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MiR-21 promotes fracture healing by activating the PI3K/Akt signaling pathway

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Abstract. – OBJECTIVE: This study aims to elucidate the potential mechanism of micro ribonucleic acid (miR)-21 in promoting fracture healing.

MATERIALS AND METHODS: 30 male Sprague-Dawley rats were randomly divided into group A (phosphate-buffered saline, PBS, n=10), group B (AntagomiR-21, n=10) and group C (AntagomiR-NC, n=10) according to the different treatments. The femoral fracture operation was performed in every rat, which was pathologically diagnosed via X-ray. After the successful modeling, 50 μL (2 nmoL) PBS, 50 μL AntagomiR-21 or 50 μL AntagomiR-NC was intraperitoneally injected into rats in group A, B or C, respectively. The above agents were injected once a week for 6 weeks. At 6 weeks, 3 rats were executed in each group, and the tissue at the fracture end was observed via hematoxylin-eosin (HE) staining. The fracture healing of rats was evaluated via imaging at 1 and 7 weeks. At the same time, the expression of miR-21 in the three groups was detected via Reverse Transcription-Polymerase Chain Reaction (RT-PCR), and the expression of phosphatidylinositol 3-hydroxy kinase (PI3K) and phosphorylated-serine/threonine-protein kinase B (p-Akt) in the three groups was detected via Western blotting.

RESULTS: According to the histological staining, the bone repair at the fracture end of rats in group B was not significant with fracture and poor continuity compared with those in group A and group C. The imaging evaluation revealed that in group B, the callus tissues were significantly reduced, the fracture line had undesirable healing. There were no displacement and loosening of internal fixation compared with group A and group C. Besides, RT-PCR showed that the miR-21 expression declined markedly in group B compared with that in group A and group C, and the differences were statistically significant ($p<0.05$). Western blotting manifested that the protein levels of PI3K and p-Akt also declined in group B compared with those in group A and group C, and there were statistically significant differences ($p<0.05$).

CONCLUSIONS: MiR-21 promotes the fracture healing in fractured rats by activating the PI3K/Akt signaling pathway.

Key Words:
MiR-21, PI3K/Akt signaling pathway, Fracture healing.

Introduction

The bone fracture is the most prevalent trauma-induced damage to the human body[1]. Although the bone has the self-reconstruction and repair ability, bone nonunion and delayed healing occur in about 10-20% fracture diseases[2]. The delayed healing often extends the time of internal fixation and greatly increases the risk of ankylosis. Therefore, it is extremely important to deeply study the mechanism of promoting fracture healing.

Micro ribonucleic acid (miRNA) is an endogenous short-stranded RNA with about 20 (18-24) bases in length, as well as one type of non-coding RNA, which, through the targeted induction of messenger RNA (mRNA) silencing or degradation, affects its stability and translation process. Functionally, miRNAs widely paticipate in regulating the expressions of the target genes[3]. Increasingly more evidence has proved that some miRNAs can promote the differentiation of bone mesenchymal stem cells into osteoblasts, and play important roles in skeletal development and homeostasis of bone[4-6]. At the same time, miRNAs are involved in the pathophysiological process of a variety of diseases, and abnormally expressed in many diseases[7-10]. For example, Jones et al[11] found that both miR-9 and miR-98 are highly expressed in human osteoarthritis tissues. A study[12] demonstrated that the changes in miRNA expression level may alter its regulatory function at the post-transcriptional level, thus leading to a series of physiological changes. Although the understanding of the function of miRNA is not comprehensive enough, it is believed that miRNA may play a key role in fracture healing in light of these findings. Recent investigagions[13] have shown that

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the expression level of miR-21 is up-regulated during the differentiation of mesenchymal stem cells into osteoblasts. Another study[14] manifested that the overexpression of miR-21 during osteogenic differentiation can promote the formation of calcium nodules.

The serine/threonine kinase (Akt) was originally reported as a proto-oncogene. Recently, it has been identified to regulate various in vivo biological processes. Multiple growth factors contribute to activating phosphatidylinositol 3-hydroxy kinase (PI3K) by binding to their receptors. The resulting product further binds to Akt, thus phosphorylating Akt and acting on downstream target proteins Bad and Caspase[9], ultimately regulating cell proliferation and differentiation[15-17]. In addition, PI3K/Akt, as a regulator during bone metabolism, can promote bone formation and differentiation of immature osteoblasts into mature osteoblasts[18].

Fracture healing is a complex biological process, which plays an important role in the development and function of osteoblasts and osteoclasts in bone formation. In this study, the animal model of fracture was established to explore the potential mechanism of miR-21 and the PI3K/Akt signaling pathway in promoting *in vivo* fracture healing.

Materials and Methods

Grouping of Laboratory Rats and Establishment of Rat Model of Fracture

30 healthy male Sprague-Dawley rats aged 12 weeks and weighing 480-550 g were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). They were randomly divided into group A (phosphate-buffered saline, PBS, n=10), group B (AntagomiR-21, n=10) and group C (AntagomiR-NC, n=10). All reagents were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The femoral fracture in the right lower limb of 30 rats was induced via operation. Briefly, rats were anesthetized with 3% pentobarbital sodium (0.1 g/kg) (Sigma-Aldrich, St. Louis, MO, USA), the limbs were fixed, and the hair was shaved off in the surgical field. After disinfection and draping in a left lateral position, the middle transverse fracture in the right femur was artificially caused using the wire saw. A small incision was made in the medial knee of the right lower limb, and a prebored hole was drilled in the intercondylar knee using the 1.0 mm Kirschner wire. Subsequently, the 1.2 mm Kirschner wire was inserted into the femoral marrow cavity after the reduction of the femoral fracture. Then the Kirschner wire was bent and sheared off using a bending tool. Finally, the wound was rinsed, and the skin was sutured layer by layer after thorough hemostasis. 50,000 U penicillin (Shanghai Xianfeng Pharmaceutical Co., Ltd., Shanghai, China, Batch No.: S100824) was intramuscularly injected into rats twice a day for 3 consecutive days after the operation. The successful establishment of rat model of fracture was confirmed via X-ray. This study was approved by the Animal Ethics Committee of the Huazhong University of Science and Technology Animal Center.

Treatment of Rat Model of Fracture in Each Group

All rats were fed in separate cages without limited activities under a constant temperature of 21°C and 12/12 h diurnal cycle. After the successful modeling, 50 μL (2 nmoL) phosphate-buffered saline (PBS), 50 μL AntagomiR-21 or 50 μL AntagomiR-NC was intraperitoneally injected into rats in group A, B and C, respectively. The above agents were injected once a week for 6 weeks.

Imaging Analysis

After the fracture model was successfully established and agents were injected, the lateral thigh X-ray (52 kV, 4.5 mAs, 0.5 s) was performed at 7 and 9 d. Callus growth, internal fixation position, fracture line healing and fracture alignment at the fracture end were observed through X-ray film. Every X-ray film was analyzed by a radiologist, a laboratory researcher and an orthopedist, independently. After the last analysis, the rats were decapitated.

The callus tissues were taken from the corpse, placed in a low-temperature tissue storage tube with liquid nitrogen added quickly, and stored in the ultra-low temperature manual climatic box.

Histological Staining

A total of 3 rats in each group were executed at 6 weeks after the operation. The skin was cut and the muscle was separated quickly. After the femur was dissociated, it was fixed with 10% formaldehyde, decalcified with 9% formic acid and embedded in paraffin. After standardized treatment, the specimens were sliced into 5 μm-thick longitudinal sections and deparaffinized, followed by hematoxylin-eosin (HE) staining to observe the tissue staining.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

The total RNA was extracted using TRIzol reagent (Sangon Biotech, Shanghai, China) and reversely transcribed using the NanoDrop 2000 device (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA samples were obtained using the TaKaRa RNA PCR kit (TaKaRa, Dalian, China) and Oligo dT primers (Invitrogen, Carlsbad, CA, USA). The miR-21 expression level was detected using SYBR mixture (TaKaRa, Dalian, China) on the LightCycler 480 device (Roche, Basel, Switzerland). Each sample was independently detected 3 times. The primer design and synthesis were shown in Table I. The miR-21 expression level was normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the data were analyzed using the 2-ΔΔCT method.

Western Blotting Analysis

Rat callus tissues were taken from washing twice with ice-cold saline and ground evenly. According to the instructions of the whole protein extraction kit, the tissues were added with lysis buffer, homogenized in the tissue homogenizer for 1 min and centrifuged at 12000 rpm and 4°C for 15 min. The supernatant was collected for determining protein concentration using the bicinchoninic acid (BCA) protein quantification kit (Pierce, Waltham, MA, USA). Extracted protein was stored at -70°C for later use. The whole protein extract was mixed with 2× loading buffer (1:1), boiled via boiling water bath for 5 min, cooled naturally and stored in a refrigerator at 4°C for later use. The separation gel in an appropriate ratio for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was prepared according to the molecular weight of the target protein and solidified for about 1 h. 5% SDS-PAGE spacer gel was prepared and solidified for about 0.5 h. The electrophoresis buffer and denatured protein samples were added into the loading well for loading based on the protein concentration (the total protein content in each well was the same). Electrophoresis was performed at a constant voltage of 220 V until the bromophenol blue reached the bottom of the gel. The gel was cut according to the molecular weight of the target protein and placed into the transfer buffer. A layer of polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland) and 6 layers of filter paper were cut according to the size of the gel. The PVDF membrane was soaked in methanol for 10 s, and then the PVDF membrane and filter paper were put into the transfer buffer. The positive electrode - 3 layers of filter paper - PVDF membrane - gel - 3 layers of filter paper - negative electrode were placed into the transfer device in order, and the edge was aligned to prevent blistering. The protein was transferred at a constant pressure of 110 V for 2 h. The protein-attached PVDF membrane was blocked with 5% skim milk powder at room temperature on a shaking table for 2 h. After washing with Tris-buffered saline with Tween-20 (TBST) for 5 min, membranes were incubated with the primary antibody in the corresponding ratio at 4°C overnight. At the other day, they were incubated again with the corresponding secondary antibody on the shaking table at room temperature for 3 h and washed again with TBST 3 times (10 min/time). The gel imager was turned on for preheating for 30 min, followed by band exposure using the enhanced electrochemiluminescence (ECL) kit (Thermo Fisher Scientific, Waltham, MA, USA). Finally, the image was analyzed using the image analysis software.

Statistical Analysis

All data in this experiment were expressed as mean ± standard error of mean (Mean ± SEM), and statistical analysis was performed for experimental results using the Statistical Product and Service Solutions (SPSS) 21.0 software (IBM, Armonk, NY, USA). The *t*-test was used for the comparison of means between the two groups. One-way analysis of variance was used for the intergroup comparison of samples, followed by the

Table I. RT-PCR primer sequences of miR-21.

Gene	Primer sequences
miR-21	5'-3' CACCTAGCTTATCAGACTGATGTTGATTTTTTG 3'-5' ATCGAATAGTCTGACTACAACTAAAAAACTCGA
U6-F	CTCGCTTCGGCA GCA CA
U6-R	AAC GCT TCA CGA ATT TGC GT

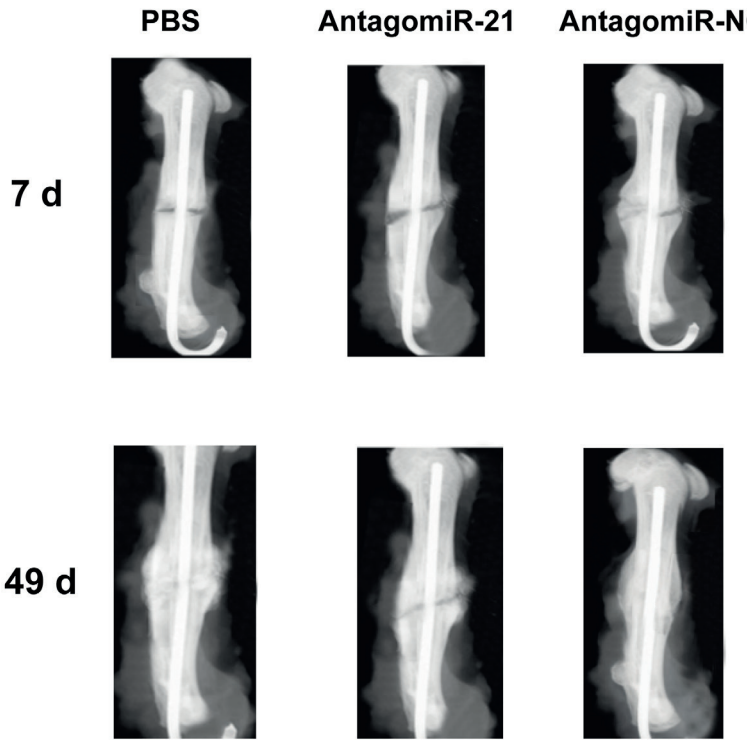

Figure 1. Imaging evaluation.

post-hoc test. $p<0.05$ suggested that the difference was significant.

Results

Imaging Analysis

The X-ray images were taken at 7 and 49 d after the operation to observe the callus formation and fracture line healing at the fracture end. The imaging evaluation revealed that in group B, the callus tissues were significantly reduced, the fracture line had undesirable healing. There were no displacement and loosening of internal fixation compared with group A and group C (Figure 1), indicating that miR-21 knockdown suppressed the fracture healing in rats.

Histological Staining

A total of 3 rats in each group were executed at 6 weeks after operation. The skin was cut and the muscle was separated quickly. After the femur was dissociated, it was fixed with 10% formaldehyde, decalcified with 9% formic acid, embedded in paraffin and sliced into sections. HE staining was performed to observe the tissues at the fracture end at 6 weeks. It was observed that in group A and group C, the bone repair at the fracture end was significant and the continuity of bone was repaired. In group B, the bone repair at the fracture end was not significant with fracture and poor continuity (Figure 2).

MiR-21 Expression in the Three Groups Detected via RT-PCR

After successful establishment of the rat model of fracture, 50 μL (2 nmoL) PBS, 50 μL AntagomiR-21 or 50 μL AntagomiR-NC was intraperitoneally injected into rats in group A, B and C, respectively. The above agents were injected once a week for 6 weeks. After the rats were decapitated, the miR-21 expression in callus tissues in the three groups was detected via RT-PCR. The results showed that the miR-21 expression had no statistically significant difference between group A and group C ($p>0.05$). However, it declined markedly in group B compared with that in group A and group C, and the differences were statistically significant ($p<0.01$) (Figure 3).

Western Blotting

The protein expressions of PI3K and p-Akt in callus tissues in the three groups were detected

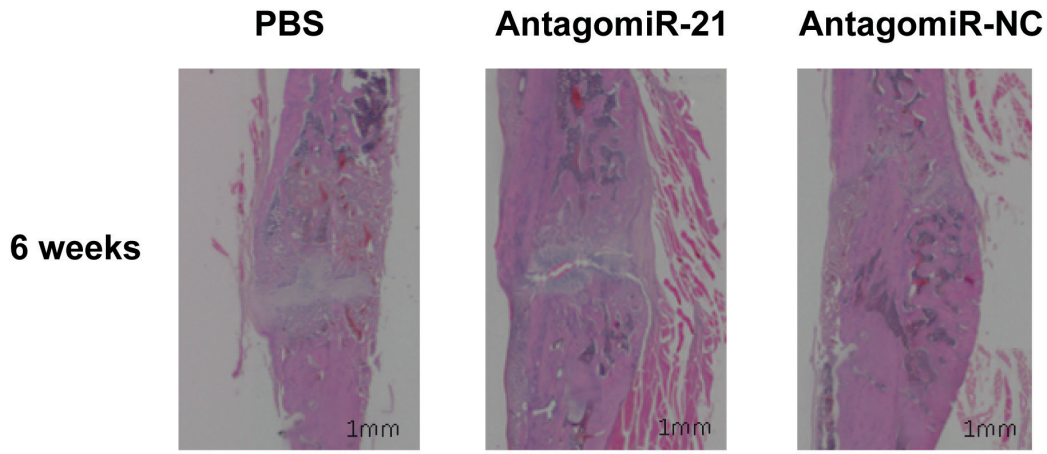

Figure 2. HE staining results in the three groups at 6 weeks (Magnification x 10). In group ***A*** and group ***C,*** the bone repair at the fracture end is significant and the continuity of bone is repaired. In group ***B,*** the bone repair at the fracture end is not significant with fracture and poor continuity.

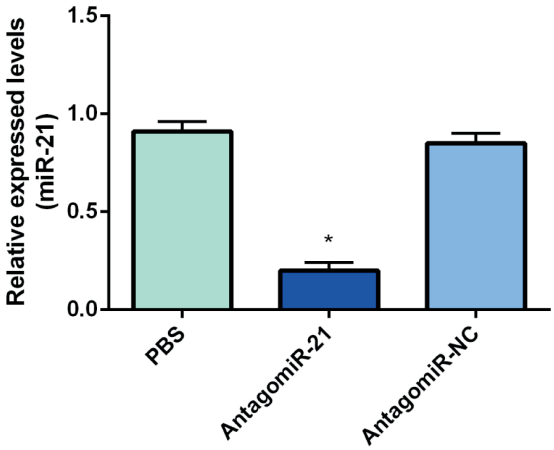

Figure 3. MiR-21 expression in the three groups detected via RT-PCR. The miR-21 expression declines markedly in group B compared with that in group A and C (*p<0.01).

key factors of osteoblast differentiation (Runx2 and TGF-β) during the differentiation of osteoblasts. Studies[21,22] have found that the bone mass and trabecular number are decreased significantly, and the ALP activity also declines in mice with miR-1792 knockout. Sun et al[23] reported that the miR-21 overexpression can promote the differentiation of bone mesenchymal stem cells into osteoblasts, thereby promoting both bone formation and fracture healing, which is consistent with our experimental results. In this work, the miR-21 expression was inhibited in the rat model of fracture and resulted in delayed fracture healing of rats, reversely proving that miR-21 can promote fracture healing. In the PI3K/Akt pathway, PI3K can stimulate the differentiation of bone mesenchymal stem cells into osteoblasts and promote the bone formation[24], which are

via Western blotting. It was found that the protein expressions of PI3K and p-Akt declined significantly in group B compared with that in group A and group C (p<0.05) (Figure 4).

Discussion

Evidence has proved that miRNAs play important roles in regulating the osteogenic differentiation and osteogenic repair in fracture healing[19]. Li et al[20] reported that miR-29 can activate the osteoblast differentiation by promoting the

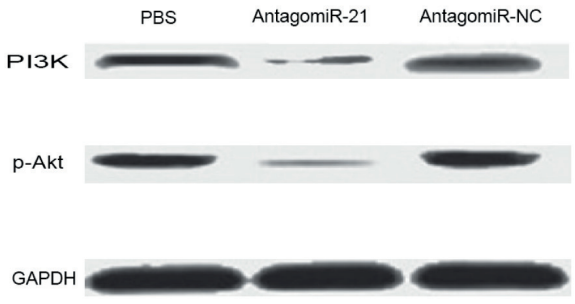

Figure 4. The protein expression of PI3K and p-Akt declines significantly in group B compared with that in group A and group C (*p<0.05).

also consistent with our experimental results. In this experiment, Western blotting revealed that the expressions of PI3K and Akt declined remarkably in group B with poor fracture healing compared with those in group A and group C with good fracture healing. It is proved that inhibiting the PI3K/Akt pathway aggravated fracture healing, and the PI3K/Akt signaling pathway may serve as a potential mechanism of promoting fracture healing.

In this work, the rat model of fracture was established. 50 μL (2 nmoL) PBS, 50 μL AntagomiR-21 or 50 μL AntagomiR-NC was intraperitoneally injected into rats in group A, B and C, respectively. X-ray imaging evaluation revealed that in group B, the formation of callus tissues was significantly insufficient, the fracture line had undesirable healing. However, there were no displacement and loosening of internal fixation compared with group A and group C. At 6 weeks after injection, HE staining showed that in group A and group C, the bone repair at the fracture end was significant and the continuity of bone was repaired. In group B, the bone repair at the fracture end was not significant with fracture and poor continuity. The miR-21 expression in the three groups was detected via RT-PCR, and the results showed that the miR-21 expression declined markedly in group B compared with that in group A and group C. Moreover, the expression levels of PI3K and Akt in the three groups were detected via Western blotting, and it was found that they also declined significantly in group B compared with those in group A and group C. The above results indicate that inhibition of miR-21 expression suppressed the fracture healing in fractured rats. It is speculated combined with HE staining and Western blotting results that miR-21 promoted fracture healing by activating the PI3K/Akt signaling pathway. To further study the mechanism of fracture healing, the living model was provided in this work, laying a foundation for the more in-depth study on human fracture healing. More experiments are still needed to deeply study the mechanism of fracture healing.

Conclusions

We observed that miR-21 promoted the fracture healing in fractured rats by activating the PI3K/Akt signaling pathway.

Conflict of interest

The authors declare no conflicts of interest.

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