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MicroRNA-92a-3p regulates the expression of cartilage-specific genes by directly targeting histone deacetylase 2 in chondrogenesis and degradation

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SUMMARY

Objective: Increased activity of histone deacetylase 2 (HDAC2) has been found in patients with osteoarthritis (OA) and cartilage matrix degradation and has been shown to mediate the repression of cartilage-specific gene expression in human chondrocytes. We aimed to determine whether microRNA-92a-3p (miR-92a-3p) regulates cartilage-specific gene expression *via* targeted HDAC2 in chondrogenesis and degradation.

Methods: miR-92a-3p expression was assessed *in vitro* in a human mesenchymal stem cells (hMSCs) model of chondrogenesis and in normal and OA primary human chondrocytes (PHCs), and in normal and OA human cartilage by *in situ* hybridization. hMSCs and PHCs were transfected with miR-92a-3p or its antisense inhibitor (anti-miR-92a-3p), respectively. PHCs were transfected with miR-92a-3p or anti-miR-92a-3p for 24 h before chromatin immunoprecipitation (ChIP) assay was performed with anti-ac-H3 antibody. Direct interaction between miR-92a-3p and its putative binding site in the 3′-untranslated region (3′-UTR) of HDAC2 mRNA was confirmed by luciferase reporter assay.

Results: miR-92a-3p expression was elevated in chondrogenic and hypertrophic hMSC, while reduced in OA cartilage compared with normal cartilage. The overexpression of miR-92a-3p suppressed the activity of a reporter construct containing the 3′-UTR and inhibited HDAC2 expression in both hMSCs and PHCs, while treatment with anti-miR-92a-3p enhanced HDAC2 expression. ChIP assays showed that miR-92a-3p enhances H3 acetylation on aggrecan (ACAN), cartilage oligomeric protein (COMP) and Col2a1 promoter, and also promotes relative cartilage matrix expression.

Conclusion: Our results suggest that miR-92a-3p regulates cartilage development and homeostasis, which directly targets HDAC2, indicating histone hyperacetylation plays an important role in increased expression of cartilage matrix.

Introduction

Osteoarthritis (OA) is a common chronic degenerative joint disease characterized by progressive articular cartilage destruction, and a leading cause of pain and disability in older adults[1,2]. There is increasing evidence that the initiation and progression of OA is contributed to various expression patterns and enhanced activation of histone deacetylase (HDAC)[3–7]. It has been reported that enhanced activation of some HDACs increases articular cartilage destruction and OA progression. For example, HDAC4-null mice displayed aberrant chondrocyte hypertrophy and subsequent premature ossification in the chondrocostal cartilage[8], and HDAC4 overexpression promote matrix-degrading enzymes level and enhanced catabolic activity of chondrocyte in osteoarthritic cartilage[9]; HDAC1 and HDAC2 share a common domain that suppresses the expression of genes encoding extracellular cartilage matrix genes such as type II collagen (Col2a1), aggrecan (ACAN), and cartilage oligomeric protein (COMP)[10]. It also has been reported that HDAC inhibitors can reduce the expression of matrix-

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degrading enzymes in chondrocytes and fibroblasts[11,12], and can inhibit arthritis in animal models[13,14]. Elevated expression of HDAC7 in OA cartilage contributed to increased MMP13 production and accelerated cartilage matrix degradation[15]. Furthermore, some researches have found the HDAC regulation maybe related with miRNAs. For example, miRNA-222 regulates MMP-13 *via* the targeting HDAC4 during OA pathogenesis[16], and miR-140 targets HDAC4 to regulate cartilage and bone formation[17]. Inhibitors of HDAC increase miRNA-146a expression and enhance negative regulation of interleukin-1β signaling in OA fibroblast-like synoviocytes[18].

MicroRNAs (miRNAs) are a class of small (22~24 nt), non-coding RNAs that can regulate gene expression at the post-transcriptional level by binding to the 3′-untranslated region (3′-UTR) of target genes and suppressing target gene expression[19]. The important regulatory roles of miRNAs during chondrogenesis were identified recently. In a previous study, we reported a 4.397-fold up-regulation in microRNA-92a-3p (miR-92a-3p), during chondrogenic differentiation in hADSCs (human adipose-derived stem cells)[20]. We found that miR-92a-3p has the potential to regulate HDAC2 expression by miRNA target prediction software. However, only a few studies have been carried out about the function of miR-92a-3p. Previous studies have revealed that miR-92a upholds Bmp (bone morphogenetic protein) signaling by targeting noggin 3 during pharyngeal cartilage formation[21]. Additionally, miR-92a decreases rapidly in macrophages once stimulated with TLR (Toll-like receptor) ligands, and miR-92a controls inflammatory response by targeting the MKK4/JNK/c-Jun pathway[22]. MiR-92a controls angiogenesis and functional recovery of ischemic tissues in mice[23].

Given the role of miRNAs in regulating cartilage homeostasis and chondrogenesis, we hypothesized that miR-92a-3p may play a role in both chondrogenic differentiation and OA pathogenesis. In this study, we aimed to determine whether miR-92a-3p regulates cartilage-specific gene expression *via* targeted HDAC2 in chondrogenesis and degradation.

Methods

This study adhered to the standards of the Ethics committee on Human Experimentation at the First Affiliated Hospital of Sun Yat-Sen University, China (IRB: 2011011) and the Helsinki Declaration (2000). All volunteers provided written informed consent.

Culture of human mesenchymal stem cells (hMSCs)

Bone marrow samples were obtained by iliac crest aspiration from six normal human donors (mean age: 37 years, range: 35–39 years, male: 3, female: 3). Isolation of hMSCs was carried out using density gradient centrifugation methods[24]. Briefly, the bone marrow aspirate was added to Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden), and centrifuged for 18 min at 1000 g. The interfacial mononuclear cells were collected and resuspended in hMSC basal medium (Alpha-modified Eagle's medium [α-MEM]) (Gibco Life Technology Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technology), 1% penicillin/streptomycin (Gibco Life Technology). Cells were cultured at 37°C under a 5% CO_2 atmosphere. The medium was changed every 3 days. When the cultures were near 80% confluence, the cells were detached by treatment with 0.05% trypsin/ethylene-diamine-tetra-acetic acid and passaged in culture.

Chondrogenesis in a micromass culture

The cultured hMSCs between passages three and five were harvested using the method described previously. For micromass culturing, the cells were resuspended in α-MEM supplemented with 10% FBS at a specified density of 10^5 cells/μL of media, and 12.5 μL of the suspended cells was dotted on the center of each well of 24-well plates to stimulate the adherence of the cells to the plate. Then they were divided into two groups. The first group was cultured in an incomplete chondrogenic induction medium (human mesenchymal stem cell chondrogenic differentiation basal medium 97 mL, dexamethasone 10 μL, ascorbate 300 μL, ITS + (insulin, transferrin, selenium) supplement 1 mL, sodium pyruvate 100 μL, proline 100 μL) (Cyagen, Guangzhou, China), and the second group in a complete chondrogenic induction medium (10 μL of TGF-β3, which convert 1 mL of incomplete chondrogenic medium into complete medium) (Cyagen) At selected time points, the cells were fixed in formalin, and stained with alcian blue 8GX (Cyagen) for the detection of glycosaminoglycans, immunohistochemistry of COL2A1 (1:200 dilution; Abcam, Cambridge, MA, USA) for the chondrogenesis, safranin O (Sigma, St. Louis, MO, USA) for the detection of matrix deposition and cell morphology, and alizarin red staining (Cyagen) for the detection ossification.

Primary chondrocyte collection, isolation, and cell culture

Degraded joint cartilage samples were obtained from OA knee joints during total knee replacement operation from patients ($n = 6$; mean ± standard deviation [SD] age: 64.2 ± 3.16 years, male: 3, female: 3). Normal cartilage samples were taken from patients ($n = 6$; mean ± SD age: 66.2 ± 2.62 years, male: 3, female: 3) with no previous history of OA or rheumatoid arthritis, who underwent total hip replacement surgery because of fractures of the femoral neck. OA cartilage was collected from gross erosion areas, while normal cartilage was collected from areas with no surface irregularity. Primary human chondrocytes (PHCs) were isolated as described previously[25]. The chondrocytes were seeded into flasks containing DMEM/F12 (Gibco Life Technology) plus 5% FBS, 2% penicillin and streptomycin and ITS + premix (BD Biosciences, Franklin Lakes, NJ, USA). During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2, and the medium was changed every 2 days.

RNA extraction, reverse transcription, and quantitative polymerase real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted with a miRNeasy Mini Kit (QIAGEN, CA, USA) following the manufacturer's instructions. cDNA was generated using the PrimeScript® miRNA cDNA Synthesis Kit (TAKARA Biotechnology, Japan) following the manufacture's instruction. QRT-PCR was performed using SYBR® Premix Ex Taq™ II (TAKARA Biotechnology) according to the manufacturer's instruction reverse transcription and the CFX96 system for miR-92a-3p was performed using the Mir-XTM miRNA First-Strand Synthesis kit and miR-92a-3p qRT-PCR SYBR® Premix Ex Taq™ II (TAKARA Biotechnology) according to the manufacturer's instructions on a CFX96 system. The specific primers are listed in Table I. The relative gene expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method. All experiments were performed in six biological replicates.

Transfection

The hMSCs and PHCs were transfected with an agomir or antagomir (RiboBio, Guangzhou, China) of miR-92a-3p at a concentration of 50 nM and PHCs were also transfected with siHDAC2 or siNC (RiboBio), Lipofectamine® 2000 Transfection Reagent (Gibco Life Technologies) was used to transfect cells according to the manufacturer's instructions. Nonspecific microRNA (miR-control; RiboBio) was used as a control.