Immunology and Inflammation

Inhibition of miR-199b-5p reduces pathological alterations in Osteoarthritis by tar-geting *Fzd6* and *Gcnt2*

Tong Feng, Qi Zhang, Si-Hui Li, Shuan-hu Zhou, Xin Wang, Jun-Meng Wang, Fan-Rong Liang, Shu-Guang Yu, Qiao-Feng Wu

Acupuncture and Tuina College, Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan, 610075, China • Chongqing Hospital of Traditional Chinese Medicine, Chongqing, 400011, China • Department of Orthopedic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, 02115, USA • Harvard Stem Cell Institute, Harvard University, Cambridge, Massachusetts, 02138, USA • Departments of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, 02115, USA • Acupuncture & Chronobiology Key Laboratory of Sichuan Province, Chengdu, Sichuan, 610075, China

Reviewed Preprint

Published from the original preprint after peer review and assessment by eLife.

About eLife's process

Reviewed preprint posted
December 4, 2023 (this version)

Posted to bioRxiv
October 3, 2023

Sent for peer review
September 27, 2023

Abstract

Osteoarthritis (OA) is a degenerative disease with a high prevalence in the elderly population, but our understanding of its mechanisms remains incomplete. Analysis of serum exosomal small RNA sequencing data from clinical patients and gene expression data from OA patient serum and cartilage obtained from the GEO database revealed a common dysregulated miRNA, miR-NA-199b-5p. In vitro cell experiments demonstrated that miRNA-199b-5p inhibits chondrocyte vitality and promotes extracellular matrix degradation. Conversely, inhibition of miRNA-199b- 5p under inflammatory conditions exhibited protective effects against damage. Local viral injec-tion of miRNA-199b-5p into mice induced a decrease in pain threshold and OA-like changes. In an OA model, inhibition of miRNA-199b-5p alleviated the pathological progression of OA. Fur-thermore, bioinformatics analysis and experimental validation identified *Gcnt2* and *Fzd6* as tar-get genes of miRNA-199b-5p. Thus, these results indicated that miRNA-199b-5p/*Gcnt2* and *Fzd6* axis might be a novel therapeutic target for the treatment of OA.

eLife assessment

The manuscript provides interesting evidence that miR-199b-5p regulates osteoarthritis and as such it may be considered as a potential therapeutic target. This finding may be **useful** to further advance the field. Although the study is considered potentially clinically relevant, the evidence provided was deemed insufficient and **incomplete** to support the conclusions drawn by the authors.

Introduction

Osteoarthritis (OA) is a degenerative disease characterized by the deterioration of articular cartilage and affecting all components of the joint, including the synovium, subchondral bone, and meniscus[1]. Knee OA (KOA) is the leading cause of disability and increased living costs among elderly patients[2]. Currently, the treatment options for osteoarthritis are limited to symptomatic relief and joint replacement. However, the insufficient relief of symptoms, potential medication side effects, and the economic burden and complications associated with joint replacement surgeries have prompted the need to identify new, effective, and safe treatment methods and explore unknown targets for individuals with KOA[3].

Exosomes are extracellular vesicles that are distributed in body fluids such as serum and play a crucial biological role by delivering molecules such as MicroRNAs (miRNAs)[4]. They serve as vital carriers for intercellular communication and transfer of genetic information. MiRNAs are a class of non-coding RNAs ranging from 18 to 24 nucleotides in length, which exert inhibitory effects on the expression of target genes through interactions with mRNA. Studies have demon-strated that miRNAs play a crucial role as a pathogenic factor in osteoarthritis (OA). [5]. For in-stance, miR-199b-5p was found to contribute to the osteogenic differentiation of bone marrow stromal cells [6]; another miR-140 showed cartilage-specific expression, and its expression was significantly reduced in OA cartilage [7];[8].recently, intra-articular injection of antisense oligonu-cleotides of miR-181a-5p produced chondroprotective effects in OA mice [9].

In this study, we initially screened miR-199b-5p as a potential key miRNA based on clinical data by detecting differentially expressed exosomal miRNAs. To elucidate the role and function of miR-199b-5p, we investigated its impact on chondrocytes, which are crucial pathogenic cells in KOA, and identified its molecular targets through in vitro experiments. Furthermore, we ex-plored its role in vivo. Hence, this article not only identifies miR-199b-5p as a potential micro-target for KOA but also provides a potential strategy for future identification of new molecular drugs.

Results

Identification and enrichment analysis of differentially expressed miRNAs in serum exo-somes

To investigate the dysregulated miRNAs in serum exosomes of KOA patients, we extracted exosomal miRNAs from serum and performed sequencing. Serum samples from 15 patients with KOA and 10 healthy subjects were collected (**Supplementary Table 2**). After extraction, the se-rum exosomes were observed under transmission electron microscopy and nanoparticle tracking analysis, revealing a diameter ranging from approximately 70 to 150 nm (Fig. S1A, B). Furthermore, the surface marker proteins CD9, CD63, and CD81 were found to be expressed on the exosomes (Fig. S1C). Next, we performed sequencing of miRNAs in serum exosomes.

The results showed that 88 miRNAs were up-regulated and 89 miRNAs were downregulated in KOA patients compared with the control group based on fold change > 1.5 and $p < 0.05$ (**Fig. 1A, B**). Afterwards, we performed bioinformatics Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses on these differentially expressed genes. Among the top 5 enriched results of up-regulated differentially expressed miRNAs, the CC (Cell, intracellular, and cytoplasm) GO terms were notable. The MF (Translation activator activity, binding to ubiq-uitin-conjugating enzymes, and possessing transferase activity) GO terms and BP (Cell differen-tiation and the stabilization of mRNA through 3'-UTR mediation) GO terms were also significant (**Fig. 1C**). The top 10 enriched KEGG pathways, PI3K-AKT signaling, Ras signaling, apoptosis, and other types of O-glycan biosynthesis are considered to be of significant importance (**Fig. 1D**).The

analysis of the top 5 down-regulated differential miRNAs reveals interesting findings. the CC (Cell, intracellular) GO terms were particularly noteworthy. The MF (RNA polymerase II transcription factor activity and sequence-specific DNA binding) GO terms, as well as the BP (collagen-activated signaling pathway) GO terms, were also deemed significant (**Fig. 1E**). More-over, the analysis of the top 10 enriched KEGG pathways revealed a strong association with the Hedgehog signaling pathway, pluripotency of stem cells, and glycosaminoglycan biosynthesis-keratan sulfate (**Fig. 1F**).

It is noteworthy that in the aforementioned results, both the up-regulated and down-regulated differential miRNA enrichment analyses are involved in processes related to cartilage synthesis, including O-glycan biosynthesis and glycosaminoglycan biosynthesis-keratan sulfate[10;11].

Dysregulated serum exosomal miRNAs were found to be expressed in both serum and car-tilage

Subsequently, we aimed to investigate whether these dysregulated miRNAs in serum exosomes show differential expression in other affected sites or tissues of KOA. Leveraging the KOA pa-tient cartilage and serum sequencing data available in the GEO database, we compared our dysregulated miRNA list with the datasets. Remarkably, we identified 169 miRNAs that exhibit-ed differential expression in the serum of KOA patients (**Fig. 2A**), suggesting their involvement in the disease. Moreover, these miRNAs were also found to be expressed in KOA patient carti-lage (**Fig. 2B**). This robust validation reinforces the reliability of our data. Consequently, we proceeded with further screening of differentially expressed genes.

miRNA-199b-5p has been identified as a potential target molecule in KOA

Based on the P-value and exosomal expression, five miRNAs(hsa-mir-3168, hsa-mir-1296-5p, hsa-mir-15b-3p, hsa-mir-338-3p and hsa-mir-199b-5p) were selected to further research and val-idated in independent human samples by RT-qPCR (hsa-mir-199b-5p, $p < 0.01$; hsa-mir-3168, $p=0.33$; hsa-mir-15b-3p, $p < 0.01$; hsa-mir-338-3p, $p=0.20$ and hsa-mir-1296-5p, $p < 0.01$) (**Fig. 2C**). To further explore the functional roles of these miRNAs, we established a mouse model of KOA to evaluate their expression in the joints. Due to species limitations, we examined the ex-pression of miR-199b-5p ($p <0.01$), miR-15b-3p ($p <0.05$) and miR-338-3p ($p <0.05$) in joint tissue samples (control *vs* M-0.5) (**Fig. 2D**). The results showed that only the expression trend of miR-199b-5p was consistent between the clinical samples and the mouse arthritis model. There-fore, we selected miR-199b-5p as the target for our subsequent research.

miR-199b-5p mimic inhibits the cell viability and extracellular matrix (ECM) of chondro-cytes and inhibitor restores LPS-induced chondrocytes damage

We first extracted mouse primary chondrocytes (**Fig. 3A-C**). In vitro experiments showed that overexpression miR-199b-5p inhibited the viability of chondrocytes($p<0.01$) (**Fig. 3D**, Fig. S2). we also find miR-199b-5p mimic increased the mRNA expressions of *MMP3* ($p=0.09$) and *ADAMTS5* ($p<0.05$) and decreased the mRNA expression of *COL2A1*($p=0.05$), *AGGRECAN* (p=0.20) and *SOX9* ($p=0.22$), which are often used as the biomarkers of chondrocytes ECM met-abolic balance. In contrast, miR-199b-5p inhibitor decreased the mRNA expression of *MMP3* ($p<0.01$) and *ADAMTS5* ($p=0.11$) and increased the mRNA expression of *COL2A1* ($p=0.07$), *AGGRECAN* ($p<0.01$) and *SOX9* ($p<0.01$) (**Fig. 3E–I**). While some gene expression changes may not be significant in statistic, but the modulation of miR-199b-5p expression has been ob-served to exert an influence on the metabolic alterations of chondrocytes.

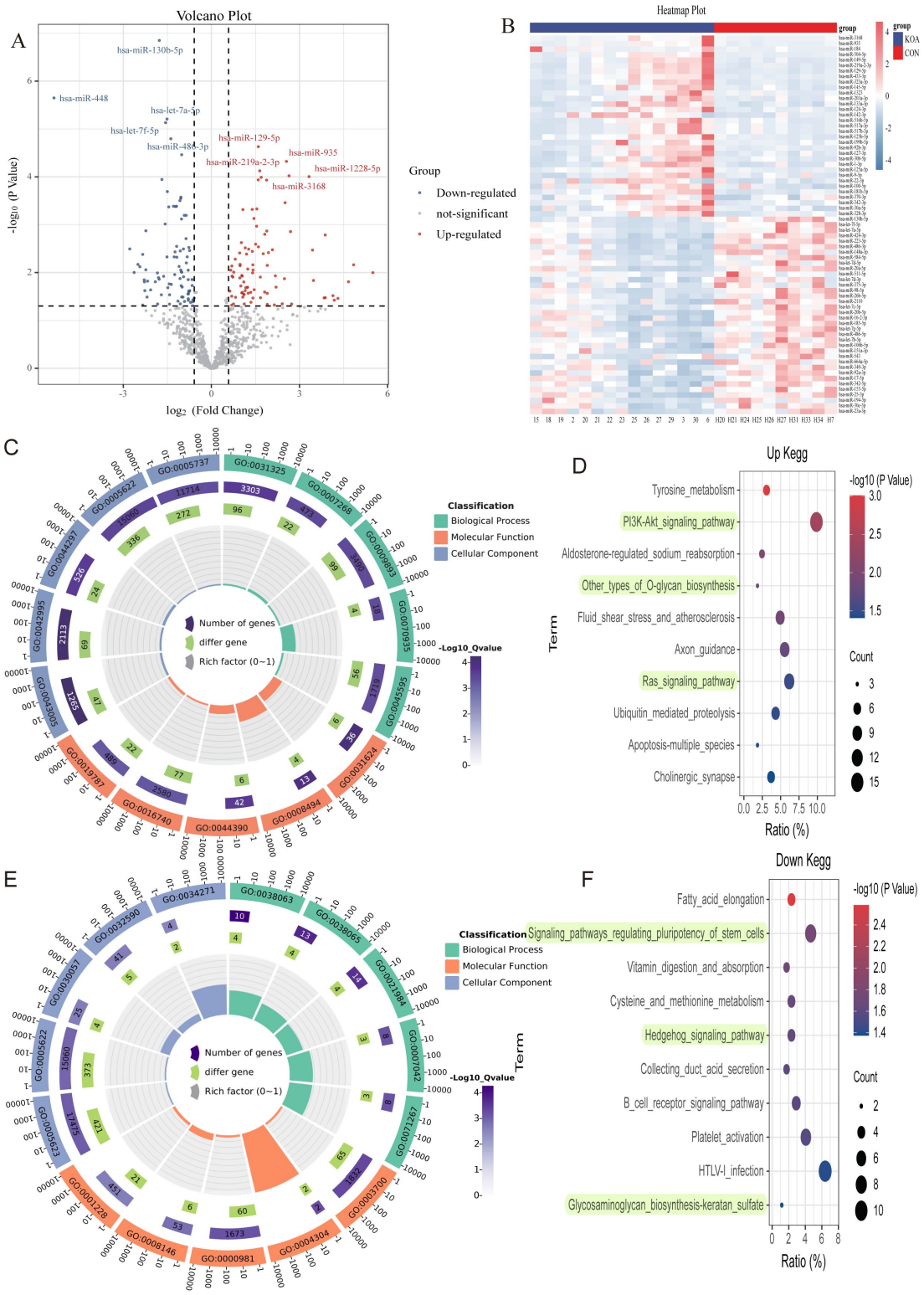

Figure 1

MiRNA expression in KOA patients.

(A, B) Volcano plot and heatmap showing dif-ferential miRNAs between KOA and healthy groups (healthy, *n* = 10; KOA, *n* = 15, | log2 Fold Change | 0.585, *P* < 0.05). **(C)** GO and KEGG enrichment analyses of upregulated genes. **(D)** GO and KEGG enrichment analyses of downregulated genes.

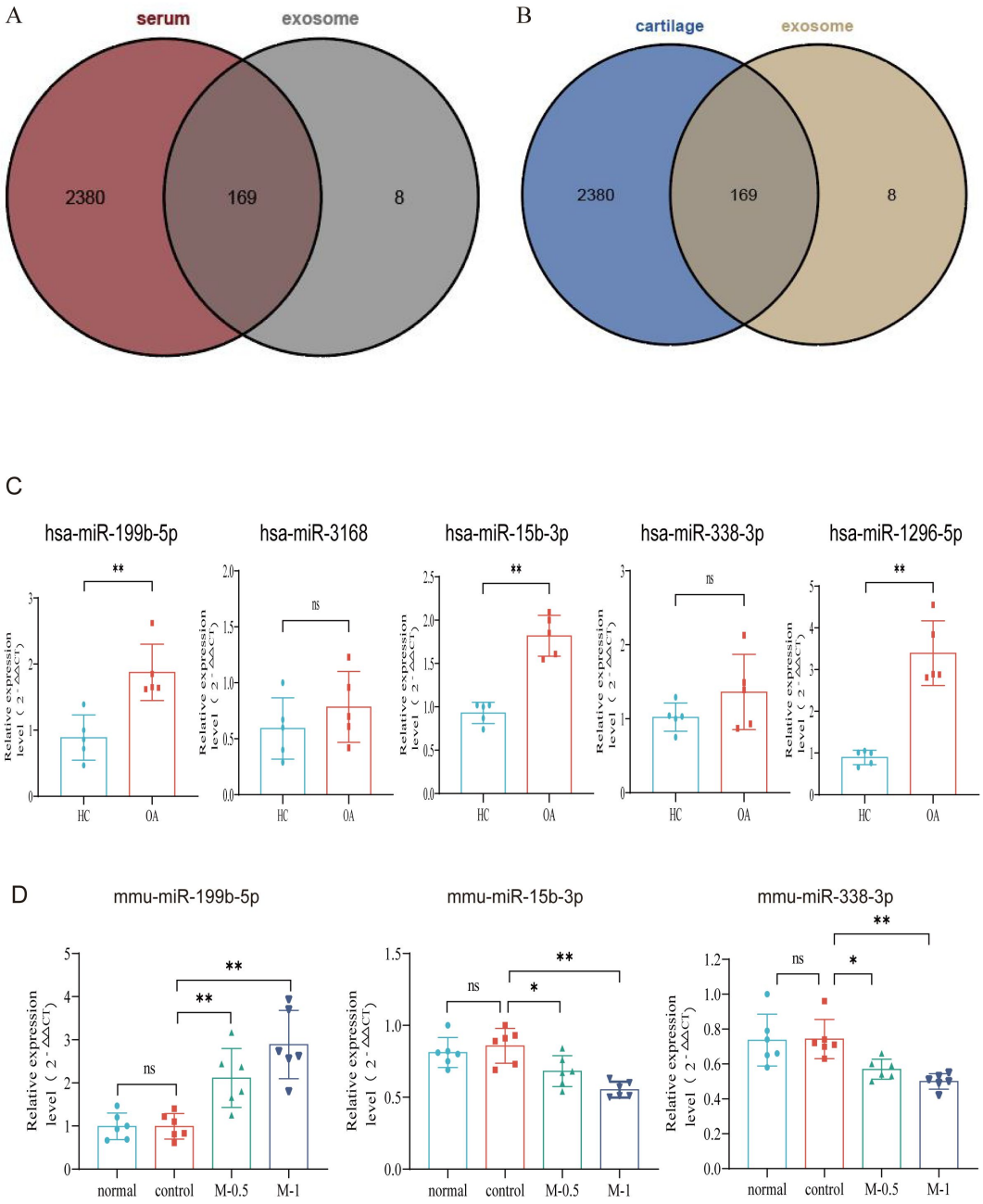

Figure 2

Verification with GEO data and miRNA screening.

(A, B) Venn plot showing GEO dataset and our result. **(C)** RT-qPCR in clinical samples to verify the expression (HC, *n* = 5, KOA, n = 5). **(D)** RT-qPCR results of mouse joint samples (*n* = 6). Data are shown as means ± SD. **P*<0.05, ***P*<0.01.

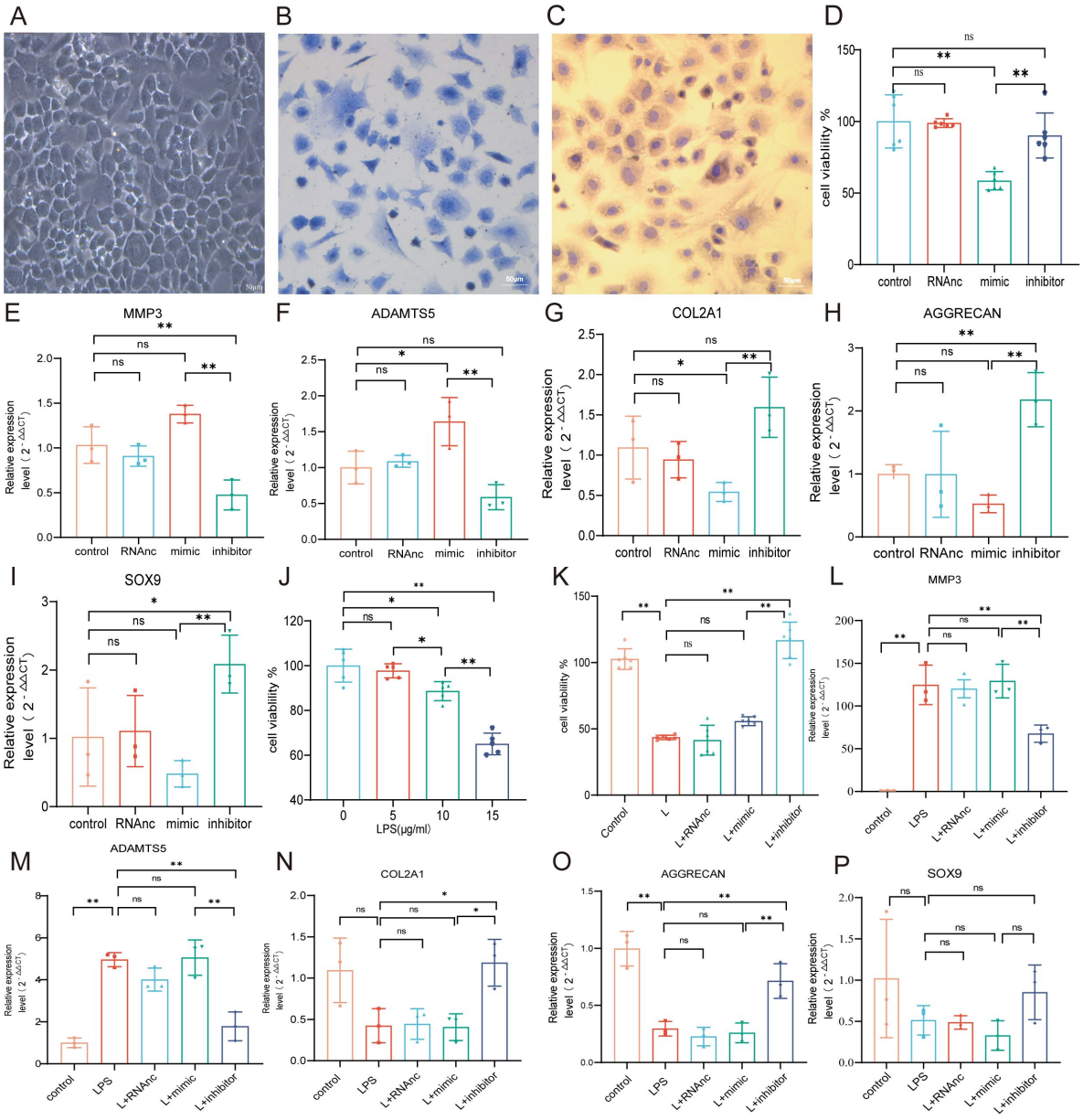

Figure 3

Chondrocyte proliferation and marker expression changes after miR-199b-5p mimic or inhibitor treatment.

(A) Second generation primary mouse chondrocytes. **(B)** Toluene blue staining. **(C)** Type II collagen immunoassay. **(D)** CCK-8 assay for cell viability (*n*=6). **(E, F)** RT-qPCR detection of *MMP-3* and *ADAMTS5* mRNA expression (*n*=3). **(G-I)** RT-qPCR detec-tion of *COL-2A1*, *AGGRECAN*, and *SOX9* mRNA expression (*n*=3). **(J)** CCK-8 cell viability assay after different doses of LPS induction (*n*=5). **(K)** CCK-8 cell viability assay after virus in-fection (*n*=6). **(L, M)** RT-qPCR detection of *MMP-3* and *ADAMTS5* mRNA expression (*n*=3). **(N-P)** RT-qPCR detection of *COL2A1*, *AGGRECAN*, and *SOX9* mRNA expression (*n*=3). Data are shown as means ± SD. **P*<0.05, ***P*<0.01.

To explore the effect of miR-199b-5p under pathological conditions, an LPS-stimulated inflammation chondrocyte cell model was established. We examined the effect of LPS at 5, 10, and 15 μg/ml on cell viability and found that cell viability was significantly decreased at 15 μg/ml(p<0.001) (**Fig. 2J**). Next, we chose 15 μg/ml of LPS to establish a chondrocyte injury model. The CCK-8 assay showed that miR-199b-5p inhibitor reversed the decrease of cell viabil-ity caused by LPS (p<0.01) (**Fig. 3K**). Also, we revealed that LPS elevated *MMP3* (p<0.01) and *ADAMTS5* (p<0.01) and decreased *COL2A1*(p=0.06), *AGGRECAN* (p<0.01), and *SOX9* (p=0.13) expression. In the presence of miR-199b-5p inhibitor, the changes in mRNA levels were re-versed (**Fig. 3L-P**). These results suggest that miR-199b-5p overexpression reduces cell viability and miR-199b-5p inhibition partly restores LPS-induced cell damage and ECM degradation.

miR-199b-5p mimic induces inflammation in normal mice, and miR-199b-5p inhibitor alle-viates symptoms in KOA mice

Now, we want to know the vivo role of miRNA-199b-5p. Firstly, we screened Adneovirus(AD) and utilized High-AD as a vector to either overexpress or inhibit the expression of miR-199b- 5p(Fig. S3). Then, miR-199b-5p mimic was injected to the joint of mice (**Fig. 4A**) and a decrease in pain threshold was found (p<0.01) (**Fig. 4B**). Four weeks later, serum *IFN-γ* (p<0.01) and *TNF-α* (p<0.01) were also significantly increased (**Fig. 4C, D**). The cartilage of mice injected with the miR-199b-5p mimic was slightly degraded (p=0.02) (**Fig. 4E, F**). Additionally, the ar-ticular surface showed insect erosion (**Fig. 4G**). interestingly, the level of serum inflammation in the miR-199b-5p inhibitor injection group was significantly decreased compared with the mimic group (*IFN-γ*, p<0.01; *TNF-α*, p<0.01). These results indicated that intra-articular injection of miR-199b-5p mimic induced inflammation response in mice.

Followingly, we established an MIA-induced KOA model to further investigate the role of miR-199b-5p (Fig. S4). We observed a decrease in pain threshold in the model group, and recovery was observed on the 10th day in the inhibitor group (p<0.01) (**Fig. 5A, B**). Joint tissues were tak-en at the fourth week, and revealed that the expression of *IFN-γ* (p<0.01) and *TNF-α* (p<0.01) decreased after inhibiting miRNA-199b-5p (**Fig. 5C, D**). Safranin-fast green staining of joints showed recovery of articular cartilage degradation (p<0.05) (**Fig. 5E, F**), and the moth erosion of the cartilage μCT was partly improved (**Fig. 5G**). These results proved that intra-articular injec-tion of the miR-199b-5p inhibitor partly recovered pain, inflammation, and cartilage degenera-tion in KOA mice.

Gcnt2 and *Fzd6* are two target genes of miR-199b-5p

In order to investigate the underlying mechanism of miRNA-199b-5p, we utilized five widely used miRNA target gene prediction tools, namely miRWalk, miRDB, TarBase, starbase, and TargetScan, to identify potential target genes. Consequently, we identified six putative target genes of miRNA-199b-5p (**Fig. 6A**). Bioinformatics analysis of the six possible target genes showed that BP is in posttranscriptional regulation of gene expression and angiogenesis; CC is in cytoplasmic vesicle and cell leading edge; and MF is in ubiquitin protein ligase binding and pro-tein domain specific binding (**Fig. 6B**).

After Mimic infected cells, we found decreases expression in *Fzd6* (P<0.01), *Gcnt2* (P<0.01), and *Caprin1* (P=0.024) in the chondrocytes (**Fig. 6C**). Conversely, after inhibitor infection, *Hif-1α* (P=0.048), *Fzd6* (P<0.01) and *Gcnt2* (P<0.01) were increased and *Atg14* (P=0.042) increased (**Fig. 6D**). Notably, we observed corresponding changes in the expression of *Fzd6* and *Gcnt2* up-on miR-199b-5p overexpression and under-expression. Moreover, we predicted potential binding sites for miRNA-199b-5p within the 3'-untranslated region (UTR) of these two target genes (**Fig. 6E, F**) and luciferase reporter assays confirmed that miR-199b-5p can bind to and suppress the expression of both *Gcnt2* (P < 0.01) and *Fzd6* (P < 0.01) *via* their complementary sequences (**Fig. 6G, H**). Furthermore, we found differential expression of *Fzd6* in both synovial tissue data

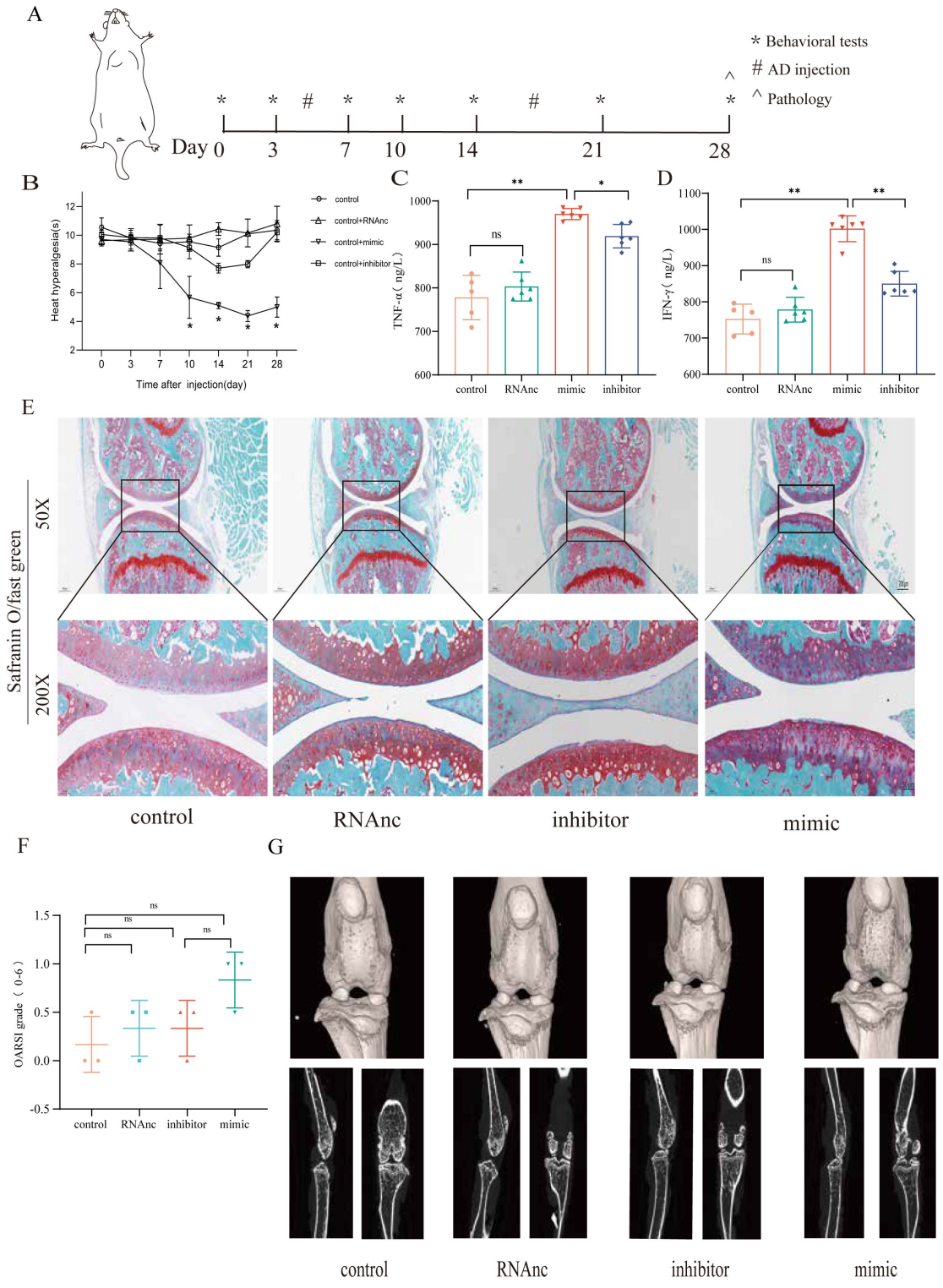

Figure 4

Injection of adenovirus expressing miR-199b-5p mimic results in inflammation and pain threshold sensitivity in mice.

(A) Animal experiment schematic. **(B)** Behavioral detection of animal thermal pain threshold. **(C, D)** Detection of serum levels of *IFN-γ* and *TNF-α* in controls by ELISA. **(E, F)** Safranin-fast green staining and semiquantitative scoring of articular cartilage. **(G)** 3D reconstruction and 2D images of joints from μCT scans. Data are shown as mean ± SD. **P*<0.05, ***P*<0.01, *n*=6.

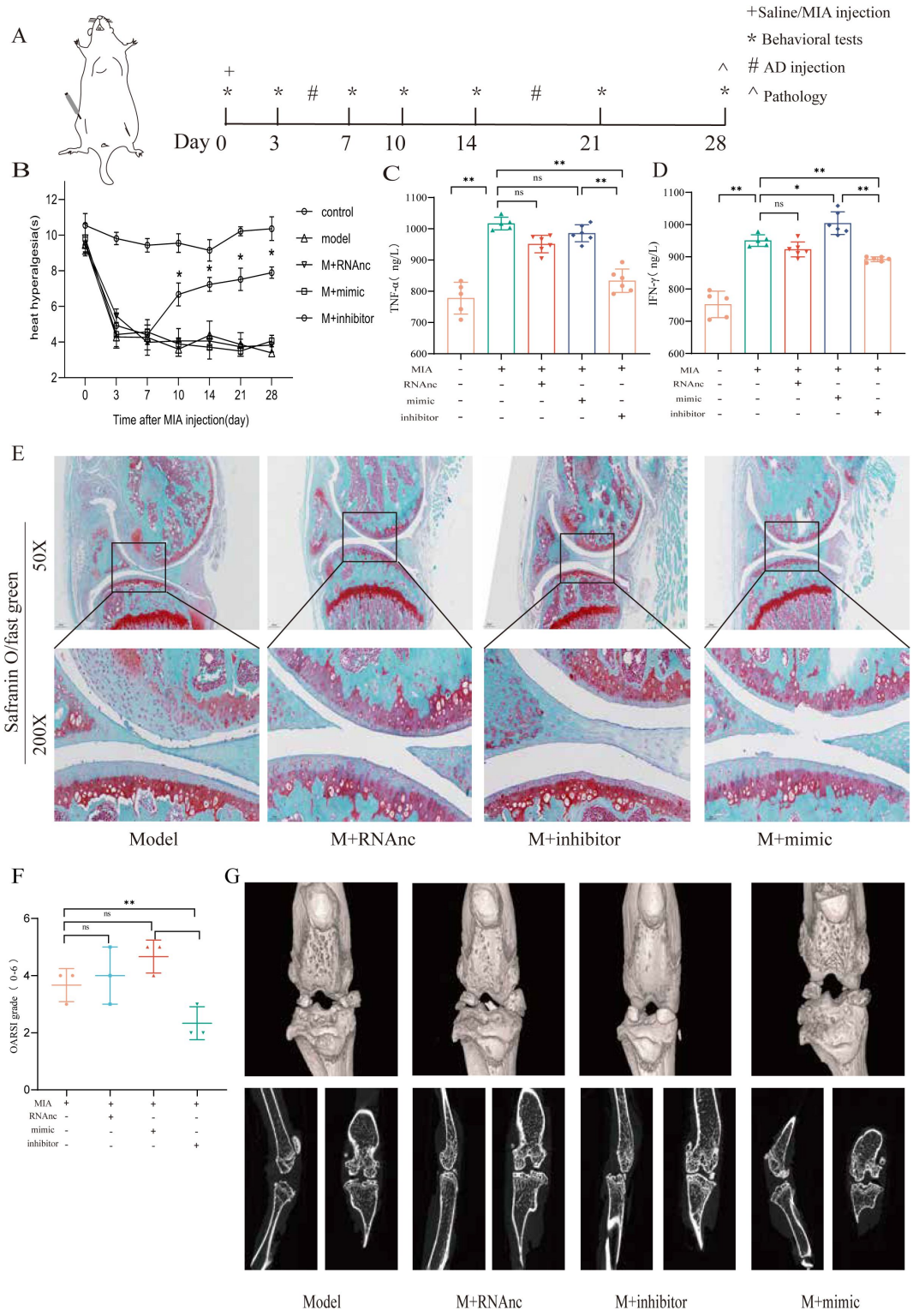

Figure 5

Injection of adenovirus expressing miR-199b-5p inhibitor partly recovered pathologi-cal changes in KOA mice.

(A) Animal experiment schematic. **(B)** Behavioral detection of animal thermal pain threshold. **(C, D)** Detection of serum levels of *IFN-γ* and *TNF-α* in controls by ELISA. **(E, F)** Safranin-fast green staining and semiquantitative scoring of articular cartilage. **(G)** 3D reconstruction and 2D images of joints from μCT scans. Data are shown as mean ± SD. **P*<0.05, ***P*<0.01, *n*=6.

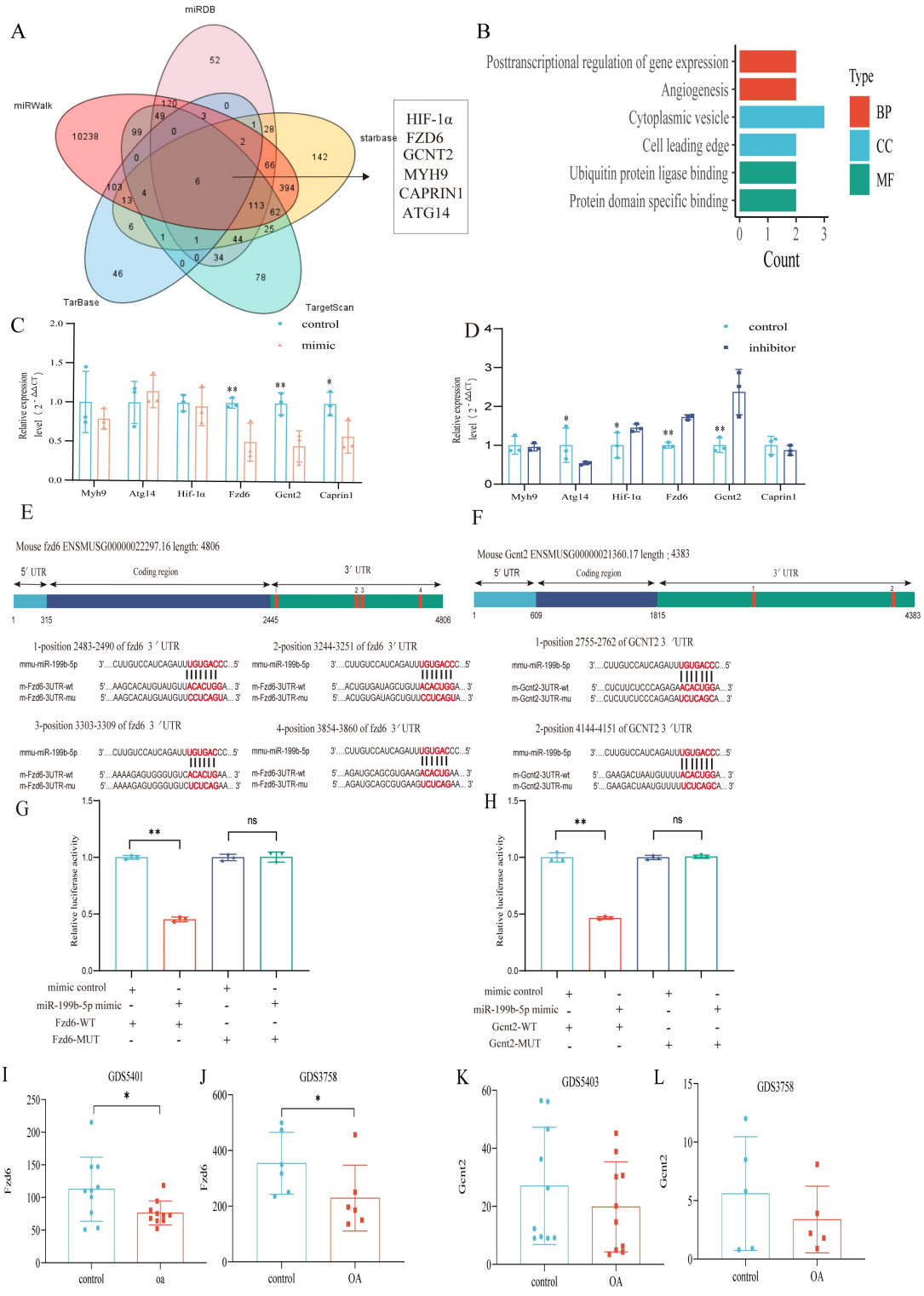

Figure 6

Validation of the miR-199b-5p target gene.

(A) Prediction of target genes of miR- 199b-5p using search sites. **(B)** Target gene GO analysis. **(C, D)** Detection of the expression of target genes under different conditions (*n*=3). **(E, F)** Predicting the binding site of miR-199b-5p and target genes. **(G, H)** Validation by luciferase reporter gene assay (n=3). Data are show as mean ± SD.*P<0.05, **P<0.01, n=6. **(I-L)** The expression of FZD6 and GCNT2 in the synovial membrane and chondrocytes of GEO Profiles KOA.

(GDS5401) and chondrocyte data (GDS3758) from KOA patients in the GEO profile (**Fig. 6I, J**). Similarly, differential expression tendency of *Gcnt2* was observed in both the synovial tissue da-ta (GDS5403) and GDS3758 from the same KOA patients (**Fig. 6K, L**). These findings provide further validation to our results. Therefore, *Fzd6* and *Gcnt2* was confirmed as an important downstream target for further research.

Discussion

In this study, we initially performed sequencing of serum exosomal miRNAs from clinical pa-tients and identified 177 dysregulated miRNAs. Subsequently, through comparison with GEO data, we found that 169 miRNAs were expressed in both KOA serum and cartilage. Following a screening process, miR-199b-5p was selected for further experiments. In cell-based assays, we discovered that miR-199b-5p can influence the viability of chondrocytes and cytokine-mediated extracellular matrix metabolism. Moreover, in vitro experiments demonstrated that it can induce inflammation and abnormal pain threshold in normal mice. Importantly, inhibition of miR-199b- 5p alleviated the pathological symptoms of KOA. Finally, these effects were achieved by target-ing *Gcnt2* and *Fzd6* (**Fig. 7**). Thus, our findings demonstrated that miR-199b-5p might be a novel potential therapeutic target for OA prevention and treatment.

miRNAs function as regulators of gene expression in biological processes by regulate mRNA translation by specifically binding to the 3'UTRs of target mRNAs [12]. Exosomes are rich in miRNAs, and various cells can secrete exosomes to target cells under physiological and patho-logical conditions, which function as delivery vehicles for miRNAs [13;14]. There is evidence sug-gesting that miRNAs can participate in various cellular processes (inflammation, cell viability, ECM dysregulation) and signaling pathways (Hedgehog signaling, PI3K-AKT signaling) rele-vant to OA[15;16]. Our differential miRNA enrichment analysis also strongly supports the correla-tion with these findings.

In cancer research, overexpression of miR-199b-5p can inhibit the proliferation, migration and invasion of prostate cancer cells in vitro and tumor growth and metastasis in vivo by targeting DDR1[17]. In addition, exogenous miR-199b-5p inhibited the growth of bone marrow mesenchy-mal stem cells (MSCs) and promoted the differentiation of bone MSCs into chondrocytes by tar-geting the *JAG1* pathway [18;19]. Our functional experiments showed that overexpression of miR- 199b-5p reduced chondrocyte viability and the expression of anabolic factors such as COL2A1, AGGREGN, and SOX9. It also increased inflammation levels and decreased pain thresholds in control mice. Although no pathological changes were observed in the articular cartilage of con-trol mice, a similar study demonstrated that pathological cartilage changes only occurred after six months in mice with miR-211 and miR-204 knockout[20]. Therefore, we speculate that the time of overexpression of miR-199b-5p in our experiment was too short, so it only caused inflammation and pain threshold response. To best of our knowledge, this is the initial investigation document-ing the involvement of miR-199b-5p in KOA.

Fzd6 is known to be up-regulated during the osteogenic differentiation of MSCs and can be regu-lated by miR-194-5p to activate the WNT signaling pathway, thereby promoting osteogenic dif-ferentiation of MSCs[21]. *Gcnt2* has been found to induce epithelial-mesenchymal transition and enhance migration and invasion of esophageal squamous cell carcinoma cells[22]. Our findings in-dicate that miR-199b-5p plays a crucial role in KOA by targeting *Fzd6* and *Gcnt2*. Future re-search should further explore the roles and mechanisms of *Fzd6* and *Gcnt2* in the context of KOA.

This study also has some limitations. We initially detected serum exosomes miRNA and later examined the miRNA through in vitro experiment and animal study. However, whether the miRNAs targeting the joints have the same role as the serum exosome miRNAs or blood miRNA has not been clear. We also performed direct intra-articular injection of adenoviral vectors. The

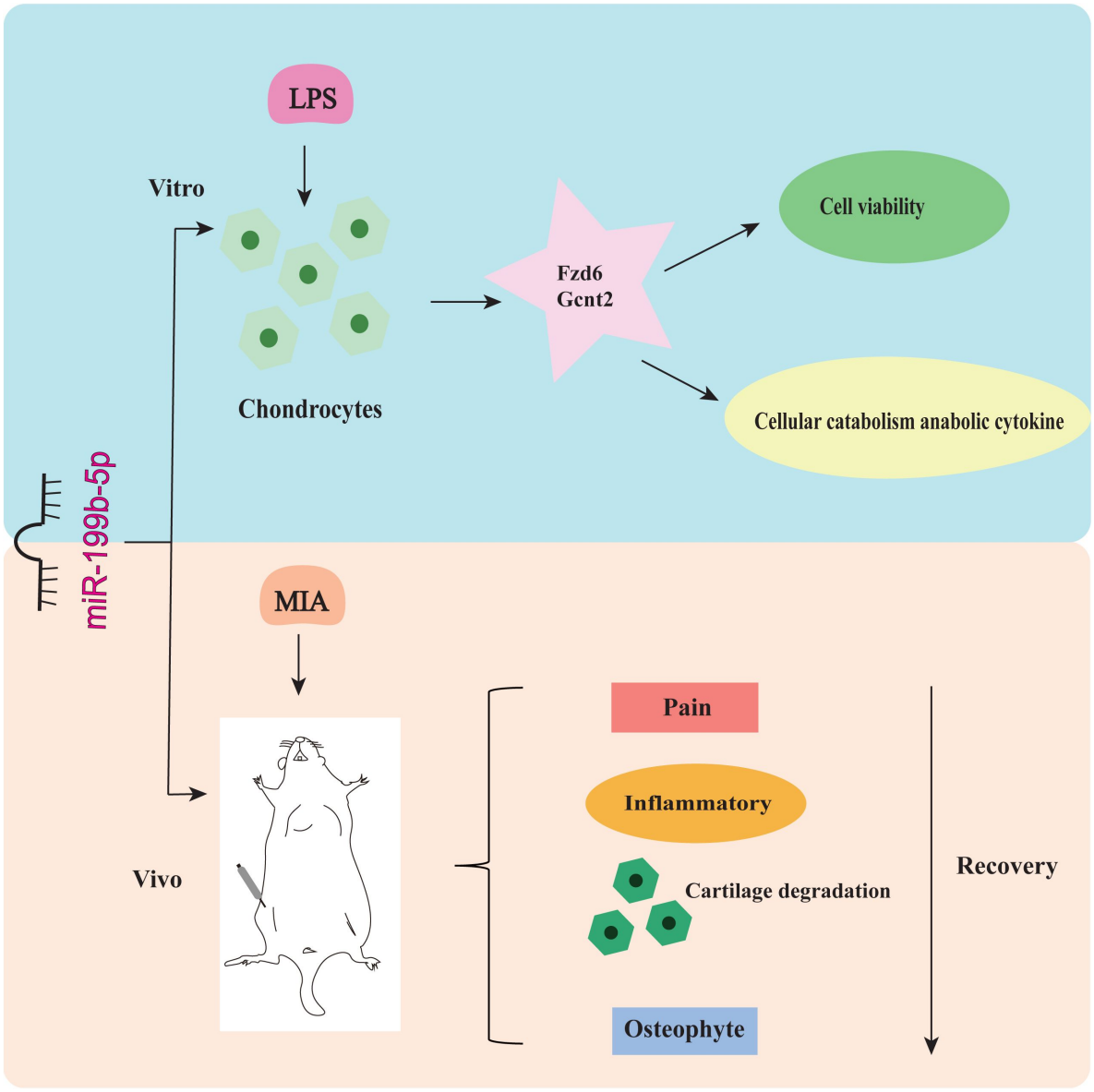

Figure 7

miR-199b-5p exerts its effects on in vitro cells and in vivo mice by targeting *Fzd6* and *Gcnt2*.

intra-articular injection can reduce the exposure of extra-articular tissue, thereby minimizing the associated side effects and improving targeting. Besides, more in-vivo study such as gene knockout mice study can be used in the future study.

In conclusion, we found that miR-199b-5p is elevated in osteoarthritis and may affect cell viabil-ity and related cytokines by targeting *Gcnt2* and *Fzd6*. Overexpression of miR-199b-5p induced OA-like pathological changes in normal mice and inhibiting miR-199b-5p alleviated symptoms in KOA mice, suggesting it may be a target for treatment of OA. Through human clinical trials, cell experiments, and animal models, we not only identified a new OA-related miR-199b-5p but also examined the biological function of miR-199b-5p in OA.

Materials and methods

Human samples

Study participants were recruited from the Hospital of Chengdu University of Traditional Chi-nese Medicine and the surrounding communities. The study was approved by the Ethics Review Committee of the Hospital at Chengdu University of Traditional Chinese Medicine (2016KL-017) and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Serum was collected from all participants and serum samples were stored at -80°C.

Human subjects

Patients were enrolled if they fulfilled the following criteria: (1) diagnosed with KOA according to the ACR; (2) age between 40 and 65 years; (3) agreed to cooperate with researchers in all re-search procedures after enrollment; (4) provided with written informed consent. Patients with any of the following conditions were excluded: (1) accompanied with other diseases such as rheumatoid arthritis, bone tumors, and bone tuberculosis; (2) treated with intra-articular gluco-corticoid or viscoelastic supplementation in the last six months; (3) knee replacement history; (4) had complicated cardiovascular disease, diabetes, skin disease, and liver or kidney impairment; (5) pregnant or breastfeeding women; (6) accompanied with mental and intellectual disabilities; or (7) undergoing other clinical trials.

Extraction and sequencing of serum exosome miRNAs

Serum samples were filtered using 0.22 μM filters. Exosomes were isolated from the serum sample using ExoQuick™ Exosome Precipitation Solution (System Biosciences) following the manufacturer's instructions. Briefly, serum was thawed on ice and centrifuged at 4000 rpm for 15 min to remove any cells or cellular debris. Next, 50 μL ExoQuick Solution was added into the 200 μL serum sample and mixed thoroughly. The exosomes were suspended in PBS.

Exosomes were characterized by electron microscopy (Tecnai G2 Spirit 120KV, FEI), nanopar-ticle tracking analysis (NTA) (NTA 3.2 Dev Build 3.2.16), and western blot analysis (for CD9, CD63 and CD81). Sequencing was performed by single-end sequencing (1×150 bp) on Illumina NextSeq 500. The libraries were sequenced on an Agilent 2100 Bioanalyzer platform. The mirdeep2 software (*https://www.mdc-berlin.de/content/mirdeep2-documentation*) was used to analyze the miRNA sequences and quantification. The heatmap was plotted based on the log2 (fold change), using Heatmap Illustrator software (Heml 1.0).

Total RNA was isolated from human serum, cell and mice

Chondrocytes using a kit (Yeasen,Shanghai, China) according to the manufacturer's instructions. Reverse transcription was performed using 1000 ng total RNA and a Prime Script RT Reagent Kit(Yeasen, Shanghai, China) or Prime Script RT Master Mix (Yeasen, Shanghai, China), which were

used for miRNA and mRNA, respectively. For miRNA, the reactions were incubated at 42°C for 15 min followed by inactivation at 85°C for 5 s. qRT-PCR amplification was assessed in a CFX Connection Real-Time System (Bio-Rad) using the SYBR Premix Ex Taq II kit (Yeasen, Shanghai,China). The following cycling conditions were used: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. All reactions were performed in duplicate and normalized to the internal reference U6 for miRNA and GAPDH mRNA for mRNAs. The $2^{-\Delta\Delta CT}$ CT method was used to evaluate the relative mRNA/miRNA expression levels.

RNA primer

Primers are listed in **Supplementary Table 1**.

Bioinformatics analysis

GO analysis was performed, involving the biological process (BP), cellular component (CC), and molecular function (MF), using DAVID[23] (*https://david.ncifcrf.gov/home.jsp*).We used the miRNA target gene prediction websites miRDB[24](*http://mirdb.org/*), miR-Walk[25](*http://mirwalk.umm.uni-heidelberg.de/*), Starbase[26] (*https://starbase*. sysu.edu.cn/), DI-ANA-TarBase[27] (*http://diana.imis.athena-innovation.gr/DianaTools/* index.php?r=tarbase/index), Targetscan[28] (*https://www.targetscan.org/vert_72/*). The target genes of mmu-miR-199b-5p were predicted, and the intersection of the target gene prediction list results of the five sites was taken.

The dysregulated miRNAs were compared to relevant published miRNA data from human KOA patients. The dataset GSE105027 was obtained from serum samples of KOA patients, while GSE175961 comprised sequencing data from KOA patient cartilage. Finally, we validated the expression of the target genes Gcnt2 and Fzd6 in KOA patients using synovial data (GDS5401, GDS5403) and cartilage data (GDS3758).

Primary mouse chondrocyte culture

Primary mouse cartilage was extracted following the protocol of Gosset et al.[29]. The cells were cultured in F12 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C and a 5% CO_2. The medium was changed every three days, and cells were cultured to the second passage for experiments. Cells were stimulated with LPS for 6 h before subsequent investigation.

Animal model of KOA

Animal experiments were performed using total of 108 8-week-old male C57BL/6 mice. The protocol was approved by the Committee on the Ethics of Animal Experiments of Chengdu University of Traditional Chinese medicine (2019-04). After randomization, the KOA mouse model was established by orthotopic injection of MIA (sigma) into the knee joint of mice. After the an-imals were anesthetized with isoflurane in a small animal anesthesia machine, the right knee joint of the mouse was shaved, the knee joint was flexed 90°, and 10 μL of MIA solution was injected into the knee joint cavity using a 28G microsyringe. The syringe needle was slowly withdrawn and the knee joint was gently moved. Before model establishment and at 3, 7, 10, 14, 21, and 28 days after model establishment. After the 3rd day and 14th day after the start of the experiment, the experimental mice were injected with adenovirus (total volume 10μL) in the knee joint, and the control mice were injected with empty adenovirus as a control (Hanbio tech, Shanghai, China)[30].

Thermal pain threshold detection

A pain threshold detection instrument was used to measure the latency of the right plantar leg raising reflex of mice under heat radiation. Three days before the experiment, mice were cut off from water for half a day and adapted to the environment for 1 h. A certain intensity of pyrogen

was used to irradiate the plantar position of the mouse's right limb, and a machine was used to automatically record the time when the mouse moved the limb from thermal pain. The resting intensity of the thermal pain stimulator was set to 10%, and the maximum duration was set to 20 s to avoid prolonged heating and burning of skin. After the light source is aimed at the sole of the mouse, we observed the mouse in real time. If paw raising, paw licking, or paw retraction was observed, the irradiation was stopped and the irradiation time of the instrument was recorded. The right limb of each mouse was tested five times, with an interval of 10 min each time; outliers were eliminated, and the average value was calculated and included in the statistics[31].

ELISA and μCT scanning

Mice were sacrificed after four weeks, and serum was collected for ELISA assay (Jiangsu Jing-mei Biotechnology Co., Ltd., Yancheng, China). The knee joint specimens of the right hindlimb of mice were scanned using a high-resolution micro-CT skysan1267 instrument (Bruker, Germa-ny). The sample was removed from fixative and dried. The scanning parameters were as follows: voltage 55 KV, current 200 μA, and filter 0.25AL. After scanning, three-dimensional reconstruc-tion was performed using NRecon1.7.4.2 software.

Safranin Fast green staining

Mouse knee joint staining was performed using the Safranin Fast Green Staining Kit (Servicebio Biological Technology, Wuhan, China). The sample was examined under a microscope, and the cartilage integrity was scored according to the OARSI grading system, in which the score ranged from 0 to 6 points[32].

Luciferase assay

The wild-type (WT) and mutant-type (MUT) sequences (according to the predicted binding site) were inserted into the pmiRGLO plasmid. HEK-293T cells were seeded in 6-well plates at 24 h before transfection. GCNT2 and FZD6 3'UTR-wt and gcnt2 and fzd6 3'UTR-mut plasmids (500 ng) and 20 nmol miR-199b-3p and NC were co-transfected with Lipofectamine 3000 (Hanbio Biotechnology, China) following the manufacturer's instructions. After 48h, firefly and Renilla luciferase activities were calculated using the Promega Dual-Luciferase system following the manufacturer's instructions (Hanbio tech, Shanghai, China). Firefly/Renilla luciferase was meas-ured to evaluate relative luciferase activity.

Statistical analysis

Data are reported as the means ± SD. Normal distribution and homogeneity of variance of data were first tested. Shapiro-Wilk test was used to verify data normality, while Levene's test or Browne-Forsythe test was adopted for assessment of variance equality. Statistical analysis was performed by unpaired two *t*-test and Tukey-corrected one-way analysis of variance (ANOVA) for comparisons between groups. Tukey-corrected two-way ANOVA for the comparison of mice thermal pain threshold data. $P < 0.05$ was considered statistically significant for all statistical calculations. PRISM 8.0 (GraphPad Software, San Diego, CA, USA) was used for data analysis.

Contributions

F.T., Z.Q., and Q.-F.W. designed the study.

F.T. performed most in vitro and in vivo experiments.

F.T. and J.-M.W. analyzed data.

Z.Q., Y.J. and S.-H.L. performed the miRNA sequencing and interpreted the data.

Z.Q., and Y.J collected and inspected human patient samples.

F.T. and Q.-F.W. wrote the manuscript.

All authors read and edited the manuscript.

F.-R.L., S.-G.Y., and Q.-F.W. supervised the study.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Acknowledgements

This work was supported by the National Key R&D Program of China (No.2019YFC1709001); Innovation Team and Talents Cultivation Program of National Administration of Traditional Chinese Medicine (No. ZYYCXTD-D-202003); Fund of Science and Technology Department of Sichuan Province, China (No. 2021ZYD0081); and National Natural Science Foundation of Chi-na (No. 82174512, 81873383).

Data Sharing Statement

All data are available in the main text or the supplementary materials.

List of Supplementary Materials

Present a list of the Supplementary Materials in the following format.

Materials and Methods

Fig S1. Identification of exosome. (A) Transmission electron microscope scanning of isolated exosomes from the serum of participants. (B) The Nano sight particle analysis of isolated exosomes from the serum of participants. (C) The Western blot analysis of symbolic surface markers of isolated exosomes from the serum of participants.

Fig S2. Fluorescence of Adenovirus-Infected Chondrocytes.

Fig S3. Expression of adenovirus in mouse knee joint.

Fig S4. Establishment of KOA model in mice by injection of MIA. (A) Behavioral detection of animal thermal pain threshold. (B) Microscopic observation of the surface of the mouse knee joint. (C) Safranin-fast green staining and semiquantitative scoring of articular cartilage. (D) 3D reconstruction images of joints from μCT scans. *P<0.05, **P<0.01, n=6.

gene	sequence
U6	GSP:5'GCTTCGGCAGCACATATACTAAAAT3'
	R:5'CGCTTCACGAATTTGCGTGTCAT3'
hsa-miR-199b-5p	GSP:5'GGGACCCCAGTGTTTAGACTAT3'
	R: 5'GTGCGTGTCGTGGAGTCG3'
hsa-miR-338-3p	GSP:5'GGGGGTCCAGCATCAGTGA3'
	R:5'GTGCGTGTCGTGGAGTCG3'
hsa-miR-15b-3p	GSP:5'GGGGCGAATCATTATTTGCT3'
	R:5' GTGCGTGTCGTGGAGTCG3'
hsa-miR-1296-5p	GSP:5'GGTATTAGGGCCCTGGCTC3'
	R:5'GTGCGTGTCGTGGAGTCG3'
hsa-miR-3168	GSP:5'TTGGGGGGGAGTTCTACAG3'
	R:5'GTGCGTGTCGTGGAGTCG3'
mmu-miR-199b-5p	GSP:5'GGGACCCCAGTGTTTAGACTAT3'
	R: 5'GTGCGTGTCGTGGAGTCG3'
mmu-miR-15b-3p	GSP:5'GGGGCGAATCATTATTTGCT3'
	R:5' GTGCGTGTCGTGGAGTCG3
mmu-miR-338-3p	GSP:5'GGGGGTCCAGCATCAGTGA3'
	R:5'GTGCGTGTCGTGGAGTCG3'
Gapdh	F: TGTTTCCTCGTCCCGTAGA
	R: ATCTCCACTTTGCCACTGC
Hif-1α	F: GGGGAGGACGATGAACATCAA
	R: GGGTGGTTTCTTGTACCCACA
Fzd6	F: AGAGGTGAAAGCGGACGGA
	R: AGAGAGTCTGGAGATGGATGCT
Gcnt2	F: TCCTGGACGGGTAACCTCAG
	R: CTGCAAGTCTCCGTTTCCATAG
Myh9	F: AGAAGTTGGTATGGGTGCCTT
	R: CCCTGAGTAGTATCGCTCCTTG
Caprin1	F: GAAGCAGATTCTCGGCGTAAT
	R: TCCCCTTTATTCATTCGTTCCTG
Atg14	F: GAGGGCCTTTACGTGGCTG
	R: AATAGACGAAATCACCGCTCTG
Mmp3	F: ACTGTGTCCCAAGGAGAGGAG
	R: AAACCATCTACACAGTTCAGACAC
Adamts5	F: ATGCAGCCATCCTGTTCACC
	R: AAGGCCAAGTAGATGCCCAATTT
Aggrecan	F: CACTGTCAAAGCACCATGCC
	R: TAGGCTGGCTCCCATTCAGT
Sox9	F: TAATTCCCCAGGCTCTTGGAT
	R: GCAGCCGGGATTTAAGGCTC
Col2a1	F: CACGCATGAGCCGAAGCTA
	R: GGGTTTCCACGTCTCACCA

Supplement table 1

Primer List

Baseline	KOA（N=15）	HC（N=10）	*P* value
Age	52.67 ± 7.35	51.50 ± 7.71	0.706
Height	1.63±0.07	1.60±0.05	0.201
Weight	65.03±9.53	61.30±8.47	0.327
BMI	24.28 ± 2.39	23.90± 2.65	0.715

Supplement table 2

Basic information for recruiting patients

References

1. Chen D, Shen J, Zhao W, et al. (2017) **Osteoarthritis: toward a comprehensive understanding of pathological mechanism** *Bone research* **5**

2. Hunter D, Osteoarthritis Bierma-Zeinstra S. (2019) **Lancet (London** *England* **393**:1745–1759

3. Ito Y, Matsuzaki T, Ayabe F, et al. (2021) **Both microRNA-455-5p and -3p repress hypoxia-inducible factor-2α expression and coordinately regulate cartilage homeostasis** *Nature communications* **12**

4. Xu W, Yang Z, Lu N (2016) **From pathogenesis to clinical application: insights into exosomes as transfer vectors in cancer** *Journal of Experimental & Clinical Cancer Research : CR* **35**

5. Ali S, Peffers M, Ormseth M, Jurisica I, Kapoor M (2021) **The non-coding RNA interactome in joint health and disease** *Nature reviews Rheumatology* **17**:692–705

6. Zhao R, Li Y, Lin Z, et al. (2016) **miR-199b-5p modulates BMSC osteogenesis via suppressing GSK-3β/β-catenin signaling pathway** *Biochemical and biophysical research communications* **477**:749–754

7. Yamashita S, Miyaki S, Kato Y, et al. (2012) **L-Sox5 and Sox6 proteins enhance chondrogenic miR-140 microRNA expression by strengthening dimeric Sox9 activity** *The Journal of biological chemistry* **287**:22206–22215

8. Miyaki S, Nakasa T, Otsuki S, et al. (2009) **MicroRNA-140 is expressed in differentiated human articular chondrocytes and modulates interleukin-1 responses** *Arthritis and rheumatism* **60**:2723–2730

9. Nakamura A, Rampersaud Y, Nakamura S, et al. (2019) **microRNA-181a-5p antisense oligonucleotides attenuate osteoarthritis in facet and knee joints** *Annals of the rheumatic diseases* **78**:111–121

10. Wu J, Yang F, Wu X, Liu X, Zheng D (2023) **Comparison of genome-wide DNA methylation patterns between antler precartilage and cartilage** *Molecular Genetics and Genomics : MGG* **298**:343–352

11. Yang X, Yip J, Harrison M, Brockhausen I (2008) **Primary human osteoblasts and bone cancer cells as models to study glycodynamics in bone** *The International Journal of Biochemistry & Cell Biology* **40**:471–483

12. Bartel DP (2004) **MicroRNAs: genomics, biogenesis, mechanism, and function** *Cell* **116**:281–297

13. Kalluri R, LeBleu V (2020) **The biology function and biomedical applications of exosomes** *Science (New York, NY).* **367**

14. Sun H, Hu S, Zhang Z, Lun J, Liao W, Zhang Z (2019) **Expression of exosomal microRNAs during chondrogenic differentiation of human bone mesenchymal stem cells** *Journal of Cellular Biochemistry* **120**:171–181

15. Ali SA, Peffers MJ, Ormseth MJ, Jurisica I, Kapoor M (2021) **The non-coding RNA interactome in joint health and disease** *Nature Reviews Rheumatology* **17**:692–705

16. Jiang Y, Zhang L, Tian H (2023) **MicroRNA-149 improves osteoarthritis via repression of VCAM-1 and inactivation of PI3K/AKT pathway** *Experimental Gerontology* **174**

17. Zhao Z, Zhao S, Luo L, et al. (2021) **miR-199b-5p-DDR1-ERK signalling axis suppresses prostate cancer metastasis via inhibiting epithelial-mesenchymal transition** *British Journal of Cancer* **124**:982–994

18. Zhang M, Yuan S, Sun H, Sun L, Zhou D, Yan J (2020) **miR-199b-5p promoted chondrogenic differentiation of C3H10T1/2 cells by regulating JAG1** *Journal of tissue engineering and regenerative medicine* **14**:1618–1629

19. Qu X, Chen Z, Fan D, et al. (2017) **MiR-199b-5p inhibits osteogenic differentiation in ligamentum flavum cells by targeting JAG1 and modulating the Notch signalling pathway** *Journal of cellular and molecular medicine* **21**:1159–1170

20. Huang J, Zhao L, Fan Y, et al. (2019) **The microRNAs miR-204 and miR-211 maintain joint homeostasis and protect against osteoarthritis progression** *Nature communications* **10**

21. Lu Y, Liu Y, Wan F, Shi S, Tao R (2022) **CircSmg5 stimulates the osteogenic differentiation of bone marrow mesenchymal stem cells by targeting the miR-194-5p/Fzd6 axis and beta-catenin signaling** *Environmental toxicology* **37**:593–602

22. Peng F, He Q, Cheng C, Pan J (2019) **GCNT2 induces epithelial-mesenchymal transition and promotes migration and invasion in esophageal squamous cell carcinoma cells** *Cell biochemistry and function* **37**:42–51

23. Sherman BT, Hao M, Qiu J, et al. (2022) **DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update)** *Nucleic Acids Research*

24. Chen Y, Wang X (2020) **miRDB: an online database for prediction of functional microRNA targets** *Nucleic acids research* **48**:D127–D131

25. Dweep H, Sticht C, Pandey P, Gretz N (2011) **miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes** *Journal of biomedical informatics* **44**:839–847

26. Li J, Liu S, Zhou H, Qu L, Yang J (2014) **starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data** *Nucleic acids research* **42**:D92–97

27. Karagkouni D, Paraskevopoulou M, Chatzopoulos S, et al. (2018) **DIANA-TarBase v8: a decade-long collection of experimentally supported miRNA-gene interactions** *Nucleic acids research* **46**:D239–D245

28. Agarwal V, Bell G, Nam J, Bartel D (2015) **Predicting effective microRNA target sites in mammalian mRNAs** *eLife* **4**

29. Pitcher T, Sousa-Valente J, Malcangio M (2016) **The Monoiodoacetate Model of Osteoarthritis Pain in the Mouse** *Journal of visualized experiments : JoVE* **111**

30. Choi W-S, Lee G, Song W-H, et al. (2019) **The CH25H-CYP7B1-RORα axis of cholesterol metabolism regulates osteoarthritis** *Nature* **566**:254–258

31. Cheah M, Fawcett J, Andrews M (2017) **Assessment of Thermal Pain Sensation in Rats and Mice Using the Hargreaves Test** *Bio-protocol* **7**

32. Glasson S, Chambers M, Van Den Berg W, Little C (2010) **The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the mouse** *Osteoarthritis and cartilage* :S17–23

Article and author information

Tong Feng
Acupuncture and Tuina College, Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan, 610075, China

Qi Zhang
Acupuncture and Tuina College, Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan, 610075, China, Chongqing Hospital of Traditional Chinese Medicine, Chongqing, 400011, China

Si-Hui Li
Acupuncture and Tuina College, Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan, 610075, China

Shuan-hu Zhou
Department of Orthopedic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, 02115, USA, Harvard Stem Cell Institute, Harvard University, Cambridge, Massachusetts, 02138, USA

Xin Wang
Departments of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, 02115, USA

Jun-Meng Wang
Acupuncture and Tuina College, Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan, 610075, China

Fan-Rong Liang
Acupuncture and Tuina College, Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan, 610075, China

Shu-Guang Yu
Acupuncture and Tuina College, Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan, 610075, China, Acupuncture & Chronobiology Key Laboratory of Sichuan Province, Chengdu, Sichuan, 610075, China
For correspondence: ysg@cdutcm.edu.cn
ORCID iD: 0000-0002-1413-8989

Qiao-Feng Wu
Acupuncture and Tuina College, Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan, 610075, China, Acupuncture & Chronobiology Key Laboratory of Sichuan Province, Chengdu, Sichuan, 610075, China
For correspondence: wuqiaofeng@cdutcm.edu.cn
ORCID iD: 0000-0002-8308-2843

Copyright

Editors

Reviewing Editor
Yousef Abu-Amer
Washington University in St. Louis, United States of America

Senior Editor
Hiroshi Takayanagi
The University of Tokyo, Japan

Reviewer #1 (Public Review):

Summary:

In this manuscript, the authors observed that miR-199b-5p is elevated in osteoarthritis (OA) patients. They also found that overexpression of miR-199b-5p induced OA-like pathological changes in normal mice and inhibiting miR-199b-5p alleviated symptoms in knee OA mice. They concluded that miR-199b-5p is not only a potential micro-target for knee OA but also provides a potential strategy for the future identification of new molecular drugs.

Strengths:

The data are generated from both human patients and animal models.

Weaknesses:

The data presented in this manuscript is not solid enough to support their conclusions. There are several questions that need to be addressed to improve the quality of this study.

The following questions that need to be addressed to improve the quality of the study.

1. Exosomes were characterized by electron microscopy and western blot analysis (for CD9, 264 CD63, and CD81). However, figure S1 only showed two sample WB results and there is no positive and negative control as well as the confused not clear WB figure.

2. The sequencing of miRNAs in serum exosomes showed that 88 miRNAs were upregulated and 89 miRNAs were downregulated in KOA patients compared with the control group based on fold change > 1.5 and $p < 0.05$. Figure 2 legend did not clearly elucidate what those represent and why the authors chose those five miRNAs to further validate although they did mention it with several words in line 108 'based on the p-value and exosomal'.

3. In Figure 3 legend and methods, the authors did not mention how they performed the cell viability assay. What cell had been used? How long were they treated and all the details? Other figure legends have the same problem without detailed information.

4. The authors claimed that Gcnt2 and Fzd6 are two target genes of miR-199b-5p. However, there is no convincing evidence such as western blot to support their bioinformatics prediction.

5. To verify the binding site on 3'UTR of two potential targets, the authors designed a mouse sequence for luciferase assay, but not sure if it is the same when using a human sequence.

Reviewer #2 (Public Review):

Summary:

The authors identified miR-199b-5p as a potential OA target gene using serum exosomal small RNA-seq from human healthy and OA patients. Their RNA-seq results were further compared with publicly available datasets to validate their finding of miR-199b-5p. In vitro chondrocyte culture with miR-199b-5p mimic/inhibitor and in vivo animal models were used to evaluate the function of miR-199b-5p in OA. The possible genes that were potentially regulated by miR-199b-5p were also predicted (i.e., Fzd6 and Gcnt2) and then validated by using Luciferase assays.

Strengths:

1. Strong in vivo animal models including pain tests.
2. Validates the binding of miR-199b-5p with Fzd6 and binding of miR-199b-5p with Gcnt2.

Weaknesses:

1. The authors may overinterpret their results. The current work shows the possible bindings between miR-199b-5p and Fzd6 as well as bindings between miR-199b-5p and Gcnt2. However, whether miR-199b-5p truly functions through Fzd6 and/or Gcnt2 requires genetic knockdown of Fzd6 and Gcnt2 in the presence of miR-199b-5p.
2. In vitro chondrocyte experiments were conducted in a 2D manner, which led to chondrocyte de-differentiation and thus may not represent the chondrocyte response to the treatments.
3. There is a lack of description for bioinformatic analysis.
4. There are several errors in figure labeling.