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# MicroRNA-30a promotes chondrogenic differentiation of mesenchymal stem cells through inhibiting Delta-like 4 expression

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ABSTRACT

*Aims:* MicroRNAs (miRNAs) play important roles in chondrogenic differentiation of mesenchymal stem cells (MSCs). However, the regulation of miR-30a during such process has not yet been well understood. The aim of the study was to investigate the effects of miR-30a on chondrogenic differentiation of MSCs and explore the underlying mechanisms.

*Materials and methods:* MSCs were isolated from rat bone marrow, and their immunophenotypes and multilineage differentiation potentials were identified. MiR-30a mimics or inhibitor were transfected into rat MSCs and SW1353 cells, respectively, and then the effects of miR-30a on chondrogenic differentiation were detected. The predicted target gene Delta-like 4 (DLL4, a ligand of the Notch signaling family) was verified by luciferase reporter assay, quantitative real time PCR and western blot.

*Key findings:* MiR-30a was significantly up-regulated during chondrogenic differentiation of rat MSCs. Additionally, transfection of miR-30a mimics remarkably promoted the differentiation of rat MSCs into chondrocytes as evidence by the notably increased mRNA and protein expression levels of chondrogenic markers Collagen II and aggrecan as well as the enhanced alcian blue staining intensity, whereas inhibition of miR-30a obviously suppressed such process. Furthermore, during chondrogenesis, DLL4 expression was found to significantly decrease at both mRNA and protein levels, which was negatively regulated by miR-30a through directly targeting the 3'UTR of DLL4.

*Significance:* Our results indicate that miR-30a acts as a key promoter for chondrogenic differentiation of MSCs by down-regulating DLL4 expression, and provide a novel insight on miRNA-mediated MSC therapy for cartilage-related disorders including osteoarthritis.



## 1. Introduction

Osteoarthritis (OA) is one of the most common forms of slowly progressive degenerative articular disease that affects millions of patients worldwide [1]. The principal pathophysiological characteristics of OA include progressive cartilage degradation, subchondral bone remodeling, synovial inflammatory response and osteophyte formation, which result in joint pain, stiffness and dysfunction [2,1]. Although traditional drug therapies for OA such as non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase (COX) inhibitors and hyaluronic acid can provide partial pain relief, these drugs can not cure OA thoroughly but cause gastrointestinal complications [3,4]. Recently, transplantation of mesenchymal stem cells (MSCs) has been considered as a new strategy for OA treatment due to their potential to differentiate into chondrocytes as well as anti-inflammatory and immunomodulatory effects [5,6]. Chondrogenic differentiation of MSCs plays critical roles in successful cartilage regeneration, because these differentiated chondrocytes are able to mediate stable cartilage formation [7]. Such cellular processes depend on complex networks involving many transcription factors and growth factors as well as multiple signaling pathways.

MicroRNAs (miRNAs) are small non-coding RNAs (approximately 22 nucleotides), which negatively regulate the expression of target genes by binding to 3′-untranslated region (3′-UTR) of mRNAs [8]. To date, hundreds of miRNAs have been identified, and their roles are being widely investigated in almost every field in biology and linked to diverse biological processes such as cell proliferation, apoptosis, differentiation, development, and tumorigenesis [9,10]. Recently, increasing evidence has shown that specific miRNAs play important regulatory roles in chondrogenic differentiation. Yang et al investigated the miRNA expression profiles of MSCs during chondrogenic differentiation, which revealed five down-regulated miRNAs and eight up-regulated miRNAs [11]. Furthermore, two of significantly up-regulated miRNAs, including miR-140 and miR-199*, have been reported to regulate chondrogenic differentiation by targeting Smad3 and Smad1, respectively [12,13]. Conversely, miR-145 and miR-495, two of obviously down-regulated miRNAs, can both inhibit chondrogenesis by targeting Sox9, the key chondrogenic transcription factor [14,15].

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Additionally, miR-30a was also found to be up-regulated in MSCs during chondrogenic differentiation [11]. However, to the best of our knowledge, how miR-30a regulates such process remains poorly understood.

In this study, we focused on the roles of miR-30a in chondrogenic differentiation of MSCs and its underlying regulatory mechanisms. A previous report identified the function of miR-30a involved in endothelial tip cell formation by targeting DLL4, a membrane-bound ligand of the Notch signaling family [16]. Moreover, our recent studies have shown that the activation of Notch signaling represses chondrogenic differentiation of MSCs by up-regulating the transcription factor Twist1 [17]. Therefore, based on the highly conserved characteristics of miR-30a as well as DLL4-Notch signaling pathway, we hypothesized that the contribution of miR-30a to chondrogenic differentiation of MSCs might be through targeting DLL4. Here, we tested this hypothesis and demonstrated that miR-30a could act as a promoter of chondrogenic differentiation via targeting DLL4.

## 2. Materials and methods

### 2.1. Isolation and culture of bone marrow MSCs and chondrosarcoma cell line culture

MSCs were isolated from rat bone marrow according to the previous studies with some medications [18]. Four-week-old female Sprague-Dawley rats (Vital River Laboratory Animal Technology Co. Ltd, Beijing, China) were sacrificed by cervical dislocation and disinfected with 75% alcohol. Aseptically, the femurs and tibiae were carefully isolated and all the muscle and connective tissues were removed. Subsequently, the ends of femur and tibia were cut and the bone marrow was harvested by flushing with Dulbecco's modified Eagle's medium (DMEM; Gibco, MD, USA). Following centrifugation at 1500 rpm for 10 min, cell pellets were re-suspended in red blood cell (RBC) lysis buffer (Solarbio, Beijing, China) to remove the RBCs. After wishing with phosphate buffered saline (PBS; Doublehelix, Wuhan, China), cells were re-suspended in DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone, UT, USA) and counted by the Trypan Blue exclusion test. Then, cells were plated in culture flasks at a density of $1 \times 10^5$ cells/ml and incubated at 37 °C with 5% $CO_2$. After 24 h, the non-adherent cells were removed and adherent cells were further cultured by changing the medium every 3–4 days. Reaching 80% confluence, primary cells were passaged by 0.25% trypsin-EDTA (Beyotime, Jiangsu, China) centrifuged at 1000 rpm for 3 min, and continued to culture in the complete medium. Following 3 passages, the cells were used for subsequent experiments. All animal protocols were approved by the Animal Care Ethics and Use Committee of China Medical University and carried out in accordance with its guidelines.

Human chondrosarcoma cell line SW1353 (Shanghai Institute of Cell Resource Center of Life Science, Shanghai, China) was cultured in RPMI-1640 medium (Gibco, MD, USA) supplemented with 10% FBS and 1% antibiotic-antimycotic solution at 37 °C in humidified atmosphere with 5% $CO_2$.

### 2.2. Flow cytometry analysis

Flow cytometry analysis was performed to identify the surface markers of isolated MSCs as previously described [19]. In brief, the MSCs were collected, mixed with different fluorescently labeled monoclonal antibodies, including anti-rat CD34-PE (Abcam, Cambridge, MA, USA), CD105-FITC (Abcam), CD45-FITC (eBioscience, San Diego, CA, USA), and CD90-FITC (eBioscience), and incubated in the dark at room temperature for 30 min. After washing three times, the cell pellet was re-suspended in PBS and determined immediately using a FACSCalibur cytometer (BD Biosciences, San Diego, CA, USA). The unstained cells ($1 \times 10^6$) served as negative control, and data analysis was conducted using BD FAC Suite software (BD Biosciences).

### 2.3. Adipogenic and osteogenic differentiation of rat MSCs

For adipogenic differentiation, the MSCs of passage 3 were collected and seeded in a 6-well plate at a density of $1 \times 10^5$/well. When reached ~80% confluence, the MSCs were incubated in adipogenic differentiation medium (Cyagen Bioscience Inc., Santa Clara, CA, USA) consisting of basal medium, FBS, penicillin-streptomycin, glutamine, insulin, isobutylmethylxanthine, rosiglitazone and dexamethasone for 21 days, and the medium was changed every three days. After washing with PBS, cells were fixed with 4% paraformaldehyde solution for 30 min at room temperature. Subsequently, the fixed-cells were stained for 1 h at room temperature in 0.6% oil red O (Sigma-Aldrich, MO, USA) solution, and observed under a phase contrast microscope (Motic, Xiamen, China).

For osteogenic differentiation, passage 3 MSCs were harvested and seeded in a 6-well plate at a density of $1 \times 10^5$/well. Reaching ~80% confluence, the MSCs were cultured in osteogenic differentiation medium (Cyagen Bioscience Inc., Santa Clara, CA, USA) consisting of basal medium, FBS, glutamine, penicillin-streptomycin, ascorbate, β-glycerophosphate and dexamethasone, and the medium was changed every two days. Then, the osteogenic differentiation potential of MSCs was assessed by Alizarin red staining, during which cells were fixed for 15 min in 4% paraformaldehyde solution followed by staining with 0.1% Alizarin red (Sigma-Aldrich, MO, USA) solution for 40 min at room temperature. Finally, the cells were observed under a phase contrast microscope.

### 2.4. Chondrogenic differentiation of MSCs

Differentiation of MSCs into chondrocytes was performed using SD Rat MSCs Chondrogenic Differentiation Kit (Cyagen Bioscience Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. Briefly, following 3 passages, cells were cultured in chondrogenic differentiation medium which consists of basal medium, dexamethasone, ascorbate, insulin transferring selenium-A, sodium pyruvate, proline and TGF-β3. During the differentiation periods, the complete chondrogenic media was replaced every two days. After three weeks of differentiation, chondrocytes were identified by Toluidine Blue staining or Alcian Blue staining. Cells were fixed with 4% paraformaldehyde solution for 30 min, washed three times, stained with 0.1% toluidine blue solution (Sigma-Aldrich) or 1% alcian blue solution (Solarbio, Beijing, China), and observed under a microscope (Olympus, Tokyo, Japan).

### 2.5. Lentivirus-mediated cell transfection

To construct the stable miR-30a-overexpressing and miR-30a-silencing cells, the recombinant lentivirus LV-miR-30a encoding miR-30a or LV-NC (negative control) and recombinant lentivirus LV-miR-30a-inhibitor or LV-inhibitor-NC were purchased from Hanbio (Shanghai, China). Lentivirus infection was conducted according to the manufacturer's protocols. In brief, the differentiated MSCs and SW1353 cells were seeded into a 6-well plate and cultured overnight to reach 70% confluence. Subsequently, cells were transfected with above different lentivirus in the presence of ploybrene (8 μg/ml; Sigma-Aldrich, MO, USA) for 24 h. The stable transfectants were used in the following experiments.

### 2.6. Luciferase reporter assay

The 3'UTR of DLL4 containing the binding site of miR-30a (DLL4 3'UTR-wt) and the control sequences containing mutated seed region (DLL4 3'UTR-mut) were synthesized and respectively cloned into pmirGLO dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA). Hsa-miR-30a mimics (sense: 5′-UGUAAACAUCCU CGACUGGAAG -3′; anti-sense: 5′-UCCAGUCGAGGAUGUUUACAUU-3′), hsa-miR-30a mutant (sense: 5′-UGAUUUCAUCCUCGACUGGAAG-3′; anti-sense: 5′-UCCAGUCGAGGAUGAAAU CAUU-3′), and scrambled RNA oligomers (NC; sense: 5′-UUCUCCGAACGUGUCACGUT T-3′; anti-