MiR-181d promotes steroid-induced osteonecrosis of the femoral head by targeting SMAD3 to inhibit osteogenic differentiation of hBMSCs

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Abstract. – OBJECTIVE: We aimed to investigate whether miR-181d may be involved in steroid-induced osteonecrosis of the femoral head (ONFH) and its underlying mechanism.

PATIENTS AND METHODS: Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used to detect the expression of miR-181d in bone marrow of 5 cases of steroid-induced femoral head necrosis and 5 cases of femoral head necrosis secondary to femoral neck fractures. Marrow-derived mesenchymal stem cells (MSCs) were obtained from bone marrow samples and identified. Subsequently, the effects of miR-181d on osteogenic differentiation were evaluated by alizarin red staining and alkaline phosphatase. Meanwhile, qRT-PCR was performed to detect the levels of osteoblast-specific genes.

RESULTS: The expression of miR-181d in the bone marrow of patients with steroid-induced osteonecrosis of the femoral head was significantly higher than that in the control group. When the expression of miR-181d in MSCs was upregulated, the ALP staining became lighter and the number of calcified nodules, as well as the expression of osteoblast-specific genes, decreased significantly. Meanwhile, the opposite results were observed when miR-181d expression was inhibited. Western blot and luciferase reporting assay proved that miR-181d could negatively regulate the expression of SMAD3.

CONCLUSIONS: MiR-181d can inhibit the differentiation of hBMSCs into osteoblasts by regulating the expression of SMAD3.

Key Words:
   MiR-181d, Bone marrow mesenchymal stem cells, Osteogenic differentiation, SMAD3, Steroid-induced femoral head necrosis.

Introduction

Femoral head necrosis (ONFH) is a progressive disease, which is characterized by the collapse of the femoral head due to necrosis of bone marrow and bone tissue. Steroid-induced femoral head necrosis is a serious clinical complication caused by the use of glucocorticoid. Currently, high doses of glucocorticoid (GC) are commonly used in the treatment of autoimmune diseases and inflammation-dependent diseases, which is considered as the only effective treatment of these diseases[1,2]. However, many investigations[2,3] have confirmed that the use of glucocorticoid has a significant correlation with the occurrence and development of femoral head necrosis. It was verified by clinical observation that most of the patients treating with the high-dose glucocorticoid had appeared osteonecrosis and its clinical symptoms within 2 years. GC-induced femoral head necrosis often involves young adults with high morbidity, seriously affecting the quality of life of patients. However, its pathogenesis is not clear. Bone marrow mesenchymal stem cells (MSCs), as a group of pluripotent stem cells, have multidirectional differentiation potential and multiple tissue-repair capabilities[4,5]. Human bone marrow-derived mesenchymal stem cells (hBMSCs) are derived from the human bone marrow cavity, characterized by a negative phenotype of hematopoietic lineage. Therefore, hBMSCs can be used as ideal seed cells for the treatment of orthopedic diseases[6,7]. At present, researches have shown that the occurrence and development of many diseases are closely related to the functional changes of hBMSCs. For example, the change of MSCs activity in femoral bone marrow can lead to non-traumatic necrosis of the femoral head. Moreover, some scholars[8,9] have found that MSCs are impaired in steroid-induced osteonecrosis due to a decrease of their proliferative ability and mitochondrial membrane potential, and an increase of reactive oxygen species. In addition, studies[2,10] have shown

that the use of glucocorticoid inhibits bone formation through the direct effect on MSCs. Therefore, the analysis of how the glucocorticoid regulates osteogenic differentiation of MSCs is the key to explain the occurrence and development of steroid-induced femoral head necrosis.

MicroRNAs, as a kind of non-coding single-stranded small RNA molecules, are about 17-25 nucleotides in length. They can promote degradation of target genes or inhibit their transcription by binding to their 3 '-UTRs region11. Various researches[2,12] have shown that miRNAs are involved in the regulation of cell functions, such as differentiation, proliferation and migration of MSCs. By now, many studies[2,13] have demonstrated that miRNAs play an important role in the regulation of GC-related pathological process. Li et al[14] found that miR-181d played an important regulatory role in human gene expression, including regulation of immunity, inflammation and cell cycle, and further affected cell apoptosis and differentiation. However, there is no research on how miR-181d regulates the function of MSCs in steroid-induced femoral head necrosis.

The primary purpose of this study was to investigate the regulating effect of miR-181d on MSCs in steroid-induced femoral head necrosis, and its underlying mechanism.

Patients and Methods

Patients

After getting examination and approval from the Medical Ethics Committee of Liuzhou Worker's Hospital and informed consent from patient, we obtained whole-bone marrow samples from the marrow cavity of patients undergoing total hip arthroplasty in our center during July 2003 and February 2017. Inclusion criteria are as follows: age between 20 and 50, and no history of smoking and alcoholism, no history of hypertension, diabetes mellitus, hyperlipidemia, heart disease, infectious disease or congenital disease. For patients with steroid-induced necrosis of the femoral head (experimental group), the intake threshold of GCs must be greater than 1800 mg or a long-term glucocorticoid therapy for more than 4 weeks must be received. Correspondingly, the patients with secondary femoral head necrosis after the old femoral neck fracture (control group) must have no history of GCs therapy. The etiology was the avascular necrosis of femoral head after trauma, so the MSCs isolated from patients with secondary femoral head necrosis after the old femoral neck

fracture were considered as control. According to the inclusion criteria, we selected 5 patients with steroid-induced osteonecrosis into the experimental group (GCs Group) and 5 patients with secondary osteonecrosis into the control group. All the subjects were comparable in terms of age, sex and grade of necrosis (Ficat staging). The clinical characteristics of all patients were summarized in Table I. Necrosis of the femoral head was diagnosed preoperatively with imaging test and postoperatively with pathological examination.

Isolation and Culture of hBMSCs

When the total hip arthroplasty was at the stage of reaming the proximal femur, 5-10 mL bone marrow were aspirated from marrow cavity of the proximal femoral using sterile syringe, and immediately placed in a sterile container and quickly transported to the ultra-clean bench. The bone marrow fluid was added to a centrifuge tube containing phosphate-buffered saline (PBS) and dissociated by pipette to mix as a cell suspension. Then, the suspension was injected into a centrifuge tube containing an equal volume of 1.073 g/mL lymphocyte separation solution, and then centrifuged at 2000 r/min for 30 min. The mononuclear cells in the white layer after centrifugation were collected and resuspended in low glucose Dulbecco's Modified Eagle Medium (DMEM) basal medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and Penicillin-Streptomycin solution. The cells were seeded at a cell density of 5000 cells/cm^2 in T25 cell culture flasks and incubated with 5% CO_2 and 95% humidity at 37°C, and the medium was changed every two or three days.

Phenotypic Characterization

Flow cytometry was used to identify the cellular phenotype of the MSCs that we isolated. MSCs were digested at cell density of 106 cells/mL, resuspended in PBS and washed twice with PBS. 500 μL of cell suspension were taken and 5 μL antibodies of CD44, CD45 were used respectively to incubate the cells in the dark at room temperature for 30 min. Afterwards, the above cells resuspended in 500 μL PBS. Flow cytometer was used to measure the fluorescence value and 10,000 cells of each tube were collected to identify the phenotype of the cells.

Induction of Osteogenic Differentiation

The cells were seeded in six-well plates, and when the degree of cell fusion reached 60-70%, the

medium was changed as complete medium for inducing hBMSCs osteogenic differentiation. About 2 weeks after the induction of differentiation, alizarin red staining was performed depending on cell morphology and growth. Osteoinductive differentiation medium was composed of basal medium, 10% FBS, 10 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate, 50 μg/mL ascorbic acid, 1% penicillin-streptomycin and 1% HEPES.

Alizarin Red Staining

After osteogenic induction, the cells were washed with PBS 1-2 times. Each well was filled with 2 mL 4% neutral formalin and fixed at room temperature for 30 min. Subsequently, the neutral formaldehyde solution was discarded and rinsed twice with PBS. 1 mL alizarin red stain was added to each well for 3-5 min and rinsed twice with PBS after the alizarin red stain was sucked out. After drying, the culture plate was placed under a microscope to observe the osteogenic staining effect, then photographed and recorded.

Alkaline Phosphatase (ALP) Staining

ALP staining experiment was performed in MSCs with different treatment for 7 days, and all staining steps were based on the instructions. Incubation solution was added on the glass slide in 6-well plate at 37°C for 15 min and then rinsed for 2 min. Counterstain of hematoxylin was used to dye the MSCs on glass slide for 5 min. Then the glass slide was rinsed for 2 min, dried and observed under an optical microscope; lastly, the photos were collected.

RNA Extraction

The cells were washed with PBS after discarding the culture medium. 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse cells, which were then transferred to a tube. 250 μL chloroform were added and the tube was shaken for 30 s, mixed and centrifuged at 4°C. The aqueous phase was aspirated and an equal volume of precooled isopropanol was added. After centrifugation, the pellet was gently washed with 75% ethanol and then dissolved in 20 μL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). RNA concentration was measured using a spectrophotometer. Extracted RNA was placed in -80°C refrigerator for later use.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

The reverse transcription reaction system was prepared on ice using PrimeScript RT re-

agent Kit (TaKaRa, Code No. RR037A, Otsu, Shiga, Japan), and complementary Deoxyribose Nucleic Acid (cDNA) was obtained after the reaction completed. The miRNA quantitative PCR was performed according to the miScript SYBR Green PCR Kit manual. The volume of the total reaction was 10 μL. PCR amplification conditions are pre-denaturation at 94°C for 5 min followed by 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min and 30 s. The primer sequences were as follows: SMAD3 (F: 5'-TGGTCAGTTGAGCAGGTCTG-3', R: 5'-GGCCC-CACTTACTTCTTCCTC-3'), RUNX2 (F: 5'-TGGTTACTGTCATGGCGGGTA-3', R: 5'TCTCAGATCGTTGAACCTTGCTA-3' glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (F: 5'-ACCCACTCCTCCACCTTTGA-3', R: 5'-CTGTTGCTGTAGCCAAATTCGT-3'). U6 (F: 5'-CTCGCTTCGGCAGCACATATA-3', R: 5' - AAATATGGAACGCTTCACGA -3 ').

Transfection of Cells

The cells with good viability in logarithmic phase were selected and transfected with miR-181d mimics or inhibitor according to Lipofectamine 2000 instruction (Invitrogen, Carlsbad, CA, USA). The medium was changed 6 h after transfection. The sequences of miR-181d mimics and inhibitor as well as negative control were as follows: miR-181d mimics (sense: 5'-AACAUUCAUUGUUGUCG-GUGGGU-3', anti-sense: 5'-CCACCGACAACA-AUGAAUGUUUU-3'); miR-181d inhibitor (sense: 5'- AAAACAUUCAUUGUUGUCGGUGG-3', anti-sense: 5'- CAGUACUUUUGUGUAGUA-CAA-3'); Negative control, (sense: 5'-UUCUCC-GAACGUGUCACGUTT-3', anti-sense: 5'-ACGU-GACACGUUCGGAGAATT-3').

Dual-Luciferase Reporter Gene Assay

The 3'UTR sequence of SMAD3 was obtained from the NCBI website and SMAD3 WT 3'UTR, the SMAD3 wild-type sequence, and SMAD3 MUT 3'UTR, the mutant sequences, were constructed. Cells were then seeded in 96-well plates, and 50 pmol/L mir-181d mimics or negative control and the constructed 80 ng SMAD3 wild-type or mutant plasmids were co-transfected in the cells. 48 hours after transfection, the dual-luciferase reporter assay system was used to detect the fluorescence intensity.

Western Blotting

Cells were collected after centrifugation, then sonicated and centrifuged again to get the super-

natant. Bromophenol blue was added (0.5 mL in each 9.5 mL protein sample) to boil 10 min, then the protein was aliquoted and stored at -20°C. The sample was dissolved directly when used. Each protein sample was added in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel in electrophoresis system. After the gel was transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), the membrane was blocked in 5% non-fat milk, incubated with primary antibody overnight, and incubated with secondary antibodies. Finally, image exposure was performed to observe the protein expression.

Statistical Analysis

The measurement data were presented as means ± standard deviation, with each experiment repeated at least three times. Statistical analysis was completed by computer software statistical product and service solutions (SPSS 21.0, Armonk, NY, USA). Differences between groups were compared using the t-test, and $p < 0.05$ was considered statistically significant. GraphPad Prism 5 software (La Jolla, CA, USA) was used to make charts for statistical analysis.

Results

The Expression of miR-181d in Bone Marrow of Patients with Glucocorticoid-Induced Osteonecrosis and the identification of hBMSCs

We detected the expression of miR-181d in 5 cases of GC-induced femoral head necrosis and 5 cases of secondary femoral neck necrosis (control group) by Real-time fluorescence quantitative PCR. The age, gender and Ficat staging of both groups were matched (Table I). The results showed that the expression of miR-181d in bone marrow of patients with steroid-induced necrosis was significantly higher than that of the control group ($p < 0.001$) (Figure 1A). After 3 days of primary culture, the numbers of erythrocytes and other small amounts of suspended cells gradually decreased. Each cell population observed under the microscope indicated that hundreds of BMSCs grew adherently in the form of fusiform or spindle (Figure 1B). Next, we examined two hBMSCs markers to distinguish them from blood cells and other monocytes. The results showed that CD44 (99.99%) was positively expressed in our cells, while CD45 (0.12%) was negatively expressed

(Figure 1C), proving that cultured hBMSCs with high purity were obtained and could be used for subsequent experiments. These results demonstrated that highly expressed miR-181d may be involved in steroid-induced ONFH.

Effects of Glucocorticoid on Osteogenic Differentiation Ability and miR-181d Expression of hBMSCs

After 7 and 14 days of osteoinductive culture, ALP staining and alizarin red staining were performed in the hBMSCs isolated from GC group and control group, respectively. HBMSCs from patients with steroid-induced osteonecrosis showed lighter ALP staining (Figure 2A) and less alizarin red-stained mineralized nodules (Figure 2B) than the control hBMSCs. These results indicated that the ability of osteogenic differentiation in hBMSCs from GC group was lower than that from control group. Subsequently, different concentrations of dexamethasone (10-8 M, 10-7 M, 10-6 M) were added into the control group, and the expression level of miR-181d was enhanced with the increase of the interference concentration accordingly (Figure 2C). The above data indicated that GCs could inhibit the ability of osteogenic differentiation of hBMSCs as well as increase the expression of miR-181d.

Up-Regulation of miR-181d Selectively Regulated SMAD3 Expression and Inhibited Osteogenic Differentiation of MSCs

The miR-181d mimics and miR-181d inhibitor were transfected into hBMSCs from control group. The findings of qRT-PCR showed that miR-181d mimics significantly increased the expression of miR-181d, while miR-181d inhibitor significantly decreased the expression of miR-181d (Figure 3A). SMAD3 was selected by bioinformatics prediction for target gene of miR-181d and the functional analysis. The results of reporter gene assay showed that luciferase was decreased in SMAD3-WT 3'-UTR group after transfected with miR-181d, but there was no significant difference in SMAD3-MUT 3'-UTR luciferase (Figure 3B). These results indicated that SMAD3 could interact with miR-181d. After overexpression of miR-181d, the expression of RUNX2, as one of the osteoblast marker genes, was detected. It was found that the expression of RUNX2 as well as SMAD3 was significantly decreased after miR-181d was overexpressed (Figure 3C, 3D). The same result was also validated at the protein level
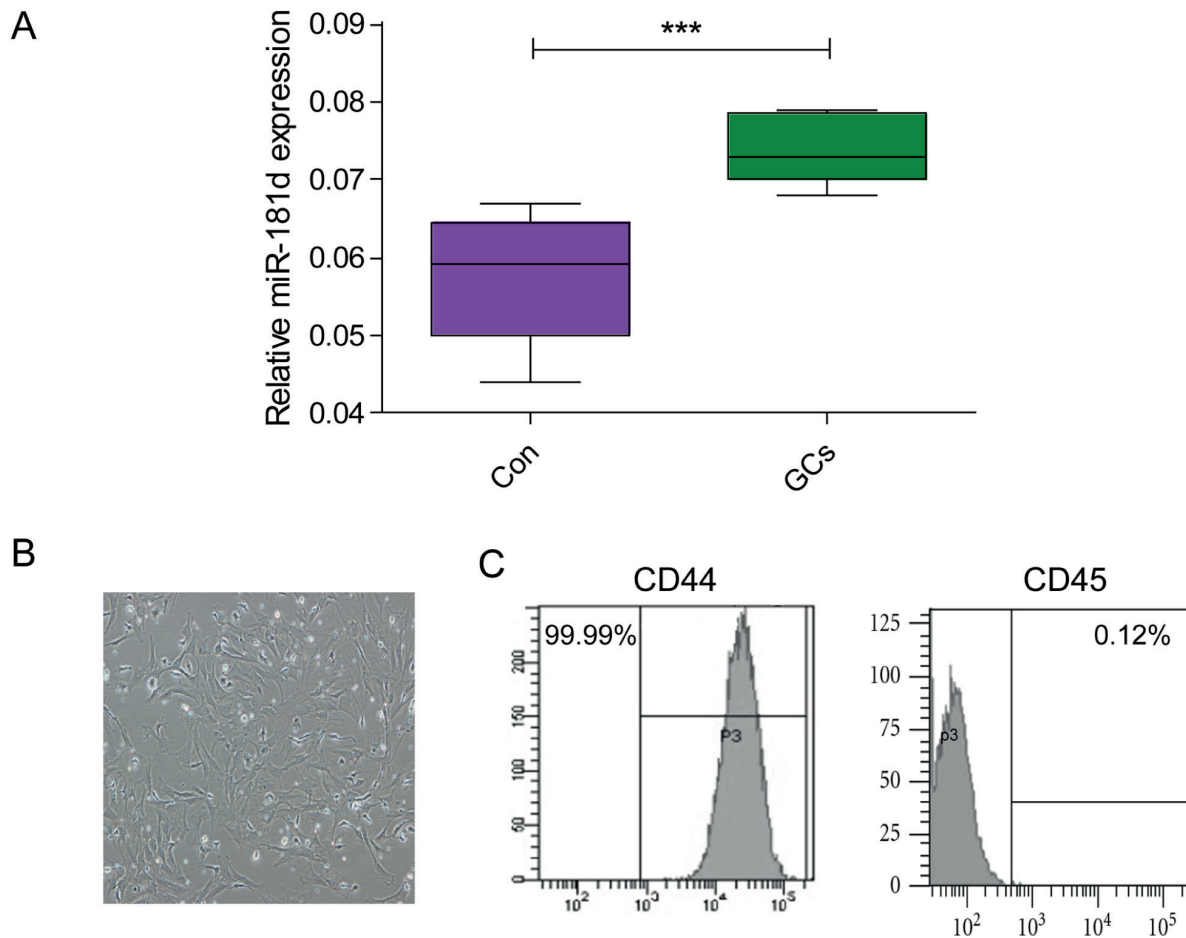
A

B

C

Figure 1. Expression of miR-181d in bone marrow of patients with steroid-induced osteonecrosis of the femoral head and the controls, and the phenotypic characterization of hBMSCs. **A,** The expression of miR-181d in bone marrow of patients with steroid-induced necrosis was significantly higher than that of the control group. **B,** The shape of hBMSCs cultured for three days. **C,** Specific surface antigens of hBMSCs identified by flow cytometry, including positive CD44 (99.99%) and negative CD44 (0.12%).

(Figure 3E). These data indicated that miR-181d may inhibit osteogenic differentiation of MSCs by targeting SMAD3.

Down-Regulation of miR-181d Promoted the Osteogenic Differentiation of hBMSCs and Increased the Expression of RUNX2 and SMAD3

In order to further verify the effect of miR-181d on osteogenic differentiation of hBMSCs, negative control and miR-181d inhibitor were transfected into hBMSCs respectively. After osteogenic induction, it was demonstrated by ALP staining assay that the expression of miR-181d was decreased and ALP staining of MSCs became darker, indicating the ability of osteogenic differentiation was increased (Figure 4A). After 14 days of osteogenic induction, alizarin red staining was per-

formed and the results showed that inhibition of miR-181d expression could enhance alizarin red staining and the ability of osteogenic differentiation (Figure 4B). At the same time, the expression of RUNX2 and SMAD3 protein was significantly increased after the expression of miR-181d was suppressed (Figure 4C). These results indicated that miR-181d could inhibit osteogenic differentiation of hBMSCs by inhibiting SMAD3.

Discussion

Osteonecrosis of the femoral head (ONFH) is a result of bone cells death caused by blood circulation disorders in the subchondral bone, which could lead to dysfunction of the hip joint[15]. ONFH can be divided into two types: traumatic

A

ALP Staining

MSC   GCs-MSC

B

Alizarin Red
Staining
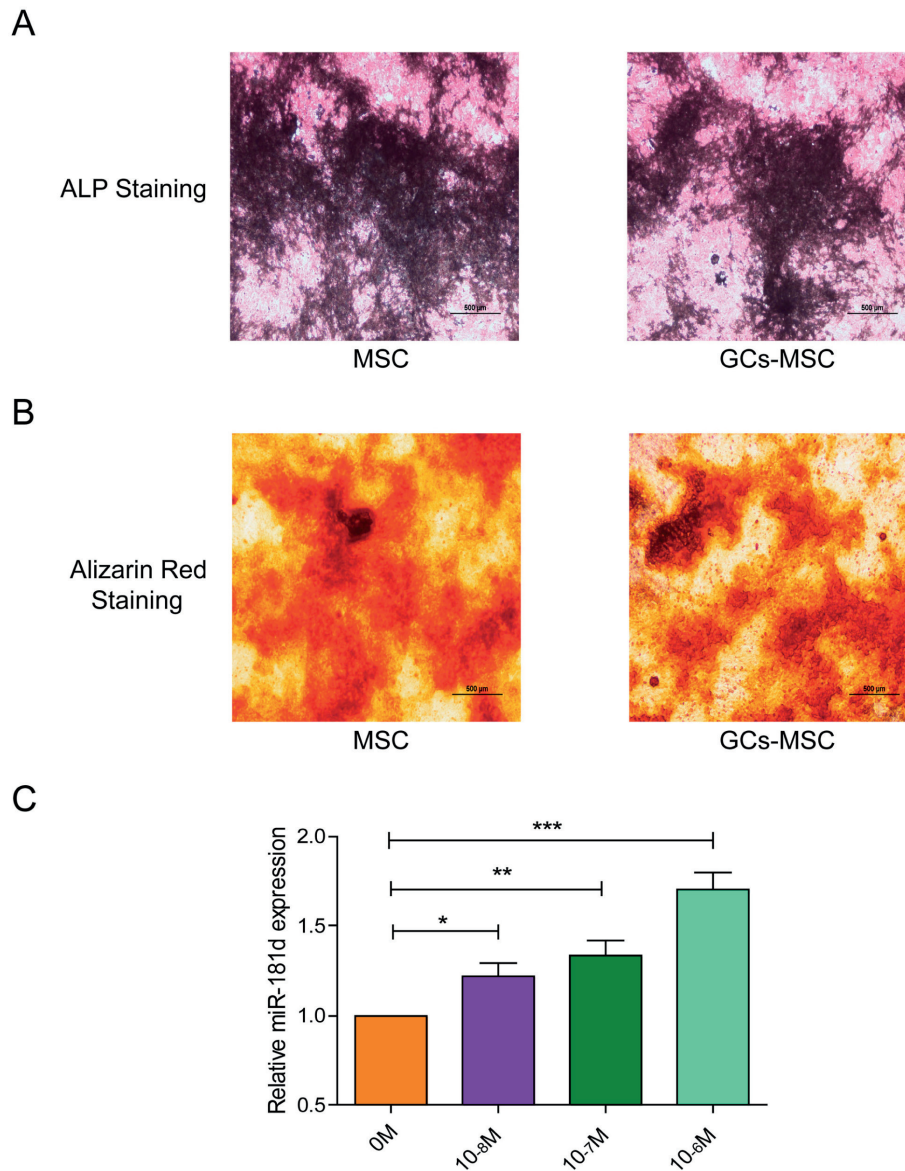
MSC   GCs-MSC

C

Figure 2. Effects of glucocorticoid on osteogenic differentiation ability and miR-181d expression of hBMSCs. *A,* HBMSCs from patients with steroid-induced osteonecrosis showed lighter ALP staining (Figure 2A) than the control hBMSCs after induction of osteogenic differentiation. *B,* HBMSCs from patients with steroid-induced osteonecrosis showed less alizarin red-stained mineralized nodules than the control hBMSCs after induction of osteogenic differentiation. *C,* The level of miR-181d expression increased with the increase of interference concentration in the non-GC group when MSCs were interfered with different concentrations of dexamethasone.

type and non-traumatic type. The most common cause of non-traumatic necrosis is the use of long-term high-dose glucocorticoid. Most patients with GC-induced ONFH have a clear history of extensive use of glucocorticoid, and the occurrence of ONFH is significantly higher when the equivalent doses are more than 2000 mg predni-solone[16]. This disorder not only seriously affects the quality of life of patients, but also brings huge and heavy economic burden to society. Therefore, timely and effective intervention of GC-induced ONFH will achieve great economic and social benefits. Unfortunately, effective early diagnosis, prevention method and specific treatment are still lacked. The major problem is that its pathogenesis has not yet been clarified.

BMSCs were first isolated and identified from bone marrow by Friedenstein et al[17]. BMSCs are
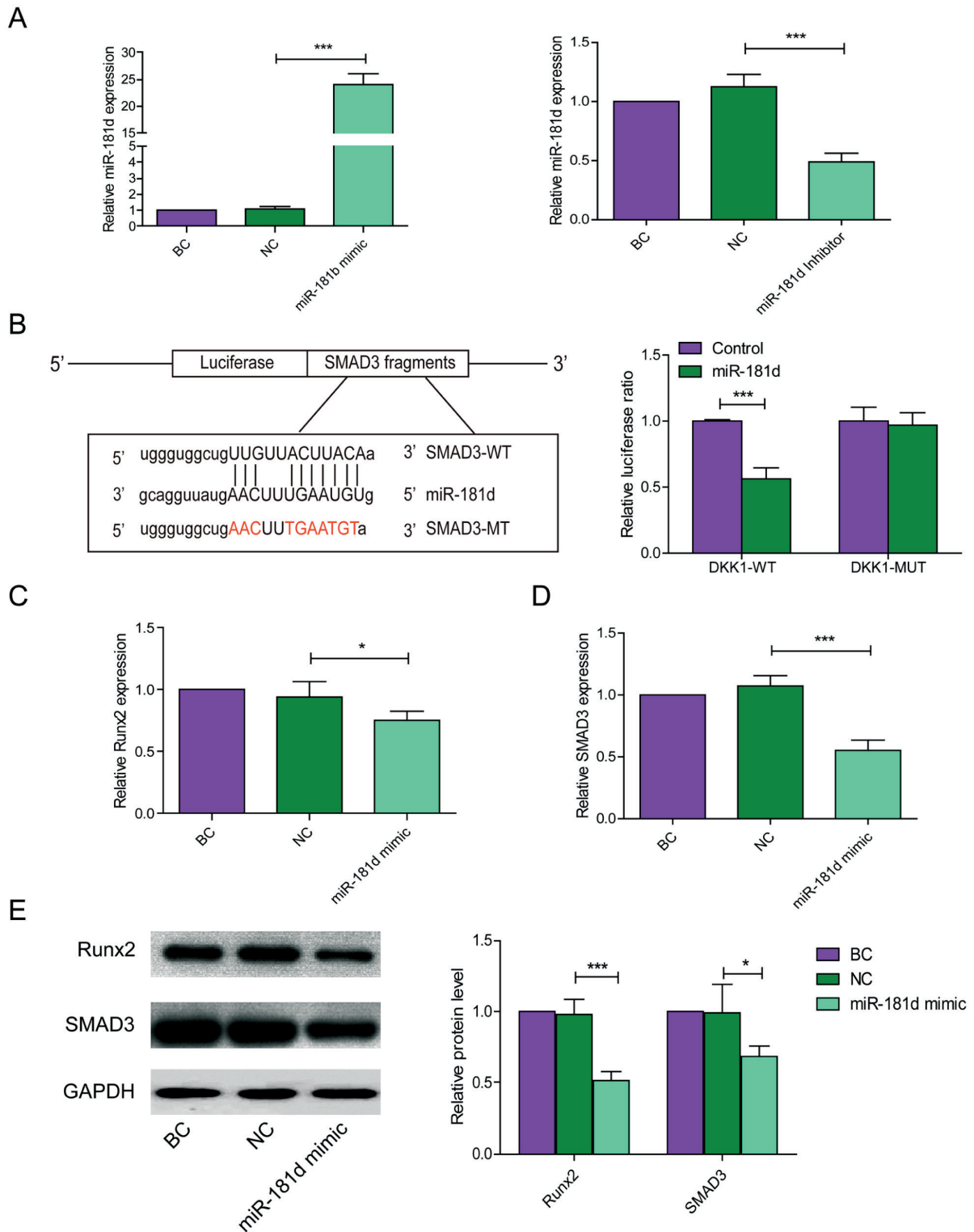
Figure 3. Up-regulation of miR-181d selectively regulated SMAD3 expression and inhibited osteogenic differentiation of MSCs. *A,* MiR-181d expression in hBMSCs after transfected with miR-181d mimic and miR-181d inhibitor. *B,* The sequence of the binding site of miR-181d with SMAD3 and the result of luciferase reporter assay. *C,* The expression of RUNX2 in hBMSCs was significantly decreased after overexpression of miR-181d and osteogenic induction. *D,* The expression of SMAD3 in hBM-SCs was significantly decreased after overexpression of miR-181d and osteogenic induction. E, The protein levels of RUNX2 and SMAD3 in hBMSCs were also significantly decreased after overexpression of miR-181d and osteogenic induction.
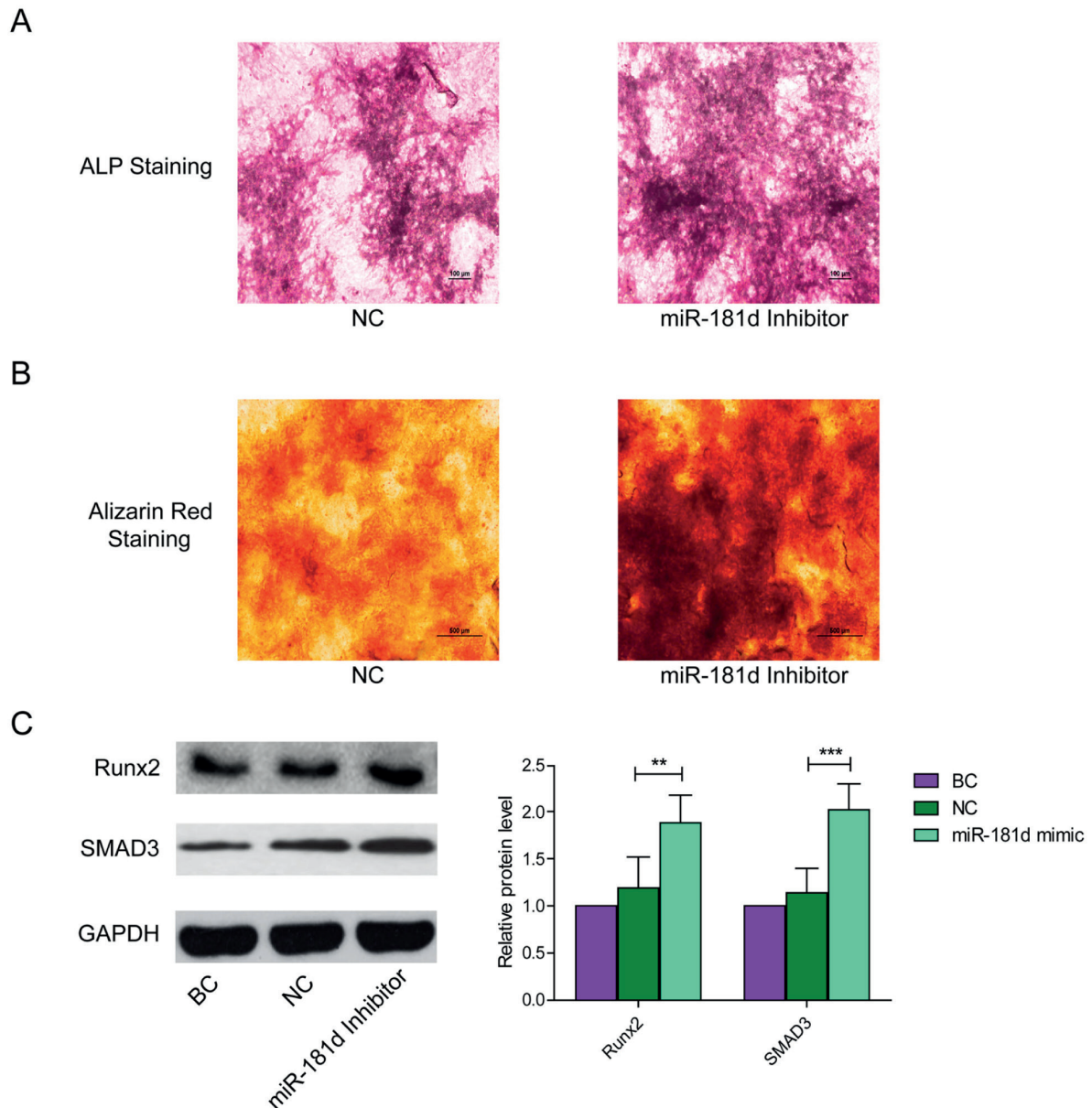
Figure 4. Down-regulation of miR-181d promoted the osteogenic differentiation of hBMSCs and increased the expression of RUNX2 and SMAD3. **A,** After miR-181d was down-regulated, ALP staining of MSCs became darker compared with the control group. **B,** After miR-181d was down-regulated, alizarin red-stained mineralized nodules increased compared with the control group. **C,** After down-regulating miR-181d, the protein levels of RUNX2 and SMAD3 increased.

easy to culture *in vitro* and can differentiate into osteoblasts under osteogenic conditions[18]. Recently, some scholars[19] have found that the ability of osteogenic differentiation of MSCs in patients with GC-ONFH is significantly decreased. However, the implantation of MSCs stimulated with colony-stimulated cytokine can partly improve the therapeutic effect of ONFH[20]. Previous *in vitro* experiments showed that although small

doses of glucocorticoids (GCs) are one of the necessary inducing factors for osteogenic differentiation of MSCs, high doses of GCs can inhibit the proliferation and differentiation of MSCs through the cellular glucocorticoid receptor and AP-1 pathway[21]. In this study, we successfully isolated, cultured, and identified MSCs from patients with 2 different types of ONFH. Induced differentiation and staining results indicated that, compared

with the MSCs from the control group, the osteogenic differentiation ability of MSCs from patients with GC-induced osteonecrosis was inhibited.

In recent years, miRNAs have been identified as a new class of molecules that regulate gene expression and play an important role in stem cell function[22]. Many miRNAs have been confirmed to be important intermediate nodes of regulating the signaling pathways involved in osteogenic differentiation. For example, researchers have found that miR-21, miR-26a and miR-196 are essential in the osteogenic differentiation of MSCs through targeting related genes and activating the corresponding signaling pathways[23-25]. Some studies[26,27] have confirmed that miR-188 and miR-194 exert great influence in the osteogenic and adipogenic differentiation of MSCs. In our study, miR-181d expression was significantly up-regulated in bone marrow of patients with GC-ONFH. In addition, its expression level was enhanced with the increasing use of GC concentration. During the process of inducing osteogenic differentiation of hBMSCs, high expression of miR-181d can inhibit the MSCs ability of osteogenic differentiation.

The SMADs protein family is an important part and checkpoint of TGF-beta signaling pathway and TGF-beta/BMP pathway, which plays a crucial role in the process of cell osteogenic differentiation. TGF-beta initiates signal transduction through Ser/Thr kinases on the type I and type II combination receptors that are on the cell membrane, followed by phosphorylation of the effector protein SMAD2/3 in the cytoplasm. The phosphorylated SMAD2/3 and SMAD4 then form complexes and are translocated into the nucleus, resulting in activation or repression of downstream genes and affecting cellular function and metabolism[28,29]. In this study, transfection of miR-181d mimics significantly increased the expression of miR-181d and reduced the expression of SMAD3. SMAD3 expression was enhanced by transfecting with miR-181d inhibitor, indicating that miR-181d can participate in the development of GC-induced osteonecrosis through SMAD3.

Conclusions

We found that miR-181d is highly expressed in patients with GC-induced osteonecrosis of the femoral head and -inhibit the differentiation of MSCs into osteoblasts through inhibiting SMAD3.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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