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Research paper

Circular RNA Atp9b, a competing endogenous RNA, regulates the progression of osteoarthritis by targeting miR-138-5p

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ABSTRACT

Osteoarthritis (OA) is the most common joint disease and is mainly characterized by degradation of the articular cartilage. Recently, circular RNAs (circRNAs), novel noncoding RNAs with different biological functions and pathological implications, have been reported to be closely associated with various diseases. Growing evidence indicates that circRNAs act as competing endogenous RNAs (ceRNAs) that bind with microRNAs (miRNAs) and regulate their downstream functions. Here, we identified a new circRNA, circRNA_Atp9b, and further investigated its function in OA using a well-established mouse chondrocyte model. We demonstrated that circRNA_Atp9b expression was significantly up-regulated in mouse chondrocytes after stimulation with interleukin-1 beta (IL-1β), and that knockdown of circRNA_Atp9b promoted the expression of type II collagen while inhibiting the generation of MMP13, COX-2 and IL-6. Moreover, there was a negative correlation between the expression levels of circRNA_Atp9b and microRNA (miR)-138-5p, indicating that miR-138-5p also played a role in IL-1β-induced chondrocytes. Bioinformatics analysis predicted circRNA_Atp9b directly target miR-138-5p, which was validated by dual-luciferase assay. Further functional experiments revealed that down-regulation of miR-138-5p partly reversed the effects of circRNA_Atp9b on extracellular matrix (ECM) catabolism and inflammation. Taken together, these results suggest that circRNA_Atp9b regulates OA progression by modulating ECM catabolism and inflammation in chondrocytes via sponging miR-138-5p. Our findings provide novel insight into the regulatory mechanism of circRNA_Atp9b in OA and may contribute to establishing potential therapeutic strategies.

1. Introduction

Osteoarthritis (OA) is a widely prevalent and age-related joint disorder that is primarily characterized by degradation of the articular cartilage, as well as subchondral bone sclerosis and osteophyte formation (Loeser et al., 2012; Vinatier et al., 2016). However, the mechanism of OA is still not fully understood, leading to a lack of effective disease-modifying therapies for clinical use.

Circular RNAs (circRNAs) are a class of endogenous RNAs characterized by covalently closed loop structures with neither 5′ to 3′ polarity nor a polyadenylated tail (Memczak et al., 2013). Recently, with the development of high-throughput sequencing and novel computational technologies, it has been revealed that circRNAs, mainly formed by a non-canonical mode of RNA splicing, are widely expressed in human cells and are involved in transcriptional and posttranscriptional gene expression regulation (Chen et al., 2017; Salzman et al., 2012; Li et al., 2015). Mechanistically, circRNAs may have many potential functions, including functioning as a microRNA (miRNA) 'sponge', binding and sequestering other RNA binding proteins (Szabo & Salzman, 2016), and one of the most studied functional roles of circRNAs is that they act as miRNA sponges and repress their downstream functions. MiRNAs are small non-coding RNAs, 20–22 nt in length, and the mechanisms underlying miRNA regulation of mRNA targets through sequence complementarity have been extensively investigated (Pasquinelli, 2012). Although a great number of miRNAs have been reported to play a critical role in the development and progression of OA (Si et al., 2017a; Wang et al., 2017; Zhang et al., 2017), the specific micro-environmental factors that are involved in this pathological process have not been fully elucidated. Therefore, studying the effects of miRNAs and their potential circRNA regulators, on OA progression

Abbreviations: OA, osteoarthritis; circRNAs, circular RNAs; ceRNAs, competing endogenous RNAs; miRNAs, microRNAs; IL-1β, interleukin-1β; ECM, extracellular matrix; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; qRT-PCR, quantitative real-time polymerase chain reaction; siRNAs, small interfering RNAs

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may help to clarify the pathogenesis of OA.

In a previous study we found that circRNA differential expression are involved in IL-1β-induced mouse chondrocyte model, and revealed that these differentially expressed circRNAs might be potential regulators of OA using bioinformatics analysis (Zhou et al., 2017). Here, we identified and characterized one circRNA derived from the mouse Atp9b gene locus, termed circRNA_Atp9b (circ_15898, according to the RNA-seq analysis, GSE107009), which is significantly up-regulated in IL-1β-induced mouse chondrocytes. More importantly, we found that circRNA_Atp9b could regulate the progression of OA by regulating extracellular matrix (ECM) catabolism and inflammation via sponging miR-138-5p. Our findings reveal a novel mechanism underlying circRNA_Atp9b in OA and may contribute to the establishment of therapeutic strategies for OA.

2. Materials and methods

2.1. Cell culture

Mouse articular chondrocytes were isolated from the knee joints according to a previously described method (Gosset et al., 2008), and then were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin–streptomycin (Hyclone, GE Healthcare Life Sciences, Logan, UT, USA) at 37 °C in a 5% CO_2 incubator. Chondrocytes were treated with 10 ng/ml recombinant human IL-1β and incubated for 12 h, 24 h and 48 h, while cells cultured in growth culture medium under the same conditions without IL-1β stimulation served as a normal control.

2.2. Quantitative real-time PCR

According to the manufacturer's instructions, total RNA was isolated from chondrocytes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse transcribed into cDNA using random primers for mRNA analysis, and a stem-loop Quantitative real-time PCR (qRT-PCR) assay was done for miRNA quantitative PCR analysis. The primers used were as follows: circRNA_Atp9b forward: 5′-GAACTTGG CAGTCTGCGAGTAA-3′, reverse: 5′-CTGGTATTGTGCTGGTCCGA-3′; miR-138-5p forward: 5′-CCCAGGGTCTGGTGCGGAGA-3′, reverse: 5′-CAGGGGCTGAGCGGTGAGGG-3′.

The reaction was performed in an ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Forster, CA, USA). Three independent experiments were conducted for each sample. CircRNA_Atp9b and mRNA expression levels were normalized to GAPDH, and miR-138-5p expression was normalized to U6. Data were analyzed by comparing the $2^{-\Delta\Delta Ct}$ value.

2.3. RNA transfection

The circRNA_Atp9b overexpression vector (p-circRNA_Atp9b) was constructed by cloning circRNA_Atp9b into pcDNA3.1 following the procedures in Hansen et al. (2013), and all vectors were verified by direct sequencing. Small interfering RNAs (siRNAs) against circRNA_Atp9b, overexpression of circRNA_Atp9b, miR-138-5p mimics and miR-138-5p inhibitor vectors were constructed and purchased from GenePharma (China). All cell transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

2.4. Dual-luciferase assay

The linear sequence of circRNA_Atp9b was cloned into the dual-luciferase reporter vector psiCHECK-2 (Promega, China), and the potential binding sites of miR-138-5p were mutated. The wild-type (WT) or mutated (MU) plasmid was cotransfected into cells with miR-138-5p mimics or negative control (NC) miRNAs together with the circRNA_Atp9b overexpression vector (p-circRNA_Atp9b) or pcDNA3.1 empty vector (EV), and changes in luciferase expression were analyzed using the Dual Glo Luciferase Assay System (Promega) following the manufacturer's instructions. Three independent experiments were performed, each with two technical replicates.

2.5. Western blotting

Chondrocytes were lysed in Radio Immunoprecipitation Assay (RIPA) protein extraction reagent supplemented with 1 mM phenylmethanesulfonyl fluoride. Protein concentration was determined by BCA protein assay (Tiangen, China). Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Massachusetts, USA). After blocking in 5% non-fat milk, the membranes were probed with the following primary antibodies overnight at 4 °C: anti-COL2, anti-MMP13, anti-IL-6, anti-COX-2 and anti-GAPDH (Santa Cruz, California, USA). Then the membranes were incubated with horse radish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz, California, USA) for 2 h at room temperature, and protein bands were visualized and detected by ECL chemiluminescence (Pierce, Rockford, IL, USA).The intensities of the bands were quantified using Image J software. GAPDH was used as an internal loading control.

2.6. Statistical analysis

All experiments were performed and analyzed in triplicate. Data analysis was performed with SPSS19.0 statistical software. All data are presented as the mean ± standard deviation (SD). Differences in the means of two groups were evaluated using *t*-tests. The means of multiple groups were compared by analysis of variance. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Expression of circRNA_Atp9b is elevated in IL-1β-induced chondrocytes

According to our previous study, we found that the parental gene of circRNA_Atp9b is located at mouse chr18:80734143–80934058(−), and after obtaining the back spliced junction reads of circRNA_Atp9b, Sanger sequencing of the RT-PCR products amplified by divergent primers further confirmed the back splice junction of circRNA_Atp9b (Fig. 1A). The gene sequence and divergent primers used for Sanger sequencing verification were provided in Supplementary. To further investigate whether circRNA_Atp9b is involved in the regulation of IL-1β-induced chondrocytes, we examined the expression pattern of circRNA_Atp9b by qRT-PCR assay. Compared with the normal control group, circRNA_Atp9b expression levels were significantly up-regulated in IL-1β-induced chondrocytes in a time-dependent manner (Fig. 1B), suggesting that circRNA_Atp9b may play an important role in IL-1β-induced chondrocytes.

3.2. Effect of circRNA_Atp9b on ECM catabolism and inflammation in IL-1β-induced chondrocytes

To determine the functional role of circRNA_Atp9b in IL-1β-induced chondrocytes, we used siRNAs against circRNA_Atp9b (si-circRNA) to suppress circRNA_Atp9b expression. Then, we assessed the effect of downregulation of circRNA_Atp9b on the expression of ECM products and inflammatory factors in chondrocytes. QRT-PCR revealed that circRNA_Atp9b was significantly downregulated by si-circRNA transfection compared with the negative control group in IL-1β-induced chondrocytes (Fig. 2A). Western blotting showed that knockdown of circRNA_Atp9b dramatically increased the synthesis of type II collagen compared with the IL-1β group, but reduced the expression of MMP13 in chondrocytes (Fig. 2B, C). Furthermore, compared with the IL-1β