Tohoku J. Exp. Med., 2025, **266**, 219-227

Long Non-coding RNA RASSF8-AS1 Promotes M1 Macrophage Polarization in Osteoarthritis via Moderating miR-27a-3p

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Long non-coding RNAs are major regulators in the pathophysiology of osteoarthritis (OA), which involves the dysfunction of cartilage and synovium. The aim of this study was, therefore, to discover the role of RASSF8-AS1 in cartilage degradation and M1 macrophage polarization during OA. Healthy and OA cartilage and synovium were collected. After measuring RASSF8-AS1 levels in tissues and lipopolysaccharide (LPS)- or IL-1β-induced cells, the role of RASSF8-AS1 in the expression of cartilage degradation markers and M1 macrophage molecules was assessed *in vitro*. The apoptotic rate of IL-1β-stimulated chondrocytes, with or without RASSF8-AS1 overexpression, was quantified using flow cytometry. RASSF8-AS1 was significantly upregulated not only in cartilage and synovium from OA patients, but also in IL-1β- or LPS-induced cells, compared to normal controls. A decrease in RASSF8-AS1 level increased the expression of chondrogenic markers, but reduced the expression of genes encoding matrix-degrading proteases, thereby reducing cell apoptosis. Downregulation of RASSF8-AS1 reduced the M1 macrophage markers in RAW264.7 cells and bone marrow-derived macrophages. RASSF8-AS1 may be a ceRNA for miR-27a-3p. These findings support the role of RASSF8-AS1 as a promoting factor of cartilage degradation and M1 macrophage polarization in OA.

Keywords: macrophages; miR-27a-3p; osteoarthritis; RASSF8-AS1
Tohoku J. Exp. Med., 2025 July, **266** (3), 219-227.
doi: 10.1620/tjem.2024.J092

Introduction

Osteoarthritis (OA) is a degenerative disease characterized by the destruction or damage of articular cartilage (Barnett 2018). As the most common form of arthritis, OA is a leading cause of disability worldwide, with an annual global increase in the age-standardized incidence rate of 0.32% and an increase of approximately 9% in the 28 years from 1990 to 2017 (Quicke et al. 2022). Inflammation, including active synovitis and systemic inflammation, plays a key role in the pathogenesis of OA (Abramoff and Caldera 2020; Zhang et al. 2023). The main clinical features observed in OA patients were joint pain and joint dysfunction in typical joints, such as the knee, hip, distal, and proximal interphalangeal joints (Abdel-Aziz et al. 2021). Long before a complete understanding of the molecular events involved in this disease, treatment options for OA are limited by the multiple disease phenotypes (Kumar et al. 2021). Recently, OA has been considered a much more complex process, involving multiple inflammatory and metabolic factors, rather than a simple disease of "wear and tear" (Molnar et al. 2021). The pathogenesis of this degenerative process involves the complex network of inflammatory and metabolic factors and cytokines that regulate cartilage degeneration, synovial inflammation, and subchondral bone thickening (O'Neill and Felson 2018).

Received May 21, 2024; revised and accepted September 1, 2024; J-STAGE Advance online publication September 12, 2024
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This opens the door to the development of new treatments for OA.

In recent years, genome-wide association studies have revealed the molecular genetics of OA (Wood et al. 2022). This has been accompanied by a refreshing wave of discoveries about epigenetic alternations in joint tissues, including regulatory RNAs, has occurred concurrently (Rice et al. 2020; Wood et al. 2022). It has become apparent is that many genetic risk signals for OA interact with epigenetic mediators, including non-coding RNAs (ncRNAs). NcRNAs, such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), can directly or indirectly interact with the OA risk genes to regulate cell functions (Allis and Jenuwein 2016; Gargano et al. 2022, 2023). LncRNAs are a group of essential functional molecules in many biological processes (Nadhan et al. 2022). Recently, the rapid development of lncRNA research has revealed the involvement of lncRNAs in the pathological process of OA (Wang et al. 2022). Dysregulation of lncRNAs has been implicated in the catabolic processes involved in OA, such as cartilage tissue destruction, inflammatory events, and apoptosis (Okuyan and Begen 2022). For example, overexpression of lncRNA POU3F3 can inhibit chondrocyte apoptosis, increase cell viability, rescue metabolic dysfunction, and alleviate IL-1β-induced injury *in vitro* (Shi et al. 2022). LncRNA H19 is involved in cartilage matrix degradation and calcification in osteoarthritis (Yang et al. 2020). Notably, lncRNAs have been reported to play important roles in regulating macrophage polarization and inflammatory responses, which are closely associated with the pathogenesis of OA (Feng et al. 2022). The most common functional mechanism for lncRNA is to act as a decoy molecule of miRNA, as a competing endogenous RNA (ceRNA), thereby interfering with the binding of miRNA and the target genes (Wang et al. 2021). The XIST/miR-376c-5p axis has been verified to act on M1 macrophages, and subsequently affect the apoptosis of cocultured chondrocytes (Li et al. 2020). Therefore, the altered expression of lncRNAs in joint tissue and synovial fluid makes them attractive candidates for the treatment of OA.

RASSF8-AS1 has been identified as an immune-related circRNA in cervical cancer (Jiang et al. 2022). Although the contributions of lncRNA RASSF8-AS1 in neoplastic and non-neoplastic conditions have been extensively studied (Xie et al. 2022), it is unknown whether RASSF8-AS1 is involved in M1 macrophages during OA. The aim of this study was to characterize the expression levels of RASSF8-AS1 in OA patients, IL-1β chondrocytes, and macrophages, and to investigate its role in inflammation, chondrocyte-mediated cartilage destruction and regulation of macrophage polarization in OA.

Materials and Methods

Database retrieval

GSE163552, GSE175960, and GSE146895 in the GEO database (https://www.ncbi.nlm.nih.gov/geo/) were searched and analyzed by GEO2R online tool. The differentially expressed lncRNAs were intersected using Venn diagram.

ENCORI (https://rna.sysu.edu.cn/encori/index.php) was used to collect the downstream miRNAs for RASSF8-AS1.

Collection of human samples

Human OA cartilage and synovial tissues were obtained from patients diagnosed with OA and undergoing total knee replacement surgery. Healthy cartilage and synovial specimens are sourced from individuals who had undergone amputation surgery due to trauma and had no history of arthritic disease. OA tissue samples were subjected to further histological examination and scored for synovitis. Patient recruitment, participation, and sample collection were approved by the Ethics Committee of The First Affiliated Hospital of Xi'an Medical University, with complete written consent before surgery.

Cell culture and stimulation

Human chondrocytes (CP-H107) were purchased from Procell Life (Wuhan, China), and cultured in human chondrocyte complete culture medium (CM-H107, Procell Life, Wuhan, China). To simulate inflammatory status, chondrocytes were stimulated with interleukin-(IL-)1β (10 ng/mL; Sigma-Aldrich) in the culture medium. RAW264.7 cells and human bone marrow-derived macrophages (hBMDMs) were obtained from the National Biomedical Experimental Cell Resource Library (Beijing, China), and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA), which contained 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). To induce M1 macrophage polarization, 100 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich, Saint Louis, MO, USA) was used to culture the RAW264.7 cells and hBMDMs.

Cell transfection

The targeted RASSF8-AS1 siRNA (siRASSF8-AS1, 5′-CCGGCAGTTGTGACTATATTCTACTCGAGTAGAATATAGTCACAACTGTTTTTG-3′), the negative siRNA control (siNC, 5′-CCGGTCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTG-3′), miR-27a-3p mimic (5′-UUCACAGUGGCUAAGUUCCGC-3′) and the negative mimic control (5′-UUCUCCGAACGUGUCACGU-3′) were transfected into cells based on the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Transfection was performed when the inoculated cells reached 80% confluence. In brief, Lipofectamine 3000 reagent was diluted using Opti-MEM medium and mixed thoroughly. siRNA was diluted using Opti-MEM medium (Gibco, Grand Island, NY, USA) to prepare siRNA premix. The siRNA premix was added to the already diluted Lipofectamine 3000 reagent and incubated for 5 min at room temperature. The incubated complex was added to the cells and incubated at 37°C for 2 days, after which the transfected cells were analyzed.

RNA measurements using quantitative real-time PCR (qRT-PCR)

Using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), total RNA was isolated from OA tissues/cells and controls. For reverse transcription, one microgram RNA was subjected to the synthesis of first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY, USA) and PCR amplification. Primers were then used to amplify each gene were used. RNA levels were measured using a QuantStudio 7 pro Real-Time PCR System (ThermoFisher, Waltham, MA, USA).

Positive cell number detection in tissues using immunocytochemistry

Histological slides of tissues were deparaffinized and hydrated using a gradient concentration of ethanol. The samples were then blocked with 5% FBS for one hour and incubated with primary antibodies (1:200, dilution) for CD80 (Proteintech, Rosemont, USA), F4/80 (Proteintech, Rosemont, USA), and iNOS (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Slides were washed with PBS and incubated with secondary antibodies. Thereafter, the nuclei were labeled with 4′,6-diamidino-2-phenylindole. The number of $F4/80^+$, iNOS-positive, and $CD80^+$ macrophages in fluorescence images was quantified as a percentage of total cells using ImageJ software.

Quantification of CD-positive cell using flow cytometry

Cells were suspended and incubated in PBS containing 1.0 mM EDTA and 5% FBS and incubated with fluorescence-labelled anti-human CD80 antibody or PE-conjugated anti-CD14/PE-conjugated anti-HLA-DR (BD Pharmingen, San Jose, CA, USA) for 30 min on ice. Data acquisition was performed on a Cytoflex S flow cytometer (Beckman Coulter, Brea, CA, USA).

Measurement of cell apoptosis using flow cytometry

Annexin V-FITC/PI Apoptosis Kit (Elabscience, Wuhan, China) was used to measure cell apoptosis. Cells (3×10^5) were resuspended. 100 μL of Annexin V Binding Buffer (1 ×) was added to resuspend the cells. Then 2.5 μL of Annexin V-FITC Reagent and 2.5 μL of PI Reagent (50 μg/mL) were added to the cell suspension. The mixture was incubated at room temperature for 20 min. Then, 400 μL of Annexin V Binding Buffer (1 ×) was added to the samples. After mixing, the sample was immediately loaded into the flow cytometer for detection.

Luciferase reporter assay

MiR-27a-3p mimic and the corresponding negative control were purchased from Genepharma (Shanghai, China). The RASSF8-AS1 sequence or the HDAC4 3′ untranslated region (3′-UTR) sequence harboring the predicted binding sites of miR-27a-3p or the designed mutant binding sites were inserted into pmirGLO reporter vectors (Promega, Madison, WI, USA) to obtain wild-type (WT) or mutant (MUT) RASSF8-AS1 or HDAC4, respectively. The day before transfection, human chondrocytes, RAW264.7 cells, and hBMDMs were seeded in a 96-well plate. The firefly luciferase reporter plasmids were co-transfected with miR-27a-3p mimic or negative control using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The firefly and renilla luciferase activities were assayed, respectively, using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA), with firefly luciferase activity/renilla luciferase activity values presented.

Statistical analysis

Sample replications were conducted with a minimum sample size of n = 3 for each experiment. Data were analyzed using a two-tailed Student's t-test or analysis of variance, with a p-value of 0.05 or less as the threshold for statistical significance.

Results

Increased RASSF8-AS1 expression in OA cartilage and chondrocytes

RASSF8-AS1 was one of the differentially expressed lncRNAs in both peripheral blood leukocytes and cartilage samples from OA patients (Fig. 1A). To determine how RASSF8-AS1 levels are altered in OA, we performed qRT-PCR analysis on cartilage samples from OA patients and healthy controls, as well as in induced or non-induced chondrocytes. In our patient cohort, expression of RASSF8-AS1 was significantly increased in cartilages from patients with OA, compared to that from healthy individuals ($p < 0.001$, Fig. 1B). RASSF8-AS1 expression was also increased in IL-1β-induced human chondrocytes, compared to that in non-induced cells ($p < 0.001$, Fig. 1C).

Increased RASSF8-AS1 expression in OA synovial tissues and macrophages

Synovitis in OA is known to accelerate cartilage loss (Sun et al. 2022). We therefore determined the expression of RASSF8-AS1 in the synovial tissues of patients with OA and healthy controls. The synovitis score in the joints of OA patients was quantified and compared with that of control subjects (Fig. 2A). The expression of RASSF8-AS1 was significantly increased in synovial tissue from OA patients compared to normal synovial tissue ($p < 0.001$, Fig. 2B). Moreover, increased macrophage accumulation was found in synovial tissue from OA patients compared to normal synovial tissue, which was characterized by a higher rate of F4/80-, iNOS-, and CD80-positive cells ($p < 0.001$, Fig. 2C). Furthermore, the expression of RASSF8-AS1 was significantly increased in LPS-induced hBMDMs and RAW264.7 cells ($p < 0.001$, Fig. 2D). In addition, as shown in Fig. 2D, the frequency of $CD14^+HLA\text{-}DR^{-/low}$ cells in the synovium of OA patients was significantly higher compared to that in healthy individuals. These results implied that RASSF8-AS1 may participate in the M1 macrophage inflammation in OA.

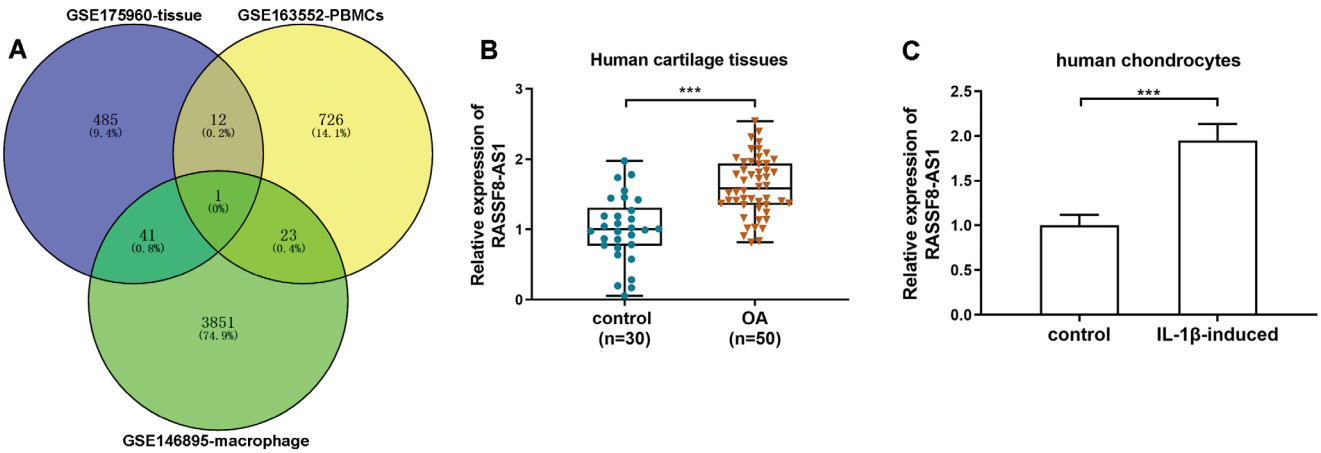

Fig. 1. RASSF8-AS1 was upregulated in human OA cartilage and human chondrocytes.
(A) RASSF8-AS1 was a dysregulated lncRNA shared by GSE163552, GSE175960 and GSE146895. (B) RASSF8-AS1 levels in healthy and OA cartilages were determined by qRT-PCR. (C) RASSF8-AS1 levels in human chondrocytes, with and without IL-1β stimulation, were determined by qRT-PCR. Values were mean ± SD. ***$p < 0.001$.

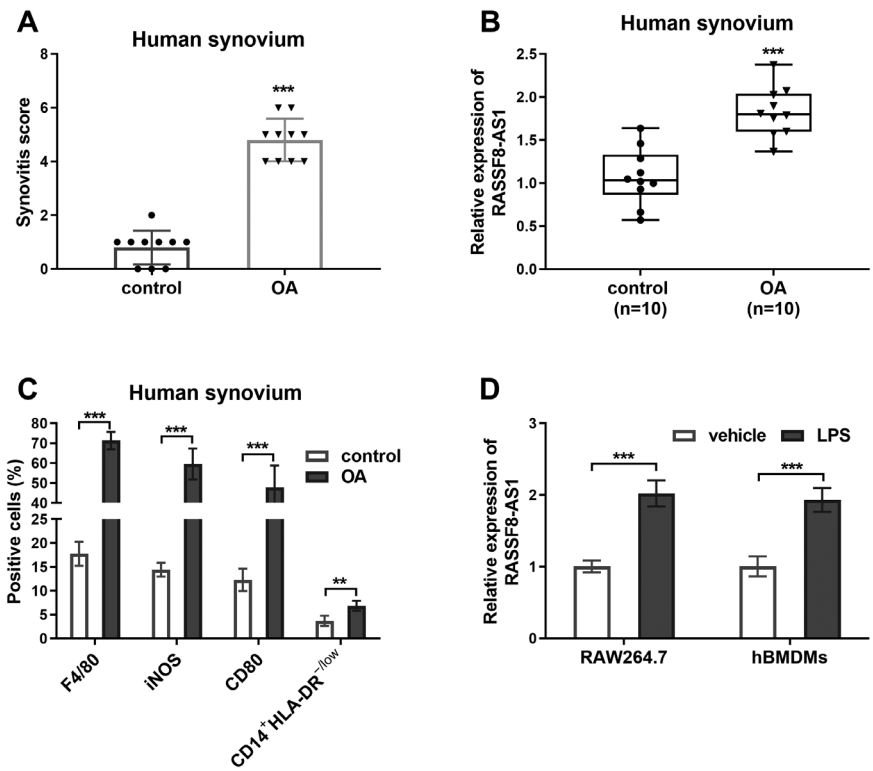

Fig. 2. RASSF8-AS1 was upregulated in human OA synovial tissues and LPS-stimulated macrophages.
(A) Synovitis scores were assessed in ten patients, randomly. (B) RASSF8-AS1 levels in healthy and OA synovial tissues were determined by qRT-PCR. (C) F4/80-, iNOS-, CD80-positive and $CD14^{+}HLA\text{-}DR^{-/low}$ cells were quantified in synovial tissues using flow cytometry. (D) RASSF8-AS1 levels in macrophages, RAW264.7 cells, and human bone marrow-derived macrophages (hBMDMs), were assayed by qRT-PCR. Values were mean ± SD. ***$p < 0.001$.

Downregulation of RASSF8-AS1 alleviated deleterious effects of IL-1β on chondrocytes

To further identify the biological functions of RASSF8-AS1 in cartilage degeneration during OA, we knocked down RASSF8-AS1 in IL-1β-induced human chondrocytes ($p < 0.001$, Fig. 3A). After RASSF8-AS1 blockade, the mRNA expression levels of the chondrogenic markers COL2A1, ACAN, SOX9 were increased ($p < 0.05$, Fig. 3B), whereas mRNA expression levels of MMP3, MMP13, and ADAMTS4, which encode matrix-degrading proteases, were reduced to near normal levels ($p < 0.05$, Fig. 3C). Furthermore, RASSF8-AS1 knockdown inhibited IL-1β-induced chondrocyte apoptosis ($p < 0.01$, Fig. 3D). These observations demonstrated that downregulation of

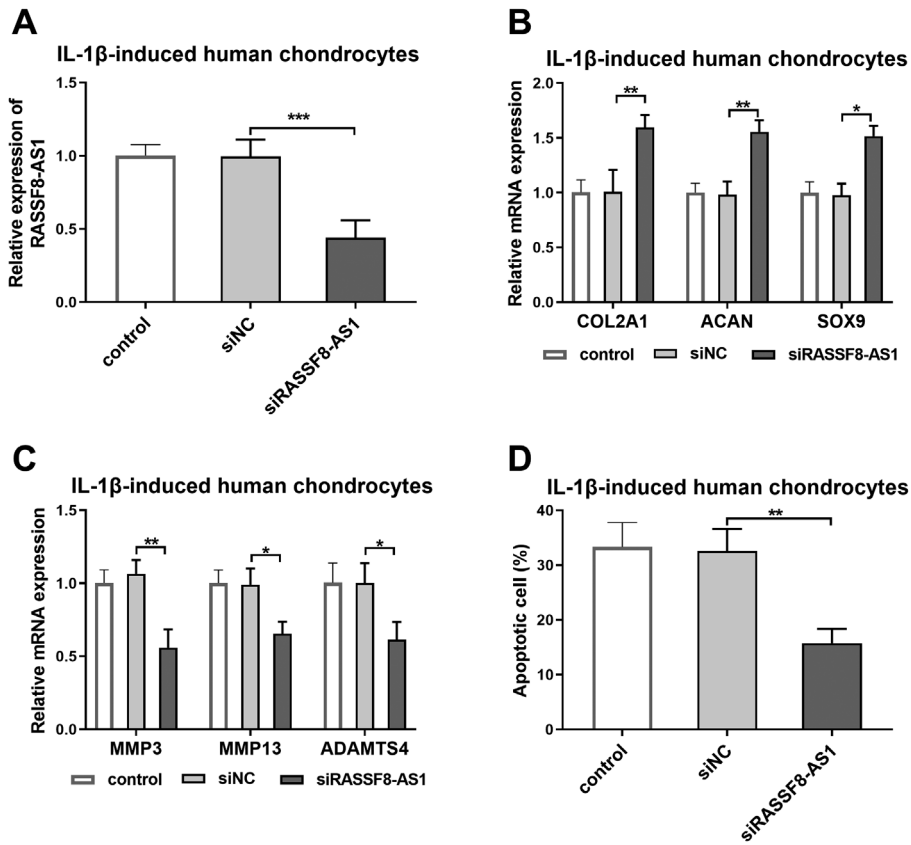

Fig. 3. Downregulation of RASSF8-AS1 alleviated chondrocytes apoptosis.
(A) RASSF8-AS1 was decreased after transfection of RASSF8-AS1 siRNA in IL-1β-stimulated chondrocytes. (B) mRNA levels of key chondrogenic markers were determined by qRT-PCR in IL-1β stimulated chondrocytes, with or without RASSF8-AS1 downregulation. (C) mRNA levels of key matrix metalloproteinases were determined by qRT-PCR in IL-1β-stimulated chondrocytes, with or without RASSF8-AS1 downregulation. (D) Cell death of IL-1β-stimulated chondrocytes, with or without RASSF8-AS1 downregulation, was quantified using flow cytometry. Values were mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

RASSF8-AS1 can alleviate cartilage extracellular matrix degeneration in OA.

Downregulation of RASSF8-AS1 inhibited M1 macrophage polarization

To investigate whether RASSF8-AS1 mediates LPS-induced macrophage polarization, RASSF8-AS1 siRNA was used *in vitro*, to knock down RASSF8-AS1 in LPS-induced RAW264.7 cells ($p < 0.001$, Fig. 4A). The proportion of $CD80^+$ cells in RAW264.7 cells was significantly decreased after RASSF8-AS1 downregulation ($p < 0.001$, Fig. 4B). The mRNA levels of M1 macrophage markers, IL1B, IL6, and TNF-α were decreased after RASSF8-AS1 downregulation in RAW264.7 cells ($p < 0.01$, Fig. 4C), as were the mRNA levels of INOS and CXCL10 ($p < 0.01$, Fig. 4D). The inhibitory effect of siRASSF8-AS1 on LPS induction of the M1 phenotype was also observed in hBMDMs ($p < 0.05$, Fig. 4E-H). Thus, the downregulation of RASSF8-AS1 can inhibit LPS-induced M1 macrophage polarization in both RAW264.7 cells and hBMDMs.

RASSF8-AS1 acted as ceRNA for miR-27a-3p

To identify the underlying mechanism by which RASSF8-AS1 functions in OA, its downstream miRNAs were searched, which appeared to be miR-27a-3p. MiR-27a-3p has been reported to be downregulated in human OA (Li et al. 2018). According to ENCORI prediction, the miR-27a-3p sequence contains complementary sites with RASSF8-AS1 (Fig. 5A). We performed a luciferase reporter assay on human chondrocytes, RAW264.7 cells, and hBMDMs. Compared with the mimic negative control group, the relative luciferase activity of WT-RASSF8-AS1 in the miR-27a-3p mimic group was significantly lower, while the relative luciferase activity of MUT-RASSF8-AS1 group did not show any significant change ($p < 0.05$, Fig. 5B-D). These results suggest that RASSF8-AS1 may function in OA via sponging miR-27a-3p.

Discussion

OA is a member of degenerative diseases that affect multiple joint tissues. Given the growing burden of OA, increased efforts are needed to provide effective and personalized treatments for individuals with this disease. Increasing evidence suggests that lncRNAs affect chondrocyte proliferation, apoptosis, inflammatory responses, and extracellular matrix degradation in OA, and thus have

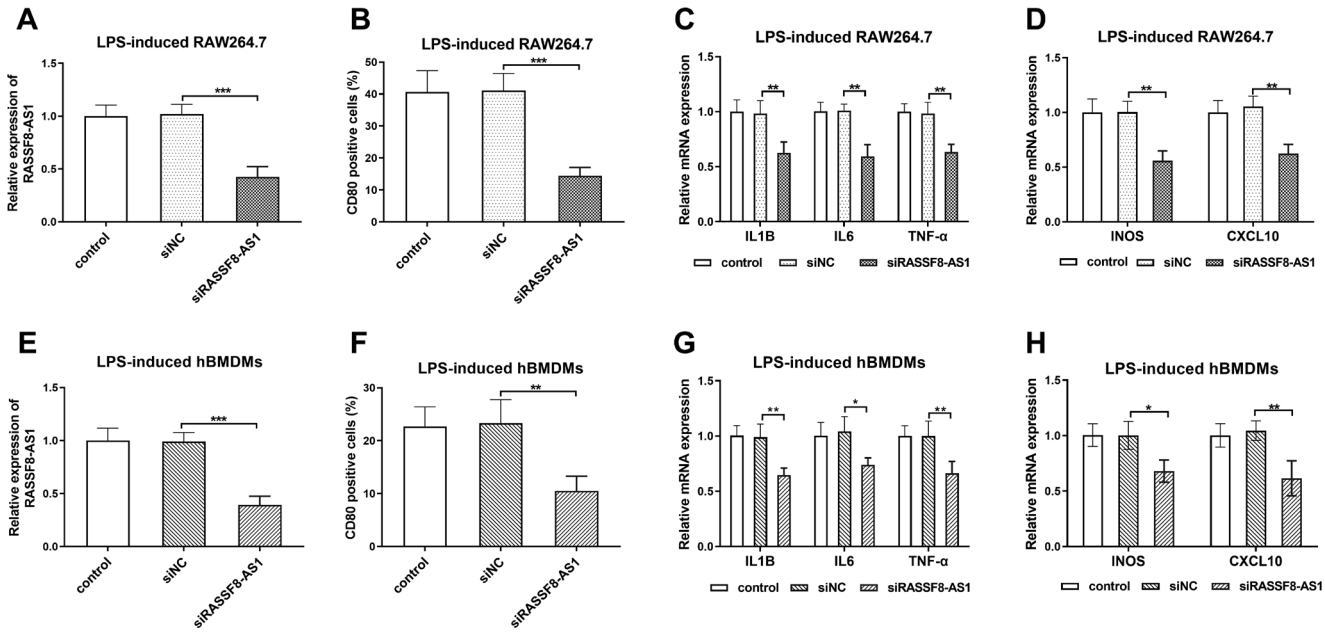

Fig. 4. Downregulation of RASSF8-AS1 inhibited M1 polarized macrophages.
(A) RASSF8-AS1 was decreased after transfection of RASSF8-AS1 siDNA in LPS-stimulated RAW264.7 cells. (B) $CD80^+$ cells were quantified using flow cytometry in LPS-stimulated RAW264.7 cells, with or without RASSF8-AS1 downregulation. (C) mRNA levels of IL1B, IL6, and TNF-α were determined by qRT-PCR in LPS-stimulated RAW264.7 cells, with or without RASSF8-AS1 downregulation. (D) mRNA levels of INOS and CXCL10 were determined by qRT-PCR in LPS-stimulated RAW264.7 cells, with or without RASSF8-AS1 downregulation. (E) RASSF8-AS1 was decreased after the transfection of RASSF8-AS1 siRNA in LPS-stimulated human bone marrow-derived macrophages (hBMDMs). (F) $CD80^+$ cells were quantified using flow cytometry in LPS-stimulated hBMDMs, with or without RASSF8-AS1 downregulation. (G) mRNA levels of IL1B, IL6, and TNF-α were determined by qRT-PCR in LPS-stimulated hBMDMs, with or without RASSF8-AS1 downregulation. (H) mRNA levels of INOS and CXCL10 were determined by qRT-PCR in LPS-stimulated hBMDMs, with or without RASSF8-AS1 downregulation. Values were mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

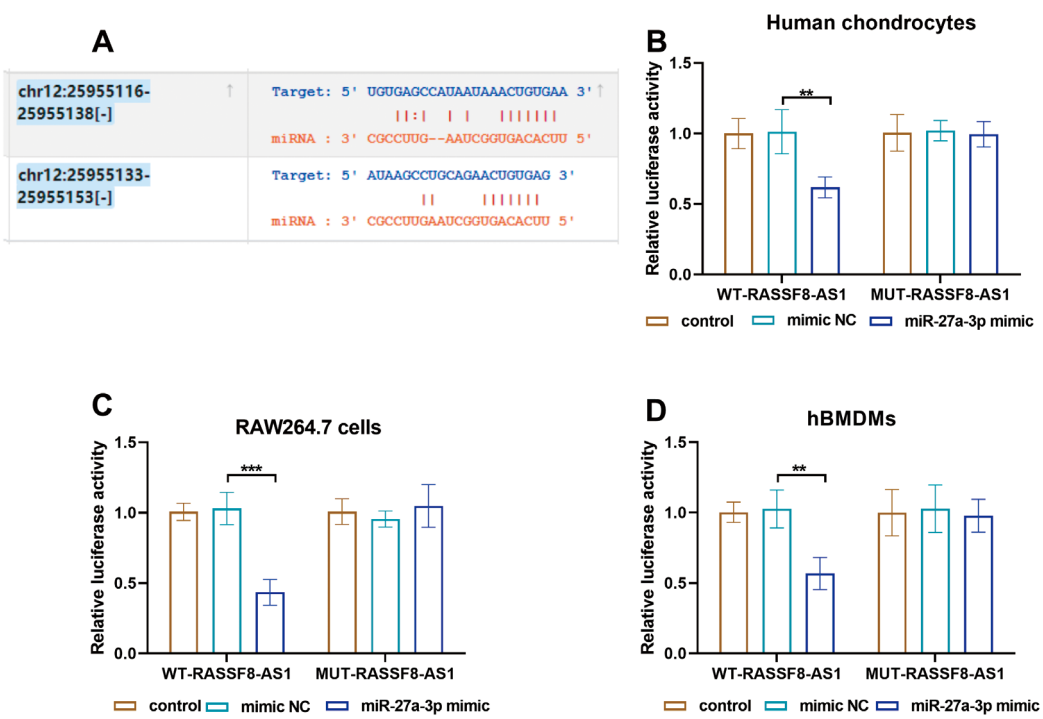

Fig. 5. RASSF8-AS1 can sponge miR-27a-3p.
(A) MiR-27a-3p showed putative binding sites with RASSF8-AS1. (B) MiR-27a-3p mimic influenced luciferase activity of mild-type RASSF8-AS1 (WT-RASSF8-AS1) in human chondrocytes (B), RAW264.7 cells (C), and human bone marrow-derived macrophages (hBMDMs) (D), using Luciferase Reporter Assay. Values were mean ± SD. $**p < 0.01$, $***p < 0.001$.

potential as therapeutic targets for OA (Tu et al. 2020). In addition, research highlights that OA is involved in a complex inflammatory response during inflammatory cell infiltration of the synovial membrane (Nedunchezhiyan et al. 2022). New approaches that can modulate macrophages into desired functional phenotypes, including the regulation of lncRNA expression, may enhance cartilage repair and regeneration, providing novel therapeutic strategies for OA (Fernandes et al. 2020). Our data demonstrated that RASSF8-AS1 was upregulated in both cartilage and synovial tissues of OA patients. This study highlights the therapeutic potential of RASSF8-AS1, which was involved in inflammation and M1 macrophage polarization in OA. The downregulation of RASSF8-AS1 was beneficial for OA, from the perspective of joint regeneration and inflammation.

OA affects articular cartilage. During OA, the osteochondral junction undergoes cellular phenotypic, and therefore functional alterations. Articular cartilage is composed of a single cell type (chondrocytes) surrounded by a dense extracellular matrix (Charlier et al. 2019). Type IIA procollagen, an alternative splice variant of type II collagen, is reported to be expressed in differentiated chondroprogenitor cells (Wang et al. 2020; Ren et al. 2024). COL2A1 encodes the extracellular matrix type II collagen proteins, including procollagens IIA and IIB. ACAN is abundant within human hyaline cartilage. The decrease in aggrecan during chondrocyte dedifferentiation is one of the readouts to assess chondrocyte phenotype, along with collagen type II (Charlier et al. 2019). SOX9 is one of the key drivers for the activation of COL2A1 gene, defined as a master chondrogenic factor required for cartilage formation (Semenistaja et al. 2023). Our *in vitro* results showed that downregulation of RASSF8-AS1 promoted the expression of key chondrogenic markers (COL2A1, ACAN, and SOX9) and reduced the expression of key extracellular matrix markers - matrix metalloproteinases (MMP3, MMP13, and ADAMTS4) in human OA chondrocytes. Interestingly, the rate of LPS-induced chondrocyte apoptosis can be reduced by RASSF8-AS1 knockdown. Therefore, RASSF8-AS1 may be a promoting factor of OA.

There is a molecular communication network between the major joint compartments, such as the synovial membrane and the cartilage of normal and OA-affected joints. During osteochondral dysfunction, the synovial unit undergoes inflammatory pathology (Semenistaja et al. 2023). At the cellular level, the synovial membrane can be influenced by release of cleavage and molecular fragments, such as extracellular matrix degradation products, from apoptotic cells in the cartilage. These “xenobiotics” trigger and maintain low-grade inflammation in the synovium. As we found in our results, the scoring of synovial tissue from OA patients showed more or less inflammation. Macrophages, as immune cells that are present in synovial lining, can polarize to pro-inflammatory (M1) phenotypes (Fernandes et al. 2020). M1 macrophages induced by LPS could increase CD80 expression and present as $CD80^+$ cells. However, our data showed that RASSF8-AS1 overexpression can lead to an increased number of $CD80^+$ cells induced by LPS. A previous study stated that the M1-associated cytokines (IL-6, IL-1β, and TNF-α) can induce destructive processes in chondrocytes, through downregulating of collagen type II and aggrecan synthesis (Fahy et al. 2014). RASSF8-AS1 is reported to be an immune-related circRNA based on E6 splicing, that is implicated in immune response regulation and the infiltration of M1 macrophages (Jiang et al. 2022). Here, we found that RASSF8-AS1 downregulation can inhibit the mRNA levels of M1-like macrophage markers, including IL-6, IL1B, CXCL10, and TNF-α in both RAW264.7 cells and hBMDMs. Thus, we speculate that RASSF8-AS1 may play a promoting role in the skewing of macrophages towards a pro-inflammatory M1 phenotype.

To discover the mechanism of RASSF8-AS1 in OA, the downstream miRNAs were investigated as miR-27a-3p. MiR-27a-3p is downregulated in OA (Li et al. 2018) and is involved in chondrocyte proliferation and migration (Ren et al. 2023). Expression of miR-27a-3p has been identified to be downregulated in synovial tissues from patients with rheumatoid arthritis (Chen et al. 2021). MiR-27a-3p contributes to fibroblast-like synoviocyte proliferation and cytokine production, making it a potential candidate for epigenetic therapy of arthritis (Bullock et al. 2023). It has been reported that M2 polarized macrophage-derived exosomes could deliver miR-27a-3p from macrophages to hemangioma stem cells, reducing the sensitivity of hemangioma stem cells to propranolol (Liu et al. 2021). MiR-27a-3p can regulate differentiation, autophagy, and release of inflammatory factors in pre-osteoblasts by targeting GLP1R (Zeng et al. 2021). Here, the Luciferase Reporter Assay confirmed the binding sites between RASSF8-AS1 and miR-27a-3p. To this extent, it can be speculated that RASSF8-AS1 may sponge miR-27a-3p, and thus play a role in OA. Of course, this speculation needs further *in vitro* and *in vivo* confirmation.

In conclusion, this study demonstrates that RASSF8-AS1 was upregulated in both cartilage and synovial tissues of OA patients. Downregulation of RASSF8-AS1 attenuated the effects of IL-1β on chondrocytes and inhibited the macrophage differentiation towards the M1 phenotype, partly at least via miR-27a-3p. These results highlight the potential of RASSF8-AS1 as a therapeutic target for OA, in particular by suppressing M1 polarized macrophages.

Author Contributions

Study concept and design: Haijun Shi, Meizhi Liu, Zhigang Chen and Yongyun Shi; analysis and interpretation of data: Haijun Shi, Meizhi Liu and Wenhan Ma; drafting of the manuscript: Haijun Shi, Meizhi Liu and Wenhan Ma; critical revision of the manuscript for important intellectual content: Zhigang Chen and Yongyun Shi; statistical analy-

sis: Wenhan Ma.

Funding

This study was funded by Hebei Provincial Administration of Traditional Chinese Medicine Scientific Research Program Project Tasks (2022640).

Conflict of Interest

The authors declare no conflict of interest.

References

Abdel-Aziz, M.A., Ahmed, H.M.S., El-Nekeety, A.A. & Abdel-Wahhab, M.A. (2021) Osteoarthritis complications and the recent therapeutic approaches. *Inflammopharmacology*, **29**, 1653-1667.

Abramoff, B. & Caldera, F.E. (2020) Osteoarthritis Pathology, Diagnosis, and Treatment Options. *Medical Clinics of North America*, **104**, 293-311.

Allis, C.D. & Jenuwein, T. (2016) The molecular hallmarks of epigenetic control. *Nat. Rev. Genet.*, **17**, 487-500.

Barnett, R. (2018) Osteoarthritis. *Lancet*, **391**, 1985.

Bullock, C.H., McAlpine, S.M., Roberts, S.E. & Derfalvi, B. (2023) MicroRNA-27a-3p enhances the inflammatory phenotype of Juvenile Idiopathic Arthritis fibroblast-like synoviocytes. *Pediatr. Rheumatol. Online J.*, **21**, 53.

Charlier, E., Deroyer, C., Ciregia, F., Malaise, O., Neuville, S., Plener, Z., Malaise, M. & de Seny, D. (2019) Chondrocyte dedifferentiation and osteoarthritis (OA). *Biochem. Pharmacol.*, **165**, 49-65.

Chen, L., Lu, Q., Chen, J., Feng, R. & Yang, C. (2021) Upregulating miR-27a-3p inhibits cell proliferation and inflammation of rheumatoid arthritis synovial fibroblasts through targeting toll-like receptor 5. *Exp. Ther. Med.*, **22**, 1227.

Fahy, N., de Vries-van Melle, M.L., Lehmann, J., Wei, W., Grotenhuis, N., Farrell, E., van der Kraan, P.M., Murphy, J.M., Bastiaansen-Jenniskens, Y.M. & van Osch, G.J. (2014) Human osteoarthritic synovium impacts chondrogenic differentiation of mesenchymal stem cells via macrophage polarisation state. *Osteoarthritis Cartilage*, **22**, 1167-1175.

Feng, F., Jiao, P., Wang, J., Li, Y., Bao, B., Luoreng, Z. & Wang, X. (2022) Role of Long Noncoding RNAs in the Regulation of Cellular Immune Response and Inflammatory Diseases. *Cells*, **11**, 3642.

Fernandes, T.L., Gomoll, A.H., Lattermann, C., Hernandez, A.J., Bueno, D.F. & Amano, M.T. (2020) Macrophage: A Potential Target on Cartilage Regeneration. *Front. Immunol.*, **11**, 111.

Gargano, G., Asparago, G., Spiezia, F., Oliva, F. & Maffulli, N. (2023) Small interfering RNAs in the management of human osteoporosis. *Br. Med. Bull.*, **148**, 58-69.

Gargano, G., Oliva, F., Oliviero, A. & Maffulli, N. (2022) Small interfering RNAs in the management of human rheumatoid arthritis. *Br. Med. Bull.*, **142**, 34-43.

Jiang, S., Zhou, Y., Ye, M., Li, X., Zhang, L. & Yang, Y. (2022) Construction of an immune-related ceRNA network in cervical cancer based on HPV E6 splicing. *Front. Oncol.*, **12**, 979884.

Kumar, A., Palit, P., Thomas, S., Gupta, G., Ghosh, P., Goswami, R.P., Kumar Maity, T. & Dutta Choudhury, M. (2021) Osteoarthritis: Prognosis and emerging therapeutic approach for disease management. *Drug Dev. Res.*, **82**, 49-58.

Li, L., Lv, G., Wang, B. & Kuang, L. (2020) XIST/miR-376c-5p/OPN axis modulates the influence of proinflammatory M1 macrophages on osteoarthritis chondrocyte apoptosis. *J. Cell Physiol.*, **235**, 281-293.

Li, X., He, P., Li, Z., Wang, H., Liu, M., Xiao, Y., Xu, D., Kang, Y. & Wang, H. (2018) Interleukin-1beta-mediated suppression of microRNA-27a-3p activity in human cartilage via MAPK and NF-kappaB pathways: A potential mechanism of osteoarthritis pathogenesis. *Mol. Med. Rep.*, **18**, 541-549.

Liu, C., Zhao, Z., Guo, S., Zhang, L., Fan, X. & Zheng, J. (2021) Exosomal miR-27a-3p derived from tumor-associated macrophage suppresses propranolol sensitivity in infantile hemangioma. *Cell Immunol.*, **370**, 104442.

Molnar, V., Matisic, V., Kodvanj, I., Bjelica, R., Jelec, Z., Hudetz, D., Rod, E., Cukelj, F., Vrdoljak, T., Vidovic, D., Staresinic, M., Sabalic, S., Dobricic, B., Petrovic, T., Anticevic, D., et al. (2021) Cytokines and Chemokines Involved in Osteoarthritis Pathogenesis. *Int. J. Mol. Sci.*, **22**, 9208.

Nadhan, R., Isidoro, C., Song, Y.S. & Dhanasekaran, D.N. (2022) Signaling by LncRNAs: Structure, Cellular Homeostasis, and Disease Pathology. *Cells*, **11**, 2517.

Nedunchezhiyan, U., Varughese, I., Sun, A.R., Wu, X., Crawford, R. & Prasadam, I. (2022) Obesity, Inflammation, and Immune System in Osteoarthritis. *Front. Immunol.*, **13**, 907750.

O'Neill, T.W. & Felson, D.T. (2018) Mechanisms of Osteoarthritis (OA) Pain. *Curr. Osteoporos. Rep.*, **16**, 611-616.

Okuyan, H.M. & Begen, M.A. (2022) LncRNAs in Osteoarthritis. *Clin. Chim. Acta*, **532**, 145-163.

Quicke, J.G., Conaghan, P.G., Corp, N. & Peat, G. (2022) Osteoarthritis year in review 2021: epidemiology & therapy. *Osteoarthritis Cartilage*, **30**, 196-206.

Ren, H., Zhang, L., Zhang, X., Yi, C. & Wu, L. (2024) Specific lipid magnetic sphere sorted CD146-positive bone marrow mesenchymal stem cells can better promote articular cartilage damage repair. *BMC Musculoskelet. Disord.*, **25**, 253.

Ren, J., Zhang, F., Zhu, S., Zhang, W., Hou, J., He, R., Wang, K., Wang, Z. & Liang, T. (2023) Exosomal long non-coding RNA TRAFD1-4:1 derived from fibroblast-like synoviocytes suppresses chondrocyte proliferation and migration by degrading cartilage extracellular matrix in rheumatoid arthritis. *Exp. Cell Res.*, **422**, 113441.

Rice, S.J., Beier, F., Young, D.A. & Loughlin, J. (2020) Interplay between genetics and epigenetics in osteoarthritis. *Nat. Rev. Rheumatol.*, **16**, 268-281.

Semenistaja, S., Skuja, S., Kadisa, A. & Groma, V. (2023) Healthy and Osteoarthritis-Affected Joints Facing the Cellular Crosstalk. *Int. J. Mol. Sci.*, **24**, 4120.

Shi, M., Sun, M., Wang, C., Shen, Y., Wang, Y. & Yan, S. (2022) Therapeutic Potential of POU3F3, a Novel Long Non-coding RNA, Alleviates the Pathogenesis of Osteoarthritis by Regulating the miR-29a- 3p/FOXO3 Axis. *Curr. Gene. Ther.*, **22**, 427-438.

Sun, Z., Liu, Q., Lv, Z., Li, J., Xu, X., Sun, H., Wang, M., Sun, K., Shi, T., Liu, Z., Tan, G., Yan, W., Wu, R., Yang, Y.X., Ikegawa, S., et al. (2022) Targeting macrophagic SHP2 for ameliorating osteoarthritis via TLR signaling. *Acta Pharm. Sin. B*, **12**, 3073-3084.

Tu, J., Huang, W., Zhang, W., Mei, J. & Zhu, C. (2020) The emerging role of lncRNAs in chondrocytes from osteoarthritis patients. *Biomed. Pharmacother.*, **131**, 110642.

Wang, C., Brisson, B.K., Terajima, M., Li, Q., Hoxha, K., Han, B., Goldberg, A.M., Sherry Liu, X., Marcolongo, M.S., Enomoto-Iwamoto, M., Yamauchi, M., Volk, S.W. & Han, L. (2020) Type III collagen is a key regulator of the collagen fibrillar structure and biomechanics of articular cartilage and meniscus. *Matrix Biol.*, **85-86**, 47-67.

Wang, R., Shiu, H.T. & Lee, W.Y.W. (2022) Emerging role of lncRNAs in osteoarthritis: An updated review. *Front. Immunol.*, **13**, 982773.

Wang, W., Min, L., Qiu, X., Wu, X., Liu, C., Ma, J., Zhang, D. & Zhu, L. (2021) Biological Function of Long Non-coding RNA (LncRNA) Xist. *Front. Cell Dev. Biol.*, **9**, 645647.

Wood, M.J., Miller, R.E. & Malfait, A.M. (2022) The Genesis of Pain in Osteoarthritis: Inflammation as a Mediator of Osteoarthritis Pain. *Clin. Geriatr. Med.*, **38**, 221-238.

Xie, K., Zheng, C., Gu, W., Jiang, Z., Luo, C., Luo, J., Diao, Y.,

Wang, G., Cong, Z., Yao, X., Hu, L. & Shen, Y. (2022) A RASSF8-AS1 based exosomal lncRNAs panel used for diagnostic and prognostic biomarkers for esophageal squamous cell carcinoma. *Thorac. Cancer*, **13**, 3341-3352.

Yang, B., Xu, L. & Wang, S. (2020) Regulation of lncRNA-H19/miR-140-5p in cartilage matrix degradation and calcification in osteoarthritis. *Ann. Palliat. Med.*, **9**, 1896-1904.

Zeng, Z., Fei, L., Yang, J., Zuo, J., Huang, Z. & Li, H. (2021) MiR-27a-3p Targets GLP1R to Regulate Differentiation, Autophagy, and Release of Inflammatory Factors in Pre-Osteoblasts via the AMPK Signaling Pathway. *Front. Genet.*, **12**, 783352.

Zhang, Q., Sun, C., Liu, X., Zhu, C., Ma, C. & Feng, R. (2023) Mechanism of immune infiltration in synovial tissue of osteoarthritis: a gene expression-based study. *J. Orthop. Surg. Res.*, **18**, 58.