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Emerging Roles of circRNA Related to the Mechanical Stress in Human Cartilage Degradation of Osteoarthritis

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Circular RNAs (circRNAs) are involved in the development of various diseases; however, knowledge on circRNAs in osteoarthritis (OA) is limited. This study aims to identify circRNA expression in different regions affected by OA and to explore the function of mechanical stress-related circRNAs (circRNAs-MSR) in cartilage. Bioinformatics was employed to predict the interaction of circRNAs and mRNAs in the cartilage. Loss-of-function experiments for circRNAs-MSR were performed in vitro. A total of 104 circRNAs were differentially expressed in damaged versus intact cartilage. Of these circRNAs, 44 and 60 were upregulated and downregulated, respectively, in the damaged tissue. circRNA-MSR expression increased under mechanical stress in chondrocytes. circRNAs-MSR were silenced using small interfering RNA, and knockdown of circRNAs-MSR could suppress tumor necrosis factor alpha (TNF-α) expression and increase extracellular matrix (ECM) formation. Our results demonstrated that circRNAs-MSR regulated TNF-α expression and participated in the chondrocyte ECM degradation process. We propose that the inhibition of circRNAs-MSR could inhibit the degradation of chondrocyte ECM and knockdown of circRNAs-MSR could be a potential therapeutic target for OA.

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease characterized by articular cartilage degradation, subchondral bone thickening, and osteophyte formation.[1,2] Cartilage cellularity is reduced in OA through chondrocyte death, in which chondrocytes are stimulated by cytokines and growth factors to undergo catabolic and abnormal differentiation, leading to extracellular matrix (ECM) degradation.[3–6] In OA, the medial compartment of the articular cartilage is the most susceptible to degeneration, whereas the lateral compartment remains relatively unaffected.[7,8] Differences in mechanical factors underlie the disparity in disease susceptibility between the medial and lateral compartments; the damaged cartilage regions are usually subjected to mechanical loading, whereas the intact regions are not. The chondrocytes in the damaged cartilage are particularly susceptible to mechanical stress, and the tensile properties of the damaged cartilage are lost because of the destructed collagen network.[9,10]

Circular RNAs (circRNAs) are a large class of noncoding RNAs that exist ubiquitously in the cytoplasm of eukaryotic cells;[11,12] these endogenous RNAs are characterized by stable structure and high tissue-specific expression.[13] Compared with linear RNAs, circRNAs can remarkably undergo non-canonical splicing without a free 3′ or 5′ end.[14,15] Recent reports show that circRNAs function as microRNA (miRNA) sponges that naturally sequester and competitively suppress miRNA activity.[16] The involvement of circRNAs is demonstrated in the development of several types of diseases, such as atherosclerosis and nervous system disorders.[17–19] However, the role of circRNAs in cartilage and their overall contribution to OA pathogenesis are still unknown.

The present study identified a small number of up- or downregulated circRNAs in different cartilage regions. We specifically identified a new circRNA, called mechanical stress-related circRNA (circRNA-MSR), involved in mechanical stress. We demonstrate that circRNA-MSR controls tumor necrosis factor alpha (TNF-α) expression and promotes the degradation of ECM.

RESULTS

circRNA Expression Profiles in Different Cartilage Regions

The cartilage was assessed by histologic examination, and the histologic scores were graded according to a modified Mankin scale. The score of <4 points was considered intact cartilage and a score of >6 represented damaged cartilage. Hierarchical clustering revealed the circRNA expression in the cartilage samples (Figure 1A). The scatter and volcano plots showed varied circRNA expressions between the damaged and intact cartilage samples (Figures 1B and 1C). We identified 104 differentially expressed circRNAs in the damaged cartilage compared to the intact cartilage. Of these circRNAs, 44 were upregulated and 60 were downregulated in the damaged tissue samples (Table S1). To validate the circRNA
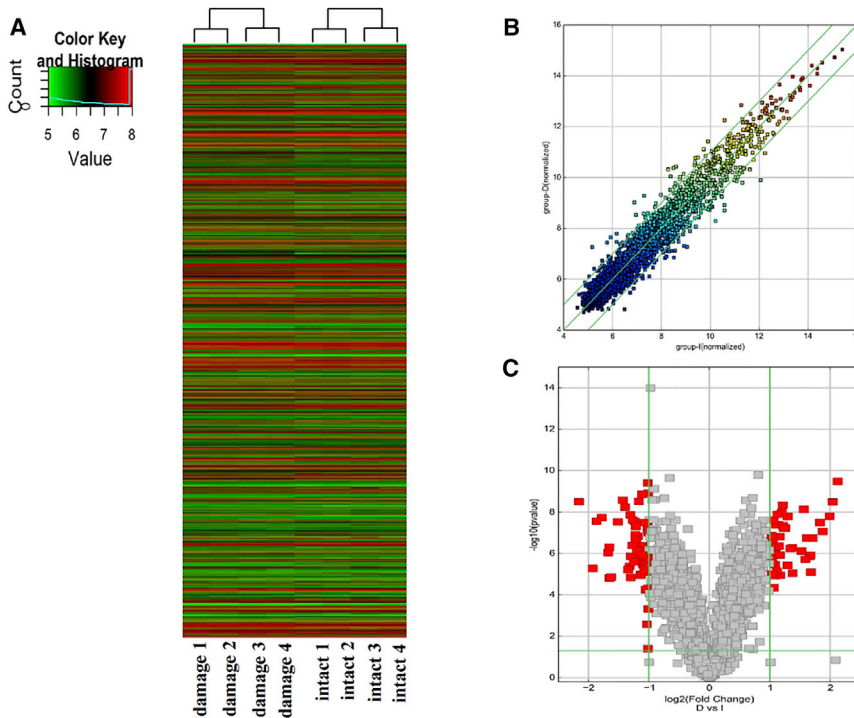
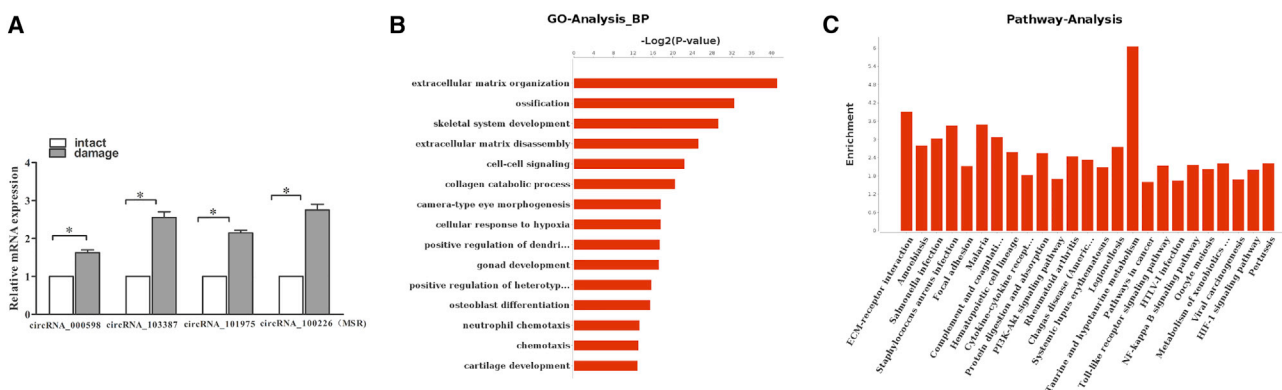**Figure 1. Differential Expression of circRNAs in Cartilage Tissues**

(A) Differentially expressed circRNAs between damaged and intact cartilages were subjected to hierarchical clustering analysis; each group contains four individuals (>2-fold difference in expression; p < 0.05). Expression values are represented in different colors, indicating expression levels above and below the median expression level across all samples. (B) The scatterplot is a visualization method for the evaluation of the variation in circRNA expression between intact (group I) and damaged (group D) samples. The values corresponding to the x and y axes in the scatterplot are the normalized signal values of the samples (log2 scaled). The green lines indicate fold changes. (C) Volcano plots were constructed using fold change and p values. The vertical lines correspond to 2.0-fold up- and downregulation between intact and damaged samples (I versus N), and the horizontal line represents a p value. The red point in the plot represents the differentially expressed circRNAs with statistical significance.

microarray results, we performed qPCR to analyze the changes in expression among the differentially expressed circRNAs. The data confirmed that circRNA_000598, circRNA_103387, circRNA_101975, and circRNA_100226 (circRNAs-MSR) were overexpressed in the damaged region compared with the intact region of the cartilage in OA (Figure 2A).

circRNA Prediction Relative to Mechanical Stress

We used gene co-expression networks to predict circRNA targets. A total of 89 circRNAs and 61 mRNAs were noted in the damaged cartilage network, whereas 94 cir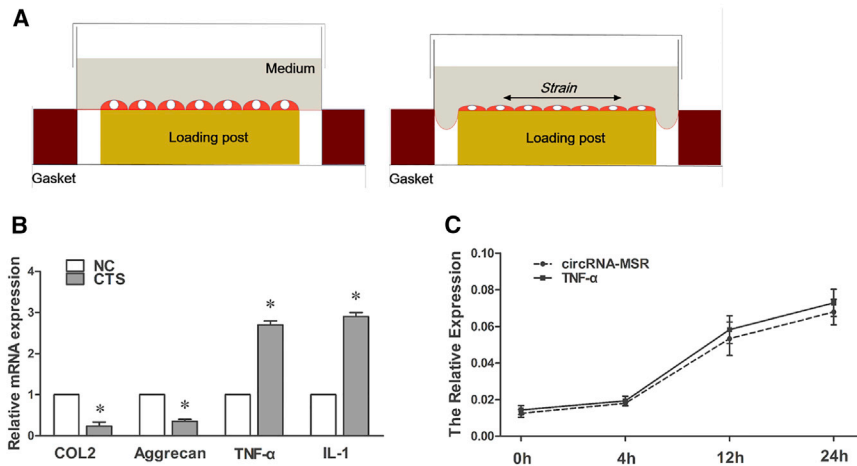cRNAs and 36 mRNAs were in the intact cartilage network. In these co-expression networks, circRNAs and mRNAs were connected by a string, indicating a tight correlation between these genes and a potential regulatory relationship (Figures S1 and S2). Furthermore, we constructed another network with circRNAs, mRNAs, and the common binding miRNAs. The differentially expressed circRNAs were annotated in detail with the miRNA interaction information (Table S1). We selected cartilage-specific mRNAs according to gene ontology (GO) and pathway analyses (Figures 2B and 2C). We constructed a network of circRNAs-miRNAs-mRNAs, with a total of 45 circRNAs, 42 mRNAs, and 42 miRNAs, by merging the commonly targeted miRNAs of circRNAs and mRNAs (Figure 3). This indicated the tight correlation and regulation relationship of these genes in the network.

Figure 2. Validation of the Differential Expression of circRNAs and Bioinformatics Analysis

(A) The expression levels of the following circRNAs were analyzed using qPCR: circRNA_000598, circRNA_103387, circRNA_101975, and circRNA_100226 (circRNAs-MSR). The presented values are the means ± SEM (*p < 0.05). (B) GO analysis of differentially expressed mRNAs. (C) Pathway analysis of differentially expressed mRNAs.

Figure 3. circRNA-miRNA-mRNA Network

The network consists of 129 genes. In the circRNA-miRNA-mRNA-Net, the circle represents mRNA, the rhombus denotes miRNA, the inverted triangle represents circRNA, and their relationship is depicted by one edge.

Upregulation of Novel circRNA-MSR in Chondrocytes Stimulated by Mechanical Stress

We assumed that circRNA-MSR was a special cartilage circRNA (hsa_circ_100226, ID circ_0005567 in circBase; http://circbase.org), with its gene located at chr1:51868106-51874004 and the symbol of the associated gene being EPS15. The circRNA-MSR length is 607 bp. The circRNA-MSR was chosen because it is one of the circRNAs indicated to be associated with mechanical stress and inflammatory response according to the bioinformatics analysis mentioned above.

To investigate the effect of mechanical stress on chondrocytes, the cells were exposed to pre-optimized magnitudes of cyclic tensile strain (CTS) using Flexcell-5000 (Figure 4A). The expression levels of COL2A1 and aggrecan were significantly downregulated, whereas the expression levels of TNF-α and IL-1 were significantly upregulated by CTS for 24 hr (Figure 4B). Moreover, we found that circRNA-MSR expression paralleled TNF-α expression under mechanical stress (Figure 4C). Therefore, we speculated the co-regulated expression of circRNA-MSR and TNF-α under mechanical stress in vitro conditions.

Effects of circRNA-MSR on TNF-α Expression in Chondrocyte ECM Degradation

To analyze the effects of circRNA-MSR on TNF-α expression in chondrocyte ECM degradation, we examined the effect of circRNA-MSR knockdown on damaged chondrocytes. The small interfering RNA (siRNA) used in this experiment was specific for circRNA-MSR. As a consequence of this inhibition, the mRNA expression for TNF-α decreased, whereas the ECM expression significantly rose by si-MSR treatment (Figure 5A). The immunofluorescence results also showed that ECM proteins were significantly elevated by the si-MSR treatment (Figures 5B and 5C).

circRNA-MSR Targeted by TNF-α-Targeting miRNAs

circRNAs have been reported to function as miRNA sponges that naturally sequester and competitively suppress miRNA activity.[16] We assumed that circRNA-MSR functions as a decoy to regulate TNF-α expression through the same mechanism. According to the informatics analysis, there were five miRNA-binding sites for circRNA-MSR, and they were miR-138, miR-145, miR-24, miR-620, and miR-875 (Figure 6A). The circRNA-MSR 3′ UTR sequence

matched these miRNAs, and the TNF-α 3′ UTR matched miR-875 (Figure 6B). Thus, we identified a common miRNA (miR-875) for circRNA-MSR and TNF-α targets.

DISCUSSION

OA is a multifactorial disease characterized by progressive inflammation, pain, and cartilage destruction in load-bearing surfaces of the knee joints.[20] Among various pathogenic factors, the biomechanical factor is critical for cartilage development, homeostasis, and functionality.[21] Aberrant mechanical stimulation results in a physiological imbalance between mechanical stress on the joint and the joint's ability to withstand such stress. Chondrocytes are directly exposed to compression force during cartilage loading; thus, the ECM of chondrocytes tends to stretch the cells during cartilage compression.[22] Many studies on OA focused on the epigenetic regulation of its pathogenesis and potential targets for therapy, including miRNAs and long noncoding RNAs (lncRNAs). However, the occurrence of circRNAs in cartilage remains largely unknown. This study profiles circRNA expression in different regions of OA cartilage. This study identified a number of aberrantly expressed circRNAs in damaged regions compared with the intact regions of OA. We found that circRNA-MSR is vital under mechanical stress in loss-of-function experiments and that it regulates the TNF-α in the process of ECM degradation in chondrocytes.

Recent studies showed that numerous exonic transcripts can form circRNAs through non-linear reverse splicing or gene rearrangement.[11] The two properties of circRNAs are the most important: first, they are highly conserved sequences; second, they show a high degree of stability in mammalian cells.[16] Compared with other noncoding RNAs, such as miRNAs and lncRNAs, these properties provide circRNAs with the potential to be used as ideal biomarkers and potential therapy targets.[23] We assumed that circRNA-MSR functioned as a decoy to regulate TNF-α expression through the same mechanism. We found that circRNA-MSR harbors miRNA-binding sites, including miR-138, miR-145, miR-24, miR-620, and miR-875. In addition, miR-875 can bind to the 3′ UTR of TNF-α. However, this

study focused on miR-875 as the only miRNA that can target both circRNA-MSR and TNF-α.

This study demonstrated that circRNA-MSR regulated TNF-α expression and participated in the chondrocyte ECM degradation process. We confirmed that the silencing of circRNA-MSR by siRNA can suppress TNF-α expression and increase ECM formation. Thus, circRNA-MSR can be used as a potential target and specific siRNAs can be used as therapeutic agents in OA therapy. The most attractive aspect of this therapeutic is the ability to target the gene(s), which may not be possible with small molecules or protein-based drugs.[24] This opens up a whole new therapeutic approach for the treatment of OA by targeting genes that are causally involved in the pathological process. Although this approach is promising, several challenges have been identified, including the lack of stability against extracellular and intracellular degradation by nucleases, poor uptake and low potency at target sites of siRNAs, and off-target effects.[25]

Collectively, our data indicate that 104 circRNAs were either over- or underexpressed in OA. The observed changes were suggested to have biologic effects and that circRNAs are key regulators of gene expression. We confirmed that circRNA-MSR is the decoy for TNF-α. The mechanism needs to be confirmed with further specific studies. Deciphering the precise molecular mechanisms of circRNA function in OA is critical to understanding OA pathogenesis and exploring new potential therapeutic targets.

MATERIALS AND METHODS

Patients and Specimens

OA cartilage was isolated from the knee joints of 30 patients undergoing total knee arthroplasty. Joint tissue was immediately shock-frozen in liquid nitrogen, and the articular cartilage was isolated within 24 hr from the condyles and tibia plateaus with the use of a surgical blade. The tissues were examined histologically. All tissue donors included in this study provided their informed consent. The study was approved by the Human Ethics Committee of the Peking University Third Hospital (China).

Histological Examination

The cartilage was removed and fixed in 4% paraformaldehyde in PBS solution (pH 7.4) for 48 hr at 4°C, and then it was demineralized in
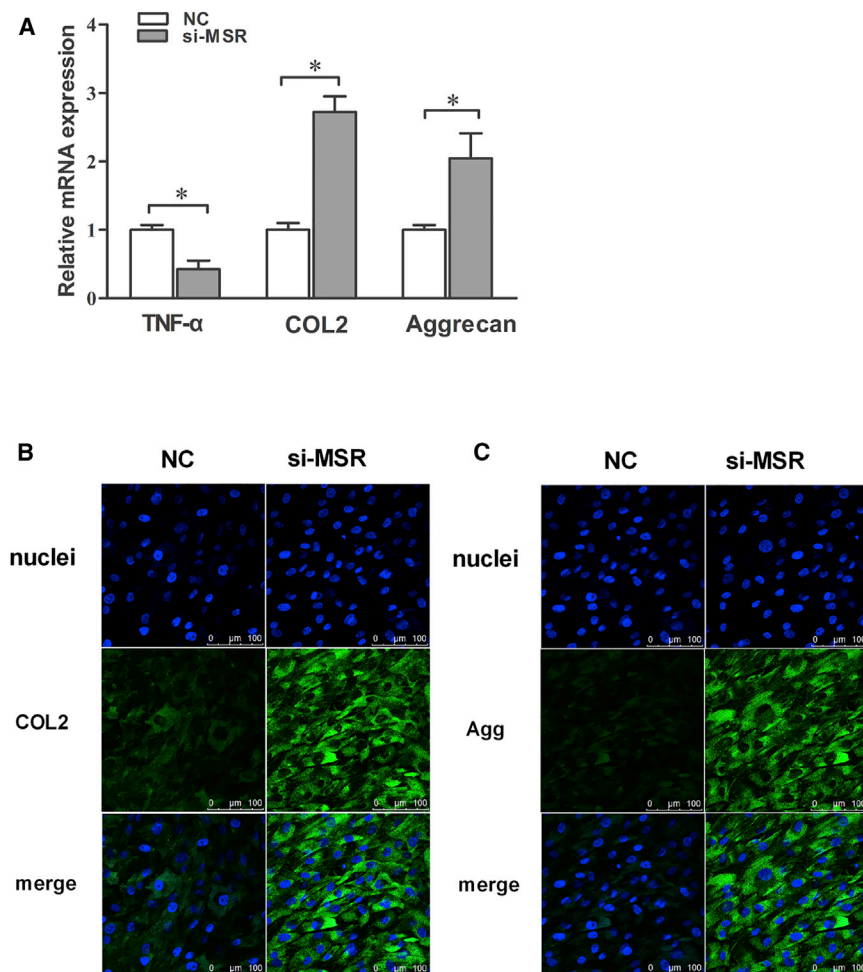
Figure 5. Effects of circRNA-MSR on Gene Expression in Human Chondrocytes

(A) TNF-α, COL2, and aggrecan mRNA expression levels were detected following the knockdown of circRNA-MSR using si-MSR. (B and C) Immunofluorescence staining for COL2 and aggrecan after transfection with si-MSR. Specific antibodies against COL2 and aggrecan were used with fluorescein isothiocyanate and Hoechst 33342 staining.

15% EDTA (pH 7.2) in PBS for 2 weeks. The cartilage specimens were dehydrated in a graded series of alcohol and xylene, and then they were embedded in paraffin and cut serially into 5 μm sagittal sections. The sections were stained with toluidine blue, Safranin-O, and H&E as per routine protocol.[26] Changes were graded according to a modified Mankin scale.[27] Scores of <4 and >6 points indicated intact and damaged cartilage, respectively.

Microarray and Quantitative Analysis

Joint tissue was immediately shock-frozen in liquid nitrogen, and then the articular cartilage was isolated from the condyles and tibia plateaus using a surgical blade within 24 hr.[28] Afterward, the samples were homogenized in TRIzol reagent (Invitrogen), and the total RNA in each sample was quantified using a NanoDrop ND-1000. Sample preparation and microarray hybridization were performed based on the Arraystar standard protocols. The total RNA from each sample was amplified and transcribed into fluorescent cRNA utilizing random primers according to the Arraystar Super RNA Labeling protocol. The labeled cRNAs were hybridized onto the Arraystar Human circRNA Array (8 × 15 K).

Bioinformatics Analysis

Differentially expressed mRNAs were associated with GO analysis. We selected specific mRNAs to construct the network according to our previous microarray data and the enrichment analyses of GO.[28] circRNAs were selected according to circRNA profiling data. Co-expression networks were constructed according to the normalized signal intensities of circRNAs and mRNAs in the original microarray data.[29] Pearson's correlation analysis was applied to measure the significance of the correlation of the expressions between each gene pair. When the expression levels of two genes were similar above a preselected threshold in the Pearson analysis, they were considered to exhibit a co-expression relationship and would be connected. Each gene corresponded to a node, and two genes were connected by a string, indicating a tight correlation. The degree of correlation determined gene importance in the network.[30,31] An mRNA-miRNA-circRNA network was constructed according to the common target miRNAs of the circRNAs and mRNAs. The interactions of circRNAs and mRNAs with miRNAs were predicted with the Arraystar miRNA target prediction software based on TargetScan and miRanda.[32,33]

Primary Culture of Chondrocytes and Exposure to Mechanical Stress

Donor chondrocytes were isolated as previously described. Chondrocytes (5 × 10⁵/well) were at passage 2 and grown on ProNectin F-coated Bioflex six-well culture plates (Flexcell International) to 80% confluence. CTS experiments were performed using an FX-5000 Flexercell system (Flexcell International). Our previous study selected the pre-optimized magnitudes of CTS, so the chondrocytes were enforced at 10% elongation (0.5 Hz) for the indicated times.[34,35]

RNAi and Transfection

The siRNAs targeting circRNA-MSR (referred to as si-circMSR) were designed and synthesized by RiboBio. The sequence of the functional si-circMSR was CCTTTTGTTGGCAATCTCT. Chondrocytes were transfected with si-circMSR using Lipofectamine 3000 (Invitrogen)
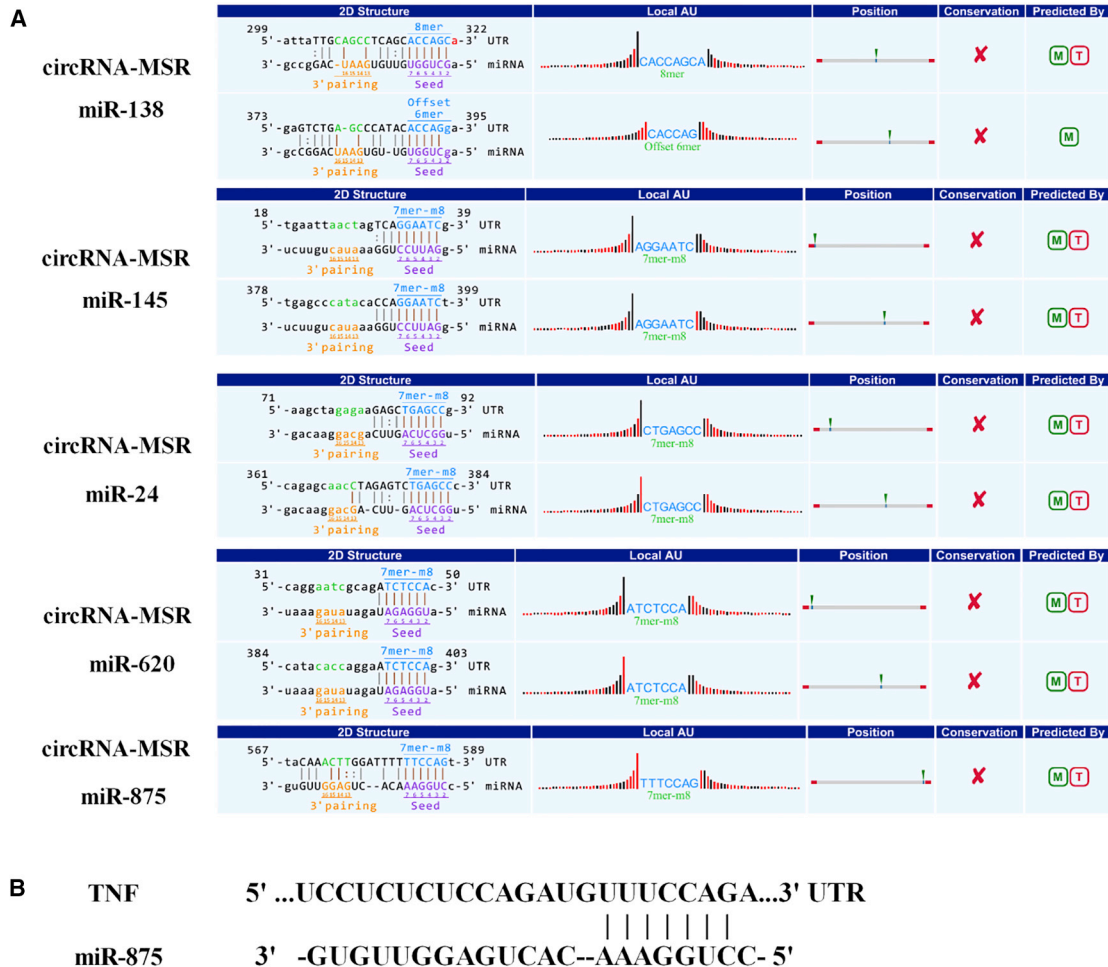
Figure 6. Targeted MicroRNAs Matched circRNA-MSR

(A) Targeted microRNAs including miR-138, miR-145, miR-24, miR-620, and miR-875. (B) miR-875 matched circRNA-MSR.

according to the manufacturer's protocol. The cells were briefly cultured with DMEM in a 96-well plate as described above. Prior to transfection, the culture medium was replaced with a medium without antibiotics, and the cells were cultured for 24 hr (Cyagen Biosciences). The RNAi Lipofectamine 3000 complex was prepared by mixing for 20 min, and the complex was then added to each cell. The cells were cultured for 48 hr at 37°C with normal DMEM.

Real-Time PCR

Total RNA was isolated from cartilage tissues or monolayer-cultured primary chondrocytes using Trizol reagent. Homogenized tissue samples were in 1 mL Trizol reagent per 50–100 mg, and the lysed cells were directly added to 1 mL Trizol reagent in a 3.5-cm diameter dish. For miRNA qPCR analysis, reverse transcription of specific miRNAs was performed with the Bulge-Loop miRNA Primer Set (RiboBio) according to the manufacturer's instructions. For mRNA analysis, total RNA was reverse transcribed using random primers.

The mRNA expression levels were reported relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), whereas miRNA expression levels were reported relative to U6. The primers used in the present study are as follows:

TNF-α forward: 5′-CCTCTCTCTAATCAGCCCTCTG-3′, reverse: 5′-GAGGACCTGGGAGTAGATGAG-3′;

circRNA-MSR forward: 5′-TCCAGTCTGATCCTTTTGTTGG-3′, reverse: 5′-CTGTTTCTTGCTGTAGACGGCT-3′;

hsa_circRNA_103387 forward: 5′-AGTCTTTCCACCTTGGC TCT-3′, reverse: 5′-TGGACAGGGTACTTCTCGTTT-3′;

hsa_circRNA_000598 forward: 5′-GTCCCTTCCCTGTCACTAC CT-3′, reverse: 5′-TCTGTTGATGCCGCCTTGG-3′;

hsa_circRNA_101975 forward: 5′-GCCCAAACCAGACCTCAC TT-3′, reverse: 5′-TCCTTCTCGGGCTCCTGA-3′; and

GAPDH forward: 5′-GGGAAACTGTGGCGTGAT-3′, reverse: 5′-GAGTGGGTGTCGCTGTTGA-3′.

Immunofluorescence Analysis

The cultured cells were rinsed in PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Goat serum was used to block nonspecific binding sites. The cultured cells were incubated with anti-COL2 (1:200 dilution) and aggrecan (1:200 dilution) at 4°C overnight. The cells were subsequently incubated for 1 hr with fluorescein isothiocyanate-conjugated AffiniPure goat anti-rabbit IgG (1:100 dilution). Finally, the samples were incubated for 5 min with Hoechst 33342 and observed with a confocal microscope (FV 1000 Olympus IX-81). Images were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics).

Statistical Analysis

Statistically significant differences from multiple groups were calculated through ANOVA. The results from two groups were evaluated using t tests. The results are reported as the mean ± SEM; $p < 0.05$ was considered statistically significant. All experiments were performed and analyzed in triplicate. Data analysis was performed using SPSS software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.omtn.2017.04.004.

AUTHOR CONTRIBUTIONS

Q.L. and X.Z. contributed equally to this work. Q.L. and L.Y. conducted the experiments. Q.L., X.Z., X.H., and Y.A. designed the experiments. Q.L., J.C., and Y.J. wrote the paper.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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