

# Over-expression of PTEN attenuates the inflammation of fibroblast-like synoviocytes in rheumatoid arthritis

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## Research

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# Abstract

**Background:** Rheumatoid arthritis (RA) is characterized by a tumor-like expansion of the synovium and the subsequent destruction of adjacent articular cartilage and bone. Recent studies have shown that phosphatase and tension homolog deleted on chromosome 10 (PTEN) might contribute to the survival of fibroblast-like synoviocytes (FLS) and the production of pro-inflammatory cytokines in RA.

**Methods :** The expression was determined in RA and adjuvant-induced arthritis (AIA) synovial tissues by immunohistochemistry. FLSs were treatment with bpv, PTEN-RNAi or over-expression plasmid in RA and AIA. FLSs migration was assessed. The ad-PTEN was also injected into the knee of AIA in vivo. Chromatin Immunoprecipitation (ChIP) and Methylation-special PCR (MSP) assay were used to study the expression of PTEN mRNA in DNA methylation.

**Results :** Down-regulated level of PTEN expression was observed in RA and AIA. Inhibition PTEN expression by bpv or PTEN-RNAi could promote the expression of pro-inflammatory cytokines, chemokines and migration of FLS with TNF- $\alpha$  in RA and AIA. Consistently, over-expression of PTEN reduced their low-expression of pro-inflammatory cytokines, chemokines and migration. Intra-articular injection of ad-PTEN in AIA knees dramatically reduced inflammatory and paw swelling in vivo. The ChIP and MSP assay has clearly detected the DNA methylation of PTEN was increased in FLS with TNF- $\alpha$ . Moreover, intraperitoneally injected 5-Aza in AIA also suppressed the inflammatory and paws swelling in vivo.

**Conclusions:** Our findings suggest that over-expression PTEN attenuates the formation of pro-inflammatory cytokines, chemokines and migration of FLS, and it may be regulated by DNA methylation in the pathogenesis of RA.

## Background

Rheumatoid arthritis (RA), a chronic and systemic autoimmune disease, is characterized by hyperplasia of synovial tissues, synovial inflammation and pannus, and subsequent destruction of adjacent articular cartilage and bone [1, 2]. It has been reported that fibroblast-like synoviocytes (FLS) [3], the major cells population invading the pannus, are actively involved in the in the various pathological events and inflammatory processes of RA [4, 5]. Activated FLS could directly secrete pro-inflammatory cytokines (tumor necrosis factor- $\alpha$ , TNF- $\alpha$  and interleukin, IL-1 $\beta$ ) [6], chemokines (monocyte chemoattractant protein-1, CCL-2) [7], matrix metalloproteinases (MMP-3, MMP-9), [5] and angiogenic factors [8] which are carried into the intra-articular synovial fluid and destroy cartilage and bone in onset of RA. It has been proved that the activation and immune dysregulation of FLS are the main factor in the pathogenesis and development of RA. Therefore, how to inhibit the activation and secretion pro-inflammatory of FLS is positive significance for RA patients, however,

Cytokines and chemokines play critical roles in the onset and progression of RA [9], particularly activation of FLS. Various pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-17A are requirement to onset and

development of RA in synoviocyte activation. It is one of the candidate methods of RA therapies that balancing these pro-inflammatory cytokines could prevent the occurrence and development of autoimmune disease[10]. TNF- $\alpha$  and IL-6, key pro-inflammatory cytokines, are present at high concentrations in the serum and synovial fluid and involved in the pathogenesis of RA. Sohn C et al. [11] have proved that prolonged TNF- $\alpha$  exposure could decrease histone levels and increase acetylation of the remaining histones through recruitment of p65 in RA FLS. Chemokines also play an important role in chronic synovitis, and macrophage inhibitory protein (MIP-1 $\alpha$ , CCL-3) and CCL-2 show abnormal expression in different stages of RA [7].

Recent studies have reported that phosphatase and tension homolog deleted on chromosome 10 (PTEN) might contribute to the survival and inflammation of FLS in RA [12, 13]. More importantly, PTEN exerts remarkable anti-inflammatory and anti-proliferation activity through blocking activation of the PI3-kinase/AKT pathway [14–16]. In addition, Wang et al. [17] also proposed that adenoviral with PTEN-human significantly reduced articular index, ankle circumference and histology scores, and also decreased the VEGF and IL-1 $\beta$  in collagen-induced arthritis. Interestingly, our previous research [15] found that over-expression of PTEN suppresses proliferation and migration of FLS in adjuvant-induced arthritis (AIA). Additionally, PTEN expression may be regulated by DNA methylation in the pathogenesis of AIA. However, potential functions of PTEN in the activation of FLS are still unknown. Hence, we hypothesized that PTEN was significantly associated with RA, especially, the pro-inflammatory cytokines, chemokines and migration of activated FLS.

To further elucidate the relationship between PTEN and the pro-inflammatory cytokines, chemokines and migration of FLS in RA, in particularly, we explored whether PTEN regulates the initiation of FLS pro-inflammatory cytokines, chemokines and migration and if it is closely associated with the activation of DNA methylation in the pathogenesis of RA.

## Materials And Methods

### Materials and reagents

Bpv (PTEN) and 5-Aza-2'-deoxycytidine (5-Aza) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Complete Freund's adjuvant was obtained from Chondrex, Inc. (Redmond, WA). Rabbit anti-PTEN, anti-TIMP metalloproteinase inhibitor 1 (TIMP-1), anti-TNF- $\alpha$  antibody and mouse anti-DNA methyltransferase 1 (DNMT1) monoclonal antibody were purchased from Abcam (Cambridge, UK). Rabbit anti-IL-1 $\beta$ , anti-IL-6 and anti-IL-17A, and mouse anti- $\beta$ -actin monoclonal antibody were obtained from Bioworld (Shanghai, China). Rabbit anti-MMP-3, anti-MMP-9, anti-AKT and anti-p-AKT antibody, and Simple CHIP<sup>®</sup> Enzymatic Chromatin IP Kit (Magnetic Beads) were purchased from Cell Signaling Technology (Danvers, MA). Peroxidase-Conjugated Goat anti-Rabbit IgG (H+L) were purchased from ZSGB-BIO (Beijing, China). The primers of PTEN, IL-1 $\beta$ , IL-6, IL-17A, IL-8, IL-10, MMP-3, MMP-9, TIMP-1, CCL-2, CCL-3, CCL-8 and  $\beta$ -actin were synthesized by Sangon Biotech Co., Ltd (Shanghai, China).

## **Human synovium and FLS**

Human synovium or FLS were extracted from patients with RA (6 patients) or OA (8 patients) undergoing total joint replacement. All patients were collected at the Department of Orthopedics, First Affiliated Hospital, Anhui Medical University, Hefei, China. And all patients with RA met the American College of Rheumatology 1987 revised criteria for seropositive RA as previously described. FLS were used between p4 and p9 passages.

## **Adjuvant-induced arthritis (AIA) of rat model**

The AIA model was induced by Sprague-Dawley rats (80-120 g, female) were treated with Complete Freund's adjuvant (Chondrex, Inc, 0.1 mL/100 g body weight) for 24 days by subcutaneously injection in the left hind paw [7, 21]. At same time, normal control rats were injected with normal saline. After 7 days, AIA rats were treatment with adenovirus carrying rattus PTEN (ad-PTEN) and 5-Aza. The 0.1 mL ad-PTEN or ad-GFP was intraarticular injected into AIA hind knees. And 5-Aza was intraperitoneal injected at a dose of 0.7 mg/kg/3 days for 21 days. The rats were provided from the Experimental Animal Center of Anhui Medical University. And efforts were made to reduce the number of animals used and their suffering in all animal experiments. All experimental protocols used on the animals were approved by the institutions' subcommittees on animal care of Anhui Medical University (approval number: 20160253).

## **Histopathology**

The synovium specimens from human and rats' knee joint were fixed by 4% paraformaldehyde for 48 h and embedded by paraffin. According to a standard procedure, hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC) and immunofluorescence (IF) were performed. And the pathological changes were assessed and photographed under CaseViewer (3DHISTECH Ltd., Hungary).

## **Enzyme-Linked Immunosorbent Assay (ELISA) assay**

After AIA treatment with ad-PTEN and 5-Aza for 21 days, the serum of rats was collected through abdominal aorta. And the RA FLS were treated for 48 h, the supernatant was collected by centrifugation. The levels of Rat IL-6 and TNF- $\alpha$ , human IL-6 and IL-8 were determined by ELISA kit (R&D, Minneapolis, USA) according to the manufacturer's protocol.

## **Isolation of peritoneal macrophages**

Peritoneal macrophages were collected from the peritoneum of AIA by douched with PBS. Cells were placed in 6 well plates at  $2.0-5.0 \times 10^6$  with high-glucose DMEM supplemented with 10 % (v/v) FBS (PAN Biotech, Germany). Adherent cells were harvested from the plates after 2 h of culture.

## **Isolation of peripheral blood mononuclear cell (PBMC)**

The blood was collected from rats AIA through abdominal aorta. And the samples were prepared with human peripheral blood lymphocyte separation fluid (Tianjin Hao Yang, China) according to the manufacturer's protocol prior to RNA extraction and protein analysis.

## **Cell culture**

FLS were derived by tissue direct separation method from rats AIA and human RA synovial and cultured in DMEM (HyClone, South Logan, UT, USA) supplemented with 20 % (v/v) FBS, 100 U/mL of penicillin, and 100 mg/mL of streptomycin (Beyotime, Shanghai, China) at 37 °C and in an atmosphere of 5 % CO<sub>2</sub>.

## **Immunocytochemistry staining**

The FLS were plated at a density of 1.0-2.0×10<sup>5</sup> cells/ml in 6-well plates for 24 h. After FLS treatment with TNF-α, immunocytochemistry staining was performed with rabbit anti-PTEN. And the Alexa Fluor 488-Conjugated Goat anti-rabbit IgG (H+L) (ZSGB-BIO, Beijing, China) and 4', 6-diamidino-2-phenylindole (DAPI; Beyotime, China) were incubated in dark. And then the cells were photographed under an Olympus BX-51 microscope (Olympus, Tokyo, Japan).

## **Small interfering RNA silencing and plasmid construction**

According to the manufacturer's instructions, FLS were transfected with small interfering RNA (RNAi; GenePharma, Shanghai, China) or over-expression plasmid (GeneChem, Shanghai) using lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). The oligonucleotide sequences were as follows: PTEN-RNAi (rat), 5'-CCGAUACUUCUCUCCAAAUTT-3' for the sense strand and 5'-AUUUGGAGAGAAGUAUCGGTT-3' for the antisense strand; PTEN-RNAi (human), 5'-CAGUAGAGGAGCCGUCAAATT-3' for the sense strand and 5'-UUUGACGGCUCCUCUACUGTT-3' for the antisense strand. A negative scrambled RNAi was used in parallel. And the FLS were transfected with rat PTEN-GV141 (rat) and PTEN-pcDNA3.1 (human) to induce the over-expression of PTEN, and with empty GV141 vector (GV141) or empty pcDNA3.1 vector as control. After transfection for 8 h, the FLS were cultured with complete medium at 37 °C for 48 h.

## **Ad-PTEN over-expression**

For recombinant adenovirus construction, over-expression of PTEN adenovirus (Ad-PTEN) and the negative control adenovirus (Ad-GFP) were obtained from Hanbio (Shanghai). The stock solutions of Ad-PTEN and Ad-GFP were 1×10<sup>10</sup> PFU/mL, respectively.

## **Methylation-specific PCR (MSP)**

DNA samples treated with a Wizard® DNA Clean-Up System (Promega, Madison, WI) according to the manufacturer's instructions. Unmethylated cytosine residues in the DNA samples were converted to uracil using a Methylamp™ DNA Modification Kit (EpiGenetek, Farmingdale, NY). Primers of methylated and unmethylated PTEN were as follows: PTEN (human, methylated) forward: 5'-GATGAGGTGATATACGTTGGCG-3' and reverse: 5'-TTTACACCGCTATCGAATCACAAT-3'; PTEN (human,

unmethylated) forward: 5'-GGATGAGGTGATATATGTTGGTGAT-3' and reverse: 5'-TTTTACACCACTATCAAATCACAATCA-3'; PTEN (rat, methylated) forward: 5'-CGGTCGGTGTTAAGTTTTTCGT-3' and reverse: 5'-AAAACGAATAATCCTCGCAACG-3'; PTEN (rat, unmethylated) forward: 5'-ATTTGGTTGGTGTTAAGTTTTTTGT-3' and reverse: 5'-AAAAACAAATAATCCTCACAACAAAC-3'.

### **Quantitative real-time PCR (q-PCR)**

Total RNA was extracted by classical TRIzol reagent (Invitrogen) from FLS and reverse transcribed to cDNA with iScript™ cDNA kit (Bio-Rad, CA). Then, the q-PCR reaction mixture comprises cDNA and SYBR Green q-PCR Master Mix (TOYOBO, Japan). Primer sequences are shown in Supplementary Table 1. All reaction was conducted 3 times, and the relative mRNA expression of target genes was obtained by normalization to the levels of  $\beta$ -actin.

### **Western blot**

The total protein was extracted by lysis buffer from FLS and denatured by boiling. Then, cell extract was isolated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blot onto PVDF membranes (Millipore, Bedford, MA). After blocking, it was incubated with primary antibody overnight. Rabbit antibodies (PTEN, IL-1 $\beta$ , IL-6, IL-17A, MMP-3, MMP-9 and TIMP-1) were used at a dilution of 1:500, and mouse anti-DNMT1 and anti- $\beta$ -actin were used at a dilution of 1:1, 000. Then, after washing, the blot was incubated with goat anti-mouse or anti-rabbit HRP-conjugated antibodies for 1 h. The protein blot bands were photographed by ChemiDoc™ MP Imaging System (Bio-Rad) with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore).

### **Wound-healing**

FLS were cultured in 24-well plate ( $5.0 \times 10^5$ /mL cells/ well). After cells reached to 60%-80%, the FLS treated with bpv, PTEN-RNAi or PTEN-GV141 for 24 h. And then, cells were serum deprived and scratched with a pipette tip. After 48 h and the cells were fixed with methanol, stained with crystal violet, and photographed by Olympus BX-51 microscope (Olympus).

### **Chromatin Immunoprecipitation (ChIP)**

The SimpleChIP® Kit (Cell Signaling Technology) was performed according to manufacturer's instructions. After FLS were cross-linked in 1% formaldehyde and lysed, it was immunoprecipitated using anti-DNMT1 monoclonal antibody overnight at 4 °C, and incubated with ChIP-grade Protein A/G Plus agarose beads for 2 h. After washing with three different buffers, the samples reverse cross-linked at 65 °C for 2 h, and detected ChIP signals by q-PCR.

### **Statistical analysis**

Data are presented as the means  $\pm$  standard deviation and analyzed using SPSS16.0 software. Statistical significances were determined by one-way ANOVA with a post-hoc Dunnett's test. In all cases, values of  $P < 0.05$  were considered to be statistically significant.

## Results

### PTEN expression is reduced in FLS of human RA and rats AIA

To confirm the role of PTEN in RA, we first studied to determine whether or not the expression of PTEN in synovial tissue. As show as Fig. 1A, the histopathological analysis (has proved that the rat AIA models were established successfully, and we also observed a significant increase in the number of infiltrating inflammatory cells. Moreover, IHC analysis indicated the clear down-regulation of PTEN expression in RA and AIA synovial tissues, very predominant in the lining but also in sublining (Fig. 1B). Of interest, while PTEN expression was negative in RA synovial and rat AIA samples, both lining and sublining were abundant for PTEN expression in most OA synovial and rat normal samples. What's more, the PTEN protein expression was also lower in RA by IF staining similarly, in rats AIA was lower than normal. In addition to reduced expression of PTEN, we also observed concomitant increased expression of IL-1 $\beta$ , IL-6, IL-17A and TNF- $\alpha$  in RA synovial and rats AIA samples, as determined by western blot. Of note, after FLS treatment with various inflammation factors *in vitro*, such as IL-1 $\beta$ , IL-6, IL-17A, TNF- $\alpha$ , IFN- $\gamma$  and LPS, expression of PTEN was decreased to different degrees in RA synovial and AIA, showed as Fig. 1F and G. And IF staining (supplementary Fig. 1 A) determined PTEN expression was low in RA and AIA FLS treatment with TNF- $\alpha$ . Collectively, these results showed that PTEN expression was clearly reduced in both human RA and AIA FLS.

### PTEN low-expression modulates pro-inflammation and migration of FLS in RA and AIA

In order to investigate the role of PTEN in RA FLS functions, we conducted its effect on the pro-inflammatory cytokines and migration of FLS by following treatment with PTEN inhibitor bpv or PTEN-RNAi in RA and AIA *in vitro*. As shown in Fig. 2A and B, the results of western blot and q-PCR showed that PTEN expression were markedly down-regulated in RA FLS with bpv or PTEN-RNAi, along with down-regulated p-AKT expression. More significantly, after treatment with TNF- $\alpha$ , western blot and q-PCR has verified expression of pro-inflammatory cytokines IL-1 $\beta$ , IL-6 and IL-17A were notably up-regulated by bpv or PTEN-RNAi in human RA FLS (Fig. 2 C and supplementary Fig. 3 E), however, IL-10 mRNA was down-regulated (Fig. 2 B). More interestingly, bpv or PTEN-RNAi also increased secretion of IL-6 and IL-8 protein in human RA FLS with TNF- $\alpha$  (Fig. 2 F, H). In addition, inhibition of PTEN expression with bpv or PTEN-RNAi also could increase chemokines IL-8, CCL-2, CCL-3 and CCL-8 mRNA and reduce IL-10 mRNA expression by q-PCR in RA FLS (Fig. 2 B, and D). As shown as supplementary Fig. 2, the expression of pro-inflammatory cytokines IL-1 $\beta$ , IL-6 and IL-17A and chemokines CCL-2 and CCL-3 mRNA were also increased markedly by bpv or PTEN-RNAi in rat AIA FLS with TNF- $\alpha$ .

More significantly, as shown in Fig. 2 E and supplementary Fig. 3 H, the mRNA and protein expression of MMP-3 and MMP-9 were up-regulated observably by bpv or PTEN-RNAi, however, TIMP-1 expression was

down-regulated in RA FLS with TNF- $\alpha$ . What's more, inhibition of PTEN by bpv or PTEN-RNAi increased the length of the scar in the migration assay in AIA FLS (Fig. 2 G). Similarly, western blot and q-PCR have verified expressions of MMP-3 and MMP-9 were also increased markedly by bpv or PTEN-RNAi, as same as TIPM-1 was down-regulated in AIA FLS with TNF- $\alpha$ , as show supplementary Fig. 3. Thus, these researches had proved that the PTEN low-expression could increase markedly pro-inflammation, chemokines and migration of FLS in RA and AIA.

### **PTEN over-expression modulates pro-inflammation and migration of FLS in RA and AIA**

Previous studies have shown that inhibited PTEN expression could increase pro-inflammatory cytokines, chemokines and migration of FLS in RA. To further determine the underlying mechanism of PTEN during pro-inflammatory cytokines, chemokines and migration of FLS, rat PTEN-GV141 and human PTEN-pcDNA3.1 vector were used to over-express PTEN *in vitro*. As shown in Fig. 3 A and B, the results of western blot and q-PCR showed the expression of PTEN were up-regulated markedly by transfected of RA FLS with human PTEN-pcDNA3.1, along with up-regulated p-AKT expression. More significantly, western blot and q-PCR has proved pro-inflammatory cytokines IL-1 $\beta$ , IL-6 and IL-17A were down-regulated markedly by PTEN-pcDNA3.1 in human RA FLS treatment with TNF- $\alpha$  (Fig. 3C and supplementary Fig. 4 G), however, IL-10 mRNA was up-regulated (Fig. 3 B). More interestingly, PTEN-pcDNA3.1 also reduced secretion of IL-6 and IL-8 protein in human RA FLS with TNF- $\alpha$  (Fig. 3F and H). In addition, after treating with PTEN-pcDNA3.1, the mRNA expression of chemokines IL-8, CCL-2, CCL-3 and CCL-8 were reduced observably in RA FLS with TNF- $\alpha$ , (Fig. 3B and D). Moreover, as shown in supplementary Fig. 4 A-D, after FLS treatment with TNF- $\alpha$ , over-expression of PTEN by PTEN-GV141 also reduced expression of pro-inflammatory cytokines and chemokines in rats AIA. More significantly, as shown in figure 3 E and supplementary Fig. 4 H, MMP-3 and MMP-9 mRNA and protein expression were down-regulated observably by PTEN-pcDNA3.1, whereas TIPM-1 was up-regulated in RA FLS with TNF- $\alpha$ . What's more, over-expression of PTEN by PTEN-GV141 decreased the length of the scar in the migration assay in AIA FLS (Fig. 3 G). Similarly, the expressions of MMP-3 and MMP-9 mRNA and protein were down-regulated markedly by PTEN-GV141, whereas TIPM-1 was up-regulated in rats AIA FLS with TNF- $\alpha$ , as show supplementary Fig. 4 E and F. Taken together, our findings suggested that over-expression of PTEN had a profound inhibitory effect on pro-inflammatory cytokines, chemokines and migration of FLS in RA *in vitro*.

In order to assess the effect of PTEN over-expression in synovial tissues *in vivo*, we have intra-articular injected adenovirus carrying rattus PTEN (ad-PTEN) into rat knees, and adenovirus carrying GFP (ad-GFP) as a control in the rats AIA knees. Over-expression of PTEN in the synovial lining was effective since PTEN expression was increased in ad-PTEN-injected joints compared with ad-GFP-injected joints (Fig. 4 A and B). Interestingly, as show as Fig. 4 C we also observed down-regulated remarkably inflammatory cells infiltrations by intra-articular injection of ad-PTEN in AIA knees. Moreover, injection of ad-PTEN could significantly reduced paw swelling by measuring secondary paw swelling compared with ad-GFP-injected joints in AIA as show Fig. 4 D. What's more, the expression of IL-6 and TNF- $\alpha$  protein were also down-regulated remarkably in AIA serum with ad-PTEN (Fig. 4 E). On the other hand, we also found the expression of pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-17A and TNF- $\alpha$  were all down-regulated markedly

by intra-articular injection of ad-PTEN in AIA synovial tissues, peritoneal macrophage and PBMC as show supplementary Fig. 5. All together, this evidences has proved over-expression of PTEN with intra-articular injection of ad-PTEN could inhibit expression of pro-inflammatory cytokines and paw swelling in AIA *in vivo*.

### **PTEN expression may be regulated by DNA methylation in RA**

It is evident that the PTEN negatively regulates the pro-inflammatory cytokines and migration of FLS in RA. To clarify the mechanism or the down-regulation of PTEN expression in RA FLS, we examine whether DNA methylation mediates its gene expression. First, we identified CpG islands near the first exon of the PTEN transcript and in the upstream region suggesting that the decrease of PTEN gene expression may be related to CpG methylation (supplementary Fig. 6 C). Moreover, we found the expression of DNMT1 protein was up-expression in RA FLS compared with OA (Fig. 5 A), same as AIA FLS compared with normal rats (supplementary Fig. 6 A). Interestingly, PTEN expression was decreased to different degrees in RA synovial and rats AIA FLS with various inflammation factors *in vitro*, showed as Fig. 5 B and supplementary Fig. 6 B. What's more, as shown in Fig. 5 C, D, and supplementary Fig. 6 D, E, the CHIP and MSP assay has clearly detected DNMT1 was recruited to the coding region of PTEN gene in RA and AIA FLS treatment with TNF- $\alpha$ . In addition, after treatment with methylation inhibitor 5-Aza in FLS, the PTEN expression was over-expression obviously, as show as Fig. 5 E and F, conversely, DNMT1 protein was up-regulated significantly in RA with TNF- $\alpha$ . As same as expected, expression of pro-inflammatory cytokines and chemokines were reduced observably by 5-Aza in RA FLS with TNF- $\alpha$  (Fig. 5 F-H). On the other hand, as shown in supplementary Fig. 6 and 7, after FLS with TNF- $\alpha$ , 5-Aza also reduced expression of pro-inflammatory, chemokines and migration of FLS in RA and AIA. Taken together, these data show that treatment of FLS with methylation inhibitor 5-Aza could suppress their pro-inflammatory cytokines, chemokines and migration *in vitro* and PTEN may have a role in methylation in RA.

In our research, 5-Aza, an inhibitor of DNMTs, was also intraperitoneally injected into rats AIA *in vivo*. First, expression of PTEN was up-regulated markedly by intraperitoneally injected 5-Aza in the synovial compared with AIA (Fig. 6 A and B). Moreover, we also observed down-regulated remarkably inflammatory cells infiltrations by intraperitoneally injected 5-Aza in AIA knees as show Fig. 6 C. Similarly, intraperitoneally injected 5-Aza could significantly reduced paw swelling by measuring secondary paw swelling compared with normal joints in AIA (Fig. 6 D). Moreover, expression of IL-6 and TNF- $\alpha$  protein were also down-regulated remarkably in AIA serum with 5-Aza (Fig. 6 E). On the other hand, we also found the expression of DNMT1 and pro-inflammatory cytokines were all down-regulated markedly by intraperitoneally injected 5-Aza in AIA synovial tissues, peritoneal macrophage and PBMC as show supplementary Fig. 8 and 9. All together, this evidences proved low-expression of DNMT1 with intraperitoneally injected 5-Aza could inhibit expression of pro-inflammatory cytokines and paw swelling in AIA *in vivo*.

## **Discussion**

The AIA model, injection Completes Freund's adjuvant, has been widely used in the mechanism research of RA [15, 18, 19]. The rats AIA model has similar characteristics with RA in histology and immunology, and it is one of the models to evaluate the treatment and research of RA. It is evident that various inflammatory cells are activated, such as innate immune cells (mast cells and macrophages, et al.), adaptive immune cells (T and B cells) and FLS in the pathogenesis of RA [1]. The exact pathogenesis of RA has not been fully described, but an important feature involves communication between FLS with inflammatory cells in the joint [20]. In addition, the activated of FLS population potentially promotes the infiltration, recruitment and retention of T lymphocyte and macrophage by producing various inflammatory cytokines (such as pro-inflammatory, extracellular matrix proteins, chemokines and cell adhesion molecules) in RA [21, 22]. These cells communicate via a network of proteins known as cytokines, some of which exert pro-inflammatory actions and others that provide anti-inflammatory or immune-regulatory effects. However, there are no drugs that target FLSs, and the mechanism that regulates FLS activation remains unclear. Therefore, how to target the inhibition pro-inflammatory cytokines and chemokines of FLSs has positive significance for RA treatment.

In this study, we provided the first detailed evidence that PTEN may regulate the formation of pro-inflammatory cytokines, chemokines and migration of FLS and it's closely associated with DNA methylation in RA pathogenesis. Many researches [23, 24] have found PTEN expression is lacking in the lining layer of RA synovium. In this paper, we also found expression of PTEN was detected in RA synovial tissues, especially, after FLS treatment with various inflammation factors in vitro in RA and AIA. Moreover, we also have measured inhibition of PTEN expression with inhibitor bpv or PTEN-RNAi could active pro-inflammation cytokines (IL-1 $\beta$ , IL-6 and IL-17A) of FLS treatment with TNF- $\alpha$  in RA and AIA. These different cytokines could play overlapping biological roles, but they may produce a complex network of function that cooperate or promote each other to perform multiple physiological effects. What's more, over-expression of PTEN could down-regulation pro-inflammation cytokines of FLS treatment with TNF- $\alpha$  in RA and AIA. More significantly, the inflammatory cells infiltrations and paw swelling were down-regulated remarkably by intra-articular injection of ad-PTEN in AIA knees in vivo. Similarly, the expression of IL-6 and TNF- $\alpha$  were also down-regulated remarkably in AIA serum. Interestingly, the expressions of pro-inflammatory cytokines were also reduced in AIA synovial tissues, peritoneal macrophage and PBMC in vivo. Riquelme SA et al. [25]also found IL-6, IL-1 $\alpha$ , IL-1 $\beta$  and KC were elevated in lung lysates of PNTN<sup>-/-</sup> mice. Thus, we speculated PTEN has a positive effect on the treatment of RA by suppressing the production of pro-inflammatory cytokines of FLS in RA.

Along with activation of FLS, migration, which is stimulated by chemokines, has also been shown to significantly aggravate inflammation and bone destruction in progression of RA[8, 26]. And these chemokines are abundantly expressed in the RA synovium, these chemokines may facilitate fibroblast recruitment to the synovium, and subsequent induce activation of FLS [6, 27]. Interestingly, we also have measured inhibition of PTEN expression with bpv or PTEN-RNAi could active the expression of chemokines (IL-8, CCL-2, CCL-3 and CCL-8) in FLS with TNF- $\alpha$ . Chemokines regulate chemotaxis in nearby responsive cells and are implicated in chronic inflammatory diseases, such as RA, atherosclerosis, and

adipose inflammation [28]. Furthermore, over-expression of PTEN could down-regulation chemokines expression of FLS treatment with TNF- $\alpha$  in RA and AIA. On the other hand, altering the expression of PTEN can also regulate the migration of FLS treatment with TNF- $\alpha$  in RA and AIA in vitro. Consequently, PTEN also negatively regulates the expression of chemokines and migration of FLS in RA and AIA.

It has been demonstrated that the expression of PTEN mRNA was associated with DNA methylation in gastric cancer [29], melanoma [30, 31]. DNA methylation is a kind of epigenetic modification that plays a critical role in regulating gene expression and potentially contributes to immune dysregulation [32, 33]. Moreover, Zhu et al. [34] found that methylation of PARP9 was correlated with mRNA level in Jurkat cells and T lymphocytes isolated from patients with RA. In this study, after FLS treatment with TNF- $\alpha$ , we also proved the expression of DNMT1 was up-regulated in RA and AIA, particularly, and recruited to the coding region of PTEN gene. What's more, with methylation inhibitor 5-Aza in FLS, the PTEN mRNA and protein expression was over-expression obviously in RA. On the other hand, the expression of pro-inflammatory cytokines, chemokines and migration of FLS were suppressed variously in RA. Interestingly, the expressions of pro-inflammatory cytokines were also reduced in FLS by intraperitoneally injected 5-Aza, AIA synovial tissues peritoneal macrophage and PBMC cells in vivo. To summarize, DNA methylation may regulate PTEN expression in the pathogenesis of RA (Fig. 7).

## Conclusion

This study is not without some limitations. The PTEN<sup>-/-</sup>-knockout mouse and various RA models could be used to elucidate the role of PTEN in RA. In summary, our findings in the present study suggested that PTEN might play a pivotal role during pro-inflammatory cytokines, chemokines and migration of FLS through activation of AKT signaling pathway. Additionally, PTEN expression may be regulated by DNA methylation in the pathogenesis of AIA. These findings indicate the potential of PTEN as a therapeutic target for RA.

## Abbreviations

ad-GFP: adenovirus carrying GFP; ad-PTEN: adenovirus carrying rattus PTEN; AIA: adjuvant-induced arthritis; CCL-2/MCP-1: monocyte chemoattractant protein-1; CCL-3/MIP-1 $\alpha$ : macrophage inhibitory protein; ChIP: Chromatin Immunoprecipitation; DNMT1: DNA methyltransferase 1; ELISA: Enzyme-Linked Immunosorbent Assay; FLS: fibroblast-like synoviocytes; HRP: horseradish peroxidase; H&E: Hematoxylin and eosin; IF: immunofluorescence; IHC: immunohistochemistry; IL: interleukin; MMPs: matrix metalloproteinases; MSP: Methylation-specific PCR; PBMC: peripheral blood mononuclear cell; PTEN: phosphatase and tension homolog deleted on chromosome 10; q-PCR: quantitative real-time PCR; RA: Rheumatoid arthritis; TIMP-1: tissue inhibitor of metalloproteinase inhibitor 1; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ .

## Declarations

## Acknowledgements

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## Authors' contributions

XF. Li performed all experiments, analyzed the data and wrote the manuscript. B Song helped to collect the RA, OA and normal synovial tissues. QQ. Xu, MW. Yang, X. Chen, SQ. Yin helped to isolated FLSs and helped to western blotting, q-PCR, histological analysis and MSP. L. Zhang participated in the design of the study. XM. Meng helped to revise the manuscript. J. Li conceived the study and revised the manuscript. All authors approved the final manuscript.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Ethics approval and consent to participate

All the animal experiments were performed in accordance with the Regulations of the Experimental Animal Administration issued by the State Committee of Science and Technology of China. Efforts were made to minimize the number of animals used and their suffering. Animals were maintained in accordance with the Guides of Center for Developmental Biology, Anhui Medical University for the Care and Use of Laboratory. Animals and all experiments used protocols approved by the institutions' subcommittees on animal care.

**Consent for publication** Not applicable.

**Competing interests** The authors declare that they have no competing interests.

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## Supplemental Information Note

### Supplementary Figure Legends

#### Supplementary Figure 1.

(A) Representative immunocytochemistry images of PTEN staining in RA FLSs with TNF- $\alpha$  (10 ng/mL).

#### Supplementary Figure 2.

(A) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the protein levels of PTEN and p-AKT were analyzed by western blot in FLSs with bpv (250 nM) in AIA. (B) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the mRNA levels of PTEN, CCL-2 and CCL-3 were analyzed by q-PCR assays in FLSs with bpv (250 nM) in AIA. (C) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the protein levels of PTEN and p-AKT were analyzed by western blot in FLSs with PTEN-RNAi in AIA. (D) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the mRNA levels of PTEN, CCL-2 and CCL-3 were analyzed by q-PCR assays in FLSs with PTEN-RNAi in AIA. (E) The protein levels of IL-1 $\beta$ , IL-6 and IL-17A of FLSs with TNF- $\alpha$  (10 ng/mL) were analyzed by western blot in FLSs with bpv (250 nM) in AIA. (F) The mRNA levels of IL-1 $\beta$ , IL-6 and IL-17A of FLSs with TNF- $\alpha$  (10 ng/mL) were analyzed by q-PCR in FLSs with bpv (250 nM) in AIA. (G) The protein levels of IL-1 $\beta$ , IL-6 and IL-17A of FLSs with TNF- $\alpha$  (10 ng/mL) were analyzed by western blot in FLSs with PTEN-RNAi in AIA. (H) The mRNA levels of IL-1 $\beta$ , IL-6 and IL-17A of FLSs with TNF- $\alpha$  (10 ng/mL) were analyzed by q-PCR in

FLSs with PTEN-RNAi in AIA. All values were expressed as mean  $\pm$  SD.  $^{##}$  P < 0.01 vs AIA group;  $^{*}$  P < 0.05,  $^{**}$  P < 0.01 vs TNF- $\alpha$  group or NC-RNAi group.

#### Supplementary Figure 3.

(A) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the protein levels of MMP-3, MMP-9 and TIMP-1 were analyzed by western blot in FLSs with bpv (250 nM) in AIA. (B) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the mRNA levels of MMP-3, MMP-9 and TIMP-1 were analyzed by q-PCR assays in FLSs with bpv (250 nM) in AIA. (C) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the protein levels of MMP-3, MMP-9 and TIMP-1 were analyzed by western blot in FLSs with PTEN-RNAi in AIA. (D) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the mRNA levels of MMP-3, MMP-9 and TIMP-1 were analyzed by q-PCR assays in FLSs with PTEN-RNAi in AIA. (E) The mRNA levels of IL-1 $\beta$ , IL-6 and IL-17A of FLSs with TNF- $\alpha$  (10 ng/mL) were analyzed by q-PCR in FLSs with bpv (250 nM) or PTEN-RNAi in RA. (F) The mRNA levels of MMP-3, MMP-9 and TIMP-1 of FLSs with TNF- $\alpha$  (10 ng/mL) were analyzed by q-PCR in FLSs with bpv (250 nM) or PTEN-RNAi in RA. All values were expressed as mean  $\pm$  SD.  $^{##}$  P < 0.01 vs AIA group;  $^{*}$  P < 0.05,  $^{**}$  P < 0.01 vs TNF- $\alpha$  group or NC-RNAi group.

#### Supplementary Figure 4.

(A) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the protein levels of PTEN and p-AKT were analyzed by western blot in FLSs with PTEN-GV141 in AIA. (B) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the mRNA levels of PTEN, CCL-2 and CCL-3 were analyzed by q-PCR assays in FLSs with PTEN-GV141 in AIA. (C) The protein levels of IL-1 $\beta$ , IL-6 and IL-17A of FLSs with TNF- $\alpha$  (10 ng/mL) were analyzed by western blot in FLSs with PTEN-GV141 in AIA. (D) The mRNA levels of IL-1 $\beta$ , IL-6 and IL-17A of FLSs with TNF- $\alpha$  (10 ng/mL) were analyzed by q-PCR in FLSs with PTEN-GV141 in AIA. (E) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the protein levels of MMP-3, MMP-9 and TIMP-1 were analyzed by western blot in FLSs with PTEN-GV141 in AIA. (F) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the mRNA levels of MMP-3, MMP-9 and TIMP-1 were analyzed by q-PCR in FLSs with PTEN-GV141 in AIA. (G) The mRNA levels of IL-1 $\beta$ , IL-6 and IL-17A of FLSs with TNF- $\alpha$  (10 ng/mL) were analyzed by q-PCR in FLSs with PTEN-pcDNA3.1 in RA. (H) The mRNA levels of MMP-3, MMP-9 and TIMP-1 of FLSs with TNF- $\alpha$  (10 ng/mL) were analyzed by q-PCR in FLSs with PTEN-pcDNA3.1 in RA. All values were expressed as mean  $\pm$  SD.  $^{##}$  P < 0.01 vs AIA group;  $^{*}$  P < 0.05,  $^{**}$  P < 0.01 vs GV141 group.

#### Supplementary Figure 5.

(A) After AIA with intra-articular injection of ad-PTEN, the protein levels of IL-1 $\beta$ , IL-6, IL-17A, TNF- $\alpha$  and PTEN were analyzed by western blot in synovial tissues. (B) After AIA with intra-articular injection of ad-PTEN, the mRNA levels of IL-1 $\beta$ , IL-6, IL-17A, TNF- $\alpha$  and PTEN were analyzed by q-PCR assays in synovial tissues. (C) After AIA with intra-articular injection of ad-PTEN, the protein levels of IL-1 $\beta$ , IL-6, IL-17A, TNF- $\alpha$  and PTEN were analyzed by western blot in peritoneal macrophages. (D) After AIA with intra-articular injection of ad-PTEN, the mRNA levels of IL-1 $\beta$ , IL-6, IL-17A, TNF- $\alpha$  and PTEN were analyzed by q-PCR

assays in peritoneal macrophages. (E) After AIA with intra-articular injection of ad-PTEN, the protein levels of IL-1 $\beta$ , IL-6, IL-17A, TNF- $\alpha$  and PTEN were analyzed by western blot in PBMC. (F) After AIA with intra-articular injection of ad-PTEN, the mRNA levels of IL-1 $\beta$ , IL-6, IL-17A, TNF- $\alpha$  and PTEN were analyzed by q-PCR assays in PBMC. All values were expressed as mean  $\pm$  SD.  $^{##}$  P < 0.01 vs normal group; \*P < 0.05, \*\*P < 0.01 vs ad-GFP group.

#### Supplementary Figure 6.

(A) The expression of DNMT1 protein was analyzed by western blot in AIA FLSs. (B) The protein level of DNMT1 was analyzed by western blot in AIA FLSs treatment with IL-1 $\beta$  (2 ng/mL), IL-6 (5 ng/mL), IL-17A (10 ng/mL), TNF- $\alpha$  (10 ng/mL), IFN- $\gamma$  (10 ng/mL) and LPS (1  $\mu$ g/mL). (C) We projected near the transcript first exon and first exon upstream found CPG Island with CG point rich form rattus and human PTEN gene. (D) MSP analysis detected CpG methylation level of PTEN reversed in AIA FLSs with TNF- $\alpha$ . (E) After incubation with anti-DNMT1 antibody, ChIP staining detected the mRNA of PTEN in AIA FLSs with TNF- $\alpha$ . (F) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the protein levels of DNMT1, PTEN and p-AKT were analyzed by western blot in FLSs with 5-Aza (2  $\mu$ M) in AIA. (G) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), mRNA levels of IL-8, IL-10 and PTEN were analyzed by q-PCR in FLSs with 5-Aza (2  $\mu$ M) in AIA. (H) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), mRNA levels of IL-8, IL-10 and PTEN were analyzed by q-PCR in FLSs with 5-Aza (2  $\mu$ M) in RA. (I) The mRNA levels of IL-1 $\beta$ , IL-6 and IL-17A of FLSs with TNF- $\alpha$  (10 ng/mL) were analyzed by q-PCR in FLSs with 5-Aza (2  $\mu$ M) in RA. All values were expressed as mean  $\pm$  SD.  $^{##}$  P < 0.01 vs AIA group; \*P < 0.05, \*\*P < 0.01 vs TNF- $\alpha$  group.

#### Supplementary Figure 7.

(A) The protein levels of IL-1 $\beta$ , IL-6 and IL-17A of FLSs with TNF- $\alpha$  (10 ng/mL) were analyzed by western blot in FLSs with 5-Aza (2  $\mu$ M) in AIA. (B) The mRNA levels of IL-1 $\beta$ , IL-6 and IL-17A of FLSs with TNF- $\alpha$  (10 ng/mL) were analyzed by q-PCR in FLSs with 5-Aza (2  $\mu$ M) in AIA. (C) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the protein levels of MMP-3, MMP-9 and TIMP-1 were analyzed by western blot in FLSs with 5-Aza (2  $\mu$ M) in AIA. (D) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the mRNA levels of MMP-3, MMP-9 and TIMP-1 were analyzed by q-PCR assays in FLSs with 5-Aza (2  $\mu$ M) in AIA. (E) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), the protein levels of MMP-3, MMP-9 and TIMP-1 were analyzed by western blot in FLSs with 5-Aza (2  $\mu$ M) in RA. (F) After FLSs treatment with TNF- $\alpha$  (10 ng/ml), the mRNA levels of MMP-3, MMP-9 and TIMP-1 were analyzed by q-PCR assays in FLSs with 5-Aza (2  $\mu$ M) in RA. (G) Representative images of migration are shown in FLSs with 5-Aza (2  $\mu$ M) in AIA. (H) After FLSs treatment with TNF- $\alpha$  (10 ng/mL), mRNA levels of CCL-2, CCL-3 and CCL-8 were analyzed by q-PCR in FLSs with 5-Aza (2  $\mu$ M) in RA. All values were expressed as mean  $\pm$  SD.  $^{##}$  P < 0.01 vs AIA group or RA group; \*P < 0.05, \*\*P < 0.01 vs TNF- $\alpha$  group.

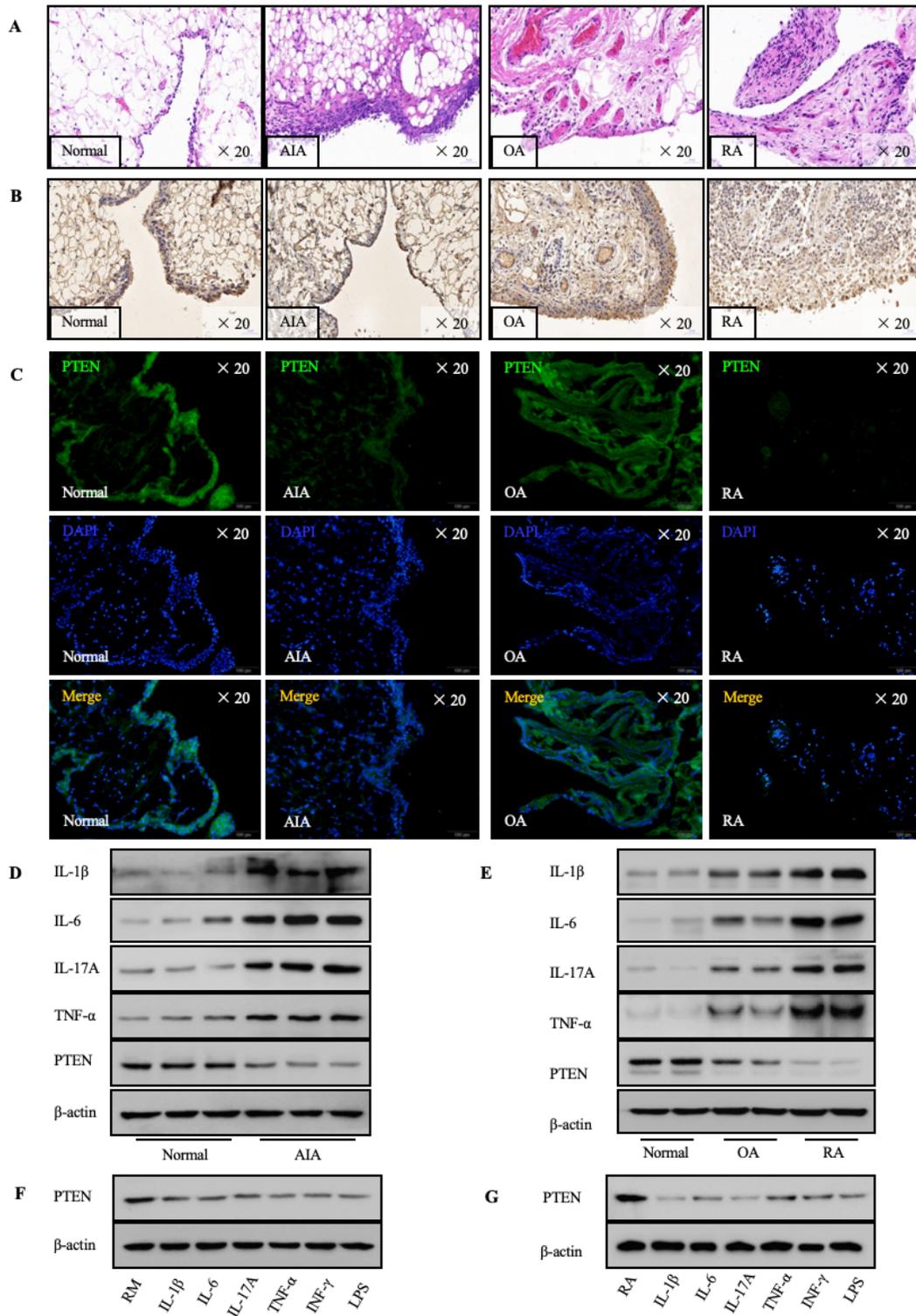
#### Supplementary Figure 8.

(A) After AIA with intraperitoneally injected 5-Aza (0.7 mg/kg), the protein levels of PTEN, TNF- $\alpha$  and DNMT1 were analyzed by western blot in synovial tissues. (B) After AIA with intraperitoneally injected 5-Aza (0.7 mg/kg), the mRNA levels of TNF- $\alpha$  and PTEN were analyzed by q-PCR assays in synovial tissues. (C) After AIA with intraperitoneally injected 5-Aza (0.7 mg/kg), the protein levels of IL-1 $\beta$ , IL-6 and IL-17A were analyzed by western blot in synovial tissues. (D) After AIA with intraperitoneally injected 5-Aza (0.7 mg/kg), the mRNA levels of IL-1 $\beta$ , IL-6 and IL-17A were analyzed by q-PCR assays in synovial tissues. (E) After AIA with intraperitoneally injected 5-Aza (0.7 mg/kg), the protein levels of PTEN, TNF- $\alpha$  and DNMT1 were analyzed by western blot in peritoneal macrophages. (F) After AIA with intraperitoneally injected 5-Aza (0.7 mg/kg), the mRNA levels of TNF- $\alpha$  and PTEN were analyzed by q-PCR assays in peritoneal macrophages. (G) After AIA with intraperitoneally injected 5-Aza (0.7 mg/kg), the protein levels of IL-1 $\beta$ , IL-6 and IL-17A were analyzed by western blot in peritoneal macrophages. (H) After AIA with intraperitoneally injected 5-Aza (0.7 mg/kg), the mRNA levels of IL-1 $\beta$ , IL-6 and IL-17A were analyzed by q-PCR assays in peritoneal macrophages. All values were expressed as mean  $\pm$  SD. <sup>##</sup> P < 0.01 vs normal group; \*P < 0.05, \*\*P < 0.01 vs AIA group.

Supplementary Figure 9.

(A) After AIA with intraperitoneally injected 5-Aza (0.7 mg/kg), the protein levels of PTEN, TNF- $\alpha$  and DNMT1 were analyzed by western blot in PBMC. (B) After AIA with intraperitoneally injected 5-Aza (0.7 mg/kg), the mRNA levels of TNF- $\alpha$  and PTEN were analyzed by q-PCR assays in PBMC. (C) After AIA with intraperitoneally injected 5-Aza (0.7 mg/kg), the protein levels of IL-1 $\beta$ , IL-6 and IL-17A were analyzed by western blot in PBMC. (D) After AIA with intraperitoneally injected 5-Aza (0.7 mg/kg), the mRNA levels of IL-1 $\beta$ , IL-6 and IL-17A were analyzed by q-PCR assays in PBMC. All values were expressed as mean  $\pm$  SD. <sup>##</sup> P < 0.01 vs normal group; \*P < 0.05, \*\*P < 0.01 vs AIA group.

## Figures



**Figure 1**

PTEN expression is reduced in FLS of human RA and rats AIA. (A) Representative H&E staining in RA and AIA synovium. (B) Representative IHC images of PTEN staining in RA and AIA synovium. (C) Representative IF images of PTEN staining in RA and AIA synovium. (D) The protein levels of IL-1 $\beta$ , IL-6, IL-17A, TNF- $\alpha$  and PTEN were analyzed by western blot in AIA FLS. (E) The protein levels of IL-1 $\beta$ , IL-6, IL-17A, TNF- $\alpha$  and PTEN were analyzed by western blot in RAFLS. (F) The protein level of PTEN was

analyzed by western blot in AIA FLS treat with IL-1 $\beta$  (2 ng/mL), IL-6 (5 ng/mL), IL-17A (10 ng/mL), TNF- $\alpha$  (10 ng/mL), IFN- $\gamma$  (10 ng/mL) and LPS (1  $\mu$ g/mL). (G) The protein level of PTEN was analyzed by western blot in RA FLS treat with IL-1 $\beta$  (2 ng/mL), IL-6 (5 ng/mL), IL-17A (10 ng/mL), TNF- $\alpha$  (10 ng/mL), IFN- $\gamma$  (10 ng/mL) and LPS (1  $\mu$ g/mL). Results are average of three different RA FLS lines. All values were expressed as mean  $\pm$  SD.

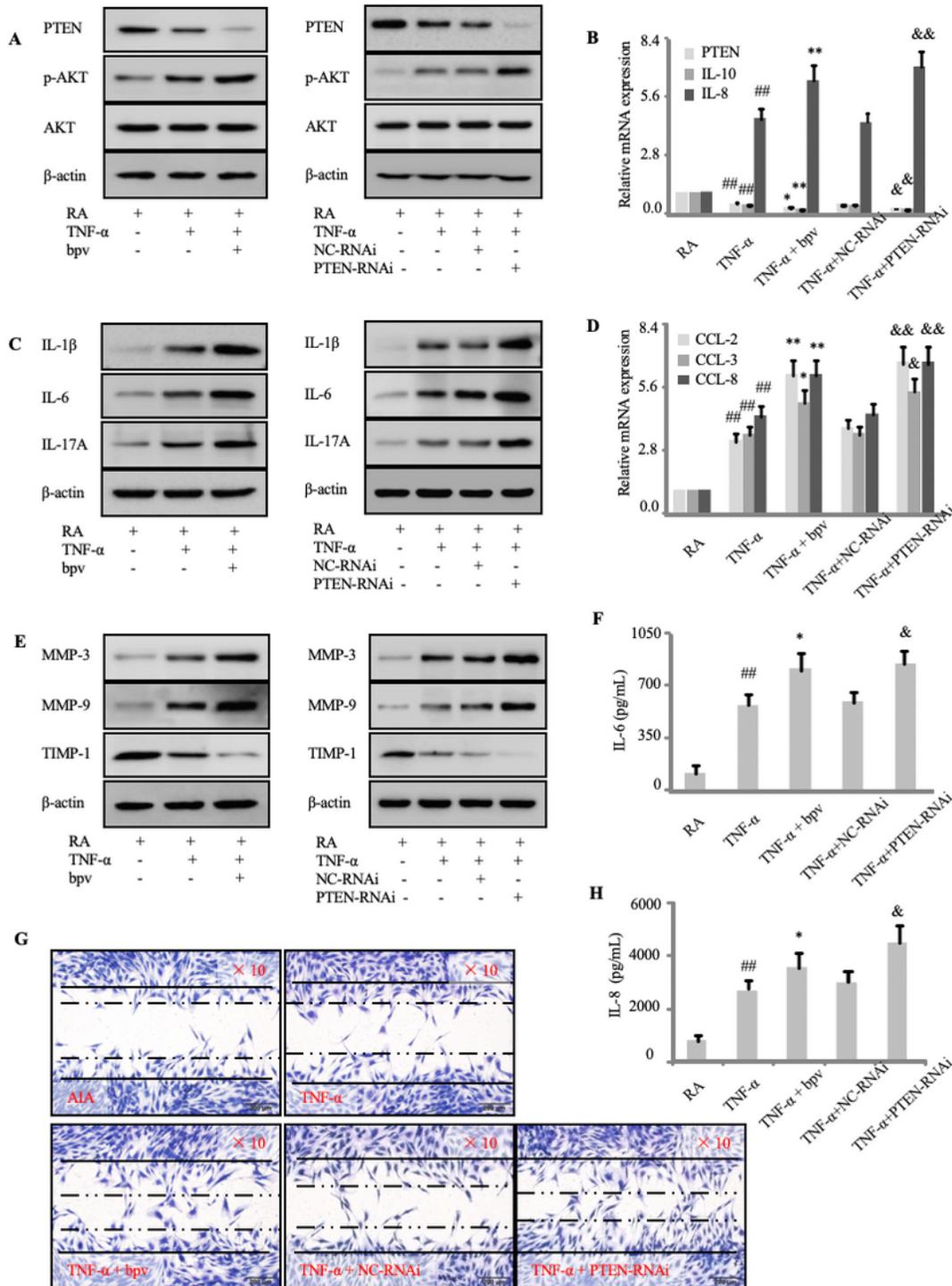
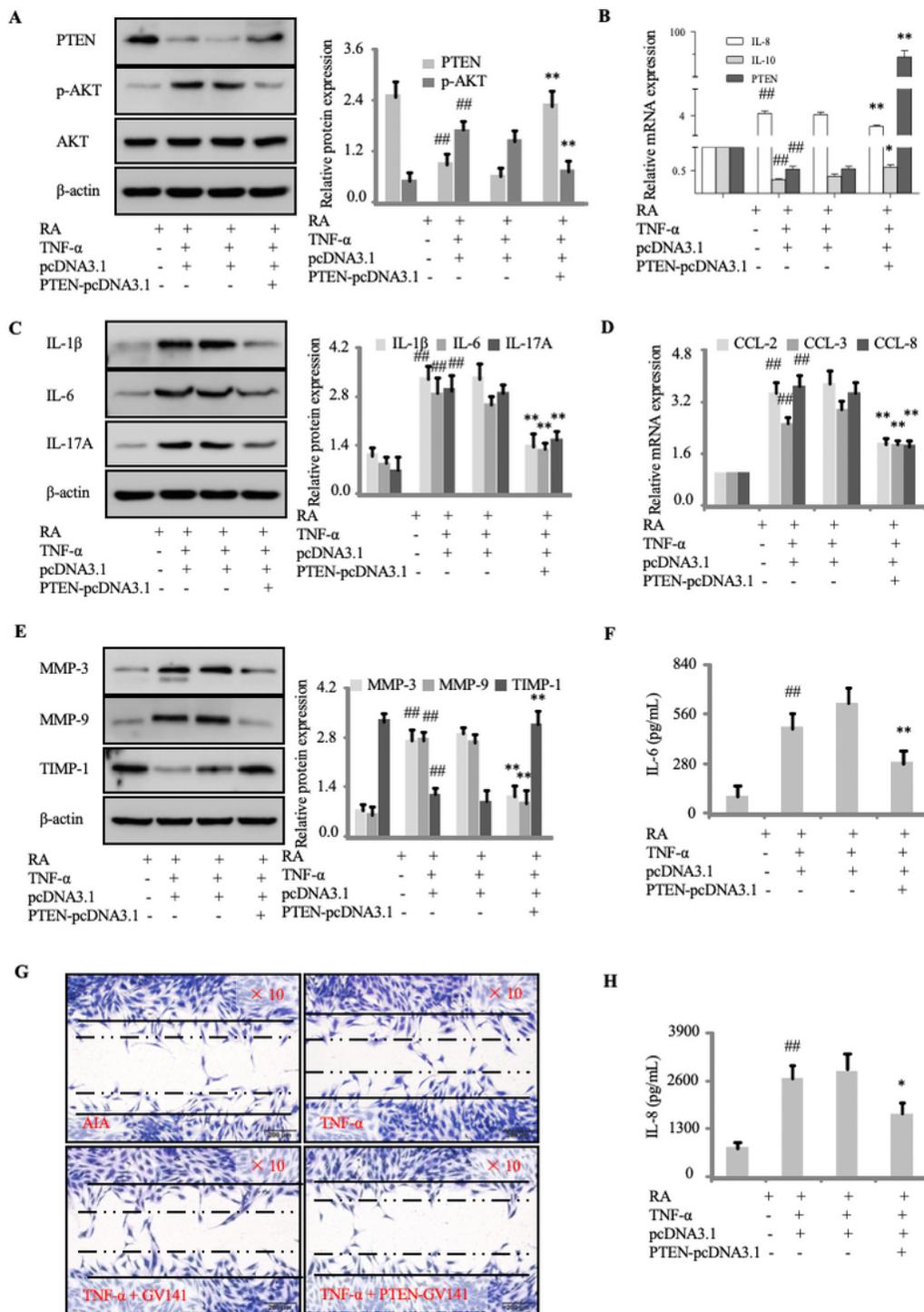


Figure 2

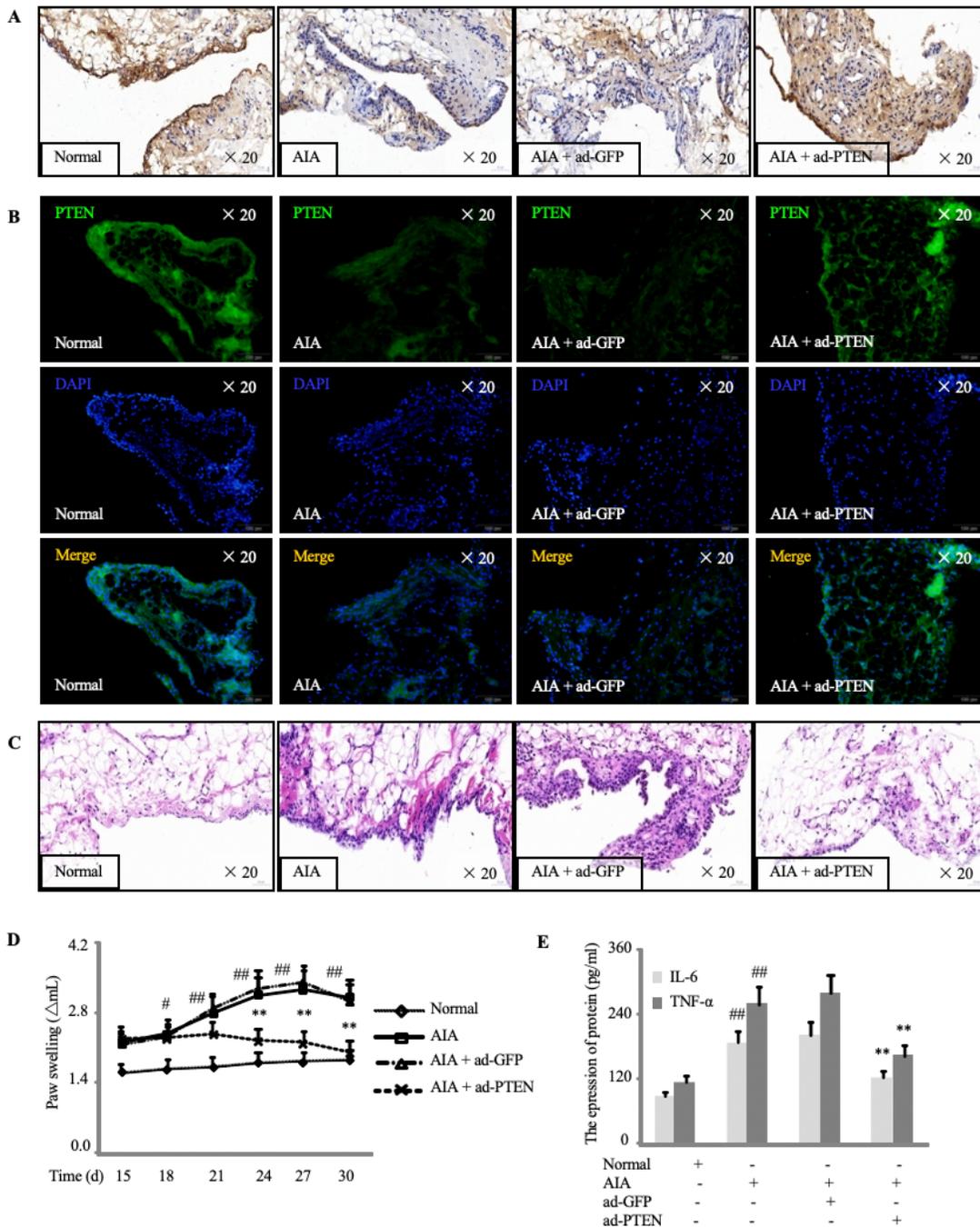
PTEN low-expression modulates pro-inflammation and migration of FLS in RA. (A) After FLS treat with TNF- $\alpha$  (10 ng/mL), the protein levels of PTEN and p-AKT were analyzed by western blot in FLS with bpv (250 nM) or PTEN-RNAi in RA. (B) After FLS treat with TNF- $\alpha$  (10 ng/mL), the mRNA levels of PTEN, IL-10 and IL-8 were analyzed by q-PCR assays in FLS with bpv (250 nM) or PTEN-RNAi in RA. (C) The protein levels of IL-1 $\beta$ , IL-6 and IL-17A of FLS with TNF- $\alpha$  (10 ng/mL) were analyzed by western blot in FLS with bpv (250 nM) or PTEN-RNAi in RA. (D) After FLS treat with TNF- $\alpha$  (10 ng/mL), mRNA levels of CCL-2, CCL-3 and CCL-8 were analyzed by q-PCR in FLS with bpv (250 nM) or PTEN-RNAi in RA. (E) After FLS treat with TNF- $\alpha$  (10 ng/mL), the protein levels of MMP-3, MMP-9 and TIMP-1 were analyzed by western blot in FLS with bpv (250 nM) or PTEN-RNAi in RA. (F) After FLS treat with TNF- $\alpha$  (10 ng/mL), the protein levels of IL-6 was analyzed by ELISA from cellular supernatant in FLS with bpv (250 nM) or PTEN-RNAi in RA. (G) Representative images of migration are shown in FLS with bpv (250 nM) or PTEN-RNAi in RA. (H) After FLS treat with TNF- $\alpha$  (10 ng/mL), the protein levels of IL-8 was analyzed by ELISA from cellular supernatant in FLS with bpv (250 nM) or PTEN-RNAi in RA. All values were expressed as mean  $\pm$  SD. ## P < 0.01 vs. RA group; \*P < 0.05, \*\*P < 0.01 vs. TNF- $\alpha$  group; &P < 0.05, &&P < 0.01 vs. NC-RNAi group.



**Figure 3**

PTEN over-expression modulates pro-inflammation and migration of FLS in RA. (A) After FLS treat with TNF- $\alpha$  (10 ng/mL), the protein levels of PTEN and p-AKT were analyzed by western blot in FLS with PTEN-pcDNA3.1 in RA. (B) After FLS treat with TNF- $\alpha$  (10 ng/mL), the mRNA levels of PTEN, IL-10 and IL-8 were analyzed by q-PCR assays in FLS with PTEN-pcDNA3.1 in RA. (C) The protein levels of IL-1 $\beta$ , IL-6 and IL-17A of FLS with TNF- $\alpha$  (10 ng/mL) were analyzed by western blot in FLS with PTEN-pcDNA3.1 in RA. (D)

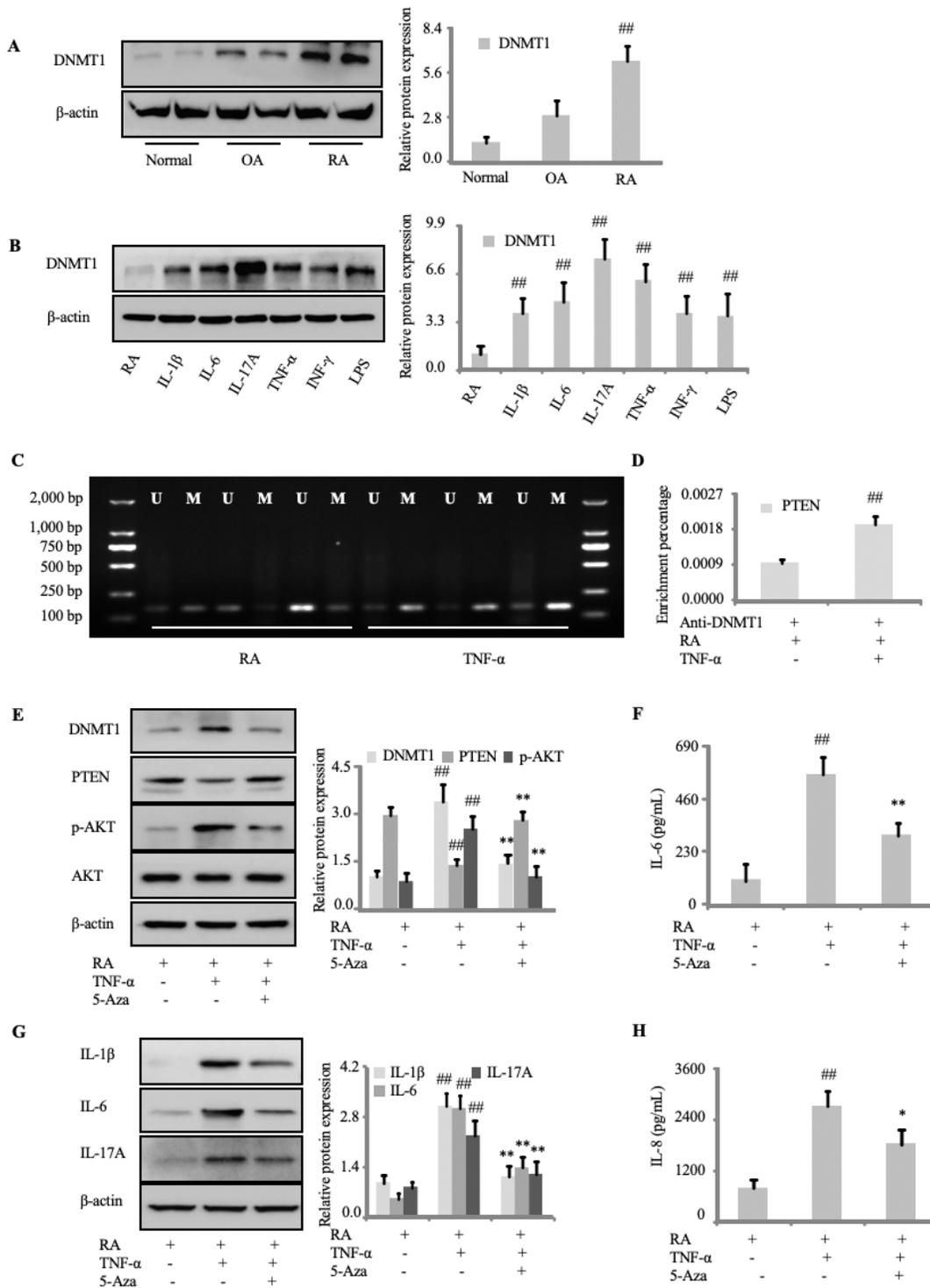
After FLS treat with TNF- $\alpha$  (10 ng/mL), mRNA levels of CCL-2, CCL-3 and CCL-8 were analyzed by q-PCR in FLS with PTEN-pcDNA3.1 in RA. (E) After FLS treatment with TNF- $\alpha$  (10 ng/mL), the protein levels of MMP-3, MMP-9 and TIMP-1 were analyzed by western blot in FLS with PTEN-pcDNA3.1 in RA. (F) The protein levels of IL-6 of FLS with TNF- $\alpha$  (10 ng/mL) was analyzed by ELISA from cellular supernatant in FLS with PTEN-pcDNA3.1 in RA. (G) Representative images of migration are shown in FLS with PTEN-pcDNA3.1 in RA. (H) The protein levels of IL-8 of FLS with TNF- $\alpha$  (10 ng/mL) was analyzed by ELISA from cellular supernatant in FLS with PTEN-pcDNA3.1 in RA. All values were expressed as mean  $\pm$  SD. ## P < 0.01 vs. RA group; \*P < 0.05, \*\*P < 0.01 vs. pcDNA3.1 group.



**Figure 4**

PTEN was over-expressed by ad-PTEN inhibits inflammation in AIA in vivo. (A) Representative IHC images of PTEN staining in AIA synovium by intra-articular injection of ad-PTEN. (B) Representative IF images of PTEN staining in AIA synovium by intra-articular injection of ad-PTEN. (C) Representative H&E images in AIA synovium by intra-articular injection of ad-PTEN. (D) The paw swelling by measuring secondary paw swelling by intra-articular injection of ad-PTEN in AIA. (E) The expression of IL-6 and TNF- $\alpha$  protein by

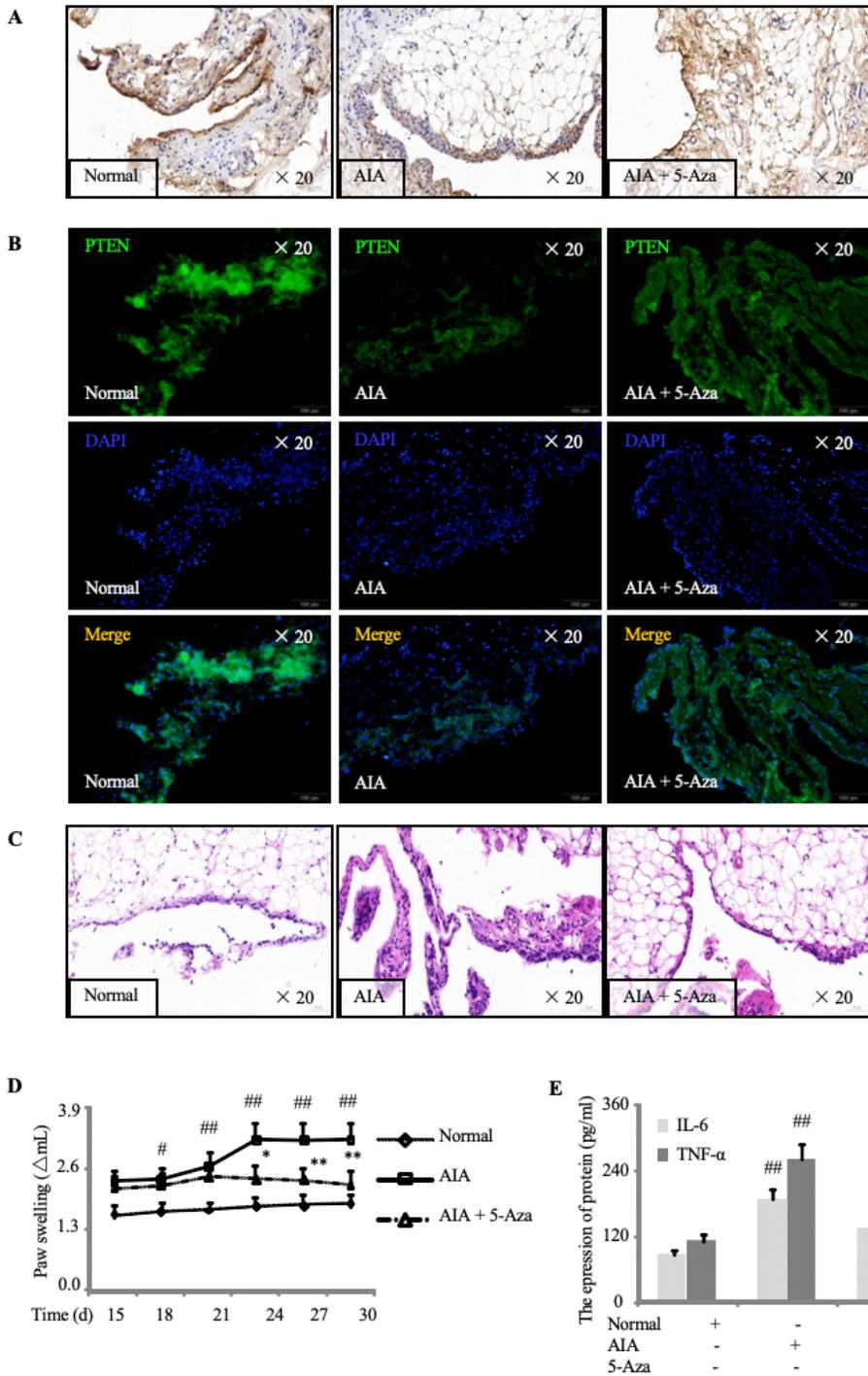
ELISA in serum from AIA with intra-articular injection of ad-PTEN. All values were expressed as mean  $\pm$  SD. #P < 0.05, ## P < 0.01 vs. Normal group; \*P < 0.05, \*\*P < 0.01 vs. ad-GFP group.



**Figure 5**

PTEN expression may be regulated by DNA methylation in RA. (A) The expression of DNMT1 protein was analyzed by western blot in RAFLS. (B) The protein level of DNMT1 was analyzed by western blot in RA FLS treat with IL-1 $\beta$  (2 ng/mL), IL-6 (5 ng/mL), IL-17A (10 ng/mL), TNF- $\alpha$  (10 ng/mL), IFN- $\gamma$  (10 ng/mL)

and LPS (1  $\mu\text{g}/\text{mL}$ ). (C) MSP analysis detected CpG methylation level of PTEN reversed in RAFLS with TNF- $\alpha$ . (D) After incubation with anti-DNMT1 antibody, ChIP staining detected the mRNA of PTEN in RAFLS with TNF- $\alpha$ . (E) After FLS treat with TNF- $\alpha$  (10 ng/mL), the protein levels of DNMT1, PTEN and p-AKT were analyzed by western blot in FLS with 5-Aza (2  $\mu\text{M}$ ) in RA. (F) After FLS treat with TNF- $\alpha$  (10 ng/mL), the protein levels of IL-6 was analyzed by ELISA from cellular supernatant in FLS with 5-Aza (2  $\mu\text{M}$ ) in RA. (G) The protein levels of IL-1 $\beta$ , IL-6 and IL-17A of FLS with TNF- $\alpha$  (10 ng/mL) were analyzed by western blot in FLS with 5-Aza (2  $\mu\text{M}$ ) in RA. (H) After FLS treat with TNF- $\alpha$  (10 ng/mL), the protein levels of IL-8 was analyzed by ELISA from cellular supernatant in FLS with 5-Aza (2  $\mu\text{M}$ ) in RA. All values were expressed as mean  $\pm$  SD. ## P < 0.01 vs. AIA group; \*P < 0.05, \*\*P < 0.01 vs. TNF- $\alpha$  group.



**Figure 6**

Intraperitoneally injected 5-Aza inhibits inflammation in AIA in vivo. (A) Representative IHC images of PTEN staining in AIA synovium by intraperitoneally injected 5-Aza (0.7 mg/kg). (B) Representative IF images of PTEN staining in AIA synovium by intraperitoneally injected 5-Aza (0.7 mg/kg). (C) Representative H&E staining in AIA synovium by intraperitoneally injected 5-Aza (0.7 mg/kg). (D) The paw swelling by measuring secondary paw swelling by intraperitoneally injected 5-Aza (0.7 mg/kg) in AIA. (E)

The expression of IL-6 and TNF- $\alpha$  protein by ELISA in serum from AIA with intraperitoneally injected 5-Aza (0.7 mg/kg). All values were expressed as mean  $\pm$  SD. #P < 0.05, ## P < 0.01 vs. Normal group; \*P < 0.05, \*\*P < 0.01 vs. AIA group.

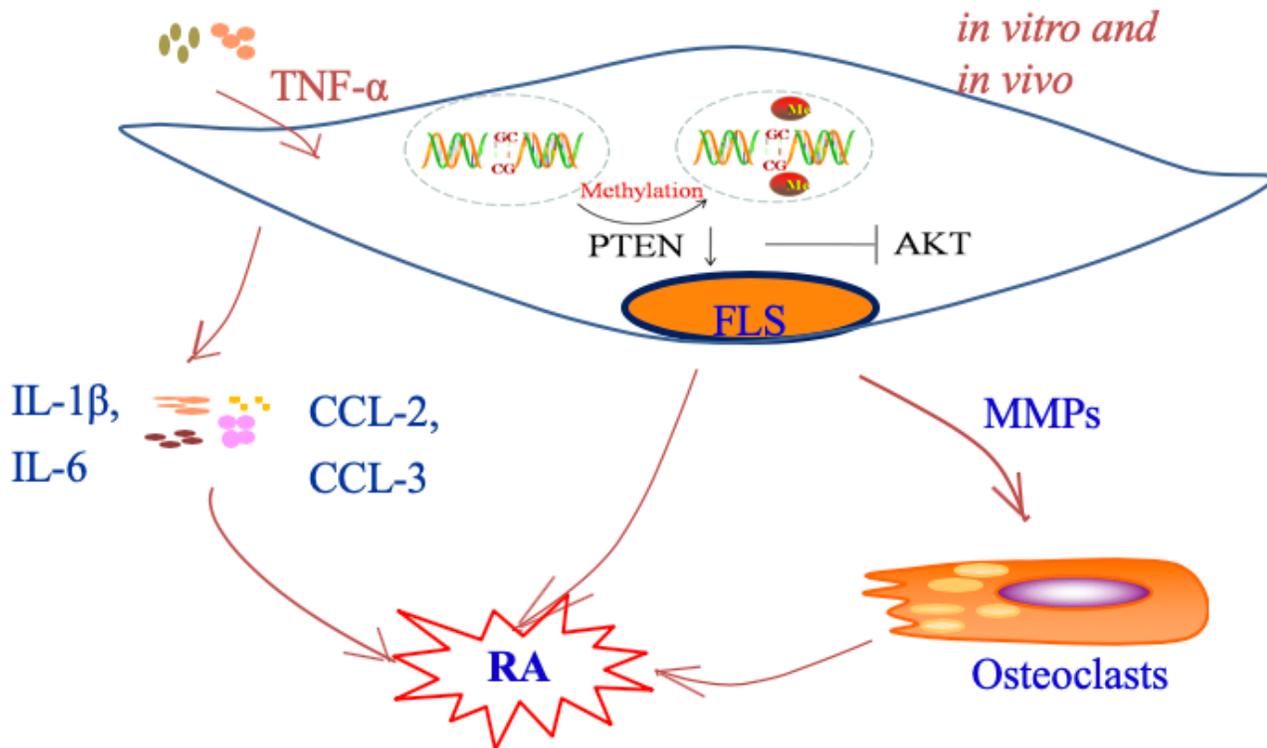


Figure 7

Downregulation of PTEN by DNA methylation promotes the inflammatory response of FLS and intensifies the development of RA.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable1.doc](#)
- [SupplementaryFigures.ppt](#)