# Long noncoding RNA FER1L4 regulates rheumatoid arthritis via targeting NLRC5

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## Abstract

### Objective

*Rheumatoid arthritis (RA) is a systematic autoimmune disease that cardinally affects the joints and other organs. Many people all over the world are suffering from the disease and no effective treatment has been established. Fibroblast-like synoviocytes (FLSs) play a critical role in the occurrence and development of RA. Long non-coding RNA Fer-1-like protein 4 (FER1L4) has been reported to participate in various cancers as a tumour suppressor. However, its clinical significance and biological role in RA is completely unknown.*

### Methods

*RT- qPCR or FISH were used to examine the expression of FER1L4 NLRC5, FER1L4 and inflammatory cytokine levels in synovial tissues (STs) from patients with RA or RA FLSs. Western blot was applied to examine the expression of NLRC5 and inflammatory cytokine levels in synovial tissues (STs) from patients with RA or RA FLSs. BrdU staining and MTT assay were used to examine the cell proliferation ability. The methylation-specific PCR was performed to analyse the methylation levels.*

### Results

*The level of FER1L4 significantly reduced in STs and FLSs, whereas the nucleotide oligomerisation domain-like receptors 5 (NLRC5) levels were increased. Overexpression of FER1L4 can decreased the level of NLRC5 and inflammatory cytokine level. The FER1L4 gene promoter was significantly methylated in RA STs and FLSs. More importantly, treatment with methylation inhibitor 5-aza-2-deoxycytidine (5-azadC) inhibited hypermethylation of FER1L4 promoter and the expression of NLRC5.*

### Conclusion

*These results indicated that FER1L4 regulates RA via targeting NLRC5 potentially. Therefore, this study may provide a candidate therapeutic target for RA.*

**Key words**

rheumatoid arthritis, fibroblast-like synoviocytes, long non-coding RNA Fer-1-like protein 4, nucleotide oligomerisation domain-like receptors 5, promoter methylation

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*Competing interests: none declared.*

## Introduction

Rheumatoid arthritis (RA) is one of the most commonly systematic autoimmune disease affecting multiple joints, with a worldwide incidence of around 1% (1). The characteristics of RA are inflammation in synovial joints as well as the progressive degradation in adjacent cartilage and bone and synovitis, synovial hyperplasia, joint space narrowing, and cartilage and bone destruction, is the major determinant of RA, however, until now no effective treatment method have been established (2, 3). RA patients have always suffered from reduction of the life quality because of the physical disability of the joint and severe chronic pain (4). Various studies have proved that smoking, diet, birthweight, socioeconomic status and location of residence are dangerous risk factors associated with RA (5, 6). RA has led to negative impact and huge economic pressures on the patients and family (7, 8). Therefore, it is of the utmost importance to identify the mechanism of RA occurrence and development and effective treatment for RA (9, 10). A growing number of researches have suggested that fibroblast-like synoviocytes (FLSs) play significant roles in both the occurrence and the progression of RA, although the precise pathogenesis of RA remains unknown. Activated FLSs hold key functions in synovial tissue transformation and joint destruction in the development of RA (11).

Long noncoding RNAs (lncRNAs) are a kind of RNAs that cannot code protein with a length of 200 nt to ~100 kb (12, 13). lncRNAs have been reported to have a variety of vital biological functions in the modulation of genes at transcriptional, post-transcriptional and chromosomal levels because of the widespread presence and performed an increasingly pivotal role in human diseases (14). Studies have shown that lncRNAs participate in osteosarcoma, Alzheimer's disease, liver cancer and other diseases (15, 16). An increasing number of studies have also shown that lncRNAs play an important role in RA. For example, lncRNA metastasis associated lung adenocarcinoma transcript 1 inhibited the proliferation and inflammation of FLSs in RA (17). Based on different RA and normal FLSs gene expression profiles, Zhang *u* identified 135 lncRNAs different expression (18). Fer-1-like protein 4 (FER1L4) is a newly identified lncRNA, which has been reported to associated with hepatocellular carcinoma, gastric cancer, endometrial carcinoma and colon cancer and FER1L4 mainly acted as a tumour suppressor in these cancers (19-22). However, the biological role and clinical function of FER1L4 in RA is still unknown. Therefore, in this study, we investigated expression of FER1L4 in RA synovial tissues (STs) and FLSs. We find that FER1L4 regulates RA via targeting nucleotide oligomerisation domain NOD-like receptors 5 (NLRC5) which has been proved to play a key role in inflammatory and autoimmune diseases. Our finding will provide new insights into the molecular function of FER1L4 in RA and hopefully may provide new diagnostic target for RA patients and human health.

## Materials and methods

### *Study subjects*

Twelve RA patients' samples and 16 trauma patients' samples were collected in this study. The 12 RA patients underwent surgical resection or joint replacement surgery at the First Affiliated Hospital of Harbin Medical University or the Second Affiliated Hospital of Harbin Medical University. The mean age of 12 RA patients are 53±4.2 years old and there were 6 males and 6 females. The mean age of the control group, 16 trauma patients, are 54±3.6 years old. The work described has been carried out in accordance with The Code of Ethics of the World Medical Association and Eligible patients were diagnostically confirmed in accordance with the American College of Rheumatology 1987 revised criteria for RA (23).

### *Cell culture and identification*

The extracted STs were finely cut into paste and digested with 0.15% filter-sterile type II collagenase (Gibco, USA) and cultured in high-glucose DMEM medium (Hyclone, Logan, UT) supplemented with 15% FBS (Aidenbach, Bavaria, Germany) for 6 days. Removed the non-adherent or dead cells. When

the cells reached 70–80% confluence, they were subcultured into a ratio of 1: 2. The FLSs at three passages were used for further experiments. In order to characterise the cytological phenotypes of synovial cultures the FLSs were identified by vimentin staining.

*Cell transfection*

The sequence of FER1L4 was using human liver cDNA (Invitrogen) and cloned into pCDNA3.1 vector (pFER1R4). NLRC5-siRNA and its control were acquired from GenePharma Corporation (Shanghai, China). FLSs were seeded into a six-well plate and grown to 60-80% confluency, then transfected plasmids or its control plasmids using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocols.

*Total RNA extraction and Quantitative real-time PCR analysis (qRT-PCR)*

Total RNA was isolated using TRIZOL reagent (Invitrogen) from STs and FLSs. According to the manufacturer's protocol. qRT-PCR was used to determine the mRNA expression of FER1L4, NLRC5, interleukin 6 (IL-6) and tumour necrosis factor-α (TNF-α). qRT-PCR was performed using SYBR-Green qPCR Master Mix (TAKARA, Japan). The primer sequences are as follows. The mRNA levels of β-actin acted as an internal control.

FER1L4 Forward: 5'- CCGTGTTGAGGTGCTGTTC -3',
FER1L4 Reverse: 5'- GGCAAGTCCACTGTCAGATG -3'.
NLRC5 Forward: 5'- CGCTCTGTGGCCACTTTCAG -3',
NLRC5 Reverse: 5'- TGCCCGCTGTGAGACTTCAT-3'.
TNF-α Forward: 5'- ACTCCCAGAAAAGCAAGCAA-3',
TNF-α Reverse: 5'- CAGTTCCACATCTCGGATCA-3'.
IL-6 Forward: 5'- GAGCCCACCAGGAACGAAAGTC-3',
IL-6 Reverse: 5'- TGTTGTGGGTGGTATCCTCTGTGAA-3'.
β-actin Forward: 5'- CCCATCTATGAGGGTTACGC-3',
β-actinReverse: 5'- TTTAATGTCACGCACGATTTC-3'.

*RNA fluorescence in situ hybridisation (FISH)*

FISH was performed according to the manufacturer's protocols. Briefly, FLSs were seeded and fixed. After 48 hr, digested cells with 1% TritonX-100 for 20 minutes and incubated cells with 2×SSC for 40 min at 37°C. Then, washed the slides and rehydrated in series alcohol, subsequently added to each slide, and hybridised for 24hr at 37°C. Finally, the sections were counterstained with DAPI for 5 minutes to stain the cell nucleus.

*ELISA assay*

IL-6 and TNF-αconcentration in the cultured supernatant were quantified by using the IL-6 ELISA KIT and TNF-α ELISA Kit (Boster, Wuhan, China) according to the manufacturer's instructions.

*MTT assay*

FLSs were seeded in 96-well culture plates at a density of 10000 per well. When the cells reached 60–80% confluence, they were transfected with the required oligonucleotide. After transfection for 24, 48, or 72 hr, the cells were stained with 20 μl of MTT (5 mg/ml), (Sigma, USA) for 4 h at 37°C. The cell medium was carefully aspirated and 150 μl of dimethyl sulfoxide was added to each well. Then, the absorbance at 490 nm was measured.

*Western blot analysis*

Total cell or tissue extracts were extracted using cell lysis buffer followed by immunoblotting with anti-NLRC5 (Abcam, USA), TNF-α (Abcam, USA), IL-6 (Abcam, USA) and anti-β-actin (Santa Cruz Biotechnology, CA). Cells were lysed in RIPA buffer with protease inhibitors (Roche Applied science) on ice for 20 min. Centrifugate for 20 minutes, 13500 rpm at 4°C. The supernatant was placed into a new tube and measured the protein concentration using assay kit (Applygen, China). 30 ug of cell lysates were resolved with 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Then, block the membranes with 5% skim milk for 1 hr at room temperature (RT). After blocking, the membranes were incubated with primary antibody for 1 hr at RT. Then, the membranes were incubated with secondary antibodies at RT for 1hr. The protein bands were visualised using enhanced chemiluminescence chromogenic substrate with horseradish peroxidase (Beijing, China).

*BrdU treatment and immunostaining*

For proliferation analysis, FLSs were treated with 2.5 μM BrdU (abcam, USA) for 2 hours and stained with anti-BrdU antibody (abcam, USA) staining. For immunostaining, the FLS cells were fixed with 4% paraformaldehyde for 20 min at RT and blocked with 5% BSA for 1hr. Then, the cells were washed three times and incubated with anti-BrdU antibody overnight at 4°C. After that, 20 μl 4′,6-diamidino- 2-phenylindole (DAPI) was added to the cells to stain the nuclear.

*Treatment with 5-azadC*

The FLSs were seeded into a six-well plate with a density of $1 \times 10^5$ cells/ml. After 12 hr, 1 μM 5-azadC was applied to cells and incubated for another 48 hr in 37°C.

*Methylation-Specific PCR (MSP)*

MSP was used to analyse the methylation level of FER1L4 promoter. Extracted the genomic DNA and amplified CpG sites of FER1L4 promoter region. The methylated and unmethylated sequence were as follows:

FER1L4-M forward: 5′-TATTTGGTTTAGTTTTTTGTTTCGT-3′;
FER1L4-M reverse: 5′-TCTAACTCAAAATTTCTTCTCCGTT-3′;
FER1L4-U forward: 5′-TTATTTGGTTTAGTTTTTTGTTTTGT-3′;
FER1L4-U reverse: 5′-TCTAACTCAAAATTTCTTCTCCATT-3′.

*Statistical analysis*

These data were expressed as mean ± standard deviation (SD), and the experiment was repeated at least three times. Statistical differences were assessed by one-way analysis of variance. Statistical differences were considered when $*p<0.05$, $** p<0.01$, $*** p<0.005$.

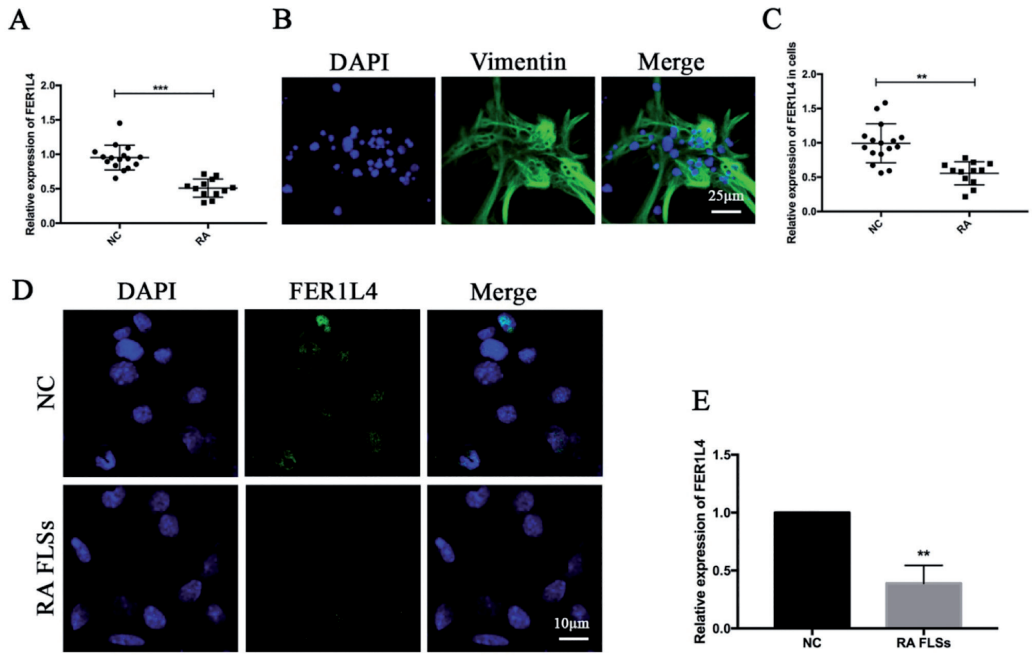


**Fig. 1.** FER1L4 expression is reduced in RA STs and RA FLSs.
**A**: The expression of FER1L4 in RA STs determined by RT-qPCR. *** $p<0.005$, NC: the control group, n=16; the RA group: rheumatoid arthritis tissues, n=12.
**B**: The FLSs were identified by vimentin in the immunofluorescence assay.
**C**: The expression of FER1L4 in RA FLSs determined by RT-qPCR. ** $p<0.01$, NC: the control FLSs group, n=16; the RA group: rheumatoid arthritis FLSs, n=12.
**D**: The expression of FER1L4 in RA FLSs measured by FISH.
**F**: Relative expression of FER1L4 in (**D**). ** $p<0.01$, NC: the control FLSs group, n=16; the RA group: rheumatoid arthritis FLSs, n=12. The experiments were independently repeated 3 times.

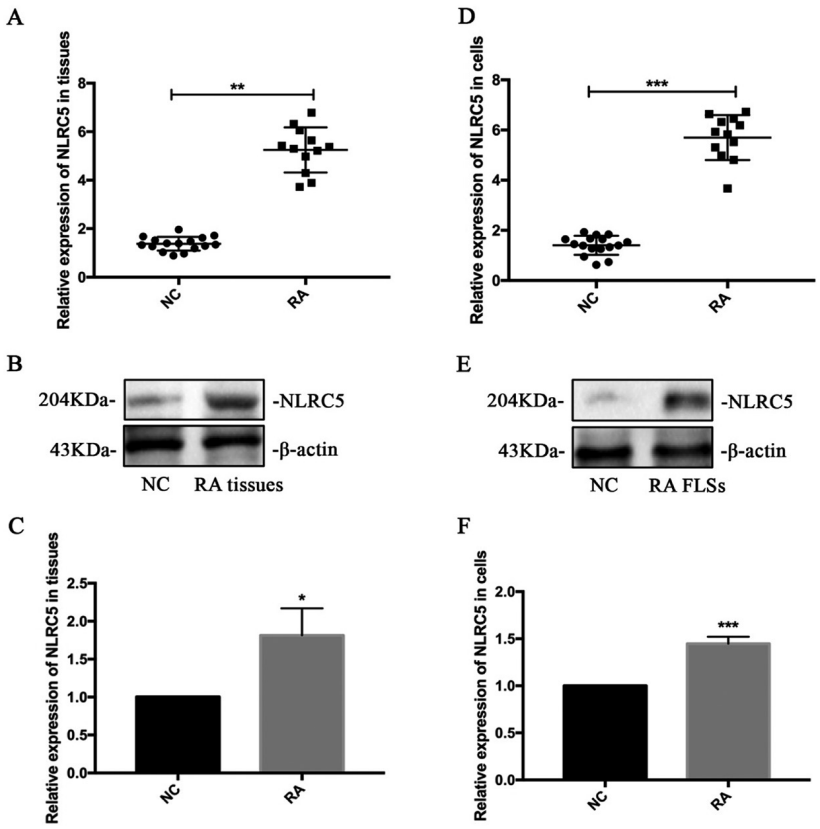


**Fig. 2.** The expression of NLRC5 in RA STs and RA FLSs.
**A**: The expression of NLRC5 in RA STs determined by RT-qPCR. ** $p<0.01$, NC: the control group, n=16; the RA group: rheumatoid arthritis tissues, n=12.
**B**: Western blot showed that the expression of NLRC5 in RA STs and control group.
**C**: Relative expression of NLRC5 in (**B**), * $p<0.05$. n=3.
**D**: The expression of NLRC5 in RA FLSs determined by RT-qPCR. *** $p<0.005$, NC: the control FLSs group, n=16; the RA group: rheumatoid arthritis FLSs, n=12.
**E**: Western blot showed that the expression of NLRC5 in RA FLSs and control group.
**F**: Relative expression of NLRC5 in (**E**), * $p<0.05$. n=3. The experiments were independently repeated 3 times.

## Results

### *The expression of FER1L4 is decreased in RA STs and RA FLSs*

Comparison of the expression of FER1L4 between the RA STs from patients and normal STs was examined via qRT-PCR. The results demonstrated that the expression of FER1L4 was significantly lower in the RA STs than in the normal STs (Fig. 1A). FLSs were identified by the morphology and vimentin staining (Fig. 1B). qRT-PCR was used to examined the expression of FER1L4 between the FLSs isolated from RA patients and normal individuals. The results showed that the expression of FER1L4 was significantly decreased in the RA FLSs (Fig. 1C). To further strength our results, fluorescence in situ hybridisation was used for FER1L4 and the results showed that the expression of FER1L4 in RA FLSs was decreased compared with normal FLSs (Fig. 1D, E). These data indicated that FER1L4 is lowly expressed in RA synovial tissue and RA FLSs.

### *NLRC5 was upregulated in RA STs and RA FLSs*

To identify the levels of NLRC5 in the RA STs and normal STs, qRT-PCR and western blot were initiated. The qRT-PCR data showed that the mRNA level of NLRC5 was higher in the RA STs compared with the normal STs (Fig.

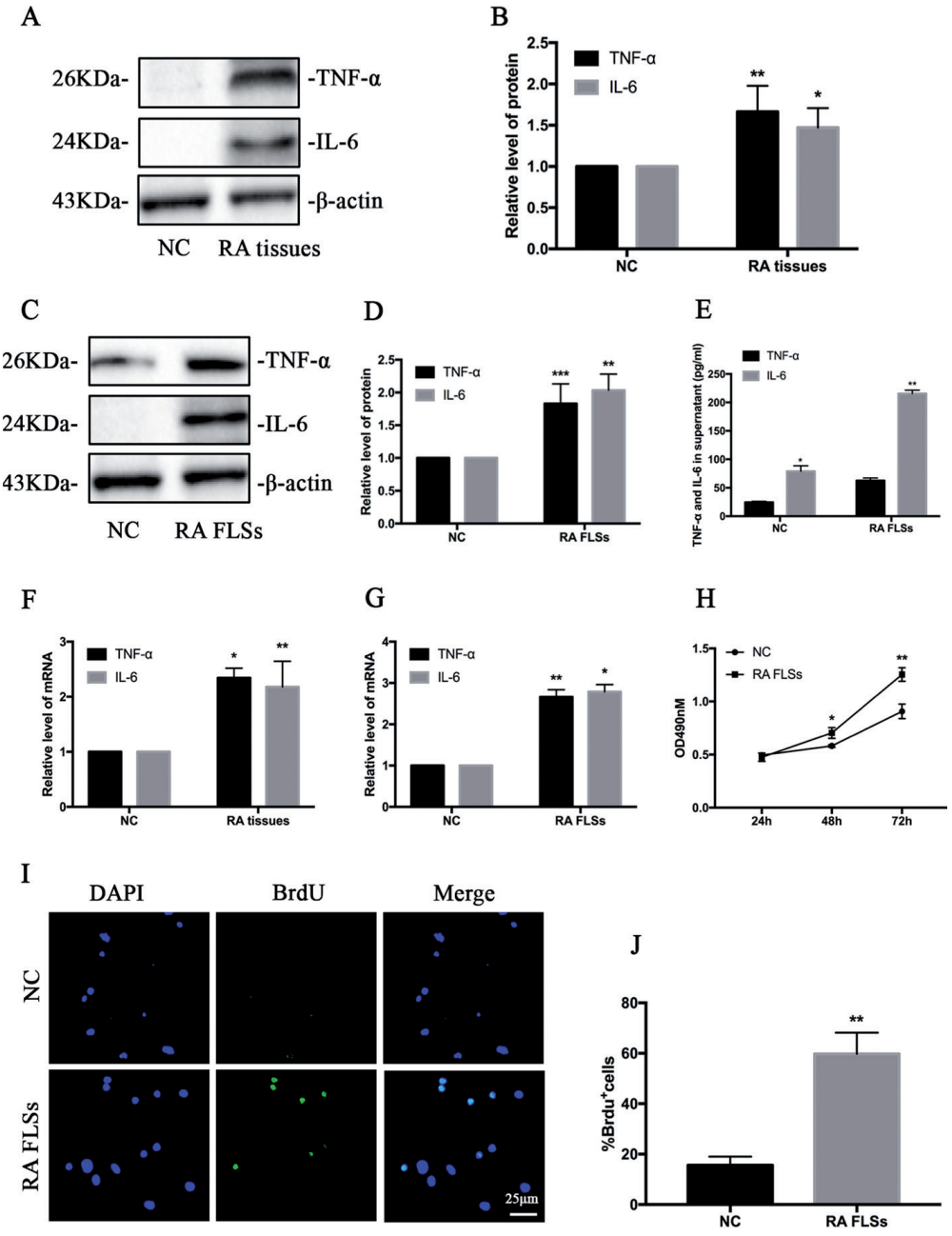


**Fig. 3.** The level of inflammatory cytokines and the proliferation of FLSs in RA.
**A**: Western blots showed the protein expression of TNF-α and IL-6 in RA STs and control group. Representative images are shown.
**B**: Relative protein levels of TNF-α and IL-6 in RA STs. The results are presented as mean ± SD, and. **p*<0.05, ***p*<0.01 *vs*. the control group.
**C**: Western blots showed the protein expression of TNF-α and IL-6 in RA STs. Representative images are shown.
**D**: Relative protein levels of TNF-α and IL-6 in RA FLSs and control FLSs. The results are presented as mean ± SD, and. ***p*<0.01, ****p*<0.005 *vs*. the control FLSs.
**E**: ELISA assay of TNF-α and IL-6 levels in cultured supernatant of RA FLSs cells. The results are presented as mean ± SD, and **p*<0.05, ***p*<0.01 *vs*. the control group.
**F**: qRT-PCR data showed the mRNA expression of TNF-α and IL-6 in RA STs and control group. The results are presented as mean ± SD, and **p*<0.05, ***p*<0.01 *vs*. the control group.
**G**: qRT-PCR data showed the mRNA expression of TNF-α and IL-6 in RA FLSs and control FLSs. The results are presented as mean ± SD, and.
**p*<0.05, ***p*<0.01 *vs*. the control FLSs.
**H**: The MTT assay showed the cell proliferation of RA FLSs and control FLSs. **p*<0.05, ***p*<0.01.
**I**: The BrdU assay showed the cell proliferation of RA FLSs and control FLSs.
**J**: The percentage of BrdU positive (BrdU$^+$) cells in RA FLSs and control FLSs. The results are presented as mean ± SD, ***p*<0.01. The experiments were independently repeated 3 times.

2A). Western blotting data showed the expression of NLRC5 protein was higher in RA STs compared with the normal STs (Fig. 2B, C). Comparison of the expression of NLRC5 between the RA FLSs and normal FLSs was also examined via qRT-PCR and western blot. The mRNA level of NLRC5 in RA FLSs was increased compared with normal FLSs (Fig. 2D). The protein level of NLRC5 in RA FLSs showed the same pattern (Fig. 2E, F). These data indicated that NLRC5 was upregulated both in RA synovial tissue and RA FLSs.

*Downregulated FER1L4 and upregulated NLRC5 was accompanied by high expression of inflammatory cytokine and proliferation of FLSs*

To explore whether FER1L4 exerted effects on the secretion of cytokines and proliferation in FLSs isolated from RA patients, western blot, qRT-PCR, MTT assay, and BrdU assay were performed. Firstly, western blot results showed that the protein level of TNF-α and IL-6 was higher in both RA STs and FLSs compared with the control group (Fig. 3A-D). The ELISA assay results showed that the level of TNF-α and IL-6 were higher in the cultured supernatant of RA FLSs compared with the control group. The qRT-PCR results showed that the mRNA levels of TNF-α and IL-6 were higher in both RA STs and FLSs compared with the control group (Fig. 3G, H). Furthermore, the MTT and BrdU assay suggested that the cell proliferation of RA FLSs was higher than that of the control group (Fig. 3I-J). The results showed that the decreased expression of FER1L4 and increased expression of NLRC5 were accompanied by high levels of inflammatory cytokine and cell proliferation of FLSs.

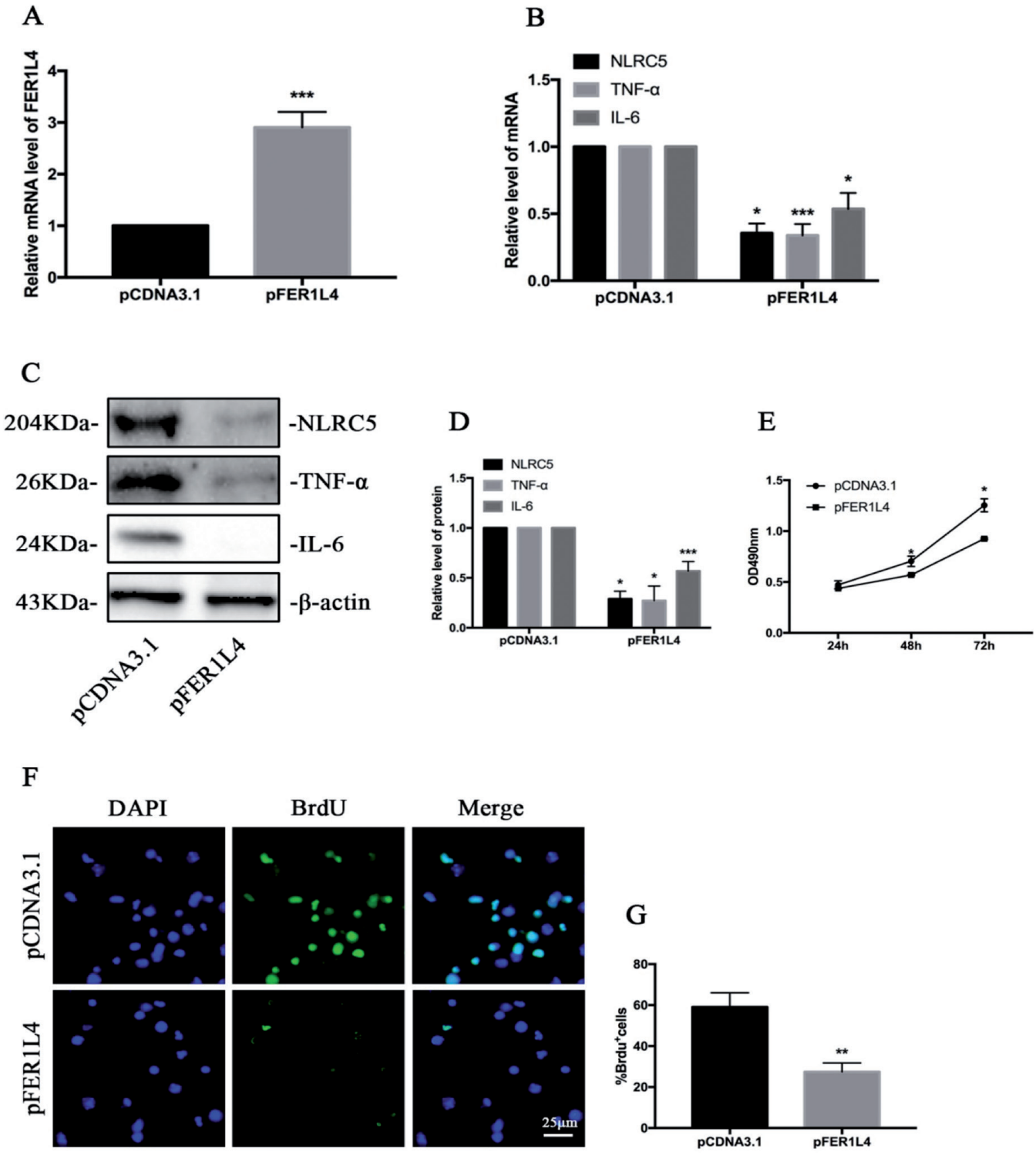


**Fig. 4.** FER1L4 was overexpressed in FLS cells by transfected with pFER1L4.
**A**: qRT-PCR data identified the mRNA level of FER1L4 after transfection. The results are presented as mean ± SD, and ***$p$<0.005.
**B**: qRT-PCR data showed the mRNA expression of NLRC5, TNF-α, and IL-6 in RA FLSs after transfected with pFER1L4 or pCDNA3.1. The data are presented as mean ± SD, *$p$<0.05, ***$p$<0.005.
**C**: Western blot showed the protein expression of NLRC5, TNF-α, and IL-6 in RA FLSs after transfected with pFER1L4 or pCDNA3.1. Representative images are shown.
**D**: Relative protein levels of TNF-α and IL-6 in RA FLSs after transfected with pFER1L4 or pCDNA3.1. The results are presented as mean ± SD, and *$p$<0.05, ** $p$<0.005.
**E**: The MTT assay showed the cell proliferation of RA FLSs after transfected with pFER1L4 or pCDNA3.1. *$p$<0.05.
**F**: The BrdU assay showed the cell proliferation of RA FLSs after transfected with pFER1L4 or pCDNA3.1.
**G**: The percent of BrdU positive ($BrdU^+$) cells in RA FLSs after transfected with pFER1L4 or pCDNA3.1. The results are presented as mean ± SD, ** $p$<0.01. The experiments were independently repeated 3 times.

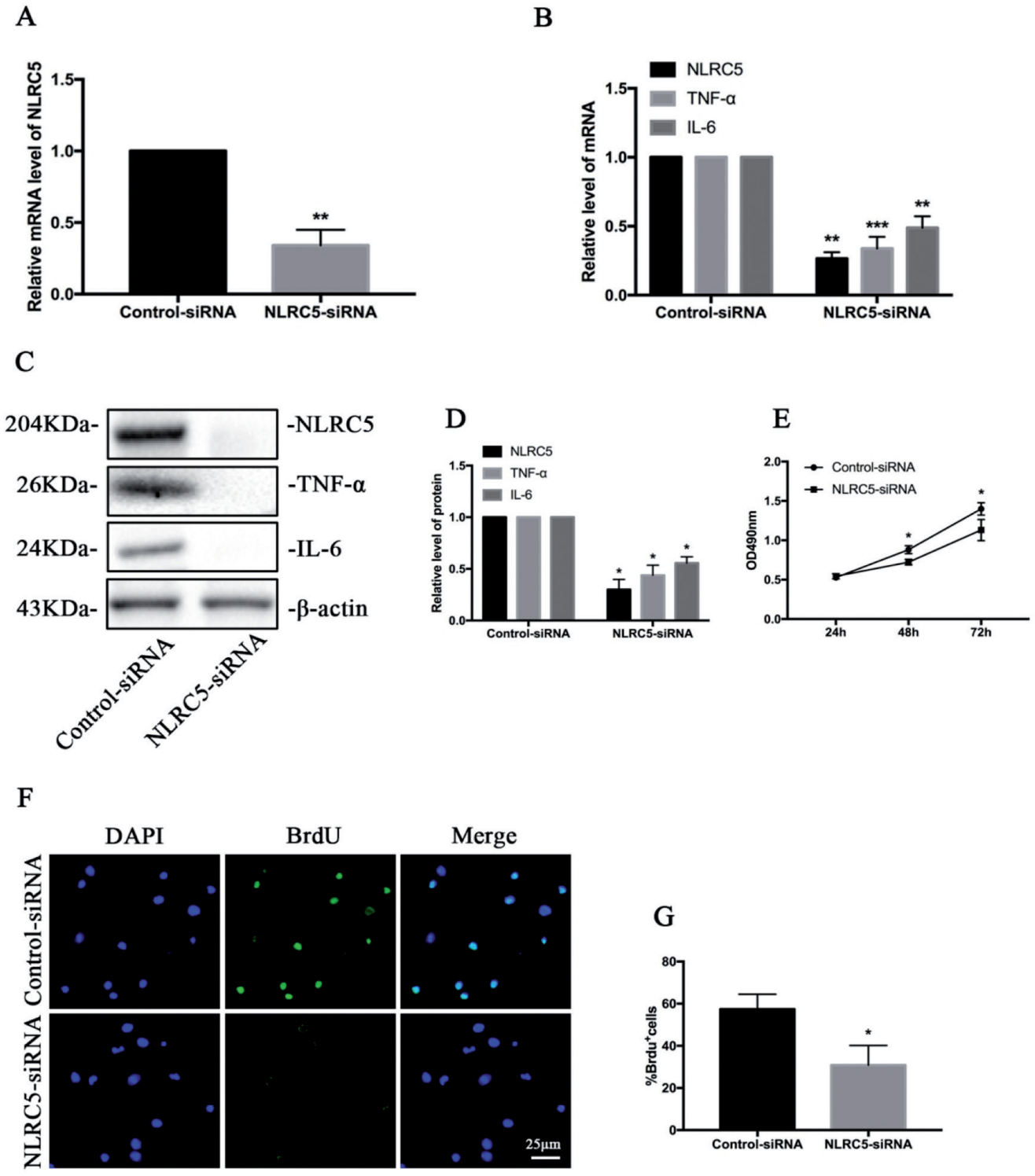


**Fig. 5.** NLRC5 was knockdown in RA FLSs by transfected with NLRC5-siRNA.
**A**: qRT-PCR data identified the mRNA level of NLRC5 after transfection. The results are presented as mean ± SD, and $^{**}p<0.01$.
**B**: qRT-PCR data showed the mRNA expression of NLRC5, TNF-α, and IL-6 in RA FLSs after transfected with NLRC5-siRNA or control-siRNA. The data are presented as mean ± SD, $^{**}p<0.01$, $^{***}p<0.005$.
**C**: Western blot showed the protein expression of NLRC5, TNF-α, and IL-6 in RA FLSs after transfected with NLRC5-siRNA or control-siRNA. Representative images are shown.
**D**: Relative protein levels of TNF-α and IL-6 in RA FLSs after transfected with NLRC5-siRNA or control-siRNA. The results are presented as mean ± SD, and. $^{**}p<0.01$, $^{***}p<0.005$.
**E**: The MTT assay showed the cell proliferation of RA FLSs after transfected with NLRC5-siRNA or control-siRNA. $^{*}p<0.05$.
**F**: The BrdU assay showed the cell proliferation of RA FLSs after transfected with NLRC5-siRNA or control-siRNA.
**G**: The percentage of BrdU positive ($BrdU^{+}$) cells in RA FLSs after transfected with NLRC5-siRNA or control-siRNA. The results are presented as mean ± SD, $^{*}p<0.05$. The experiments were independently repeated 3 times.

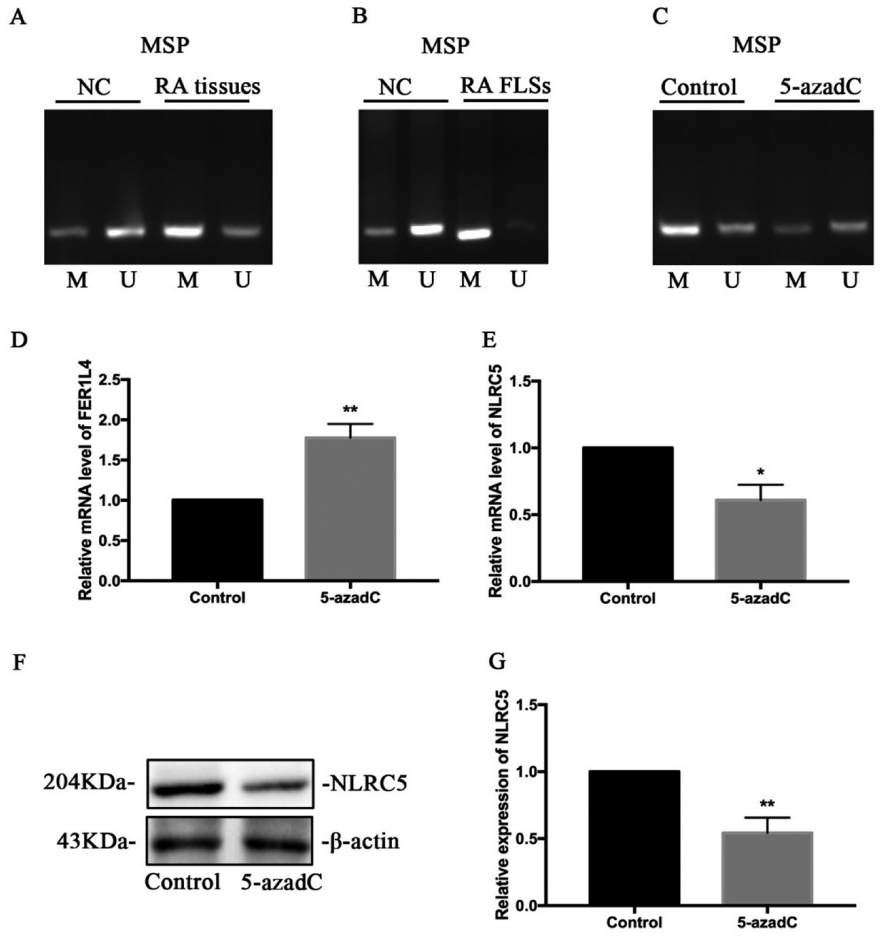


**Fig. 6.** Detection of FER1L4 methylation in AA rat by MSP.
**A**: MSP showed the methylation levels of FER1L4 in STs of control and RA patients, M referred to PCR products of methylated alleles and U referred PCR products of unmethylated alleles, respectively.
**B**: MSP showed the methylation levels of FER1L4 in FLSs of control and RA patients.
**C**: Treatment with 5-azadC for 48 hr, the methylation levels of FER1L4 in FLSs of the control and RA group.
**D**: qRT-PCR showed the mRNA expression of FER1L4 in RA FLSs after treatment with 5-azadC for 48 hr. The results are presented as mean ± SD, $^{**}p<0.01$.
**E**: qRT-PCR showed the mRNA expression of NLRC5 in RA FLSs after treatment with 5-azadC for 48 hr. The results are presented as mean ± SD, $^{*}p<0.05$.
**F**: Western blot showed the expression of NLRC5 in RA FLSs after treatment with 5-azadC for 48 hr. Representative images are shown.
**G**: Relative protein levels of NLRC5 after treatment with 5-azadC for 48 hr. The results are presented as mean ± SD, and $^{**}p<0.01$. The experiments were independently repeated 3 times.

*Enforced expression of FER1L4 decreased the expression of NLRC5, inflammatory cytokine and cell proliferation of FLSs*

To investigate the potential influence of FER1L4 in NLRC5 expression, FER1L4 was enforced overexpression in RA FLSs by transfected with pFER1L4 or its control plasmid pCDNA3.1. The transfection was identified via qRT-PCR (Fig. 4A). Firstly, the qRT-PCR data showed that the mRNA level of NLRC5, TNF-α and IL-6 were decreased in the pFER1L4 group compared with the pCDNA3.1 group (Fig. 4B). Western blot results presented that the protein expression of NLRC5 and inflammatory cytokine (TNF-α and IL-6) was were also decreased in the pFER1L4 group compared with the pCDNA3.1 group (Fig. 4C-D). The MTT and BrdU assay showed that the cell proliferation of the pFER1L4 group was inhibited compared with the pCDNA3.1 group (Fig. 4E-G). The results indicated that FER1L4 overexpression decreased NLRC5 expression and inhibited inflammatory cytokine expression and cell proliferation of FLSs.

*Knockdown the expression of NLRC5 inhibited inflammatory cytokine expression and cell proliferation of FLSs*

To explore the effect of NLRC5 knockdown, NLRC5 expression was silenced in FLS cells by transfected with NLRC5-siRNA or its control-siRNA. The transfection was identified via qRT-PCR (Fig. 5A). Then, qRT-PCR, western blot, MTT assay, and BrdU assay were performed. The qRT-PCR data showed a significant decrease of NLRC5, TNF-α, and IL-6 in the NLRC5-siRNA group compared with the NLRC5-control group (Fig. 5B). Western blot data indicated that the protein level of NLRC5, TNF-α, and IL-6 were also significantly decreased in the NLRC5-siRNA group compared with the NLRC5-control group (Fig. 5C-D). Likewise, the MTT and cell BrdU assay suggested that the cell proliferation ability of the NLRC5-siRNA group was inhibited compared with the NLRC5-control group (Fig. 5E-G). The results suggested that Knockdown the expression of NLRC5 inhibited the expression of inflammatory cytokine and cell proliferation of FLSs.

*DNA methylation contributed to the downregulation of FER1L4*

MSP was performed to analyse the contribution of methylation in alteration of FER1L4. The results suggested that FER1L4 promoter was significantly methylated in RASTs and RA FLSs, whereas the control group had an unmethylated FER1L4 promoter region (Fig. 6A-B). The FER1L4 promote methylation level was reduced after transfection with methylation inhibitor 5-azadC (Fig. 6C). qRT-PCR was performed to show whether the methylation inhibitor 5-azadC had a significant influence on the mRNA expression of FER1L4 and the results showed that the mRNA level of FER1L4 was increased in FLSs after treatment with 5-azadC compared with control group (Fig. 6D). Furthermore, the qRT-PCR and western blot results showed that the mRNA level and protein levels of NLRC5 were lower in FLSs after stimulation with 5-azadC (Fig. 6E-G). In addition, the western blot data showed stimulation

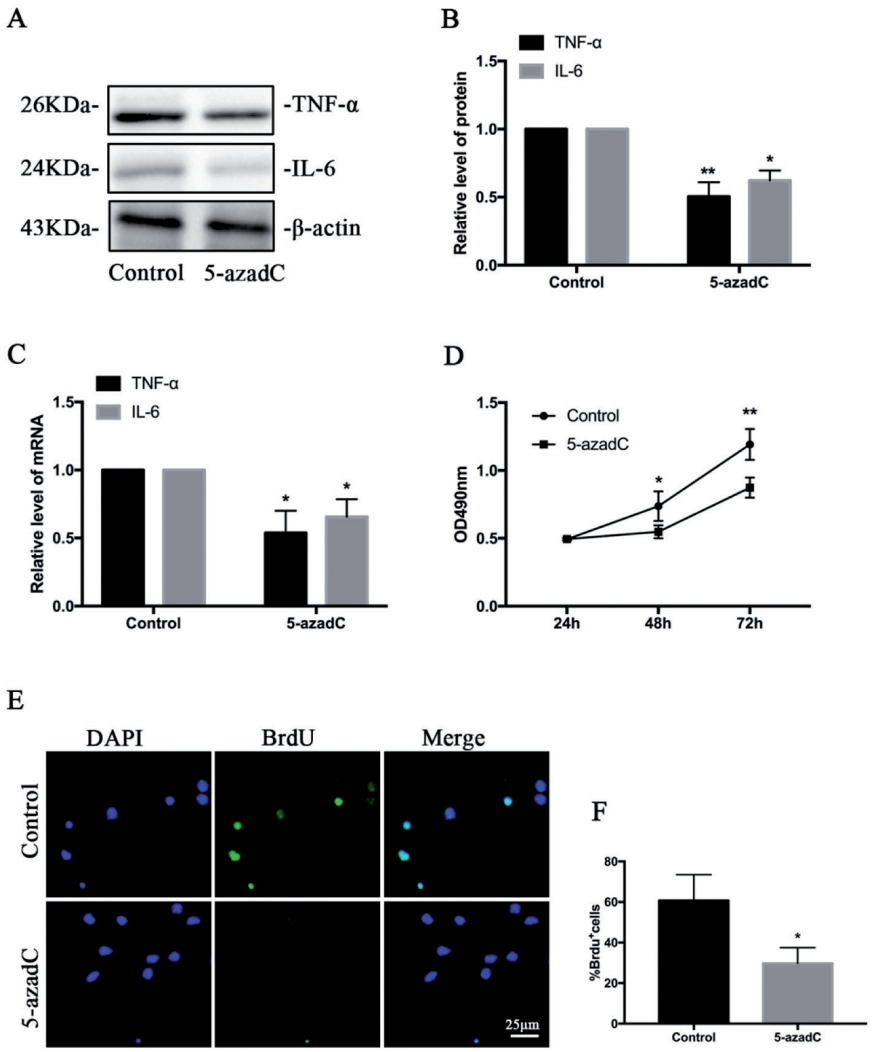


**Fig. 7.** The expression of inflammatory cytokine and FLSs proliferation after treatment with methylation inhibitor 5-azadC.

**A**: The protein expression of TNF-α and IL-6 in FLSs after treatment with 5-azadC and the representative images are shown.

**B**: Relative protein levels of TNF-α and IL-6after treatment with 5-azadC for 48 hr. The results are presented as mean ± SD, and. *$p$<0.05, **$p$<0.01.

**C**: qRT-PCR showed the mRNA expression of TNF-α and IL-6 in FLSs after treatment with 5-azadC. The results are presented as mean ± SD. *$p$<0.05.

**D**: The MTT assay illustrated that stimulated with 5-azadC could significantly inhibit cell proliferation of FLSs. The results are presented as mean ± SD. *$p$<0.05, **$p$<0.01.

**E**: The BrdU assay illustrated the proliferation of FLSs after treatment with 5-azadC. **F**: The percent of BrdU positive ($BrdU^+$) cells in FLSs after treatment with 5-azadC. The results are presented as mean ± SD, *$p$<0.05. The experiments were independently repeated 3 times.

with 5-azadC inhibited the expression of TNF-α and IL-6 (Fig. 7A-B) and the mRNA levels of TNF-α and IL-6 were also decreased after stimulation with 5-azadC (Fig. 7C). Importantly, MTT assay and BrdU analysis illustrated that stimulation with 5-azadC could significantly inhibit cell proliferation of FLSs (Fig. 7D-F).

These results suggested that DNA methylation contributed to the down-regulation of FER1L4.

## Discussion

RA, an autoimmune disorder, characterised by hyperplastic growth of FLSs as well as chronic inflammation of the STs, affects roughly 1% of the adult population all over the world (24-26). However, the exact mechanism underlying RA still remains unknown. It is urgent to explore the pathogenesis of RA (27-29). Multiple etiologies could cause the pathogenesis of RA such as epigenetic, heredity, immune disorders, cytokine factors and environmental factors and the drugs commonly used for treating RA also have significant side effects (30-32). Therefore, search for a better treatment approach for RA is very important (33). FLSs, which have the ability to invade and destroy cartilage and bone during the course of RA (34), are considered to play a significant role in the pathogenesis of RA (35-37). Previous studies focus on protein-coding genes to investigate the pathogenesis of RA until lncRNAs came into our sight (38-40). Numerous lncRNAs have been reported to be expressed differentially in RA patients, and act as central regulators of the inflammatory response (41, 42). Furthermore, lncRNAs have been identified as potent biomarkers and therapeutic targets in RA (43, 44). For example, lncRNA NR024118 was proved decreased in RA FLSs and enforced expression of lncRNA NR024118 contributed to suppress the inflammation in RA FLSs (45). In this study, we demonstrated that enforced expression of FER1L4 inhibited the proliferation and inflammatory progress of FLSs in RA.

Numerous studies suggested that activated FLSs have similar behavior like cancer cells such as migration, invasion (46, 47). So, we hypothesised that FER1L4 affect the proliferation of FLSs and develop this research. qRT-PCR results showed that the mRNA level of FER1L4 was decreased in RA STs and FLSs, indicating a potential function of FER1L4 in RA development. To further explore the functional of FER1L4 in RA, FER1L4 was overexpressed to observe the effect on inflammatory factors TNF-α and IL-6 expression and the proliferation ability of FLSs. We transfected FLSs with pFER1L4 or pCDNA3.1 for 48 hr and the data showed that transfection of pFER1L4 significantly decreased inflammatory factors expression and inhibited cell proliferation ability of FLSs, indicating that FER1L4 inhibits RA development through the potential inhibition of inflammatory factors and cell proliferation ability.

Pattern recognition receptors (PRRs) family is composed of C-type lectin receptors, Toll-like receptors, retinoid

acid-inducible gene I-like receptors and the nucleotide binding and NOD-like receptors (NLRs) and helps the innate immune system to recognise the intrusion of exotic microorganism and cellular stress (48-50). NLRC5 is the largest member of the NLR family, which plays an important role in adaptive immune responses and regulating innate (51-53). In this study, the protein and mRNA level of NLRC5 was shown increased in RA STs and FLSs. Therefore, we suspected that FER1L4 may participate in the development of RA by regulating NLRC5. To explore whether FER1L4 regulates the expression of NLRC5 during the development of RA, we detected the effect of FER1L4 overexpression on NLRC5 expression. We transfected pFER1L4 into FLSs for 48 hr, and the results showed that transient transfection of pFER1L4 decreased NLRC5 expression. In all, these data suggested that FER1L4 targeting NLRC5 participate in the development and progression of RA.

Previous research showed that DNA methylation plays a key function in genome dynamics and affect many biological processes related to human disease (54, 55). Thus, it is speculated that the loss of FER1L4 in RA STs and FLSs might have a relationship with promoter hypermethylation. In our study, we found that FER1L4 promoted hypermethylation in RA STs and FLSs. Furthermore, the 5-azadC, a methylation inhibitor, remarkably decreased the hypermethylation of the FER1L4 and the expression of NLRC5, which indicated that FER1L4 potentially regulates the expression of NLRC5.

All in all, our research in this study showed that overexpression of FER1L4 and silencing of NLRC5 decreased FLS proliferation ability and inflammatory reaction. The loss of FER1L4 we observed in RA STs and FLSs were mainly caused by the hypermethylation of the FER1L4 gene promoter. Methylation inhibitor 5-azadC could offset the hypermethylation of FER1L4 and decrease NLRC5 expression. These results indicated FER1L4 might regulate the expression of NLRC5 during the development and progression of RA. These results give us a novel insight that FER1L4 regulates RA by potentially targeting NLRC5. Our finding provides new insights into the molecular function of FER1L4 in RA and hopefully may provide new diagnostic target for RA patients and human health.

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