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MiR-25 overexpression promotes fracture healing by activating the Wnt signaling pathway

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Abstract. – OBJECTIVE: To study the mechanism of micro-ribonucleic acid (miR)-25 in regulating the fracture healing in rats.

MATERIALS AND METHODS: A total of 45 male Sprague-Dawley (SD) rats were selected and randomly divided into group A [Phosphate Buffered Saline (PBS), n=15], group B (mimics NC, n=15) and group C (miR-25 mimics, n=15). The fracture model in rats was established *via* operation in all groups. From 1 d after the successful modeling, 50 μL (2 nmoL) of PBS was intraperitoneally injected into rats in group A, an equal amount of mimics NC was injected into rats in group B, and an equal amount of miR-25 mimics was injected into rats in group C. The above agents were injected once a week for consecutive 6 weeks. Fracture healing in rats was evaluated *via* X-ray imaging. At the same time, miR-25 expression in the three groups was detected *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Protein expressions of β-catenin, proliferating cell nuclear antigen (PCNA) and bone morphogenetic protein-2 (BMP-2) in the three groups were detected *via* Western blotting. The OCN-, PCNA- and BMP-2-positive osteoblasts in the three groups were detected *via* immunohistochemical staining and were further quantified. Moreover, the biomechanical properties of femoral fracture healing in the three groups were analyzed *via* the 4-point bending flexural test.

RESULTS: The X-ray examination of the femoral fracture healing at postoperative 1 and 7 weeks revealed that the fracture line disappeared, and both callus formation and fracture healing were good in miR-25 mimics group. In PBS group and mimics NC group, a few fracture lines could be observed, and both callus formation and fracture healing were poor. RT-PCR data showed that the expression level of miR-25 significantly increased in the miR-25 mimics group compared with that in the other two groups, and the differences were statistically significant ($p<0.01$). Western blotting analyses showed upregulated levels of β-catenin, PCNA and BMP-2 in the miR-25 mimics group compared with those in the control group, and the differences were statistically significant ($p<0.01$). Immunohistochemical staining manifested that the numbers of OCN-, PCNA- and BMP-2-positive osteoblasts in miR-25 mimics group markedly increased compared with that in the other two groups ($p<0.01$), suggesting that osteoblast differentiation in miR-25 mimics group was affected. The above immunohistochemical results indicated that the osteoblast differentiation at the fracture end in miR-25 mimics group was markedly enhanced compared with that in control groups. The results of the biomechanical test of femur specimens at 7 weeks after operation showed that in miR-25 mimics group, the maximum load, fracture energy and stiffness increased by 188%, 333% and 90%, respectively, compared with those in the PBS group ($p<0.01$). It is indicated that miR-25 promoted the mechanical properties of fracture healing.

CONCLUSIONS: The overexpression of miR-25 in the fracture in rats promotes fracture healing by activating the Wnt signaling pathway.

Key Words:
MiR-25, Wnt signaling pathway, Fracture healing.

Introduction

Fracture is the most common trauma in people of all ages, which is generally caused by increased bone fragility due to trauma or disease. Fracture often leads to disability and related diseases, especially in the elderly[1]. Healing after fracture is a complex regeneration process that restores bone function. In the case of stable fracture fixation,

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the granulation tissues gradually ossify, ultimately healing the fracture through intramembranous and endochondral ossification. However, studies[2-4] have demonstrated that about 5-10% fractures could not be able to fully heal, including delayed fracture healing or nonunion. The poor fracture healing significantly reduces the life quality of affected patients, bringing a huge burden on family members and the society. Therefore, it is urgently needed to develop therapeutic strategies to accelerate bone regeneration during fracture healing.

Micro-ribonucleic acids (miRNAs) are endogenous, non-coding and single-stranded RNAs with 19-25 nucleotides in length[5], first discovered by Lee et al[6] in *Caenorhabditis elegans*. MiRNAs affect the expressions of hundreds of genes through the targeted regulation of mRNAs to produce RNA-induced silencing complex, thereby inhibiting or directly destroying translation[5]. According to latest reports, miRNAs regulate nearly 60% genes in the human genome[7]. MiRNAs exert biological functions by directly inhibiting transcription and demethylation, or degrading various mRNA targets. Moreover, miRNAs regulate the activation or inhibition of many osteoblast-related genes, and some miRNAs promote osteoblast differentiation. For example, miR-15b enhances osteoblast differentiation of human MSCs by inhibiting the BMP-binding endothelial regulator[8]. In addition, some miRNA markers can reflect the correlation of the miRNA with bone disease, osteoporosis and osteoarthritis. For example, miR-503 is significantly down-regulated, while miR-133a is remarkably up-regulated in peripheral blood mononuclear cells of patients with postmenopausal osteoporosis[9].

The Wnt signaling pathway plays an important role in the coordination of various biological processes, including proliferation, tissue development, repair and metabolism[10]. According to recent studies, the expression of Wnt ligand protein increases at the fracture end during fracture healing. The Wnt/β-catenin signaling pathway is activated and widely involved in the fracture repair process[11], which is critical for the accumulation of normal bone mass, and controls almost all aspects of osteoblast maturation and function. To improve the therapeutic effect, therefore, various drugs have been studied to promote fracture healing, among which the Wnt/β-catenin signaling pathway-related drugs have been well concerned[12,13]. In this work, fracture model in rats was established to study the potential mechanism of *in vivo* miR-25 and Wnt signaling pathway in fracture healing in rats.

Materials and Methods

Laboratory Animals

A total of 45 adult healthy female Sprague-Dawley rats aged 8 weeks and weighing 200-220 g were selected (Shanghai LSAC Laboratory Animal Co. Ltd., Shanghai, China). They were fed under the environment with appropriate humidity at the room temperature of 20-22°C, with a photoperiod of 12 h. All rats were randomly divided into group A [Phosphate Buffered Saline (PBS), n=15], group B (mimics NC, n=15) and group C (miR-25 mimics, n=15). To avoid confusion, rats in each group were fed in separate cages, and had free access to food and water. The breeding environment was regularly disinfected and kept clean. This study was approved by the Animal Ethics Committee of Zhejiang Chinese Medical University Animal Center.

Establishment of the Rat Model of Fracture

After successful anesthesia *via* intraperitoneal injection of 2% pentobarbital sodium (Shanghai Xianfeng Pharmaceutical Co., Ltd., Shanghai, China, batch No.: S100724) (30 mL/kg), rats were placed on a supine position, and the skin was prepared in the right hind limb. After disinfection and draping, an incision was made in front of the patella to expose the femoral intercondylar notch. A 1.2 mm Kirschner wire was inserted from the femoral intercondylar notch, and the middle femoral fracture was caused using blunt scissors. Finally, the incision was sutured after hemostasis and washing. After the operation, 50000 U penicillin (Shanghai Xianfeng Pharmaceutical Co., Ltd., Shanghai, China, batch No.: S100835) was intramuscularly injected twice a day for consecutive 3 days. Fracture model in rats was confirmed *via* X-ray. From 1 d after modeling, 50 μL (2 nmoL) of PBS (Gibco, Grand Island, NY, USA) was intraperitoneally injected into rats in group A, an equal amount of mimics NC was injected into rats in group B, and an equal amount of miR-25 mimics was injected into rats in group C. The above agents were injected once a week for 6 weeks.

Imaging Analysis

At 1 d after modeling, the anteroposterior X-ray photograph was taken in the right femur, confirm-

ing the successful establishment of the fracture model. Rats were executed at 1 and 7 weeks after the operation. The right femur specimen was taken, the full-length femur was retained, and the Kirschner wire was removed. Anteroposterior X-ray photography was conducted using the X-ray machine (Bangsheng Medical Equipment Co., Ltd., Tianjin, China) (100 mA, 50 kv, projection distance: 90 cm). The femoral fracture healing and callus formation were observed.

Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

At 2 weeks, 4 specimens were taken from each group, and the femoral callus tissues were taken, smashed and mixed evenly, from which the total RNA was extracted. The expression level of miR-25 among group A, B and C was detected *via* RT-PCR. The total RNA was reversely transcribed by TRIzol (B512421, Sangon Biotech, Shanghai, China) using NanoDrop 2000 device (Thermo Fisher Scientific, Waltham, MA, USA), and the complementary deoxyribose nucleic acid (cDNA) samples were obtained using the TaKaRa RNA PCR kit (C506668, TaKaRa, Dalian, China) and Oligo dT primers (Invitrogen, Carlsbad, CA, USA). The miR-25 expression level was detected using the SYBR mixture (TaKaRa, Dalian, China) on a LightCycler 480 device (Roche, Switzerland). Each sample was measured 3 times. The reaction conditions were as follows: 95°C for 5 min for 1 cycle, followed by 40 cycles at 95°C for 45 s, 60°C for 50 s, and 72°C for 40 s. The primer design and synthesis were shown in Table I. MiR-25 expression level was normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Western Blotting Analysis

The complete callus tissues were lysed with radioimmunoprecipitation assay (RIRA) lysis buffer (Beyotime Biotechnology, Shanghai, China) to obtain the total protein, and the protein was detected according to the instructions of the bicinchoninic acid (BCA) protein assay kit (Pierce, Waltham, MA, USA). GAPDH (D4C6R) primary antibody (97564, Cell Signaling Technology, Danvers, MA, USA) diluted at 1:1000 with buffer (Beyotime Biotechnology, Shanghai, China) was added and transferred into an incubator with the membrane at 4°C overnight. After washing, the secondary antibody was added for incubation at room temperature for 1 h, followed by color development and fixation according to the instructions of the Supersignal West Dura Extended Duration Substrate Kit (Millipore, Billerica, MA, USA). The optical density value of the band was analyzed using BandScan 5.0 software 3 times.

Immunohistochemical Staining

At 2 weeks, 4 specimens were taken in each group for immunohistochemical staining. The femur specimens of rats were taken, decalcified in 10% ethylenediaminetetraacetic acid (EDTA) solution (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 1 week, washed with running water for 2 h, dehydrated with gradient alcohol and embedded into paraffin. After cooling, the paraffin block was sliced into 4 μm-thick tissue sections using a microtome, followed by routine deparaffinization and hydration. After the endogenous peroxidase was blocked, sections were incubated with 3% H_2O_2 at room temperature for 30 min, washed with PBS twice (2 min/time), and incubated again with complex enzyme digestive solution at 37°C for 30 min. After washing again with PBS 3 times (2 min/time) for antigen retrieval, sections were sealed with serum for 1 h. After the solution was dried, 50 μL of specific primary antibody working solution was added in each specimen for incubation in a refrigerator at 4°C for more than 17 h, followed by washing with PBS 3 times (5 min/time) and incubation with secondary antibody at 37°C for 1 h. After washing with PBS 3 times (5 min/time), incubation with streptavidin-biotin complex (SABC) reagent (Boster, Wuhan, China) at 37°C for 30 min was performed. One drop of diaminobenzidine (DAB) developing solution was added (Boster, Wuhan, China), the developing effect was observed under a microscope, and the color development was terminated with distilled water. Methyl green counterstaining for 5 min, routine dehydration, transparentization, sealing and microscopic observation and photography were finally conducted.

Biomechanical Test

At 7 weeks, 6 specimens were taken in each group, followed by imaging examination and biomechanical test. The specimens were stored at -80°C. On the evening before the test, all specimens were taken out and naturally thawed at room temperature, and then all soft tissues on the surface of the specimen were removed. The 4-point bending test was performed for all specimens using the biomechanical tester (temperature: 20-25°C, humidity: 70%, lower supporting

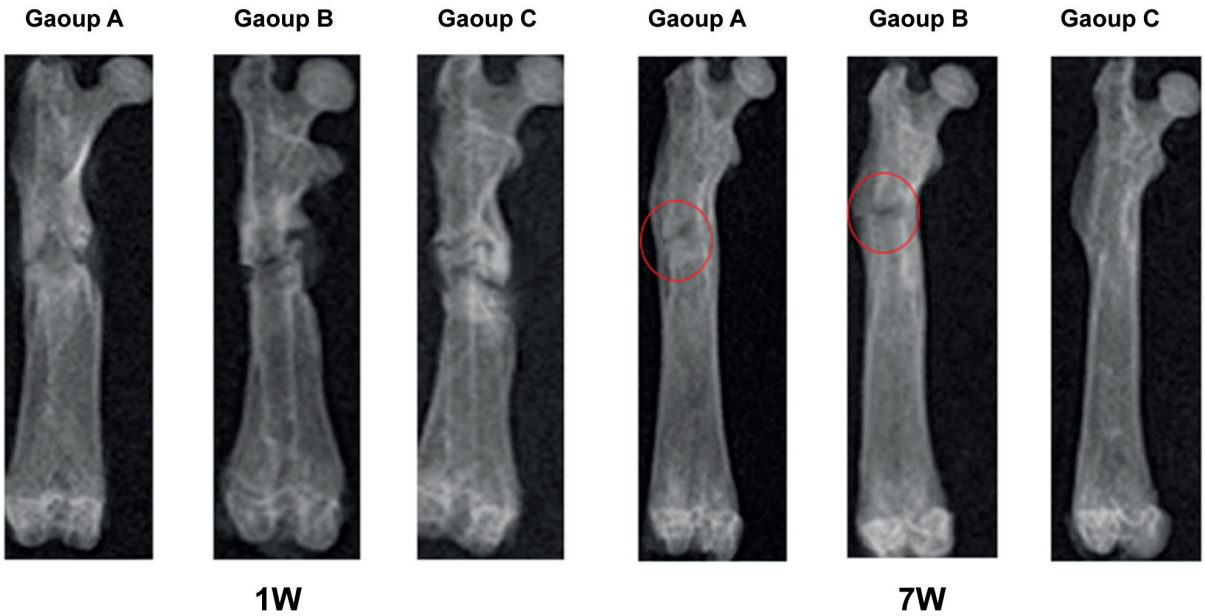

Figure 1. X-ray analysis of femoral fracture healing in rats at 1 and 7 weeks. The fracture line disappears, and both callus formation and fracture healing are good in group C. In group A and B, a few fracture lines can be observed, and both callus formation and fracture healing are poor.

point span: 20 mm, upper supporting point span: 8 mm). In the biomechanical test, the femur specimens were loaded from top to bottom at a rate of 2 mm/min in the same position with the fracture site as the center. The corresponding biomechanical indexes, including stiffness (N/mm, defined as the slope of the pressure-displacement curve), maximum load (N) and fracture energy (N/mm, defined as the area under the pressure-displacement curve), were automatically calculated using the in-built software.

Statistical Analysis

All data in this experiment were expressed as mean ± standard error of mean (Mean ± SEM), and the experimental results were statistically analyzed using Statistical Product and Service Solutions (SPSS) 21.0 software (IBM, Armonk, NY, USA). The *t*-test was used for the mean comparison between the two groups. One-way analysis of variance was used for the intergroup comparison of mean samples, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

Imaging Analysis

At 1 and 7 weeks after successful modeling, the anteroposterior X-ray photograph was taken in the right femur, and femoral fracture healing and callus formation among group A, B and C were compared to detect whether there were differences in these indexes. The results revealed that the alignment of femoral fracture was good without significant fracture displacement in all groups. In group C, the callus diameter was larger, and the density on both sides of callus was higher. In group A and B, there were callus formation and increased callus density, but the fracture line could still be observed. Fracture healing was better and there was more and better callus formation in group C compared with the other two groups, displaying significant differences (Figure 1).

MiR-25 Expression in the Three Groups Detected Via RT-PCR

At 2 weeks, 4 specimens were taken from each group, and the femoral callus tissues were taken, smashed and mixed evenly, from which the total RNA was extracted. MiR-25 expression among group A, B and C was detected *via* RT-PCR. As shown in Figure 2, there was no statistically significant difference in the expression of miR-25 between group A and B ($p>0.05$). Compared with that in group A and B, the expression of miR-25 markedly increased in group C, and the differences were statistically significant ($p<0.01$).

Western Blotting Results

At 7 weeks after the operation, the protein expressions of β-catenin, PCNA and BMP-2 were detected in group A, B and C. The results showed that the protein expressions of β-catenin, PCNA and BMP-2 in group C were remarkably higher than those in group A and B (p<0.01) (Figure 3).

Immunohistochemical Staining Results

A variety of factors are involved in the regulation during osteoblast differentiation. For example, OCN, PCNA and BMP-2 are markers for the osteoblast differentiation and maturation, which are used as markers for bone formation. Immunohistochemical staining results manifested that the number of OCN-, PCNA- and BMP-2-positive osteoblasts in miR-25 mimics group markedly increased compared with that in the other two groups, and there were statistically significant differences (p<0.01). It is indicated that the osteoblast differentiation in miR-25 mimics group was affected. The above immunohistochemical results suggested that the osteoblast differentiation at the fracture end in the miR-25 mimics group was remarkably enhanced compared with that in the control groups (Figure 4-6).

Biomechanical Test

At 7 weeks after the operation, the biomechanical test was performed for femur specimens. It was found that in the miR-25 mimics group, the maximum load, fracture energy and stiffness increased by 188%, 333% and 90%, respectively, compared with those in the PBS group (p<0.01) (Table II). It is indicated that miR-25 promoted the mechanical properties of fracture healing. No significant differences in the maximum load, fracture energy and stiffness were found between the PBS group and mimics-NC group (Table II).

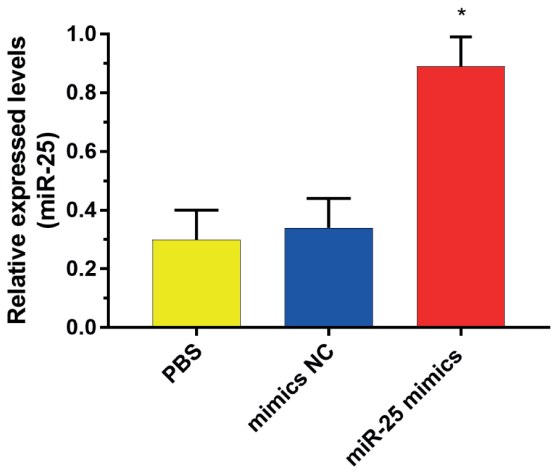

Figure 2. MiR-25 expression in the three groups detected *via* RT-PCR. MiR-25 expression in miR-25 group, PBS group and mimics NC group (*p<0.01).

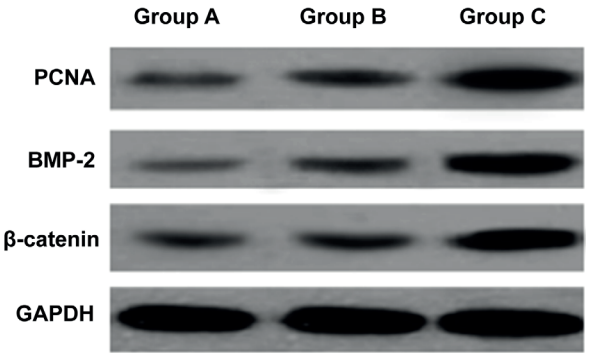

Figure 3. Protein expressions of β-catenin, PCNA and BMP-2 analyzed *via* Western blotting. The protein expressions in group C are markedly higher than those in group A and B, showing statistically significant differences (p<0.01).

Discussion

Fracture is a very common disease, but the concrete mechanism of fracture healing has not been studied clearly. Poor fracture healing is the most common complication after fracture. Therefore, it is of great significance to study the mechanism of promoting fracture healing. With the deepening of research on biomolecules and gene levels, it has been found that miRNAs can promote osteogenic differentiation of bone marrow MSCs[14], thus regulating the fracture healing. To explore the potential mechanism of fracture healing at a new level, miR-25 was screened as the miRNA we studied.

In this work, RT-PCR was performed for callus tissues in group A, B and C at 2 weeks after the operation. The results manifested that the ex-

Table I. Primer sequences.

Gene	Primer sequence
miR-25-F	CAU UGC ACU UGU CUC GGU CUG A
miR-25-R	UUC AAG UAA UCC AGG AUA GGC U
U6-F	CTC GCT TCG GCA GCA CA
U6-R	AAC GCT TCA CGA ATT TGC GT
GAPDH-F	TCA CTG CCA CCC AGA AGA
GAPDH-R	AAG TCG CAG GAG ACA ACC

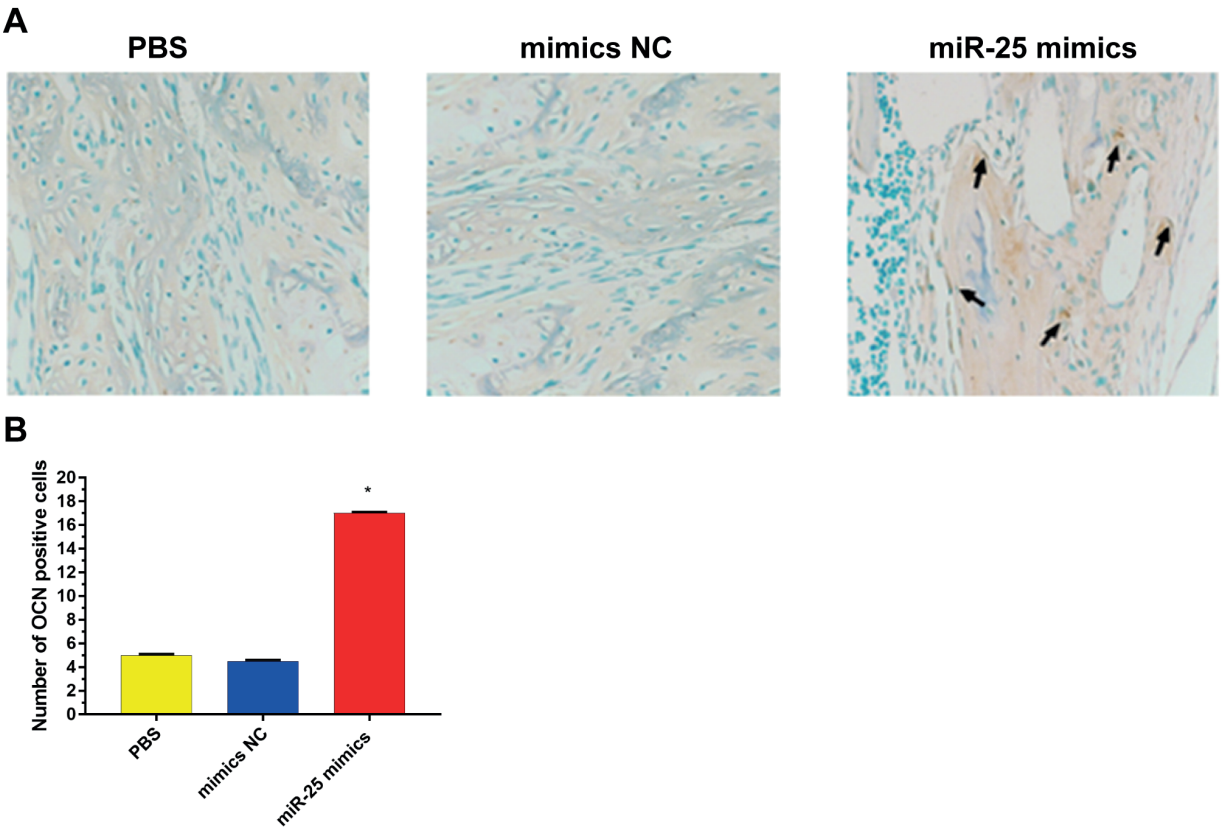

Figure 4. ***A,*** Immumohistochemical staining results and ***B,*** quantitative values of OCN in femur in miR-25 mimics group and control groups (× 400, **p*<0.01 *vs.* Control).

pression of miR-25 was detected in all groups, but it was significantly higher in group C than that in group A and B ($p<0.01$), indicating that the miR-25 mimics injected into rats in group C successfully overexpressed miR-25. To compare the fracture healing and callus formation, the anteroposterior X-ray photograph was taken in the right femur in all groups at 1 and 7 weeks after operation, and it was found that the alignment of femoral fracture was good without significant fracture displacement in all groups. In group C, the callus diameter was larger, and the density on both sides of callus was higher. In group A and B, there were callus formation and increased callus density, but the fracture line could still be observed, suggesting that the fracture healing was better in group C and poor in group A and B. A variety of factors are involved in the regulation during osteoblast differentiation. For example, OCN, PCNA and BMP-2 are markers for the osteoblast differenti-

Table II. Biomechanical analysis of femur.

Parameter	Time	n	PBS	miR-25 mimics	mimics-NC
Load	7w	6	48.2±6.12	139±11.09	45.3±5.88
Energy	7w	6	34.2±4.63	148±9.56	35.7±4.63
Stiffness	7w	6	81.2±7.41	154±12.18	81.2±7.41

At 7 weeks after the operation, the biomechanical properties of femoral healing are analyzed via the 4-point bending test. The maximum load (load), fracture energy (energy) and stiffness (stiffness) when the fracture occurs again are calculated according to the load and displacement curves. Data are expressed as mean ± standard deviation.

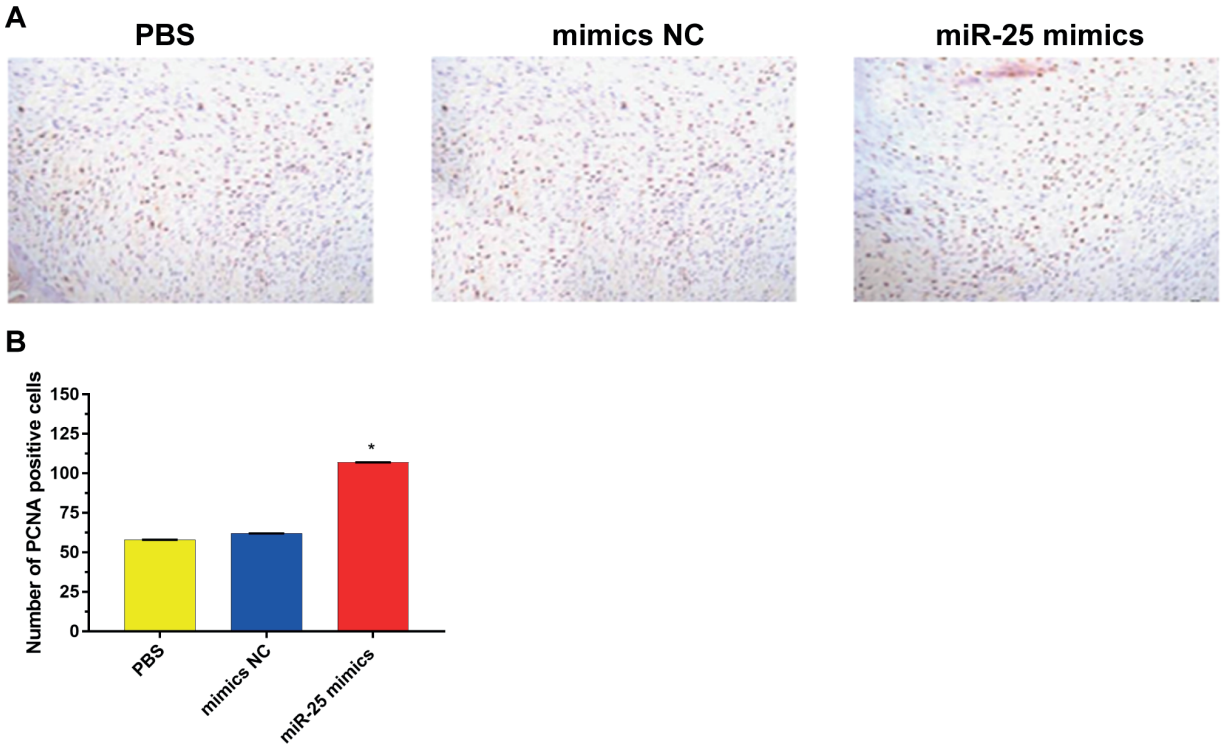

Figure 5. Immunohistochemical analysis of PCNA expression. ***A,*** PCNA-labeled cells in miR-25 mimics group and control groups at 2 weeks after operation, ***B,*** the number of PCNA-positive cells, mean ± standard deviation. (×400, *p<0.01 *vs.* Control).

ation and maturation, considering as markers for bone formation. Immunohistochemical staining was performed for determining OCN, PCNA and BMP-2. The results manifested that the number of OCN-, PCNA- and BMP-2-positive osteoblasts in group C was remarkably increased compared with that in the other two groups (p<0.01), indicating that the overexpression of miR-25 in group C promoted osteoblast differentiation. It is believed that miR-25 is involved in regulating the fracture healing process, and the overexpression of miR-25 promotes fracture healing. Li et al[15] also studied and found that miR-25 can promote osteogenic differentiation of bone marrow mesenchymal stem cell, which is consistent with the experimental results in this study.

The Wnt signaling pathway plays an important role in the coordination of various biological processes, including proliferation, tissue development, repair and metabolism[10]. Recent studies have demonstrated that the Wnt/β-catenin signaling pathway is activated and widely involved in the fracture repair process[11]. Therefore, the downstream proteins of the Wnt signaling pathway, including β-catenin, PCNA and BMP-2, were analyzed *via* Western blotting. The results showed that the expressions of β-catenin, PCNA and BMP-2 were significantly higher in group C than those in group A and B (p<0.01), clearly indicating that miR-25 promoted the expression of downstream proteins by activating the Wnt signaling pathway, and the overexpression of miR-25 can markedly activate the Wnt signaling pathway, significantly enhancing the expression of downstream proteins (β-catenin, PCNA and BMP-2).

To further verify the fracture healing in all groups, the biomechanical test was performed for femur specimens. It was found that in group C, the maximum load, fracture energy and stiffness increased by 188%, 333% and 90%, respectively, compared with those in group A (p<0.01). We believed that the overexpression of miR-25 remarkably promoted the mechanical properties of fracture healing.

Conclusions

MiR-25 closely related to fracture healing was screened in this study, and it was found

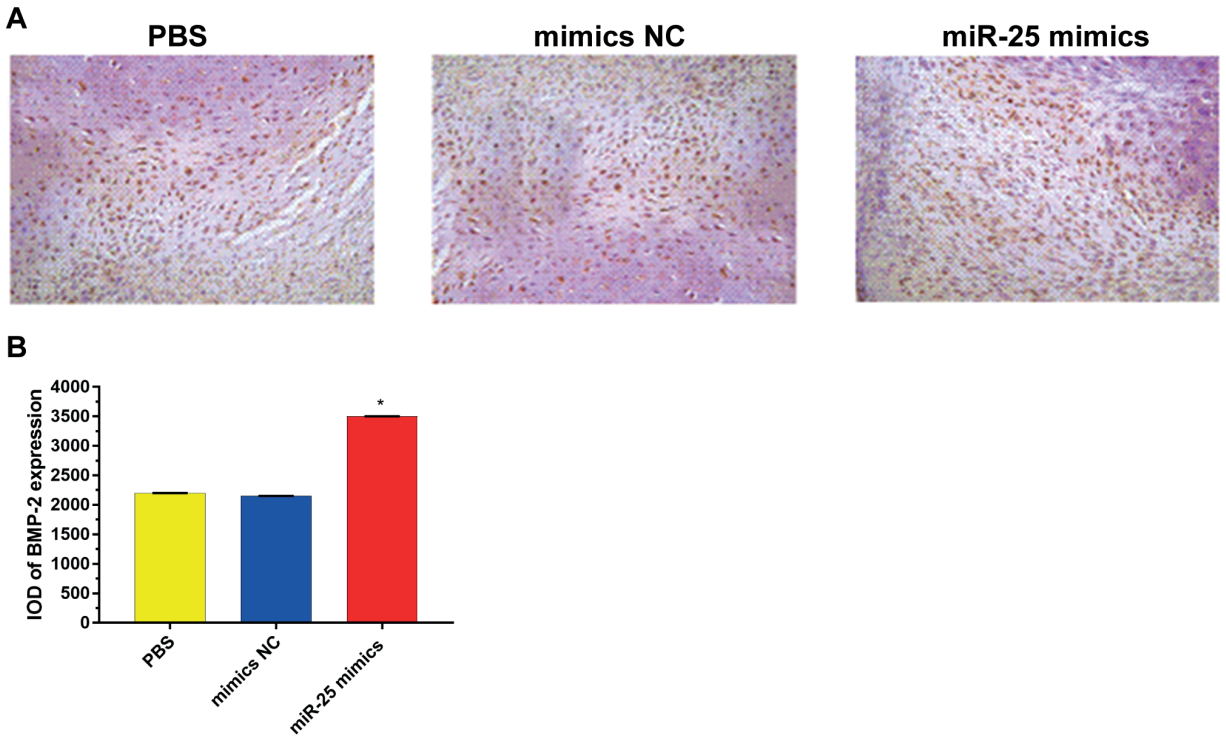

Figure 6. ***A,*** Immunohistochemical staining of BMP-2 expression in miR-25 mimics group and control groups at 2 weeks after operation. ***B,*** IOD analysis of BMP-2 expression in callus, mean ± standard deviation. (×400, **p*<0.01 *vs.* Control).

that the changes in the miR-25 expression in the rat model of fracture had close correlation with the expression of downstream proteins of the Wnt signaling pathway (β-catenin, PCNA and BMP-2), proving that the overexpression of miR-25 can activate the Wnt signaling pathway to promote fracture healing. This work provides *in vivo* basis on the mechanism of fracture healing, and lays a foundation for more in-depth studies on the mechanism of human fracture healing, which remains to be explored by further experiments.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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