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Original Paper

Over-Expression of PTEN Suppresses the Proliferation and Migration of Fibroblast-Like Synoviocytes in Adjuvant-Induced **Arthritis**

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Key Words

Adjuvant-induced arthritis • PTEN • Proliferation • Migration • DNA methylation

Abstract

Background/Aims: 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 surviva of fibroblast-like synoviocytes (FLSs) and the production of pro-inflammatory cytokines in RA. The purpose of this study was to explore the functions and underlying mechanisms of PTEN in the proliferation and migration of FLSs. *Methods:* FLSs were obtained from adjuvant-induced arthritis (AIA) and normal rats. The expression levels of PTEN, c-Myc, cyclin D1, PCNA, and MMP-9 were detected by guantitative-real-time-PCR and western blot assay. A BrdU proliferation assay, cell cycle analysis, and a wound-healing assay were used to study the role of PTEN in FLSs treated with PTEN inhibitor bpv, specific small interfering RNA targeting PTEN (PTEN-RNAi) or a PTEN over-expression vector (PTEN-GV141). Chromatin immunoprecipitation and methylation-special PCR assays were used to study the expression of PTEN mRNA in the presence of DNA methylation. *Results:* PTEN expression was downregulated in AIA FLSs in comparison to normal rats. Moreover, inhibition of PTEN expression by bpv or PTEN-RNAi could promote the proliferation and migration of FLSs, and increase the expression of c-Myc, cyclin D1, PCNA, and MMP-9 in AIA FLSs, but had no effect on TIMP-1 expression. In addition, transfection of AIA FLSs with PTEN-GV141 reduced their proliferation and migration. Further study indicated that DNA methylation could regulate PTEN expression in AIA. **Conclusion:** Our findings suggest that PTEN might play a pivotal role

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in the proliferation and migration of FLSs through the activation of the AKT signaling pathway. Additionally, PTEN expression may be regulated by DNA methylation in the pathogenesis of AIA.

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease of the joints characterized by synovial inflammation, hyperplasia of synovial tissues, synovial pannus, and subsequent destruction of adjacent articular cartilage and bone [1-3]. At the same time, angiogenesis is required to maintain the chronic inflammatory state by transporting inflammatory cells to the site of synovitis and supplying nutrients to the pannus [4]. Among the various pathological events in the synovium, fibroblast-like synoviocytes (FLSs), the major cell population invading the pannus, are reported to participate actively in the inflammatory processes of RA [5]. FLSs directly invade the extracellular matrix and secrete pro-inflammatory cytokines, chemokines, matrix metalloproteinases (MMPs), and angiogenic factors into the synovial fluid, which destroycartilage and bone, and exacerbate joint damage, such as tumor necrosis factor- α (TNF- α) [6], interleukin (IL)-6 [7], chemokines ligand (CCL)-2, MMP-3 [8], and vascular endothelial growth factor (VEGF- α). One of main mechanisms underlying RA is the excessive proliferation, migration and activation of FLSs that invade adjacent cartilage and bone [1-3]. Therefore, inhibiting the proliferation and migration of FLSs is an ideal target for the treatment of RA. Although the exact causes of RA remain unknown, the proliferation and migration of FLSs have been shown to be involved in the inflammation and synovial cell proliferation that result in joint destruction in patients with RA [8]. However, little is currently known about the molecular mechanisms underlying the proliferation and migration of activated FLSs.

Recent studies have suggested that phosphatase and tension homolog deleted on chromosome 10 (PTEN) might contribute to the surviva of FLSs in RA [9-11]. PTEN, one of the most important tumor suppressors in mammals, is a ubiquitous modulator of cell growth, proliferation and inflammation, which are mainly associated with its lipid phosphatase activity [12, 13]. More importantly, PTEN exerts remarkable anti-inflammatory and anti-proliferation activity by blocking the activation of the PI3-kinase/AKT pathway [13]. In addition, Wang et al. [11] proposed that treatment with adenoviral vectors encoding human PTEN significantly reduced ankle circumference, articular index scores and histology scores, and also decreased the levels of VEGF and IL-1 β in collagen-induced arthritis. However, the potential functions of PTEN in activated FLSs are still unknown. Hence, we hypothesized that PTEN is significantly associated with RA, especially, the proliferation and migration of activated FLSs.

To further elucidate the relationship between PTEN and the proliferation and migration of FLSs in RA, we explored whether PTEN regulates the initiation of FLSs proliferation 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), 5-aza-2'-deoxycytidine (5-Aza) and Cell Proliferation Enzyme-Linked Immunosorbent Assay (ELISA), and BrdU (colorimetric) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Complete Freund's adjuvant was purchased from Chondrex, Inc. (Redmond, WA). Rabbit anti-PTEN anti- TIMP metallopeptidase inhibitor 1 (TIMP-1), anti- DNA methyltransferase 3 alpha (DNMT3a), and anti--DNA methyltransferase 3 beta (DNMT3b) monoclonal antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-c-Myc, anti-cyclinD1 and anti-proliferating cell nuclear antigen (PCNA) monoclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-MMP-9 antibody was purchased

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from Merck Millipore (Billerica, MA). Mouse anti-DNMT1 and anti-vimentin (Alexa Fluor 594 Conjugated) antibodies were purchased from Cell Signaling Technology, and mouse anti-β-actin monoclonal antibody was purchased from Bioworld (Shanghai, China). A horseradish peroxidase (HRP)-labeled secondary antibody for goat anti-rabbit immunoglobulin (IgG) was purchased from Beijing Zhongshan Biotechnology Corporation (Beijing, China). A SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) was purchased from Cell Signaling Technology. PTEN, MMP-9, TIMP-1, c-Myc, cyclinD1 and β-actin primers were synthesized by Shanghai Sangon Biological and Technological Company (Shanghai, China).

Rat model of adjuvant-induced arthritis

Female adult Sprague-Dawley rats (80-120 g) were treated with complete Freund's adjuvant (Chondrex, Inc) for 24 days at 0.1 mL /100 g body weight by intradermal injection in the left hind paw to induce rat adjuvant-induced arthritis (AIA), a model of RA [8, 14]. Normal control rats were injected with 0.1 mL normal saline/ 100 g body weight at same time. The rats were provided by the Experimental Animal Center of Anhui Medical University. All 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. The animals were maintained in accordance with the guidelines of the Center for Developmental Biology, Anhui Medical University for the Care and Use of Laboratory Animals. All experimental protocols used on the animals were approved by the institutions' subcommittees on animal care.

Histopathology

Synovial specimens were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Hematoxylin and eosin (H&E) staining and immunohistochemistry were performed according to a standard procedure. Pathological changes were assessed and photographed under an Olympus BX-51 microscope (Olympus, Tokyo, Japan).

Cell culture

FLSs were derived from the synovial tissues of AIA and control rats. The cells were cultured in highglucose Dulbecco's modified Eagle's medium (DMEM; HyClone, South Logan, UT, USA) supplemented with 20 % (v/v) heat-inactivated fetal bovine serum (FBS; PAN-Biotech, Aidenbach, Germany), 100 U/mL penicillin, and 100 mg/ml of streptomycin (Beyotime, Shanghai, China) in cell culture flasks. Cell cultures were maintained at 37 °C and in an atmosphere of 5 % CO₂.

Immunofluorescence staining

Cultured FLSs were plated in DMEM supplemented with 20 % FBS at a density of

1.02.0×10⁵ cells/mL. Immunofluorescence staining was performed with rabbit anti-PTEN and anti-Vimentin (Alexa Fluor 594 conjugated) antibodies. Alexa Fluor 488-conjugated AffiniPure goat antirabbit IgG (H+L) (Beijing Zhongshan Biotechnology Corporation) was used as a secondary antibody. Counterstaining of nuclei was performed with 4', 6-diamidino-2- phenylindole (DAPI; Beyotime).

Small interfering RNA (siRNA, RNAi) silencing

FLSs were transfected with 100 nM RNAi(GenePharma, Shanghai, China) using Lipofectamine[™] 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The oligonucleotide sequences were as follows: PTEN-RNAi (rat), 5'-CCGAUACUUCUCUCCAAAUTT-3 for the sense strand and 5'-AUUUGGAGAGAAGUAUCGGTT-3' for the antisense strand. A negative scrambled RNAi was used in parallel. Thw cells were cultured at 37 °C for 6 h, and quantitative real-time (q)-PCR and western blot analyses were performed 48 h after transfection.

Plasmid construction

A PTEN-GV141 was obtained from GeneChem (Shanghai, China). FLSs were transfected with PTEN-GV141 to induce the ectopic expression of PTEN, and with the empty GV141 vector (GV141) as a control. Transfection was performed using a Lipofectamine[™] 2000 according to the manufacturer's instructions.

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q-PCR

Total RNA was extracted from cultured FLSs using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and reverse transcribed to cDNA using an iScript^M cDNA kit (Bio-Rad, Hercules, CA). The reaction mixture was prepared according to the manufacturer's instructions using SYBRGreen q-PCR Master Mix (Vazyme Biotech, Nanjing, China). The expression of PTEN, MMP-9, TIMP-1, c-Myc, cyclinD1 and β -actin mRNA was detected byq-PCR using the following primers: PTEN forward: 5'-CCATAACCCACCACAG-3" and reverse:

5'-CAGTCCGTCCTTTCC-3' c-Myc forward 5"-TGCTCTCCGTCCTATGTTGCG-3' and reverse 5'-CAGTCCTGGATGATGATGTTCTTGA-3' cyclinD1 forward 5'-CAGCGGTAGGGATGAAATAGTGA-3' and reverse 5'-GGAATGGTTTTGGAACATGGAGA-3'; MMP-9 forward 5'-CACTGTAACTGGGGGCAACT-3' and reverse 5'-CACTTCTTGTCAGCGTCGAA-3' TIMP-1 forward 5'-CATCTCTGGCCTCTGGCATC-3' and reverse 5'-CATAACGCTGGTATAAGGTGGTCTC-3'; and β-actin forward 5'-CCCATCTATGAGGGTTACGC-3' and reverse

5'-TTTAATGTCACGCACGATTTC-3'. PCR was performed at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min by using a Thermo Fisher Step One^M PCR system. The reactions were conducted three times, and threshold cycle values were normalized to the expression of β -actin mRNA. The specificity of the products was determined by melting curve analysis. Relative mRNA expression of the target genes was obtained by normalization to the control group and to the level of β -actin.

Western blot analysis

Cultured FLSs were lysed in lysis buffer for western blot (Beyotime). The Whole-cell extracts (20 mg protein) were fractionated by electrophoresis through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blot ted onto PVDF membranes (Millipore, Bedford, MA). After blocking nonspecific protein binding, membranes were incubated for 12 h with primary antibodies diluted in primary antibody dilution buffer (Beyotime). Rabbit antibodies (PTEN, MMP-9, TIMP-1, c-Myc, cyclinD1, and PCNA) were used at a dilution of 1:500, and mouse anti-DNMT1 and anti- β -actin antibodies were used at a dilution of 1:1, 000. After incubation with the primary antibodies, the membranes were washed four times in Trisbuffered saline (TBS)/Tween-20 before incubation for 1 h in goat anti-mouse or anti-rabbit HRP-conjugated antibodies at a dilution of 1:10, 000 in TBS/Tween-20 containing 5 % skim milk. After washing four times with TBS/Tween-20, the protein bands were detected using the ImmobilonTM Western Chemiluminescent HRP Substrate (Millipore).

Cell cycle analysis

To analyze intracellular DNA content, FLSs were fixed in 70 % ethanol at 4 °C overnight after 48 h treatment with bpv, PTEN-RNAi or PTEN-GV141. FLSs were centrifuged at 1, 000×g for 5 min and resuspended in phosphate-buffered saline. The cells were stained with 0.5 mL propidium iodide staining buffer (Beyotime) contains 200 mg/mL RNase A and 50 μ g/mL propidium iodide, at room temperature for 30 min in the dark. Fluorescence-activated cell sorting (FACS) was performed on a FC 500 (Beckman Coulter, Brea, CA).

BrdU proliferation assay

FLSs (1000 cells/well) were seeded in 96-well plates with DMEM and 20 % FBS and cultured FOR 2 days. The cells were exposed to bpv, PTEN-RNAi or PTEN-GV141 for 48 h. The cells were labeled with 20 μ L/well of a BrdU labeling solution as described previously and incubated with 200 μ L/well FixDenat. After incubation with 100 μ L/well of anti-BrdU-POD working solution for 90 min and washing 3 times, substrate solution (TMB) was added and the absorbance of each well was read at 370 nm with an ELISA plate reader (BioTek, Winooski, VT).

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Wound-healing assay

FLSs were cultured in 24-well plate (5.0×10^5 cells /mL / well) in DMEM and 20% FBS. After the cells reached 90-95% confluence, thwy were treated with bpv, PTEN-RNAi or PTEN-GV141 for 24 h. The cells were serum deprived and scratched with a pipette tip. After 48 h, the cells were fixed with methanol, stained with crystal violet, and viewed under an Olympus BX-51 microscope.

Chromatin Immunoprecipitation assay

A chromatin immunoprecipitation (ChIP) assay (Cell Signaling Technology) was performed according to the manufacturer's instructions. The FLSs were cross-linked in 1% formaldehyde for 10 min at room temperature. The cell pellets were lysed, re-suspended, and subjected to sonication on ice. Five microliters of sonicated chromatin was stored as a 2% input sample. The remaining samples were immunoprecipitated overnight at 4 °C on a rocking platform using an anti-DNMT1 monoclonal antibody. Following overnight incubation, ChIP-grade Protein A/G Plus agarose beads were added to the lysate and incubated for 2 h at 4 °C. The samples were washed with three different buffers and eluted in a buffer containing 5 M NaCl and 20 mg/mL proteinase K. The immunoprecipitated and input samples were then reverse cross-linked at 65 °C for 2 h. Finally, the eluates were used to detect ChIP signals by q-PCR.

Methylation-specific PCR (MSP)

DNA samples were 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 (EpiGentek, Inc., Farmingdale, NY). The primers of methylated and unmethylated PTEN were as follows: PTEN (rat, methylated) forward:

5'-CGGTCGGTGTTAAGTTTTTCGT-3' and reverse:

5'-AAAACGAATAATCCTCGCAACG-3'; and PTEN (rat, unmethylated) forward: 5'-ATTTGGTTGGTGTTAAGTTTTTTGT-3' and reverse: 5'-AAAAAACAAATAATCCTCACAACAAAC-3'.

Statistical analysis

Data are presented as the mean ± standard deviation (SD) and were 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 down-regulated in AIA FLSs

To confirm the role of PTEN in RA, a rat model of AIA was established by injection with the complete Freund's adjuvant. Histopathological analysis (Fig. 1A) confirmed that the AIA model was established successfully, and we observed a significant increase in the number of infiltrating inflammatory cells. Immunohistochemical analysis indicated the clear downregulation of PTEN expression in AIA FLSs compared with normal cells (Fig. 1B). Analysis of Vimentin expression (Fig. 1C) indicated that the cells derived from synovial tissues were FLSs. Moreover, immunofluorescence staining demonstrated that PTEN protein expression was lower in AIA FLSs than in normal FLSs (Fig. 1C). Similarly, western blot and q-PCR analyses showed that PTEN mRNA and protein levels (Fig. 1D, E) were significantly downregulated in FLSs isolated from AIA rat synovium. Thus, these results indicated that PTEN expression was clearly reduced in AIA FLSs.

A PTEN inhibitor increases FLS proliferation and migration

To identify the significance of PTEN in RA FLSs, we measured its effect on the proliferation and migration of AIA FLSs following treatment with the PTEN inhibitor bpv. Western blot and q-PCR analyses showed that PTEN expression was markedly down-regulatedby varying degrees with 125 or 250 nM bpv in AIA FLSs (Fig. 2). More significantly, MMP-9 mRNA and protein expression was notably upregulated by bpv in AIA FLSs, whereas TIMP-1 expression



Fig. 1. PTEN expression is downregulated in AIA FLSs. (A) Representative H&E staining of AIA and normal synovial tissues in rat (original magnification, $20 \times$). (B) PTEN expression in AIA and normal synovial tissues was analyzed by immunohistochemical staining in rats (original magnification, $20 \times$). (C) PTEN and vimentin expression was analyzed by double immunofluorescence staining in rat AIA and normal FLSs (original magnification, $40 \times$). (D) PTEN protein levels were analyzed by western blot in AIA and normal FLSs. (E) PTEN mRNA levels were analyzed by q-PCR in AIA and normal FLSs. All values are expressed as the mean \pm SD. ## P<0.01 vs. normal group.



Fig. 2. Inhibition of PTEN expression with bpv increases the migration-associated protein of FLSs. (A) PTEN, MMP-9 and TIMP-1 protein levels were analyzed by western blot in AIA FLSs treated with bpv. (B) PTEN, MMP-9 and TIMP-1 mRNA levels were analyzed by q-PCR in FLSs treated with bpv. All values were expressed as the mean ± SD. [#]P<0.05, ^{##}P<0.01 vs. normal group; ^{*}P<0.05, ^{**}P<0.01 vs. AIA group.



Fig. 3. Inhibition of PTEN expression with bpv increases the migration of FLSs. FLSs were treated with bpv (250 nM), and migration into the wound scratch at 48 h was photographed (original magnification, 10×).



Fig. 4. Inhibition of PTEN expression with bpv increases the proliferation -associated protein of FLSs. (A) After AIA FLSs were incubated with bpv, the protein levels of c-Myc, cyclinD1, and PCNA were analyzed by western blot. (B) AIA FLSs were treated with bpv, and the mRNA levels of c-Myc and cyclinD1 were analyzed by q-PCR. All values were expressed as the mean ± SD. ^{##} P<0.01 vs. normal group; ^{*}P<0.05, ^{**}P<0.01 vs. AIA group.

was down-regulated (Fig. 2A and B). Remarkably, a wound-healing assay indicated that the migration of AIA FLSs was promoted by bpv (Fig. 3). In addition, western blot and q-PCR analyses verified that the expression of the proto-oncogene c-Myc, cyclin protein cyclinD1 and PCNA was up-regulated markedly by 125 and 250 nM bpv in AIA FLSs (Fig. 4). PCNA is an essential cofactor for DNA replication and repair [15]. Similarly, cell cycle analysis (Fig. 5) also suggested that treatment of FLSs with bpv increased the number of cells in the G2/M



Fig. 5. Inhibition of PTEN expression with bpv regulates the cell cycle of FLSs. FACS analysis of the cell cycle of FLSs incubated with bpv (250 nM) for 48 h.

Fig. 6. Inhibition of PTEN expression with PTEN-RNAi increases the proliferation of FLSs. FLSs treated with bpv (250 nM) for 48h. All values were expressed as the ## P<0.01 vs. normal group; *P<0.05 vs. AIA group.





Fig. 7. Inhibition of PTEN expression with PTEN-RNAi increases the migration-associated protein of FLSs. (A) The protein levels of PTEN, MMP-9 and TIMP-1 were analyzed by western blot in AIA FLSs transfected with PTEN-RNAi in. (B) The mRNA levels of PTEN, MMP-9, and TIMP-1 were analyzed by q-PCR in FLSs transfected with PTEN-RNAi. All values are expressed as mean ± SD. #P<0.05, ## P<0.01 vs. normal group; *P<0.05, **P<0.01 vs. AIA group.

phase. Furthermore, BrdU cell proliferation ELISA showed that the proliferation of AIA FLSs was promoted by 250 nM bpv (Fig. 6). Thus, these findings demonstrated that the PTEN inhibitor bpv increased the proliferation and migration of FLSs in AIA rats.

PTEN-RNAi increases FLS proliferation and migration

In order to provide additional evidence that PTEN is involved in the proliferation and migration of RA FLSs, specific siRNA targenting rat PTEN was used to knockdown its expression in AIA FLSs. AIA FLSs transfected with normal control siRNA (NC-RNAi) or PTEN-RNAi (100 nM) werecultured for 48 h following transfection, and the levels of PTEN mRNA and protein were reduced remarkably in the PTEN-RNAi transfected cells compared with the NC-RNAi transfected cells (Fig. 7). As expected, MMP-9 mRNA and protein expression was also clearly upregulated by PTEN-RNAi; however, TIMP-1 expression was down-regulated in AIA FLSs (Fig. 7). In addition, a wound-healing assay (Fig. 8) indicated that migration was notably promoted in AIA FLSs following transfectionwith PTEN-RNAi. Furthermore, the mRNA and protein expression c-Myc, cyclinD1, and PCNA were upregulated by PTEN-RNAi in





Fig. 8. Inhibition of PTEN expression with PTEN-RNAi increases the migration of FLSs. FLSs were transfected with PTEN-RNAi, and migration into a wound scratch at 48 h was photographed (original magnification, 10×).



Fig. 9. Inhibition of PTEN expression with PTEN-RNAi increases the proliferation -associated protein of FLSs. (A) After AIA FLSs were transfected with PTEN-RNAi, the protein levels of c-Myc, cyclinD1, and PCNA were analyzed by western blot. (B) AIA FLSs were transfected with PTEN-RNAi, and the mRNA levels of c-Myc and cyclinD1 were analyzed by q-PCR. All values are expressed as mean ± SD. ^{##} P<0.01 vs. normal group; *P<0.05, **P<0.01 vs. AIA group.



Fig. 10. Inhibition of PTEN expression with PTEN-RNAi regulates the cell cycle of FLSs. FACS analysis of the cell cycle of FLSs transfected with PTEN-RNAi for 48 h.

AIA FLSs (Fig. 9). Similarly, cell cycle analysis also suggested that treatment of AIA FLSs with PTEN-RNAi resulted in an increased number of cells in the G2/M phase (Fig. 10). Remarkably, a BrdU cell proliferation ELISA demonstrated that PTEN-RNAi promoted the **Fig. 11.** Inhibition of PTEN expression with PTEN-RNAi increases the proliferation of FLSs. FLSs transfected with PTEN-RNAi for 48h. All values are expressed as ^{##} P<0.01 vs. normal group; *P<0.05 vs. AIA group.



proliferation of AIA FLSs (Fig. 11). Taken together, our findings suggested that the inhibition of PTEN expression could increase the proliferation and migration of FLSsin AIA rats and may contribute to the progression of RA.



+

AIA

GV141

+

+

+

+

PTEN-GV141 PTEN-GV141 + PTEN-GV141 + + Fig. 12. Over-expression of PTEN expression with PTEN-GV141 inhibits the migration-associated protein of FLSs. (A) The protein levels of PTEN, MMP-9 and TIMP-1 were analyzed by western blot in AIA FLSs transfected with PTEN-GV141. (B) The mRNA levels of PTEN, MMP-9, and TIMP-1 were analyzed by q-PCR in FLSs transfected with PTEN-GV141. All values are expressed as mean ± SD. ## P<0.01 vs. normal group; *P<0.05, **P<0.01 vs. AIA group.

+

4 +

AIA

GV141

+

AIA

GV141

+



Fig. 13. Over-expression of PTEN expression with PTEN-GV141 inhibits the migration of FLSs. FLSs were transfected with PTEN-GV141, and migration into a wound scratch at 48 h was photographed (original magnification, 10×).



Fig. 14. Over-expression of PTEN expression with PTEN-GV141 inhibits the proliferation -associated protein of FLSs. (A) After FLSs were transfected with PTEN-GV141, and the protein levels of c-Myc, cyclinD1, and PCNA were analyzed by western blot. (B) AIA FLSs were transfected with PTEN-GV141, and the mRNA levels of c-Myc and cyclinD1 were analyzed by q-PCR. All values are expressed as mean ± SD. ## P<0.01 vs. normal group; *P<0.05, **P<0.01 vs. AIA group.

PTEN over-expression vector inhibits FLS proliferation and migration

In order to provide additional evidence that PTEN is involved in the proliferation and migration of RA FLSs, a PTEN-GV141 vector was used to over-express PTEN in AIA FLSs. Western blot and q-PCR analyses showed that PTEN expression was upregulated by transfection of AIA FLSs with PTEN-GV141 (Fig. 12). In addition, the mRNA and protein expression of MMP-9 was also downregulated by PTEN-GV141; conversely, TIMP-1-1 expression was upregulated (Fig. 12). A woundhealing assay demonstrated that the migration of AIA FLSs was suppressed by PTEN-GV141 (Fig. 13). Furthermore, after



Fig. 15. Over-expression of PTEN expression with PTEN-GV141 inhibits the cell cycle of FLSs. FACS analysis of the cell cycle of FLSs transfected with PTEN-GV141 for 48 h.

Fig. 16. Over-expression of PTEN expression with PTEN-GV141 inhibits the proliferation of FLSs. FLSs transfected with PTEN-GV141 for 48h. All values are expressed as ^{##} P<0.01 vs. normal group; ^{*}P<0.05 vs. AIA group.





Fig. 17. DNA methylation regulates PTEN expression. (A) The protein levels of DNMT1, DNMT3a, and DNMT3b were analyzed by western blot in AIA and normal FLSs. (B) DNMT1 protein was recruited to the coding region of the PTEN gene in AIA FLSs according to a ChIP assay. PTEN mRNA levels were analyzed by q-PCR. All values are expressed as the mean ± SD. ## P<0.01 vs. normal group.

transfection with PTEN-GV141, the mRNA and protein expression of c-Myc, cyclinD1 and PCNA was downregulated in AIA FLSs (Fig. 14). As further support for ourhypothesis, the over-expression of PTEN by PTEN-GV141 resulted in a significant decrease in the number of cells in G2/M phase, as shown by FACS analysis in AIA rats (Fig. 15). Consistent with the cell proliferation results, a BrdU cell proliferation ELISA showed that the proliferation of AIA FLSs was suppressed by PTEN-GV141 (Fig. 16). These data proved that transfection of AIA FLSs with PTEN-GV141 had a profound inhibitory effect on their proliferation and migration, indicating that PTEN may be involved in the proliferation and migration of FLSs; and plays a pivotal role in the pathogenesis of RA.

DNA methylation regulates PTEN expression

From our findings, it is evident that PTEN negatively regulates the proliferation and migration of FLSs in RA. To clarify the mechanism for the down-regulation of PTEN expression in AIA FLSs, we examined whether DNA methylation mediated its expression. First, we identified four 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. Moreover, we found that the expression of DNMT1, DNMT3a, and DNMT3b proteins was upregulated in AIA FLSs compared with cells from normal rats (Fig. 17). Furthermore, ChIP assay demonstrated that DNMT1 was recruited to the coding region of the PTEN gene in AIA FLSs (Fig. 17 B). In addition, after treatment of AIA FLSs with the methylation inhibitor 5-Aza, PTEN expression was clearly increased (Fig. 18); conversely,





MMP-9

TIMP-1

the mean ± SD. ## P<0.01 vs. normal group. *P<0.05, **P<0.01 vs. AIA group.



Fig. 19. 5-Aza inhibits the migration of FLSs. (A) FLSs were treated with 5-Aza, and migration into a wound scratch at 48 h was photographed (original magnification, 10×). (B) MSP indicating that the CpG methylation level of PTEN was reversed in AIA FLSs.



Fig. 20. 5-Aza inhibits the proliferation-associated protein of FLSs. (A) After AIA FLSs were incubated with 5-Aza, the protein levels of c-Myc, cyclinD1, and PCNA were analyzed by western blot in AIA. (B) AIA FLSs were treated with 5-Aza, and the mRNA levels of c-Myc and cyclinD1 were analyzed by q-PCR. All values are expressed as the mean ± SD. ## P<0.01 vs. normal group. *P<0.05, **P<0.01 vs. AIA group.

DNMT1 protein was significantly downregulated. Moreover, MSP indicated that PTEN DNA methylation was increased in AIA FLSs (Fig. 19 B). As expected, the expression of MMP-9 mRNA and protein in AIA FLSs was also reduced by 5-Aza; conversely, TIMP-1 was upregulated (Fig. 18). A woundhealing assay indicated that the migration of AIA FLSs was suppressed with 4µM 5-Aza (Fig. 19 A). In addition, after treatment with5-Aza, the mRNA and protein expression of c-Myc, cyclinD1 and PCNA was downregulated in AIA FLSs (Fig. 20). In agreement with our hypothesis, treatment with 5-Aza significantly decreased the number of cells in the G2/M phase in AIA rats, as determined FACS analysis (Fig. 21). Taken 1457





Fig. 21. 5-Aza inhibits the proliferation protein of FLSs. FACS analysis of the cell cycle of FLSs incubated with 5-Aza (4 µM) for 48 h.

together, these data show that treatment of FLSs with the methylation inhibitor 5-Aza could suppress their proliferation and migration and PTEN mayhave a role in methylation.

PTEN modulates FLS proliferation and migration and may be associated with the AKT signaling pathway

PTEN is known to be closely associated with the AKT signaling pathway in pathophysiological processes. To investigate the effect of PTEN on the AKT signaling pathway in FLSs proliferation and migration, we examined the expression profiles of phosphorylated (p)-AKT, a major component of the PI3K/AKT pathway. Western blot analysis showed the clear upregulation of p-AKT in AIA FLSs compared with cells from normal rats. Moreover, inhibition of PTEN with bpv or PTEN-RNAi significantly enhanced p-AKT expression in AIA FLSs (Fig. 22 A and B). In particular, PTEN-GV141 suppressed AKT signaling, and the expression of p-AKT was decreased substantially in AIA FLSs (Fig. 22 C). In addition, treatment with 5-Aza, significantly reduced the expression of p-AKT (Fig. 22 D). Taken together, these results indicated that PTEN could modulate the proliferation and migration of FLSs and may be closely associated with the AKT signaling pathway.

Discussion

The results of this study show that: 1) PTEN expression was down-regulated substantially in RA FLSs; 2) the PTEN inhibitor bpv and PTEN-RNAi increased proliferation and migration of AIA FLSs; 3) the PTEN-GV141 over-expression vector suppressed the proliferation and migration of AIA FLSs; 4) DNA methylation regulated PTEN expression in AIA FLSs; and 5) PTEN modulated the proliferation and migration of FLSs and may be closely associated with the AKT signaling pathway.

AIA is a model of experimental RA that is induced by the injection of complete Freund's adjuvant. AIA in rats has similar characteristics to RA from the perspectives of histology and immunology, and is a useful test system for evaluating therapies for RA [16]. Therefore, we chose AIA rats, rather than RA rats, to affirm the role of PTEN. The exact pathogenesis of RA remains yet to be fully elucidated, but an important feature involves communication between FLSs and inflammatory cells in the joint. In addition, the hyperplastic FLS population potentially promotes the infiltration [17], recruitment and retention of T lymphocytes and macrophages by producing cytokines [18], chemokines [19], extracellular matrix proteins [17] and cell adhesion molecules [20]. The migration of activated FLSs into cartilage and bone is a critical event during invasive pannus formation in RA synovium [21]. However, there are no approved drugs that are known to target FLSs in RA, and the underlying mechanisms driving FLS activation remain unresolved. Hence, the targeted suppression of the proliferation and migration of FLSs may potentially complement the current therapeutics without major deleterious effects on adaptive immune responses.

PTEN is a novel tumor suppressor that exhibits tyrosine phosphatase activity as well as homology to the cytoskeletal proteins tensin and auxilin [9, 10]. In this paper, we agree with et al. [22] that PTEN is clearly reduced in AIA FLSs compared with normal cells, according to immunohistochemical and western blot analyses. Moreover, we measured PTEN expression

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by immunofluorescence staining and found that it was lower in AIA FLSs than in normal cells. Furthermore, inhibition of PTEN expression with bpv or PTEN-RNAi led to the overexpression of MMP-9, the protooncogene c-Myc, the cyclin protein cyclin D1, and PCNA, whereas TIMP-1 expression was downregulated. Cvclin D1, c-Mvc [23], and PCNA [24] are common downstream molecules of this pathway and have a role in cell proliferation. In addition, numerous experimental and clinical studies [25] have indicated that the expression of MMP-9, which is a member of a family of zinc-dependent endopeptidases, provides suitable conditions for cell migration and invasion. Conversely, the action of all MMPs is regulated by a group of endogenous TIMPs. Interestingly, a recent study [26] reported that a mouse model of breast cancer with a germline deletion of the PTEN gene displayed normal development of the mammary gland, but had increased breast tumorigenicity and lung metastasis. More significantly, cycle analysis, BrdU cell cell proliferation ELISA, woundhealing assay and cell invasion assay also suggested that the inhibition of PTEN significantly expression increased FLS proliferation and migration in AIA rats.

It is necessary to consider whether PTEN over-expression of PTEN could improve or regulate FLS proliferation and migration. As a result, transfection of FLSs with the PTEN-GV141 over-expression vector had a profound inhibitory effect on their proliferation and migration in AIA rats. In this study, our results showed that PTEN overexpression could downregulate the expression of c-Myc, cyclin D1, PCNA, and MMP-9, and upregulate TIMP-1 expression. Furthermore, our latest research (unpublished data) found a significant downregulation



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Fig. 22. PTEN modulates FLS proliferation and migration and may be closely associated with the AKT signaling pathway. (A) The protein level of p-AKT was analyzed by western blot in FLSs treated with bpv. (B) The protein level of p-AKT was analyzed by western blot in FLSs treated with PTEN-RNAi. (C) The protein level of p-AKT was analyzed by western blotting in FLSs treated with PTEN-GV141. (D) The protein level of p-AKT was analyzed by western blot in FLSs treated with 5-Aza. All values are expressed as the mean \pm SD. ^{##}P<0.01 vs. normal group. ^{**}P<0.01 vs. AIA group.

of inflammatory cell infiltration following the intra-articular injection of AIA knees with

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an adenovirus carrying rat PTEN. To summarize, the over-expression of PTEN significantly reduced the proliferation and migration of FLSs in AIA.

DNA methylation is a kind of epigenetic modification that plays a critical role in regulating gene expression and potentially contributes to immune dysregulation [27, 28]. Several studies have demonstrated that DNA methylation is closely associated with RA [28]. For example, Nakano et al. [28] found that differentially methylated genes could contribute to the pathogenesis of RA. We also proved that DNMT1 was over-expressed in AIA FLSs, and recruited to the coding region of the PTEN gene in AIA rats. Similarly, et al. [29] confirmed that the loss of PTEN expression is associated with a distinct methylation signature, and the methylated CpG sites may mediate tumor progression when PTEN is deleted in prostate cancer. In particular, treatment of AIA FLSs with the methylation inhibitor, 5-Aza significantly increased PTEN mRNA and protein expression. Conversely, the expression of c-Myc, cyclin D1, PCNA, and MMP-9 was upregulated, but TIMP-1 expression was not. To summarize, DNA methylation may regulate PTEN expression in the pathogenesis of AIA.

It is clear that PTEN mediates cellactivation by negatively regulating the PI3K/AKT signaling pathway [13]. The loss of PTEN can result in the activation of AKT kinases, which play key roles in cell growth, proliferation and invasion [30]. Our results also demonstrated that inhibition of PTEN by bpv or PTEN-RNAi, significantly increased p-AKT expression in AIA FLSs. In particular, PTEN-GV141 suppressed AKT signaling, with a substantial decrease of p-AKT expression in AIA FLSs. Taken together, these data suggested that PTEN mediated the proliferation and migration of FLSs and might regulate the activation of the PI3K/AKT signaling pathway.

Conclusion

In summary, the findings of the present study suggested that PTEN might play a pivotal role in the proliferation and migration of FLSs through the activation of the 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. Further research is needed to clarify if PTEN can be used as a diagnostic marker and prognostic indicator of RA.

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Disclosure Statement

The authors declare to have no competing interests.

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