

## circ-PTTG1IP/miR-671-5p/TLR4 axis regulates proliferation, migration, invasion and inflammatory response of fibroblast-like synoviocytes in rheumatoid arthritis

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**Abstract.** Circular RNAs (circRNAs) are related to rheumatoid arthritis (RA) development. However, the function and mechanism of circRNA pituitary tumor-transforming 1 interacting protein (circ-PTTG1IP) in RA are unknown. The expression of circ-PTTG1IP in synovial tissues of RA patients and fibroblast-like synoviocytes from RA patients (RA-FLSs) were detected by RT-qPCR. The results uncovered that circ-PTTG1IP was overexpressed in RA patients and RA-FLSs, and circ-PTTG1IP knockdown suppressed cell proliferation, migration, invasion and inflammatory response in RA-FLSs. Besides, we found that circ-PTTG1IP could directly bind to miR-671-5p, and toll-like receptor 4 (TLR4) was a target of miR-671-5p, which was confirmed by dual-luciferase reporter assay. miR-671-5p inhibitor attenuated the effects of circ-PTTG1IP knockdown on RA-FLSs, while the effects of miR-671-5p mimic on RA-FLSs were partly reversed by TLR4 overexpression. Furthermore, circ-PTTG1IP could upregulate TLR4 expression by miR-671-5p. Thus, circ-PTTG1IP knockdown repressed cell proliferation, migration, invasion and inflammatory response in RA-FLSs by regulating the miR-671-5p/TLR4 axis.

**Key words:** Rheumatoid arthritis — Fibroblast-like synoviocytes — circ-PTTG1IP — miR-671-5p — TLR4

### Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease with the characteristics of progressive cartilage and bone damage (Smolen et al. 2018). The synovial joints are important elements for the body (Pap et al. 2020), and the proliferative synovial lining tissue is a main pathological feature of RA. Fibroblast-like synoviocytes (FLSs) are one class of key cell lines, which are associated with the formation of invasive synovial tissue (Harre and Schett 2017), play a pivotal role in the pathogenesis of RA (Karami et al. 2020). Hence, exploring the pathological mechanism underlying FLSs in RA may contribute to finding novel strategies for the treatment of RA.

Noncoding RNAs can function as promising biomarkers for the treatment of RA (Wang et al. 2019a). Circular RNAs (circRNAs) are a group of covalently closed non-coding RNAs produced by back-splicing (Kristensen et al. 2019). Additionally, circRNAs exert multifunctional roles in human cancers and diseases (Mumtaz et al. 2020). More importantly, circRNAs were reported to function as important biomarkers for the treatment of autoimmune diseases, including RA (Zhou et al. 2019). The circRNA pituitary tumor-transforming 1 interacting protein (circ-PTTG1IP), termed hsa\_circ\_0001200 in circBase, is significantly up-regulated in peripheral blood mononuclear cells from RA patients (Wen et al. 2020). However, the function and mechanism of circ-PTTG1IP in the pathological course of RA remain largely unknown.

Previous research indicates that circRNAs function as miRNAs sponge to regulate the expression of downstream genes targeted by miRNAs (Kristensen et al. 2019). Micro-

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RNAs (miRNAs) are closely associated with the progression and treatment of RA (Tavasolian et al. 2018; Evangelatos et al. 2019). A previous study reports that miR-671-5p is a down-regulated miRNA in RA (Tang et al. 2019), whereas no studies have reported the function of miR-671-5p in the pathological course of RA. Toll like receptors (TLRs) play important roles in the regulation of the function of fibroblast-like synoviocytes in RA (Elshabrawy et al. 2017; Luo et al. 2020). TLR4 is a representative member of TLRs, which is linked to the development of RA (Lee et al. 2017; Arjumand et al. 2019). Increasing researches suggest that TLR4 contributes to cell proliferation, migration, invasion and inflammatory response in fibroblast-like synoviocytes in RA (Shi et al. 2016; Qian et al. 2019; Yan et al. 2019; Wei et al. 2020). However, whether TLR4 participates in the regulatory function of circ-PTTG1IP and miR-671-5p in RA pathological process is unknown.

In this research, we examined circ-PTTG1IP expression in RA patients and fibroblast-like synoviocytes from RA patients (RA-FLSs). Moreover, the functional effects of circ-PTTG1IP on cell proliferation, migration, invasion and inflammatory response in RA-FLSs were detected, and the regulatory mechanism of circ-PTTG1IP was further investigated.

## Materials and Methods

### Ethics approval and consent to participate

The present study protocol was approved by the Ethics Committee of the General Hospital of Central Theater Command

**Table 1.** The clinical features of patients

Parameters	RA group ( <i>n</i> = 29)	Normal group ( <i>n</i> = 23)
Age (years)	49.35 ± 12.3	40.26 ± 9.2
Sex (male/female)	7/22	9/14
Duration of disease (years)	5.74 ± 4.31	NA
DAS28	6.33 ± 1.71	NA
ESR (mm/h)	48.35 ± 25.62	NA
CRP (mg/l)	29.65 ± 11.2	NA
RF (IU/ml)	463.57 ± 135.6	NA
L (10 <sup>9</sup> /l)	1.62 ± 0.63	2.15 ± 0.33
M (10 <sup>9</sup> /l)	0.45 ± 0.13	0.32 ± 0.1
N (10 <sup>9</sup> /l)	5.96 ± 2.12	3.49 ± 0.72
Anti-CCP (U/ml)	121.23 ± 105.36	7.88 ± 6.36

Data were expressed as mean ± standard deviation (SD). RA, rheumatoid arthritis; NA, not available; DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RF, rheumatoid factor; L, lymphocyte; M, monocyte; N, neutrophil; Anti-CCP, cyclic citrullinated peptide antibody.

and carried out following the NIH guidelines. All patients provided written informed consent.

### Patient tissue collection

Synovial tissues (*n* = 29) were collected from RA patients undergoing knee joint replacement surgery. Normal synovial tissues were obtained from 23 patients with traumatic knee. All patients were recruited from General Hospital of Central Theater Command, and written informed consent was obtained from patients before surgery. The clinical features of patients were shown in Table 1. The tissues were frozen in liquid nitrogen and then stored at -80°C for the further experiments.

### Cell culture

The human fibroblast-like synoviocytes from RA patients (RA-FLSs; cat. no. 408RA-05a) or normal fibroblast-like synoviocytes (control; cat. no. 408-05a) were purchased from Cell Applications (San Diego, CA, USA), and were cultured in human fibroblast-like synoviocytes (HFLS) growth medium (Cell Applications) at 37°C and 5% CO<sub>2</sub>. Cells were provided at passage 2, and all experiments were conducted on cells at passage 3–6, in accordance with the protocols as reported previously (Jones et al. 2017).

### Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted with Trizol reagent (Applygen, Beijing, China). The RNA in cytoplasm or nucleus was isolated using a Cytoplasmic & Nuclear RNA Purification kit (Norgen Biotek, Thorold, Canada) according to the manufacturer's instructions. To detect circRNA and mRNA expression, 1 µg RNA was reverse transcribed to cDNA using the M-MLV Reverse Transcriptase kit (Thermo Fisher Scientific, Waltham, MA, USA). To detect miRNA expression, 1 µg RNA was reverse transcribed using a TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific). All RT-PCR reactions were performed 3 times. Then cDNA was amplified by RT-qPCR with SYBR (Takara, Tokyo, Japan) and specific primer pairs (Sangon, Shanghai, China). The primer sequences were listed as follows: circ-PTTG1IP (hsa\_circ\_0001200) (sense, 5'-AGGAACGGAGAGCA-GAGATG-3'; antisense, 5'-GCGTGCAGAGCTCAATT-TAC-3'), PTTG1IP (sense, 5'-GTGCTGGAGTCGGAGTT-GTA-3'; antisense, 5'-GCAAGACGCTTGTAACTGGG-3'), TLR4 (sense, 5'-GATAGCGAGCCACGCATTC-3'; antisense, 5'-TTAGGAACCACCTCCACGCA-3'), miR-671-5p (sense, 5'-GCCGAGAGGAAGCCCTGGAG-3'; antisense, 5'-CAGTGCCTGTCGTGGAGT-3'), U6 (sense, 5'-CTCGCTTCGGCAGCACA-3'; antisense,

5'-AACGCTTCACGAATTTGCGT-3'), and GAPDH (sense, 5'-AATGGGCAGCCGTTAGGAAA-3'; antisense, 5'-GCGCCCAATACGACCAAATC-3'). U6 or GAPDH was used as an internal reference, and relative RNA levels were calculated by the  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen 2001).

#### *circRNA stability*

To detect the stability of circ-PTTG1IP, total RNA was treated with 4 U/ $\mu$ g RNase R (Geneseed, Guangzhou, China) at 37°C for 30 min. Then, RNA was collected, and the expression levels of circ-PTTG1IP and its linear transcript PTTG1IP were analyzed by RT-qPCR.

#### *Cell transfection*

circ-PTTG1IP overexpression vectors were constructed using the pcDNA3.1 circRNA mini vector (Addgene, Watertown, MA, USA), with the empty vector as negative control (vector). TLR4 overexpression vectors were constructed using the pcDNA3.1 vector (Thermo Fisher Scientific), and the empty vector were used as negative control (pcDNA). The small interference RNA (siRNA) against circ-PTTG1IP (si-circ-PTTG1IP, 5'-ATATGTGTCTTTG-GTGAACA-3'), and its negative control (si-NC) (5'-AA-GACAUUGUGUGUCCGCCTT-3'), miR-671-5p mimics (5'-AGGAAGCCCUGGAGGGGCUGGAG-3'), mimics negative control (miR-NC, 5'-UUCUCCGAACGUGU-CACGU-3'), miR-671-5p inhibitor (anti-miR-671-5p, 5'-CUCCAGCCCCUCCAGGGCUUCCU-3'), and inhibitor negative control (anti-miR-NC, 5'-CUAACGCAUG-CACAGUCGUACG-3') were synthesized by GenePharma (Shanghai, China). Cell transfection was performed in RA-FLSs using Lipofectamine 2000 reagent (Thermo Fisher Scientific). After transfection for 24 h, the cells were collected, and the transfection efficiency was determined by RT-qPCR or Western blot.

#### *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)*

Cell viability was analyzed by MTT assay.  $1 \times 10^4$  transfected RA-FLSs were plated into 96-well plates. After the following culture for 72 h, the medium was replaced with fresh medium containing 0.5 mg/ml MTT (Beyotime, Shanghai, China), and cultured for another 4 h. Then the medium was removed, and 100  $\mu$ l of dimethyl sulfoxide (Solarbio, Beijing, China) was added to each well. The absorbance at 570 nm was detected with a microplate reader (Potenov, Beijing, China). Cell viability was normalized to the control group (100%). A total of three biological replicates, and three technical replicates of each repetition were conducted.

#### *Flow cytometry*

Flow cytometry assay was conducted to evaluate cell cycle distribution and apoptosis. For analysis of cell cycle distribution,  $2 \times 10^5$  transfected RA-FLSs were inoculated into 6-well plates, and incubated for 72 h. Then cells were fixed with 70% ethanol (Aladdin, Shanghai, China) at 4°C overnight. Then the cells were stained with 10  $\mu$ g/ml RNase (Thermo Fisher Scientific) and 50  $\mu$ g/ml propidium iodide (PI; Thermo Fisher Scientific) in the dark for 1 h. Then, a flow cytometer (Agilent, Beijing, China) was used to analyze cell cycle distribution. A total of three biological replicates were performed.

Cell apoptosis was detected using an Annexin V-FITC apoptosis kit (Yeasen, Shanghai, China) according to the manufacturer's protocol. In brief,  $1 \times 10^5$  transfected RA-FLSs were inoculated into 12-well plates. After incubation for 72 h, cells were harvested and incubated with Annexin V binding buffer, followed by staining with 5  $\mu$ l Annexin V-FITC and 5  $\mu$ l PI in the dark for 30 min. The apoptotic rate (the percentage of cells in right lower and upper quadrants) was analyzed using the flow cytometer (Agilent, Beijing, China). A total of three biological replicates were performed.

#### *Transwell analysis*

Cell migratory and invasive abilities were analyzed using transwell chamber (Costar, Corning, NY, USA). To detect the migratory ability,  $1 \times 10^4$  RA-FLSs in HFLS medium without serum were plated into the upper chamber; to investigate the invasive ability,  $5 \times 10^4$  RA-FLSs in non-serum medium were added in the upper chamber coated with Matrigel (Solarbio). The lower chamber was supplemented with 600  $\mu$ l HFLS medium plus 10% serum. After incubation for 24 h, cells that migrated or invaded through pores to the lower chamber side of the membrane were fixed, and stained with 0.2% crystal violet (Beyotime). The migratory or invasive cells that were randomly selected from 3 fields, were observed under microscope (Olympus, Tokyo, Japan), magnification  $\times 100$ .

#### *Enzyme-linked immunosorbent assay (ELISA)*

The secretion of inflammatory cytokines was detected by ELISA assay.  $1 \times 10^5$  RA-FLSs were placed in 12-well plates, and nurtured for 72 h, followed by stimulation with 50 ng/ml tumor necrosis factor alpha (TNF- $\alpha$ ; Genscript; Nanjing, China) for 1 h (Liu et al. 2017). Next, cell medium was collected, and interleukin (IL)-8, IL-1 $\beta$  and IL-6 levels in cell medium were evaluated in triplicate using human IL-8, IL-1 $\beta$  or IL-6 ELISA kit (Thermo Fisher Scientific). The absorbance was measured using a microplate reader with a wavelength of 450 nm. The concentration of IL-8, IL-1 $\beta$  or IL-6 was calculated according to the standard curve. A total of three biological replicates were performed.

### Western blot

Protein was extracted using RIPA buffer (Beyotime) with 1% protease inhibitor, and concentration was determined with a BCA kit (Amyjet, Wuhan, China) following the manufacturer's protocols. The protein sample (20  $\mu$ g/lane) was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the nitrocellulose membrane (Solarbio). Then, the membranes were blocked with 5% non-fat milk for 1 h, and then incubated with primary antibodies, including TLR4 (ab13556, 1:500 dilution, Abcam, Cambridge, MA, USA) and GAPDH (ab245355, 1:3000 dilution, Abcam) overnight at 4°C. Then the membranes were incubated with goat anti-rabbit horse-radish peroxidase (HRP)-conjugated secondary antibody (ab205718, 1:8000 dilution, Abcam) for 2 h. Finally, the blots were visualized using ECL reagent (Solarbio). The grey values were analyzed by Image J software (NIH, Bethesda, MD, USA). Relative protein expression was normalized to the control group, with GAPDH as a loading control.

### Dual-luciferase reporter assay

The targets of circ-PTTG1IP and miR-671-5p were predicted by StarBase (<http://starbase.sysu.edu.cn/>). The sequences of circ-PTTG1IP or TLR4 3'UTR containing wild type (WT) or mutant type (MUT) miR-671-5p binding sites were cloned into the pmirGLO vectors (Promega, Madison, WI, USA) to construct WT-circ-PTTG1IP, MUT-circ-PTTG1IP, TLR4

3'UTR-WT and TLR4 3'UTR-MUT. These constructs and miR-671-5p mimic or miR-NC were co-transfected into the RA-FLSs for 24 h. The luciferase activity was examined using a luciferase analysis kit (Promega) by GloMax 20/20 Luminometer (Promega).

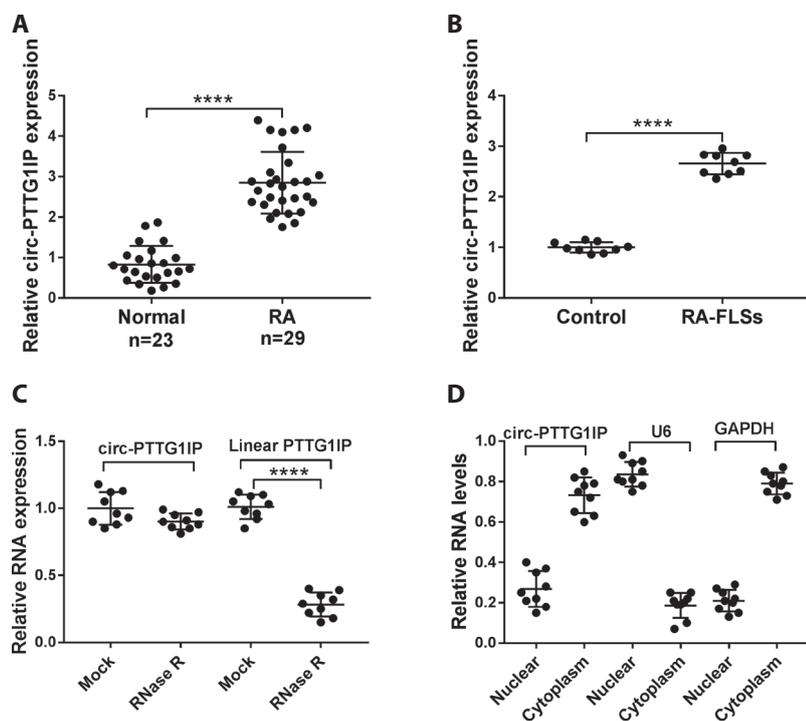
### Statistical analysis

The cellular experiments were performed 3 biological replicates with 3 technical replicates. Each dot means one date. Statistical analysis was conducted using GraphPad Prism 6 (GraphPad Inc., La Jolla, CA, USA) and the data were represented as mean  $\pm$  standard deviation (SD). Pearson correlation analysis was performed to detect the correlation between miR-671-5p and circ-PTTG1IP or TLR4. Difference between the two groups was analyzed *via* Student's *t*-test. Differences between multiple groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test. *p* value < 0.05 was statistically significant.

## Results

### *circ-PTTG1IP* expression is increased in RA patients and RA-FLSs

To investigate the functional effects of circ-PTTG1IP in RA, we first measured the expression of circ-PTTG1IP in synovial tissues from RA patients and RA-FLSs. The results



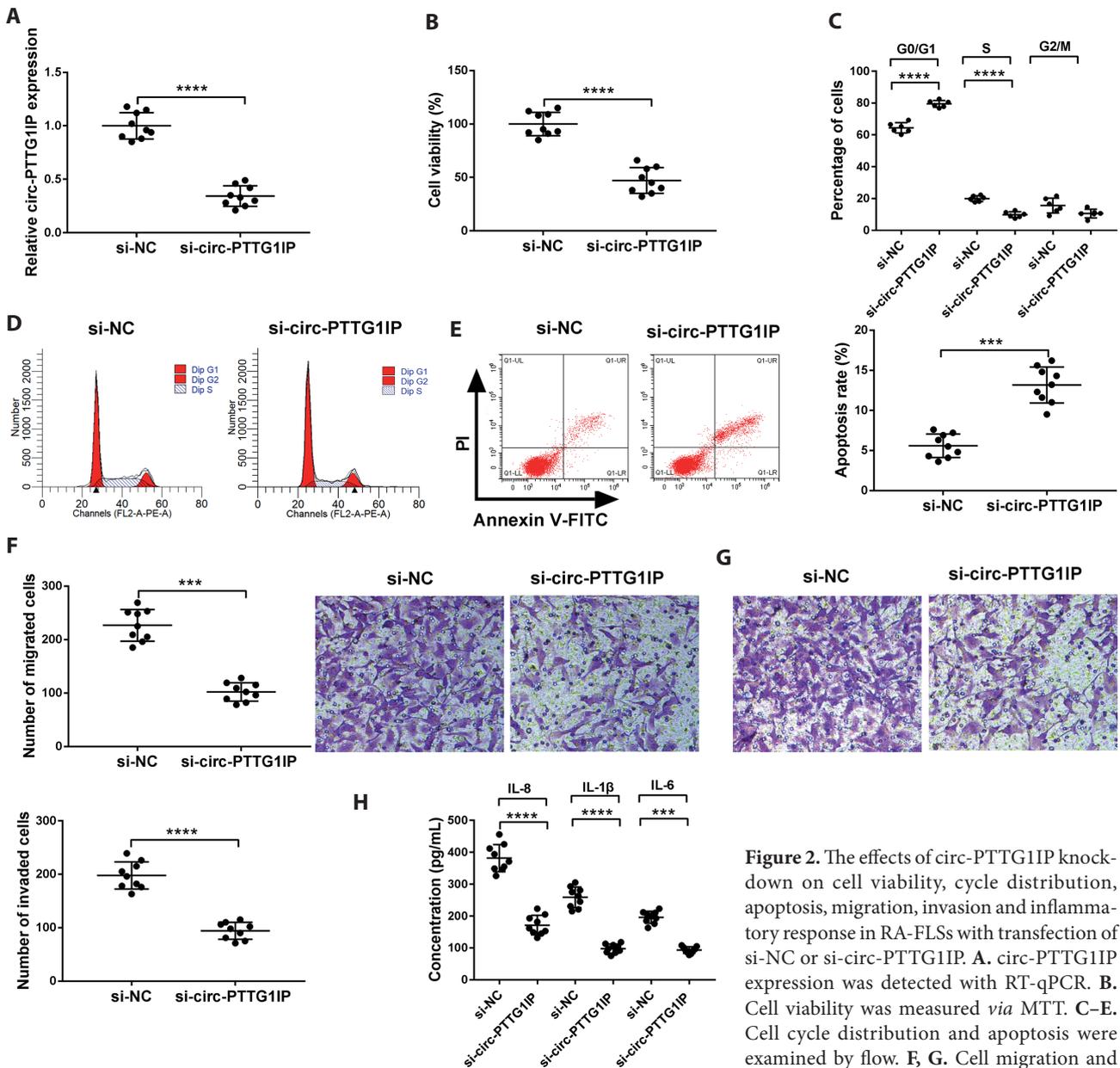
**Figure 1.** circ-PTTG1IP expression in RA patients and RA-FLSs detected by RT-qPCR. **A.** circ-PTTG1IP expression in synovial tissues from RA patients ( $n = 29$ ) and normal patients ( $n = 23$ ). **B.** circ-PTTG1IP expression in control and RA-FLSs. **C.** circ-PTTG1IP and linear PTTG1IP levels in RA-FLSs with or without RNase R treatment. **D.** circ-PTTG1IP level in cytoplasm and nucleus. \*\*\*\*  $p < 0.0001$ . PTTG1IP, pituitary tumor-transforming gene 1 protein-interacting protein; RA, rheumatoid arthritis; FLSs, fibroblast-like synoviocytes; Mock, mock treated with water.

exhibited that circ-PTTG1IP expression was enhanced in RA patients ( $n = 29$ ) compared with the normal synovial tissues ( $n = 23$ ) (Fig. 1A). Moreover, circ-PTTG1IP level in RA-FLSs was 2.67-fold higher than that in the control group (Fig. 1B). Besides, circ-PTTG1IP was resistant to RNase R, whereas the linear PTTG1IP was significantly reduced in RA-FLSs treated with RNase R (Fig. 1C). Furthermore, the level of circ-PTTG1IP in cytoplasm and nuclear was detected, and results showed that circ-PTTG1IP was mainly located in the cytoplasm (Fig. 1D), indicating

it mainly plays the corresponding biological functions in the cytoplasm.

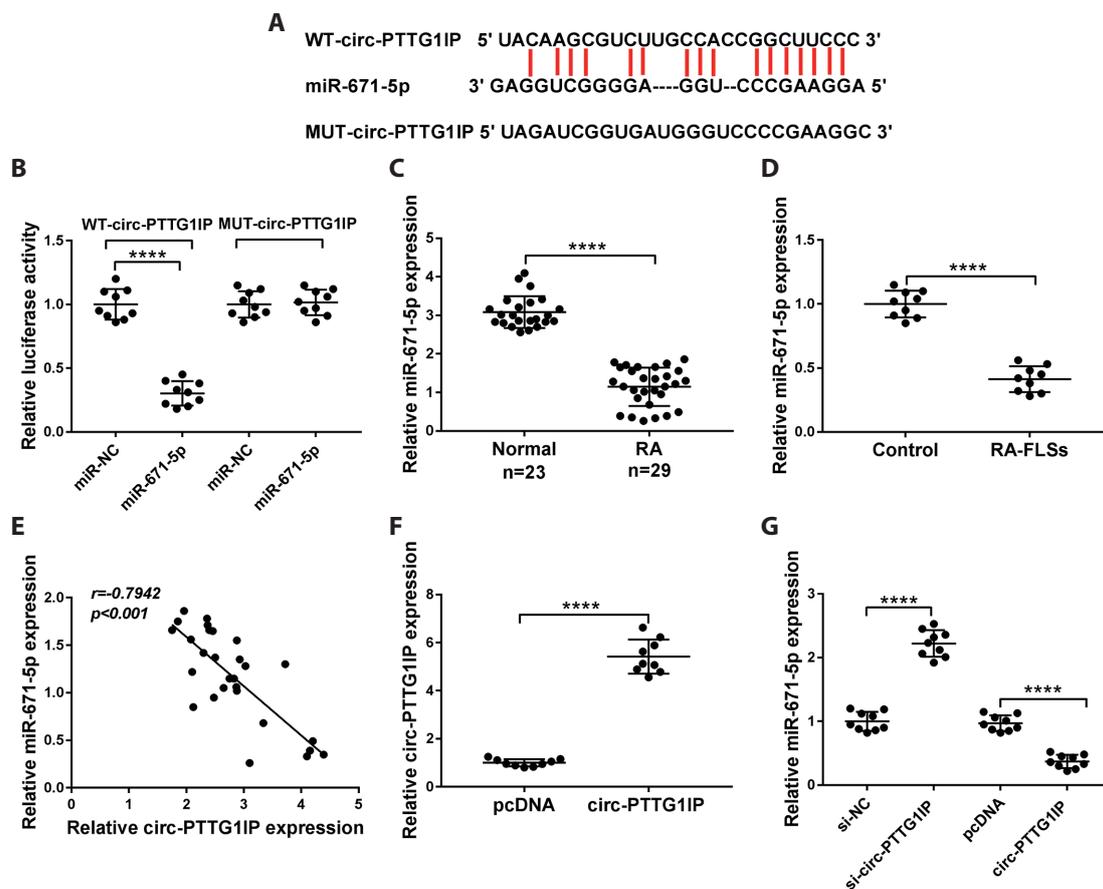
*circ-PTTG1IP knockdown induces apoptosis and inhibits cell proliferation, migration, invasion and inflammatory response in RA-FLSs*

To further study the function of circ-PTTG1IP in RA-FLSs, siRNA targeting circ-PTTG1IP was used to knock down its expression. As displayed in Figure 2A, transfection of si-



**Figure 2.** The effects of circ-PTTG1IP knock-down on cell viability, cycle distribution, apoptosis, migration, invasion and inflammatory response in RA-FLSs with transfection of si-NC or si-circ-PTTG1IP. **A.** circ-PTTG1IP expression was detected with RT-qPCR. **B.** Cell viability was measured *via* MTT. **C–E.** Cell cycle distribution and apoptosis were examined by flow. **F, G.** Cell migration and invasion were examined using transwell analysis. **H.** The secretion of inflammatory cytokines was detected by ELISA. \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . si-NC, siRNA negative control; PI, propidium iodide; IL, interleukin. For more abbreviations, see Fig. 1.

analysis. **H.** The secretion of inflammatory cytokines was detected by ELISA. \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . si-NC, siRNA negative control; PI, propidium iodide; IL, interleukin. For more abbreviations, see Fig. 1.



**Figure 3.** circ-PTTG1IP negatively correlated with miR-671-5p. **A.** The binding sites between circ-PTTG1IP and miR-671-5p. **B.** Dual-luciferase reporter assay was performed in RA-FLSs with co-transfection of WT-circ-PTTG1IP or MUT-circ-PTTG1IP and miR-NC or miR-671-5p mimic. **C.** miR-671-5p expression was examined in synovial tissues from RA patients ( $n = 29$ ) and normal patients ( $n = 23$ ). **D.** miR-671-5p level was measured in control and RA-FLSs. **E.** Correlation between circ-PTTG1IP and miR-671-5p levels in 29 cases of RA synovial tissues was analyzed by Pearson correlation analysis. **F.** circ-PTTG1IP expression was detected by RT-qPCR in RA-FLSs with transfection of vector or circ-PTTG1IP overexpression vector. **G.** miR-671-5p level was examined using RT-qPCR in RA-FLSs with transfection of si-NC, si-circ-PTTG1IP, vector or circ-PTTG1IP overexpression vector. \*\*\*\*  $p < 0.0001$ . WT, wild type; MUT, mutant type; miR-NC, miRNA mimic negative control; si-NC, siRNA negative control. For more abbreviations, see Fig. 1.

circ-PTTG1IP induced a 65% reduction in circ-PTTG1IP level in RA-FLSs compared with the si-NC group. Moreover, circ-PTTG1IP downregulation markedly decreased cell viability (Fig. 2B) and induced cell cycle arrest at G0/G1 phase (Fig. 2C,D), which suggested that circ-PTTG1IP knockdown repressed the proliferation of RA-FLSs. Besides, the apoptosis rate of RA-FLSs with circ-PTTG1IP knockdown was significantly elevated compared with the si-NC group (Fig. 2E). Additionally, the effects of circ-PTTG1IP knockdown on cell migration and invasion were investigated by transwell assay. As shown in Figure 2F and G, circ-PTTG1IP knockdown significantly restrained RA-FLSs migration and invasion. Besides, ELISA assay indicated that circ-PTTG1IP interference significantly reduced the secretion of IL-8, IL-1 $\beta$  and IL-6 in RA-FLSs upon TNF- $\alpha$  stimulation (Fig. 2H).

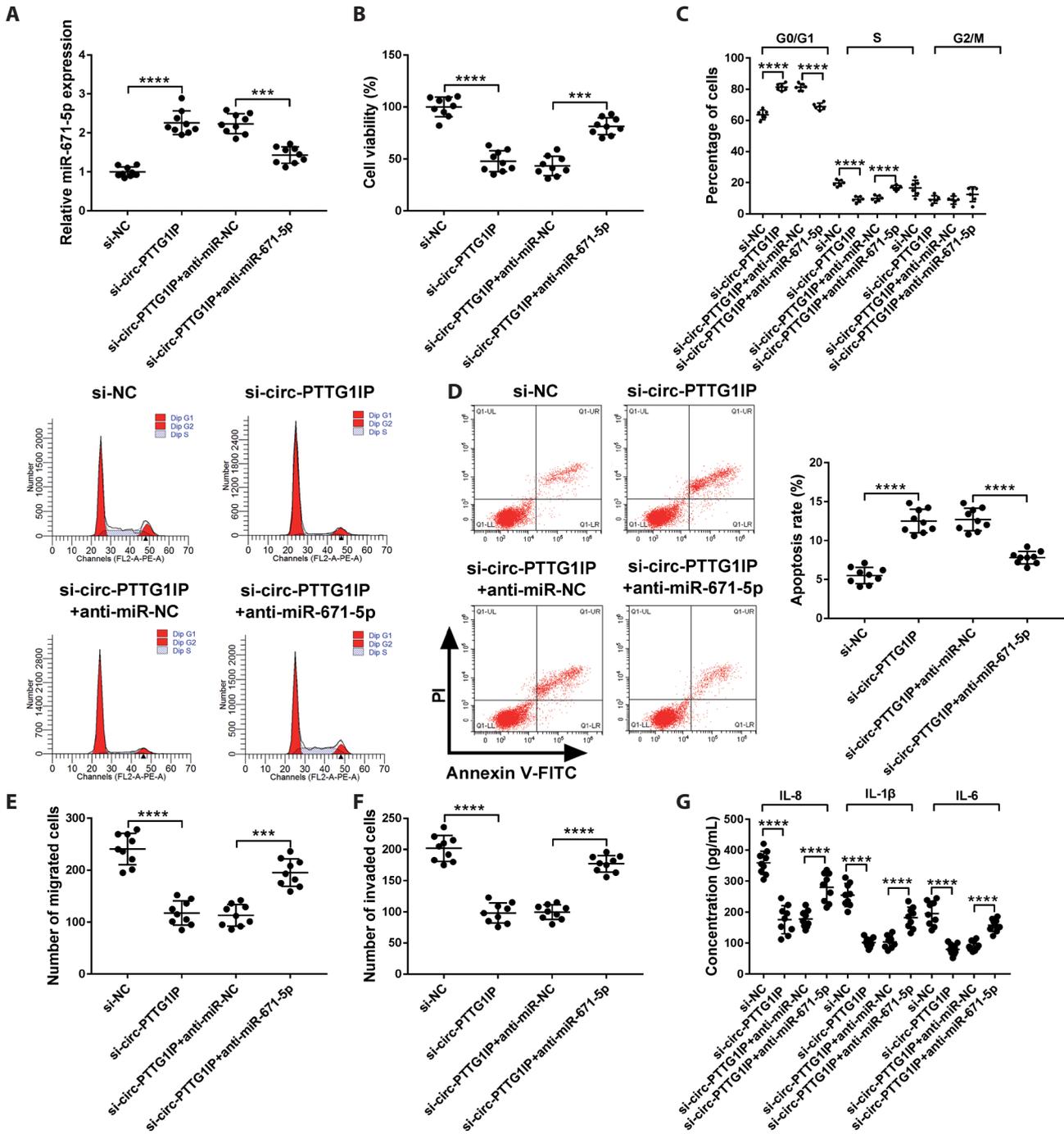
These data indicated that circ-PTTG1IP knockdown induced apoptosis and suppressed proliferation, migration, invasion and inflammatory response of RA-FLSs.

#### *circ-PTTG1IP acts as an endogenous sponge for miR-671-5p*

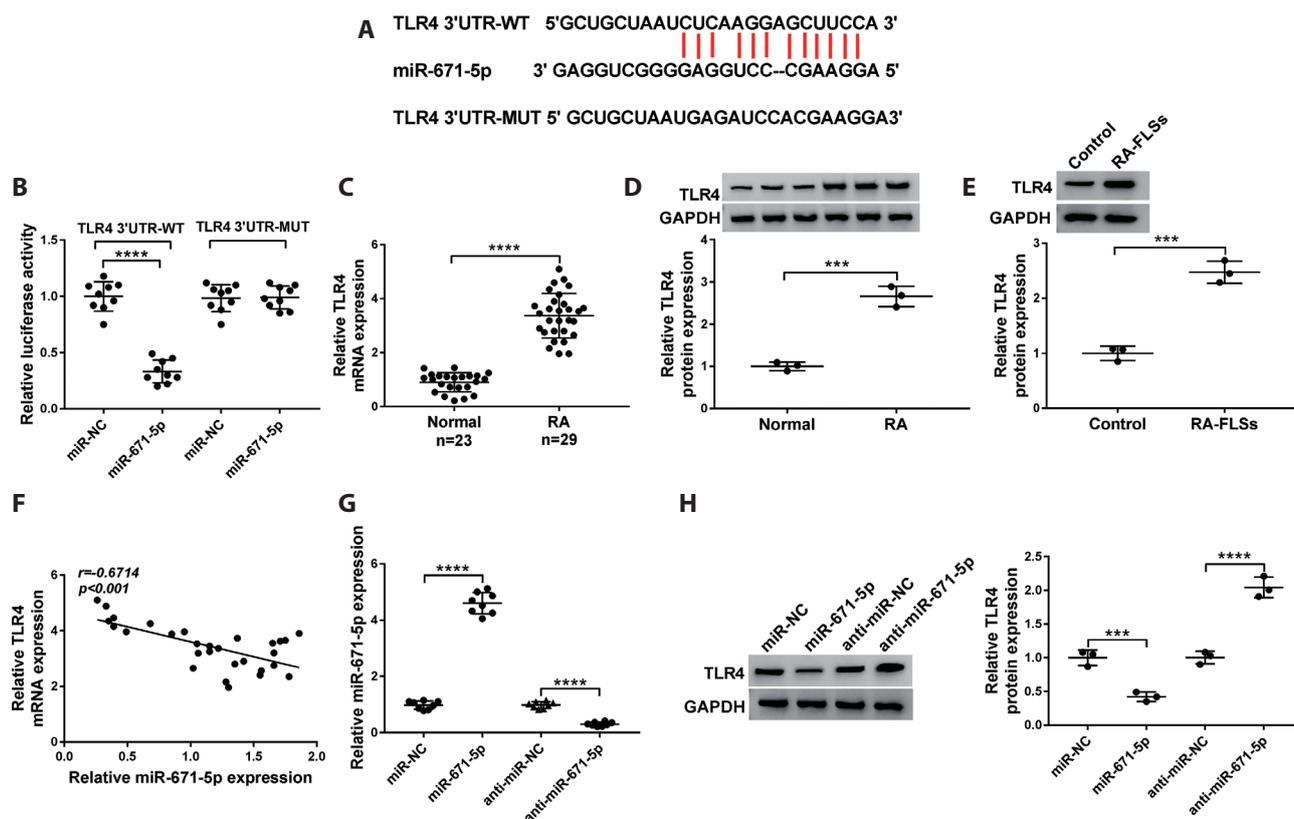
Given that circ-PTTG1IP may function as a miRNA sponge, we analyzed the targets of circ-PTTG1IP using StarBase bioinformatics, which predicted that miR-671-5p was the potential target of circ-PTTG1IP. To confirm the target relationship between circ-PTTG1IP and miR-671-5p, circ-PTTG1IP sequences contained WT or MUT miR-671-5p binding sites were amplified to construct WT-circ-PTTG1IP and MUT-circ-PTTG1IP luciferase reporter vectors (Fig. 3A). Dual-luciferase reporter assay displayed that the luciferase

activity of WT-circ-PTTG1IP group was decreased ~70% by miR-671-5p mimic, while the luciferase activity of MUT-circ-PTTG1IP group was not in cells with miR-671-5p transfection

(Fig. 3B). Furthermore, miR-671-5p expression in RA patients and RA-FLSs were detected. As shown in Figure 3C and D, miR-671-5p level was significantly decreased in synovial tis-



**Figure 4.** The effects of miR-671-5p on circ-PTTG1IP-mediated regulation in cell viability, cell cycle distribution, apoptosis, migration, invasion and inflammatory response in RA-FLSs. miR-671-5p expression (A), cell viability (B), cycle distribution (C), apoptosis (D), invasion (E), migration (F), and inflammatory cytokine secretion (G) were examined in RA-FLSs with transfection of si-NC, si-circ-PTTG1IP, si-circ-PTTG1IP + anti-miR-NC or anti-miR-671-5p. \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . anti-miR-NC, miRNA inhibitor negative control. For more abbreviations, see Fig. 2.



**Figure 5.** The association between miR-671-5p and TLR4. **A.** The binding sites between miR-671-5p and TLR4. **B.** Dual-luciferase reporter assay was conducted in RA-FLSs with co-transfection of TLR4 3'UTR-WT or TLR4 3'UTR-MUT and miR-NC or miR-671-5p mimic. **C, D.** TLR4 expression was examined in synovial tissues from RA patients ( $n = 29$ ) and normal patients ( $n = 23$ ). **E.** TLR4 protein level was examined in control and RA-FLSs. **F.** The linear correlation between miR-671-5p and TLR4 levels in RA synovial tissues. **G.** miR-671-5p expression was examined *via* RT-qPCR in RA-FLSs with transfection of miR-NC, miR-671-5p mimic, anti-miR-NC or anti-miR-671-5p. **H.** TLR4 protein level was measured using Western blot in RA-FLSs with transfection of miR-NC, miR-671-5p mimic, anti-miR-NC or anti-miR-671-5p. \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . TLR4, Toll-like receptor 4; 3'UTR, 3'-untranslated regions; anti-miR-NC, miRNA inhibitor negative control. For more abbreviations, see Fig. 3.

sues from RA patients and RA-FLSs. Meanwhile, miR-671-5p level in 29 cases of RA patients was negatively correlated ( $r = -0.7942$ ,  $p < 0.001$ ) with circ-PTTG1IP expression (Fig. 3E). Additionally, circ-PTTG1IP expression was elevated by more than five times in cells with circ-PTTG1IP overexpression vector transfection (Fig. 3F). We found that miR-671-5p level increased in RA-FLSs with circ-PTTG1IP knockdown, while it decreased in cells in RA-FLSs with circ-PTTG1IP overexpression (Fig. 3G). These results indicated that circ-PTTG1IP acted as a sponge for miR-671-5p.

*miR-671-5p knockdown reversed the effect of circ-PTTG1IP knockdown on cell proliferation, apoptosis, migration, invasion and inflammatory response in RA-FLSs*

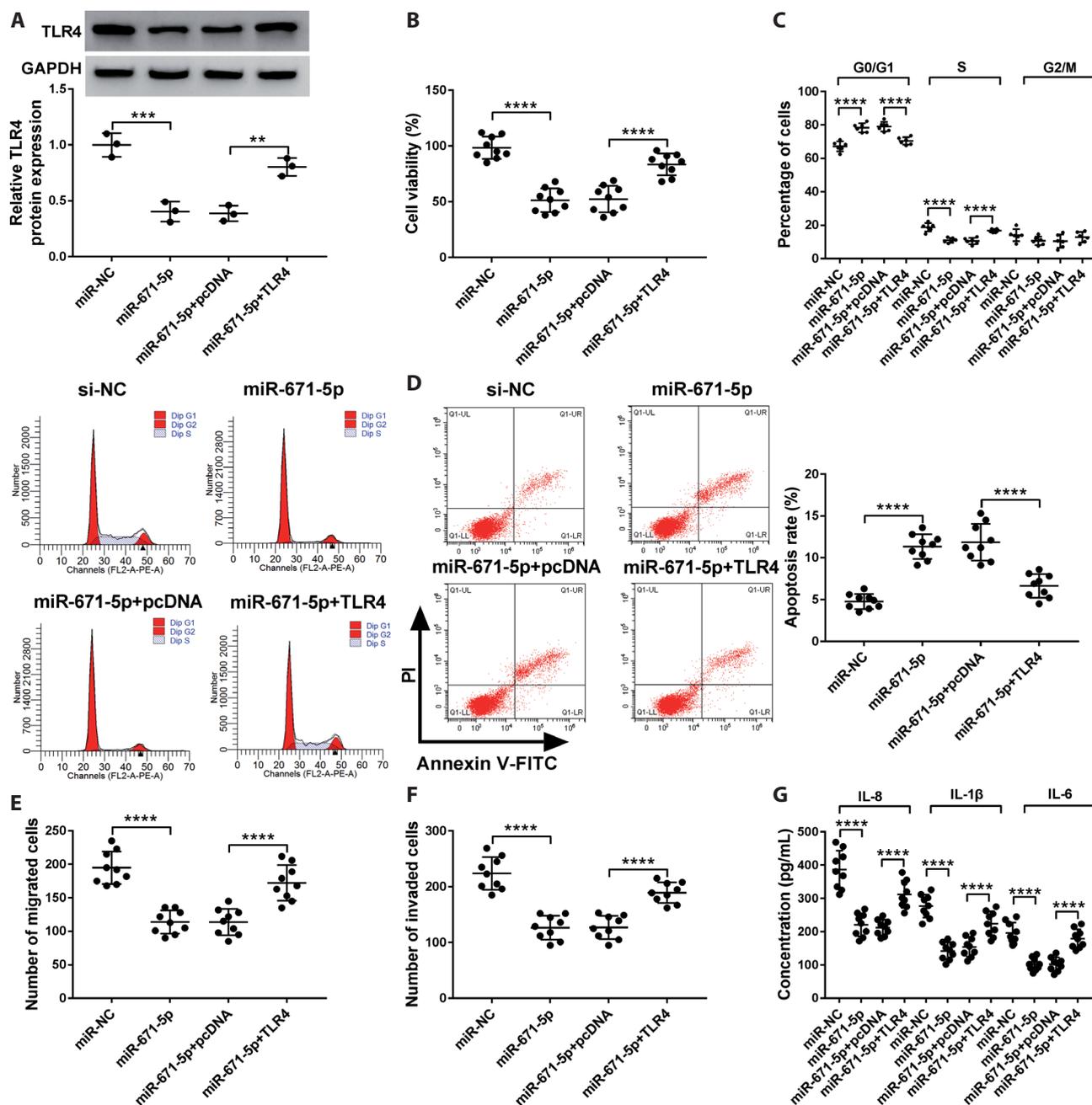
To explore whether miR-671-5p is involved in the function of circ-PTTG1IP in RA-FLSs, RA-FLSs were transfected with

si-NC, si-circ-PTTG1IP, si-circ-PTTG1IP + anti-miR-NC or anti-miR-671-5p. As displayed in Figure 4A, miR-671-5p expression increased in RA-FLSs after circ-PTTG1IP knockdown, whereas this tendency was reversed by anti-miR-671-5p. Besides, miR-671-5p downregulation weakened circ-PTTG1IP knockdown-mediated proliferation suppression through increasing cell viability and regulating cell cycle distribution (Fig. 4B,C). Furthermore, the promotion effect of circ-PTTG1IP on apoptosis was partly reversed by anti-miR-671-5p (Fig. 4D). Additionally, miR-671-5p knockdown partly mitigated the suppression effects of si-circ-PTTG1IP on cell migration and invasion (Fig. 4E,F). Moreover, miR-671-5p interference alleviated the inhibition effects of circ-PTTG1IP on IL-8, IL-1 $\beta$  and IL-6 reduction in RA-FLSs with TNF- $\alpha$  stimulation (Fig. 4G). These data suggested that circ-PTTG1IP regulated RA-FLSs proliferation, apoptosis, migration, invasion and inflammatory response through sponging miR-671-5p.

*TLR4 is targeted by miR-671-5p*

To further investigate the regulatory network underlying circ-PTTG1IP/miR-671-5p axis in RA-FLSs, starBase bioinformatics was used to explore the targets of miR-671-5p.

TLR4 was predicted to harbor the complementary binding sites of miR-671-5p (Fig. 5A). To identify this prediction, dual-luciferase reporter assay was conducted by using the TLR4 3'UTR-WT and TLR4 3'UTR-MUT luciferase reporter vectors. miR-671-5p mimic induced a 65% reduction in lu-



**Figure 6.** The effects of miR-671-5p/TLR4 axis on cell viability, cycle distribution, apoptosis, migration, invasion and inflammatory response in RA-FLSs. TLR4 expression (A), cell viability (B), cycle distribution (C), apoptosis (D), invasion (E), migration (F), and inflammatory cytokine secretion (G) were measured in RA-FLSs with transfection of miR-NC, miR-671-5p mimic, miR-671-5p mimic + pcDNA or TLR4 overexpression vector. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . miR-NC, miRNA mimic negative control; TLR4, toll-like receptor 4. For more abbreviations, see Fig. 2.

ciferase activity of TLR4 3'UTR-WT group, but it had little effect on the TLR4 3'UTR-MUT group (Fig. 5B). Besides, TLR4 mRNA and protein levels were significantly upregulated in synovial tissues from RA patients and RA-FLSs (Fig. 5C–E). In addition, TLR4 level was negatively associated with ( $r = -0.6714$ ,  $p < 0.001$ ) miR-671-5p level in 29 cases of RA patients (Fig. 5F). Furthermore, miR-671-5p expression was 4.6-fold elevated by miR-671-5p mimic, but was suppressed by 72% in RA-FLSs with miR-671-5p inhibitor transfection (Fig. 5G). Moreover, we found that miR-671-5p negatively regulated TLR4 expression in RA-FLSs, as identified by the decreased protein level of TLR4 in RA-FLSs with miR-671-5p mimic transfection and the elevated protein level of TLR4 in RA-FLSs after miR-671-5p inhibitor transfection (Fig. 5H). These data indicated that TLR4 was a target of miR-671-5p and was negatively regulated by miR-671-5p.

#### miR-671-5p mediates cell proliferation, migration, invasion and inflammatory response by regulating TLR4 in RA-FLSs

The function of miR-671-5p on TLR4 and RA-FLSs processes was investigated by transfecting RA-FLSs with miR-NC, miR-671-5p mimic, miR-671-5p mimic + pcDNA or TLR4 overexpression vector. As exhibited in Figure 6A, TLR4 protein level was reduced 60% by miR-671-5p overexpression, which was partly restored by transfection of TLR4 overexpression vector. Moreover, miR-671-5p overexpression repressed cell viability, induced cell cycle arrest and apoptosis in RA-FLSs, whereas overexpression of TLR4 partly

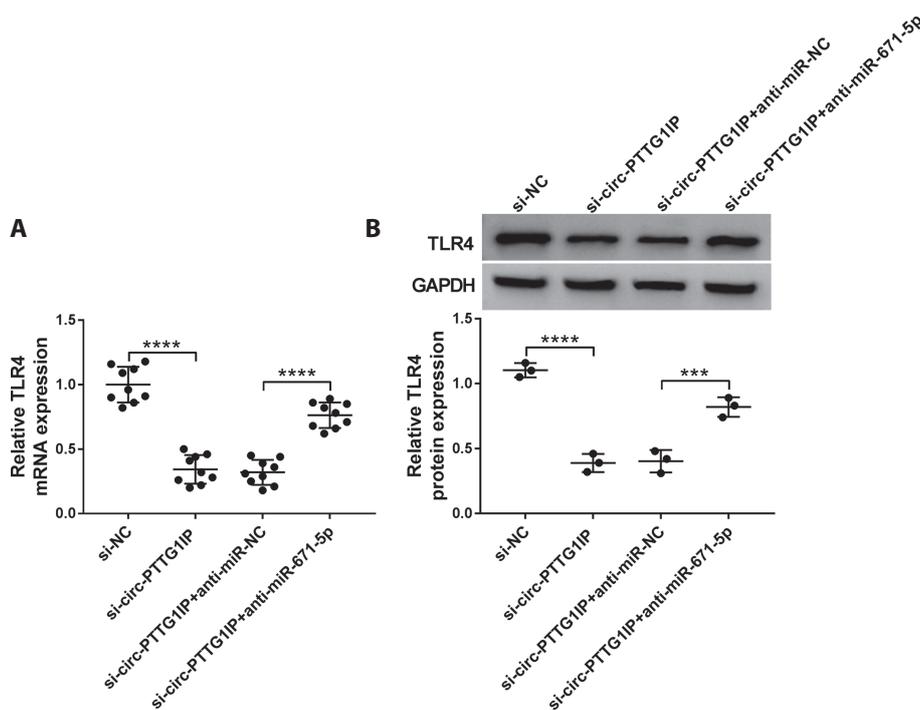
reversed these effects (Fig. 6B–D). In addition, miR-671-5p overexpression significantly constrained cell migration and invasion, and this effect was abolished by TLR4 upregulation (Fig. 6E,F). Furthermore, miR-671-5p overexpression significantly decreased the levels of IL-8, IL-1 $\beta$  and IL-6 in RA-FLSs with TNF- $\alpha$  stimulation, and this effect was mitigated by TLR4 overexpression (Fig. 6G). These results suggested miR-671-5p regulated proliferation, apoptosis, migration, invasion and inflammatory response of RA-FLSs through regulating TLR4.

#### circ-PTTG1IP upregulates TLR4 expression by sponging miR-671-5p in RA-FLSs

To explore whether circ-PTTG1IP promotes TLR4 expression by sponging miR-671-5p, RA-FLSs were transfected with si-NC, si-circ-PTTG1IP, si-circ-PTTG1IP + anti-miR-NC or anti-miR-671-5p, respectively. The results showed that TLR4 mRNA and protein levels were significantly reduced by si-circ-PTTG1IP, while it was elevated by co-transfection of anti-miR-671-5p (Fig. 7A,B). These results indicated that circ-PTTG1IP could regulate TLR4 expression by sponging miR-671-5p.

## Discussion

RA is a chronic inflammatory disease with a peak incidence at 50 to 60 years old (Sparks 2019). The fibroblast-like synovi-



**Figure 7.** The effect of circ-PTTG1IP and miR-671-5p on TLR4 expression. TLR4 mRNA (A) and protein levels (B) were detected in RA-FLSs with transfection of si-NC, si-circ-PTTG1IP, si-circ-PTTG1IP + anti-miR-NC or anti-miR-671-5p. \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . si-NC, siRNA negative control; TLR4, toll-like receptor 4; anti-miR-NC, miRNA inhibitor negative control. For more abbreviations, see Fig. 1.

ocytes are the key cells for the formation of invasive synovial tissue, which are closely associated with the pathogenesis of RA (Karami et al. 2020). The proliferation, migration, invasion and inflammatory response of fibroblast-like synoviocytes are reported to contribute to RA development (Wang et al. 2019c). And thousands of dysregulated circRNAs are involved in the thousands of RA (Wang et al. 2019b; Yang et al. 2019). However, the research on the roles of circRNAs in RA-FLSs is limited. Here, we first proposed that circRNA circ-PTTG1IP regulates the proliferation, apoptosis, migration, invasion and inflammatory response of RA-FLSs, and confirmed the regulatory network of circ-PTTG1IP/miR-671-5p/TLR4 axis in RA-FLSs.

Consistent with a previous study (Wen et al. 2020), circ-PTTG1IP expression was increased in RA patients and RA-FLSs, suggesting the abnormally expressed circ-PTTG1IP might participate in RA development. To investigate the effect of circ-PTTG1IP in RA-FLSs, loss-of-function experiments were conducted by transfecting RA-FLSs with si-NC or si-circ-PTTG1IP. Our research uncovered that circ-PTTG1IP knockdown promoted apoptosis in RA-FLSs, but repressed RA-FLSs proliferation by decreasing cell viability and inducing cell cycle arrest. Moreover, the migration and invasion of RA-FLSs are associated with the joint destruction and RA pathology (Zhu et al. 2019; Mosquera et al. 2020). Transwell assay disclosed that circ-PTTG1IP knockdown inhibited RA-FLSs migration and invasion. IL-8, IL-1 $\beta$  and IL-6 are the important pro-inflammatory cytokines in RA progression (Alam et al. 2017). Our study showed that circ-PTTG1IP knockdown suppressed the inflammatory response of RA-FLSs by decreasing the secretion of IL-8, IL-1 $\beta$  and IL-6. Thus, circ-PTTG1IP promotes RA-FLSs progression by regulating RA-FLSs proliferation, apoptosis, migration, invasion and inflammatory response.

circRNAs could act as a miRNA sponge to absorb functional miRNAs, decreasing the abundance of miRNA in the cytoplasm, thereby promoting mRNA translation, which mainly occurs in the cytoplasm (Kristensen et al. 2019). In current research, circ-PTTG1IP is mainly localized in cytoplasm. This led us to hypothesize that circ-PTTG1IP function as a miRNA sponge in RA-FLSs. And miR-671-5p was predicted to be the target of circ-PTTG1IP in RA-FLSs. A previous study suggested that miR-671-5p mitigated osteoarthritis progression (Zhang et al. 2019). However, the role of miR-671-5p in RA progression is largely unknown. In current research, miR-671-5p was expressed at a low level in RA, and circ-PTTG1IP negatively regulated miR-671-5p in RA-FLSs. Furthermore, we found that miR-671-5p function as a suppressor in RA, as identified by the decreasing RA-FLSs proliferation, migration, invasion and inflammatory response, as well as the elevated apoptosis in RA-FLSs, which was consistent with previous (Qiu et al. 2018; Zhang

et al. 2018; Xin et al. 2019; Song et al. 2020). In addition, miR-671-5p knockdown partly reversed the effects of circ-PTTG1IP knockdown on RA-FLSs progression, indicating that circ-PTTG1IP regulated RA-FLSs progression by sponging miR-671-5p.

Moreover, we further explored the downstream mechanism of miR-671-5p in RA-FLSs, and found that TLR4 was targeted by miR-671-5p. As an upstream regulator of the nuclear factor-kappa B (NF- $\kappa$ B) signaling, TLR4 promoted RA-FLSs proliferation, migration, invasion and inflammatory response by activating the NF- $\kappa$ B signaling pathway in RA (Shi et al. 2016; Liu et al. 2018; Wang et al. 2018; Yan et al. 2019; Zhang et al. 2020). Furthermore, TLR4 facilitated RA progression in a rat model (Acheh et al. 2019; Cao et al. 2019). These reports indicated that TLR4 exerted a promoting role in RA progression. Similarly, our study uncovered that TLR4 partly reversed the suppressive effects of miR-671-5p on RA-FLSs processes. Additionally, circ-PTTG1IP upregulated TLR4 expression through absorbing miR-671-5p. Thus, circ-PTTG1IP regulates RA-FLSs progression by targeting the miR-671-5p/TLR4 axis.

In conclusion, circ-PTTG1IP knockdown constrained RA-FLSs proliferation, migration, invasion and inflammatory response by regulating the miR-671-5p/TLR4 axis. This study provides a novel insight into RA progression, and indicates a novel target for the treatment of RA.

**Conflict of interest.** The authors declare that they have no financial conflicts of interest.

**Availability of data and materials.** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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