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RBM5-AS1 participates in fracture healing and inhibits apoptosis of bone cells through the up-regulation of β-catenin

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Abstract. – OBJECTIVE: The aim of this study was to detect the expression of RBM5-AS1 during fracture healing, and to explore its possible mechanism.

MATERIALS AND METHODS: A mice tibia fracture model was constructed in this study. Mice in the control group and experimental group were sham-operated on the left tibia and were operated in the right tibia, respectively. The tibia bones of both groups were obtained at 4 d, 8 d, 12 d, 16 d, 20 d, and 24 d after the operation. Quantitative polymerase chain reaction (qPCR) was used to detect the expression of RBM5-AS1 in tibiae. After interfering with the expression of RBM5-AS1 in bone cells, Cell Counting Kit-8 (CCK-8) was used to detect cell proliferation ability, and flow cytometry was applied to detect apoptosis. Western blot was used to measure the protein expression of b-catenin and RBM5 after down-regulating RBM5-AS1. Finally, b-catenin was interfered in osteoblasts to explore the relationship between RBM5-AS1 and b-catenin.

RESULTS: Compared with the control group, the expression of RBM5-AS1 in the experimental group was significantly increased on the 4 d, 8 d, 12 d, and 16 d after fracture surgery. However, no statistical difference was observed on the 20 d and 24 d between the two groups. After interfering with RBM5-AS1, the apoptosis of chondrocytes and osteoblasts was significantly increased in both mouse and human cells, while the expression of b-catenin was strikingly decreased. Further up-regulation of b-catenin could reduce the apoptosis of bone cells. The expression of RBM5, which was a natural antisense transcript of RBM5-AS1, was increased after down-regulating RBM5-AS1.

CONCLUSIONS: RBM5-AS1 can inhibit the apoptosis of bone cells by promoting the expression of b-catenin and can be used as a biomarker for fracture healing.

Key Words:
Fracture healing, RBM5-AS1, b-catenin, Apoptosis.

Introduction

In recent years, the incidence of fractures caused by trauma has been increasing. The occurrence of comminuted fractures of bone and soft tissue defects resulted from high energy damage has increased as well[1]. Although the healing capacity of bone tissues is as high as 90%-95%, 5%-10% of the fractures still cannot be healed[2]. Delayed bone healing and fracture nonunion are common post-operative complications of fractures, which may be eventually accompanied by high morbidity. Nonunion of fracture means that the fractures cannot heal within the normal healing time after treatment. It is the termination of all repair processes of bone tissues. The current diagnostic criteria for fracture nonunion was established by the U.S. Food and Drug Administration (FDA) in 1986, in which fracture nonunion was specifically defined as follows: "Bone injury and fracture have happened for at least 9 months, and there has no healing tendency for at least 3 months". Bone nonunion is still one of the most important problems to be solved in orthopedics clinics[3]. The nonunion of bones can lead to pain during walking, inconvenient mobility of limbs and even mental disorders in patients[4], eventually bringing great economic burden to patients and the society. Therefore, an in-depth understanding of the molecular mechanism of fracture healing plays a significant role in the treatment and prevention of bone healing and bone nonunion.

It is well known that multiple non-coding RNAs (ncRNAs), including long non-coding RNAs (lncRNAs), play an irreplaceable role in protein translation[5]. According to the statistics of the LNCipedia3.1 database, the number of known lncRNAs has now exceeded 100,000[6]. According to their location in the genome, lncRNAs can be divided

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into five types, namely, lncRNA sense, antisense lncRNA, bidirectional lncRNA, intronic lncRNA, and intergenic lncRNA[7,8]. Unlike microRNAs, lncRNAs have a complex and high-level structure that may interact with multiple molecules simultaneously, such as DNA, RNA, and proteins. Meanwhile, lncRNAs participate in a variety of molecular regulatory pathways[9,10]. LncRNAs mainly function through inducting, activating and scaffolding molecules[11]. In addition, lncRNAs play an important role in normal physiological processes as well as abnormal pathological processes including the development of tumors[12,13].

In this study, we mainly explored the expression of antisense lncRNA *RBM5-AS1* during fracture healing, and further discussed its role in fracture healing and the possible underlying mechanism.

Materials and Methods

Construction of the Tibia Fracture Model in Mice

Totally 36 male Sprague Dawley (SD) mice (6-8 weeks old, 16.8-25.3 g in weight) were used for modeling. Mice were anesthetized with 1% pentobarbital (1 mL/kg) by intraperitoneal injection. Subsequently, mice in the control group were treated with sham surgery on the left tibia, while mice in the experimental group were treated with surgery on the right tibia. Meanwhile, for mice in the experimental group, a fixed intramedullary needle was inserted into the cannulation after the operation. On the 4 d, 8 d, 12 d, 16 d, 20 d, and 24 d after the operation, bone tissues of the left and right temporal bones were collected. TRIzol (Invitrogen, Carlsbad, CA, USA) was used for total RNA extraction according to the instructions. The samples were stored in a -80°C freezer. This study was approved by the Animal Ethics Committee of Zhangjiagang Hospital Affiliated to the Animal Center of Soochow University, Zhangjiagang, China.

Cell Culture

Mouse chondroblasts (ATDC5), osteoblasts (MC3T3-E1), human articular chondrocytes (HCa), and osteoblasts (hFOB1.19) were all purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA).

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were incubated in a 37°C, 5% CO_2 incubator. The culture medium was changed every two or three days. Cells passage was performed when the cell fusion reached 80%-90%.

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

Total RNA was extracted, and reverse transcription was performed according to the PrimeScript RT reagent Kit instructions (TaKaRa, DRR047A, Otsu, Shiga, Japan). The complementary deoxyribose nucleic acid (cDNA) synthesized by reverse transcription was diluted to an appropriate concentration. Subsequently, cDNA was mixed with primers, pre-mixed solutions and ultrapure water to make the final volume of 20 μL. After centrifugation, the mixture was performed with quantitative fluorescence PCR. The relative expression was calculated according to the ΔΔCT method. Primers used in this study were as follows: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) F: 5'-GAAGGTGAAGGTCGGAGT, R: 5'-GAAGATGGTGATGGGATTTC; (*RBM5-AS1*) F: 5'-GCTTCAACACTGCGTGACAA, R: 5'-CGTGGAATCAAATGGAGTGG.

Western Blot Analysis

The cells were lysed with the radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China) containing a protease inhibitor to obtain total cellular protein. The concentration of proteins was measured by using a spectrophotometer (NanoDrop 2000). Then, the protein samples were denatured by adding a 5x loading buffer. The extracted proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and were subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were incubated with relevant primary and secondary antibodies. The protein expression was finally analyzed according to standard procedures.

Cell Counting Kit-8 (CCK-8) Assay

The cells were seeded in a 96-well plate. After the adherence of cells reached 80%, they were synchronized for 12 hours and the original medium was discarded. The total reaction volume of each well was 200 μL with 6 replicates of each sample. After adding 20 μL CCK8 reaction solution (Dojindo, Kumamoto, Japan), the cells were incubated at 37°C in the dark for 2 h. Then, the cells were vibrated on a micro-vibrator for 10

min. The absorbance at 450 nm wavelength was measured by a microplate reader.

Cell Apoptosis

After the cell supernatant was collected in a pre-labeled flow tube, the cells were digested with EDTA-free trypsin. After the suspension, the cells were pipetted into a corresponding flow tube and centrifuged twice with phosphate-buffered saline (PBS). Subsequently, 200 μL calcium-containing binding buffer was added after centrifugation. Meanwhile, 10 μL annexin V-FITC fluorescent probe and 5 μL propidium iodide (PI; Abcam, Cambridge, MA, USA) were added. The cells were incubated in the dark for another 5 min. Finally, FL1 and FL3 dual-channel wavelength detection were used for the flow cytometry.

Cell Transfection

LncRNA *RBM5-AS1* knockdown experiments were performed by using four different probes for lncRNA *RBM5-AS1* and negative controls. The pcDNA3-*RMB5-AS1* plasmid was transfected into cells for *RBM5-AS1* overexpression. The oligonucleotide sequence was designed by the Exiqon's GapmeR design algorithm and its LNATM spike pattern was as follows: (http://www.exiqon.com/ls/Pages/GDTSequenceInput.aspx?SkipCheck=true)

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 Software (IBM, Armonk, NY, USA) was used for all statistical analysis. GraphPad Prism 6.0 (La Jolla, CA, USA) was used for image editing. Measurement data were compared by using the *t*-test. Measured data were expressed as mean ± standard deviation ($\bar{x} \pm s$). The chi-square test was used to compare the difference of classified data. $p<0.05$ was considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

The Expression of RBM5-AS1 Was Markedly Increased During Fracture Healing

To explore the expression of *RBM5-AS1* during fracture healing, we collected tibia tissues from the control and the experimental group at 4 d, 8 d, 12 d, 16d, 20 d, and 24 d after the operation, respectively. Results of qRT-PCR showed that the expression of *RBM5-AS1* in the experimental group was markedly increased (Figure 1A) at 4 d, 8 d, 12 d, and 16 d compared with that of the control group. However, there was no significant difference in *RBM5-AS1* expression at 20 d and 24 d between the two groups. Therefore, we speculated that *RBM5-AS1* might be a driving factor for fracture healing.

RBM5-AS1 Could Promote the Proliferation of Osteoblasts and Chondroblasts in Both Mouse and Human Cells

We used the locked nucleic acid (LNA) RNA GapmeR technique to knock down the expression of *RBM5-AS1* in mouse bone cell lines to explore the role of *RBM5-AS1* in fracture healing (Figure 2A). CCK8 results indicated that the proliferation of ATDC5 and MC3T3-E1 cells was both inhibited after reducing the expression of *RBM5-AS1* (Figure 2B). In the HC-a and hFOB1.19 cell lines, we also knocked down the expression of *RBM5-AS1* (Figure 2C). Results found that the knockdown of *RBM5-AS1* expression exhibited the same effect on human cell lines, with a decrease in cell proliferation (Figure 2D). Therefore, we believed that *RBM5-AS1* could promote the proliferation of osteoblasts and chondroblasts in both mouse and human cells.

RBM5-AS1 Overexpression Could Inhibit the Apoptosis of Osteocytes and the Expression of Its Natural Antisense Transcript RBM5

We further overexpressed *RBM5* in the HC-a and hFOB1.19 cell lines to explore the effect of *RBM5-AS1* on bone cells (Figure 3A-B).

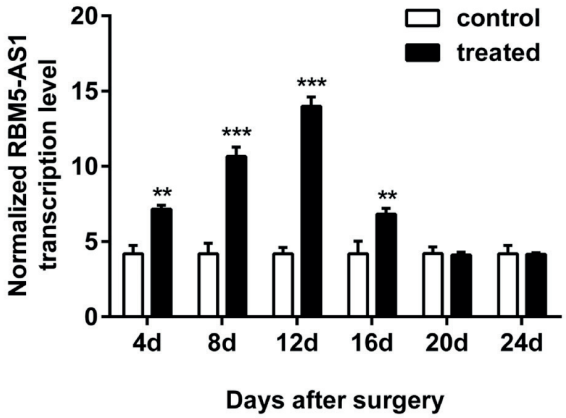

Figure 1. Expression of *RBM5-AS1* in fracture healing sites of mice was significantly elevated. Fluorescence quantitative PCR results showed that, compared with the control group, the expression of *RBM5-AS1* in the experimental group was significantly increased on 4 d, 8 d, 12 d, and 16 d.

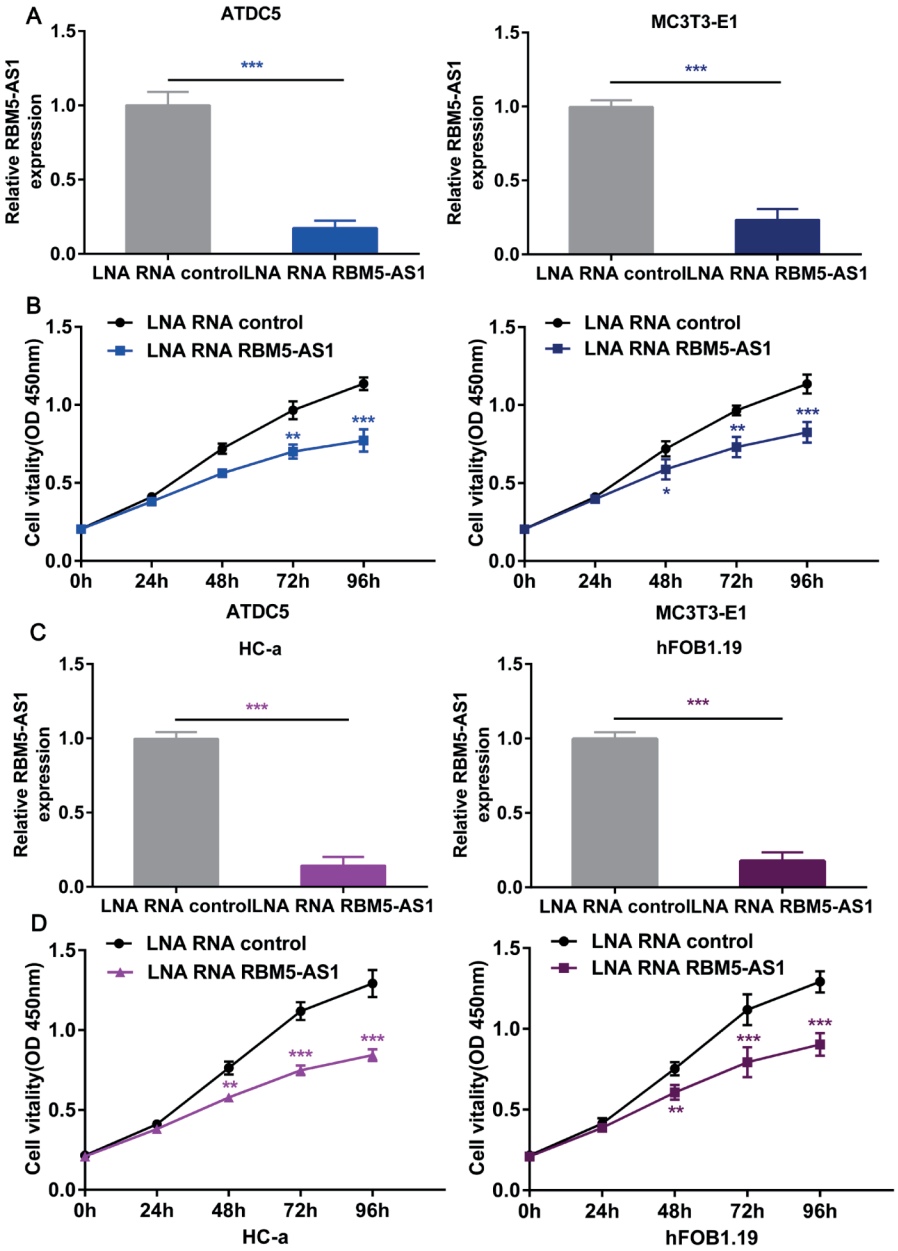

Figure 2. After the knockdown of *RBM5-AS1*, the proliferation of chondrocytes and osteoblasts in mice and human cell lines was significantly decreased. ***A,*** In mouse ATDC5 and MC3T3-E1 cell lines, LNA RNA *RBM5-AS1* significantly reduced *RBM5-AS1* expression. ***B,*** Decreased expression of *RBM5-AS1* significantly inhibited the proliferation of ATDC5 and MC3T3-E1 cells. ***C,*** LNA RNA *RBM5-AS1* significantly reduced the expression of *RBM5-AS1* in human HC-a and hFOB1.19 cell lines. ***D,*** The proliferation of HC-a and hFOB1.19 cells was significantly inhibited after *RBM5-AS1* knock-down.

Results demonstrated that the elevated expression of *RBM5-AS1* could strikingly reduce the apoptosis of osteocytes (Figure 3C-D). Since *RBM5* was the natural antisense transcript of *RBM5-AS1*, we tested the expression of *RBM5* as well. Western blot results demonstrated that the protein expression of *RBM5* was strikingly decreased when *RBM5-AS1* was overexpressed (Figure 3E). These results suggested that *RBM5-AS1* overexpression could inhibit the apoptosis of osteocytes and the expression of its natural antisense transcript *RBM5*.

RBM5-AS1 Inhibited the Apoptosis of Osteocytes by Ð-Catenin

β-catenin is widely existed in cells, and plays a significant role in cell apoptosis. We then detected the expression of *β-catenin* in hFOB1.19 cells

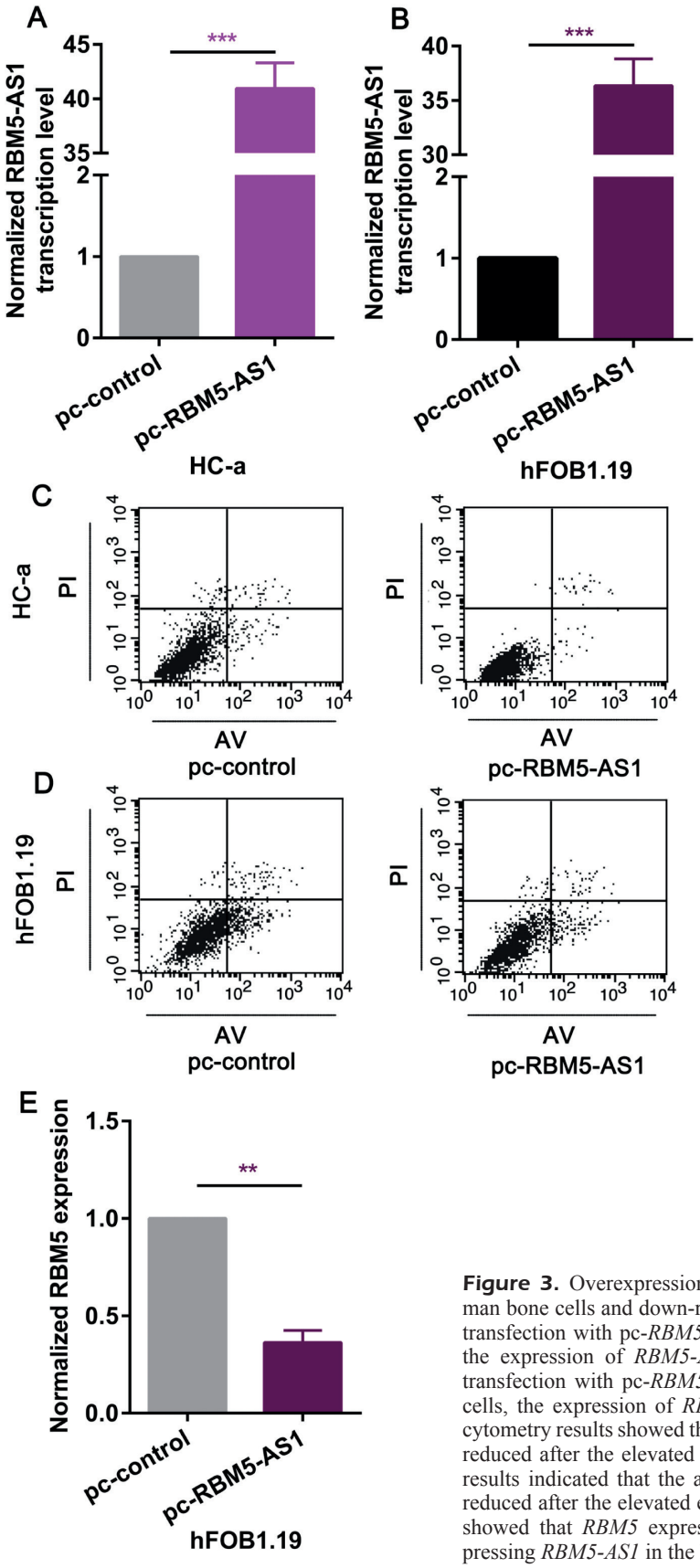

Figure 3. Overexpression of *RBM5-AS1* inhibited the apoptosis of human bone cells and down-regulated the expression of *RBM5*. ***A,*** After the transfection with pc-*RBM5-AS1* in HC-a and human chondrogenic cells, the expression of *RBM5-AS1* was significantly elevated. ***B,*** After the transfection with pc-*RBM5-AS1* in hFOB1.19 and human chondrogenic cells, the expression of *RBM5-AS1* was significantly elevated. ***C,*** Flow cytometry results showed that the apoptosis of HC-a cells was significantly reduced after the elevated expression of *RBM5-AS1*. ***D,*** Flow cytometry results indicated that the apoptosis of hFOB1.19 cells was significantly reduced after the elevated expression of *RBM5-AS1*. ***E,*** QRT-PCR results showed that *RBM5* expression was significantly reduced after overexpressing *RBM5-AS1* in the human osteoblast line hFOB1.19.

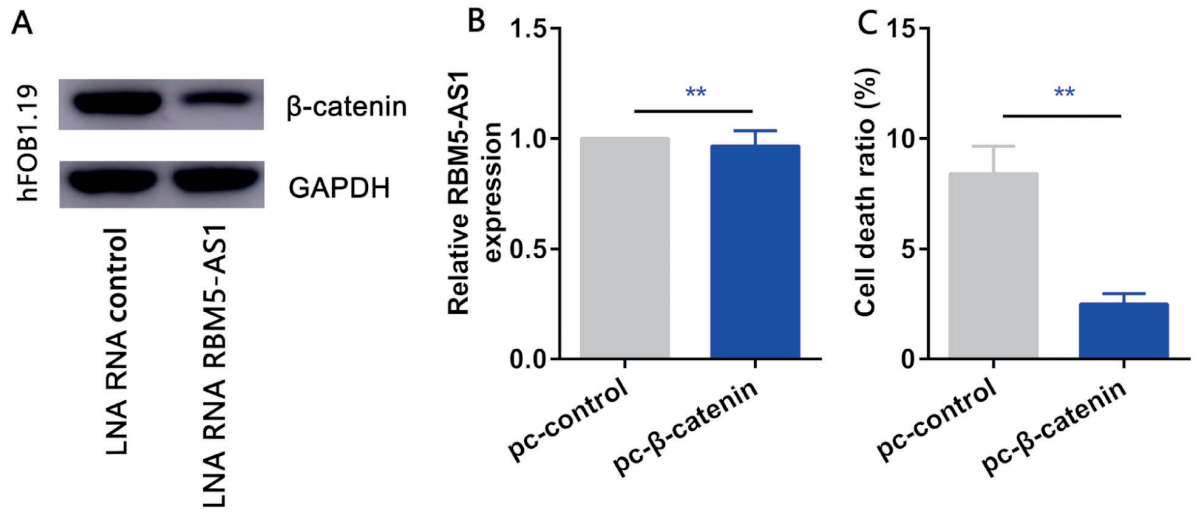

Figure 4. The expression of *β-catenin* decreased with the down-regulation of *RBM5-AS1*. ***A,*** The expression of *β-catenin* was significantly decreased after *RBM5-AS1* knock-down in hFOB1.19 cells. ***B,*** No significant change in expression of *RBM5-AS1* was found when the expression of *β-catenin* was increased in hFOB1.19 cells. ***C,*** Cell apoptosis was significantly decreased after the overexpression of *β-catenin* in hFOB1.19 cells.

after knocking down the expression of *RBM5-AS1* (Figure 4A). Western blot results demonstrated that the protein expression of β-*catenin* decreased significantly with the decreased expression of *RBM5-AS1* in human osteoblasts (Figure 4A). Subsequently, we found that the expression of *RBM5-AS1* was not remarkably altered after *β-catenin* overexpression in hFOB1.19 cells (Figure 4B), while cell apoptosis was decreased (Figure 4C). Hence, these results suggested that *RBM5-AS1* inhibit the apoptosis of osteocytes by *β-catenin*.

Discussion

Delayed or non-healing of fractures is a common complication in the treatment of bone defects[14], which is also a difficult task for bone orthopedists. At present, the clinical treatment is complicated and difficult[15]. Meanwhile, the patients are often faced with the risk of amputation[16]. Bone tissue has a strong self-repair ability[17], and the fractured end can be completely replaced by new bones, eventually restoring its original structure and function. Unlike the healing of other tissues, fracture healing generally does not leave scars[18]. However, the occurrence of delayed fracture healing or non-healing due to trauma is increasing year by year, accounting for about 3% of the total prevalence of bone diseases. Therefore, searching for the key molecules in fracture healing will provide important clues for improving the status of delayed bone healing and nonunion.

With the completion of the Human Genome Project, scientists have found that only 2% RNAs can encode proteins, and 98% of them are non-coding RNAs (ncRNAs). According to the length of the transcript, ncRNAs can be divided into short ncRNAs and long ncRNAs (lncRNA)[19,20]. LncRNAs are non-coding RNAs with transcripts longer than 200 nt. They are considered as "the noise of gene transcription" and have no biological function. However, recent researches[21-23] have demonstrated that lncRNAs can participate in many biological processes, including genetic regulation, transcriptional regulation and post-transcriptional regulation. LncRNAs also may be involved in the fracture healing process[24]. Therefore, studying the regulatory mechanism of lncRNAs in fracture healing will provide new ideas for the treatment of fracture nonunion/delayed healing.

Our study demonstrated that the expression of *RBM5-AS1* was markedly increased on the 4 d, 8 d, 12 d, and 16 d after bone fracture in mice. After down-regulating *RBM5-AS1*, the apoptosis of chondrocytes and osteoblasts in both mouse and human cells were statistically increased. However, up-regulating the expression of *RBM-AS1* exhibited the opposite results. We supposed that *RBM-AS1* could promote the proliferation

of chondrocytes and osteoblasts in both mouse and human cell lines. In addition, *RBM5-AS1* significantly inhibited the expression of its natural antisense transcript *RBM5* and up-regulated *β-catenin*, resulting in the decreased apoptosis of bone cells. *RBM5-AS1* interference significantly decreased the expression of *β-catenin* in the osteoblast cell line, while over-expressing *β-catenin* could renew the apoptosis of bone cells.

Conclusions

We found that *RBM5-AS1* can be used as a biomarker for fracture healing, and can inhibit the apoptosis of bone cells by up-regulating *β-catenin* expression.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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