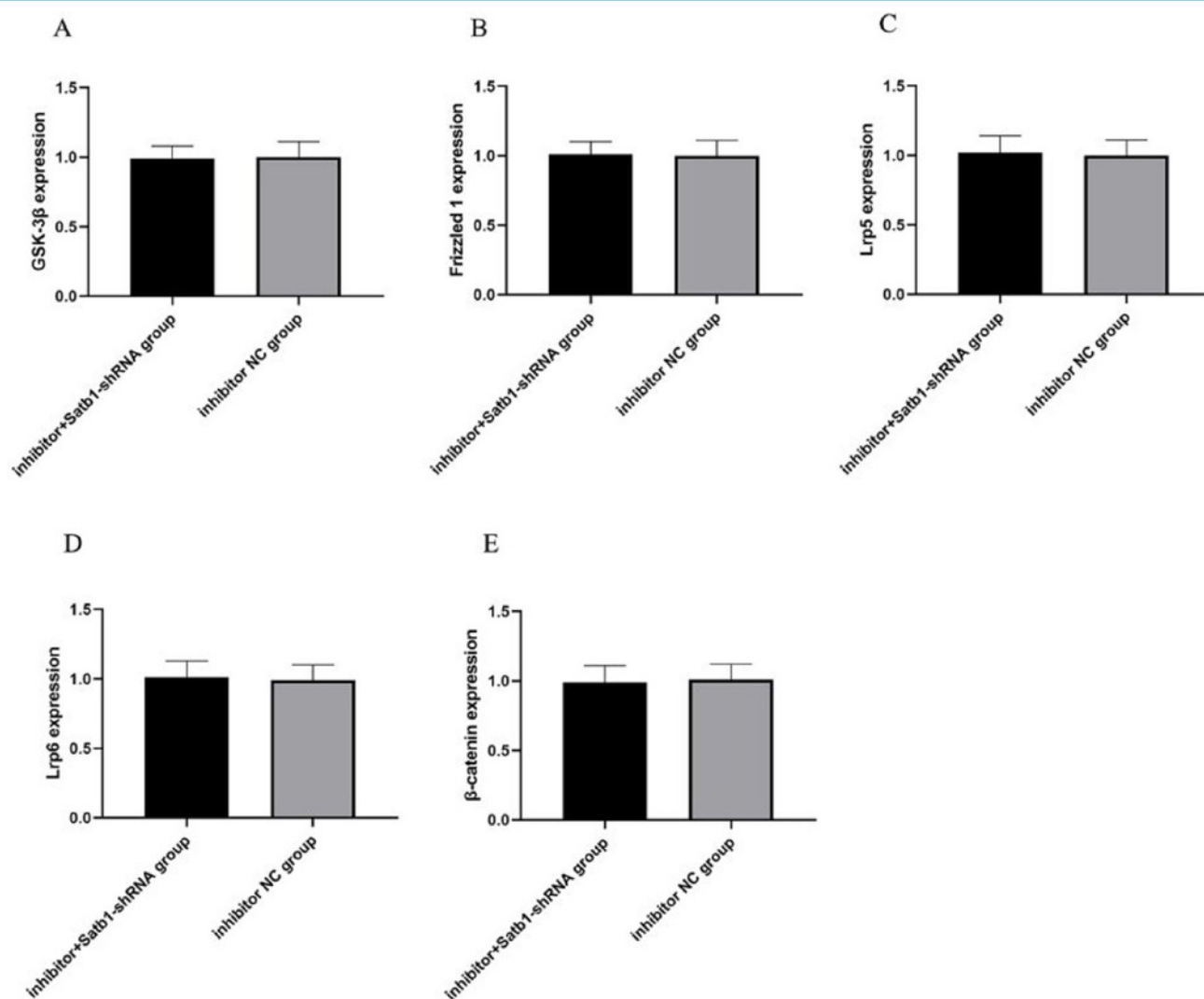


Figure 7. Expression levels of Wnt/ β -catenin pathway related proteins after transfection of miR-31 inhibitor and Satb1 shRNA to bone mesenchymal stem cells. A: GSK-3 β ; B: Frizzled 1; C: Lrp5; D: Lrp6; E: β -catenin.

help in developing effective diagnostic and treatment methods for this disease.

miRNAs are the most abundant RNA found in serum, and have been shown to play a crucial regulatory role in the occurrence and development of bone metabolic diseases. In a recent study, Lin et al.¹⁶ found that miR-338 was significantly enriched in the serum of postmenopausal patients with OP. Functional experiments confirmed the regulatory effect of the Runx2/Sox4/miR-338 positive feedback pathway on osteoblast differentiation, suggesting that miR-338 in serum could serve as a potential diagnostic and therapeutic target in postmenopausal patients with OP. A study conducted by Tang et al.¹⁷ revealed that miR-144 expression was significantly higher in clinical serum samples from postmenopausal patients with OP, as compared to postmenopausal women with normal BMD. Functional experiments demonstrated that

miR-144 promotes BMSCs proliferation, inhibits apoptosis, and induces osteogenic differentiation, thereby participating in the regulation of osteogenesis mechanism in OP. Wang et al.¹⁸ have shown in cellular experiments that miR-765 mediates the BMP6/Smad1/5/9 signaling pathway, and inhibits the osteogenic differentiation of BMSCs by targeting BMP6. In this study, we investigated the expression levels of miR-31 and Satb2 gene mRNA in patients with OP, and found that miR-31 was significantly lower in patients with OP than in healthy controls, while mRNA of downstream miR-32 gene was significantly higher in patients with OP, with a significant negative correlation between miR-31 and Satb2 gene mRNA. These results suggest that the differential expression of miR-31 and Satb2 gene may be related to the occurrence of OP. However, it is not clear whether miR-31 and Satb2 are involved in regulating the osteogenic differentiation through

specific mechanisms. To clarify the regulatory mechanism of miR-31 targeting *Satb2* gene in osteogenic differentiation, we validated the targeting relationship between miR-31 and *Satb2* gene. The dual luciferase reporter results confirmed that *Satb2* is a target gene for miR-31. *Satb2* is a matrix binding region binding protein for tissue-specific expression, which plays an important role in the regulation of multiple biological processes¹⁹. Studies²⁰ have shown that the decrease in *Satb2* expression could inhibit the osteogenic differentiation of BMSCs and promote the progression of OP.

To further validate the role of miR-31 in the pathogenesis of osteoporosis, we conducted *in vitro* cellular assays using BMSCs. BMSCs were transfected with miR-31 mimics and inhibitors, followed by osteogenic induction, and ALP staining was performed after 21 days. The results revealed a significant reduction in ALP activity in the miR-31 over-expression group and a significantly higher ALP activity in the miR-31 inhibit group. Furthermore, the expression levels of osteogenic marker genes, including *RUNX2*, *OCN*, and *BMP*, showed a significant decrease in the miR-31 over-expression group, which also significantly decreased in the silencing group. These results suggest that the increase of miR-31 expression suppresses the osteogenic differentiation of BMSCs and, thus, may be involved in the regulation that promotes the occurrence and development of OP.

Previous studies have shown that miR-31 is associated with the occurrence and development of multiple bone-related diseases. Wang et al.¹² showed that miR-31 is highly expressed in peripheral blood monocytes from patients with ankylosing spondylitis, which is associated with the disease activity. Through *in vitro* experiments, Costa et al.¹¹ found that miR-31 can cooperate with miR-33a-5p in the proliferation and differentiation of osteoblasts and chondrocytes, and can serve as a new biomarker for osteoarthritis.

Furthermore, we investigated whether miR-31 could reduce the expression of Wnt/ β -catenin pathway related proteins by suppressing *Satb2* gene expression. The results revealed that 48 hours post-transfection the miR-31 overexpression group showed significant reductions in the expression levels of *Satb2* gene and Wnt/ β -catenin pathway-related proteins, including *GSK-3*, *Frizzled 1*, *Lrp5*, *Lrp6*, and β -catenin, whereas their expression significantly increased in the miR-31 silencing group. These findings suggest that miR-31 can decrease the expression of *Satb2* gene, and subsequently inhibit Wnt/ β -catenin pathway by targeting, leading to the inhibition of osteogenic differentiation of BMSCs.

In conclusion, our study found that miR-31 expression was increased in postmenopausal women with OP. We also observed that miR-31 can decrease the expression of *Satb2* gene, which then down-regulates the Wnt/ β -catenin pathway, leading to the inhibition of the osteogenic differentiation of BMSCs and promoting the development of the disease. These findings provide an important reference for understanding the pathogenesis of OP and identifying potential targets for early diagnosis and treatment.

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Authors' contributions

XO and SL conceived and designed the study, and drafted the manuscript. XO, SL, YD, FX and ML collected, analyzed and interpreted the experimental data. XO and YD revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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