European Review for Medical and Pharmacological Sciences 2018; 22: 17-24

MiR-214-3p inhibits β-catenin signaling pathway leading to delayed fracture healing

J.-W. TENG[1], P.-F. JI[2], Z.-G. ZHAO[3]

[1]Department of Microscopic Orthopedics, Affiliated Hospital of Shandong University of Chinese Medicine, Shandong, China
[2]Department of Orthopedics, Qihe People's Hospital of Shandong Province, Shandong, China
[3]Department of Orthopedics, Anqiu People's Hospital, Shandong, China

Jiawen Teng and *Pengfei Ji* contributed equally to this work

Abstract. – OBJECTIVE: To investigate the effect of micro ribonucleic acid (miR)-214-3p on the fracture healing process of mice and its mechanism.

MATERIALS AND METHODS: 90 mice were selected and randomly divided into three groups to establish the right tibial fracture model. AgomiR-214-3p or agomiR negative control (agomiR-NC), or the same volume of phosphate-buffered saline (PBS), was injected locally at 0 d, 7 d, 14 d and 21 d after operation, respectively. At the end of the experiment, the imageological observation, histological observation and the detection of callus osteocalcin level were conducted for mice in each group to evaluate the fracture healing. At the same time, Real-time polymerase chain reaction (RT-PCR) and Western blotting were used to detect the expression of β-catenin at different time points in each group.

RESULTS: Imageological and histological observations showed that the fracture lines of mice in the PBS injection group and the agomiR-NC injection group were found to be healed at 28 d after fractures, while fuzzy fracture lines could be seen in mice with fewer calluses in the agomiR-214-3p injection group, and the expression level of osteocalcin at each time point in the agomiR-214-3p injection group was decreased compared with that in the control group. In addition, RT-PCR and Western blotting results revealed that the expression level of the miR-214-3p target gene, β-catenin, was decreased at each time point in the agomiR-214-3p group compared with that in the control group.

CONCLUSIONS: MiR-214-3p delays the fracture healing by inhibiting the Wnt/β-catenin signaling pathway.

Key Words:
Fracture healing, miR-214-3p, Wnt/β-catenin signaling pathway.

Introduction

The fracture is the most serious complication of osteoporosis and is one of the most common diseases in orthopedic clinics. Once it occurs, it will seriously affect the quality of life of patients and even threaten their lives[1]. Fractures trigger a complex and highly regulated bone regeneration process, whose purpose is to restore the original structure and function of bones. The process of fracture healing is similar to the long bone development in the embryonic period, and its original structure and mechanical function can be basically restored through tissue remodeling with no scar tissues[2]. Fracture healing is affected by a variety of factors, including the trauma energy of fractured ends, local load size, fracture type, stability of fractured ends, compression or displacement degree and the degree of soft tissue injury[3-5]. However, from the perspective of molecular biology, functions of osteoblasts play important roles in the process of fracture healing. Osteoblasts secrete and synthesize procollagens into the extracellular space under the action of various cytokines such as bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), platelet-derived growth factors (PDGFs) and fibroblast growth factors (FGFs), so as to form Type I collagen network. Then, these procollagens were deposited on its grid in the form of calcium phosphate crystals, thus forming a mineralized bone matrix[6-8]. On the other hand, osteoblasts complete new bone remodeling by secreting receptor activator for nuclear factor-κB ligands (RANKLs) and inducing the maturation of osteoclasts[9]. However, the regulation mechanism of osteoblast functions in the process of fracture

Corresponding Author: Zhigang Zhao, MD; e-mail: shoushi43064@163.com

healing remains to be further studied. Micro ribonucleic acids (miRNAs) are a class of endogenous single-strand non-coding RNA molecules with the length of 19-25 nucleotides. MiRNA can degrade proteins or inhibit the translation of messenger RNA (mRNA) after transcription by binding to it, playing an important role in physiology, development and pathology. The differentiation and function of osteoblasts are also regulated by miRNAs. It has been reported that miR-26a, miR-133, miR-135, miR-29, miR-141, etc., can inhibit the differentiation of osteoblasts by regulating the key proteins in the differentiation process of osteoblasts[10-14]; miR-2861/3960 can promote the differentiation of osteoblasts[15,16], and miR-93 can inhibit the mineralization of osteoblasts by inhibiting the expression of the transcription factor, Sp7[17]. In addition, researchers found that in bone tissues of aging female patients with osteoporosis, the highly expressed miR-214-3p in osteoclasts can be secreted to the extracellular site in the form of encapsulated exosomes, which inhibits the activity of osteoblasts, thus reducing the osteogenic capability and delaying fracture healing[18]. Therefore, we established a model of fracture healing in mice with highly expressed miR-214-3p on the basis of the animal model of internal fixation of tibial fractures, observed the effect of miR-214-3p on the fracture healing *in vivo*, and investigated its potential mechanism.

Materials and Methods

Experimental Animals and Methods

90 clean male C57BL/6 mice at the age of 10-12 weeks old were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). They were randomly divided into 3 groups, 30 mice in each group, using a random number table. Group A was the phosphate-buffered saline (PBS) control group, Group B was the agomiR negative control (agomiR-NC) group, and Group C was the agomiR-214-3p group. The model of right tibial fractures in mice was established according to the method in the following literature. The mice were weighed after they were numbered, and then they were injected with 10% chloral hydrate for anesthesia. After the mice were completely anesthetized, they were fixed on the operating table, and their hair in the surgical area was cut. Conventional iodophor disinfection and draping were conducted, and then the middle of the knee was longitudinally incised with about 2 cm skin incision. The surrounding tissues were separated, and the tibia was exposed. The tibia was sawed off using a metal saw at about 1 cm under the knee. The action should be gently performed to reduce hemorrhage. After the well reduction of fractured ends, the assistant used a drill to insert a 1.0 mm Kirschner wire for intramedullary fixation. After the firm fixation, the Kirschner wire was cut off. After the wound was washed with normal saline, it was sutured layer by layer. The penicillin sodium was intramuscularly injected after operation for continuous 3 d to prevent infection. The whole operation was conducted in strict accordance with the principle of sterility. The mice were placed in a cage under the close observation until they woke up, and then they freely ate food and drank water. The suture was not taken out after operation, so that it came off naturally. 50 μL (2 nmol) agomiR-214-3p or agomiR-NC, or the same volume of PBS, was injected locally in mice of each group at 0 d, 7 d, 14 d and 21 d after fractures. This study was approved by the Animal Ethics Committee of Shandong University of Chinese Medicine Animal Center.

Radiological Analysis

At 7 d and 28 d after operation, mice were anesthetized (6 in each group) until their muscles were loose, then the mice were fixed. After anesthesia, the mice were fixed on A3 papers with medical tapes with the abduction and external rotation of two lower limbs. X-ray shooting: 41 KV, 160 mA and 8 mAh were set as fixed parameters of X-ray instrument, and the right tibial lateral X-ray of each mouse was shot. Radiograph reading and analysis: analysis contents included the location of fractures, fracture types, density and size of the callus and the state of fracture lines; the results were independently evaluated by three persons from the Department of Orthopedics, Department of Radiology and the Research Laboratory of Metabolic Bone Disease. They exchanged views and reached a unified conclusion so as to assess the model stability and fracture healing. After the radiological analysis, the mice were sacrificed by the cervical dislocation method and placed on the foam board covered by the sterile gauze. Callus tissues at the bone incision were completely taken and placed in the tissue cryopreservation tube, and the liquid nitrogen was quickly injected into the tube for storage and stand-by application.

Extraction of the Total RNA and Detection by Real-Time Polymerase Chain Reaction (RT-PCR)

The mortar was precooled using liquid nitrogen and added with 2-3 mL TRIzol. Callus tissues or tissues in the middle tibial stem froze by the liquid nitrogen were taken and placed in the mortar and ground to powder. The powder was transferred into a 1.5 mL Eppendorf (EP) tube, and the total RNA was extracted using TRIzol method. 1 μL total RNA was taken out for quality inspection. An ultraviolet spectrophotometer was used to detect the concentration of total RNA and A260/A280, and RNA was preserved at -70°C for standby application. The extracted total RNA was reversely transcribed using Taqman miRNA reverse transcription kit manufactured by Applied Biosystems (Fosters City, CA, USA). The levels of mmu-miR-214-3p and osteocalcin in callus tissues were measured by quantitative RT-PCR (qRT-PCR) at 7 d, 14 d, 21 d and 28 d after modeling with U6 and 18S as internal references, respectively. Nanjing Jinsirui Biotechnology Co., Ltd. was commissioned to synthesize and purify primers. Primer sequences: miR-214-3p: upstream: GACAGCAGGCACAGACA and downstream: GTGCAGGGTCCGAGG; U6: upstream: CTCGCTTCGGCAGCACA3 and downstream: AACGCTTCACGAATTTGCGT; Osteocalcin: upstream: CTGACAAAGCCTTCATGTCCAA and downstream: GCGCCGGAGTCTGTTCACTA.

Extraction of the Total Protein in Callus Tissues

Callus tissues at different points after fractures were placed in the liquid nitrogen for grinding, after which they were placed in a 1.5 mL EP tube, and each tube was added with 120 μL lysate. After phacofragmentation and ultrasonic splitting on the ice (on and off with 2 s each; 10 cycles), the standing was performed for 15 min; cell lysates were centrifuged at 4°C at the rate of 12000 rpm for 10 min; the supernatant was transferred to the 1.5 mL EP tube and stored at -70°C. The bicinchoninic acid (BCA) kit was used to quantify the protein concentration. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted for separation, polyvinylidene fluoride (PVDF) was used for transfer membranes, and 5% skim milk was used for sealing at room temperature for l h. β-catenin proteins were identified by β-catenin rabbit anti-mouse monoclonal antibodies (Abcam, Cambridge, MA, USA 1:1000) and goat anti-rabbit secondary antibodies (1:2000) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control. After 4 h of incubation with primary antibodies, the Tris-buffered saline and Tween 20 (TBST-20) was used to wash membranes for 6 min×5 times. After 1 h incubation with secondary antibodies, the TBST was used for washing membranes for 6 min×5 times, and water was dried using the filter paper. The enhanced chemiluminescence (ECL) reagent kit was used to enhance the color development.

Hematoxylin and Eosin (HE) Staining

Each tibial specimen was placed in a 5 mL centrifuge tube with a lid containing 4 mL paraformaldehyde for fixation for 24 h. The specimens were washed with phosphate-buffered saline (PBS) for three times, and then steeped in 10% ethylenediaminetetraacetic acid (EDTA) decalcifying solution for decalcification. The solution was changed once every 5-7 days until the tibia became soft, and then specimens were embedded in paraffin and sliced. They were toasted at 60°C till the wax was melted. Turpentine was used for dewaxing, ethanol for dehydration, HE for staining, and neutral gum for sealing. The results were observed under an inverted microscope, which showed that the nucleus was dark blue, and the cytoplasm and fibrous tissues became red at different degrees.

Statistical Analysis

All data in this experiment were expressed as mean ± standard deviation ($\bar{x} \pm s$), and processed using Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA). The intergroup data were detected by one-way analysis of variance. Pairwise comparisons of the mean were conducted, and $p < 0.05$ represented that the difference was statistically significant.

Results

X-ray Manifestations of Mice with Overexpressed agomiR-214-3p on the 7th Day and 28th Day After Fractures

The miR-214-3p mimic, agomiR-214-3p, was locally injected at the fracture site of mice to observe the effect of the change in miR-214-3p expression on fracture healing of mice. There were no differences among the three groups on the 7th d after fractures. On the 28th d after fractures, the

X-ray images of mice injected with PBS or agomiR-NC showed that fracture lines disappeared, calluses were relatively small and the density was high. In the agomiR-214-3p injection group, fuzzy fracture lines could be seen in mice with fewer calluses, suggesting that agomiR-214-3p inhibits fracture healing of mice (Figure 1).

Histological Manifestations of Mice with Overexpressed agomiR-214-3p on the 7th and 21st Day After Fractures

After agomiR-214-3p, agomiR-NC and PBS were locally injected at the fracture site of mice, HE staining was conducted at the fracture site of mice on the 7th and 21st d after fractures. It was found that bone trabecula of mice in the agomiR-NC injection group and the PBS injection group were regularly arranged on the 21st d after fractures, and some mature tabular bones were observed under the microscope. However, in the agomiR-214-3p injection group, bone trabecula of mice were arranged in disorder and discontinuous (Figure 2).

Change in the Expression Level of Callus Osteocalcin in Mice with Overexpressed agomiR-214-3p After Fractures

The expression levels of osteocalcin and miR-214-3p at the fracture site were detected by qRT-PCR at 1 d, 7 d, 14 d, 21 d and 28 d after frac-

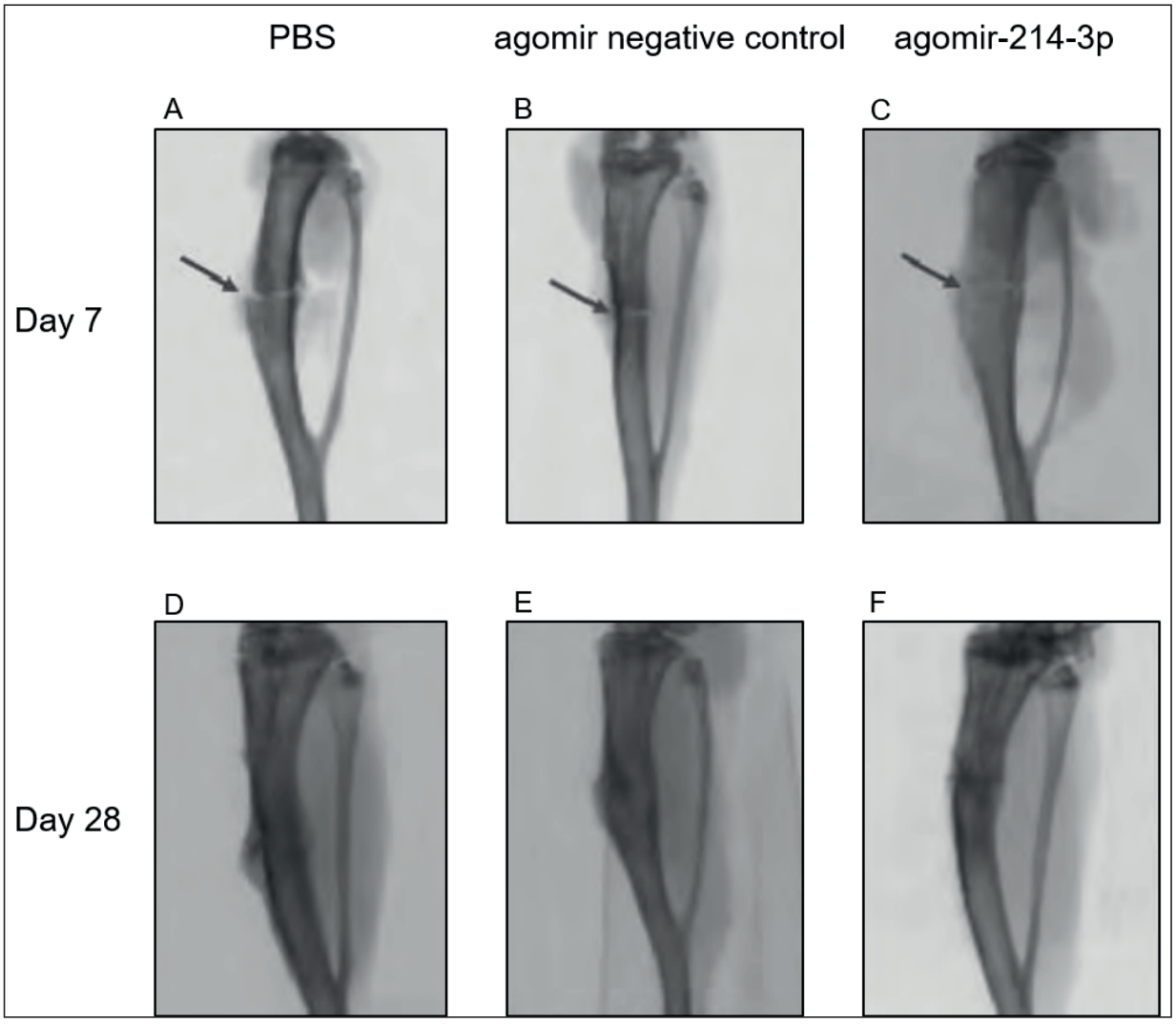

Figure 1. X-ray appearance of mice with overexpressed agomiR-214-3p on the 7th and 28th day after fractures. ***A-C,*** Little external calluses are formed on the 7th day after operation, and there is no difference. ***D-F,*** X-ray images of mice in the PBS injection group and the agomiR-NC injection group show that fracture lines disappear, but they can be indistinctly seen in the agomiR-214-3p injection group.

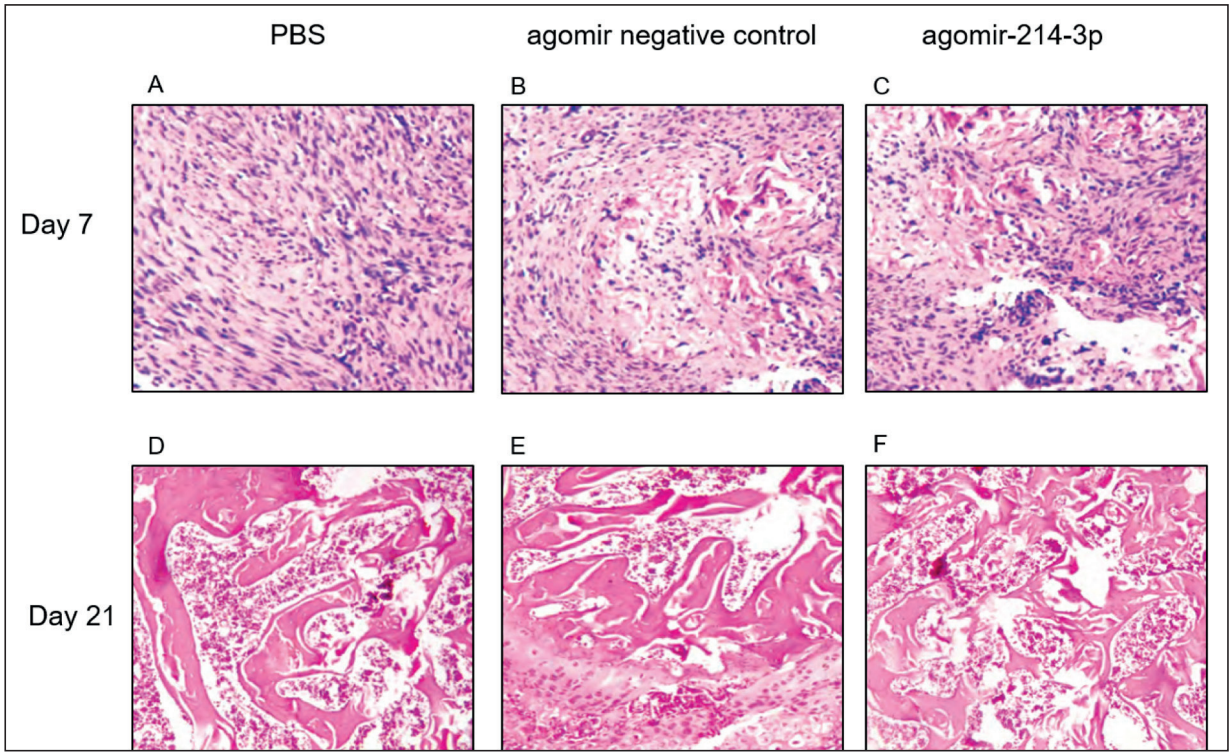

Figure 2. HE staining (100×) of tissues at the fracture site of mice with overexpressed agomiR-214-2p on the 7th day and 28th day after fractures. ***A-C,*** At 7 d after operation, granulation and fibrous tissues grow in callus tissues in each group. ***D-F,*** On the 28th day after operation, bone trabecula of mice in the agomiR-NC injection group and the PBS injection group grow vigorously and are regularly arranged, and some mature tabular bones are observed under the microscope. However, in the agomiR-214-3p injection group, bone trabecula of mice are still arranged in disorder.

tures. The expression level of miR-214-3p was increased gradually after the local injection with agomiR-214-3p at the fracture site (Figure 3A). Compared with that of mice in the PBS injection group, the expression level of osteocalcin in the agomiR-NC injection group was not different, but that of mice in the agomiR-214-3p injection group after fractures was decreased compared with that in the control group (Figure 3B).

Change in the Expression of β-catenin in Mice with Overexpressed agomiR-214-3p After Fractures

The expression level of β-catenin at the fracture site was detected by qRT-PCR at 1 d, 7 d, 14 d, 21 d and 28 d after fractures. The results showed that the expression level of β-catenin in mice injected with agomiR-NC and PBS after fractures was compensatively up-regulated, thus promoting the fracture healing. However, the level of β-catenin in the agomiR-214-3p injection group was progressively declined, and was significantly lower than that in the control group (Figure 4A). In addition, the expression level of β-catenin proteins in callus tissues on the 21st day after fractures was detected using Western blotting. The results revealed that the level of β-catenin protein was significantly down-regulated in callus tissues after the injection with agomiR-214-3p (Figure 4B).

Discussion

In recent years, more and more miRNAs have been shown to play important roles in orthopedic diseases. In a study on the regulation of miRNAs in bone and cartilage tissues, miR-214 has been found to be a new factor regulating osteogenic transformation, which inhibits the osteogenic differentiation of mouse myoblasts[19]. In osteoblasts, miR-214 inhibits osteoblast activity and matrix mineralization process by the target regulation of the expression of transcriptional activators, thus promoting the occurrence and development of osteoporosis[20]. In order to further study the function of miR-214, this investigation aimed to explore its role in the development of the fracture healing and

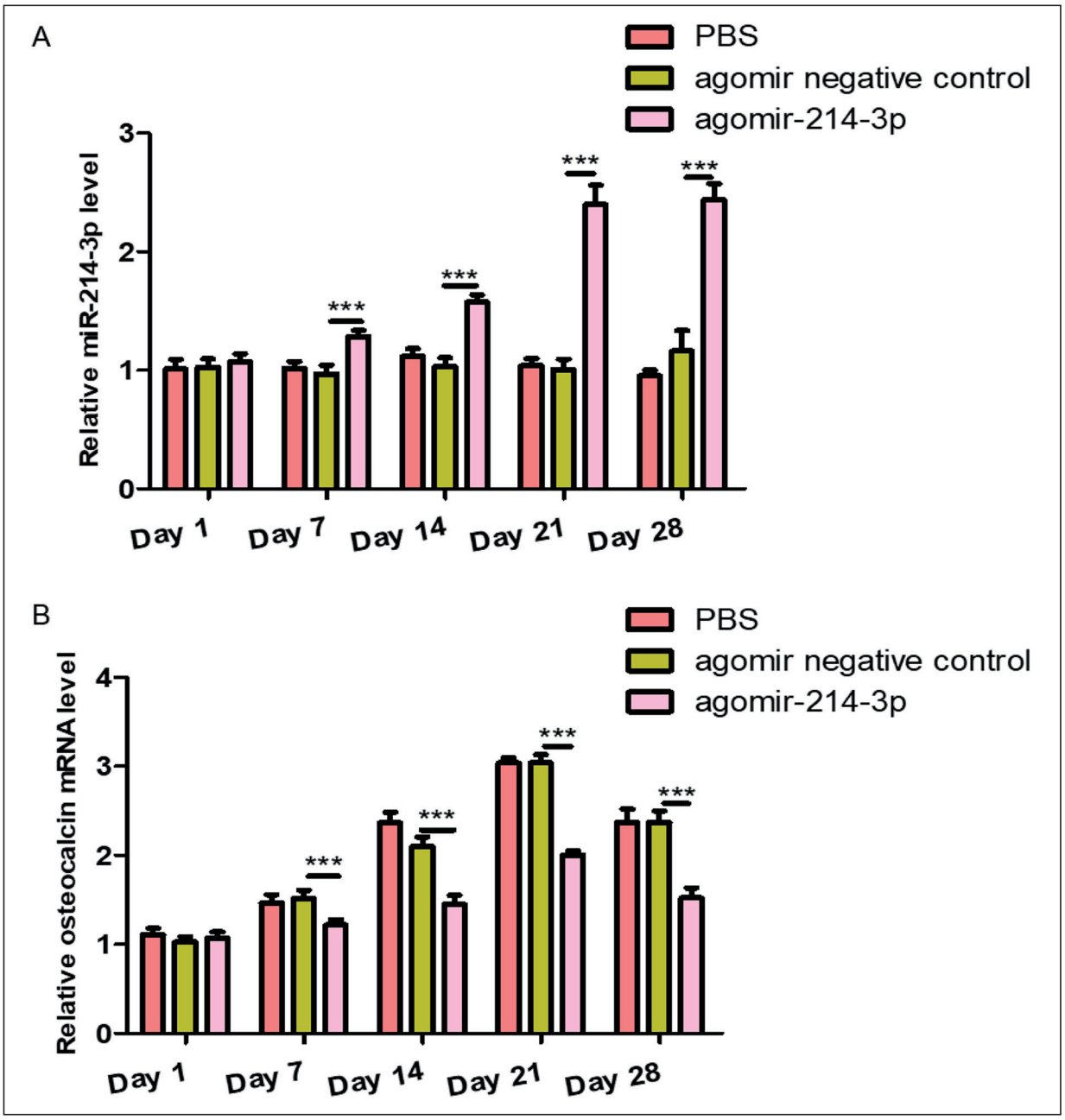

Figure 3. Change in the expression level of callus osteocalcin in mice with overexpressed agomiR-214-3p after fractures. ***A,*** The expression level ofmiR-214-3p at the fracture site is gradually increased after the injection with agomiR-214-3p. ***B,*** qRT-PCR is used to detect the expression level of osteocalcin of mice in each group at 1 d, 7 d, 14 d, 21 d and 28 d after fractures. The expression level of osteocalcin of mice in the agomiR-214-3p injection group after fractures at each time point is decreased compared with that in the control group, ***$p < 0.001$.

its mechanism. The fracture healing is a complex regeneration process similar to the development of human long bones in the embryonic period. The difference is that the fracture healing process began in the inflammatory response. Holmen et al[21] showed that Wnt signaling pathway plays an important role in bone development and maturation in the embryonic period. More and more researches have revealed that the activation of Wnt/β-catenin leads to increased phenotypic phenotypes, which shows a promising prospect in the treatment of low bone mass disease. β-catenin is a hinge molecule of the Wnt signaling system that mediates the transmission of Wnt signals

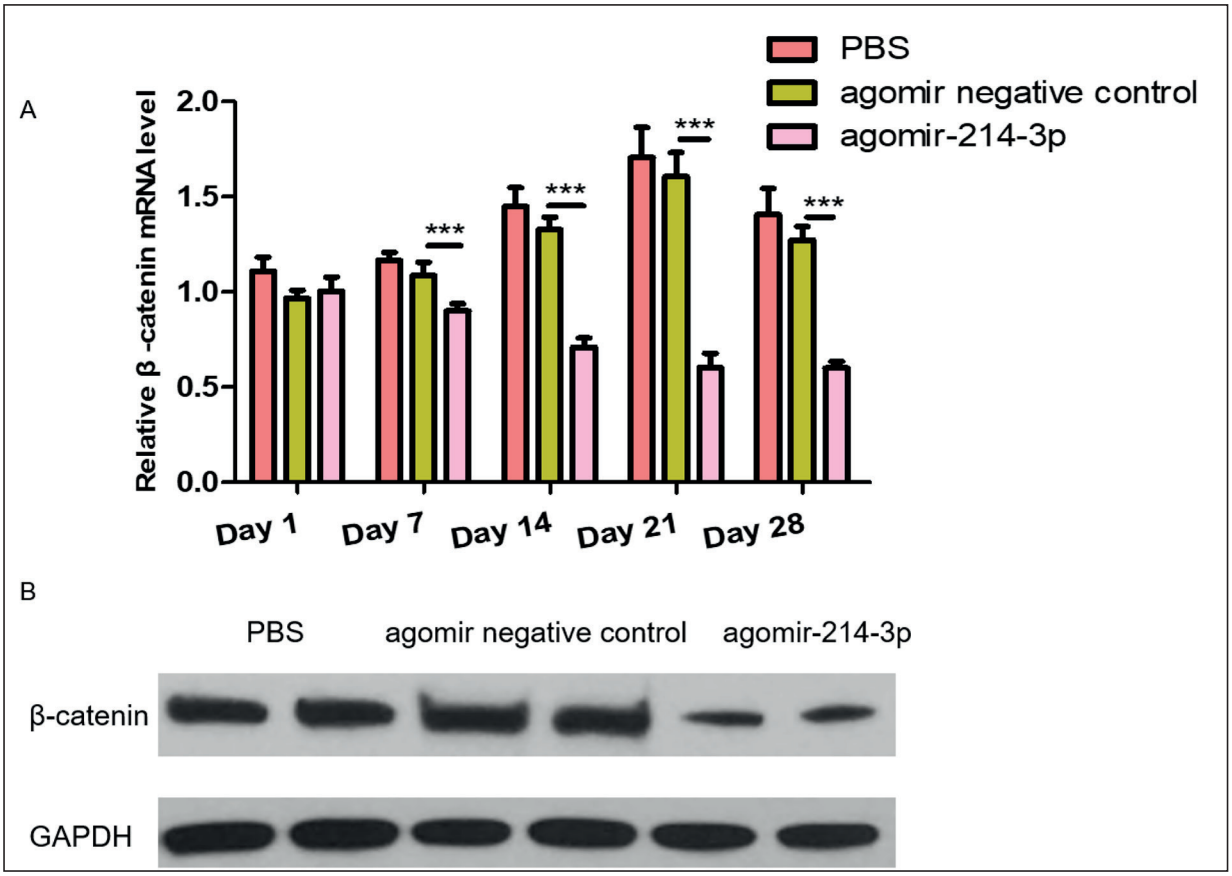

Figure 4. Change in the expression of β-catenin in mice with overexpressed agomiR-214-3p after fractures. ***A,*** The expression level of β-catenin in mice injected with agomiR-NC and PBS after fractures is compensatively up-regulated, while that in the agomiR-214-3p injection group is progressively declined and significantly lower than that in the control group. ***B,*** The expression level of β-catenin proteins in callus tissues after the injection with agomiR-214-3p is significantly down-regulated, ***$p < 0.001$.

from the membrane to the cytoplasm. Previously, Chen et al[22], constructed transgenic mice [type II collagen a1 (Cor2a1)-isotope-coded affinity tag (ICAT)] with Cor2a1 as the promoter, which can specifically express β-catenin and lymphoid enhancer-binding factor-inhibitor/T-cell factor (LEF-I/TCF) [(inhibitor of β-catenin and TCF, isotope-coded affinity tag (ICAT)] in chondrocytes. It has been found that β-catenin can affect chondrocyte hypertrophy during bone development, thus affecting endochondral ossification process. The fracture healing is thought to be the recurrence of long bone development in the embryonic period, and whether the Wnt signaling pathway affects the fracture healing process was one of the topics of this study. In this work, the tibial fracture model of mice with overexpressed miR-214-3p was established. The results showed that fuzzy fracture lines at the fracture site of mice could be seen with fewer calluses at 28 d after the high expression of miR-214-3p. HE staining revealed that there were relatively more polyfibrous and cartilaginous calluses that are partly calcified, and the expression level of osteocalcin was decreased compared with that in the control group. However, in the PBS injection group and the agomiR-NC injection group, fracture sites were found to be well healed, suggesting that miR-214-3p inhibits the fracture healing of mice. At the same time, change in the expression level of β-catenin in calluses was detected, and it was found that the expression levels of β-catenin mRNAs and proteins were gradually decreased after the high expression of miR-214-3p. Previous researches have confirmed that inhibiting β-catenin signaling pathways blocks blood vessels in callus tissues for a long term, so that osteoclasts cannot be gathered in time around calluses, thus reducing the secretion of matrix metalloproteinases and resulting in delayed absorption and alteration of callus tissues.

Conclusions

The results of this work suggested that highly expressed miR-214-3p inhibits β-catenin signaling pathway and the endochondral ossification process in the fracture healing, eventually leading to delayed fracture healing.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Solomon DH, Johnston SS, Boytsov NN, McMorrow D, Lane JM, Krohn KD. Osteoporosis medication use after hip fracture in U.S. patients between 2002 and 2011. J Bone Miner Res 2014; 29: 1929-1937.
2) Bigham-Sadegh A, Oryan A. Basic concepts regarding fracture healing and the current options and future directions in managing bone fractures. Int Wound J 2015; 12: 238-247.
3) Pope MH, Murphy MC. Fracture energy of bone in a shear mode. Med Biol Eng 1974; 12: 763-767.
4) Song Y, Han GX, Chen L, Zhai YZ, Dong J, Chen W, Li TS, Zhu HY. The role of the hippocampus and the function of calcitonin gene-related peptide in the mechanism of traumatic brain injury accelerating fracture-healing. Eur Rev Med Pharmacol Sci 2017; 21: 1522-1531.
5) McKibbin B. The biology of fracture healing in long bones. J Bone Joint Surg Br 1978; 60-B: 150-162.
6) Kells AF, Coats SR, Schwartz HS, Hoover RL. TGF-beta and PDGF act synergistically in affecting the growth of human osteoblast-enriched cultures. Connect Tissue Res 1995; 31: 117-124.
7) Bosetti M, Leigheb M, Brooks RA, Boccafoschi F, Cannas MF. Regulation of osteoblast and osteoclast functions by FGF-6. J Cell Physiol 2010; 225: 466-471.
8) de Jong DS, Steegenga WT, Hendriks JM, van Zoelen EJ, Olijve W, Dechering KJ. Regulation of Notch signaling genes during BMP2-induced differentiation of osteoblast precursor cells. Biochem Biophys Res Commun 2004; 320: 100-107.
9) Fumoto T, Takeshita S, Ito M, Ikeda K. Physiological functions of osteoblast lineage and T cell-derived RANKL in bone homeostasis. J Bone Miner Res 2014; 29: 830-842.
10) Luzi E, Marini F, Sala SC, Tognarini I, Galli G, Brandi ML. Osteogenic differentiation of human adipose tissue-derived stem cells is modulated by the miR-26a targeting of the SMAD1 transcription factor. J Bone Miner Res 2008; 23: 287-295.
11) Kapinas K, Kessler C, Ricks T, Gronowicz G, Delany AM. MiR-29 modulates Wnt signaling in human osteoblasts through a positive feedback loop. J Biol Chem 2010; 285: 25221-25231.
12) Li Z, Hassan MQ, Volinia S, van Wijnen AJ, Stein JL, Croce CM, Lian JB, Stein GS. A microRNA signature for a BMP2-induced osteoblast lineage commitment program. Proc Natl Acad Sci U S A 2008; 105: 13906-13911.
13) Marsh DR, Li G. The biology of fracture healing: optimising outcome. Br Med Bull 1999; 55: 856-869.
14) Itoh T, Nozawa Y, Akao Y. MicroRNA-141 and -200a are involved in bone morphogenetic protein-2-induced mouse pre-osteoblast differentiation by targeting distal-less homeobox 5. J Biol Chem 2009; 284: 19272-19279.
15) Li H, Xie H, Liu W, Hu R, Huang B, Tan YF, Xu K, Sheng ZF, Zhou HD, Wu XP, Luo XH. A novel microRNA targeting HDAC5 regulates osteoblast differentiation in mice and contributes to primary osteoporosis in humans. J Clin Invest 2009; 119: 3666-3677.
16) Hu R, Liu W, Li H, Yang L, Chen C, Xia ZY, Guo LJ, Xie H, Zhou HD, Wu XP, Luo XH. A Runx2/miR-3960/miR-2861 regulatory feedback loop during mouse osteoblast differentiation. J Biol Chem 2011; 286: 12328-12339.
17) Yang L, Cheng P, Chen C, He HB, Xie GQ, Zhou HD, Xie H, Wu XP, Luo XH. MiR-93/Sp7 function loop mediates osteoblast mineralization. J Bone Miner Res 2012; 27: 1598-1606.
18) Li D, Liu J, Guo B, Liang C, Dang L, Lu C, He X, Cheung HY, Xu L, Lu C, He B, Liu B, Shaikh AB, Li F, Wang L, Yang Z, Au DW, Peng S, Zhang Z, Zhang BT, Pan X, Qian A, Shang P, Xiao L, Jiang B, Wong CK, Xu J, Bian Z, Liang Z, Guo DA, Zhu H, Tan W, Lu A, Zhang G. Osteoclast-derived exosomal miR-214-3p inhibits osteoblastic bone formation. Nat Commun 2016; 7: 10872.
19) Shi K, Lu J, Zhao Y, Wang L, Li J, Qi B, Li H, Ma C. MicroRNA-214 suppresses osteogenic differentiation of C2C12 myoblast cells by targeting Osterix. Bone 2013; 55: 487-494.
20) Wang X, Guo B, Li Q, Peng J, Yang Z, Wang A, Li D, Hou Z, Lv K, Kan G, Cao H, Wu H, Song J, Pan X, Sun Q, Ling S, Li Y, Zhu M, Zhang P, Peng S, Xie X, Tang T, Hong A, Bian Z, Bai Y, Lu A, Li Y, He F, Zhang G, Li Y. MiR-214 targets ATF4 to inhibit bone formation. Nat Med 2013; 19: 93-100.
21) Holmen SL, Zylstra CR, Mukherjee A, Sigler RE, Faugere MC, Bouxsein ML, Deng L, Clemens TL, Williams BO. Essential role of beta-catenin in postnatal bone acquisition. J Biol Chem 2005; 280: 21162-21168.
22) Chen M, Zhu M, Awad H, Li TF, Sheu TJ, Boyce BF, Chen D, O'Keefe RJ. Inhibition of beta-catenin signaling causes defects in postnatal cartilage development. J Cell Sci 2008; 121: 1455-1465.