The role of PTEN up-regulation in suppressing glomerular mesangial cells proliferation and nephritis pathogenesis

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Abstract. – OBJECTIVE: Over-proliferation of mesangial cells is the major pathological change of mesangial proliferative glomerulonephritis (MPGN). PTEN-PI3K/AKT pathway plays a role in regulating proliferation of mesangial cells. Anti-thymocyte serum nephritis (ATSN) is a widely used animal model for studying MPGN. This study established ATSN model, on which the role of PTEN-PI3K/AKT signal pathway in MPGN pathogenesis was investigated.

MATERIALS AND METHODS: ASTN rat model was established in parallel with control group. Protein expressions of PTEN, p-AKT, PCNA, Cyclin D1 and Bcl-2 were quantified, along with glomerular mesangial cell (GMC) counting. Rat mesangial cell (RMC) was treated with 0 or 10 ng/mL IL-6, followed by flow cytometry analysis for apoptosis, cycle and PCNA expression. Expressions of PTEN, p-AKT, PCNA, Cyclin D1 and Bcl-2 were measured. RMC was treated with pSicoR-PTEN and/or LY294002, followed by the treatment of 10 ng/mL IL-6 for 48 h. Cell apoptosis, cycle, PCNA expression and protein expression were measured.

RESULTS: Lower PTEN expression was found in renal cortex of ATSN rats, along with increasing levels of p-AKT, PCNA, Cyclin D1, Bcl-2, and higher GMCs, compared to that in control rats. IL-6 treatment increased protein expression in RMC, facilitated cell proliferation and cycle progression and suppressed apoptosis. Over-expression of PTEN and/or LY294002 remarkably decreased protein expression in RMC, inhibited the effect of IL-6 on proliferation, and induced cell apoptosis and cycle arrest.

CONCLUSIONS: The down-regulation of PTEN played a role in enhancing PI3K/AKT pathway activity, facilitating GMC proliferation and MPGN pathogenesis.

Key Words:
PTEN, PI3K/AKT, Mesangial proliferative glomerulonephritis, ATSN.

Introduction

Mesangial proliferative glomerulonephritis (MPGN) is a sort of glomerulonephritis commonly found in clinics[1]. Over-proliferation of glomerular mesangial cells (GMCs) and abnormally higher level of extracellular matrix (ECM) significantly participated in patho-physiological changes in MPGN pathogenesis, and led to fibrosis and eventually of glomerulus cirrhosis, which destructed renal functions and compromised patient's life[2]. Phosphatidylinositol-3Kinase[1-3] kinase (PI3K) is characterized as an important growth factor receptor-related kinase, and can be activated by various external stimulus, thus modulating biological processes including cell proliferation, cycler and apoptosis via the activation of downstream protein kinase B (PKB/AKT)[3,4]. PI3K/AKT signaling pathway contributed to pro-proliferation and anti-apoptosis function and played important roles in occurrence and progression of multiple inflammatory diseases including rheumatoid arthritis[5], systemic lupus erythematosus (SLE)[6], osteoarthritis[7] and lupus nephritis (LN)[8]. Various studies showed the participation of PI3K/Akt signaling pathway in the regulation of inflammatory factors on GMC proliferation[9,10], synthesis and expression of ECM, such as type I collagen[11]. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) has drawn extensive attention for the critical anti-tumor functions. It can de-phosphorylate PIP3 to PIP2, and antagonize phosphorylation effects of PI3K kinase on PIP2, thus exerting a suppressor of AKT via the modulation of PI3K[12]. Previous study[13] showed the role of PTEN in regulating GMC proliferation, and the correlation between PTEN and LN pathogenesis. However, the impact and mechanism of the

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abnormal expression or function of PTEN on the onset of MPGN remain unclear. Anti-thymocyte serum nephritis (ATSN) belongs to the nephritis induced by anti-rat thymus cell antibody and is featured with abnormal proliferation of rat mesangial tissues. Due to similar pathological change in human MPGN, ATSN, as an animal model, is widely used for the study of MPGN[14]. Therefore, this study generated an ASTN animal model and sought to determine the role of PTEN-PI3K/AKT signal pathway in MPGN pathogenesis.

Materials and Methods

Major Reagent and Materials

Male and female SD rats (6-8 weeks, body weight 220-240 g) and male New Zealand rabbits (7-8 weeks, body weight 2.0-2.2 kg) were purchased from Shaanxi Medical Animal Center (Xi'an, Shanxi, China). Rat mesangial cell (RMC) was obtained from ATCC (Manassas, VA, USA). 1640 culture medium and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA). RNA extraction kit SPLIT RNA was purchased from Lexogen (Vienna, Austria). Fluorescent quantification kit QuantiTect SYBR Green RT-PCR Kit was purchased from Qiagen (Hilden, Germany). PCR primer was synthesized by Sango (Shanghai, China). Cytokine IL-6 was purchased from Peprotech (Rocky Hill, NJ, USA). Radioimmunoprecipitation assay (RIPA) lysis buffer and apoptosis test kit were purchased from Beyotime (Beijing, China). PE labeled proliferation cell nuclear antigen (PCNA) was purchased from BD Biosciences (San Jose, CA, USA). Antibody of PTEN, p-AKT, PCNA, Cyclin D1, Bcl-2 and β-actin was acquired from Abcam (Cambridge, MA, USA). Horseradish Peroxidase (HRP) labeled secondary antibody was purchased from Boster (Wuhan, Hubei, China). ViaFect transfection reagent was acquired from Promega (Madison, WI, USA). Over-expression plasmid pSicoR-GFP was purchased from Addgene (Cambridge, MA, USA). Rat urea protein assay kit was obtained from Jingkang Bio (Shanghai, China). PI3K/AKT specific inhibitor LY294001 was obtained from Selleck (Houston, TX, USA). PI and RNase A were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of The Kidney Internal Medicine of Xianyang First People's Hospital.

Preparation of Anti-thymocyte Serum (ATS)

Female SD rats were anesthetized by ether for collecting thymocytes, which were mixed with incomplete Freund's adjuvant to immunize male New Zealand rabbits. 14 days later, booster immunization was performed using thymocytes equivalent to 1/5 of initial dosage. 10 days later, anti-serum was collected.

ATSN Model Generation

Male SD rats with normal urea protein contents were divided into two groups (N=24 each). In ATSN model group, ATS (0.5 mL/100 g) was injected once vie tail vein. In control group, equal saline (0.5 mL/100 g) was injected via the tail vein. Renal cortical tissues were collected at 3, 5 and 7 day to extract protein and to prepare paraffin-based sections.

PAS Staining for Mesangial Cells Counting

Paraffin sections were de-waxed and oxidized by iodic acid. After using Schiff's dye staining, hematoxylin nuclear staining, HCl-ethanol differentiation, ammonia processing, gradient ethanol dehydration, xylene immersing and resin mounting, 20 nuclear cells in glomerulus were counted under light field microscope to calculate the average value.

24-h Urea Protein Assay

24-h total protein content in rat urea at 3, 5 and 7 days after model generation was measured by biuret approach and was presented as mg/24 h.

Construction of PTEN Over-Expression Plasmid

Using rat RMA cDNA as the template, CDS region of PTEN gene was amplified by PCR, and was ligated into pSicoR plasmid at 3:1 ratio. After screening by Amp resistance, sequencing and sub-cloning, plasmid DNA was extracted.

RMC Culture and IL-6 Treatment

RMC was cultured in 1640 medium containing 10% FBS and 1% streptomycin. When cells were at log-growth phase, 10 ng/mL IL-6 was used to treat cells for 48 h. Control group was recruited without IL-6 treatment.

RMC Culture and Grouping

ViaFect transfection reagent was used to transfect PTEN over-expression plasmid (pSi-

Table I. Mesangial cell count and 24 h urea protein comparison.

	Group	Day 3	Day 5	Day 7
Mesangial cell per glomerulus	Control	52.2±4.8	55.7±5.1	53.9±5.2
	ATSN	68.1±5.9[a]	85.6±7.1[ab]	97.7±8.4[abc]
Urea protein (mg/24 h)	Control	11.7±1.2	13.1±1.4	12.4±1.3
	ATSN	56.8±4.7[a]	41.6±3.9[ab]	32.5±3.7[abc]

Note: [a], $p<0.05$ compared to control group; [b], $p<0.05$ compared to day 3; [c], $p<0.05$ compared to day 5.

coR-PTEN) or blank plasmid (pSicoR-Blank) into RMC. 6 h later, 1640 medium containing 10% FBS was used for 72 h incubation. Then, cells were collected for demonstrating transfection efficiency.

qRT-PCR for Gene Expression

SPLIT RNA Extract Kit was added into 20 mg renal cortical tissues for lysing cells. RNA was extracted following manual instruction. One-step qRT-PCR was performed using QuantiTect SYBR Green RT-PCR Kit to test gene expression.

Western Blot for Protein Expression

600 μL RIPA lysis buffer was added into 20 mg renal cortical tissues. After complete lysing, protein concentration was quantified in the supernatant. 40 μg protein samples were separated in 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After membrane transfer, primary antibody (PTEN at 1:200, p-AKT at 1:100, Cyclin D1 at 1:200, Bcl-2 at 1:200, PCNA at 1:300, β-actin at 1:600) was added for 4°C overnight incubation. After three times of rinsing in PBST, HRP labeled secondary antibody (1:5000) was used for 60 min room temperature incubation. Enhanced chemiluminescence (ECL) approach was used to test protein expression.

Flow cytometry for cell apoptosis

Cells from all treatment groups were collected. Following manual instruction of Annexin V-FITC/PI apoptosis test kit, 5 μL Annexin V-FITC and 5 μL PI was added for dark incubation. Flow cytometry was used to test cell apoptosis.

Flow Cytometry for Cell Proliferation

Cells were collected and digested. After permeabilization in Triton X-100, 2 μL PE-PCNA antibody was added into 100 μL cell suspension. After 30 min dark incubation at 4°C, flow cytometry was used to quantify PCNA expression reflecting cell proliferation level.

Flow Cytometry for Cell Cycle

Cells were digested with trypsin and fixed in 70% ethanol. After PBS washing, propidium iodide (PI) and RNase A staining buffer were added for 30 min dark incubation at 37°C. Flow cytometry was measured by flow cytometry.

Statistical Analysis

SPSS 18.0 (SPSS Inc., Chicago, Il, USA) was used for data analysis. Measurement data were presented as mean±standard deviation (SD). Comparison of measurement data between groups was performed by student *t*-test. Statistical significance was defined when $p<0.05$.

Results

Sever Kidney Damage and Mesangial Cell Proliferation in ASTN Model Rats

Pathology examination of renal tissues showed no significant change of GMCs in control rats, whilst ATSN model rats had significantly more mesangial cells at day 3, 5, and 7, and higher GMCs number than control rats at the same time point (Table I). Urea protein test showed no significant change of protein content at day 3, 5, and 7, whilst decreasing trends of protein urea in ASTN model was observed in a time-dependent manner, but the levels were all higher than control group, suggesting severe glomerular dysfunction (Table I).

Decreased PTEN Expression and Potentiated PI3K/AKT Pathway Activity in ATSN Model Rat Renal Tissues

Western blot results showed that, the expression of PTEN protein in renal cortical tissues of ATSN model rats was gradually decreased as time went by, the levels of which were lower than that of control rat at the same time point. In contrast, protein expressions of p-AKT, PCNA, Cyclin D1 and Bcl-2 were gradually increased, and were all

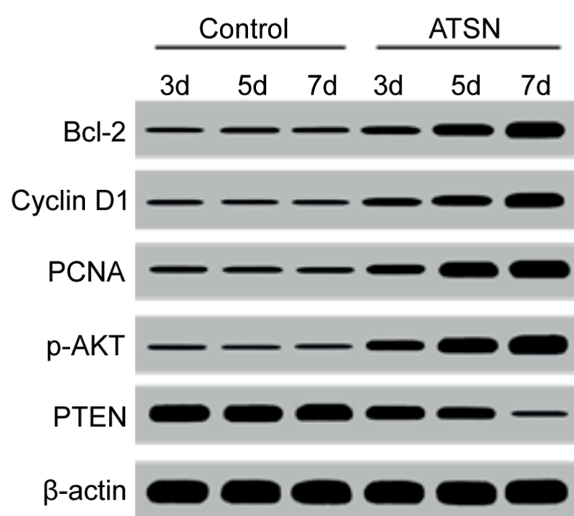

Figure 1. Western blot for protein expression in rat renal cortical tissues.

higher than that of control group at the same time (Figure 1).

IL-6 Treatment Significantly Decreased PTEN Expression, Potentiated AKT Activity and Facilitated Cell Proliferation in RMC

Compared to control group, in the treatment of IL-6, PCNA expression was significantly elevated in RMC, indicating that cell proliferation was induced (Figure 2A). Flow cytometry results showed significantly lower apoptotic rate in RMC treated with IL-6 than those without the treatment of IL-6 (Figure 2B). Moreover, PI staining results showed remarkably arrested RMC cell cycle at G0/G1 phase (Figure 2C). The result of qRT-PCR showed that IL-6 suppressed the expression of PTEN mRNA in RMC, and increased the mRNA levels of Cyclin D1 and Bcl-2 (Figure 2D). Also, Western blot results showed lower PTEN protein in RMC than that in control group, whilst protein expressions of p-AKT, Cyclin D1 and Bcl-2 were upregulated (Figure 2E).

PTEN Up-Regulation in Habited AKT Activity and Suppressed IL-6 Induced Pro-Proliferation Effects on RMC

After over-expression of PTEN in RMC, the levels of p-AKT, cyclin D1 and Bcl-2 were significantly decreased (Figure 3A), whilst IL-6 treatment weakened cell proliferation potency (Figure 3B), increased cell apoptosis (Figure 3C), and enhanced G0/G1 phase arresting (Figure 3D). Treatment of PI3K/AKT specific inhibitor, LY294002, significantly suppressed p-AKT, Cyclin D1 and Bcl-2 expressions in RMC, weakened pro-proliferation induced by IL-6, enhanced cell apoptosis and G0/G1 phase arrest. Combined treatment using both PTEN over-expression and inhibitor LY294002 enhanced the inhibitory effect on proliferation, apoptosis induction and cycle arresting of RMC, compared to single treatment of PTEN over-expression or LY294002.

Discussion

PI3K/AKT represented a signal transduction pathway widely distributed in multiple tissues and cells. Under the stimulus of growth factors and mitogens, PI3K occurred to conformational change, the activation of which phosphorylated PIP2 to PIP3. It activated AKT and facilitated transcription and expression of multiple genes. Activated PI3K/AKT signal transduction pathway was vital in maintaining cell cycle process, inhibiting cell apoptosis, and facilitating cell growth and proliferation[10]. Bcl-2 exerted inhibitory effects on mitochondrial dependent apoptotic transduction pathway via multiple mechanisms including suppressing mitochondrial Cyt C release, formation of free oxygen radicals and peroxidation of fatty acids[15]. Cell cycle protein Cyclin D1 was a type of highly conserved protein with rhythmic expression during cell cycle, and played important roles in accelerating G1/S phase transition and facilitating cell mitosis[16]. Various investigations attributed Bcl-2 and Cyclin D1 as important target genes for PI3K/AKT signal pathway regulation[17,18]. Multiple studies revealed important roles of PI3K/AKT signal pathway in the induction of pro-proliferation and anti-apoptotic effects in occurrence and progression of multiple inflammatory diseases including rheumatoid arthritis[5], systemic lupus erythematosus (SLE)[6], osteoarthritis[7] and lupus nephritis (LN)[8]. It has been shown that over-activation of PI3K/AKT signal pathway in inflammatory factor induced GMC proliferation[9,10]. Previous researches[13] revealed the role of PTEN in regulating GMC proliferation and LN disease occurrence. Our study showed that the decrease of PTEN enhanced PI3K/AKT signal pathway activity, facilitated cell cycle progress, decreased cell apoptosis, and promoted mesangial cell proliferation. Liu et al[13] observed significantly decreasing expression of PTEN in renal tissues of

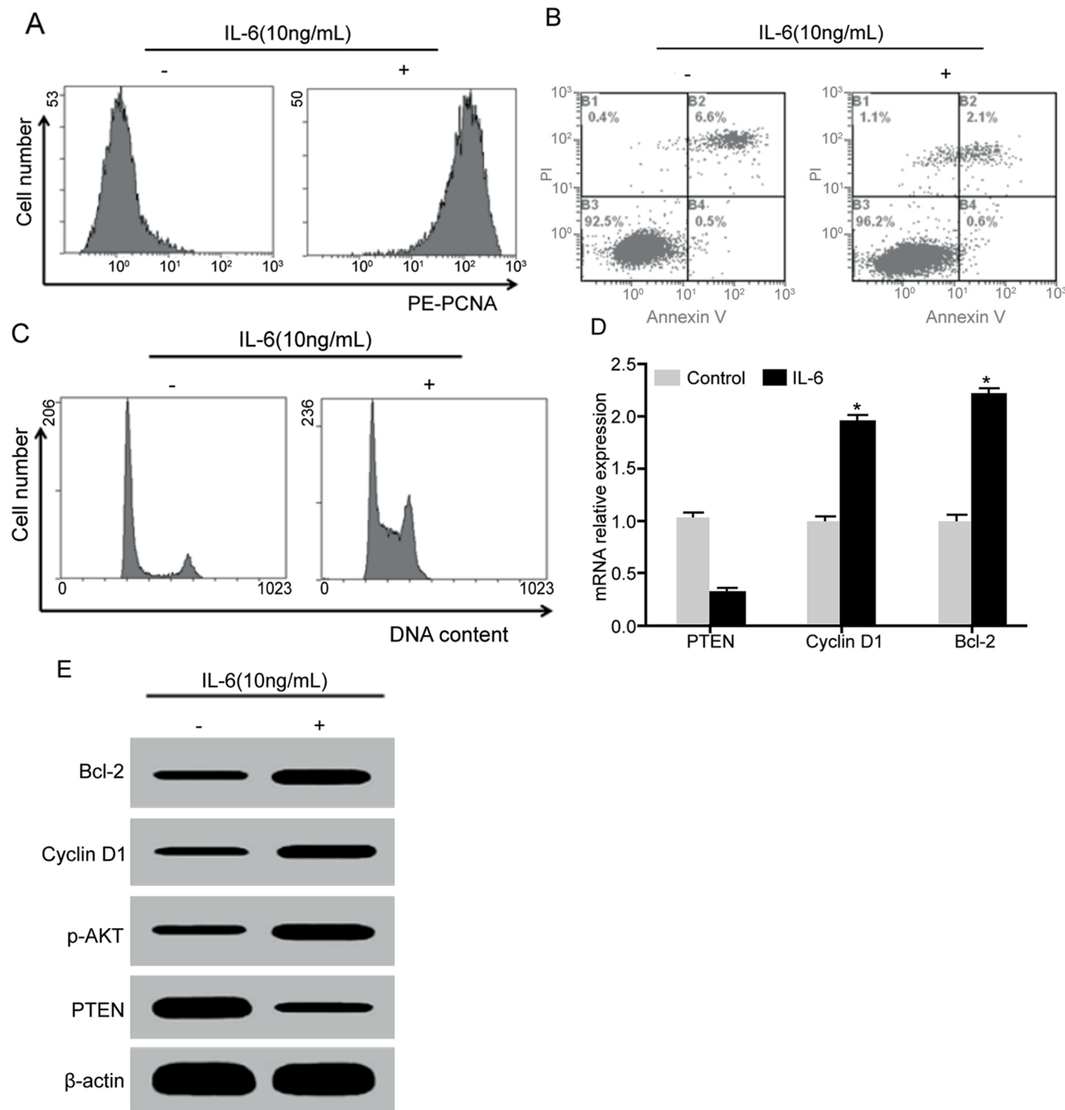

Figure 2. IL-6 treatment significantly decreased PTEN expression, potentiated AKT activity and facilitated cell proliferation in RMC. ***(A)*** flow cytometry for PCNA expression; ***(B)*** flow cytometry for cell apoptosis; ***(C)*** flow cytometry for cell cycle; ***(D)*** qRT-PCR for gene expression; ***(E)*** Western blot for protein expression. *, $p<0.05$ compared to control group.

nephritis model of mouse, and it was negatively correlated with proliferation index of mesangial cells. Bao et al[19] found significantly lower PTEN expression in IgA nephritis renal tissues featured with diffused mesangial cell proliferation and mesangial matrix, and verified the correlation between mesangial cell over-proliferation and PTEN reduction. In this study, we found significantly reduced level of PTEN in rat renal tissues, as similar with Liu et al[13] and Bao et al[19] findings. Takemura et al[20] showed important roles of abnormally elevated Bcl-2 expression in glomerulus in inducing glomerular nephritis[20]. Our work found significantly higher Bcl-2 expression in rat renal tissues compared to that in control group, as similar with Takemura et al[20] results. IL-6 can facilitate mesangial cell proliferation, and is an inflammatory factor closely correlated with glomerular nephritis[21]. Therefore, in this study, we added IL-6 to rat glomerular mesangial cells to mimic *in vivo* inflammatory environment. It has been demonstrated that the elevation of PTEN and inactivation of PI3K/Akt signaling inhibited the proliferation, migration, and invasion of human lung adenocarcinoma cells[22]. Of note, our findings showed that IL-6 treatment significantly decreased PTEN expression in mesangial cells, potentiated AKT phosphorylated activa-

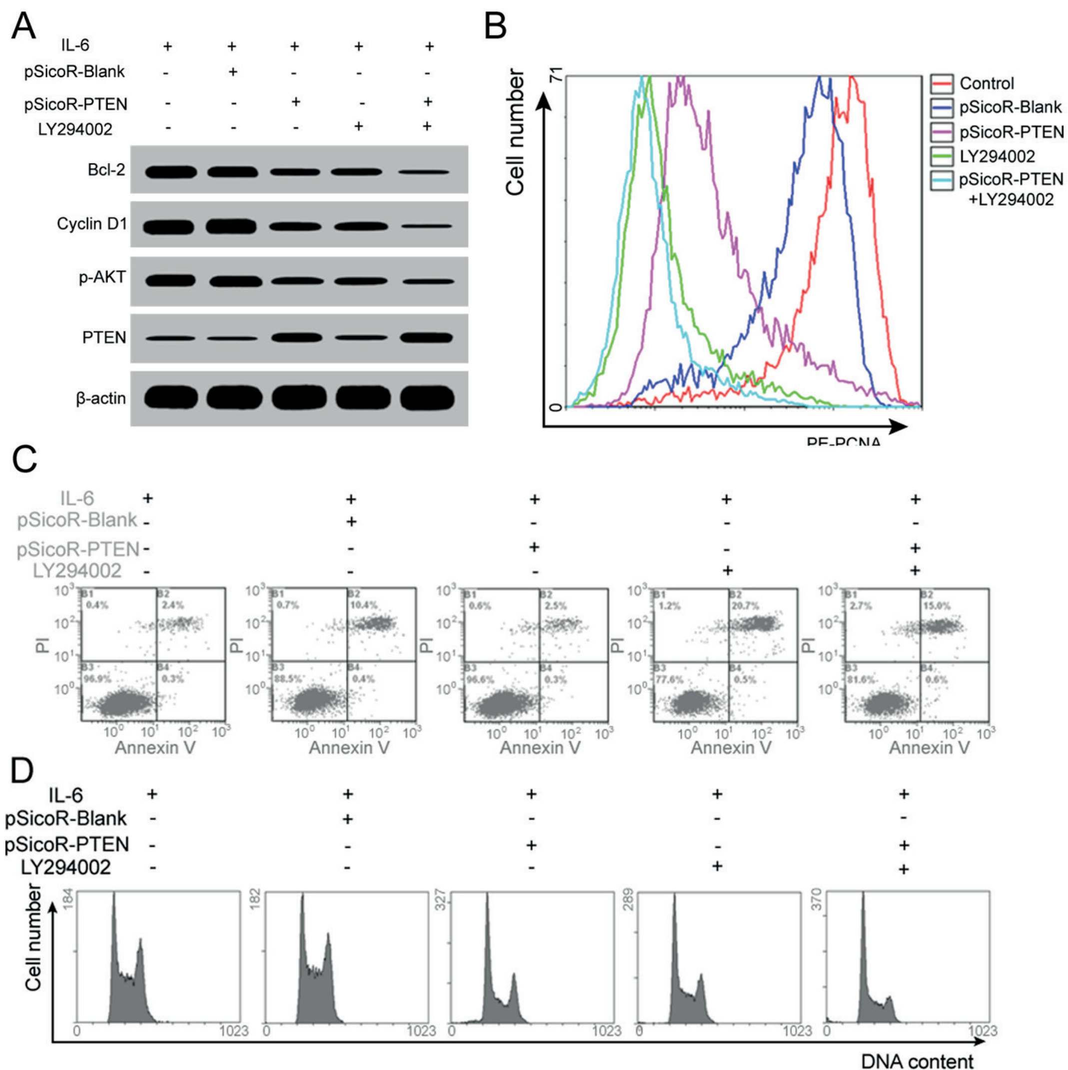

Figure 3. PTEN upregulation in habited AKT activity and suppressed pro-proliferation effects on RMC induced by IL-6. ***(A)*** Western blot for protein expression; ***(B)*** flow cytometry for PCNA expression; ***(C)*** flow cytometry for cell apoptosis; ***(D)*** flow cytometry for cell cycle.

tion and expression of downstream target genes Bcl-2 and Cyclin D1, decreased cell apoptosis, facilitated cell cycle progress and induced cell proliferation. Results demonstrated the role of PTEN on the activity of PI3K/AKT signal pathway and mesangial cell proliferation caused by IL-6. Wang et al[23] suggested that platelet-derived growth factor significantly facilitated glomerular mesangial cell proliferation and inflammatory factor via activating PI3K/AKT signal pathway. Also, this study validated the correlation between IL-6-induced over-proliferation of mesangial cells and PTEN down-regulation and enhancing activity of PI3K/AKT signal pathway. Further assays showed that PTEN over-expression or LY294002 treatment significantly suppressed PI3K/AKT pathway activity, and decreased IL-6 related pro-proliferation effects to induce cell cycle arresting and apoptosis. Feng et al[10] showed that over-expression of PTEN remarkably inhibited PI3K/AKT signal pathway activity, and decreased inflammatory factor HMGB1-induced mesangial cell proliferation. Liu et al[13] showed that up-regulation of PTEN expression significantly weakened proliferation potency of mesangial cells, alleviated renal damage in nephritis model of mice and improved renal function. Wang et al[23] found that small molecule blocker of PI3K/AKT signal pathway remarkably weakened pro-proliferation effects

of PDGF-BB on mesangial cells proliferation, in addition to decrease expression and release of inflammatory mediators such as TNF-α and MCP-1 in mesangial cells. The limitation in this study was that biological effects on *in vivo* glomerular mesangial cells and renal function changes required to be further investigated, although *in vitro* studies for the effects of PTEN on PI3K/AKT pathway activity, proliferation, cycle and apoptosis of glomerular mesangial cells, were elucidated.

Conclusions

The reduction of PTEN up-regulated the activity of PI3K/AKT pathway, facilitated proliferation of glomerular mesangial cells and induced MPGN pathogenesis.

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Conflict of interest
The authors declare no conflicts of interest.

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