miRNA-101 promotes chondrogenic differentiation in rat bone marrow mesenchymal stem cells

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Abstract. Effect and related mechanisms of miR-101 on the chondrogenic differentiation of rat bone marrow mesenchymal stem cells (MSCs) were investigated. The expression level of miR-101 was detected during chondrogenic differentiation. Three groups were established to study the potential function between miR-101 and chondrogenic differentiation: miR-NC group (negative control), miR-101 mimics (BMSCs transfected by miR-101 mimics) and mimics + inhibitor (BMSCs transfected by miR-101 mimics and inhibitor), after the induction of chondrogenic differentiation, the cell viability of MSCs and chondrogenic markers were determined, further, the expression level of Sox9 and Runx2 were detected. In our present research, miR-101 was found upregulated during chondrogenic differentiation of MSCs. Compared with the miR-NC group, the cell viability of MSCs was enhanced and the expression level of chondrogenic markers were respectively gained. The expression level of Sox9 was increased but the expression level of Runx2 was decreased by treatment of miR-101 mimics after induction of chondrogenic differentiation. However, these variations of the indicators were reversed by the intervention using the miR-101 inhibitor. Collectively, our research revealed promotion function of miR-101 on chondrogenic differentiation of MSCs, indicating that miR-101 could be a potential therapeutic strategy for the treatment of osteoarthritis (OA).

Introduction

Osteoarthritis (OA) is characterized by gradual degeneration of articular cartilage due to various causes of injury, hyperplasia at the edge of joint, formation of osteophytes, and synovial inflammatory response (1). OA is the most common cause of joint disease, especially in the elderly, and its clinical manifestations are joint pain and dysfunction (2,3). Although there are many causes of OA in humans, including changes in subchondral bone, ligamentous laxity and synovial fluid inflammation, articular cartilage injury is still an important pathological factor leading to OA, and studied most frequently (3,4). Reasons for articular cartilage injury mainly include biomechanical and biochemical factors, which also interact with each other. The structure of articular cartilage is different from other tissues, and is almost impossible to repair when it is damaged due to degeneration or trauma, ultimately developing to pathological changes in OA (5,6). Therefore, there is a lack of effective clinical treatment means of OA currently. The early conservative treatment is mainly based on oral administration of drugs and physical therapy, but they can only relieve the symptoms, but cannot delay the progression of disease. OA in the late stage can only be treated with surgery to relieve joint pain and improve function. As the incidence of OA is very common, a large number of medical resources are consumed each year. Therefore, prevention and intervention of the disease are particularly important.

The number of mesenchymal stem cells (MSCs) in articular cartilage is very small, accounting for only 3-4% of that of articular chondrocytes. When OA occurs in human, the differentiation of MSCs in articular cartilage, despite its active proliferation, into osteoblasts or cartilages is very limited. The importance of using tissue engineering technique to overexpress the target gene, promoting the proliferation and differentiation of MSCs into cartilages when OA occurs, maintaining the dynamic balance between cartilage tissue injury and repair, and restoring the self-regulation and self-repair capacities of articular chondrocytes in OA is inestimable in the treatment of OA (7,8). At present, the treatment method is generally culturing cytokines *in vitro* first to make tissue engineering components, and then implanting them into the body, but the stability is poor with higher cost, so it is not suitable to be applied directly (9). Therefore, introducing the target gene into cells combined with transgenic technology can repair the degraded or damaged cartilage through the high expression of target gene in cells to promote the proliferation and differentiation of MSCs into cartilages, so the research on these target genes has important application prospect and value in the treatment of human OA.

Micro ribonucleic acid (miRNA) is a class of non-coding single-stranded RNA with approximately 18-22 nucleotides in length, which negatively regulates the post-transcriptional

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expression of target gene by binding specific sequences to messenger RNA (mRNA). In recent years, studies have shown that miRNA is not only involved in the regulation of articular cartilage development, but also closely related to the occurrence and development of OA (10-11).

The regulatory effect of miR-101 on differentiation of MSCs in articular cartilage was studied, investigating whether miR-101 can regulate the differentiation of MSCs into chondrocytes, aiming elucidation of the interaction mechanism of miRNA and protein-coding genes during the differentiation process of MSCs induced by osteogenic microenvironment, and to lay a foundation for the regulation of biological behavior of chondrocytes using miRNA in clinic, which is expected to provide valuable clues for the treatment of OA.

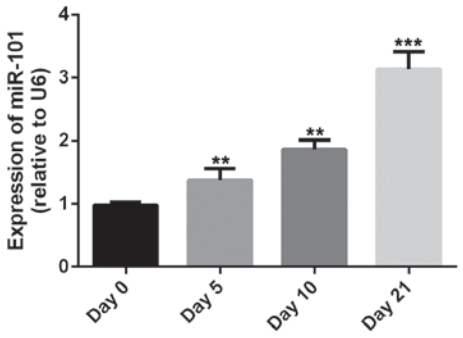

Figure 1. Expression of miR-101 during the chondrogenic differentiation. **P<0.01; ***P<0.001 compared with day 0.

Materials and methods

Isolation and culture of MSCs. A total of 60 healthy Sprague-Dawley (SD) rats (male=30; female=30) aged 12 weeks (meanweight=400 g) were selected and sacrificed under anesthesia. The rats were housed in a temperature controlled room (21±2˚C) on a 12:12-h light/dark cycle (lights on at 06:00). All rats had free access to water and food. This study was approved by the Animal Ethics Committee of Jilin University Animal Center (Changchun, China). Bilateral lower extremities of rats were isolated, the epiphyseal ends were cut off, and the bone marrow in marrow cavity was poured out. MSCs were gently blown and beaten using phosphate-buffered saline (PBS) to be prepared into single-cell suspension, and centrifuged at 1,050 x g for 10 min. After the supernatant was discarded, cells were resuspended in F12/Dulbecco's modified Eagle medium (DMEM), inoculated into a 25 cm^2 culture flask, and cultured under 5% CO_2 at 37˚C. The solution was replaced once every 2 days. The cell morphology and growth status were observed under an inverted microscope (AZ100; Nikon Corporation, Tokyo, Japan). When cells were fused, they were digested using 0.25% trypsin, followed by passage at a ratio of 1:2.

Cell transfection and treatment. BMSCs at passage 3 were pre-cultured in a 24-well plate for 24 h, miR-101 mimics and inhibitor were synthesized and transfected to MSC cells to analyze biological function of miR-101. Then three groups were established to study the potential relevance between miR-101 and chondrogenic differentiation: miR-NC group (negative control), miR-101 mimics (BMSCs transfected by miR-101 mimics) and mimics + inhibitor (BMSCs transfected by miR-101 mimics and inhibitor). The above material were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China), and transfected by using Lipofectamine RNAiMAX (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. At 36 h after transfection, the chondrogenic differentiation of bone marrow-derived MSCs into chondrocytes was induced, and cartilage micelles were constructed via centrifugation at 2,500 x g for 5 min.

Induced chondrogenic differentiation of bone marrow-derived MSCs into chondrocytes. The cell suspension was prepared and the cell concentration was adjusted into $1x10^9$/l. Then cell suspension was transferred into a centrifuge tube for centrifugation at 2,500 x g for 5 min, and the supernatant was discarded. Cartilage-inducing liquid (containing 50 mg/l ascorbic acid, 1% fetal bovine serum, 100 nmol/l dexamethasone, 100 mg/l sodium pyruvate, 1.0% indometacin, 40 mg/l L-proline, and low-glucose DMEM) was added, cell mass was not dispersed, and cells were cultured under 5% CO_2 at 37˚C. The cartilage-inducing liquid was replaced once every 3 days, and cartilage mass was formed after induction for 3 weeks.

Cell proliferation. At 21 days after induction of chondrogenic differentiation, cells were harvested and inoculated into 96-well plates at a density of $2x10^3$ cells for 48 h, MTT solution [5 mg/ml; Multisciences (Lianke) Biotech Co., Ltd., Hangzhou, China] was appended to each well after 4 h incubation. Then, 150 μl of dimethyl sulfoxide (DMSO) was added to each well for solubilizing the formazan. After 30 min, the absorbance was measured by a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) set at 490 nm.

Alcian blue staining and quantification. At 21 days after induction of chondrogenic differentiation, the medium was discarded. Cells were washed twice with PBS, and 0.5 ml alcian blue dye solution prepared by 0.1 mol/l hydrochloric acid was added into each well. After the mixture was placed at room temperature overnight, the dye solution was discarded. Then cells were washed with double distilled water several times, photographed under the inverted microscope (AZ100; Nikon Corporation), added with 6 mol/l guanidine hydrochloride, and placed at room temperature for 2 h. After the washing solution was removed, the optical density was measured by a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) set at 630 nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was procured by TRIzol reagent in accordance with the manufacturer's protocol. SYBR-Green qPCR assay was used to measure the level of collagen II, aggrecan, SRY-related high mobility group-box gene9 (Sox9) and Runt-related transcription factor 2 (Runx2) expression and endogenous controlled by GAPDH. TaqMan miRNA assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to measure the level of miR-101 expression normalized to miRNA U6. Primers used in PCR reaction were listed as

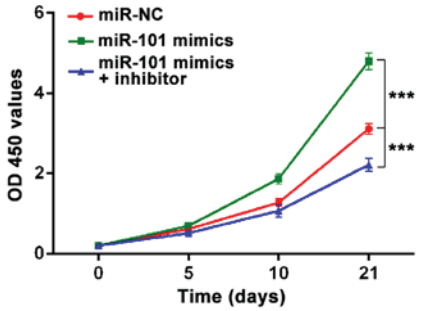

Figure 2. The effect of miR-101 on the cell viability of MSCs. Upregulation of miR-101 improved the cell viability of MSCs while downregulation of miR-101 by inhibitor suppressed the cell viability. Data are presented as mean ± standard deviation. ***P<0.001 compared with the miR-NC group.

follows: Sox9 forward, 5'-ATGGAAATCACGGAAGAGCG TC-3'; reverse, 5'-GTGCTGAAGGGCTACGACTGGA-3'. Runx2 forward, 5'-ACCAGCAGCACTCCATATCTCTAC-3'; reverse, 5'-CTTCCATCAGCGTCAACACCATC -3'. MiR-101 forward, 5'-GCGGGCGTAGTGATAA-3'; reverse, 5'-GTG CAGGTCCGAGGT-3'. GAPDH forward, 5'-ACCTCAACT ACATGGTCTAC-3'; reverse, 5'-TTGTCATTGAGAGCAA TCC-3'. With U6 as endogenous control, the relative expression level of miR-101 was calculated by $2^{-\Delta\Delta Cq}$ method (12).

Western blot analysis. At 21 days after induction of chondrogenic differentiation, cells were collected and lysed using radioimmunoprecipitation assay (RIPA) buffer. The total protein was extracted, and its concentration was determined according to instructions of the bicinchoninic acid (BCA) protein concentration kit. The same amount of total protein (30 ug proteins per lane) was separated via sodium dodecyl sulfate-polyacrylamide gel electropheresis (10% SDS-PAGE), transferred onto a nitrocellulose membrane, sealed with 5% skim milk and incubated with rabbit anti-rat Sox9 and Runx2 primary antibodies (1:1,000) at 4˚C overnight. Primary mouse monoclonal Runx2 antibody (dilution, 1:500; cat. no. ab76956); rabbit monoclonal Sox9 antibody (dilution, 1:500; cat. no. ab185966); rabbit polyclonal GAPDH antibody (dilution, 1:500; cat. no. ab37168) and secondary goat anti-rabbit (HRP) IgG antibody (dilution, 1:2,000; cat. no. ab6721) were all purchased from Abcam (Cambridge, MA, USA). After the membrane was fully washed with Tris-buffered saline with Tween-20 (TBST), anti-rabbit secondary antibody (coupled by horseradish peroxidase) was added for incubation at room temperature for 2 h, followed by development via enhanced chemiluminescence (ECL) (Merck Millipore, Billerica, MA, USA), expo-sure in gel imaging system, fixation and observation of results. With β-actin as an internal reference, the relative changes in protein expression were detected. Image J software (Version 1.38; National Institutes of Health, Bethesda, MA, USA) was used for protein quantification.

Immunohistochemical staining. At 21 days after induction of chondrogenic differentiation, the medium was discarded. Cells were washed twice with PBS, and fixed with 4% paraformaldehyde for 30 min. Immunohistochemical staining was performed for Sox9 and Runx2 according to protocol of the kit, and they were photographed under the microscope.

Statistical analysis. Statistical Product and Service Solutions (SPSS) 19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. All quantitative data are expressed as mean ± standard deviation. Comparison between groups was done using one-way ANOVA test followed by post hoc

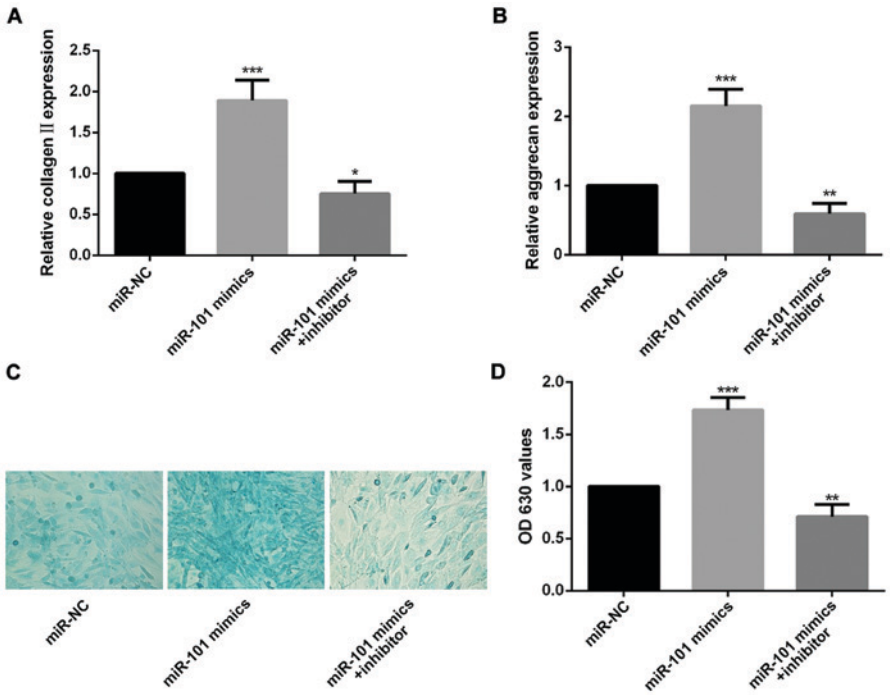

Figure 3. The effect of miR-101 on the expression of chondrogenic markers. (A) Expression of collagen II determined by RT-qPCR. (B) Expression of aggrecan determined by RT-qPCR. (C) Glycosaminoglycan expression detected by alcian blue staining (original magnification x400). (D) Quantification of C. Data are presented as mean ± standard deviation. *P<0.05; **P<0.01; ***P<0.001 compared with the miR-NC group.

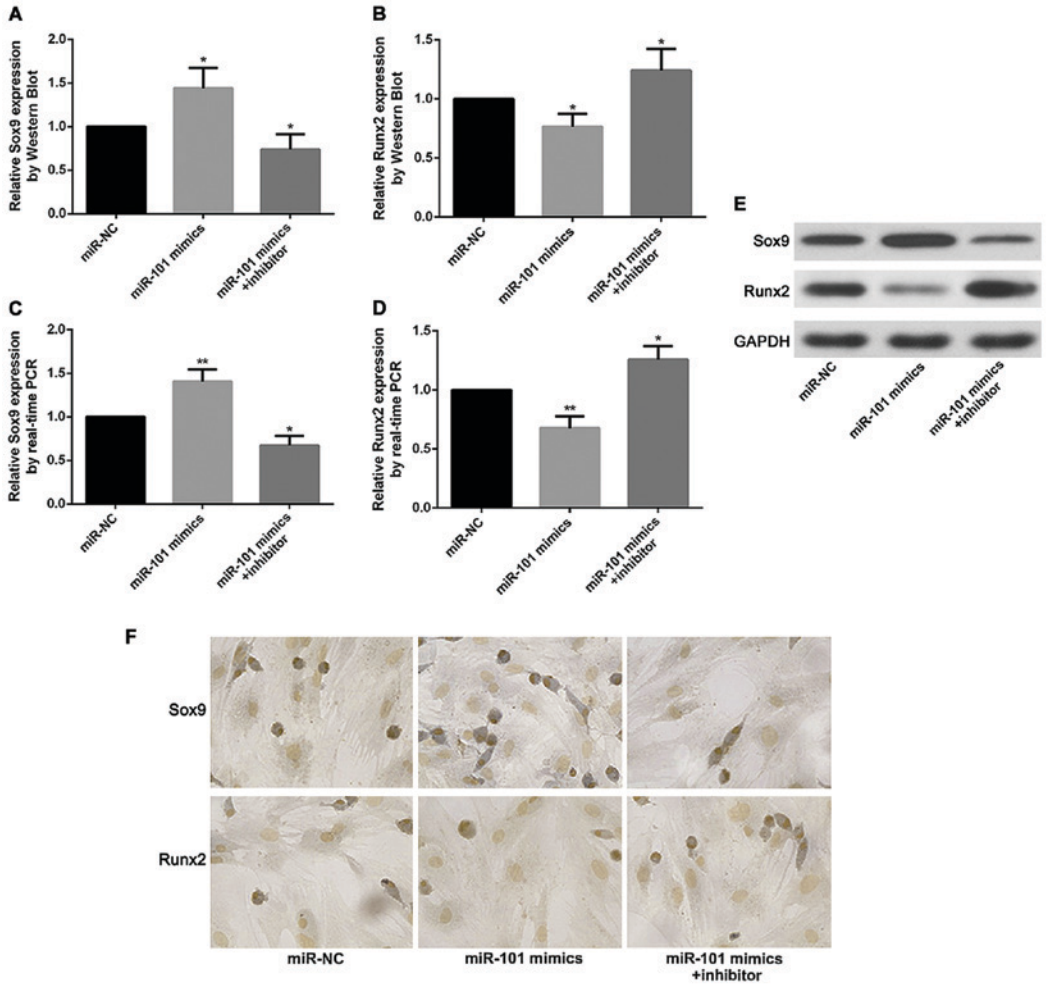

Figure 4. Effect of miR-101 on the expression of Sox9 and Runx2. (A) Expression of Sox9 determined by RT-qPCR. (B) Expression of Runx2 by RT-qPCR. (C and D) Statistical analysis of E. Data are presented as mean ± standard deviation. (E) Protein expression of Sox9 and Runx2 determined by western blot analysis. (F) Expression of Sox9 and Runx2 detected by immunocytochemical staining (original magnification x400). *P<0.05, **P<0.01 compared with the miR-NC group.

test (least significant difference). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-101 is upregulated during chondrogenic differentiation of MSCs. The present study demonstrated significant upregulation of miR-101 expression during chondrogenic differentiation (13). In our preliminary experiment, the expression of miR-101 was detected after the induction of chondrogenic differentiation of MSCs by RT-qPCR, consistent with the literature, the expression level of miR-101 was increased during the chondrogenic differentiation of MSCs (Fig. 1). The results indicated that miR-101 may have a regulating effect on the chondrogenic differentiation of MSCs (Fig. 1).

miR-101 promotes the cell viability of MSCs. To examine the function of miR-101 on proliferation of MSCs, we used MTT assay to detect the cell viability at 0, 5, 10 and 21 day of chondrogenic differentiation. The results from the MTT assay revealed that miR-101 obviously enhanced MSC viability, whereas downregulation of miR-101 by inhibitor markedly suppressed MSC viability after four days incubation (Fig. 2).

miR-101 increases the expression of chondrogenic markers. Collagen II and aggrecan have been designed as unique chondrogenic markers (14). RT-qPCR was used to analyze the effect of miR-101 on expression of chondrogenic markers. the results show that both expression level of collagen II and aggrecan could be upregulated by intervened with miR-101 mimics, and on the contrary, mRNA levels of two chondrogenic markers were restrained by miR-101 inhibitor (Fig. 3A and B).

Alcian blue staining was a method to detect the glycosaminoglycans in cells. After induction of chondrogenic differentiation for 21 days, alcian blue staining was performed, the intensity of the cells in miR-101 mimics group was markedly increased by comparing with the miR-NC group. Notably, the intensity was reduced by adding miR-101 inhibitor (Fig. 3C and D).

The above results suggest that miR-101 has a positive effect during chondrogenic differentiation of MSCs.

miR-101 increases the expression level of Sox9 and decrease the expression level of Runx2. Recent studies have demonstrated that expression levels of Sox9 and Runx2 directly impact the progress of OA. In the pathogenesis of OA, Sox9 promotes the differentiation of chondrocytes and has inhibitory effects on

OA, while Runx2 mediates the degradation of chondrocytes and promotes the OA. In our research, expression of Sox9 was found substantially increased after treatment with miR-101 mimics by comparing with the miR-NC group. However, the effect of miR-101 has been counteracted by addition of miR-101 inhibitor. As expected, the opposite trend was found in the expression of Runx2 measuring by RT-qPCR, western blot analysis and immunohistochemical staining (Fig. 4).

Discussion

OA is the most common chronic disease among all types of degenerative joint diseases, which can affect tissues and joints in the whole body (15). There are not many treatment methods for OA, and the effects of drugs and non-surgical treatment are not satisfactory, so joint replacement surgery is a last choice for OA in the late stage. Currently, MSCs used to treat various degenerative skeletal muscle diseases provide a new therapeutic strategy for OA (8).

In the process of cartilage formation, Sox9 gene plays an important role in the cartilage differentiation and has a protective effect on cartilage tissues. However, when the degradation rate of cartilage tissues exceeds its synthesis rate, it will develop into the pathological process of OA. In addition, endochondral ossification also plays a crucial role in the pathogenesis of OA (16). The pathological process of OA actually begins from the transformation from chondrocytes to mastocytes, which transforms cartilage tissues from low-oxygen tissues without vessels to bone tissues with blood supply through the expressions of type X collagen, vascular endothelial growth factor, metalloproteinase 13 and other molecular substances, accompanied by matrix degeneration, vascular endothelial invasion, endochondral ossification at the center of joint, and marginal osteophyte formation. Runx2 is an important factor initiating this pathological process.

Sox9 gene is expressed in all pre-chondrocytes and differentiated chondrocytes, excluding hypertrophic chondrocytes, which not only has important positive regulatory and control effects on the differentiation and maturation of cartilage, but also is essential for the normal physiological development process of cartilage growth plates and endochondral ossification (17,18). The differentiation of MSCs into cartilage and the maintenance of chondrocyte phenotype depend on the stable expression of Sox9 gene (19-22). Runx2 is highly expressed in pre-hypertrophic chondrocytes and hypertrophic chondrocytes in mammals. Runx2 is involved in both endochondral ossification and chondrocyte hypertrophy. Besides, Runx2 can be activated in many signaling pathways on osteogenic differentiation, such as transforming growth factor-β (TGF-β) (23), Wnt/β-catenin (24), Notch (25) and bone morphogenetic protein 2 (BMP2) (26), activating a series of downstream genes, and making cells differentiate into osteoblasts. It is found from the above that during the pathogenesis of OA, Sox9 inhibits OA through promoting chondrocyte differentiation, while Runx2 mediates the process of chondrocyte hypertrophy and controls the degradation of chondrocytes, thus promoting OA.

In the present study, the expression of miR-101 on differentiation of MSCs in articular cartilage was analyzed, and it was found that the expression level of miR-101 was upregulated during chondrogenic differentiation of MSCs. After induction of chondrogenic differentiation for 21 days. MTT assay, alcian blue staining, RT-qPCR, western blot analysis and immunohistochemical staining were performed, and it was found that miR-101 could promote the cell viability of MSCs and elevate the expression of chondrogenic markers such as collagen II, aggrecan and glycosaminoglycans. Furthermore, the expression level of Sox9 which has promotion effect in chondrogenic differentiation was more noticeable in MSCs in miR-101 group, while the expression level of Runx2, which is a obstructive factor in chondrogenic differentiation, was decreased by miR-101 intervention. All effects of miR-101 on chondrogenic differentiation of MSCs could be suppression by miR-101 inhibitor.

In conclusion, in the present study, miR-101 was found upregulated during chondrogenic differentiation and play an important role to deaccelerate chondrogenic differentiation of MSCs, and its function depends on regulating the expression of Sox9 and Runx2, our results revealed that miR-101 could be a potential therapeutic strategy for the treatment of OA.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

FG and MW designed the study and performed the experiments, FG, CZ and SZ established the animal models, CP and SZ collected the data, FG and CZ analyzed the data, FG and MW prepared the manuscript. All authors read and approved the final study.

Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of Jilin University Animal Center (Changchun, China).

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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