m\(^6\)A-mediated upregulation of AC008 promotes osteoarthritis progression through the miR-328-3p–AQP1/ANKH axis

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Long noncoding RNAs (lncRNAs) have emerged as important regulators of osteoarthritis (OA), but the biological roles and clinical significance of most lncRNAs in OA are not fully understood. Microarray analysis was performed to identify differentially expressed lncRNAs, mRNAs, and miRNAs between normal and osteoarthritic cartilage. We found that AC00840.5 (abbreviated AC008), as well as AQP1 and ANKH, were highly expressed in osteoarthritic cartilage, whereas miR-328-3p was expressed at a low level in osteoarthritic cartilage. Functional assays showed that ectopic expression of AC008, AQP1, and ANKH significantly decreased chondrocyte viability and promoted chondrocyte apoptosis and extracellular matrix (ECM) degradation, whereas knockdown of AC008, AQP1, and ANKH resulted in the opposite effects. Moreover, miR-328-3p overexpression increased chondrocyte viability and attenuated chondrocyte apoptosis and ECM degradation, whereas inhibition of miR-328-3p resulted in the opposite effects.

Bioinformatics analysis, RNA immunoprecipitation (RIP), and luciferase assays revealed that AC008 functioned as a competing endogenous RNA (ceRNA) to regulate miR-328-3p, which specifically targeted the AQP1 and ANKH genes. In addition, miR-328-3p significantly ameliorated MIA-induced OA, whereas AC008 accelerated OA progression in vivo. Furthermore, fat mass and obesity-associated (FTO)-mediated N6-methyladenosine demethylation downregulated AC008 transcription, while lower FTO expression led to upregulation of AC008 transcription in OA. In conclusion, our data reveal that AC008 plays a critical role in OA pathogenesis via the miR-328-3p–AQP1/ANKH pathway, suggesting that AC008 may be a potential therapeutic target for OA.

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INTRODUCTION

Osteoarthritis (OA) is an age-related or posttraumatic degenerative joint disease\(^1\) that is characterized by extracellular matrix (ECM) degradation, chondrocyte hypertrophy, and apoptosis\(^2–4\). Patients with OA experience many problems, such as joint pain, temporary stiffness, periarticular tenderness, swelling, and limited joint function, which may lead to difficulty with activities of daily living\(^5\). Current treatments for OA are limited to pain suppression and alleviation of inflammation, but these measures do not modify disease progression\(^6\). Therefore, a better knowledge of the underlying mechanisms that underlie the development and progression of OA is needed.

Noncoding RNAs are a class of RNA molecules that lack protein-coding potential. Based on the nucleotide sequence length, noncoding RNAs are classified as small noncoding RNAs (18–200 nt) and long noncoding RNAs (lncRNAs, >200 nt)\(^7,8\). MicroRNAs (miRNAs), a class of small noncoding RNAs ~22 nucleotides in length, negatively regulate target gene expression either by inhibiting mRNA translation or by promoting mRNA degradation\(^9\). miRNAs have been well recognized to play important roles in OA pathogenesis. For example, two homologous miRNAs, miR-204 and miR-211, maintain joint homeostasis to suppress OA progression by targeting RUNX2\(^10\), miR-141/200c promotes OA development by targeting SIRT1, thereby activating the IL-6/STAT3 pathway\(^11\). A growing body of evidence indicates that dysregulation of lncRNAs is involved in the progression of OA by affecting various essential cellular features of chondrocytes, such as their proliferation, apoptosis, inflammation, and ability to contribute to ECM degradation\(^12,13\). For example, HOTAIR aggravates ECM degradation and chondrocyte apoptosis by sponging miR-17-5p and upregulating FUT2 expression\(^14\). LncRNA-XIST promotes OA progression by regulating chondrocyte viability, chondrocyte apoptosis, and ECM degradation. Mechanistically, LncRNA-XIST functions as a competing endogenous RNA (ceRNA) by competing for binding with miR-149-5p to regulate DMR1/3 expression\(^15\). LncRNA-CRNDE regulates chondrogenic differentiation of bone marrow mesenchymal stem cells and promotes cartilage repair in OA by binding with SIRT1, thus increasing its protein stability\(^16\). However, due to the large number of lncRNAs and their complex mechanisms of action, studies on lncRNAs in OA are still very limited.

In the present study, we performed microarray analysis to identify differentially expressed lncRNAs, mRNAs, and miRNAs between normal and osteoarthritic cartilage. We identified a novel...
In conclusion, our study elucidated that AC008 plays a critical role in OA pathogenesis via the miR-328-3p–AQP1/ANKH pathway and suggested that AC008 may be a promising therapeutic target for OA.

MATERIALS AND METHODS

Microarray analysis

Total RNAs isolated from three normal cartilage samples and three osteoarthritic cartilage samples were sent to CapitalBio Corporation (Beijing, China) for microarray analysis. CapitalBio Technology Human LncRNA Array V4 containing probes for approximately 41,000 human IncRNAs and approximately 34,000 human mRNAs, as well as an Affymetrix miRNA 4.0 Array containing probes for 30,424 miRNAs from various species, including humans, mice, and rats, were simultaneously used to identify the differentially expressed IncRNAs, mRNAs and miRNAs between normal and osteoarthritic cartilage. Genes with P < 0.05 and a fold change ≥ 2 or ≤ 0.5 were considered to be significantly differentially expressed.

Clinical samples

Human osteoarthritic cartilage was collected from OA patients undergoing total knee replacement surgery (n = 39, aged 49–83 years). Normal cartilage was obtained from patients with no prior medical history of OA undergoing amputation or trauma surgery (n = 39, aged 45–91 years). The cartilage donor information is listed in Supplementary Table 1. The study was approved by the Research Ethics Committee of Nanjing Medical University. All participants read and signed an informed consent form.

Cell lines and cell culture

Primary human articular chondrocytes were isolated from normal articular cartilage, as previously described. In brief, articular cartilage was dissected into small sections and was then digested with 0.2% type II collagenase (Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco’s modified Eagle’s medium/nutrient mixture F12 (DMEM/F12) (Gibco, Carlsbad, CA, USA) at 37 °C for 16 h. The chondrocytes were then filtered through a 70 µm cell strainer and maintained in DMEM/F12 containing 10% fetal bovine serum (Gibco) in an incubator at 37 °C with 5% CO2. Primary chondrocytes at passage 2 were used for subsequent experiments. HEK293T cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum in a humidified atmosphere with 3% CO2 at 37 °C.

Plasmid construction, RNAi, and cell transfection

The AC008 overexpression plasmid was constructed by inserting the full-length AC008 sequence into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). The AQP1, ANKH, and FTO overexpression plasmids and the empty vector were purchased from Sino Biological Inc. (Beijing, China). RiboBio (Guangzhou, China) chemically synthesized the miR-328-3p mimic, miR-328-3p inhibitor, and corresponding negative controls (Mimic-NC, Inhibitor-NC); the small interfering RNAs (siRNAs) against AQP1 and ANKH (siAQP1, siANKH) and negative control siRNA (siNC); and the antisense oligonucleotide (ASO) targeting AC008 (AC008 ASO) and negative control ASO (Control ASO). For the luciferase reporter assay, the wild-type AC008 sequence and the mutant AC008 sequence (in which only the putative miR-328-3p binding site was mutated) were synthesized and cloned into the psiCHECK-2 vector (Promega, Madison, WI, USA). The AQP1 and ANKH 3’UTRs and their respective mutant sequences (in which only the predicted miR-328-3p binding sites were mutated) were amplified and inserted into the pGL3-Promoter vector (Promega). Supplementary Table 2 lists the primers used. Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s instructions.

Total RNA isolation and quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA from articular cartilage and cultured cells was isolated using TRIzol reagent (Takara, Otsu, Japan). Complementary DNA was synthesized from 1 µg of total RNA using HiScript II Q Select RT SuperMix (Vazyme Biotech, Nanjing, China). The expression levels of mature miRNAs were detected by stem-loop qRT-PCR. First, the stem-loop RT primer bound to miRNA molecules and cDNA was synthesized with reverse transcriptase. Then, the RT products were amplified using a miR-328-3p-specific forward primer and a universal reverse primer. Real-time PCR was performed using FastStart Universal SYBR Green Master Mix (Roche, Indianapolis, IN, USA) in a Roche LightCycler 96 Real-Time PCR System. U6 RNA was used as an endogenous reference for miR-328-3p, and β-actin mRNA was used as an internal reference for other genes. All primers used for PCR amplification are listed in Supplementary Tables 3–5.

Western blotting analysis

Cells were washed with phosphate-buffered saline (PBS) and harvested in radiolabeled protein isotope assay buffer (Beyotime, Haimen, China) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Equal amounts of protein were loaded on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel for electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was probed with specific antibodies overnight at 4 °C and then incubated with secondary antibodies at room temperature for 1 h. Each protein band was visualized with an ECL reagent (Millipore). Primary antibodies used for the following proteins were used: Bax, Bcl-2, cleaved Caspase 3, cleaved PARP1, and Survivin (all from Proteintech Group, Wuhan, China), cleaved Caspase 3 (Affinity, Cincinnati, OH, USA), ANKH (A8clonal, Wuhan, China), AQP1, β-actin, MMP13, COL2A1, and ADAMTS-5 (all from Bioss, Beijing, China).

Cell counting kit-8 (CCK-8) assay

Cell viability was assessed using a CCK-8 assay (Yeasen, Shanghai, China). Cells were seeded in 96-well plates at an initial density of 4 × 103 cells per well. On Days 0–4, cells were treated with CCK-8 reagent at 37 °C for 2 h, and the optical density was measured at 450 nm with a microplate reader (BioTek, Winooski, VT, USA). All experiments were repeated three times with six replicates per experiment.

Flow cytometric analysis

Apoptosis was detected using a fluorescein isothiocyanate (FITC)-Annexin V/Propidium Iodide (PI) Apoptosis Detection Kit (Vazyme Biotech). Briefly, cells from different treatment groups were trypsinized and resuspended in 100 µL of binding buffer containing FITC-Annexin V/PI for 15 min. Apoptosis was analyzed by flow cytometry with a BD FACS calibur instrument (BD Biosciences, Franklin Lakes, NJ, USA).

RNA immunoprecipitation (RIP)

RIP was performed using an EZ-Magna RIP Kit (Millipore) according to the manufacturer’s protocol. Briefly, the whole-cell lysate was incubated with RIP buffer containing magnetic beads conjugated to an anti-Ago2 antibody (Millipore) or anti-m5A antibody (Epigentek, Farmingdale, NY, USA) at 4 °C for 6 h. Immunoglobulin G (IgG) was used as the negative control. The beads were washed and then were incubated with 0.1% SDS/0.5 mg/mL proteinase K at 55 °C for 30 min to remove proteins. Finally, the coprecipitated RNAs were evaluated by qRT-PCR.

Dual-luciferase reporter assay

HEK293T cells were cotransfected with the corresponding plasmids and the miR-328-3p mimic or its negative control using Lipofectamine 2000. After 48 h of transfection, cells were lysed, and luciferase activity was measured with a microplate reader (BioTek, Winooski, VT, USA).
assessed using a Dual-Luciferase Reporter Assay Kit (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

Histological analysis and immunohistochemical (IHC) staining of murine knee joints and human cartilage

Human cartilage sections and murine joint sections were fixed with 4% paraformaldehyde, embedded in paraffin, and sliced into 5-μm sections. Sections were deparaffinized in xylene, hydrated through a graded ethanol series, and stained with safranin-O/fast green (Yeasen). For the IHC assay, sections were treated with the appropriate primary antibodies at 4 °C overnight and incubated with secondary antibodies at 37 °C for 1 h.

Fluorescence in situ hybridization (FISH)

Primary chondrocytes were fixed with 4% formaldehyde for 10 min and washed with PBS. Then, the cells were permeabilized in 0.5% Triton X-100 at 4 °C for 5 min, washed with PBS, and prehybridized at 37 °C for 30 min before hybridization. Anti-AC008 (RiboBio, Cat. No. Inc11101301), anti-U6 (RiboBio, Cat. No. Inc1110101), and anti-18S rRNA (RiboBio, Cat. No. Inc1110102) oligodeoxynucleotide probes were used in the hybridization solution for incubation at 37 °C overnight in the dark. The next day, the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Solarbio, Beijing, China) and imaged using a confocal laser scanning microscope.

Subcellular fractionation assay

Nuclear and cytoplasmic RNAs from primary chondrocytes were separated using a Cytoplasmic and Nuclear RNA Purification Kit (Norgenbiotek Corporation, Thorold, ON, Canada). The expression level of AC008 in the nucleus and cytoplasm of primary chondrocytes was analyzed by qRT-PCR.

Mouse model of monosodium iodoacetate (MIA)-induced OA

All animal procedures were approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University, Nanjing, China. In brief, 10-week-old male C57BL/6 mice were randomly divided into six groups: normal group (n = 5); MIA group (n = 5); MIA + agomir-NC group (n = 5); MIA + agomir-328-3p group (n = 5); MIA + AAV-NC group (n = 5); and MIA + AAV-AC008 group (n = 5). For induction of OA, mice were given an intra-articular injection of MIA (Sigma-Aldrich) in the knee. One week later, agomir-328-3p or negative control agomir (RiboBio) and adeno-associated virus expressing AC008 (AAV-AC008) or negative control virus (GeneChem, Shanghai, China) were injected into the knee joints of mice in the appropriate groups. Treatments were administered at 20 μL per joint per mouse once per week for 5 consecutive weeks. Six weeks later, the mice were euthanized, and the knee joints were collected for safranin-O/fast green staining and IHC analysis. All sections were observed and evaluated by three authors. Histological evaluation of plug cartilage was performed using the modified Mankin score.

Fig. 1 AC008 is upregulated in human osteoarthritic cartilage. a A cluster heat map showing the differentially expressed lncRNAs, mRNAs, and miRNAs between normal and osteoarthritic cartilage (n = 3). b The expression level of AC008 in normal and osteoarthritic cartilage was evaluated by qRT-PCR (n = 39). c Safranin-O staining was performed to assess ECM degradation in normal and osteoarthritic cartilage samples (scale bar = 500 μm). d IHC staining was conducted to evaluate the expression levels of COL2A1 and MMP13 in normal and osteoarthritic cartilage samples (scale bar = 200 μm). The quantification of cells positive for COL2A1 and MMP13 staining is shown in the right panel. OA osteoarthritis. The data are presented as the means ± SDs. Statistical differences were determined by unpaired two-tailed Student’s t test (b, d). **P < 0.01; ***P < 0.001.
Statistical analysis

The data are presented as the mean ± standard deviation values. Statistical analysis was carried out using GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA). Unpaired two-tailed Student’s t test was used to determine the significance of differences between two groups, while two-way analysis of variance (ANOVA) was used for multiple comparisons. Spearman correlation analysis was used to identify correlations. A P value of less than 0.05 was considered statistically significant.

RESULTS

AC008 is highly expressed in human osteoarthritic cartilage

We performed microarray analyses to identify the differentially expressed IncRNAs, mRNAs, and miRNAs between normal and osteoarthritic articular cartilage. In total, we identified 1194 IncRNAs, 1172 mRNAs, and 105 miRNAs that were differentially expressed between normal and osteoarthritic cartilages (fold change ≥ 2 or ≤ 0.5 and P < 0.05; Fig. 1a). Based on the differentially expressed gene data, we performed an integrated analysis to construct the potential IncRNA-miRNA-mRNA network. We found that AC008440.5 (abbreviated AC008), a novel IncRNA, potentially interacted with miR-328-3p. In addition, AQP1 and ANKH, two OA-associated genes18,19, were found to be the potential targets of miR-328-3p. Therefore, we selected AC008, miR-328-3p, AQP1, and ANKH for further study. First, we examined the expression of AC008 in a cohort of 39 osteoarthritic cartilage samples. Consistent with the microarray data, AC008 was significantly upregulated in osteoarthritic cartilage samples compared to normal cartilage samples (Fig. 1b). Severe cartilage loss was observed in osteoarthritic cartilage compared to normal cartilage, as indicated by the reduced safranin-O staining (Fig. 1c). In addition, osteoarthritic cartilage displayed decreased expression of COL2A1 and high expression of MMP13, implying ECM degradation (Fig. 1d). These results indicate that AC008 may be involved in the pathogenesis of OA.

AC008 inhibits chondrocyte viability and promotes chondrocyte apoptosis and ECM degradation

To investigate the potential effects of AC008 on the biological activity of chondrocytes, we constructed an AC008 overexpression vector and designed an ASO targeting AC008. qRT-PCR analysis revealed successful overexpression and knockdown of AC008 in primary chondrocytes (Fig. 2a). To exclude the possibility of gDNA or vector DNA contamination in the cDNA, an RT SuperMix kit

Fig. 2 AC008 inhibits chondrocyte viability and promotes chondrocyte apoptosis and ECM degradation. a qRT–PCR was performed to verify the overexpression and knockdown efficiencies of AC008 in chondrocytes transfected with pcDNA-AC008 or AC008 ASO. b A CCK-8 assay was used to identify the viability of chondrocytes with AC008 overexpression or knockdown. c Chondrocyte apoptosis was detected with FITC-Annexin V/PI double staining using flow cytometry following AC008 overexpression or knockdown. d The expression levels of apoptosis-associated proteins were evaluated by western blotting. e The expression levels of ECM proteins (Aggrecan and COL2A1) and cartilage-degrading enzymes (MMP13 and ADAMTS-5) were analyzed by western blotting in chondrocytes following AC008 overexpression or knockdown. The data are presented as the means ± SDs. Statistical differences were determined using unpaired two-tailed Student’s t test (a, c) or two-way ANOVA (b). * P < 0.05; ** P < 0.01; *** P < 0.001.
containing 4× gDNA wiper Mix was used. To further verify the absence of contamination, we performed PCR using primers designed from the cytoplasmic and nuclear fractions respectively. Scale bar: 10 μm. c RIP experiments were performed in primary chondrocytes, and the coprecipitated RNAs were subjected to qRT-PCR to analyze AC008 expression. The fold enrichment of AC008 in the anti-Ago2 precipitate is shown relative to that in the corresponding IgG control precipitate. d The potential miR-328-3p binding sites in AC008 were predicted with the RNAhybrid database. e The expression level of miR-328-3p in normal and osteoarthritic cartilage was evaluated by qRT-PCR (n = 39). f The association between AC008 and miR-328-3p expression in osteoarthritic cartilage was assessed by Spearman correlation analysis.

Fig. 3 AC008 interacts with miR-328-3p. a The expression level of AC008 in the nucleus and cytoplasm of primary chondrocytes was analyzed by qRT-PCR. β-actin and U6 were used as internal controls for the cytoplasmic and nuclear fractions, respectively. b FISH was performed to determine the subcellular localization of AC008 in chondrocytes. 18S rRNA and U6 were labeled with cyanine 3 (Cy3, red), and nuclei were stained with DAPI (blue). 18S rRNA and U6 were used as cytoplasmic and nuclear markers, respectively. Scale bar: 10 μm. c RIP experiments were performed in primary chondrocytes, and the coprecipitated RNAs were subjected to qRT-PCR to analyze AC008 expression. The fold enrichment of AC008 in the anti-Ago2 precipitate is shown relative to that in the corresponding IgG control precipitate. d The potential miR-328-3p binding sites in AC008 were predicted with the RNAhybrid database. e The expression level of miR-328-3p in normal and osteoarthritic cartilage was evaluated by qRT-PCR (n = 39). f The association between AC008 and miR-328-3p expression in osteoarthritic cartilage was assessed by Spearman correlation analysis. Top: the designed wild-type AC008 sequence and mutant AC008 sequence (in which only the putative miR-328-3p binding site was mutated). Bottom: luciferase reporter assay in HEK293T cells transfected with psiCHECK2-AC008 (WT or Mut) and the miR-328-3p mimic or mimic control. h qRT-PCR was performed to determine the expression level of AC008 after transfection with pcDNA-AC008 or AC008 ASO. OA osteoarthritis, WT wild-type, MUT mutant, ns no significant difference. The data are presented as the means ± SDs. Statistical differences were determined using unpaired two-tailed Student's t test (c, e, g, h) or Spearman correlation analysis (f). ** P < 0.01; *** P < 0.001.
attenuated chondrocyte viability, whereas AC008 silencing increased chondrocyte viability (Fig. 2b). Next, we assessed the effects of AC008 on chondrocyte apoptosis. Flow cytometric analysis indicated that the apoptosis rate was increased after AC008 overexpression and was reduced following AC008 knockdown (Fig. 2c). In addition, after AC008 overexpression, the expression of antiapoptotic proteins, including Bcl-2 and Survivin, was markedly downregulated, while the expression of proapoptotic proteins, such as Bax, cleaved PARP, cleaved Caspase-3, and cleaved Caspase-9, was upregulated. In contrast, AC008 knockdown resulted in the opposite effects on the expression of these antiapoptotic and proapoptotic markers (Fig. 2d). Since the degradation of articular cartilage is a hallmark of OA pathogenesis, we investigated the impact of AC008 on ECM degradation. AC008...
promoted the expression of major cartilage-degrading enzymes, including MMP13 and ADAMTS-5, and inhibited the expression of ECM proteins, such as COL2A1 and Aggrecan, whereas AC008 knockdown exerted the opposite effects on the expression of these genes (Fig. 2e). Taken together, these results suggest that abnormal expression of AC008 might affect OA progression by regulating chondrocyte viability, chondrocyte apoptosis, and ECM degradation.

AC008 acts as a sponge for miR-328-3p

To explore the mechanism of action of AC008 in OA, we initially determined the subcellular localization of AC008 in chondrocytes. qRT-PCR analysis showed that AC008 was predominantly localized in the cytoplasm of primary chondrocytes (Fig. 3a), consistent with the results of FISH (Fig. 3b). Given the cytoplasmic distribution of AC008, we hypothesized that AC008 might exert its effects by acting as a ceRNA. Argonaute 2 (Ago2) is a core component of the RNA-induced silencing complex (RISC), which is involved in miRNA-mediated mRNA destabilization or translational repression. To confirm whether AC008 is included in the RISC complex, we performed RIP using an anti-Ago2 antibody. As shown in Fig. 3c, endogenous AC008 was preferentially enriched in the anti-Ago2 immunoprecipitates compared with the IgG immunoprecipitates, suggesting that AC008 might directly bind to miRNAs and function as a ceRNA. By using the LncBase v2 database, we identified 17 miRNAs that may potentially interact with AC008 (Supplementary Table 6). Among these 17 miRNAs, miR-328-3p was significantly downregulated in osteoarthritic cartilage compared to normal cartilage, based on our microarray data. RNAhybrid database analysis further revealed the potential interaction between miR-328-3p and AC008 (Fig. 3d). We selected miR-328-3p for further study, as the expression of miR-328-3p was notably lower in osteoarthritic cartilage than in normal cartilage and was negatively correlated with AC008 expression in osteoarthritic cartilage (Fig. 3e, f). In addition, two OA-associated genes, AQP1 and ANKH, which were identified as upregulated genes by microarray analysis, were potential targets for miR-328-3p (Fig. 3c). Furthermore, the dual-luciferase reporter assay revealed that overexpression of miR-328-3p markedly reduced the luciferase activity of the wild-type AC008 reporter gene but not that of the AC008 mutant vector (Fig. 3g), indicating direct binding between AC008 and miR-328-3p. In addition, we found that overexpression of AC008 dramatically decreased miR-328-3p expression; in contrast, knockdown of AC008 markedly increased miR-328-3p expression (Fig. 3h). These results indicate that AC008 directly sponges miR-328-3p.

We further performed miR-328-3p overexpression and inhibition experiments to determine the function of miR-328-3p in primary chondrocytes (Supplementary Fig. 3a). The CCK-8 assay and flow cytometric analysis demonstrated that miR-328-3p overexpression increased chondrocyte viability (Supplementary Fig. 3b) and attenuated chondrocyte apoptosis (Supplementary Fig. 3c). After miR-328-3p overexpression, the expression levels of Bcl-2 and Survivin were increased, while the expression levels of Bax, cleaved PARP, cleaved Caspase-3, and cleaved Caspase-9 were reduced (Supplementary Fig. 3d). Moreover, miR-328-3p promoted the expression of COL2A1 and Aggrecan and decreased the expression of MMP13 and ADAMTS-5 (Supplementary Fig. 3e). The opposite effect was observed following inhibition of miR-328-3p (Supplementary Fig. 3b–e).

AC008 accelerates OA progression via the miR-328-3p–AQP1/ANKH axis

Considering the regulatory relationship between AC008 and miR-328-3p as well as that between miR-328-3p and AQP1/ANKH, we hypothesized that AC008 might modulate AQP1 and ANKH expression by acting as a sponge for miR-328-3p. Indeed, overexpression of AC008 increased the expression of AQP1 and ANKH, and this increase in AQP1 and ANKH expression was partially abolished by the miR-328-3p mimic (Fig. 6a). Moreover, the miR-328-3p mimic reversed the attenuating effect of AC008 on chondrocyte viability (Fig. 6b) and abrogated the proapoptotic...
effect of AC008 (Fig. 6c, d). In addition, the miR-328-3p mimic reversed the decreases in COL2A1 and Aggre can expression and abolished the increases in MMP13 and ADAMTS-5 expression induced by AC008 overexpression (Fig. 6e). Taken together, these data indicate that AC008 promotes OA progression by modulating the miR-328-3p–AQP1/ANKH axis.

Since mouse-derived and human-derived mature miR-328-3p have the same sequence, we used MIA-induced OA mice given treatment with agomir-328-3p, AAV-AC008 and their correspond ing control constructs (Fig. 6f). The in vivo delivery efficiencies of agomir-328-3p and AAV-AC008 were validated by qRT–PCR. In addition, the expression levels of AQP1 and ANKH in knee cartilage from MIA + AAV-AC008 mice were higher than those in knee cartilage from MIA + AAV-NC mice but were lower in knee cartilage from MIA + agomir-328-3p mice than in knee cartilage from MIA + agomir-NC mice (Supplementary Fig. 5). Intra-articular injection of MIA induced ECM degradation that resembled the pathological changes of OA, as evidenced by the reduced safranin-O/fast green staining, increased modified Mankin score, decreased expression of COL2A1, and high expression of MMP13, whereas treatment with agomir-328-3p ameliorated MIA-induced OA. Agomir-328-3p effectively reversed the loss of COL2A1 expression and the increase in MMP13 expression in the MIA model. However, treatment with AAV-AC008 resulted in more cartilage destruction than observed in the control group (AAV-NC) of mice with MIA-induced OA (Fig. 6g–i). Thus, consistent with our in vitro findings, these results demonstrated that the AC008–miR-328-3p–AQP1/ANKH axis mediates OA progression, suggesting that AC008 may be a promising therapeutic target for OA.

**FTO-dependent m^6^A demethylation mediates the upregulation of AC008 in OA.** Recent studies have suggested that m^6^A modification modulates all phases of RNA metabolism, including RNA folding, stability, splicing, nuclear export, translational modulation, and degradation^{23,24}. Therefore, we sought to determine whether m^6^A participates in the upregulation of AC008 in OA. According to the results from the online bioinformatics tool SRAMP (http://www.cuilab.cn/sramp/) \(^7\), we found an RRACU m^6^A sequence motif in the second exon of AC008 RNA (from position 383–387, Fig. 7a). MeRIP-qPCR showed that m^6^A was highly enriched within the AC008 sequence in chondrocytes (Fig. 7b). FTO is a key m^6^A demethylase ("eraser") in mammalian cells and has been reported to be associated with OA^{25}. Further analysis demonstrated that...
FTO was significantly downregulated in osteoarthritic cartilage compared to normal cartilage (Fig. 7c). To determine whether FTO-mediated m^6^A demethylation is involved in AC008 upregulation in OA, we overexpressed FTO in chondrocytes (Fig. 7d) and found that the m^6^A level of AC008 was decreased (Fig. 7e). In addition, FTO overexpression led to a decrease of approximately 70% in the expression level of AC008 (Fig. 7f).

FORTO expression in OA improves AC008 RNA stability, thus contributing to the upregulation of AC008 in OA.

**DISCUSSION**

Since lncRNAs play critical roles in the development of cartilage, they are recognized as novel biomarkers for OA diagnosis and prognosis. In addition, several IncRNAs, such as MEG3 and H19, are considered therapeutic targets for OA. In the present study, we investigated the IncRNA expression profiles of normal and osteoarthritic cartilage using microarray analysis. We focused on the role and potential mechanism of a novel IncRNA, termed
Fig. 7  m^6A modification is involved in the upregulation of AC008 in OA. a The m^6A modification site in AC008 was predicted with the online tool SRAMP (http://www.cuilab.cn/sramp). b The m^6A level of AC008 in chondrocytes was determined by MeRIP-qPCR. c The expression level of FTO in normal and osteoarthritic cartilages was evaluated by qRT-PCR (n = 39). d qRT-PCR was performed to verify the overexpression efficiency of FTO in chondrocytes transfected with the FTO overexpression plasmid. e The m^6A level of AC008 was evaluated in chondrocytes transfected with the indicated plasmids. f The expression level of AC008 was evaluated in chondrocytes transfected with the indicated plasmids. g Cells were treated with actinomycin D for the indicated times, and qRT-PCR was performed to evaluate the expression level of AC008. h Proposed model of the mechanism underlying the expression and function of AC008 in OA progression. The data are presented as the means ± SDs. Statistical differences were determined using unpaired two-tailed Student’s t test (b–f). *P < 0.05; **P < 0.01; ***P < 0.001.
AC008, which was aberrantly upregulated in osteoarthritic cartilage. Functionally, we demonstrated that AC008 overexpression decreased chondrocyte viability and induced chondrocyte apoptosis and ECM degradation in vitro and that knockdown of AC008 exerted the opposite effects. Consistent with our in vitro findings, AC008 overexpression induced cartilage matrix loss and accelerated OA progression in a model of MIA-induced OA.

As perturbation of antisense RNA transcription can directly or indirectly regulate the expression of sense genes, we also investigated the effect of AC008 on the expression of its coding counterpart, MYADM. Overexpression of AC008 had no obvious effect on MYADM mRNA expression (Supplementary Fig. 6). The cellular localization of IncRNAs is important for their biological functions. Generally, cytoplasmic IncRNAs participate in posttranscriptional regulation by interacting with miRNAs or RNAs, whereas nuclear IncRNAs mediate RNA processing, transcriptional regulation, and chromatin interactions. Herein, we identified that AC008 was mainly localized in the cytoplasm and could interact with Ago2, suggesting that AC008 may function as an endogenous miRNA sponge. Bioinformatic analyses and luciferase reporter assays revealed that AC008 interacted with miR-328-3p. In addition, AC008 negatively regulated miR-328-3p expression. However, the pri-miR-328-3p level was not obviously affected by AC008 overexpression (Supplementary Fig. 7), suggesting that AC008 regulates the expression of miR-328-3p but not at the transcriptional level. We speculated that AC008 may regulate miR-328-3p expression by influencing the processing, transport, and/or stability of miR-328-3p.

To date, miR-328-3p has been reported to play important roles in several types of tumors, but the role of miR-328-3p in OA is poorly understood. Our data revealed that ectopic miR-328-3p expression increased chondrocyte viability, suppressed chondrocyte apoptosis, and attenuated ECM degradation, whereas inhibition of miR-328-3p exerted the opposite effects. In addition, our experiments in the mouse model of MIA-induced OA further verified the role of miR-328-3p in suppressing the development of OA in vivo.

LncRNAs serve as miRNA sponges to regulate the expression of miRNA target genes. According to bioinformatics analysis, miR-328-3p may bind to the 3′UTRs of the AQP1 and ANKH genes; we confirmed these direct associations by dual-luciferase reporter assays. AQP1 is a member of the AQP family and is abundantly expressed in the synovium in rheumatoid arthritis. Abnormal expression of AQP1 is closely related to chondrocyte apoptosis and ECM degradation, whereas inhibition of miR-328-3p exerted the opposite effects. In addition, our experiments in the mouse model of MIA-induced OA further verified the role of miR-328-3p in suppressing the development of OA in vivo.

miR-328-3p is involved in the control of AQP1/ANKH axis, in OA. We also found that miR-328-3p regulation of AC008 accounts for the upregulation of AC008 in OA (Fig. 7h). Our study reveals that AC008 plays an important role in OA progression and highlights the potential of AC008 as a therapeutic target for OA.

DATA AVAILABILITY

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

C.Y.M. designed the study. J.S.Y., M.Z. and D.W.Y. performed most of the experiments and analyzed the data. Y.F.M. and Y.T.T. performed part of the experiments. D.W.Y. collected the clinical samples. M.Y.X., L.Y.L., L.C. and Y.C.J. analyzed and interpreted the data. Y.C.J. and C.Y.M. wrote and revised the paper. All authors read and approved the final paper.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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