SCIENTIFIC ARTICLE

Differentiation Antagonizing Non-protein Coding RNA Knockdown Alleviates Lipopolysaccharide-Induced Inflammatory Injury and Apoptosis in Human Chondrocyte Primary Chondrocyte Cells Through Upregulating miRNA-19a-3p

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Objective: To confirm the role of long noncoding RNA differentiation antagonizing non-protein coding RNA (DANCR) in chondrocyte inflammatory injury in osteoarthritis (OA) in vitro, as well as its molecular mechanism.

Methods: Human primary chondrocytes were treated with lipopolysaccharide (LPS) to construct a chondrocyte inflammatory injury in human OA cell model. Gene expression was detected using real-time quantitative polymerase chain reaction. Cell inflammatory injury was evaluated by Cell Counting Kit-8 assay, flow cytometry, and enzyme-linked immunosorbent assay. The interplay between miRNA-19a-3p (miR-19a) and DANCR was validated by dual-luciferase reporter assay and RNA immunoprecipitation.

Results: Expression of DANCR was upregulated, and miR-19a was downregulated in human OA cartilage and LPS-treated primary chondrocytes in vitro. Moreover, DANCR expression was inversely correlated with miR-19a in OA patients. LPS reduced cell viability and increased the apoptotic rate and secretion of interleukin (IL)-1β, IL-6, IL-8, as well as tumor necrosis factor (TNF)-α in primary chondrocyte cells in vitro, suggesting an inflammatory injury model of OA. Functionally, knockdown of DANCR could attenuate LPS-induced apoptosis and inflammatory response, as evidenced by improved cell viability, and reduced apoptotic rate and products of IL-1β, IL-6, IL-8, and TNF-α. Notably, DANCR negatively regulated miR-19a expression, presumably via sponging. Furthermore, miR-19a deletion eliminated the effect of DANCR knockdown on apoptosis and the inflammatory response of primary chondrocytes under LPS stress.

Conclusion: Differentiation antagonizing non-protein coding RNA silencing could protect human chondrocyte cells against LPS-induced inflammatory injury and apoptosis through targeting miR-19a, suggesting a vital role of the DANCR/miR-19a axis in OA.

Key words: DANCR; miR-19a; Lipopolysaccharide; Inflammatory injury; Osteoarthritis

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**Introduction**

Osteoarthritis (OA) is the most common chronic, degenerative joint disease. It causes inflammation in the joints, leading to pain and difficulty of movement. OA is a classic age-related disease and is a major problem in the elderly. Articular chondrocytes are the only cell type present in articular cartilage. However, chondrocytes could regulate the fate of articular cartilage and affect the chondrocyte metabolism, thus leading to destruction of the articular cartilage tissue. Therefore, articular chondrocyte abnormality is an important cause of OA, even though chondrocytes comprise no more than 3% of the total cartilage volume. Significant correlation between chondrocyte apoptosis and OA grade has been reported. In addition, inflammatory cytokines, including tumor necrosis factors (TNF) and interleukins (IL), could be secreted by chondrocytes after stimulation, further promoting the apoptosis of chondrocytes. Thus, it is important to investigate the chondrocyte inflammatory response and apoptosis to better understand the pathogenesis of OA.

Recent studies have demonstrated that extracellular genomic material, such as long noncoding RNA (lncRNA) and microRNA (miRNA) could serve as biomarkers of OA. LncRNA are RNA transcripts longer than 200 nucleotides, and lncRNA are expressed in inflammation-related diseases, including OA. Cen et al. demonstrated that IncRNA take part in the survival of chondrocytes and synoviocytes, arthritis-associated factors, and angiogenesis, thus indicating the potential role of lncRNA in diagnosis, therapy, and prognosis of OA. Several IncRNA have been suggested to participate in the onset and progression of OA, such as PMS2L2, PCGEM1, CIR, and THRIL.

Differentiation antagonizing non-protein coding RNA (DANCR) is an oncogenic IncRNA in cancers. Accumulating evidence has proven that DANCR could enhance cancer stemness features and facilitate tumor progression. Alteration of IncRNA is involved in regulating chondrogenesis and osteogenesis, and eventually affects the homeostasis of cartilage and bone. Xiao et al. identified that DANCR was highly expressed in knee cartilage from patients with OA. Recently, the functional role of DANCR in OA chondrocytes has been reported. However, the influence of DANCR on the progression of OA chondrocytes remains to be fully illuminated.

**Materials and Methods**

**Clinic Cartilage Tissue Specimens**

A total of 25 patients with OA and 12 patients with femoral neck fractures from the Wanging Hospital, Chinese Academy of Traditional Chinese Medicine were included in this study. Written informed consent was obtained from each patient. The OA cartilage and normal cartilage were isolated from the knee joints during elective joint replacement surgery. The samples were stored for cryopreservation for further total RNA extraction or cell isolation. OA was diagnosed according to the clinical and radiological evaluation criteria published by the American College of Rheumatology. The control patients did not have OA, rheumatic arthritis, or signs of cartilage degeneration. This study was performed after receiving approval from the ethics committee of the Wangling Hospital, Chinese Academy of Traditional Chinese Medicine.

**Cell Culture**

Human chondrocyte cells were isolated from this cohort of normal cartilage specimens \( n = 12 \). Minced cartilage fragments \( (2-3 \text{ mm}^3) \) were digested in 2 mg/mL of Collagenase IV for 12 h at 37 °C, and cultured in Dulbecco’s modified eagle’s medium (DMEM; Gibco, Life Technologies, Carlsbad, CA, USA). The complete medium consists of DMEM, 10% heat-inactivated fetal bovine serum (Gibco), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The cells between passages 1 and 3 were collected for further total RNA extraction with and without LPS treatment.

**Lipopolysaccharide Stimulation**

Lipopolysaccharide was purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock concentration of LPS was 5 mg/mL in ultrapure water, in accordance with the instructions. The working concentrations of LPS were 2.5, 5, and 7.5 μg/mL diluted in DMEM, and human primary chondrocytes were incubated with LPS for 12 h. Treatment of DMEM without LPS \( (0 \mu g/mL) \) was performed as control.

**Cell Viability Assay**

The viability of primary chondrocytes was determined by Cell Counting Kit-8 (CCK-8; Beyotime Biotechnology, Shanghai, China). In brief, after LPS stimulation or no stimulation, the primary chondrocytes were reseeded into 96-well plates (Corning, NY, USA) at a density of \( 1 \times 10^3 \) cells/well in normal medium for 24 h. Then, 10 μL of CCK-8 solution was added to each well for another 2 h. The cell viability was reflected by optical density (OD) measured by a microplate reader at 450 nm, comparing to control cells without LPS treatment (i.e. relative cell viability): \( (%) = 100 \times \frac{\text{OD}_{\text{experiment}}}{{\text{OD}_{\text{control}}} \times \text{OD}_{\text{group}}} \). The cell groups were in triplicate.

**Flow Cytometry for Apoptotic Rate**

Lipopolysaccharide-induced chondrocytes were seeded into 6-well plates (Corning) with \( 1 \times 10^5 \) cells per well for 24 h...
and analyzed using an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (Beyotime) on flow cytometry. In short, after LPS stimulation or no stimulation, the adherent cells were harvested, washed, and stained in FITC-annexin V and PI for 1 h in the dark. Then, the fluorescence was analyzed on a flow cytometer (Beckman-Coulter Electronics, Jiangsu, China). Apoptotic cells were sorted in annexin V+/PI− and au8 nxenix V+/PI+ quadrants, and the apoptotic rate was calculated using the following equation: apoptotic rate (%) = 100% × apoptotic cells/total cells.

**Enzyme-Linked Immunosorbent Assay**

The primary chondrocyte cells were seeded into 24-well plates (Corning) for 24 h. After LPS stimulation, the culture supernatant was collected for estimation of inflammatory factors using commercial enzyme-linked immunosorbent assay (ELISA) kits in accordance with the manufacturer’s instructions. The ELISA kits, including a human IL-1β kit (ab100562), a human IL-6 kit (ab46027), a human IL-8 kit (ab46032), and a human TNF-α kit (ab46087), were purchased from Abcam (Cambridge, UK). The OD values at 450 nm were read, and the levels of IL-1β, IL-6, IL-8, and TNF-α were determined according to a representative standard curve of OD/quantity (pg/mL).

**Real-Time Quantitative Polymerase Chain Reaction**

Total RNA from cartilage tissues and LPS-treated primary chondrocytes was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). A reverse transcription kit (Abcam) and the SYBR Green Master Mix Kit (Qiagen, Hilden, Germany) were used to amplify special gene expression on the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The expression level of DANCR and miR-19a were calculated using comparative threshold cycle value (2−ΔΔCT) method, compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 small nuclear RNA (U6), respectively. Polymerase chain reaction (PCR) primers were as follows: DANCR: 17 5'-GGCCGACTATGTAGCGGGTT-3' (sense) and 5'-TCAATGGCTTGTGCTGTAGTT-3' (antisense); U6: 5'-CTCGCTTGGGACGAC-3' (sense) and 5'-AAGCCTTCAAGATGTGGC-3' (antisense); GAPDH: 5'-AAGAAGGTGTTGACAGGAC-3' (sense) and 5'-CTCACCACCTCTTTGTCGTA-3' (antisense). The primers for miRNA-19a-3p (miR-19a) were purchased from Qiagen. All experiments were performed in at least three wells, and relative gene expression was normalized to control groups (as unit 1).

**Cell Transfection**

The primary chondrocyte cells were seeded into 6-well plates (Corning) at a density of 5 × 10^4/well prior to transfection for 24 h. DANCR siRNA (si-DANCR), miR-19a mimic, and anti-miR-19a were purchased from Genepharma (Shanghai, China), as well as the negative controls, including si-NC, miR-NC mimic, and anti-miR-NC. Cell transfection of oligonucleotides was performed using Lipofectamine 3000 reagent (Invitrogen) for 24 h prior to further study.

**Dual-Luciferase Reporter Assay**

According to the prediction of StarBase software, the potential binding site of hsa-miR-19a on DANCR was cloned by PCR method into Fluc reporter plasmid (pMiR-report miRNA vector; Promega, Madison, WI, USA), defined as wild type of DANCR (DANCR-WT). Similarly, the mutant type of this putative sequence was inserted into the pMiR vector, named DANCR-MUT. The primary chondrocyte cells in 24-well plates (Corning) at 1 × 10^4 cells/well were co-transfected with 20 nM of miR-19a/NC mimic and 20 ng of DANCR-WT or DANCR-MUT for 48 h, as well as 20 pM of Rlu reporter plasmid (pRL-TK; Promega). The relative luciferase was measured using the Dual-Luciferase Reporter Assay System (Promega). The transfection was repeated in triplicate. Fluc luciferase activity was measured with normalization to Rlu (internal control), and relative luciferase activity was further normalized to control groups (as unit 1).

**RNA Immunoprecipitation**

RNA immunoprecipitation (RIP) was performed with cell extract of primary chondrocytes after transfection with miR-19a/NC mimic, and cell extract was collected. A Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) was chosen to detect expression of DANCR in the samples bound to magnetic beads conjugated with Ago2 or IgG antibody. The RNA-protein complex was digested with Proteinase K prior to total RNA isolation and real-time quantitative PCR (RT-qPCR) assay. The relative enrichment of DANCR was compared to total input and then normalized to the control group (miR-NC transfection chondrocytes, as unit 1).

**Spearman’s Rank Correlation Analysis**

The bivariate correlations between DANCR and miR-19a expression in this cohort of OA tissues (n = 25) were determined using Spearman’s rank correlation analysis, because this test is suitable for the relatively small sample size. The regression coefficient (R) represented the strength of a monotonic relationship between two paired variables. The P-value (two-tailed) was measured.

**Statistical Analysis**

Data is presented as mean ± standard deviation for three separate experiments. Student’s t-test was used to compare the significance between two groups on GraphPad Prism 6.0 (GraphPad Software, La Jolla, USA). P-value <0.05 was statistically significant.
Results

Differentiation Antagonizing Non-Protein Coding RNA Expression was Upregulated in Human Osteoarthritis Cartilage and Lipopolysaccharide-Treated Chondrocytes

To investigate the role of DANCR in chondrocyte injury during OA, DANCR expression was measured in cartilage tissues from 25 OA patients and 12 control people. Consistently, there was a significant upregulation of DANCR in OA cartilage (Fig. 1A), highlighting its pivotal role in OA development.

In vitro, DANCR was aberrantly expressed in a model of OA in human primary chondrocyte cells after LPS (2.5–7.5 μg/mL) treatment for 12 h (Fig. 1B and Fig. S1). As shown in Fig. 1B, LPS obviously upregulated expression of DANCR in a dose-dependent manner. These results indicated the potential role of DANCR in LPS-induced inflammatory injury in primary chondrocytes.

Lipopolysaccharide Could Induce Chondrocyte Inflammatory Injury and Apoptosis in Primary Chondrocyte Cells

First, the effects of LPS in human chondrocytes were determined in vitro. Increasing concentrations of LPS were used to stimulate primary chondrocyte cells for 12 h. Cell viability was significantly reduced by LPS stimulation (by 2.5–7.5 μg/mL), paralleled with control cells (Fig. 2A). Moreover, 5 μg/mL LPS was used for subsequent experiments. As shown in Fig. 2B, apoptotic cells in primary chondrocytes were dramatically increased under LPS treatment. Meanwhile, expression of pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α was markedly higher in primary chondrocytes when treated with LPS (Fig. 2C–F). These data illustrated that LPS could evoke apoptosis and inflammatory injury in human chondrocyte cells (Fig. S1), suggesting the success of the cell model of OA.

Knockdown of Differentiation Antagonizing Non-Protein Coding RNA Attenuated Lipopolysaccharide-Induced Chondrocyte Inflammatory Injury and Apoptosis in vitro

Next, we analyzed the effect of DANCR on LPS-treated chondrocyte cells. DANCR was forcedly low expressed in the primary chondrocyte cells by transfection with si-DANCR, and subsequently exposed to 5 μg/mL LPS for 12 h (Fig. 3A). Figure 3B,C shows that si-DANCR transfection significantly reduced the cell viability inhibition and apoptotic rate during LPS treatment. Meanwhile, LPS-mediated upregulated expression of IL-1β, IL-6, IL-8, and TNF-α was attenuated with DANCR knockdown (Fig. 3D–G). These results showed the suppressive effect of DANCR silencing on LPS-induced chondrocyte inflammatory injury in human primary chondrocytes (Fig. S1).

Differentiation Antagonizing Non-Protein Coding RNA Negatively Regulated the Expression of miRNA-19a-3p through Sponging

In this study, we observed a putative miR-19a target site in DANCR by computational predictions on StarBase software (http://starbase/miRNA&lncRNA/=DANCR/=hsa-miR-19a-3p). As shown in Fig. 4A, the potential binding site of miR-19a on the 3′-UTR of DANCR wild type (DANCR-WT) was validated with a dual-luciferase reporter assay. Relative luciferase activity of DANCR-WT was remarkably declined in the presence of miR-19a mimic compared to miR-NC mimic (Fig. 4B); however, there was no difference in the DANCR-MUT group. Furthermore, RIP assay identified this binding in primary chondrocyte cells. Expression levels of DANCR from RIP-Ago2 were largely enriched after transfection with miR-19a, normalized by miR-NC (Fig. 4C). Subsequently, the regulatory effect of DANCR on miR-19a expression was detected. The miR-19a expression level was reduced when transfected with pcDNA-DANCR and increased when transfected with si-DANCR (Fig. 4D). These data suggested DANCR as a sponge for miR-19a via target binding.

Fig. 1 Expression of differentiation antagonizing non-protein coding RNA (DANCR) in osteoarthritis (OA). Levels of DANCR were detected by real-time quantitative polymerase chain reaction in (A) cartilage of patients with OA and healthy people and in (B) 0, 2.5, 5, and 7.5 μg/mL lipopolysaccharide-treated primary chondrocytes at 12 h. *P < 0.05; ***P < 0.001.
Fig. 2 Effects of lipopolysaccharide in human chondrocytes in vitro. (A) Cell proliferation was measured by CCK-8 assay after treatment with 0, 2.5, 5, and 7.5 μg/mL LPS for 12 h. (B–F) Primary chondrocyte cells were treated with 5 μg/mL LPS for 12 h. (B) Apoptotic cells were determined by flow cytometry. (C–F) Expression of IL-1β, IL-6, IL-8, and TNF-α was measured by ELISA. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 3 Role of differentiation antagonizing non-protein coding RNA (DANCR) in lipopolysaccharide-induced inflammatory injury in chondrocytes. The primary chondrocytes were transfected with si-DANCR or si-NC, followed by treatment of 5 μg/mL LPS for 12 h. (A) DANCR expression level was examined by real-time quantitative polymerase chain reaction. (B) Cell proliferation was measured by Cell Counting Kit-8 assay. (C) Apoptotic cells were determined by flow cytometry. (D–G) Expression of IL-1β, IL-6, IL-8, and TNF-α was measured by ELISA. *P < 0.05; **P < 0.01; ***P < 0.001.
miRNA-19a-3p was Downregulated in Human Osteoarthritis Cartilage and Lipopolysaccharide-Treated Chondrocytes

Expression of miR-19a was also measured by RT-qPCR in inflammatory injuries both in vivo and in vitro. In contrast to DANCR, the levels of miR-19a were downregulated in OA cartilage (Fig. 5A) and LPS-induced primary chondrocytes (Fig. 5B and Fig. S1). Furthermore, there was a moderate negative correlation between DANCR and miR-19a expression in OA tissues ($R = -0.6251$, $P = 0.0008$; Fig. 5C). These outcomes demonstrated the potential effect of miR-19a in OA development and LPS-induced chondrocyte inflammatory injury.
Deletion of miRNA-19a-3p Abolished the Suppressive Effect of Differentiation Antagonizing Non-Protein Coding RNA Knockdown on lipopolysaccharide-induced Chondrocyte Inflammatory Injury In Vitro

To explore the influence of miR-19a on DANCR knockdown-mediated effects, anti-miR-19a was transfected into primary chondrocyte cells. According to the RT-qPCR results, the expression level of miR-19a in primary chondrocytes was increased with the transfection of si-DANCR and decreased with the transfection of anti-miR-19a (Fig. 6A). Furthermore, miR-19a deletion significantly blocked the promoted cell viability when DANCR was inhibited (Fig. 6B). Simultaneously, the reduced apoptotic rate was reversed in the presence of si-DANCR and anti-miR-19a (Fig. 6C). We observed that secretions of IL-1β, IL-6, IL-8, and TNF-α were relieved in primary chondrocytes with DANCR silencing, which could be abolished by downregulation of its target gene miR-19a (Fig. 6D–G). The above results showed that DANCR knockdown could protect LPS-induced chondrocyte inflammatory injury in primary chondrocyte cells through upregulating miR-19a (Fig. S1).

Discussion

Low-grade inflammation has been demonstrated to be linked to OA progression28. LPS induces inflammatory response in vitro and in vivo27, and LPS-induced OA cell models have been widely used to detect new therapeutic targets and medicines. In the present study, LPS was used to mediate inflammatory injury in chondrocyte primary chondrocyte cells to mimic an in vitro model of OA. As a result, 2.5–7.5 μg/mL LPS notably reduced the viability of primary chondrocyte cells and induced apoptosis and secretion of pro-inflammatory cytokines. Expression of DANCR was significantly upregulated in OA cartilage and LPS-induced primary chondrocyte cells. Moreover, knockdown of DANCR could protect primary chondrocyte cells against LPS-induced injury, as evidenced by the increase in cell viability and the decrease of apoptosis and expression of IL-1β, IL-6, IL-8, and TNF-α. More importantly, DANCR functioned as a sponge for miR-19a, and silencing of miR-19a eliminated the protective effect of DANCR downregulation under LPS stimulation.

In cancer, DANCR is an oncogenic gene and is upregulated in numerous human cancers through serving as a miRNA sponge. For example, DANCR enhanced cell proliferation and metastasis in non-small cell lung cancer29, colorectal cancer30, and cervical cancer31 through sponging miR-138, miR-577, and miR-665. Furthermore, cancer stemness features were promoted by upregulated DANCR in osteosarcoma via targeting miR-33a-5p, thus suggesting that DANCR was a crucial tumor-promoting gene of osteosarcoma, as well as an independent prognosis predictor16. In bone disease, Zhang
et al. showed that DANC\(_R\) was linked to the proliferation and osteogenic differentiation of human bone-derived marrow mesenchymal stem cells. Similarly, DANC\(_R\) increased Smad3 and STAT3 expression to positively regulate human synovium-derived stem cell proliferation and chondrogenesis. Xiao et al. found 96 novel differently expressed lncRNA in OA, compared with previous lncRNA expression profiles. Among them, four differently expressed lncRNA (DANC\(_R\), SNHG5, ZFAS1, and GAS5) were analyzed using protein–protein interactions and ceRNA regulatory networks. Consistent with lncRNA sequencing, DANC\(_R\) was indicated to be significantly upregulated in cartilage tissues from OA patients. Moreover, inhibition of DANC\(_R\) induced apoptosis and suppressed cell proliferation and expression of IL-6 and IL-8 on mRNA level in primary chondrocytes, which were isolated from knee joints of OA patients. In this study, we observed a higher expression of DANC\(_R\) in OA cartilage and LPS-induced chondrocytes in primary chondrocyte cells. Functionally, DANC\(_R\) knockdown protected primary chondrocyte cells from LPS-induced reduced cell viability, and elevated apoptosis rate and secretion of IL-1\(\beta\), IL-6, IL-8, and TNF-\(\alpha\) by targeting miR-19a. Notably, to the best of our knowledge, this is the first report to discuss the role of DANC\(_R\) in LPS-induced inflammation. However, the downstream target gene(s) of DANC\(_R\)/miR-19a remains to be uncovered, as well as the signaling pathways, such as JAK2/STAT3, NF-\(\alpha\)B, and TLR pathways.

Emerging studies have shown that miR-19a is closely linked to inflammation. For example, Singh et al. established miR-19a as important regulators of type 2 innate lymphoid cell (ILC2) homeostasis and function in allergic inflammation. In that research, miR-19a promoted IL-13 and IL-5 production and inhibited SOCS1 and A20 expression. Anti-inflammatory effects of miR-19a in LPS-induced endometritis was demonstrated by Yin et al. and miR-19a suppressed the expression of TNF-\(\alpha\), IL-6, IL-1\(\alpha\) and the phosphorylation of NF-\(\alpha\)B p65 and IkB\(\alpha\). In rheumatoid arthritis (RA), data from Philippe et al. pointed to an important role for miR-19a/b in the regulation of RA inflammation in fibroblast-like synovioocytes (FLS). In parallel with TLR2 expression, IL-6 and MMP-3 release was inhibited in FLS when transfected with either mimic. Overexpressed miR-19a promoted cell viability and migration of a human chondrocyte line and primary human chondrocytes, while suppressed miR-19a exhibited the opposite effects. Furthermore, the potential regulation mechanism of miR-19a in chondrocytes was discovered through upregulating SOX9 expression and promoting activation of the NF-\(\alpha\)B signaling pathway. In this study, we illustrated the lower expression of miR-19a in cartilage tissues from OA patients and LPS-induced chondrocytes in primary chondrocyte cells. Its downregulation resulted in cell viability inhibition, apoptosis, and secretion of IL-1\(\beta\), IL-6, IL-8, and TNF-\(\alpha\). miR-19a was determined to be sponged by DANC\(_R\) during OA. However, the impact of this miRNA on MMP expression and signaling pathways is unclear, and this should be further explored.

**Conclusion**

In conclusion, DANC\(_R\) expression was upregulated in OA, accompanied by downregulated miR-19a. Silencing of DANC\(_R\) could relieve LPS-induced chondrocyte inflammatory injury in primary chondrocyte cells through sponging and inhibiting miR-19a (Fig. S1). Our results provide novel evidence for DANC\(_R\) and miR-19a as potential biomarkers and targets for the treatment of OA.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article on the publisher’s web-site:

**Fig. S1** The flow chart of DANC\(_R\)/miR-19a axis in human chondrocytes in OA.

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