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

Qiang Liu,1 Xin Zhang,1 Xiaqing Hu,1 Lan Yuan,2 Jin Cheng,1 Yanfang Jiang,1 and Yingfang Ao1

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 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 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

Received 25 October 2016; accepted 5 April 2017; http://dx.doi.org/10.1016/j.omtn.2017.04.004.

Correspondence: Dr. Yingfang Ao, Institute of Sports Medicine, Peking University Third Hospital, 49 North Garden Road, Haidian District, Beijing 100191, People’s Republic of China.

E-mail: aoyingfang@163.com

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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 circRNAs 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.
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. 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. Among various pathogenic factors, the biomechanical factor is critical for cartilage development, homeostasis, and functionality. 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. 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. 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. Compared with other non-coding RNAs, such as miRNAs and IncRNAs, these properties provide circRNAs with the potential to be used as ideal biomarkers and potential therapy targets. 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. 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.

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...
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. Changes were graded according to a modified Mankin scale. 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. 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. 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. 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. 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.

**Primary Culture of Chondrocytes and Exposure to Mechanical Stress**

Donor chondrocytes were isolated as previously described. Chondrocytes (5 x 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.

**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).
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**: forward: 5’-CCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC

![Figure 6. Targeted MicroRNAs Matched circRNA-MSR](image-url)
**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 fluoroscein 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., J.C., and Y.J. designed the experiments. Q.L., J.C., and Y.J. wrote the paper.

**CONFLICTS OF INTEREST**

The authors declare no conflict of interest.

**ACKNOWLEDGMENTS**

This work was supported by grants from the National Natural Science Foundation of China (90919022, 81101390, and 81330040), Specialized Research Fund for the Doctoral Program of Higher Education (20110001130001), and National Natural Science Foundation of Beijing (7154248).

**REFERENCES**

1. Hayami, T., Pickarski, M., Zhuo, Y., Wesolowski, G.A., Rodan, G.A., and Duong, I.T. (2006). Characterization of articular cartilage and subchondral bone changes in the rat anterior cruciate ligament transaction and meniscectomized models of osteoarthritis. Bone 38, 234–243.

2. Dieppe, P., and Kirwan, J. (1994). The localization of osteoarthritis. Br. J. Rheumatol. 33, 201–203.

3. Goldring, M.B. (2006). Update on the biology of the chondrocyte and new approaches to treating cartilage diseases. Best Pract. Res. Clin. Rheumatol. 20, 1003–1025.

4. Kühn, K., D’Lima, D.D., Hashimoto, S., and Lotz, M. (2004). Cell death in cartilage. Osteoarthritis Cartilage 12, 1–16.

5. Goldring, M.B. (2000). The role of the chondrocyte in osteoarthritis. Arthritis Rheum. 43, 1916–1926.

6. Lotz, M. (2001). Cytokines in cartilage injury and repair. Clin. Orthop. Relat. Res. (391, Suppl), S108–S115.

7. Geyer, M., Grüssel, S., Straub, R.H., Schett, G., Dinser, R., Grift, I., Gay, S., Neumann, E., and Müller-Ladner, U. (2009). Differential transcriptome analysis of intraarticular lesional vs intact cartilage reveals new candidate genes in osteoarthritis pathophysiology. Osteoarthritis Cartilage 17, 328–335.

8. Meng, J., Ma, X., Ma, D., and Xu, C. (2005). Microarray analysis of differential gene expression in temporomandibular joint condylar cartilage after experimentally induced osteoarthritis. Osteoarthritis Cartilage 13, 1115–1125.

9. Kawakita, K., Nishiyama, T., Fujishiro, T., Hayashi, S., Kanzaki, N., Hashimoto, S., Takebe, K., Iwasa, K., Sakata, S., Nishida, K., et al. (2012). Akt phosphorylation in human chondrocytes is regulated by p53R2 in response to mechanical stress. Osteoarthritis Cartilage 20, 1603–1609.

10. Thomas, R.S., Clarke, A.R., Duance, V.C., and Blain, E.J. (2011). Effects of Wnt3A and mechanical load on cartilage chondrocyte homeostasis. Arthritis Res. Ther. 13, R203.

11. Salzman, J., Gavud, C., Wang, P.L., Lacayo, N., and Brown, P.O. (2012). Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types. PLoS ONE 7, e30733.

12. Conn, S.L., Pillman, K.A., Toubia, J., Conn, V.M., Salmanidis, M., Phillips, C.A., Roslan, S., Schreiber, A.W., Gregory, P.A., and Goodall, G.J. (2015). The RNA binding protein quaking regulates formation of circRNAs. Cell 160, 1125–1134.

13. Menczak, S., Jens, M., Elefson, A., Torti, F., Krueger, J., Rybak, A., Maier, L., Mackowiak, S.D., Gregersen, L.H., Munchsauer, M., et al. (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. Nature 495, 333–338.

14. Huntze, M.W., and Preiss, T. (2013). Circular RNAs: splicing’s enigma variations. EMBO J. 32, 923–925.

15. Vicens, Q., and Westhof, E. (2014). Biogenesis of Circular RNAs. Cell 159, 13–14.

16. Hansen, T.B., Jensen, T.I., Clausen, B.H., Branssen, J.B., Finsen, B., Damgaard, C.K., and Kjems, J. (2013). Natural RNA circles function as efficient microRNA sponges. Nature 495, 384–388.

17. Burd, C.E., Jeck, W.R., Liu, Y., Sanoff, H.K., Wang, Z., and Sharpless, N.E. (2010). Expression of linear and novel circular forms of an INK4/ARF-associated non-coding RNA correlates with atherosclerosis risk. PLoS Genet. 6, e1001233.

18. Chen, Y.T., Rettig, W.J., Yenamandra, A.K., Kozak, C.A., Chaganti, R.S., Posner, J.B., and Old, L.J. (1990). Cerebellar degeneration-related antigen: a highly conserved neuroectodermal marker mapped to chromosomes X in human and mouse. Proc. Natl. Acad. Sci. USA 87, 3077–3081.

19. You, X., Vlatkovic, I., Babic, A., Will, T., Epstein, L., Tushev, G., Akbalik, G., Wang, M., Gloc, C., Quedenau, C., et al. (2015). Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity. Nat. Neurosci. 18, 603–610.

20. Huang, J., Ballou, L.R., and Hasty, K.A. (2007). Cyclic equibiaxial tensile strain induces both anabolic and catabolic responses in articular chondrocytes. Gene 404, 101–109.

21. Roos, E.M., and Dahlberg, L. (2005). Positive effects of moderate exercise on glycosaminoglycan content in knee cartilage: a four-month, randomized, controlled trial in patients at risk of osteoarthritis. Arthritis Rheum. 52, 3507–3514.

22. Holmwall, K., Camper, L., Johannsson, S., Kimura, J.H., and Lundgren-Akerlund, E. (1995). Chondrocyte and chondrosarcoma cell integrins with affinity for collagen type II and their response to mechanical stress. Exp. Cell Res. 221, 496–503.

23. Li, P., Chen, S., Chen, H., Mo, X., Li, T., Shao, Y., Xiao, B., and Guo, J. (2015). Using circular RNA as a novel type of biomarker in the screening of gastric cancer. Clin. Chim. Acta 444, 132–136.

24. Lam, J.K., Chow, M.Y., Zhang, Y., and Leung, S.W. (2015). siRNA Versus miRNA as Therapeutics for Gene Silencing. Mol. Ther. Nucleic Acids 4, e252.

25. Barata, P., Sood, A.K., and Hong, D.S. (2016). RNA-targeted therapeutics in cancer clinical trials: Current status and future directions. Cancer Treat. Rev. 50, 35–47.

26. Strehin, I.A., and Elisseef, J.H. (2009). Characterizing ECM production by cells encapsulated in hydrogels. Methods Mol. Biol. 522, 349–362.

27. Mankin, H.J., Dorfman, H., Lippelio, L., and Zarins, A. (1971). Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. J. Bone Joint Surg. Am. 53, 523–537.
28. Liu, Q., Zhang, X., Dai, L., Hu, X., Zhu, J., Li, L., Zhou, C., and Ao, Y. (2014). Long noncoding RNA related to cartilage injury promotes chondrocyte extracellular matrix degradation in osteoarthritis. Arthritis Rheumatol. 66, 969–978.

29. Pujana, M.A., Han, J.D., Starita, L.M., Stevens, K.N., Tewari, M., Ahn, J.S., Rennert, G., Moreno, V., Kirchhoff, T., Gold, B., et al. (2007). Network modeling links breast cancer susceptibility and centrosome dysfunction. Nat. Genet. 39, 1338–1349.

30. Prieto, C., Risueño, A., Fontanillo, C., and De las Rivas, J. (2008). Human gene coexpression landscape: confident network derived from tissue transcriptomic profiles. PLoS ONE 3, e3911.

31. Barabási, A.L., and Oltvai, Z.N. (2004). Network biology: understanding the cell’s functional organization. Nat. Rev. Genet. 5, 101–113.

32. Enright, A.J., John, B., Gaul, U., Tuschl, T., Sander, C., and Marks, D.S. (2003). MicroRNA targets in Drosophila. Genome Biol. 5, R1.

33. Pasquinelli, A.E. (2012). MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat. Rev. Genet. 13, 271–282.

34. Agarwal, S., Deschner, J., Long, P., Verma, A., Hofman, C., Evans, C.H., and Piesco, N. (2004). Role of NF-kappaB transcription factors in antiinflammatory and proinflammatory actions of mechanical signals. Arthritis Rheum. 50, 3541–3548.

35. Long, P., Gassner, R., and Agarwal, S. (2001). Tumor necrosis factor alpha-dependent proinflammatory gene induction is inhibited by cyclic tensile strain in articular chondrocytes in vitro. Arthritis Rheum. 44, 2311–2319.