

# Inhibition of PCSK6 May Play a Protective Role in the Development of Rheumatoid Arthritis

Feifei Wang, Lin Wang, Huiyu Jiang, Xiaotian Chang, and Jihong Pan

**ABSTRACT. Objective.** To assess the effect of proprotein convertase subtilisin/kexin type 6 (PCSK6) in the synovial fibroblasts of rheumatoid arthritis (RA). PCSK6 is a proteinase implicated in the proteolytic activity of various precursor proteins and involved in the regulation of protein maturation.

**Methods.** PCSK6 expression was detected in the synovial tissue of 10 patients with RA, 10 controls with osteoarthritis, and 10 controls with ankylosing spondylitis using Western blotting and quantitative real-time PCR. Genotyping of 67 tag single-nucleotide polymorphisms (SNP) was performed using an Illumina VeraCode (Illumina) microarray in a case-control study including 267 patients with RA and 160 healthy controls. Genotyping of 4 other tag SNP was performed using a TaqMan probe genotyping assay in 1056 healthy controls and 1151 patients with RA. Cultured RA synovial fibroblasts (RASf) were transfected with PCSK6 small interfering RNA to study changes in the proliferation, invasion, migration capacity, secretion of inflammatory cytokines, cell cycle, and expression profiles of the RASf.

**Results.** Expression of PCSK6 mRNA and protein was significantly higher in the synovial tissues of individuals with RA than in control tissues. One SNP, rs8029797, was significantly associated with RA ( $p = 0.011$ ). Knockdown of PCSK6 by RNA interference significantly decreased proliferation, invasion, and migration of RASf. These changes in RASf appeared to be related to reduced tumor necrosis factor- $\alpha$  secretion, G0/G1 arrest, and altered expression of various proteins including those involved in angiogenesis (matrix metalloproteinase 9, nitric oxide synthase trafficking), hypoxia (hypoxia-inducible factor- $\alpha$ , thioredoxin domain containing 5), proliferation (chromosome 10 open reading frame 116), and inflammation [CCL7, chemokine (C-X-C motif) ligand 9, interleukin 26].

**Conclusion.** PCSK6 is upregulated in the synovial tissues of patients with RA and has a genetic effect on the risk of RA. Inhibition of PCSK6 may play a protective role in the development of RA. (First Release Dec 1 2014; J Rheumatol 2015;42:161–9; doi:10.3899/jrheum.140435)

## Key Indexing Terms:

PCSK6

RHEUMATOID ARTHRITIS

RASf

siRNA

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the synovium and hyperplasia of synovial fibroblasts. This inflammation can erode adjacent cartilage and bone, and cause subsequent joint destruction<sup>1</sup>. The destructive process may be mediated, at least in part, by fibroblast-like synovio-

cytes (FLS) from the synovium. Indeed, FLS from patients with RA can attach to and invade normal cartilage in a severe combined immunodeficiency mouse complementation model<sup>2</sup>. Further, FLS are potent players in all aspects of the pathogenesis of RA<sup>3</sup>. Thus, FLS in the rheumatoid synovium are widely accepted as aggressive and proliferative, and are known to attack the cartilage. They possess characteristics similar to those of transformed cells<sup>3</sup> such as anchorage-independent growth<sup>4</sup>, insensitivity to apoptosis, and increased proliferation<sup>5</sup>.

FLS in culture express large amounts of proteinases that can degrade extracellular matrix components such as collagens. One family of proteinases expressed by FLS is the matrix metalloproteinase (MMP). FLS express MMP 1, 2, 3, 9, and 10, and the expression of these MMP correlates with invasion<sup>6</sup>. However, MMP are inactive precursors that must be processed by proprotein convertases (PC), which cleave at single basic or paired basic residues of the proproteins, to produce biologically active proteins. To date, 9 PC have been identified: PC1/PC3, PC2, proprotein convertase subtilisin/kexin type 6 (PCSK6)/PACE4, PC4, PC5/PC6, PC7/PC8/LPC, Furin, PCSK8, and PCSK9.

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Furin displays high expression levels in the synovium of patients with RA and may protect against RA<sup>7</sup>. PCSK6, on the other hand, plays a major role in promoting the progression of prostate tumors to a higher aggressive status<sup>8</sup>. Interestingly, tumor tissue and the RA synovium share common features, including extreme angiogenesis, excessive fibrin deposition, disordered cell proliferation, and high coagulation activity<sup>9</sup>. Thus, PCSK6 was proposed to play an important role in RA differing from that of Furin. Further, a variant of *PCSK6* was strongly associated with reduced pain in knee osteoarthritis (OA), offering some insight as to why, in the presence of the same structural damage, some individuals developed chronic pain and others were protected. Studies in *PCSK6* null mice also implicated PCSK6 in pain<sup>10</sup>. However, to our knowledge, a role for this protein in RA has not yet been reported.

In our study, we quantitatively analyzed the expression of PCSK6 in the synovial tissues of patients with RA and control individuals at both transcriptional and translational levels. Cases and controls were also genotyped for 71 tag single-nucleotide polymorphisms (SNP) of PCSK6 to identify contributions of genetic variants of PCSK6 to RA. PCSK6 knockdown was performed in isolated RA synovial fibroblasts (RASf) to identify changes in proliferation, migration, invasion capacity, and cell cycle progression.

## MATERIALS AND METHODS

**Participants and samples.** Synovial tissues were collected during knee joint replacement surgery from patients with RA, OA, and ankylosing spondylitis (AS). Synovial samples were dissected from connective tissues and immediately stored at -80°C until use. Patients with RA (n = 10), OA (n = 10), and AS (n = 10) all met the American College of Rheumatology classification criteria. A second cohort of patients with RA (n = 267) and health control (n = 160) were selected for genotyping with the Illumina platform (Illumina). For TaqMan probe assay (Applied Biosystems Ltd.), another cohort of RA (n = 1151) and control (n = 1056) was selected. Cases and controls were frequently age-matched and were from the same geographical area of Northern China, and controls did not have a family history of RA. Peripheral blood samples were collected from all participants into S-Monovette tubes containing 3.8% sodium citrate. Demographic characteristics are presented in Supplementary Table 1 (available online at jrheum.org).

Participants provided written informed consent to participate in our study and to allow their biological samples to be genetically analyzed. The Ethical Committee of Shandong Academy of Medicinal Sciences approved our study.

**Genomic DNA extraction.** Genomic DNA was extracted from peripheral blood leukocytes using the DNA Blood Mini Kit from Omega (Omega) according to the manufacturer's guidelines. DNA concentration was measured using Thermo Nanodrop (Thermo Scientific).

**SNP selection.** Illumina Golden Gate assays (Illumina) were performed on genotype tag SNP within PCSK6 in 267 patients with RA and 160 healthy control individuals. Tag SNP were selected from HapMap data with a pairwise  $r^2 \geq 0.8$  and minor allele frequencies (MAF) over 0.05. A total of 96 SNP served as candidates for Illumina Golden Gate design and were submitted to Illumina for a design score evaluation. The Illumina Assay Design Tool (Illumina) filtered out SNP unsuitable for the Illumina platform, such as insertions/deletions, tri- and tetra-allelic SNP, and SNP that were not uniquely localized. Finally, 67 SNP with a design score of 1,

spanning 0.224 Mb of the chromosome, and covering the full *PCSK6* gene were selected.

**Genotyping using microarray.** Genotyping was performed using custom-designed Illumina VeraCode microarrays (Illumina) in Dr. Zhang Feng's laboratory at the Beijing Institute of Genomics. A BeadXpress Reader using Illumina VeraCode Golden Gate Assay Kit was used. A total of 500 ng of the sample DNA were used per assay. Genotype clustering and calling were performed using BeadStudio software (Illumina).

**Genotyping using TaqMan SNP assay.** For the 29 SNP not included in the Illumina platform, we estimated the power and sample size needed for further analysis. To get a p value of < 0.01, the MAF should be about 0.3 when 1000 samples were considered. Only 4 of those 29 tag SNP met the criteria for MAF; those 4 tag SNP were genotyped using TaqMan probe genotyping assays in a cohort of patients with 1151 individuals with RA and 1056 healthy controls. TaqMan probe and primer mixes were designed and synthesized by ABI Ltd. Assays were run on a LightCycler 480 Instrument (Roche) and evaluated according to the manufacturer's instructions. Reactions were carried out in a total volume of 10  $\mu$ l using the following amplification protocol: denaturation at 95°C for 10 min, then 40 cycles of denaturation at 95°C for 10 s, followed by annealing at 60°C for 1 min, and extension at 72°C for 1 s. The fluorescence signal was obtained in the extension procedure. The genotype of each sample was determined by measuring allele-specific fluorescence using SDS 2.3 software for allelic discrimination (Roche). Duplicate samples and negative controls were included to check the accuracy of genotyping.

**Culture and identification of synovial fibroblasts.** The RA synovial tissue was finely chopped and incubated with type II collagenase (1 mg/ml, Sigma-Aldrich) in Dulbecco modified Eagle's medium (DMEM, HyClone, Thermo Scientific) for 6 h in a 37°C, 5% CO<sub>2</sub> incubator (Thermo Scientific). The tissue was treated with 0.25% trypsin (Solabio) in phosphate buffered saline (PBS) solution, in a volume equivalent to DMEM. Cells were filtered and cultured overnight in DMEM, supplemented with 10% fetal bovine serum (FBS, HyClone) as well as penicillin (100 IU/ml) and streptomycin (100  $\mu$ g/ml, Gibco) for 3 passages. RASf at passage 4-6 were used for our study; they were negative for CD14, CD3, CD19, and CD56 expressions as identified by flow cytometry analyses.

**Inhibition of PCSK6 expression with small interfering RNA (siRNA).** Because *PCSK6* has several variants, we aligned all transcripts to get a consensus sequence. The consensus sequence was used as the template for the design of siRNA. siRNA targeting PCSK6 (target mRNA sequence 5'-CCC AUC UCC ACG AUA UCA UTT-3') was designed and synthesized by GenePharma Co. Ltd. (Shanghai). Cultured RASf were transfected with siRNA at 160 nmol/l using a HiPerFect transfection reagent (QIAGEN) according to the manufacturer's protocol. The cells were harvested for analysis at 48 h following the transfection. A negative siRNA (5'-UUC UCC GAA CGU GUC ACG UTT-3') was used as the negative control; reagent-only transfection was used as the Mock group.

**Quantitative real-time PCR (qRT-PCR).** Total RNA was extracted from the cultured cells and the human tissue using Total RNA Kit (OMEGA) and reverse-transcribed using a ReverTra Ace qPCR RT Kit (Toyobo) according to the manufacturers' protocol. qRT-PCR was conducted using the LightCycler 480 (Roche) with the following amplification protocol: denaturation at 95°C for 10 min, then 40 cycles of denaturation at 95°C for 10 s, followed by annealing at 60°C for 1 min, and extension at 72°C for 1 s. The comparative cycle threshold method was used to analyze the relative expression of mRNA. The relative target gene expression was normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Primers for qRT-PCR were also designed according to the consensus sequence. The primers for the amplification of *PCSK6* were as follows: forward primer 5'-AAG CAA GGG AAG TTG AAA GA-3' and reverse primer 5'-CAC TGA AGG TGT GGT ACG-3'. The primers for the amplification of GAPDH were as follows: forward primer 5'-CAC CAT CTT CCA GGA GC-3' and reverse primer 5'-AGT GGA CTC CAC GAC GTA-3'. For all PCR, standard curves, dissociation curves, and migration

Table 1. Genotyping data for PCSK6 tag SNP by TaqMan probes.

SNP	Allele	Frequency		Allele		95% CI	Case Frequency			Genotype Control Frequency			Fisher's p
		Case	Control	p	OR		1/1	1/2	2/2	1/1	1/2	2/2	
rs8029797	T	0.372	0.352	0.19205	0.919498	0.810569–1.04306	0.422	0.413	0.165	0.419	0.458	0.123	0.011*
rs4965877	A	0.313	0.314	0.96658	0.99487	0.782128–1.26548	0.102	0.421	0.476	0.104	0.42	0.476	0.997862
rs12592414	C	0.330	0.670	0.46842	1.091716	0.861162–1.383995	0.097	0.466	0.437	0.110	0.401	0.489	0.250710

\* p < 0.05. SNP: single-nucleotide polymorphism; PCSK6: proprotein convertase subtilisin/kexin type 6.

of PCR products on acrylamide gels were done to confirm the specificity of the products. The specificity of the qRT-PCR assay was evaluated by melting curve analysis, which showed that the *PCSK6* amplification product generated a melting peak at  $81.20 \pm 0.34^\circ\text{C}$  without primer-dimers or nonspecific products.

**Western blotting.** Tissue samples of patients with RA, OA, and AS and the cultured cells were homogenized in radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology) with protease inhibitors on ice for 30 min to extract the protein. The preparation was centrifuged at 14,000 rpm for 30 min at  $4^\circ\text{C}$ . The protein concentration was measured with a Bio-Rad protein assay system. Twenty-five micrograms of the total protein were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a PVDF membrane (Amersham Pharmacia Biotech) with wet transfer, blocked in 10 mmol/l Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 (TBST) and 5% (w/v) nonfat milk, and then underwent immunoblotting with specific primary antibodies [the anti-human PCSK6 antibody diluted at 1:2000 (Anti-PACE4 antibody ab39877, Abcam); anti-human GAPDH antibody diluted at 1:1000 (Anti-GAPDH antibody sc47724); anti-MMP2 antibody ab37150 diluted at 1:500 and anti-MMP9 antibody ab137867 diluted at 1:1000 (both from Abcam, both can detect the pro and mature MMP); anti-p27<sup>KIP1</sup> antibody diluted at 1:500 (D69C12, Cell Signaling Technology)] overnight at  $4^\circ\text{C}$ . After being washed with TBST, the membrane was incubated with each corresponding secondary antibody for 1 h at  $37^\circ\text{C}$ . Detection was performed using an ECL chemiluminescence kit (Thermo Scientific). The film was scanned and analyzed with SmartView software (Furi).

**Cell proliferation assay.** The RASF were seeded onto 96-well culture plates and cultured at  $37^\circ\text{C}$  to 80% confluence. The cultures were treated with PCSK6 siRNA (160 nmol/l). After incubation for 24 h, 48 h, or 72 h, 20  $\mu\text{l}$  of 5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] prepared with PBS were added to each well, and cultures were incubated for 4 h at  $37^\circ\text{C}$  in the incubator. MTT solution was removed and 150  $\mu\text{l}$  dimethyl sulfoxide were added to extract the MTT-formazan products at room temperature for 10 min. The absorbance was measured in triplicate at 490 nm with a spectrophotometer.

**Cell invasion assay and migration measurements.** Cell invasion ability was tested using the transwell apparatus (Becton Dickinson). RASF were plated at a density of  $3 \times 10^4$  cells/well and incubated  $37^\circ\text{C}$  with 160 nmol/l of siRNA in the upper chamber of the transwell apparatus for 8 h. Both the upper and the lower chambers were cultured in medium without FBS for 12 h. After that, the lower chamber was filled with 20% FBS in DMEM for 24 h. The noninvaded cells from the upper chamber were removed with cotton swabs; the invaded cells on the lower chamber were stained with Giemsa (Solabio). The cell number that invaded through the filter was quantified in 5 random fields at 100 $\times$  magnification. The average of the 5 fields was calculated.

A wound-healing assay was performed to test the migration ability of the RASF. Cells were plated onto 24-well plates and cultured at  $37^\circ\text{C}$  to 80% confluence. The plates were scratched linearly in multiple areas with a cell scraper, and then cells were treated with 160 nmol/l siRNA of PCSK6

or control. After 24 h, the cell number that migrated into the line was calculated in the same method as described above.

**Microarray analysis.** Gene expression profiles were analyzed in the RASF after knockdown of PCSK6 on a Gene Chip Human Gene 2.0 ST Array (Affymetrix) by Life Technology Ltd. Mock-treated RASF were used as control. Microarray hybridization and the data collection were performed on the Affymetrix platform.

**Measuring interleukin (IL) 1 $\alpha$ , IL-1 $\beta$ , IL-17, and tumor necrosis factor (TNF)- $\alpha$  levels with an ELISA.** RASF were treated with 160 nmol/l siRNA for 24 h, and the culture medium was collected and centrifuged at 1000 rpm for 5 min at  $4^\circ\text{C}$ . A 100  $\mu\text{l}$  medium was added to a 96-well microplate (Costar), which was stored overnight at  $4^\circ\text{C}$ . After gently washing with PBST, 1% bovine serum albumin plus 5% sucrose was used for blocking for 1 h at  $37^\circ\text{C}$ . Following 3 PBS washes, antibodies against IL-1 $\alpha$ , IL-1 $\beta$ , IL-17, and TNF- $\alpha$  (all from Abcam, dilution 1:1000) were applied to the plate for overnight incubation. Plate was washed, blocked, treated with a 1:2000 dilution of anti-rabbit Ig-G horseradish peroxidase antibody (Proteintech), and was incubated for 3 h at  $37^\circ\text{C}$ . Staining was developed by TMB Kit (CW BIO). The absorbance at 450 nm was measured with a plate reader (Synergy HT). RASF-only-plus transfection reagent was used as control.

**Cell cycle analysis.** Cells were plated onto 6-well culture plates (Costar) at  $1.0 \times 10^6$  cells/well and the cells were treated with siRNA as described above. After removal of the cultured medium, the cells were harvested at 24 h by trypsinization, washed twice with ice-cold PBS, and fixed overnight with 70% cold ethanol. Before analysis, the fixed cells were rinsed, resuspended in PBS, and stained for 30 min at  $37^\circ\text{C}$  with 1 ml 0.05 mg/ml propidium iodide solution (Beijing Bingguo Biotechnology Co. Ltd.) containing 10  $\mu\text{g/ml}$  RNase for 30 min at  $37^\circ\text{C}$ . Cells were detected with a Coulter Epics XL flow cytometer (Beckman Coulter) and analyzed for DNA content by EXPO32 software (Beckman Coulter).

**Immunofluorescence.** Cells grown on chamber slides (NEST) were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.3% Triton X-100 for 10 min after indicated treatment. Goat serum was used for blockage for 1 h at room temperature. Cells were then incubated with primary antibodies against p27 (1:500, CST) at  $4^\circ\text{C}$  overnight. After being washed with PBS 3 times, cells were incubated with Alexa Fluor 488-conjugated secondary antibodies (1:1000, Invitrogen) for 1 h at room temperature. The cells were mounted with prolong gold antifade reagent with DAPI (Invitrogen) for 10 min. Samples were analyzed with a fluorescence microscope (Nikon).

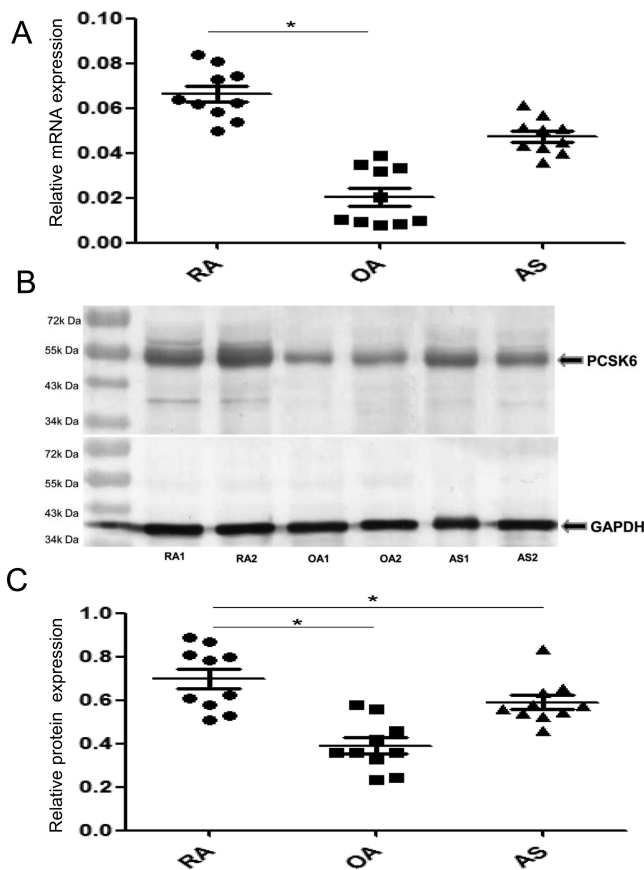
**Statistical analysis.** Genotyping data of SNP markers were evaluated for significant deviation with the Hardy-Weinberg equilibrium (HWE). Calculations were performed using the software Plink 1.07 ([pngu.mgh.harvard.edu/~purcell/plink](http://pngu.mgh.harvard.edu/~purcell/plink)). Bonferroni correction was applied for multiple comparisons.

All results were confirmed in at least 3 independent experiments. Except for genotyping data, the other results are expressed as mean  $\pm$  SD. Multiple comparisons were conducted using 1-way ANOVA. All statistical analyses were performed using SPSS 17.0 software (SPSS). P values < 0.05 were considered statistically significant for all results.

## RESULTS

**PCSK6 expression in the synovial tissue from patients with RA.** We initially investigated the expression of PCSK6 in human synovial tissues obtained from patients with RA, OA, and AS by qRT-PCR and Western blotting analyses. The relative PCSK6 mRNA expression in the synovium of patients with RA was significantly higher than that of patients with OA ( $p = 0.027$ ), but no significant difference was detected between RA and AS synovial tissues (Figure 1A). Consistent with the mRNA expression level, levels of PCSK6 protein (Figures 1B and 1C) were highest in the RA group ( $p = 0.01$ ).

**Genotyping of SNP located in PCSK6.** SNP in PCSK6 were genotyped among 2 other cohorts of patients with RA and healthy controls to identify variants associated with RA. No association correlation was found in the 67 tag SNP genotyped by Illumina microarray. In contrast, the TaqMan probe assays of 4 tag SNP identified one, rs8029797, as



**Figure 1.** PCSK6 expression in synovial tissues using qRT-PCR and Western blot analysis. The tissues were collected from patients with RA, OA, and AS. A. Relative mRNA expression level of PCSK6 was significantly different between patients with RA and OA. B. The protein expression level of PCSK6 (56 kD) was normalized to GAPDH (37 kD). C. Relative protein level was significantly higher in RA. Values are mean  $\pm$  SD. \*  $p < 0.05$ . qRT-PCR: quantitative real-time PCR; RA: rheumatoid arthritis; OA: osteoarthritis; AS: ankylosing spondylitis; PCSK6: proprotein convertase subtilisin/kexin type 6.

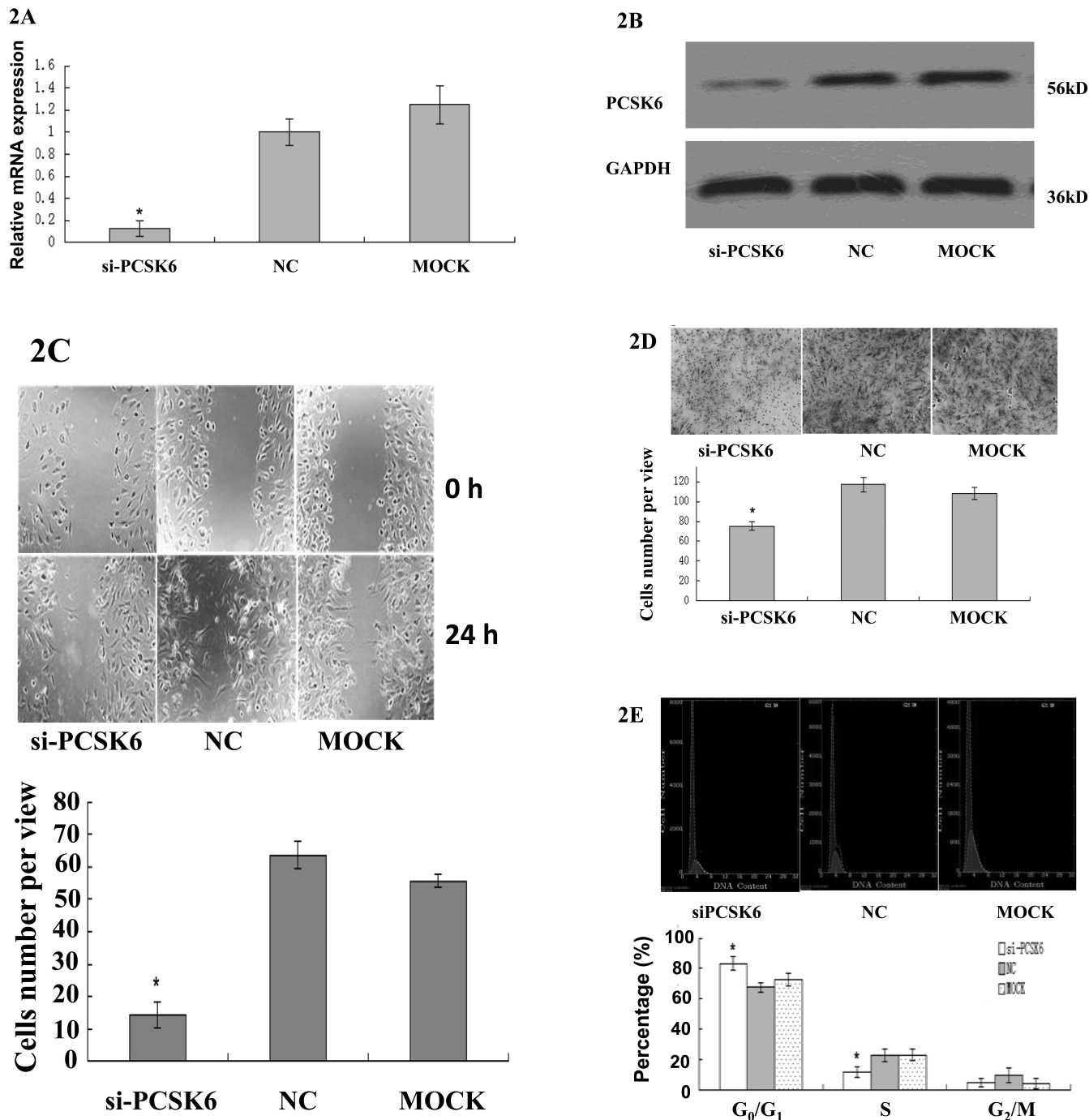
associated with RA ( $p = 0.011$ ; Table 1). However, the CI crossed 1, and therefore the statistical significance was not clear. Genotypes of another SNP, rs7166284, were not in the HWE, and this SNP was discarded from the analysis.

**Knocking down PCSK6 affected RASF proliferation, migration, and invasion in vitro.** Because at least 1 SNP in PCSK6 was significantly associated with RA, RNA interference was used to knock down PCSK6 expression and to assess the resultant effects on RASF. PCSK6 expression was analyzed in the RASF by both qRT-PCR and Western blot 24 h after the transfection with either control siRNA or PCSK6 siRNA. Expression of PCSK6 decreased significantly at both mRNA ( $p = 0.0001$ ) and protein levels ( $p = 0.001$ ; Figures 2A and 2B) following the siRNA transfection, demonstrating effective knockdown by the siRNA. Therefore, to evaluate the function of PCSK6 on proliferation, migration, and the invasion of RASF, we used MTT, wound healing, and transwell assays after PCSK6 knockdown. siRNA-PCSK6 resulted in reduced proliferation of RASF compared to controls with normal PCSK6 expression ( $p = 0.0001$ ). For cell migration assay, the RASF were scratched before treatment with siRNA-PCSK6 and then incubated with siRNA for 24 h. Significantly fewer RASF were present in the wounded area when PCSK6 was knocked down ( $p = 0.0001$ ; Figure 2C). Further, significantly fewer RASF invaded through the transwell filter when PCSK6 was knocked down ( $p = 0.0001$ ; Figure 2D).

**Knockdown of PCSK6 decreased TNF- $\alpha$  secretion in RASF.** Because RA is a condition of chronic inflammation, pro-inflammatory cytokines play prominent roles in the disease. In particular, TNF- $\alpha$ , IL-1 $\beta$ , and IL-17 are important pro-inflammatory cytokines related to synovitis and joint destruction<sup>5</sup>. TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , and IL-17 levels were compared in the supernatant of RASF cultures media following treatment with 160 nmol/l PCSK6 siRNA or control siRNA. The secretion of TNF- $\alpha$  was significantly lower ( $p = 0.0001$ ) in the siRNA-PCSK6 RASF as compared with the controls.

**Cell cycle arrested in RASF following PCSK6 knockdown.** To further study the effects of PCSK6 on the proliferation of RASF, cell cycle was analyzed by flow cytometry analysis. The ratio of G0/G1 phase cell was significantly higher in RASF with PCSK6 knockdown in comparison with controls after 12 h, 48 h, and 72 h (Figure 2E), which suggested that abnormal PCSK6 could disturb cell cycle in RASF.

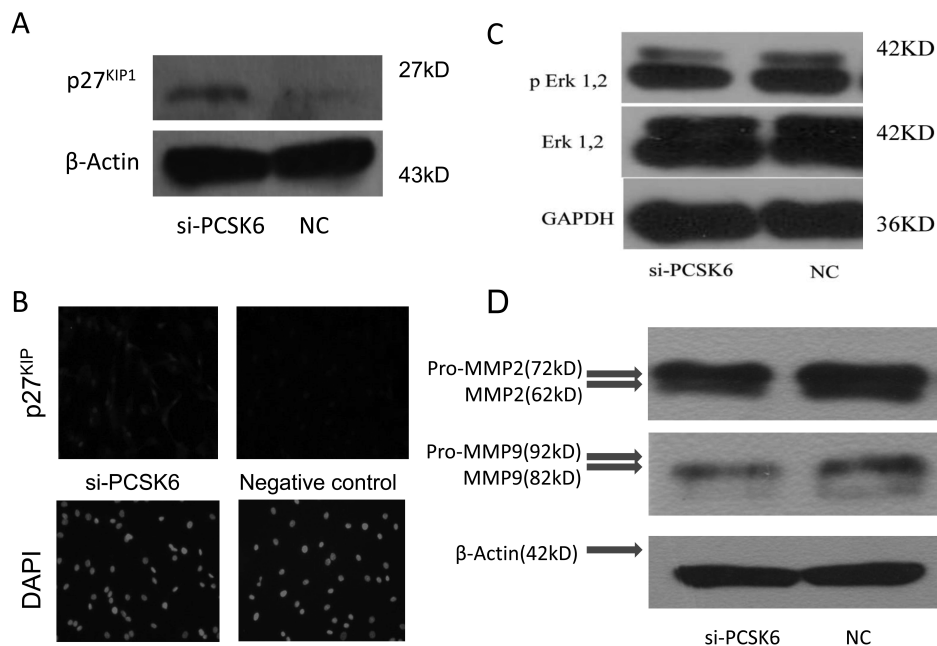
To understand the apparent delay in cell cycle progression, we also tested the expression of p27<sup>KIP</sup>, a cyclin-dependent kinase inhibitor protein implicated in blocking the cell cycle transition from the G0/G1 to S phase during cell quiescence and subsequently degraded on mitogenic signaling by Western blotting and immunofluorescent staining<sup>11</sup>. Importantly, expression of p27<sup>KIP</sup> was significantly increased (about 4-fold,  $p = 0.01$ ) after the silencing of PCSK6 in RASF (Figure 3A).



**Figure 2.** RASF were transiently transfected with siRNA targeting PCSK6. Resultant expression was detected using qRT-PCR (A) and Western blot (B). The PCSK6 mRNA level and protein levels were both normalized to GAPDH. The PCSK6 expression was significant between the siRNA treatment and negative control and only transfection-reagent control. C. Wound-healing assay was used to test the cell migration. D. The invasive ability was performed by transwell assay and the average number of cells invaded through the filter was quantified. Experiments were done in triplicate. Data are presented as mean  $\pm$  SD. \*  $p < 0.05$ . (E) Effect of anti-PCSK6 siRNA on the cell cycle in RASF. Examples of cell cycle distribution treatment were measured by flow cytometry. Results of statistics analyses of 3 independent determinations. NC indicates the negative control, and MOCK indicates the transfection-reagent control. Values are mean  $\pm$  SD. \*  $p < 0.05$ . RASF: rheumatoid arthritis synovium fibroblasts; siRNA: small interfering RNA; PCSK6: proprotein convertase subtilisin/kexin type 6; qRT-PCR: quantitative real-time PCR.

Silencing of PCSK6 reduced extracellular signal-regulated kinase (ERK) activity. The ERK1/2 isoforms play a major role in the signaling of cell proliferation. The phosphory-

lation of ERK results in the catalytic activation of ERK<sup>12</sup>. To investigate the pathway by which PCSK6 directly or indirectly regulates the cell proliferation, we examined the



**Figure 3.** Western blotting (A) and immunofluorescence (B) against cell cycle markers p27<sup>KIP1</sup> in PCSK6-silenced RASF relative to negative control RASF. C. Silencing of PCSK6 reduced ERK1/2 activity in RASF. D. Silencing of PCSK6-reduced MMP-2/9 expression and activity in RASF. PCSK6: proprotein convertase subtilisin/kexin type 6; RASF: rheumatoid arthritis synovium fibroblasts; ERK: extracellular signal-regulated kinase; MMP: matrix metalloproteinase; NC: negative control; DAPI: diamidino phenylindole.

ERK1/2 activity in RASF. Silencing of PCSK6 decreased levels of the phosphorylated forms of ERK1/2 in RASF (Figure 3B).

*Silencing of PCSK6 reduced MMP expression and activity in RASF.* MMP-2 and MMP-9 are especially important in collagen degradation, which are the main factors in invasion of the synovium to articular cartilage. Expression and activity of these 2 MMP contributes to the development of RA. As a proteolytic enzyme, PCSK6 participates in the activation of MMP-2 and MMP-9<sup>13</sup>. To test the effect of siRNA-PCSK6 on the function of PCSK6, activities of MMP-2 and MMP-9 were assayed in RASF by Western blotting. Silencing of PCSK6 resulted in a slightly lower expression of both pro-MMP-2/9 and MMP-2/9 at the same time (Figure 3C).

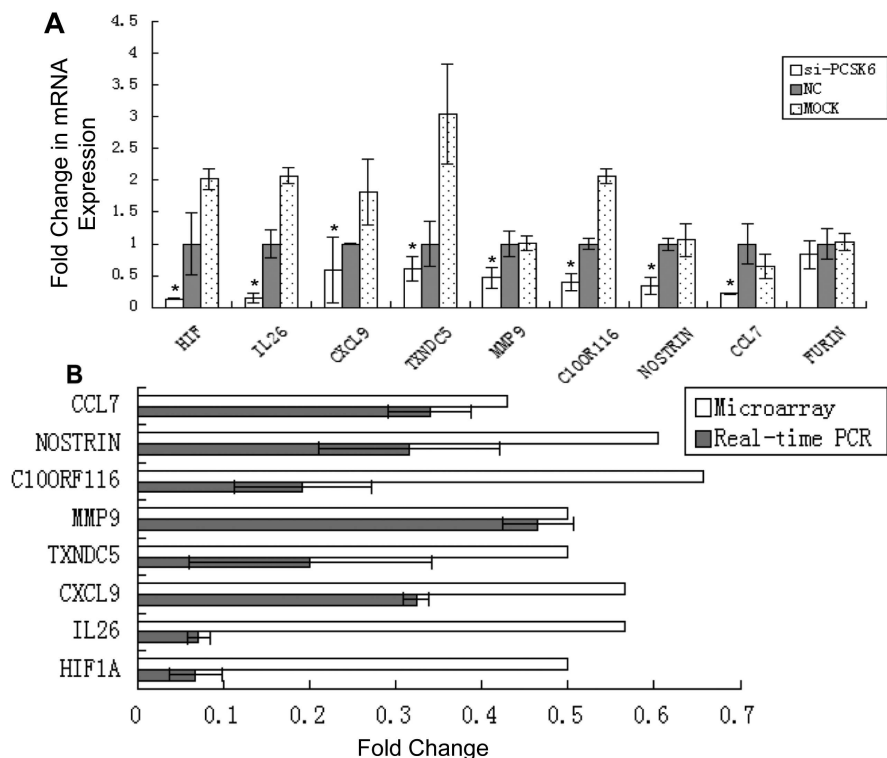
*Gene expression profiling after PCSK6 silencing in RASF.* To gain further insight into the role of PCSK6 in the pathology of RA, microarray analysis was applied to RASF with and without silencing of PCSK6. Differentially expressed genes are listed in Supplementary Table 2 and Supplementary Table 3 (available online at jrheum.org). Of them, 27 genes were downregulated and 25 genes were upregulated by 2-fold or more. Gene ontology analysis identified the biological functions of these genes. Some genes were related to proliferation of cells, such as chromosome 10 open reading frame 116 (*C10orf116*). Some were related to inflammation, such as *CCL7*, *CCL1*,

chemokine (C-X-C motif) ligand (*CXCL*) 9, and *CXCL11*. Others were related to angiogenesis, such as nitric oxide synthase trafficking (*NOSTRIN*), and cell invasion, such as *C4ORF8*. Interestingly, some were closely related to hypoxia, which is the main contributor to RA<sup>14,15</sup>, such as hypoxia-inducible factor 1α (*HIF-1α*) and thioredoxin domain containing 5 (*TXNDC5*). Changes in gene expression were validated by qRT-PCR (Figures 4A and 4B).

We also tested the expression of Furin, which has a protective role in immune response-induced arthritis<sup>8</sup>, following PCSK6 knockdown. No significant difference was observed in the expression of Furin following PCSK6 silencing (Figure 4A).

## DISCUSSION

PCSK6 is a neuroendocrine-specific mammalian subtilisin-related endoprotease<sup>16</sup> that exhibits a C-terminal cysteine-rich region<sup>17</sup> and functions in the secretory pathway. In our study, we observed an increased expression of PCSK6 in RA synovial tissues at both mRNA and protein levels, which suggests that abnormal PCSK6 expression may promote the pathological changes observed in RA. *PCSK6*, located on chromosome 15q26.3, includes about 224.6 kb. In our present study, we genotyped tag SNP of *PCSK6* covering the whole region. One SNP, rs8029797, may be associated with RA, indicating a possible genetic effect of PCSK6 on RA risk.



**Figure 4.** Microarray analysis confirmation by qRT-PCR (A) mRNA levels of the key genes expression after treatment of siRNA-PCSK6 in RASF. B. Confirmation of expression data obtained from microarrays by qRT-PCR (SYBR Green I dye). The expression levels, measured by qRT-PCR (black bars), were normalized using GAPDH as internal control. The white bars indicate expression values obtained from microarray. Values are mean  $\pm$  SD. \*  $p < 0.05$ . qRT-PCR: quantitative real-time PCR; siRNA: small interfering RNA; PCSK6: proprotein convertase subtilisin/kexin type 6; RASF: rheumatoid arthritis synovium fibroblasts; NC: negative control; MOCK: transfection-reagent control; MMP: matrix metalloproteinase; IL: interleukin; CXCL9: chemokine ligand 9; TXNDC5: thioredoxin domain containing 5; C10orf116: chromosome 10 open reading frame 116; NOSTRIN: nitric oxide synthase trafficking; HIF-1 $\alpha$ : hypoxia-inducible factor 1 $\alpha$ .

Hyperplasia of synovial fibroblasts contributes to the pathogenesis of RA and is capable of eroding adjacent cartilage and bone, and causing subsequent joint destruction. Because PCSK6 had been indicated to playing a critical role in tumor progressions<sup>8,18</sup>, we attempted to determine how PCSK6 functions in RASF. Notably, significant decreases in proliferation, migration, and invasion capacities were observed after PCSK6 silencing in RASF, which suggests that PCSK6 may play an important role in the hyperplasia and erosion capacity of synovial fibroblasts. Further analysis revealed a G0/G1 arrest in PCSK6-silenced RASF. In support of this finding, increased expression of the cyclin-dependent kinase inhibitor, p27<sup>KIP</sup>, was observed in PCSK6-silenced RASF. This result was consistent with a previous finding<sup>8</sup> in prostate cancer. A lack of mitogenic activity is associated with higher cell quiescence states characterized by the increased expression of p27<sup>KIP</sup><sup>11</sup>. In addition, previous studies have shown that mitogen-activated protein kinases p42MAPK and p44MAPK (ERK1/2) are required for fibroblast proliferation to pass the

G1 restriction point and enter S phase<sup>19</sup>. In our study, we found that the phosphorylated forms of ERK1/2 were reduced after PCSK6 was silenced. In total, our data indicate that PCSK6 promotes the proliferation of RASF by disturbing cell cycle arrest through the targeting of p27<sup>KIP</sup> or the activity of the MAPK pathway.

RA is characterized as a chronic inflammatory disease. The proinflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-17 contribute to synovitis and joint destruction. TNF- $\alpha$  can promote fibrosis of synovial tissues and immune response<sup>20</sup>. IL-26, a member of the IL-10 cytokine family, is over-expressed in patients with RA and fibroblast-like synovio-cytes are its major source. Importantly, IL-26 in RASF can induce the production of the proinflammatory cytokines, including TNF- $\alpha$ <sup>21</sup>. We found that PCSK6 knockdown led to decreased IL-26 and TNF- $\alpha$  levels in RASF, which suggests that PCSK6 exacerbates inflammation by regulating the expression or secretion of cytokines such as IL-26 and TNF- $\alpha$  in RASF.

PCSK6 knockdown also led to altered gene expression

patterns in RASF. Among the downregulated genes, many of them had important biological functions closely related with main features of RA, such as hyperplasia, hypoxia, cartilage erosion, inflammation, and angiogenesis. Several specific molecules are therapeutic targets in RA<sup>22</sup>, such as TNF- $\alpha$ , IL-6, and cytotoxic T-lymphocyte-associated protein 4. Recently, an important step in understanding the development and progression of RA was the recognition that chemokines are expressed in the synovium of RA joints and aggravate the inflammation degree<sup>23,24</sup>. Of them, CCL7 and CXCL9 were found to be overexpressed in preinflamed synovium and could induce immune-related cells to migrate into the sites of inflammation<sup>25</sup>. In our study, we observed that silencing of PCSK6 could downregulate the expression of CCL7 and CXCL9 in RASF, which further supports the proinflammatory role of PCSK6 in RA.

A slight decrease in MMP-9 expression was also observed after PCSK6 knockdown in RASF, which was consistent with a report<sup>26</sup> that PCSK6 could process immature MMP-9 to its mature form. Abnormal angiogenesis in synovium is an important feature of RA, and remodeling of the extracellular matrix by MMP is important in angiogenesis<sup>27</sup>. While MMP-9 is able to degrade extracellular matrix, MMP-9 is also implicated in the development of cartilage and bone erosions in RA<sup>28</sup>. A study by Perisic, *et al*<sup>29</sup> demonstrated that PCSK6 mRNA levels are positively correlated with typical markers of inflammation and apoptosis, like IL-1 $\beta$ , TNF- $\alpha$ , and proteins involved in matrix degradation, such as MMP-9 in vascular disease, which confirm our results and further validate the prometastasis and angiogenesis functions of PCSK6 in RA. In addition, NOSTRIN, another protein that plays an important role in developmental angiogenesis<sup>30</sup>, was downregulated after PCSK6 knockdown in RASF, further suggesting a potential central role in RA.

Hypoxia in the synovial tissue of inflamed joints is one of the most important characteristics of RA<sup>31</sup>. HIF-1 $\alpha$ , expressed under hypoxic conditions, is most strongly expressed in the sublining layer of the RA synovium and its expression is related to proliferation, angiogenesis, and inflammation in the synovium from patients with RA<sup>12</sup>. In addition, our previous study showed that increased TXNDC5 expression could also be induced by hypoxia and led to abnormal proliferation of RASF<sup>13</sup>. In our study, we found that *HIF-1 $\alpha$*  and *TXNDC5* expressions are regulated, directly or indirectly, by PCSK6. Thus, we hypothesized that PCSK6 may be involved in the regulation of the hypoxic synovium microenvironment. Further, *C10orf116*, an adipocyte lineage-specific nuclear factor, regulates master adipogenesis transcription factors during early differentiation. Chen, *et al*<sup>32</sup> reported the *in vitro* effect of *C10orf116* on cell proliferation and apoptosis in preadipocytes. In our study, decreased expression of *C10orf116* (or adipogenesis regulatory factor) was detected after silencing

PCSK6. All of the above support the function of PCSK6 in the disordered proliferation observed in RA.

Another important member of the PC family, Furin, plays a protective role in immune response-induced arthritis<sup>8</sup>. We found that Furin expression was unperturbed after knockdown of PCSK6. Thus, the role of PCSK6 may be opposite that of Furin in RA.

Our present study demonstrated that PCSK6 is more highly expressed in the tissues of patients with RA and might play important roles in the process of cell proliferation, migration, invasion, and angiogenesis. In addition, PCSK6 might contribute to proinflammatory cytokine expression and hypoxia of synovial fibroblasts. These findings suggest that PCSK6 may be an important therapeutic target in RA.

## ONLINE SUPPLEMENT

Supplementary data for this article are available online at [jrheum.org](http://jrheum.org).

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