Inhibitory role of long non-coding RNA OIP5-AS1 in rheumatoid arthritis progression through the microRNA-448–paraoxonase 1–toll-like receptor 3–nuclear factor κB axis

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Abstract
Rheumatoid arthritis (RA) is an autoimmune disorder with dysregulation of long non-coding RNAs (lncRNAs) possibly involved. This study aimed to inquire into the roles of lncRNA OIP5-AS1 in RA progression. A rat model of RA was induced. Overexpression of OIP5-AS1 was introduced in the model rats, and the changes in paw swelling, RA severity and the inflammatory factors interleukin (IL)-1β, IL-10, IL-6 and tumour necrosis factor α were measured. Fibroblast-like synoviocytes (FLSs) from RA patients were collected for in vitro experiments. A gain- and loss-of function study of OIP5-AS1, miR-448 and paraoxonase 1 (PON1) was performed to explore their roles in RA-FLS growth, apoptosis and inflammation. A toll-like receptor 3 (TLR3)-specific agonist, polyinosine-polycytidylic acid, or a nuclear factor κB (NF-κB)-specific antagonist, QNZ, was administrated in RA-FLSs. Consequently, overexpression of OIP5-AS1 reduced the symptom severity and the levels of inflammatory factors in RA rats. OIP5-AS1 could bind to miR-448 to up-regulate PON1 expression. Further overexpression of miR-448 reversed the effects of OIP5-AS1, while overexpression of PON1 inhibited RA-FLS growth and inflammation. In addition, TLR3 activation promoted RA progression. To conclude, this study evidenced that lncRNA OIP5-AS1 may mitigate RA progression through the miR-448–PON1 axis and through the inactivation of the TLR3–NF-κB signalling pathway.

KEYWORDS
long non-coding RNA OIP5-AS1, microRNA-448, paraoxonase 1, rheumatoid arthritis, TLR3/NF-κB signalling pathway

1 | INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic autoimmune disorder that features severe synovial proliferation, inflammation, rheumatoid pannus formation and autoantibody production (Hoxha, 2018). The disease is attributed to a complex interaction between genes and environment, resulting in an impairment in immune tolerance, increased synovial inflammation, matrix destruction, and damage to bone and cartilage in a characteristic symmetric pattern (Pala, Diaz, Blomberg, & Frasca, 2018). Currently the first-line therapies...
for RA are conventional synthetic disease-modifying anti-rheumatic drugs (DMARDs), mainly methotrexate and sulfasalazine, and some target-specific DMARDs blocking pro-inflammatory cytokines (Lau et al., 2019). Increased secretion of inflammatory factors, mainly tumour necrosis factor-α (TNF-α) and several interleukins (ILs), is often observed in inflamed synovium of patients and is closely linked with RA progression (Kaczynski et al., 2019). In addition, fibroblast-like synoviocytes (FLSs), an important component of the synovial hyperplasia closely linked with invading cartilage in RA, have been recognized as a crucial target for RA therapy (Ganesan & Rasool, 2017).

Emerging evidence has indicated significant roles of non-coding RNAs (ncRNAs) in inflammation and autoimmune regulation, mainly including long non-coding RNAs (lncRNAs), microRNAs (miRNAs) and circular RNAs (Wang et al., 2019). Abnormal expression of lncRNAs and miRNAs has been revealed in RA-FLSs and is closely linked with RA-FLS proliferation and cytokine production as well as RA pathogenesis (Karami et al., 2020). In addition, ncRNAs may compete with mRNAs for the same limited pools of miRNAs by binding to the shared miRNA response elements, termed competitive endogenous RNA (ceRNA) (Smillie, Sirey, & Ponting, 2018). Several ceRNA networks have been validated in RA progression (Bi et al., 2019; Li et al., 2019). Interestingly, down-regulation of opa-interacting protein 5 antisense RNA 1 (OIP5-AS1), a lncRNA with oncogenic functions in many human cancer types, has been documented to facilitate osteoarthritis inflammation (Zhi et al., 2020). This attracted our attention to its potential role in RA. Here, we identified miR-448 as a target of OIP5-AS1, and paraoxonase 1 (PON1) as a target of miR-448. miR-448 was found to be abundant in regulatory T cell-depleted mice (a representative model of autoimmune disease) and in RA patients (Jin et al., 2018). PON1 is an esterase that metabolizes oxidized lipids and organophosphates involved in the maintenance of oxidative balance, and its activity was reported to be reduced in RA patients (Bae & Lee, 2019). But the exact roles of miR-448 or PON1 remain unknown in RA. In addition, toll-like receptors (TLRs) and a component of one of their important downstream signalling pathways, nuclear factor κB (NF-κB), play important roles in inflammatory and autoimmune diseases including RA (Ain, Batool, & Choi, 2020; Andreakos, Sacre, Foxwell, & Feldmann, 2005; Joob & Wiwanitkit, 2018). Compared to TLR4, the most studied of the TLRs, TLR3 has been less investigated with regard to its role in RA pathogenesis. Taking the above together, we hypothesized that lncRNA OIP5-AS1 could alleviate RA progression through interactions with miR-448 and PON1. Artificial up-regulation or down-regulation of these molecules was carried out to identify their specific roles in RA, and the involvement of the TLR3–NF-κB signalling pathway was explored as well.

2 METHODS

2.1 Ethical approval

The study was ratified and supervised by the Clinical Ethical Committee of West China Hospital of Sichuan University. Signed informed consent was received from each eligible participant. This study conformed to the standards of the Declaration of Helsinki except for registration in a database. Animal experiments were approved by the Animal Experiment Ethical Committee of West China Hospital of Sichuan University (Approval No. 2017034A). All experimental procedures were conducted in line with the Animals in Research: Reporting In Vivo Experiments (ARRIVE) guidelines. Great effort was made to minimize the suffering and usage of animals.

2.2 Reagents, lentiviral vectors and mimic antibodies

Freud’s complete adjuvant (FCA), used to establish the rat RA model, was purchased from Merck KGaA (Darmstadt, Germany). The antibodies to surface biomarkers of FLSs used for flow cytometry were CD44–fluorescein isothiocyanate (CD44-FITC, cat. no. 338803, BioLegend, San Diego, CA, USA), CD14–FITC (cat. no. 367115, BioLegend), CD90–FITC (cat. no. 206105, BioLegend), CD68–FITC (cat. no. 367115, BioLegend), CD55–phycoerythrin (CD55-PE, cat. no. 311308, BioLegend). Primary antibodies used in western blot analysis and immunohistochemistry were TLR3 (cat. no. 6961, Cell Signaling Technology, Danvers, MA, USA), CD44–fluorescein isothiocyanate (CD44-FITC, cat. no. 338803, BioLegend, San Diego, CA, USA), CD14–FITC (cat. no. 367115, BioLegend), CD90–FITC (cat. no. 206105, BioLegend), CD68–FITC (cat. no. 367115, BioLegend), CD55–phycoerythrin (CD55-PE, cat. no. 311308, BioLegend). Primary antibodies used in western blot analysis and immunohistochemistry were TLR3 (cat. no. 6961, Cell Signaling Technology, Danvers, MA, USA), CD44–fluorescein isothiocyanate (CD44-FITC, cat. no. 338803, BioLegend, San Diego, CA, USA), CD14–FITC (cat. no. 367115, BioLegend), CD90–FITC (cat. no. 206105, BioLegend), CD68–FITC (cat. no. 367115, BioLegend), CD55–phycoerythrin (CD55-PE, cat. no. 311308, BioLegend).
antagonist 4-N-[2-(4-phenoxyphenyl)ethyl]quinazoline-4,6-diamine (QNZ) from Abcam (cat. no. ab141588). The sequences of OIP5-AS1 and PON1 transcripts were obtained from the GenBank Database (https://www.ncbi.nlm.nih.gov/), and the lentiviral (Lv) vectors for RNA overexpression and small interfering RNAs (siRNAs), as well as the negative control (NC) vector and the scramble siRNA were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). miR-448 mimic and the negative control (NC) vector and the scramble siRNA were synthesized by Sangon Biotech Co., Ltd (Shanghai, China).

2.3 | A rat model with RA

Forty specific-pathogen-free grade male Sprague–Dawley rats (6–8 weeks old, 200 ± 20 g) were acquired from the Laboratory Animal Center of Anhui Medical University. All rats were healthy without drug administration history. The animals were housed in animal facilities at a constant temperature (18–22°C) and humidity (40–60%) in a 12 h light–dark cycle with free access to food and water, and the ambient noise was maintained at the lowest level. After 1 week of acclimation, the rats were allocated into the sham group, RA group, Lv-NC group and Lv-OIP5-AS1 group (n = 10 in each group). RA was induced by intracutaneous injection of 0.1 ml of FCA (Li, Cai, Hu, Wu, & Li, 2015), while rats in the sham group were given an equal volume of liquid paraffin for control. RA rats in the Lv-NC and Lv-OIP5-AS1 groups were given 0.2 ml of Lv-NC (10^9 plaque forming units (PFU) ml^-1) or Lv-OIP5-AS1 (10^9 PFU ml^-1) through the caudal vein on the third day after FCA administration, and rats in the sham and RA groups were given an equal volume of normal saline. Animals were anaesthetized and blood was collected from the orbital vein and centrifuged at 3221 g to collect the supernatant for ELISA. In addition, exponentially growing RA-FLSs (see below) were suspended and seeded into 24-well plates (1×10^5 cells/well). After 24 h of culture, the supernatant of the culture medium was collected and centrifuged at 1812 g at 4°C for 1 min with the supernatant collected, and then the levels of inflammatory factors were determined.

2.5 | Haematoxylin and eosin staining

Rat synovial tissues were collected, fixed in 4% paraformaldehyde, embedded in paraffin, and cut into consecutive sections (5 μm). Next, the sections were dewaxed, hydrated in ethanol, and then treated with haematoxylin. Next, the sections were rinsed with water for 2 min, differentiated in HCl–ethanol for 15 s, washed with ammonia for 10 min and then stained with eosin for 5 min. Thereafter, the sections were fixed in glycerol and observed under an inverted microscope (IX51, Olympus, Tokyo, Japan).

2.6 | Detection of inflammatory factors

The protein levels of inflammatory factors IL-1β, IL-6, IL-10 and TNF-α in rat serum and cell extracts were measured using the specific ELISA kits. In brief, 4 weeks after RA model establishment, rats were anaesthetized and blood was collected from the orbital vein and centrifuged at 3221 g to collect the supernatant for ELISA. In addition, exponentially growing RA-FLSs (see below) were suspended and seeded into 24-well plates (1×10^5 cells/well). After 24 h of culture, the supernatant of the culture medium was collected and centrifuged at 1812 g at 4°C for 1 min with the supernatant collected, and then the levels of inflammatory factors were determined.

2.7 | Subcellular localization of OIP5-AS1

An RNA fluorescence in situ hybridization (FISH) kit was used according to the instructions of the kit (RiboBio Co., Ltd, Bio, Guangzhou, Guangdong, China). In brief, a probe for OIP5-AS1 was designed and synthesized by the RiboBio Company. The probe was transfected into the RA-FLSs from RA patients, and the cells were collected 48 h later and observed under a microscope (BX41, Olympus) for fluorescence determination. In addition, an in situ hybridization (ISH) assay was performed using an ISH kit (RiboBio). Synovial tissues from rats were collected, embedded in paraffin, and cut into sections (5 μm). An HRP-labelled probe of OIP-AS1 was synthesized by RiboBio as well. The staining intensity was quantified using Image-ProPlus 6.0 (Media Cybernetics, Rockville, MD, USA) according to 10 non-overlapping areas in each section.

2.8 | RT–quantitative PCR

Total tissue and cell RNA was extracted using an RNAeasy mini Kit (Qiagen GmbH, Hilden, Germany) in line with the manufacturer’s protocols. RNA was reverse-transcribed to cDNA using a QuantiTect reverse transcription Kit (Qiagen GmbH) at 42°C for 2 h. The primers were purchased from Thermo Fisher Scientific and are listed in Table 1. The q-PCR volume was 25 μl, and the reaction conditions were as follow: pre-denaturation at 95°C for 2 min, followed by 45 cycles...
### TABLE 1  Primer sequences in RT-qPCR

| Gene        | Primer sequence (5’-3’) |
|-------------|------------------------|
| OIP5-AS1    | F: GAGGACGTGCTGGGGCT   |
|             | R: CTGAGTCTCATGGCGGAATC |
| PON1        | F: C CGCTGATATCAAGAGGGTAGT |
|             | R: AA TCCACATCGGCAGGATAG |
| miR-448     | F: CCCC CGACCGATTTCAAGGGTAGT |
|             | R: GAAGTCTGGGAATCGATCTGG |
| GAPDH       | F: TATGATGATATCAAGAGGGTAGT |
|             | R: TGTATCGAAACTCATGCTAC |
| U6          | F: GCTTGCGGAGCACATATACTAAAAT |
|             | R: CGCTCAGGAATTTGGCGCTCAT |

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PON1, paraoxonase 1.

...of denaturation at 95°C for 30 s and annealing at 59°C for 30 s, and a final extension at 72°C for 30 s. The SYBR® Premix Ex Taq™ contained 50 ng cDNA, 200 μM dNTP, 2.5 units of Taq DNA polymerase (Takara Biotechnology Co., Ltd, Dalian, China) and 200 μM primers (Takara). Relative miRNA or mRNA expression was calculated using the 2^−ΔΔCt method (Livak & Schmittgen, 2001) with GAPDH and U6 as the internal references.

### 2.9 Clinical sample collection

A total of 16 RA patients who underwent knee replacement, debridement of synovium in knee joints or meniscus repair surgeries from April 2017 to February 2019 in the West China Hospital of Sichuan University were enrolled in this study with the synovial tissue samples collected. The patients were diagnosed with the criteria of the American college of Rheumatology without other joint abnormality or systemic diseases. All samples were obtained from the resected tissues.

### 2.10 RA-FLS culture and transfection

The redundant fat, blood vessels and fibrous tissues were removed, after which the synovial tissues from RA patients were washed in phosphate-buffered saline (PBS), chopped into 1 mm³ fragments, and loaded into culture flasks. The flasks were filled with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and placed in an incubator at constant 37°C with 5% CO₂. In general, FLSs would grow out from the tissue fragments on the second or the third day and reach a 95% primary cell confluence within 3 weeks. Next, the medium was renewed every 2 or 3 days, and the suspended cells were washed away with 0.25% trypsin–EDTA when the adherent cells reached an 80% confluence. The adherent cells were then resuspended and cultured in medium, and FLSs at passage 3–6 were harvested for the following experiments.

Well growing FLSs were allocated into 12 groups: (1) Lv-NC group (cells were infected with 100 nM Lv-NC), (2) Lv-OIP5-AS1 group (cells were infected with 100 nM Lv-OIP5-AS1), (3) siRNA-Scr group (cells were transfected with 50 nM siRNA scramble), (4) si-OIP5-AS1-1–3 group (cells were transfected with 3 siRNAs targeting OIP5-AS1), (5) Lv-OIP5-AS1 + mock group (cells were further transfected with 100 nM mock in the presence of OIP5-AS1 overexpression), (6) Lv-OIP5-AS1 + miR-448 group (cells were further transfected with 100 nM miR-448 mimic in the presence of OIP5-AS1 overexpression), (7) si-OIP5-AS1 + Lv-NC group (cells were further transfected with 100 nM Lv-NC in the presence of OIP5-AS1 down-regulation), (8) si-OIP5-AS1 + Lv-PON1 group (cells were further transfected with 100 nM Lv-PON1 in the presence of OIP5-AS1 down-regulation), (9) Lv-OIP5-AS1 + DMSO group (cells were further treated with dimethyl sulfoxide (DMSO) in the presence of OIP5-AS1 overexpression), (10) Lv-OIP5-AS1 + PIC group (cells were further treated with 10 ng ml⁻¹ of the TLR3-specific agonist PIC in the presence of OIP5-AS1 overexpression), (11) si-OIP5-AS1 + DMSO group (cells were further treated with DMSO in the presence of OIP5-AS1 down-regulation) and (12) si-OIP5-AS1 + QNZ group (cells were further treated with 0.5 μM of the NF-κB-specific antagonist QNZ in the presence of OIP5-AS1 down-regulation).

### 2.11 Identification of FLSs

Passage 3 FLSs were observed under an optical microscope, and the LFS biomarkers (CD55, CD90, CD44, CD14 and CD68) on the cells were measured on a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA, USA). The data were collected and analysed with Cell Quest Software (BD Biosciences).

### 2.12 Cell viability detection

Well-growing FLSs were seeded into 96-well plates at 3 × 10³ cells/100 μl. The cell number and viability were measured on a Cell Titer Glo Luminescent Cell Viability Assay System (Promega Corp., Madison, WI, USA). The luminous signal was measured with an LMax II (Molecular Devices, San Jose, CA, USA).

### 2.13 5-Ethynyl-2′-deoxyuridine labelling assay

Cells were seeded into 24-well plates at 5 × 10⁴ cells ml⁻¹. 5-Ethynyl-2′-deoxyuridine (EdU) labelling was performed using a Cell-Light EdU DNA Replication Assay Kit (cat. no. C10310-1, RiboBio) in line with the kit’s instructions. Cells were incubated with EdU (1:1000) for 8 h, washed with PBS, fixed in paraformaldehyde, osmosed in 0.3% Triton X-100–PBS for 10 min, washed, and then incubated with Apollo labelling reaction solution in the dark for 30 min. After that, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min, and...
the labelling was observed and captured under a laser confocal microscope (Olympus).

2.14 | Hoechst 33248 staining

Exponentially growing FLSs were seeded into six-well plates at 5 × 10⁶ cells per well and cultivated at 37°C for 24 h. Next, the medium was removed, and cells were fixed in 4% paraformaldehyde for 10 min, stained with Hoechst 33258 reagent (at a final concentration of 5 mg l⁻¹) in the dark for 30 min, washed, sealed and then observed under a fluorescence microscope.

2.15 | Caspase-3 activity detection

Activity of caspase-3 in cells was assessed utilizing a Caspase-Glo Kit (Promega) (Xu et al., 2015). In brief, FLSs were seeded into 96-well plates at a density of 1 × 10⁶ cells per well. Then the cells were given recombinant Caspase3-Glo reagent for 2 h, and the luminous signal produced by the caspase 3-DVED reaction was determined using a luminometer (Luminoskan Ascent, Thermo Electron, Helsinki, Finland).

2.16 | Flow cytometry

Well-growing FLSs were detached in trypsin and centrifuged to discard the supernatant. Then the FLSs were washed in cold PBS at 4°C, centrifuged at 179 g for 5 min, resuspended to 1 × 10⁶ cells ml⁻¹, washed with 1 ml cold PBS, centrifuged and resuspended again. Next, the cell suspension was treated with 2 μl annexin-V–FITC (20 μg ml⁻¹) and placed on ice in a dark environment for 15 min and then transferred to the flow tubes. Next, each tube was filled with 300 μl PBS and 1 μl propidium iodide (PI; 50 μg ml⁻¹), and run on the flow cytometer. The ratio of apoptotic cells was measured within 30 min.

2.17 | Dual luciferase reporter gene assay

The binding sites between miR-448 and OIP5-AS1 and between miR-448 and PON1 were first predicted on StarBase (http://starbase.sysu.edu.cn/). Then, the sequence containing the bindings sites between miR-448 and the 3′-untranslated region of OPI5-AS1 or PON1 were synthesized and inserted into the pMIR-REPORT™ luciferase reporter vector (Ambion, Company, Austin, TX, USA) to construct the wild-type (WT) vectors (OIP5-AS1-WT or PON1-WT), and then the corresponding mutant type (MUT) vectors were constructed as well. Exponentially growing cells were seeded into 96-well plates until 70% confluence. Next, well-constructed WT or MUT vectors were co-transfected either with mimic control or miR-448 mimic into cells using a Lipofectamine™ 2000 kit (Thermo Fisher Scientific). Forty-eight hours later, the cells were collected and lysed, and the relative luciferase activity was measured using a fluorimeter (Glomax20/20, Promega) and a Luciferase Assay Kit (BioVision, San Francisco, CA, USA).

2.18 | Western blot analysis

FLSs in each group were washed in PBS and lysed to collect total protein. The concentration of protein was measured by a DC protein detection kit (Bio-Rad Laboratories, Hercules, CA, USA). Next, cell lysates containing 50 μg protein was run on 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with Tris-buffered saline–Tween buffer for 1 h and cultured with the primary antibodies overnight at 4°C. Then, the membranes were washed 3 times and cultured with HRP-labelled secondary antibody at 37°C for 1 h. The protein bands were detected using the enhanced chemiluminescence method on a X-AR Film (Eastman Kodak Company, Rochester, NY, USA).

2.19 | Statistical analysis

Each experiment in vitro was independently performed no less than three times. SPSS Statistics 22.0 (IBM Corp., Armonk, NY, USA) was utilized for data analysis. A Kolmogorov–Smirnov test was used to check the data were normally distributed. Measurement data were expressed as means ± standard deviation (SD). Differences between two groups were evaluated using Student’s t test, while differences among multiple groups were compared using one-way or two-way analysis of variance (ANOVA). Tukey’s multiple comparisons test was used for the pairwise comparison after ANOVA analysis. P was obtained from a two-tailed test and P < 0.05 was considered to present a statistically significant difference.

3 | RESULTS

3.1 | Overexpression of OIP5-AS1 alleviates RA severity in rats

As shown in Figure 1a, rats appeared with red and swollen limbs from the second week after model establishment, and this symptom was partly alleviated in the fourth week; the corresponding statistical summary is shown in Figure 1b,c. Compared to the sham-operated rats, rats in the other groups presented red and swollen limbs on the first, second, third and fourth weeks, indicating a successful RA induction. Next, haematoxylin and eosin (HE) staining of rat synovial tissues identified significant infiltration of immune cells in RA model rats (Figure 1d). The protein levels of inflammatory cytokines including IL-1β, IL-6, IL-10 and TNF-α, according to the ELISA results, were
Overexpression of OIP5-AS1 alleviates RA severity in rats. (a) RA rat limbs became red and swollen from the second week after model establishment. (b,c) The scoring of limb swelling (b) and RA (c). (d) Infiltration of immune cells in rat synovial tissues measured using HE staining. (e) Protein levels of IL-1β, IL-6, IL-10 and TNF-α in rat serum measured using ELISA. (f) OIP5-AS1 expression in rat synovial tissues detected using RT-qPCR. (g) OIP5-AS1 expression in rat synovial tissues after Lv-OIP5-AS1 overexpressing vector transfection detected using RT-qPCR. Data are expressed as means ± SD. In (b,c) data were analysed using two-way ANOVA; in (d,e) data were analysed using one-way ANOVA with Tukey’s multiple comparison test for the post hoc test after ANOVA; and in (f,g) data were analysed by the unpaired t test. (b–f) *P < 0.05, **P < 0.01 vs. sham group; #P < 0.05 vs. Lv-NC group; (g) **P < 0.01 vs. Lv-NC group.

3.2 OIP5-AS1 serves as a sponge for miR-448 to up-regulate PON1 expression and inactivates the TLR3–NF-κB signalling pathway

The mitigating effects of OIP5-AS1 on RA in rats prompted us to explore the underlying molecular mechanisms. We first predicted the subcellular localization of OIP5-AS1 on a bioinformatics website (http://www.rna-society.org/rnalocate/index.html), which suggested a cytoplasmic localization. This was validated by an ISH assay, which suggested that OIP5-AS1 was mainly localized in cytoplasm in rat synovial tissues (Figure 2a). Next, we predicted miR-448 as a target of OIP5-AS1 by StarBase (http://www.starbase.org) (Figure 2b), and a following luciferase assay validated this binding relationship since co-transfection of miR-448 mimic and OIP5-AS1-WT led to decreased luciferase activity in cells (Figure 2c). Likewise, we identified PON1 mRNA as a target of miR-448 in a same manner (Figure 2d,e). Next, the expression of miR-448 and PON1 in rats was determined. It was found that the miR-448 expression was notably increased while PON1 expression was decreased in synovial tissues in RA rats. The subsequent overexpression of OIP5-AS1 led to a decline in miR-448 expression while there was a notable increase in PON1 expression in rats (Figure 2f,g). In addition, we measured TLR3 expression and NF-κB p65 phosphorylation in rat synovial tissues using western blot analysis. The results showed that TLR3 expression and NF-κB p65 phosphorylation were increased in RA rats, and overexpression of OIP5-AS1 inhibited TLR3 expression and NF-κB p65 phosphorylation (Figure 2h).

3.3 Overexpression of OIP5-AS1 decreases proliferation of RA-FLSs

To further identify the function of OIP5-AS1, in vitro experiments were performed on RA-FLSs extracted from 16 RA patients. According to the flow cytometry with regard to the FLS biomarkers CD55, CD90, CD44, CD14 and CD68, the extracted cells were confirmed as RA-FLSs (Figure 3a). To validate the synteny of OIP5-AS1 in
human and rat cells, OIP5-AS1 subcellular localization in RA-FLSs was determined using a FISH assay. Similarly, a cytoplasmic localization of OIP5-AS1 was identified (Figure 3b). Next, artificial up-regulation or down-regulation of OIP5-AS1 was induced in cells by infecting OIP5-AS1 Lv-overexpressing vectors, or transfecting siRNAs (siRNA-1, siRNA-2, siRNA-3) targeting OIP5-AS1 and the corresponding scramble siRNA were supplied to RA-FLSs, and then RT-qPCR was performed to measure OIP5-AS1 expression. (d) Number of cells in S stage (EdU-labelled cells) determined using EdU labelling assay. (e) Cell viability detected using a Cell Titer-Glo Kit. Three independent experiments were performed; data are presented as means ± SD; in (c,d) data were analysed using one-way ANOVA, and in (e) data were analysed using two-way ANOVA, and Tukey’s multiple comparison test was used for the post hoc test after ANOVA. *P < 0.05, **P < 0.01 vs. Sham group; #P < 0.05, ##P < 0.01 vs. Scr group
Figure 4: Overexpression of OIP5-AS1 promotes RA-FLS apoptosis and inhibits expression of inflammatory factors in vitro. (a,b) Apoptosis of RA-FLSs determined using Hoechst 33258 staining (a) and flow cytometry (b), respectively. (c) Caspase-3 activity in RA-FLSs detected using a Caspase-3 Assay Kit. (d) Levels of IL-1β, IL-6, IL-10 and TNF-α in extracted RA-FLSs evaluated using ELISA. Three independent experiments were performed; data are presented as means ± SD; in all panels data were analysed using one-way ANOVA and Tukey’s multiple comparison test. *P < 0.05, **P < 0.01 vs. Lv-NC group; #P < 0.05, ##P < 0.01 vs. Scr group.

3.4 Overexpression of OIP5-AS1 promotes RA-FLS apoptosis and inhibits expression of inflammatory factors in vitro

Apoptosis of RA-FLS was determined. Hoechst 33258 staining suggested that overexpression of OIP5-AS1 promoted RA-FLS apoptosis, while the cell apoptosis was decreased when OIP5-AS1 was down-regulated (Figure 4a). Flow cytometry presented similar trends, which suggested that both early and late apoptotic RA-FLSs were increased following OIP5-AS1 overexpression, but transfection of si-OIP5-AS1 led to the reverse results (Figure 4b). Moreover, overexpression of OIP5-AS1 was also found to decrease the caspase-3 activity in RA-FLSs (Figure 4c). We further detected the levels of IL-1β, IL-6, IL-10 and TNF-α in extracted RA-FLSs using ELISA, and the results suggested that the levels of these factors were notably decreased in vitro as well after OIP5-AS1 overexpression, and correspondingly, down-regulation of OIP5-AS1 increased the levels of inflammatory cytokines (Figure 4d).

3.5 Overexpression of miR-448 (or overexpression of PON1) partially reverses the effect of OIP5-AS1 (or si-OIP5-AS1) on RA-FLSs

To confirm the involvement of miR-448 and PON1 in the OIP5-AS1-regulated events, miR-448 mimic was further transfected into RA-FLS in the presence of stable OIP5-AS1 overexpression, while Lv-PON1 overexpressing vector was infected into RA-FLSs with silenced OIP5-AS1. The RT-qPCR results showed that the transfection was successfully performed (Figure 5a). Next, the EdU labelling and Cell Titer-Glo assay results identified that the cell proliferation and viability inhibited by OIP5-AS1 was partially reversed by overexpression of miR-448, while the proliferation and viability of RA-FLS enhanced by si-OIP5-AS1 were repressed following PON1 overexpression (Figure 5b,c). Also, further overexpression of miR-448 led to a significant decrease in cell apoptosis and caspase-3 activity in RA-FLSs in the presence of OIP5-AS1 overexpression, but overexpression of PON1 led to opposite trends in RA-FLSs with silenced OIP5-AS1 (Figure 5d,e). Further overexpression of miR-448 (or overexpression of PON1), similarly, presented reversed effects against overexpression of OIP5-AS1 (or silencing of OIP5-AS1) in RA-FLS regarding the levels of inflammatory factors IL-1β, IL-6, IL-10 and TNF-α (Figure 5f).
FIGURE 5  Overexpression of miR-448 (or overexpression of PON1) partially reverses the effect of OIP5-AS1 (or si-OIP5-AS1) on RA-FLS. (a) miR-448 mimic was further transfected into RA-FLSs with stably overexpressed OIP5-AS1, while Lv-PON1 overexpression vector was transfected into RA-FLSs with silenced OIP5-AS1, and then expression of miR-448 and PON1 in cells was determined using RT-qPCR. (b) Number of cells in S stage (EdU-labelled cells) determined using EdU labelling assay. (c) Cell viability detected using a Cell Titer-Glo Kit. (d) Apoptosis of RA-FLSs determined using flow cytometry. (e) Caspase-3 activity in RA-FLSs detected using a Caspase-3 Assay Kit. (f) Levels of IL-1β, IL-6, IL-10 and TNF-α in RA-FLSs evaluated using ELISA Kits. Three independent experiments were performed; data are presented as mean ± SD. In (a) data were analysed using unpaired t test; in (b,d,e,f) data were analysed using one-way ANOVA; and in (c) data were analysed using two-way ANOVA, and Tukey’s multiple comparison test was used for the post hoc test after ANOVA. *P < 0.05, **P < 0.01 vs Lv-OIP5-AS1 + mock group; #P < 0.05, ##P < 0.01 vs. si-OIP5-AS1 + Lv-NC group

3.6 TLR3-specific agonist PIC (or the NF-κB-specific antagonist QNZ) reverses the effects of OIP5-AS1 (or si-OIP5-AS1)

Considering overexpression of OIP5-AS1 led a TLR3–NF-κB defect in RA rat synovial tissues, a TLR3-specific agonist, PIC, was administrated into RA-FLSs with overexpressed OIP5-AS1, while a NF-κB-specific antagonist, QNZ, was introduced into RA-FLSs with silenced OIP5-AS1. It was found that PIC administration increased cell viability and proliferation but decreased apoptosis of RA-FLSs. Conversely, QNZ administration decreased cell viability and proliferation but increased apoptosis following OIP5-AS1 expression (Figure 6a–d). In addition, activation of TLR3 led to increased levels of IL-1β, IL-6, IL-10 and TNF-α, and accordingly inactivation of NF-κB resulted in a decrease in these cytokines (Figure 6e).

4 DISCUSSION

Conventional regimens based on non-targeted systemic immune suppression have inevitable side effects, and gene therapy, which holds considerable potential in future RA treatment, has aroused wide concern (Deviatkin et al., 2020). FLS concentration has been recognized as an emerging paradigm in the pathogenesis of RA, with dysregulation of IncRNAs and miRNAs suggested to mediate the proliferation and cytokine production in FLSs (Karami et al., 2020). Here, we identified that OIP5-AS1 bestowed the capacity to alleviate RA symptoms, and its up-regulation led to a decrease in FLS proliferation and viability and the production of inflammatory factors with the possible involvement of the miR-448–PON1 axis and the inactivation of the TLR3–NF-κB signalling pathway.

The initial finding of the study was that OIP5-AS1 was expressed at a low level in synovial tissues in RA rats. Aberrant expression of many lncRNAs has been suggested to be involved in RA pathogenesis. For instance, LINC00152 was reported to promote proliferation while inhibiting apoptosis of RA-FLS (Wang et al., 2020c). LncRNA HOTAIR has been documented to serve as a miR-138 sponge and further inactivate the NF-κB signalling pathway to alleviate inflammation in RA (Zhang et al., 2017). Overexpression of IncRNA FER1L4 was demonstrated to inhibit the levels of inflammatory cytokines in RA-FLSs through down-regulating nucleotide oligomerization domain-like receptors 5 (Wang et al., 2020c). As for OIP5-AS1, it has been mainly identified as an oncogene in several human malignancies (Wang et al., 2020b; Wang, Qian, Xia, & Ye, 2020a). Importantly, OIP5-AS1 has been suggested to exert beneficial effects on OA by reducing inflammatory responses in chondrocyte cell lines (Zhi et al., 2020). Here, our study found that artificial up-regulation of OIP5-AS1 by Lv-OIP5-AS1 overexpressing vectors reduced the severity of RA and decreased immune cell infiltration in model rats, diminished proliferation, increased apoptosis of extracted FLS from...
FIGURE 6 A TLR3-specific agonist, PIC (or a NF-κB-specific antagonist, QNZ) reverses the effects of OIP5-AS1 (or si-OIP-AS1). (a) Number of cells in S stage (EdU-labelled cells) determined using EdU labelling assay. (b) Cell viability detected using a Cell Titer-Glo Kit. (c) Apoptosis of RA-FLS determined using flow cytometry. (d) Caspase-3 activity in RA-FLSs detected using a Caspase-3 Assay Kit. (e) Levels of IL-1β, IL-6, IL-10 and TNF-α in RA-FLS evaluated using ELISA kits. Three independent experiments were performed; data are presented as means ± SD. In (a,c,d,e) data were analysed using one-way ANOVA, and data in (b) data were analysed using two-way ANOVA, and Tukey’s multiple comparison test was used for the post hoc test after ANOVA. *P < 0.05, **P < 0.01 vs. Lv-OIP5-AS1 + DMSO group; #P < 0.05, ##P < 0.01 vs. si-OIP5-AS1 + DMSO group.

RA patients, and decreased secretions of inflammatory cytokines including IL-1β, IL-10, IL-6 and TNF-α in both rat synovial tissues and FLSs. In addition, despite the pro-inflammatory functions, IL-6 and TNF-α have also been noted to cooperatively promote cell cycle progression and proliferation of RA-FLS (Kaneshiro et al., 2019). These findings implied that OIP5-AS1 may be a promising target in RA treatment.

The findings above prompted us to further elucidate the potential mechanical molecules involved. Importantly, the online prediction and dual luciferase reporter gene assays suggested miR-448 as a target of OIP5-AS1, and PON1 was confirmed as a target mRNA of miR-448 in a similar manner. The findings that miR-448 expression was negatively, while PON1 expression was positively associated with OIP5-AS1 expression in RA rat synovial tissues further corroborated the possible binding relationships. OIP5-AS1 has been documented to target miR-448 to promote lung adenocarcinoma progression (Deng, Deng, Liu, Liang, & Wang, 2018). Importantly, miR-448 was suggested as one of the miRNAs that were significantly expressed in animal models with autoimmune disease and in RA patients (Jin et al., 2018). Here in this study, we validated that miR-448 was up-regulated in RA rat synovial tissues. On this basis, we further confirmed that overexpression of miR-448 reversed the inhibitory effects of OIP5-AS1 on RA-FLS progression and inflammatory cytokine production. On the other hand, overexpression of PON1 retarded growth and inflammation in RA-FLSs with silenced OIP5-AS1. PON-1 is an antioxidant enzyme that has key functions in high-density lipoprotein-mediated cardio-protection and prevents the oxidization of low-density lipoproteins as well as the inactivation of formed oxidized phospholipids, with its activity found to be decreased in RA (Rodriguez-Carrio et al., 2016a; Rodriguez-Carrio et al., 2016b).

In addition, our study identified that overexpression of OIP5-AS1 decreased TLR3 activity and NF-κB p65 phosphorylation in RA rat synovial tissues. The TLRs–NF-κB signalling pathways have been recognized to participate in RA pathogenesis, among which the roles of TLR4 have been largely investigated (Kim, Bang, Son, Baek, & Kim, 2018; Samaripita, Kim, Rasool, & Kim, 2020). Likewise, TLR3 up-regulation and NF-κB activation have been evidenced in synovial fibroblasts in a mouse model with RA (Yu, Xie, Jiang, Sun, & Huang, 2018). Engagement of TLR3 was also suggested to induce vascular endothelial growth factor and IL-8 in human RA-FLSs (Moon et al., 2010). Additionally, the experimental results that administration of PIC reversed the effects of OIP5-AS1, while QNZ treatment inhibited the RA-FLS growth and inflammatory cytokine production following OIP5-AS1 silencing further identified that inhibited TLR3 expression as well as NF-κB inactivation were involved in the OIP5-AS1-mediated protective events.
**CONCLUSION**

To conclude, the current study provided evidence that OIP5-AS1 could alleviate RA pathogenesis in both in vivo model rats and extracted RA-FLSs. It mitigated RA symptoms by sponging miR-448 and up-regulating PON1 activity, and the TLR3-NF-κB signaling was suppressed during this process. (Figure 7). We hope these findings may provide novel insights into RA treatment.

**COMPETING INTERESTS**

The authors declare no competing interests.

**AUTHOR CONTRIBUTIONS**

PY.Q. is the guarantor of integrity of the entire study and contributed to the concepts; Y.L. contributed to the design and definition of intellectual content and is responsible for research governance of this study; PY.Q. and Y.L. contributed to the experimental studies, data acquisition and statistical analysis; PY.Q. contributed to the manuscript preparation. Both authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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