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Downregulated SOX9 mediated by miR-206 promoted cell apoptosis in Legg-Calvé-Perthes disease

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Abstract. Legg-Calvé-Perthes disease (LCPD) commonly onsets in adolescents, and threatens their health. However, the potential mechanism underlying LCPD remains unclear. MicroRNA (miR)-206 and SRY-box 9 (SOX9) serve an important role in chondrocytes; however, their role in LCPD remains ambiguous. In the present study, whether miR-206 and SOX9 mediated cell apoptosis in dexamethasone (DEX)-induced LCPD was investigated. The chondrocytes of the LCPD and normal control group were isolated from clinical tissues. Reverse transcription-quantitative polymerase chain reaction was used to evaluate the expression of miR-206 and SOX9 mRNA. Western blotting was used to measure the protein level of SOX9. A combination of Annexin V-fluorescein isothiocyanate flow cytometry was used to assess cell apoptosis. The association between miR-206 and SOX9 was detected using a luciferase reporter assay. miR-206 was overexpressed while SOX9 was downregulated in chondrocytes treated with DEX obtained from patients with LCPD. miR-206 targeted SOX9 to regulate its expression. Overexpression of miR-206 promoted cell apoptosis in TC28, while it was reversed by SOX9 overexpression. TC28 cells pretreated with DEX significantly promoted cell apoptosis, while cells transfected with miR-206 inhibitor significantly reversed the effect; however, downregulated SOX9 abolished the effects of miR-206 inhibitor. SOX9 mediated by miR-206 possibly contributed to the pathogenesis of LCPD. The results of the present study suggest that miR-206 and SOX9 function as important therapeutic targets for the future of clinical therapy.

Introduction

Legg-Calvé-Perthes disease (LCPD) is a common disease of femoral head necrosis that mainly affect children's health at the age of 2-12 years old (1). Study confirmed that boys diagnosed with LCPD was much more than girls (2). The clinical presentation of the early stage is the painful synovitis in the knees, and the follow-up symptoms presented that the femoral head ossific nuclei of LCPD are much smaller than those normal children with similar age, which made the traversing blood vessels are more vulnerable (3). The prognosis of LCPD in early onset is optimistic, while the treatment for advanced LCPD had poor outcome. Despite several consensus asserted that LCPD resulted from the uncoupling of bone metabolism, the exact pathophysiology remains elusive and the exploration of its mechanism remains a challenge.

SRY-related high-mobility-group box 9 (SOX9), widely accepted as a transcription factor, and governed a strong transactivation domains (4). Various studies have demonstrated that SOX9 is related to diseases. For example, downregulated SOX9 inhibited tumorigenesis in prostate; SOX9 mutations served as a therapeutic target in colorectal carcinoma (5). It is known that SOX9 is expressed in chondrocytes, and the expression level of SOX9 involved in the differentiation and formation of cartilage (6,7). Previous reports have revealed that SOX9 restrained chondrocytes to osteoblast lineage in the pathogenesis of Campomelic Dysplasia (8). SOX9 directly administrated growth plate chondrocytes in intervertebral disc (9). However, whether SOX9 acted as an important regulators in LCPD was still unclear.

MicroRNA (miRNA or miR) is a class of non-coding RNA with the length of 18-22 nucleotides, and recognized as the crucial gene regulators that target mRNAs by binding to its 3'-untranslated region (3'UTR), and further governed the degradation and translation processes (10). Its well known that miRNAs regulates gene expression, cell proliferation and apoptosis and signaling transduction in both animals and plants and as well as human. The aberrant expression of miRNA might result in the disorder of cells and tissues. Some miRNAs served as carcinogenic genes, while several miRNAs acted as tumor suppressors. For example, miR-182-5p served as an oncgenic gene in human bladder cancer by targeting Smad4 and RECK (11). While, miR-34a functioned as tumor suppressor in neuroblastoma by promoting cell apoptosis (12). Mounting evidences showed that miRNA played a vital role in the mechanism of diseases, and many miRNAs were acted as

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the biomarkers in disease diagnosis and therapy. miR-206 has been frequently presented in many diseases, and acted as tumor suppressor or oncogenic gene in different cancers. For example, miR-206 was downregulated in breast cancer, and suppressed cell proliferation by targeting cyclinD2 (13). Moreover, miR-206 also mediated cardiac hypotrophy (14) and oxidative stress (15). Study has proved that miR-206 mediated in the osteogenic differentiation in steroid-induced avascular necrosis of femoral head. While, whether it involved in the LCPD was still unknown.

Thus, in this study, several patients with LCPD were enrolled, combining with clinical test and *in vitro* experiments. Our aims were to explore the potential pathogenesis mechanism of LCPD, which might important for further LCPD therapy.

Materials and methods

Patients and ethical approval. Human femoral head cartilage tissues were collected from patients with LCPD (n=20, age 2-15), normal chondrocytes species were collected from patients with repair surgery after fracture (n=20, age 2-15). Cartilage tissues were immediately stored in -80˚C refrigerator and transferred to the laboratory. Patients with several diseases such as primary osteoarthritis, ankylosing spondylitis, systemic lupus erythematosus and inflammatory diseases were excluded.

This study was performed in accordance with The Third Hospital of Hebei Medical University (Shijiazhuang, China). All patients were assigned the informed consent.

Chondrocyte isolation and culture. The tissues of femoral head cartilage with LCPD and its normal control were disserted with trypsin for 30 min. Then collagenase II was used to digest the tissues overnight. The chondrocytes were collected using a mesh screen. The single-cell isolated from the suspension using an centrifuge (Eppendorf) following the instruction.

Human cartilage cell line TC28 were purchased from American Type Culture Collection. Both the isolated cells and TC28 were maintained in a 96-well plate supplemented with Dulbecco's modifed Eagle's medium (DMEM) at 37˚C with 5% CO_2.

Normal chondrocytes were cultured in DMEM pretreated with 0.01 mM dexamethasone (DEX) for 2 h. The level of miR-206 and SOX9 were determined to evaluate the effects of DEX.

Cell transfection. TC28 cells were cultured in DMEM for 24 h. Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was utilized to the transient transfection assay. The miR-206 mimic, miR-206 inhibitor and its negative control were transfected into cells, individually. The transfection efficiency was determined 24 h later. The SOX9 was overexpressed or downregulated in TC28 cells using retrovirus transduction. Briefly, the SOX9 expression plasmid accompanied with murine leukemia virus gap and pSV2-β-galactosidase plasmid were co-transfected into TC28 cells. The cells were harvested after 48 h. And the transfection efficiency was detect using quantitative polymerase chain reaction (qPCR). The empty plasmid served as control. The sequence of si-SOX9: 5'-ACAGAAUUGUGUUAUGUG ATT-3'.

qPCR. Total genome RNA was isolated from cells by using of TRIzol reagent kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. The RNA quality was determined by 1% agarose gel. Then 1 μg of RNA was taken out for the synthesize of cDNA using a Reverse Transcription kit (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was carried out on an ABI 7900 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative expression of mRNA was normalized by GAPDH. The primers' sequences were showing as following: SOX9: forward 5'-ATGAATCTCCTGGACCCCTT-3', reverse 3'-AACTTTGCCAGCTTGCACGT-5'; GAPDH: Forward 5'-TGAACGGGAAGCTCACTGG-3', reverse 3'-TCCACC ACCCTGTTGCTGTA-5'; miR-206: Forward 5'-CAAGAT GGCGACTTACGGATG-3', reverse 3'-GTGCAAACAGGA TGGACGTC-5'.

Western blot analysis. Cells were digested by lysis buffer, the protein was extracted using s supercentrifuge (Eppendorf). The protein concentrations were determined using a bicinchoninic acid assay kit (BCA; Beyotime Institute of Biotechnology, Haimen, China). SDS-PAGE were performed to separate protein extracts with equal amounts. Then the protein extracts were transferred to polyvinylidene fluoride (PVDF) membranes, and incubated with the primary antibodies (anti-SOX9, 1:500, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; anti-β-actin, 1;500, R&D Systems, Inc., Minneapolis, MN, USAD) at 4˚C for 24 h. the PVDF membranes were washed by PBS for twice and incubated for secondary antibodies for another 2 h at room temperature. The proteins were visualized using an enhanced chemiluminescence method. The quantity of protein was normalized by β-actin.

Apoptosis analysis. Cell apoptosis was evaluated by using a Annexin V-fluorescein isothiocyanate (FITC) cell apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instruction. Briefly, chondrocytes cells were maintained in the DMEM, then the cells were washed by PBS and resuspended with Annexin V-FITC and PI. The rate of cell apoptosis was evaluated by flow cytometry.

Luciferase reporter assay. The sequence of SOX9 was searched from Genbank and http://www.microrna.org/microrna/home.do. For luciferase reporter assay, cells were co-transfected with specific mutant-type or wild-type vector, and cotransfected with miR-206 inhibitor, mimics and their negative control (NC), respectively, using Lipofectamine® 2000 transfection kit (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were collected after transfection for 24 h. relative luciferase activity was analyzed using dual-luciferase reporter assay (Promega Corporation, Madison, WI, USA).

Statistical analysis. Data were presented as means ± standrad deviation for three individual experiments. Data analysis were processed using SPSS 18.0. Statistical differences were

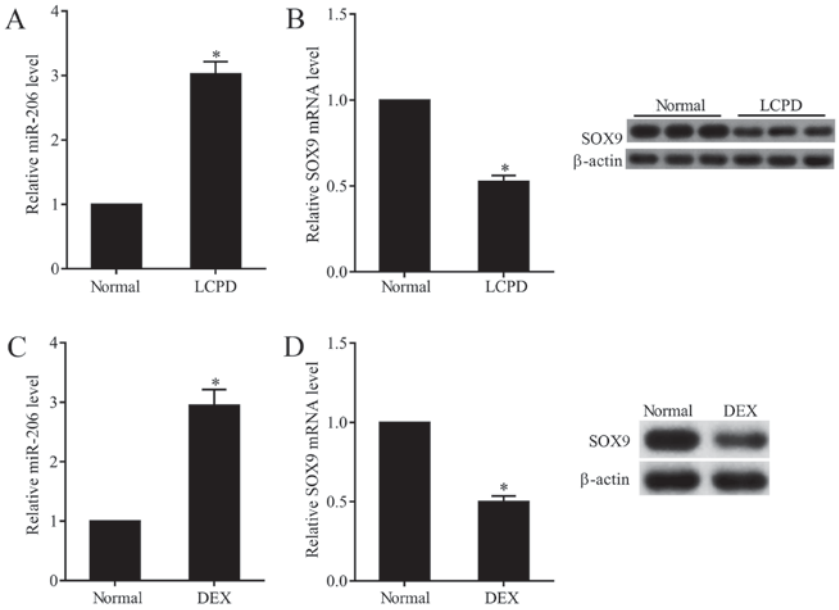

Figure 1. The expression pattern of miR-206 and SOX9 in LCPD cells. In chondrocytes that isolated from LCPD patients, (A) miR-206 expression level was detected using qPCR; (B) mRNA and protein levels of SOX9 were determined by qPCR and western blot. in chondrocytes that pretreated by DEX, (C) miR-206 expression level was measured using qPCR; (D) mRNA and protein levels of SOX9 were determined by qPCR and western blot. *P<0.05 vs. normal chondrocytes. β-actin served as internal control. miR. microRNA; SOX9, SRY-box 9; LCPD, Legg-Calvé-Perthes disease; qPCR, quantitative polymerase chain reaction.

analyzed by Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Overexpressed miR-206 and downregulated SOX9 in chondrocytes of LCPD patients. In this study, we observed that the expression of miR-206 in chondrocytes that isolated from LCPD patients was 3-folds that of normal chondrocytes (Fig. 1A), while the mRNA and protein expression of SOX9 was 50.9% that of normal chondrocytes (Fig. 1B). To further explore the effect of DEX on the expression of miR-206 and SOX9, normal chondrocytes (TC28) were subjected to 0.01 mM DEX. Results presented that DEX stimulation dramatically increased the expression of miR-206 (Fig. 1C) and decreased the mRNA and protein expression of SOX9 (Fig. 1D).

Overexpressed miR-206 promoted cell apoptosis. To evaluate the effect of miR-206 overexpression on cell apoptosis of chondrocytes, cells of chondrocytes that isolated from patients with repair surgery after fracture were transfected with miR-206 mimic, and 48 h later, cell apoptosis were determined by flow cytometry. As is presented in Fig. 2, miR-206 overexpression significantly promoted cell apoptosis.

miR-206 targeted SOX9 to regulate its expression. Online prediction found that miR-206 bound with the 3'UTR of SOX9 mRNA (Fig. 3A). To test whether miR-206 targeted SOX9 by binding 3'UTR, we constructed dual-luciferase reporter plasmid and transfected into TC28 cells. Our results revealed that miR-206 mimic significantly inhibited the relative luciferase activity of SOX9-3'UTR-WT. Moreover, miR-206 overexpression significantly decreased the expression of

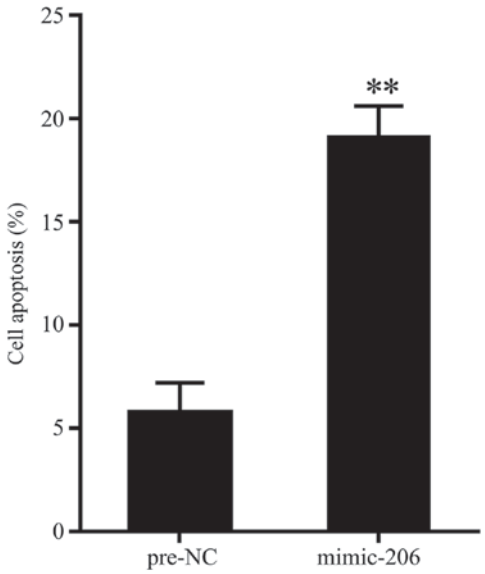

Figure 2. Flow cytometry analysis of Annexin V-FITC revealed that chondrocytes transfected with miR-206 mimic significantly promoted cell apoptosis. **P<0.01 vs. pre-NC. β-actin served as internal control. FITC, fluorescein isothiocyanate; NC, negative control.

SOX9 (Fig. 3B). In addition, miR-206 inhibitor dramatically increased the relative luciferase activity of SOX9-3'UTR-WT, and miR-206 downregulation promoted the expression of SOX9 (Fig. 3C).

Interaction effects of miR-206 and SOX9 on cell apoptosis. To investigate the effects of miR-206 and SOX9, cells of TC28 were co-transfected with miR-206 mimic and pcDNA-SOX9. As is shown in Fig. 4A, overexpressed miR-206 obviously promoted cell apoptosis, while the effect was abolished by

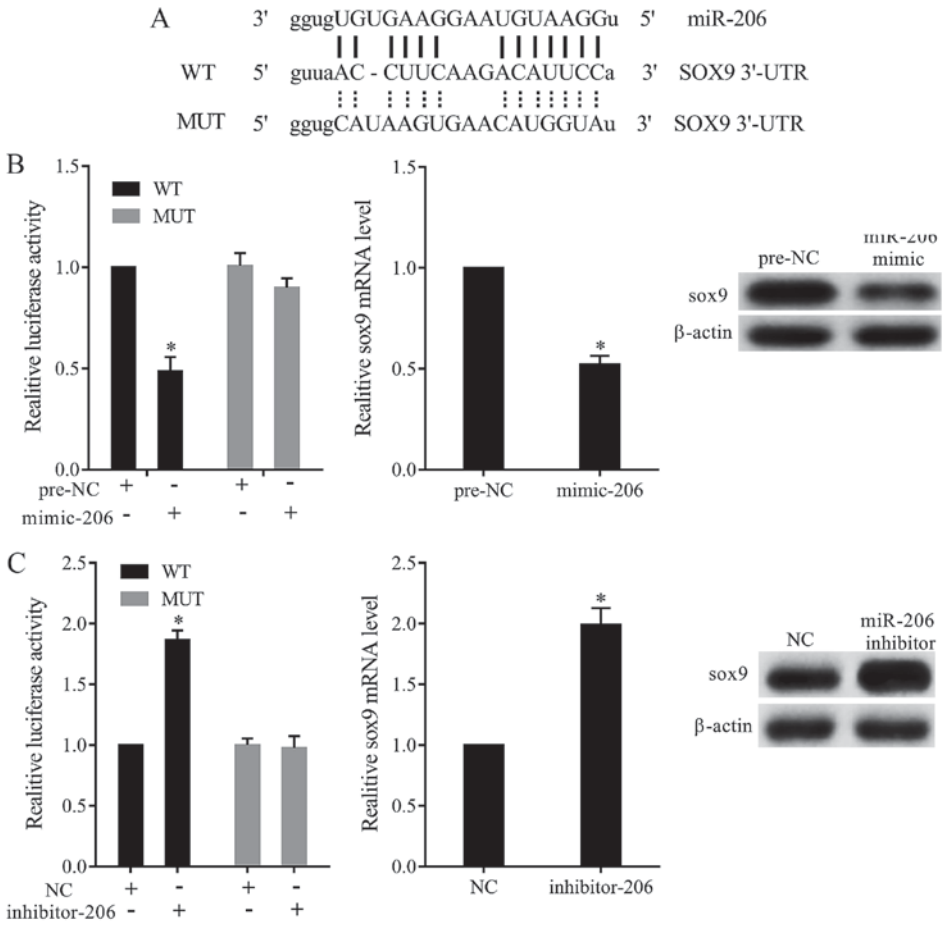

Figure 3. SOX9 is the direct target of miR-206. (A) SOX9 3'UTR bound the site of miR-206. (B) miR-206 mimic significantly decreased the relative luciferase activity of WT-SOX9 3'UTR, but not the MUT-SOX9 3'UTR. qPCR and western blotting determined the expression level of SOX9 in chondrocytes by pretreated with miR-206 mimic. (C) miR-206 inhibitor significantly promoted the relative luciferase activity of WT-SOX9 3'UTR, but note the MUT-SOX9 3'UTR. qPCR and western blotting measured the expression level of SOX9 in chondrocytes by pretreated with miR-206 inhibitor. *P<0.05 vs. pre-NC or NC. β-actin served as internal control. miR. microRNA; SOX9, SRY-box 9; 3'UTR, 3'-untranslated region; qPCR, quantitative polymerase chain reaction; WT, wild-type; MUT, mutant.

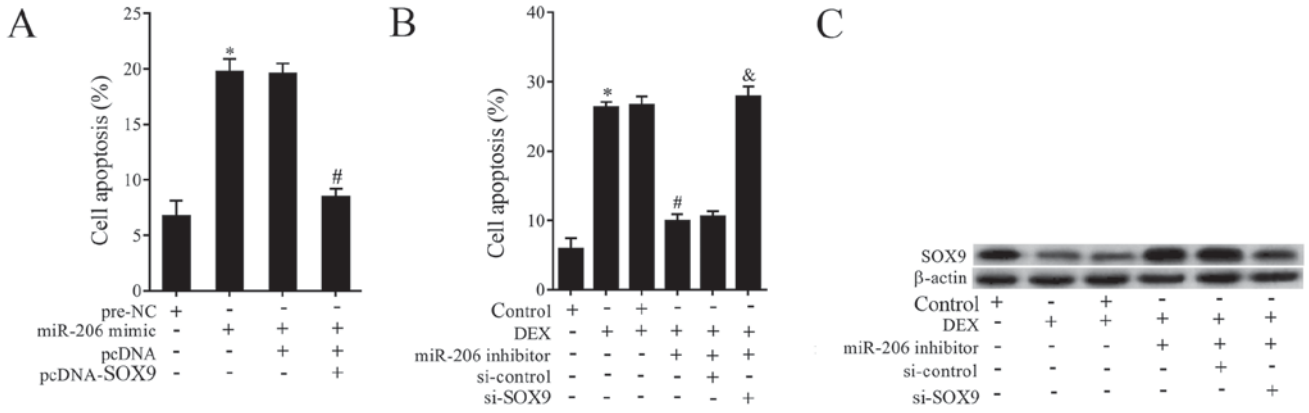

Figure 4. SOX9 expression was regulated by miR-206. (A) chondrocytes co-transfected with miR-206 mimic and pcDNA-SOX9, flow cytometry analysis of Annexin V-FITC detect cell apoptosis. Chondrocytes pretreated by DEX and co-transfectd with miR-206 inhibitor and si-SOX9, (B) flow cytometry analysis of Annexin V-FITC detect cell apoptosis, (C) western blotting measured the expression of SOX9. *P<0.05 vs. pre-NC or control; #P<0.05 vs. miR-206 mimic+ pcDNA or DEX+control; &P<0.05 vs. DEX+miR-206 inhibitor+si-control. β-actin served as internal control. miR. microRNA; SOX9, SRY-box 9; FITC, fluorescein isothiocyanate; DEX, dexamethasone.

overexpressed SOX9. Then to explore the effects of miR-206 inhibotor, si-SOX9 and DEX on the expression of SOX9 and cell apoptosis. Cells were pretreated with DEX and then co-transfected with miR-206 inhibitor and si-SOX9. The results demonstrated that DEX supplementation promoted cell apoptosis, miR-206 inhibitor transfection reversed the increased apoptosis, while si-SOX9 abolished the effects of miR-206 down-regulation (Fig. 4B). On the other hand, DEX

supplementation dramatically suppressed the expression of SOX9, miR-206 inhibitor reversed the effect of DEX, while si-SOX9 abolished the effect of miR-206 inhibitor (Fig. 4C).

Discussion

Recent years, the excessive usage of glucocorticoids has resulted in increasingly incidence of femoral head necrosis. Previous studies have tried to prevent the generation of steroid-induced femoral head osteonecrosis (SANFH), for example, enoxaparin and nitrate patch could use to prevent the occurrence of SANFH (16,17), while the potential mechanism was not fully understand. Liu *et al* found that miR-206 regulated connexin43 that involved in the osteogenic differentiation in SANFH (13). DEX was an anti-inflammatory drug, and was widely used in various serious disease (18). Study has reported that DEX could inhibit osteoblast differentiation (19,20). In this study, we aims to evaluate whether DEX supplementation could induce the similar effects in normal chondrocytes as LCPD chondrocytes.

There has been many publications reported that miRNAs exhibited a specific expression pattern in different tissues and development stages as well as lesion tissues. miR-206 was widely expressed in osteoblasts, and its expression was related to the differentiation of osteoblasts (21). Previous studies have demonstrated that overexpressed miR-206 suppressed the differentiation of osteoblast (13). While in this study, we found that comparing with the normal control, the expression of miR-206 was significantly increased in LCPD patients. In addition, 0.01 mM DEX subjected to normal chondrocytes significantly promoted the expression of miR-206. All those results suggested that miR-206 was overexpressed in both LCPD and DEX-pretreated chondrocytes. SOX9 is an important family member of SOX that mediated cell proliferation process (22). SOX9 is crucial on bone development, and is expressed in proliferating chondrocytes. Thus, in this study, we evaluated the expression of SOX9 to analysis the proliferation and apoptosis of chondrocytes in LCPD. Our study revealed that, the expression of SOX9 in chondrocytes of LCPD was decreased, DEX supplementation significantly decreased its expression. To further confirm the potential relationship between miR-206 and SOX9, both online TargetScan and http://www.microrna.org/microrna/home.dowere used to selected the sequences, results found that miR-206 could bind SOX9 in the site of 3'UTR. Then luciferase reporter assay revealed that miR-206 targeted SOX9 to regulate it expression. Thus we inferred that both miR-206 and SOX9 played an vital role in LCPD. Next, to verify the relationship of miR-206 and SOX9, TC28 cells were transfected with miR-206 mimic, and we found that overexpressed miR-206 significantly promoted cell apoptosis, while the effect was abolished by overexpressed SOX9.

To better understand the role of miR-206 and SOX9 in chondrocytes, the possible molecular mechanism was also investigated. We found that DEX stimulation significantly increased the expression of SOX9 and promoted cell apoptosis, while miR-206 inhibitor reversed it, however, si-SOX9 abolished the effects of downregulated miR-206. However, our study was accordance with the previous study of Liu *et al*, who also found that miR-206 was higher in SANFH (13), indicating the reliability of our results. Taken together, all those results indicated that miR-206 and SOX9 was mediated the mechanism of DEX-induced LCPD, which was important for the further clinical therapy of glucicorticoid- induced LCPD.

In summary, this study revealed that miR-206 was highly expressed while SOX9 was low expressed in chondrocytes in LCPD. SOX9 mediated by miR-206 was probably mediated the pathogenic mechanism of LCPD. Further clinical therapy of LCPD based on the regulation of miR-206 and SOX9 expression is important. The present study provided firm foundation for exploration of the mechanism of LCPD.

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