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Expression of miR-182 in patients with fracture of tibial plateau and its regulative effects on the fracture healing

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Abstract. – OBJECTIVE: To investigate the expression of miR-182 in patients with fracture of tibial plateau (FTP) and its effects on osteoblasts and fracture healing.

PATIENTS AND METHODS: The patients with fracture of tibial plateau treated in our hospital and healthy subjects who received physical examination from January 2017 to January 2018 were collected. The expression of miR-182 in the serum was detected. The osteoblasts from SD rats were cultured and transfected with miR-182, anti-miR-182, miR-NC or anti-miR-NC using transfection reagent LipofectamineTM 2000. The proliferation, alkaline phosphatase (ALP) activity, calcification and osteogenic gene expression of osteoblasts were detected. The rat models with fracture of tibial plateau were divided into control group, fracture group, fracture+miR-182 group, and fracture+anti-miR-182 group. The levels of vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and transforming growth factor β (TGFβ) in serum were detected by enzyme-linked immunosorbent assay (ELISA).

RESULTS: Compared with the controls, the expression of miR-182 in serum was significantly elevated in patients with fracture of tibial plateau. Overexpression of miR-182 inhibited the proliferation of osteoblasts, while the knockdown of miR-182 increased the proliferation. MiR-182 could decrease the ALP activity of osteoblasts, while anti-miR-182 increased the ALP activity. Osteoblast calcification ability was significantly decreased by overexpression of miR-182. Knockdown of miR-182 increased the calcification ability of osteoblasts and the expression of osteogenic genes. MiR-182 could inhibit the expression of osteogenic genes. The levels of VEGF, EGF and TGFβ in the fracture group were higher than those in the control group, while the levels in the fracture+miR-182 group were higher than those in the fracture group. The levels of VEGF, EGF and TGFβ in the anti-miR-182 group were lower than those in the fracture group.

CONCLUSIONS: MiR-182 is elevated in patients with fracture of tibial plateau, which can inhibit the proliferation and differentiation of osteoblasts and affect the fracture healing. The knockdown of miR-182 might be a new method for treating fracture healing.

Key Words:
Fracture of tibial plateau, Fracture healing, MiRNA, Osteoblast, Proliferation, Differentiation.

Introduction

Tibial plateau, the contact surface between the lower end of the femur and the upper end of the tibia, is an important load structure for the knee joint. Fracture of tibial plateau (FTP) is a common type of intra-articular fracture in clinical practice, mainly caused by a high-energy external attack, such as traffic accidents, crushing, fall[1,2]. When FTP occurs in a patient, soft tissue, meniscus, and ligament injuries usually occur; joint surface compression, displacement, or col-

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lapse to varying degrees will occur; the knee joint will be involved, and the patient will experience knee pain, swelling, and limited mobility[3,4]. If the patient does not get a timely consultation, there would be complications, such as acute, traumatic osteoarthritis, knee stiffness, delaying the fracture site healing of the patient, affecting the alignment, stability and movement of the knee joint, and even affecting the quality of life of the patient[5]. Therefore, the treatment of FTP is particularly important. At present, the classic treatment method for tibial plateau is traditional incision and internal fixation, which is ideal for reduction. It has disadvantages of large incision, much bleeding, difficult healing of fracture site and poor prognosis. Therefore, it is of great clinical significance to find new methods for healing of FTP.

The healing process of fracture includes bone reconstruction and repair processes. Osteoblast proliferation and differentiation play a vital role in the process of bone reconstruction and bone tissue regeneration. In addition, the bone-specific related indicators of runt transcription-related factors 2 (Runx2), Osterix, and Osteocalcin (OCN) play important regulatory roles in fracture healing[6,7]. MicroRNA (miRNA), an endogenous noncoding RNA, presents in eukaryotes with a class of approximately 22 to 25 nucleotides in length[8]. MiRNA regulates its target genes, thereby regulating gene expression abundance, gene structure, occurrence, and development of diseases[9]. It is reported that miR-182 is involved in bone-related diseases, such as osteoporosis, for example, reducing miR-182 to promote osteoblast proliferation and differentiation in osteoporotic rats by regulating the Rap1/MAPK signaling pathway. Overexpression of miR-182 inhibits chondrogenic differentiation of bone marrow mesenchymal stem cells by reducing parathyroid hormone-like hormone (PTHLH), but no research has shown whether miR-182 is involved in the bone healing process[10-12]. Therefore, this study explores the expression of miR-182 in patients with FTP and its role in regulating osteoblasts and fracture healing, and provides new ideas for clinical treatment of FTP healing.

Patients and Methods

Clinical Information

Serum samples were collected from 80 patients with FTP who underwent surgery in Orthopedic Department of our hospital from January 2017 to January 2018. There were 19 males and 21 females. The age range was (50 to 80) years, and the average age was (68.92 ± 6.12) years old. When patients with fractures were admitted to the hospital, 5 mL of whole blood was drawn from the patients. The serum was separated, and stored in a freezer at -80°C (Thermo, Waltham, MA, USA), named as the FTP group. Inclusion criteria: patients with admission examination, X-ray, CT, and confirmed with FTP by MRI examinations; patients who accepted various examinations after admission, and received surgical treatment within 1 week; patients who participated in this subject voluntarily and signed informed consent. Exclusion criteria: patients with tumors or other major diseases; patients with endocrine diseases, rheumatic immune system diseases and other systemic metabolic bone diseases; patients not willing to participate in the study; patients with incomplete data; patients with open and pathological or other types of fractures; patients combined with other knee joint injuries; patients with fractures with severe nerve or vascular injury; patients with disturbance of consciousness and abnormal blood coagulation.

In control group, serum samples were taken from 80 healthy volunteers who underwent physical examinations in our hospital during the same period. Inclusion criteria: patients without clear history of trauma; patients without swelling, pain, and other symptoms of knee joints; patients without sores and wounds in need of healing; patients who participated in this subject voluntarily and signed informed consent. Exclusion criteria: patients who were limited in activities; patients with fractures; patients with the history of knee surgery; patients with old fractures; patients combined with other knee joint injuries; patients with disturbance of consciousness and abnormal blood coagulation; patients with surgical wounds, burn wounds, infected wounds, skin and soft tissue injury wounds, cosmetic suture wounds, negative pressure wounds, incurable wounds in cancer patients and other wounds. Five mL of whole blood from patients in the control group was collected. The serum was separated, and stored in a freezer at -80°C (Thermo Fisher Scientific, Waltham, MA, USA), named as the CTL group. All operations in this experiment met the requirements of the Ethics Committee of the Third Affiliated Hospital of Qiqihar Medical University and were approved by the Ethics Committee.

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

qRT-PCR experiment was used to detect the expression of miR-182 or osteogenic genes. When detecting miR-182, the miRNA extraction kit (217004, Qiagen, Beijing, China) was used to extract total miRNA, and reverse transcription and RT-PCR experiments were performed to detect the relative expression of miR-182. When detecting osteogenic genes, each sample was added with 1 mL of TRIzol reagent (15596-018, Thermo Fisher Scientific, Waltham, MA, USA) to lyse total RNA, reverse transcription kit (11141ES10, Applied Biosystems, Carlsbad, CA, USA) was used in reverse transcription reaction, and SYBR reagent (4913914001, Roche, Basel, Switzerland) was used in RT-PCR reaction. The reaction conditions were at 95°C for 30 s, at 95°C for 5 s, at 60°C for 30 s, and at 7°C for 30 s, for a total of 40 cycles, and at 72°C for 30 s. The relative expression of the target gene was calculated by the $2^{-\Delta\Delta CT}$ method. All primers were purchased from Invitrogen (Carlsbad, CA, USA), all primer sequences were shown in Table I.

Culture and Passage of Rat Osteoblasts

The trypsin-II collagenase digestion method was used to isolate and extract primary osteoblasts in SD suckling rats. DMEM medium (Corning, Corning, New York, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin (Corning, Corning, New York, USA) was added, and the cells were cultured in an incubator at 37°C with 5% CO_2. When the cells grew and covered about 90%, trypsin (Biyuntian, Beijing, China) was used for digestion and passage.

MiRNA Transfection

MiR-182, anti-miR-182, miR-NC, or anti-miR-NC was transfected into the third-generation osteoblasts respectively with transfection reagent Lipofectamine™ 2000 (Roche, Basel, Switzerland). After washing with PBS, Lipofectamine™ 2000 was added to special Opti-MEM medium for transfection without serum and antibodies, and incubated for 5 min, mixed gently, and kept standing on the ice bag for 20 min. It was added to the cells and incubated for 6 h. The transfection mixture was discarded, and the culture solution was added to continue to be cultured.

Cell Counting Kit-8 (CCK-8) Experiment

CCK-8 assay was used to detect cell proliferation. First, osteoblasts were seeded in 96 wells at 1×10^3 cells/well. In the plate, after cultured for 24 h, miR-182, anti-miR-182, miR-NC, or anti-miR-NC was transfected. After 48 h, the mixture of medium and CCK-8 reagent were added into each well by the ratio of 10:1, with 110 μL for each well, and incubated at 37°C for 2 h. Finally, the cells were placed in a multifunctional microplate reader, and the absorbance value (A value) of each well was detected at a wavelength of 450 nm.

Detection of Alkaline Phosphatase (ALP) Activity

Osteoblasts were seeded in 12-well plates at a density of 1×10^4 cells/well. After culturing for 48 h, miR-182, anti-miR-182, miR-NC or anti-miR-NC were transfected; after 48 h, investigations were performed according to the instructions of ALP kit (Nanjing Jiancheng, Nanjing, Jiangsu, China). After the addition of the stop buffer, the cells were placed on a multifunctional microplate reader to detect the A value of each well at a wavelength of 420 nm.

ALP Stain

Osteoblast mineralization was detected by ALP staining. Osteoblasts were seeded at a density of 1×10^3 per well in a 24-well plate. After 48 h, ALP incubation solution was added according to the literature[13]. After incubation at 37°C for 4 h, 1% cobalt nitrate was added to

Table I. All primer sequences for RT-PCR experiment.

Primer	Upstream (5 'to 3')	Downstream (5 'to 3')
miR-182	TTTGGCAATGGTAGAACTCACACT	GCTTTGGCAATGGTAGAACTCACACT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
Runx2	TAACGGTCTTCACAAATCCT	GGCGGTCAGAGAACAAACTA
Osterix	CAATGACTACCCACCCTTTCC	TGCCCACCACCTAACCAA
OCN	CACAGGGAGGTGTGTGAG	TGTGCCGTCCATACTTTC
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG

continue incubating for 1 min, and washed 3 times with PBS. Finally, the cells were placed under a microscope (Nikon, Japan) to be observed, and 10 fields were randomly selected to take pictures.

Alizarin Red (ARS) Staining

Osteoblast mineralization was detected by ARS staining. Osteoblasts were seeded at a density of 1×10^3 per well in a 24-well plate. After transfection for 48 h, ARS staining solution (Saiye, Guangzhou, Guangdong, China) was added, and incubated at room temperature for 30 min, then, washed three times with PBS. Finally, the cells were placed under a microscope to be observed, and 10 fields were selected randomly to take photos.

Animal Experiment

Sixty SD rats were randomly divided into 4 groups including control group, fracture group, fracture + miR-182 group, fracture + anti-miR-182 group, with 15 rats in each group. After one week of SD rats acclimatization, the rat model of FTP was constructed. Anesthesia was performed using 2% pentobarbital sodium (Sigma-Aldrich, St. Louis, MO, USA) solution at a dose of 5 mL/kg. After supine position, the rats were fixed with medical adhesive cloth; after routine disinfection, depilation and peeling, the tibial plateau was exposed. Ophthalmic scissors, electric drill, wire saw, Kirschner needle and syringe were used to cause fracture of the right tibial plateau, and the incision was sutured. The incision in the control group did not cause FTP, and the rest of the operation was the same as above. Osteoblasts overexpressing miR-182 were injected into the tibial plateau of rats in the fracture + miR-182 group. Osteoblasts knocked down miR-182 were injected into the tibial plateau of the fracture + miR-182 group.

ELISA Experiment

The serum of clinical patients and rats was taken. A multifunctional microplate reader was used to detect the A value of each sample referring to vascular endothelial growth

Factor (VEGF) ELISA kit (Shanghai Jido Biotechnology Co., Ltd. Company, Shanghai, China), epidermal growth factor (EGF) ELISA Kit (MultiSciences, Hangzhou, Zhejiang, China), transforming growth factor β (TGFβ) ELISA Kit (Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd., Shanghai, China). The serum VEGF, EGF and TGFβ levels in clinical patients and rats in each group were calculated referring to the standard curve. All operations in this experiment met the requirements of the Ethics Committee of The Third Affiliated Hospital of Qiqihar Medical University and were approved by the Ethics Committee (QMU-AECC-2017-36).

Statistical Analysis

GraphPad software was used for statistical analysis. Measurement data were expressed as mean ± standard deviation. Compared data in the group were analyzed by paired *t*-test. Comparison between groups was analyzed by single factor analysis of variance. $p < 0.05$ was considered to be statistically significant.

Results

Expression of MiR-182 in Patients with FTP

The tibial plateau of the two groups of patients is shown in Figure 1A. The shape of tibia and fibula in the control group was normal, the bone cortex was continuous. The inner trabecular bone had uniform density and normal shape, and the surrounding soft tissue density was normal. No significant abnormal changes were found. In the FTP group, the cortical continuity of tibial plateau bone was interrupted, the bone trabecular was broken, and there was bone dislocation in the tibial plateau, and separated. The density of soft tissue around the tibia was reduced, and the soft tissue was swollen and contused (1A). As shown in Figure 1B, compared with the control group, serum miR-182 expression significantly increased in patients with FTP (Figure 1B). As shown in Figure 1C, compared with the control group, the expression of VEGF, EGF and TGFβ in the serum of patients with FTP was significantly increased (Figure 1C).

Effects of Overexpression of MiR-182 on Proliferation Activity and ALP Activity of Osteoblasts

The results of CCK-8 showed that compared with miR-NC, the proliferation activity of osteoblasts composed of transfected miR-182 was significantly decreased (Figure 2A). The results of ALP activity test showed that compared with miR-NC, ALP activity of transfected miR-182 osteoblasts was significantly decreased (Figure 2B).

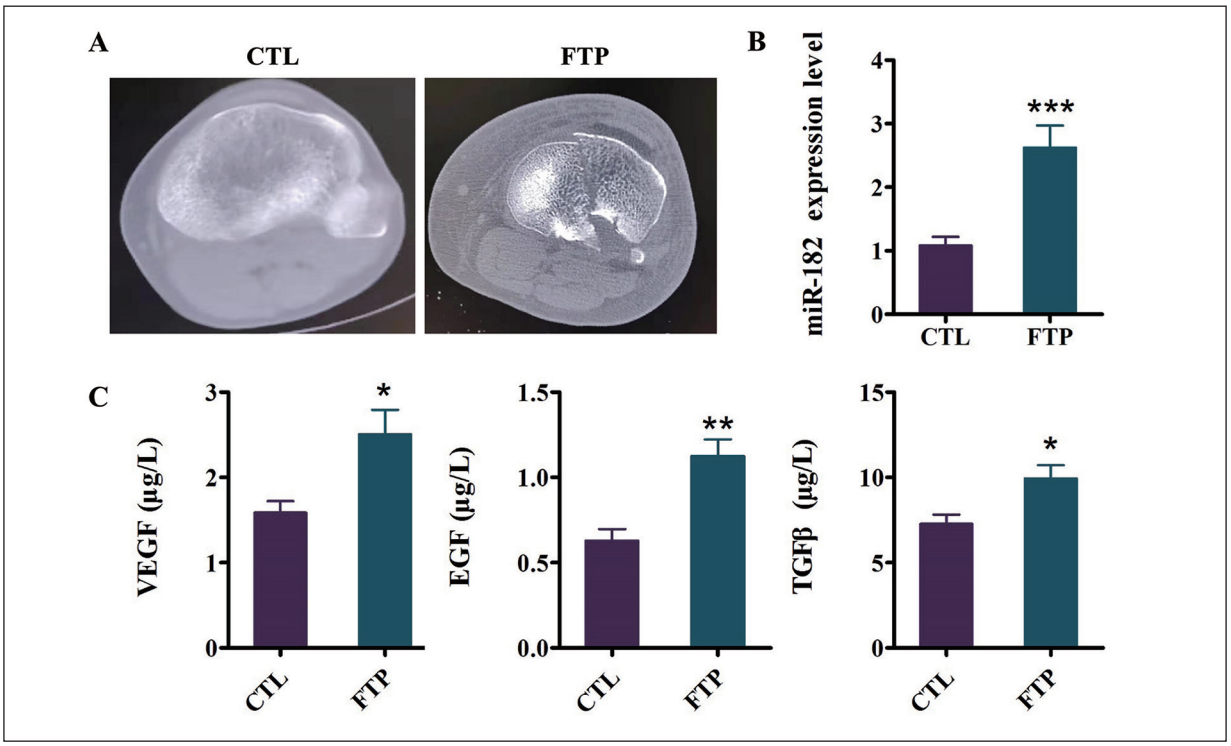

Figure 1. MiR-182 expression in patients with FTP. **A**, Radiographic images. **B**, RT-PCR test to detect the relative expression level of miR-182. **C**, ELISA test to detect VEGF, EGF and TGFβ levels in the serum of patients with tibial plateau.

Effects of Overexpression of MiR-182 on Osteoblast Differentiation

The results of ALP staining and ARS staining showed that compared to miR-NC, the overexpression of miR-182 significantly inhibited osteoblast calcification (Figure 3A and 3B). The results of RT-PCR showed that the overexpression of miR-182 osteogenic gene in osteoblasts was significantly lower than that in the control group (Figure 3C).

Effects of Knockdown of MiR-182 on Osteoblast Proliferation Activity and ALP Activity

The results of CCK-8 showed that compared with anti-miR-NC, the proliferation activity of

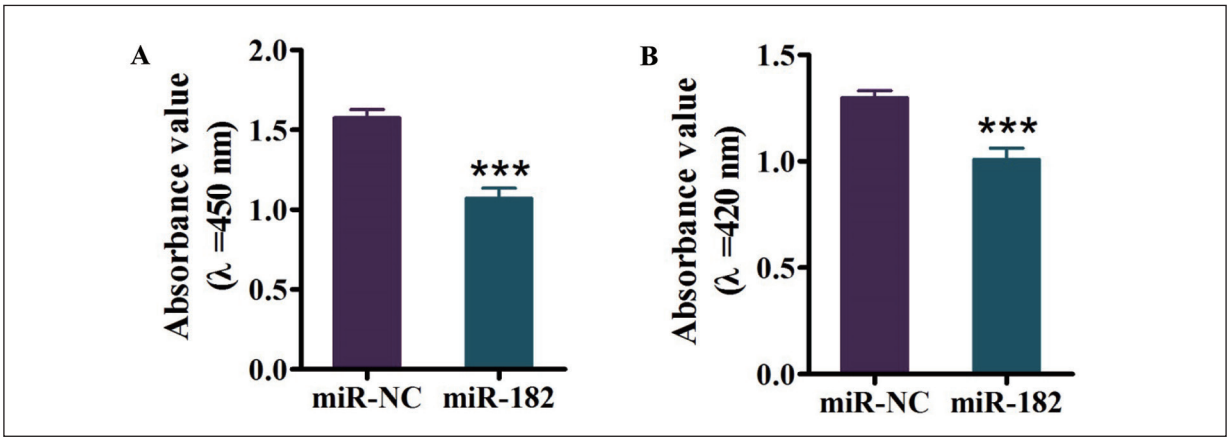

Figure 2. Effects of miR-182 on osteoblast proliferation activity and ALP activity. **A**, CCK-8 experiment to detect cell proliferation activity. **B**, ALP activity to detect osteoblast ALP activity.

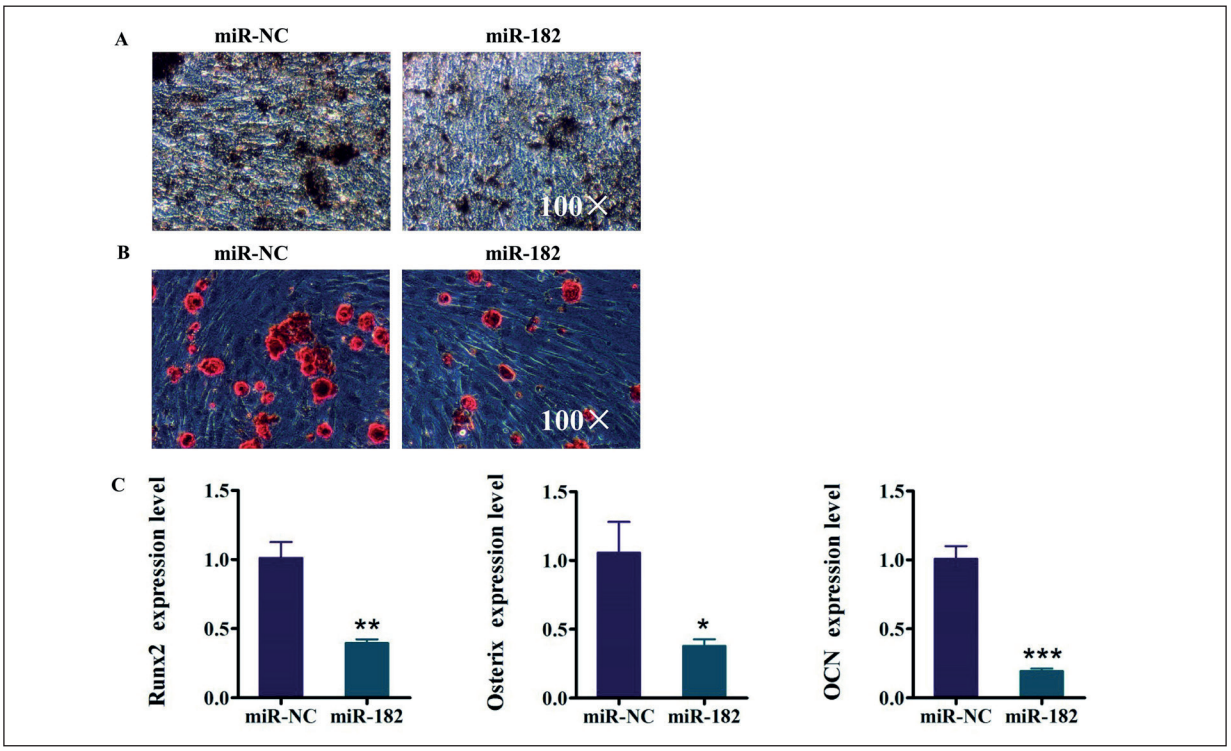

Figure 3. Effects of miR-182 on osteoblast differentiation. **A**, ALP staining test to detect cell calcification. **B**, ARS staining test to detect cell calcification. **C**, RT-PCR experiment to detect osteoblast gene expression.

osteoblasts composed of transfected anti-miR-182 was significantly increased (Figure 4A). The results of ALP activity test showed that after transfection with anti-miR-182, the ALP activity of osteoblasts was higher than that of the anti-miR-NC group (Figure 4B).

Effects of Knockdown of MiR-182 on Osteoblast Differentiation

As shown in Figure 5A and 5B, knockdown of miR-182 significantly promoted osteoblastic calcification (Figure 5A and 5B). As shown in Figure 5C, knockdown of miR-182 significantly promotes osteogenic gene expression in osteoblasts (Figure 5C).

Expression of VEGF, EGF and TGFβ in Rats with FTP

The results of ELISA test showed that compared with the control group, the levels of VEGF, EGF and TGFβ were increased in rats of the fracture group, while the levels of VEGF, EGF

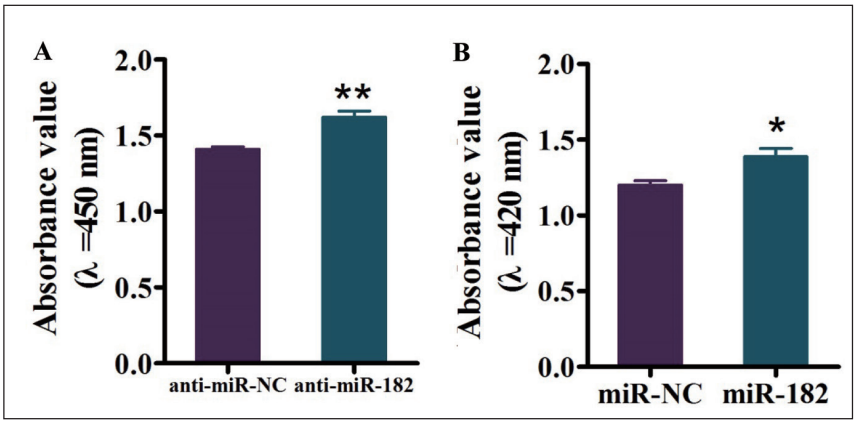

Figure 4. Effects of anti-miR-182 on osteoblast proliferation activity and ALP activity. **A**, CCK-8 experiment to detect cell proliferation activity. **B**, ALP activity to detect osteoblast ALP activity.

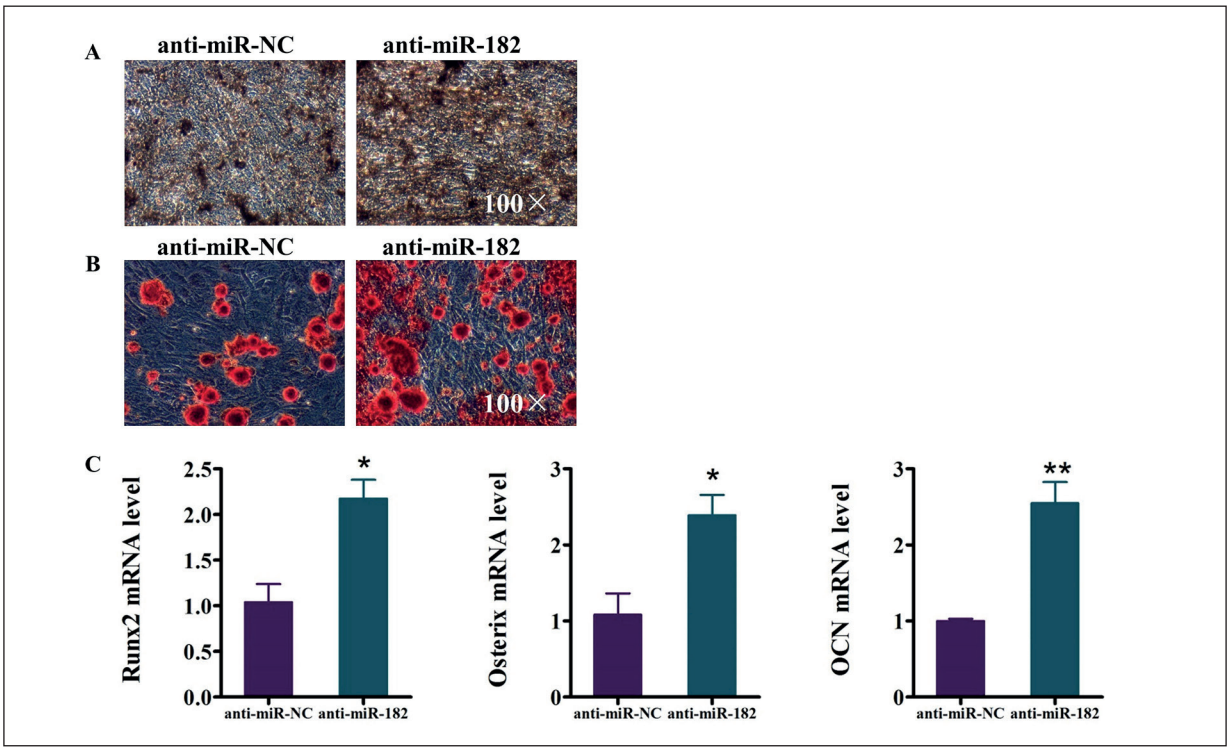

Figure 5. Effects of anti-miR-182 on osteoblast differentiation. **A**, ALP staining test to detect cell calcification. **B**, ARS staining test to detect cell calcification. **C**, RT-PCR experiment to detect osteoblast gene expression.

and TGFβ in the fracture + miR-182 group were higher than those in the fracture group. In addition, the levels of VEGF, EGF, and TGFβ in the anti-miR-182 group were lower than those in the fracture group, but higher than those in the control group (Figure 6A to 6C).

Discussion

Fracture healing is a continuous and complex process, which mainly includes three stages: the period of hematoma inflammation mechanism, the period of primitive epiphyseal formation, and

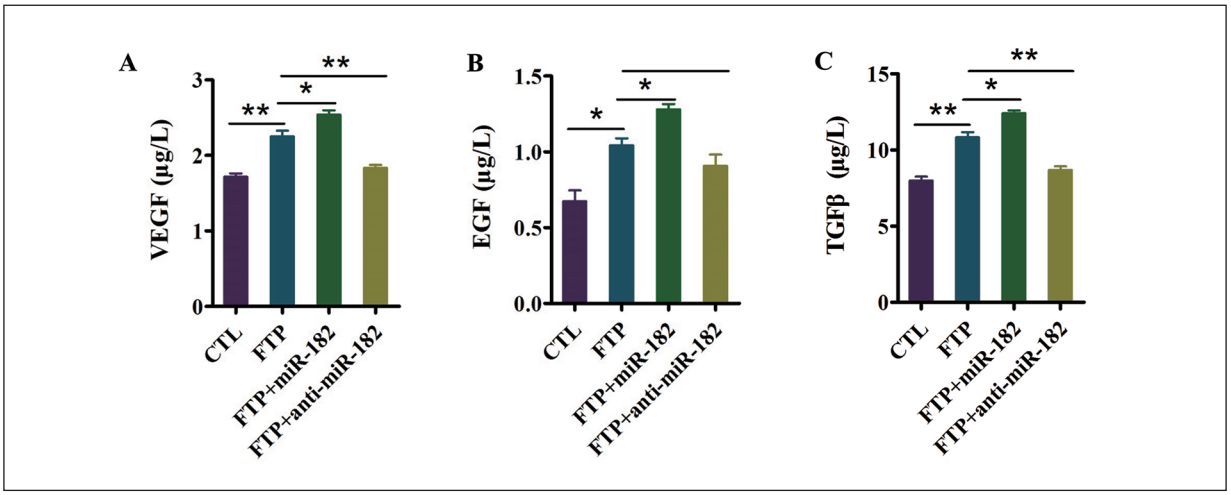

Figure 6. VEGF, EGF, and TGFβ levels in rat serum. **A**, ELISA test to detect VEGF levels in rat serum. **B**, ELISA test to detect EGF levels in rat serum. **C**, ELISA test to detect rat serum TGFβ level.

the period of bone plate formation. In these three processes, the main function of osteoblasts is to synthesize bone matrix, promote the formation of bone, and promote fracture healing[14-16]. Among them, the proliferation and differentiation of osteoblasts play a key role in fracture healing.

In this study, we collected serum samples from 80 patients with FTP who underwent surgical treatment in the Orthopedic Department of our hospital from January 2017 to January 2018. The serum of 80 healthy volunteers were taken as control group. They accepted examination at the same period in our hospital to screen differentially expressed miRNAs. The results showed that miR-182 in the serum of patients with FTP was significantly higher than that of normal people, indicating that miR-182 may be involved in bone healing in patients with FTP. In addition, miR-182 has been better conserved in humans and rats. A large number of samples were collected. MiR-182 is significantly differently expressed in healthy humans and patients with FTP, and the results are true and reliable. Therefore, we continue to explore the effects of miR-182 on the proliferation and differentiation of rat osteoblasts. Experimental results show that overexpression of miR-182 can inhibit proliferation activity and ALP activity of osteoblasts, while knockdown of miR-182 can improve proliferation activity and ALP activity of osteoblasts. Alizarin red (ARS) staining and alkaline phosphatase (ALP) staining were used to investigate the effect of miR-182 on osteoblast differentiation. ARS staining can reveal the number, size and morphology of mineralized nodules in osteoblast culture. The dark red color compounds observed under the microscope are chromogenic substances formed by the combination of ARS and calcium to judge the differentiation ability of osteoblasts. In this experiment, a large amount of orange calcium salt deposits were observed in the miR-NC group, the calcium salt deposits in the miR-182 group were decreased, suggesting that miR-182 could inhibit osteoblastic differentiation and reduce cellular calcification. ALP is a marker of osteoblasts and bone tissue. Experimental results show that overexpression of miR-182 can reduce osteoblast calcification and the differentiation, and knockdown of miR-182 can improve the differentiation ability of osteoblasts. Pan et al[10] showed that reducing the expression of miR-182 promotes osteoblast proliferation and differentiation and bone formation by regulating the Rap1/MAPK pathway. Bai et al[11] showed that overexpression of miR-182 promotes osteoclast formation, and then, inhibits bone formation by targeting PTHLH. Consistent with the results of this article, it is suggested that miR-182 plays a negative regulatory role in bone formation[10-12].

Fracture healing is the restoration of bone structural strength and the restoration of bone continuity, and multiple factors are involved in fracture healing. It is reported that vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and transforming growth factor β (TGFβ) are typical factors involved in fracture healing[17,18]. VEGF has a certain correlation with angiogenesis, it can promote the formation of new blood vessels, improve the body's local supply of oxygen and nutrition. When the patient is fractured, the soft tissue around the bone and bone are damaged, the blood vessels are broken, and the body's feedback secretion of VEGF can play a role in promoting angiogenesis[19]. EGF is a cytokine secreted by inflammatory cells and monocytes, which can play a certain repair role, promote cell division, proliferation and bone cell transformation, and accelerate the repair of fractures[20]. TGFβ is an important cytokine in the body and has many functions, which can promote wound healing, improve scar, and fibrosis. When fractures occur in the human body and the bone microenvironment is destroyed, TGFβ is activated, which can induce the activity of osteoblasts and promote bone repair and fracture healing[21]. In healthy people, VEGF, EGF and TGFβ were mostly low expressed, but under stress conditions, such as fracture, these factors were significantly increased[22-24]. To investigate the effects of miR-182 on fracture healing, we constructed SD rat models of FTP *in vivo*, grouped into control group, fracture group, fracture + miR-182 group and fracture + anti-miR-182 group. Osteoblasts that overexpressed miR-182 were injected into the tibial plateau of the fracture + miR-182 group, and osteoblasts subtracting miR-182 were injected into the tibial plateau of the rats in the fracture + miR-182 group. In the experiment, we found that if miR-182 or anti-miR-182 transfected cells were injected into FTP site in rats, cell survival rate was as high as 60% to 70%. In addition, previous studies[25-28] have reported that it is feasible to explore the regulation of bone-related diseases by transplanting cells. Therefore, we injected cells transfected with miR-182 or anti-miR-182 into SD rat models of FTP. The results showed that compared with the control group, VEGF, EGF and TGFβ levels were increased in the rats of the

fracture group. The levels of VEGF, EGF, and TGFβ in the fracture + miR-182 group were higher than those in the fracture group, indicating that miR-182 can delay fracture healing. In addition, the levels of VEGF, EGF and TGF in the fracture + anti-miR-182 group were lower than those in the fracture group, but higher than those in the control group, suggesting that miR-182 knockdown could accelerate fracture healing. This study is the first to investigate the differentially expressed miRNAs in patients with tibial plateau fractures. The differentially expressed miR-182 is significantly increased in tibial plateau patients. MiR-182 can be an important marker for early prediction of tibial plateau fractures. Detecting the expression of miR-182 in patients can predict the risk of fractures in patients. When patients have a higher risk of fractures, they should pay attention to timely preventive measures, such as calcium supplementation, light exposure, and prevention of falls. This study was the first to investigate the effect of miR-182 on osteoblast proliferation and differentiation, and the first to investigate the effect on fracture healing in rats, providing new ideas for the treatment of clinical tibial plateau fractures.

Conclusions

In summary, miR-182 expression is increased in patients with FTP, and miR-182 affects fracture healing by regulating the proliferation and differentiation of osteoblasts. Knocking down miR-182 can promote osteoblast proliferation and differentiation and accelerate and promote fracture healing. Therefore, affecting miR-182 expression may become a new direction and new method for the treatment of fracture of tibial plateau in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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