Effects of polycystin-1 N-terminal fragment fusion protein on the proliferation and apoptosis of rat mesangial cells

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Abstract. Mesangial proliferative glomerulonephritis (MsPGN) is characterized by widespread mesangial cell proliferation and an accumulation of extracellular matrix (ECM) in the mesangial area. In a previous study we developed a polycystin-1 N-terminal fragment (PC-1 NF) fusion protein that inhibits the proliferation of cyst-lining epithelial cells in autosomal dominant polycystic kidney disease. In addition, the PC-1 NF fusion protein arrests the cell cycle of cancer cells at the G_0/G_1 phase, inhibiting their proliferation. In the present study, the effect of the PC-1 NF fusion protein on MsPGN was investigated. It was found that the PC-1 NF fusion protein inhibited the proliferation of rat mesangial cells and induced G_0/G_1 phase arrest and apoptosis *in vitro*. PC-1 NF fusion protein treatment also resulted in a decrease in mRNA expression levels of proliferating cell nuclear antigen, cyclin D1 and B-cell lymphoma-2 (Bcl-2) and an increase in mRNA expression levels of Bcl-2-associated X protein (Bax) and $p21^{Waf1}$. Furthermore, a decrease in Bcl-2, c-fos, c-jun and protein kinase C-α protein levels was observed, whereas Bax protein levels increased. Additionally, PC-1 NF fusion protein induced ECM degradation and inhibited ECM expansion. The results also demonstrated that PC-1 NF fusion protein treatment resulted in a decrease in type IV collagen and tissue inhibitor of metalloproteinase mRNA levels but an increase in matrix metalloproteinase 2 mRNA levels. In combination, these results suggest that the PC-1 NF fusion protein inhibits proliferation, promotes apoptosis and induces ECM degradation in MsPGN rats. This study offers novel perspectives for the treatment of MsPGN.

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Introduction

Mesangial proliferative glomerulonephritis (MsPGN) is the most common form of primary glomerular disease, and it is characterized by mesangial cell proliferation and activation and extracellular matrix (ECM) expansion in the mesangial area (1). However, despite the severity and incidence of MsPGN, there is a lack of effective therapies.

Polycystin-1 (PC-1) is encoded by the polycystic kidney disease gene 1 (PKD1) (2). Structural and functional abnormalities in PC-1 account for 85-90% of all cases of autosomal dominant polycystic kidney disease (ADPKD) (3). In previous studies we developed a novel PC-1 N-terminal fragment (PC-1 NF) fusion protein, which encodes part of the leucine-rich repeat (LRR) and the cell wall integrity and stress response component (CWI&SRC) domains of the PC-1 extracellular region, and we found that this fusion protein may inhibit the proliferation of ADPKD cyst-lining epithelial cells (4,5). A previous study by Zheng *et al* (6) demonstrated that, when transfected with PC-1, the cancer cell lines HepG2, A549 and SW480 exhibited a significant increase in the levels of apoptosis compared with the respective control cell lines. This increase in apoptosis was accompanied by cell cycle arrest at the G_0/G_1 phase (6). An excessive proliferation of ADPKD cyst-lining epithelial, cancer and mesangial cells is characteristic of ADPKD, cancer and MsPGN, respectively. It was found that the PC-1 NF fusion protein inhibits the progression of ADPKD cyst-lining epithelial cells and cancer cells; however, little is known about the role of the PC-1 NF fusion protein in mesangial cells in MsPGN. Therefore, in this study the role of the PC-1 NF fusion protein in MsPGN was investigated.

Materials and methods

Materials. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Dulbecco's modified Eagle's medium (DMEM)/F12 medium, TRIzol®, SuperScript® II Reverse Transcriptase and RNAsin®

were obtained from Gibco®-BRL (Carlsbad, CA, USA). Fetal bovine serum was obtained from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). The BrdU-ELISA kit was obtained from Roche Diagnostics (Indianapolis, IN, USA) and the fluorescence quantitative polymerase chain reaction (qPCR) detection kit was obtained from Takara Bio, Inc. (Shiga, Japan). The ELISA regimen of collagen IV was obtained from the Cell Bank at the Chinese Academy of Science (Shanghai, China). Monoclonal rat anti-GAPDH antibody was obtained from Ambion® (Carlsbad, CA, USA). The secondary antibody was purchased from Beijing Zhongshan Corp. (Beijing, China). Polyclonal rabbit anti-protein kinase Cα (PKCα), -Bcl-2-associated X protein (Bax) and -B-cell lymphoma-2 (Bcl-2) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Enhanced chemiluminescence detection reagents and [γ-^{32}P]ATP were obtained from Amersham Pharmacia Biotech (Braunschweig, Germany). Diethylpyrocarbonate and other reagents were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany).

Immunohistochemistry. To determine the role of the PC-1 NF fusion protein in MsPGN, the distribution of PC-1 in normal and IgA nephropathy (Haas II and III class) renal tissues was investigated. The study was approved by the Ethics Committee of Zhongshan Hospital, Xiamen University, Xiamen, China and consent was obtained from patients. Tissue sections were dewaxed and the endogenous peroxidases were inactivated. The sections were then immersed in 0.01 M citrate buffer (pH 6.0), heated in a microwave oven to retrieve antigenicity and blocked for non-specific binding using 5% bovine serum albumin in phosphate-buffered saline (PBS) for 20 min. Blocked sections were incubated with mouse anti-PC-1 monoclonal antibody at 4˚C overnight. Following washing with PBS, the sections were treated with horseradish peroxidase-conjugated goat anti-mouse IgG at 37˚C for 20 min. Secondary antibody incubation was followed by color development using 3,3'-diaminobenzidine and counterstaining, as described above. Incubation with an irrelevant non-immune mouse IgG primary antibody served as the negative control.

Cell culture. The rat mesangial cell (RMC) line was established in the Division of Nephrology, Center of Kidney Disease, Changzheng Hospital, Second Military Medical University, Shanghai, China (7). The cells were cultured in DMEM supplemented with 10% fetal bovine serum/F12 medium at 37˚C with 5% CO_2. The cells were treated with PC-1 NF fusion protein at various concentrations (0, 0.5, 1, 2 and 4 μg/ml) in media for 24, 48, 72 and 96 h.

Cell cycle analysis. The cell cycle phase was examined using fluorescence-activated cell sorting (FACS) analysis. The cells were trypsinized, washed with PBS and fixed in 70% pre-cooled ethanol overnight at -20˚C. Cells were then washed twice with PBS and stained with propidium iodide for FACS analysis in the dark. The FACS data were analyzed using Multicycle AV DNA Content and Cell Cycle Analysis software (De Novo Software, San Diego, CA, USA). The proliferation index (PI) was determined using the following equation: PI (%) = (S+G2/M)/(G0/G1+S+G2/M)x100.

qPCR. The effect of the PC-1 NF fusion protein on the proliferation, cell cycle and apoptosis of RMCs, as well as changes in the mRNA levels of proliferating cell nuclear antigen (PCNA), cyclin D1, p21^{Waf1}, Bax, Bcl-2, matrix metalloproteinase 2 (MMP2) and tissue inhibitor of metalloproteinase 1 (TIMP1) were analyzed using qPCR. Total RNA was extracted from cells in the logarithmic growth phase, and the cells were treated with 4 μg/ml PC-1 NF fusion protein for 48 h using TRIzol. Genomic DNA contaminants were removed by digestion with DNase (Takara Bio, Inc.). Briefly, cDNA was synthesized from a total of 2 μg RNA in a 20-μl reaction mixture that included 20 units RNAsin, 100 units SuperScript II Reverse Transcriptase, 4 μl 5X 1st strand buffer, 0.5 μmol/l primers and 1 μl 10 mmol/l deoxynucleotide triphosphate. The mixture was incubated at 25˚C for 10 min, 42˚C for 1 h and 52˚C for 15 min. The reverse transcriptase was inactivated by incubation at 70˚C for 15 min. The primer sequences used for the qPCR are shown in Table I. qPCR was performed in a 25-μl reaction mixture [1 μl cDNA, 2.5 μl 10^{-3} buffer, 0.3 μl 250 mM Mg^{2+}, 0.3 μl 25 mM dAGCU, 1 μl 10 μmol/primers, 1 μl 10^{-3}X calibration buffer, 0.8 μl 25X SYBR Green (Bio-Rad, Hercules, CA, USA), 1.25 units Taq polymerase and 0.4 units uracil-N-glycosylase]. qPCR was performed under the following cycle conditions: Predenaturation at 95˚C for 90 sec; five cycles of denaturation at 95˚C for 5 sec, annealing at 55˚C for 15 sec, extension at 72˚C for 20 sec; 35 additional denaturing cycles at 95˚C for 5 sec; annealing at 60˚C for 30 sec to reach the fluorescent signal detection point; another 40 cycles of denaturing at 95˚C for 1 min and finally annealing at 55˚C for 1 min (increasing 0.5˚C/cycle every 10 sec). The target gene expression was calculated from the respective standard curves, and quantitative expression was normalized to GAPDH using an iCycler Thermal Cycler (Bio-Rad).

Analysis of apoptosis. Following treatment with PC-1 NF fusion protein for 48 h, the cells were harvested by trypsin digestion, fixed in 4% paraformaldehyde and 2% glutaraldehyde for 4 h and then washed in PBS. Cells were clotted by the addition of plasma and 1% osmium acid for 2-3 h, rinsed with PBS for 30 min and embedded in Epon 812 at 37˚C overnight. Ultrathin sections were stained with 4% uranyl acetate for 30 min. The sections were then washed and subjected to lead citrate staining for 5-10 min, prior to further washing and drying. Ultrastructural changes in the epithelia were observed using a transmission electron microscope (Hitachi H800, Hitachi, Tokyo, Japan). Cell preparation and analysis methods for the FACS apoptosis index (AI) analysis were the same as those employed for the cell cycle analysis with the exception that the samples included suspension cells in the media.

Western blot analysis. Following individual incubations, as described in the cell culture section, the medium was removed and the cells were scraped into ice-cold PBS supplemented with 1 mM sodium vanadate, 1 mM sodium fluoride and 1 mM phenylmethylsulfonyl fluoride. The cells were centrifuged at 700 x g for 10 min at 4˚C and resuspended in 200 ml lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl

Table I. Quantitative polymerase chain reaction primers.

Name	Sequence	Length (bp)
PCNA	Forward: 5'-CAT GGG CGT GAA CCT CAC-3' Reverse: 5'-CAC AGC TGT ACT CCT GTT CTG G-3'	206
Cyclin D1	Forward: 5'-GCT GGC CAT GAA CTA CCT G-3' Reverse: 5'-GCC TCT GGC ATT TTG GAG-3'	275
$P21^{Waf1}$	Forward: 5'-CCG ATC CTG GTG ATG TCC G-3' Reverse: 5'-TCC GAA CAC GCT CCC AGA C-3'	201
Type IV collagen	Forward: 5'-GCC CTA CGT TAG CAG ATG TAC C-3' Reverse: 5'-TAT AAA TGG ACT GGC TCG GAA T-3'	217
MMP2	Forward: 5'-TTC AAC GGT CGG GAA TAC A-3' Reverse: 5'-GGA AGC GGA ACG GAA ACT-3'	188
TIMP1	Forward: 5'-AAG TCC CAG AAC CGC AGC-3' Reverse: 5'-TCC AGT TTG CAR GGG ATG G-3'	197
Bax	Forward: 5'-GCT GAT GGC AAC TTC AAC TG-3' Reverse: 5'-CAG CCA CAA AGA TGG TCA CT-3'	235
Bcl-2	Forward: 5'-GTG GAG GAA CTC TTC AGG GAT-3' Reverse: 5'-CAG CCA GGA GAA ATC AAA CAG-3'	246
GAPDH	Forward: 5'-TGC TGA GTA TGT CGT GGA GTC-3' Reverse: 5'-TGC TGA CAA TCT TGA GGG AG-3'	173

PCNA, proliferating cell nuclear antigen; MMP2, matrix metalloproteinase 2; TIMP1, tissue inhibitor of metalloproteinase 1; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2.

fluoride, 1 mM sodium vanadate and 1 mM sodium fluoride; pH 8.0). Cell debris was sonicated using a Branson sonifier® (10 sec; duty cycle, 100%; output control, 1) and centrifuged (14,000 x g, 15 min), and the protein content was measured. To ensure equal protein loading, 150 mg protein was mixed with the same volume of SDS sample buffer (125 mM Tris HCl, 2% SDS, 10% glycerin, 1 mM dithiothreitol and 0.002% bromophenol blue; pH 6.9) and boiled for 5 min. Proteins were resolved on 10% SDS-polyacrylamide gels and were blotted onto nitrocellulose membranes. The membranes were blocked using 10 mM Tris (pH 7.5), 100 mM NaCl and 0.1% Tween-20 (TBST) containing 5% non-fat dry milk for 1 h at room temperature. The membranes were then incubated with polyclonal rabbit anti-PKCα, -Bax and -Bcl-2 antibodies and monoclonal rat anti-GAPDH antibody (1:2,000) at 4˚C overnight. The membranes were subjected to three 15-min TBST washes prior to being incubated at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,500). Following further washes with TBST, signals were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Amersham, UK).

ELISA. The concentration of type IV collagen in the medium was measured using an ELISA kit in accordance with the manufacturer's instructions. The absorbance at 492 nm was determined using a microplate reader (Wellscan MK3, Labsystems Dragon, Helsinki, Finland).

Statistical analysis. The data are presented as the mean ± standard deviation. Comparisons between results from different groups were performed using a Student's t-test or a one-way analysis of variance, as appropriate. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using the SPSS 18.0 software package (SPSS, Inc., Chicago, IL, USA).

Results

Distribution and expression level of PC-1 in renal tissues. PC-1 protein was expressed predominantly in the medullary and cortical collecting ducts and the distal convoluted tubules in the normal adult and IgA nephropathy kidney tissues, which is consistent with the expression pattern observed in rats in the preliminary experiment. Although only weak expression of PC-1 was observed in the glomerular area, the difference was great among the individual subjects. As shown in Fig. 1, positive staining appeared as brown granules throughout the cytoplasm. The amount of positive staining observed in normal renal tissue was greater than that in the IgA nephropathy (Haas III) renal tissue.

PC-1 NF fusion protein inhibits the proliferation of RMCs and affects the proliferation-related mRNA expression levels of PCNA. The results indicated that proliferation was progressively inhibited by treatment with increasing concentrations of PC-1 NF fusion protein. Treatment with 4 μg/ml PC-1 NF fusion protein for 24 h effectively inhibited the proliferation of RMCs ($P<0.01$). This inhibitory effect was time-dependent and reached a peak at 96 h of treatment (Table II). In addition, treatment with 4 μg/ml PC-1 NF fusion protein for

Table II. Concentration- and time-dependent inhibition of rat mesangial cell proliferation by the PC-1 NF fusion protein.

PC-1 NF fusion protein concentration (μg/ml)	Proliferation rate (%)			
	24 h	48 h	72 h	96 h
0.0	29.0±2.0	36.0±2.0	58.0±3.0	106.0±12.0
0.5	28.0±3.0	33.0±5.0	53.0±3.0	89.0±4.0[a,c]
1.0	27.0±3.0	32.0±4.0	49.0±4.0	81.0±4.0[b,c]
2.0	26.0±4.0	28.0±4.0	42.0±3.0	65.0±5.0[b,c]
4.0	21.0±2.0[b]	23.0±2.0[b,c]	24.0±4.0[b,c]	41.0±5.0[b,c]

After 96 h, PC-1 NF fusion protein treatment at 0.5 μg/ml inhibited proliferation relative to treatment with PC-1 NF fusion protein-free medium ([a]P<0.05). PC-1 NF fusion protein treatment at 4 μg/ml inhibited proliferation relative to treatment with PC-1 NF fusion protein-free medium after 24 h ([b]P<0.05), 48 h ([b]P<0.01), 72 h ([b]P<0.01) and 96 h ([b]P<0.01); the effect at 48 h was greater than that at 24 h ([c]P<0.01). At 96 h, all concentrations of the PC-1 NF fusion protein inhibited proliferation relative to PC-1 NF fusion protein-free medium ([b]P<0.01) and relative to the proliferation observed at 24, 48 and 72 h with the same concentrations of PC-1 NF ([c]P<0.01). PC-1 NF, polycystin-1 N-terminal fragment.

Table III. Effect of PC-1 NF fusion protein treatment (48 h) on cell cycle rate and PIs of rat mesangial cells.

PC-1NF fusion protein concentration (μg/ml)	Cell cycle rate (%)			PI (%)
	G_0-G_1	S	G_2-M	
0.0	27.1±2.1	44.1±6.7	28.8±2.0	72.9
1.0	31.2±3.4	41.7±2.8	27.1±3.0	68.8
2.0	36.5±1.6[a]	36.6±1.8[a]	26.8±2.1	63.4
4.0	42.0±2.3[b]	31.6±4.5[b]	26.4±1.8	58.0

[a]P<0.05, [b]P<0.01 vs. control group. PC-1 NF, polycystin-1 N-terminal fragment; PI, proliferation index.

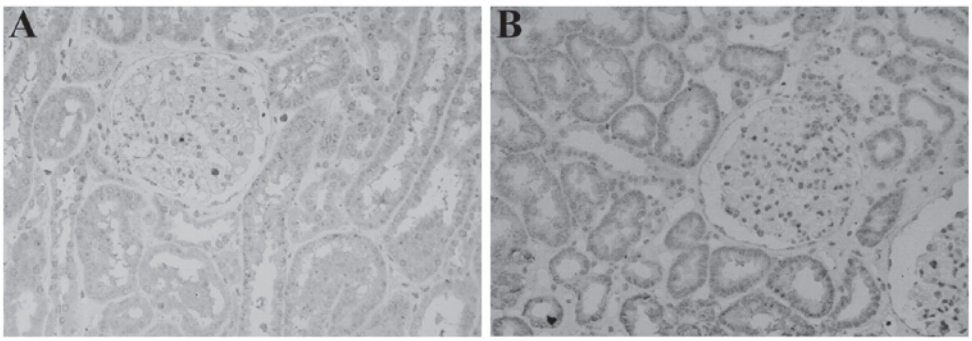

Figure 1. Distribution of PC-1 (magnification, x200). Distribution of PC-1 in (A) normal renal tissue and (B) immunoglobulin A nephropathy renal tissue (Haas III class). PC-1, polycystin-1.

48 h decreased the mRNA levels of PCNA in RMCs, from (8.26±1.01)x10^3 to (3.58±1.16)x10^3 copies per million GAPDH (P<0.01).

PC-1 NF fusion protein inhibits cell cycle progression of RMCs and affects the mRNA levels of cell cycle regulatory genes. PC-1 NF fusion protein treatment for 48 h reduced the number of RMCs in S and G_2/M phases, resulting in the cells remaining at G_0/G_1 phases in a concentration-dependent manner. Similarly, the PI gradually decreased with increasing concentrations of PC-1 NF fusion protein (Table III). Furthermore, qPCR and immunocytochemical analysis revealed that treatment with 4 μg/ml PC-1 NF fusion protein for 48 h decreased the level of cyclin D1 (Fig. 2A), whereas the $p21^{Waf1}$ levels increased with PC-1 NF fusion protein treatment (Fig. 2B and Table IV). Additionally, the expression of c-fos and c-jun decreased (Fig. 2C and D). The expression of PKCα also gradually decreased following treatment with increasing concentrations of PC-1 NF fusion protein (Fig. 2E).

PC-1 NF fusion protein induces the apoptosis of RMCs and affects the levels of apoptosis regulatory genes and proteins. Following treatment with 2 μg/ml PC-1 NF fusion

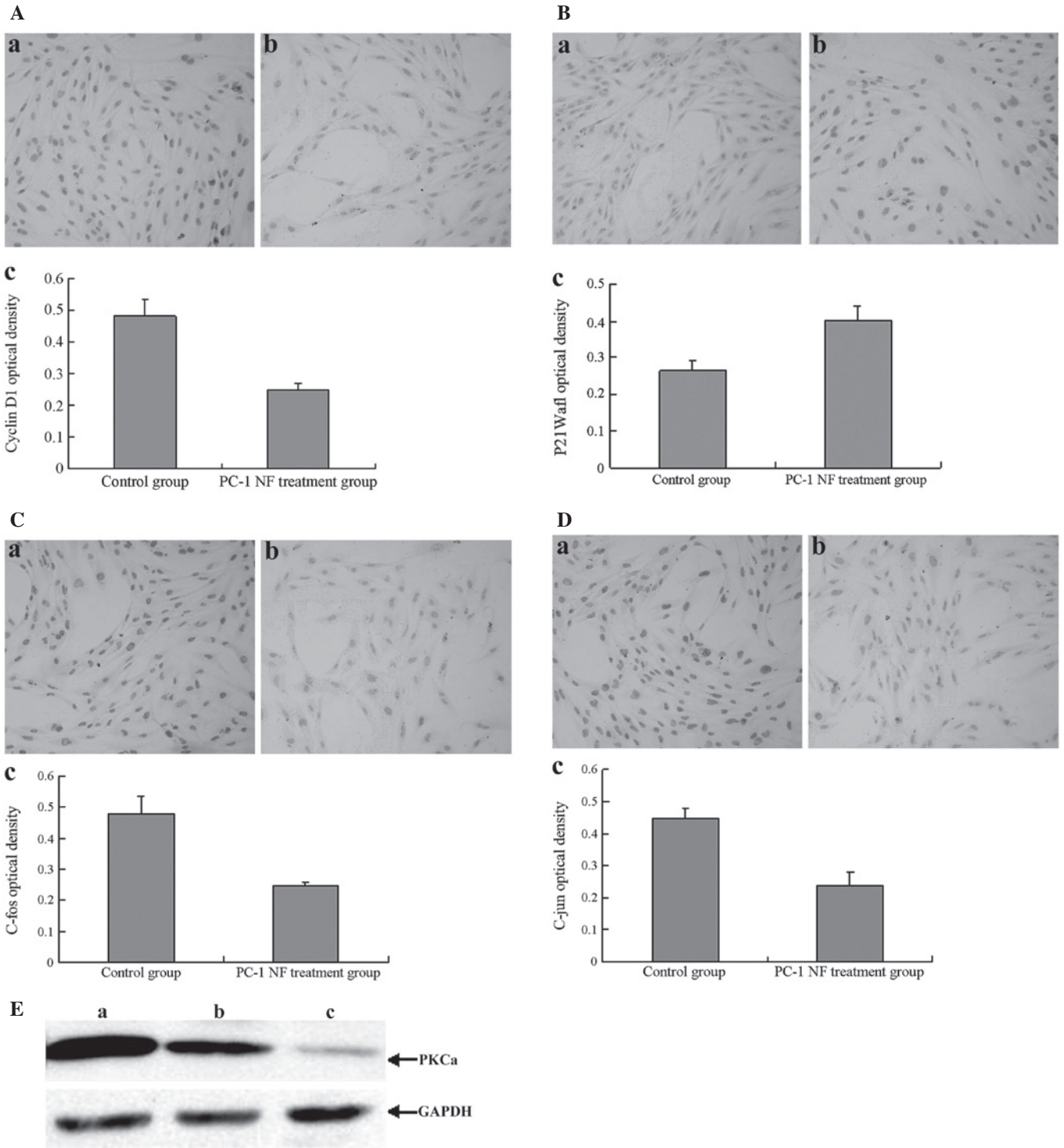

Figure 2. Effect of PC-1 NF fusion protein treatment (48 h) on the expression of cyclin D1, $P21^{Waf1}$, c-jun, c-fos and PKCα genes in RMCs. (A) Expression of cyclin D1 in RMCs treated with (a) 0 μg/ml and (b) 4 μg/ml PC-1 NF (magnification, x200). (c) Quantitative analysis of the expression of cyclin D1 in RMCs. (B) Expression of $P21^{Waf1}$ in RMCs treated with (a) 0 μg/ml and (b) 4 μg/ml PC-1 NF (magnification, x200). (c) Quantitative analysis of the expression of $P21^{Waf1}$ in RMCs. (C) Expression of c-jun in RMCs treated with (a) 0 μg/ml and (b) 4 μg/ml PC-1 NF (magnification, x200). (c) Quantitative analysis the expression of c-jun in RMCs. (D) Expression of c-fos in RMCs treated with (a) 0 μg/ml and (b) 4 μg/ml PC-1 NF (magnification, x200). (c) Quantitative analysis the expression of c-fos in RMCs. (E) Western blot analysis of PKCα in RMCs. Culture medium alone in a flask served as the control. GAPDH was used as an internal control to analyze the relative level of PKCα. (a-c) RMCs treated with (a) 0, (b) 2 and (c) 4 μg/ml PC-1 NF fusion protein. The PKCα to GAPDH optical density ratios were 96.67% (29:30), 64.29% (18:28) and 20.69% (6:29), respectively. PC-1 NF, polycystin-1 N-terminal fragment; RMC, rat mesangial cell; PKCα, protein kinase Cα.

protein for 48 h, a number of previously irregularly shaped cells became elliptical or rounded, and a number of cells were suspended with the intact cellular membranes from adherent cells. Examination by electron microscopy revealed typical apoptotic changes following PC-1 NF fusion protein treatment, including the emergence of numerous cytoplasmic vacuoles and fissures. Additionally, the nuclei were observed to have become irregular and to contain concentrated skirted

Table IV. Effect of PC-1 NF fusion protein treatment (4 μg/ml for 48 h) on the expression of cell cycle regulatory genes in rat mesangial cells.

	mRNA levels (copies/million GAPDH)	
Group	Cyclin D1	$p21^{Waf1}$
Control	$(4.10\pm0.32)\times10^{4}$	$(0.85\pm0.18)\times10^{2}$
PC-1 NF-treatment	$(2.44\pm0.27)\times10^{4a}$	$(3.73\pm0.46)\times10^{2b}$

[a]P<0.05, [b]P<0.01 vs. control group. PC-1 NF, polycystin-1 N-terminal fragment.

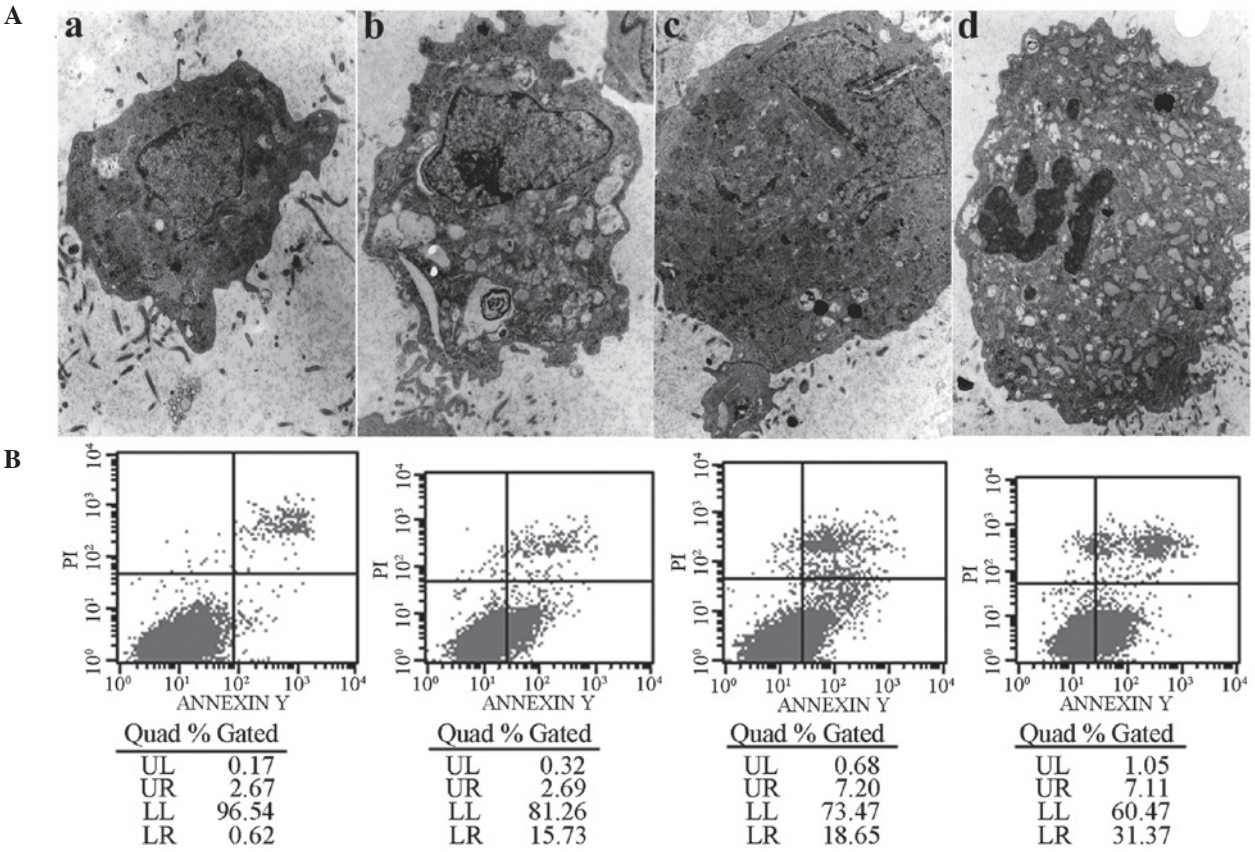

Figure 3. Changes in PC-1 NF fusion protein-treated RMCs. (A) Ultrastructural changes of PC-1 NF fusion protein-treated RMCs: (a) Normal structure of control cells; (b) numerous cytoplasmic vacuoles and fissures; (c) concentrated and skirted chromatin is apparent and typical apoptotic bodies are visible in the cytoplasm; (d) concentrated and skirted chromatin is visible and nuclear fragmentations are apparent. (B) Rate of apoptosis in RMCs treated with various concentrations of the PC-1 NF fusion protein. The rate of apoptosis in RMCs increased gradually with increasing concentrations of PC-1 NF fusion protein. After 48 h of PC-1 NF fusion protein treatment, the rates of apoptosis at 0, 1, 2 and 4 μg/ml were 0.62, 15.73, 18.65 and 31.37%, respectively. PC-1 NF, polycystin-1 N-terminal fragment; RMCs, rat mesangial cells; PI, propidium iodide.

chromatin and, in certain cells, typical apoptotic bodies were observed (Fig. 3). AIs were detected following treatment with 1, 2 and 4 μg/ml PC-1 NF fusion protein for 48 h and the corresponding AIs were 15.73, 18.65 and 31.37%, respectively. The protein expression of the apoptosis-related genes Bax and Bcl-2 was measured. The expression of Bax increased, whilst that of Bcl-2 decreased following treatment with 4 μg/ml PC-1 NF fusion protein for 48 h, which is consistent with the mRNA expression levels (Fig. 4).

PC-1 NF fusion protein induces ECM degradation in RMCs through increasing the ratio of MMP2 to TIMP1 gene expression. After 48 h of treatment with 0, 1, 2 and 4 μg/ml PC-1 NF fusion protein, the concentration of type IV collagen in the supernatant was 68.62±8.11, 57.16±7.88, 49.12±6.38 and 36.76±4.49 pg/μg, respectively (Fig. 5). The TIMP1 mRNA level was significantly decreased; however MMP2 mRNA expression levels increased significantly (Fig. 5).

Discussion

MsPGN is characterized by widespread mesangial cell proliferation and the accumulation of ECM in the mesangial area. A recent study demonstrated that the highly proliferative mesangial

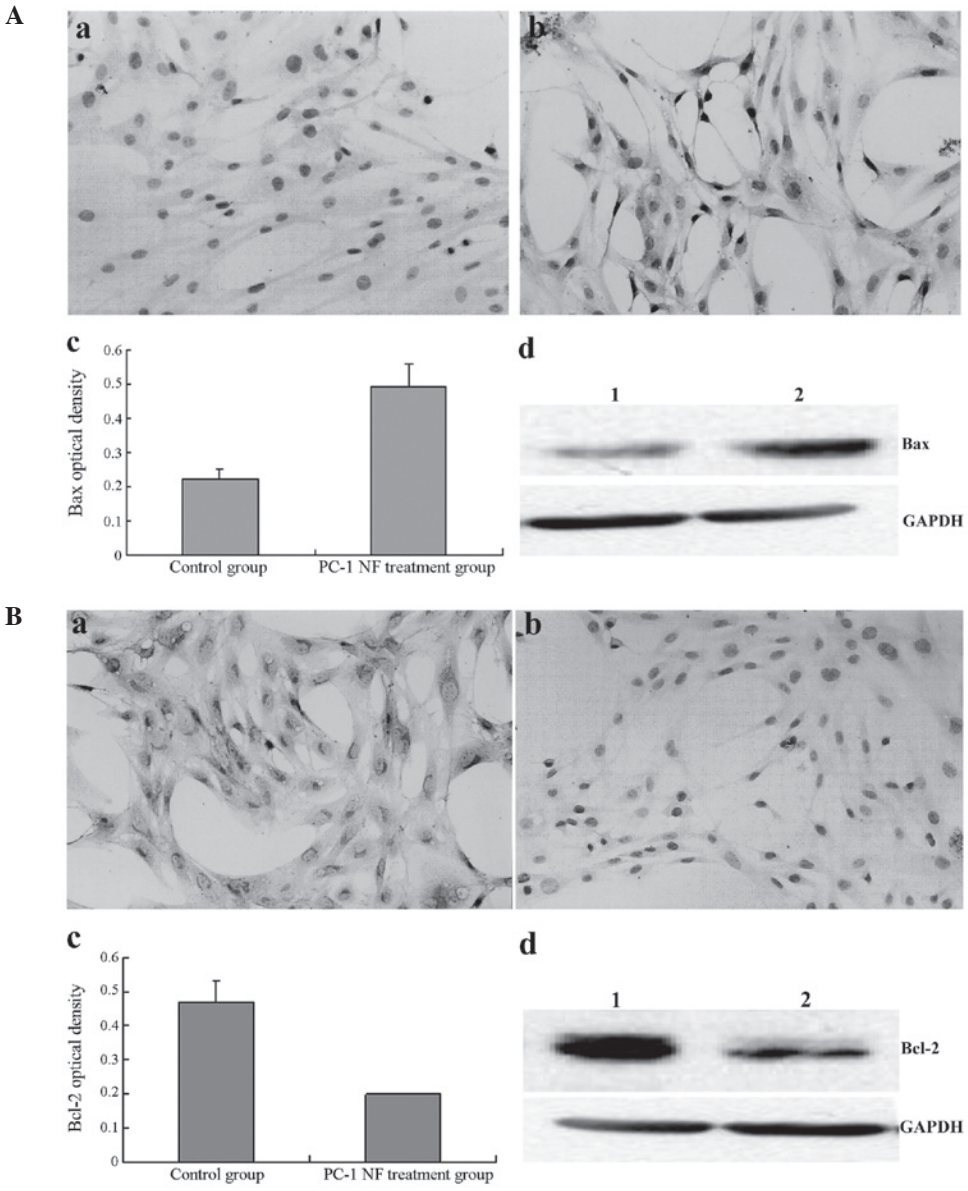

Figure 4. Effects of PC-1 NF fusion protein treatment (48 h) on the expression of Bax and Bcl-2 protein in RMCs. (A) Expression of the Bax protein in RMCs treated with (a) 0 and (b) 4 μg/ml PC-1 NF (magnification, x200). (c) Quantitative analysis of the expression of Bax in RMCs. (d) Western blot analysis of Bax expression in RMCs treated with (Lane 1) 0 and (Lane 2) 4 μg/ml PC-1 NF. The optical density ratio values are presented beneath each band. (B) Expression of Bcl-2 protein in RMCs treated with (a) 0 and (b) 4 μg/ml PC-1 NF (magnification, x200). (c) Quantitative analysis of the expression of Bcl-2 in RMCs. (d) Western blot analysis of Bcl-2 expression in RMCs treated with (Lane 1) 0 and (Lane 2) 4 μg/ml PC-1 NF. PC-1 NF, polycystin-1 N-terminal fragment; RMC, rat mesangial cell; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein.

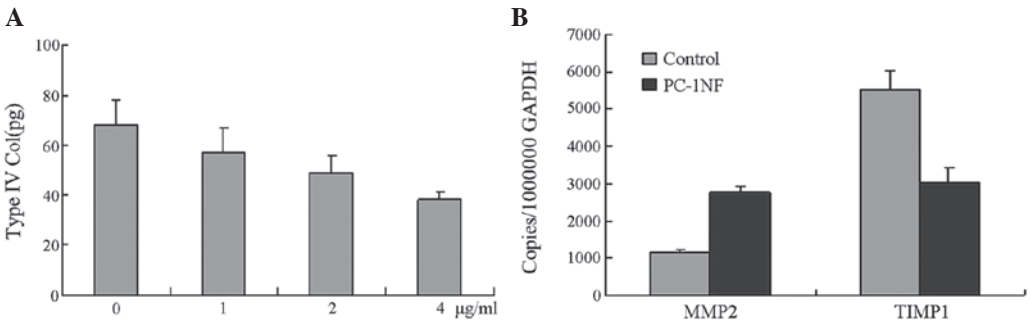

Figure 5. PC-1 NF fusion protein induces extracellular matrix degradation in RMCs and increases the ratio of MMP2 to TIMP1 gene expression. (A) Inhibition of type IV collagen in RMCs treated with various concentrations of PC-1 NF fusion protein. The inhibition of type IV collagen increased gradually with increasing concentrations of PC-1 NF fusion protein. Following incubation with 0, 1, 2 and 4 μg/ml PC-1 NF fusion protein for 48 h, the concentration of type IV collagen in the supernatants was 68.62±8.11, 57.16±7.88, 49.12±6.38 and 36.76±4.49 pg/μg, respectively. (B) Effect of PC-1 NF fusion protein treatment (4 μg/ml for 48 h) on the expression of MMP2 and TIMP1 genes in RMCs. PC-1 NF, polycystin-1 N-terminal fragment; RMC, rat mesangial cell; MMP2, matrix metalloproteinase 2; TIMP1, tissue inhibitor of metalloproteinase 1.

cells in MsPGN rats aggravate disease progression (8). The antiproliferative activity of PC-1 has been demonstrated in Madin Darby canine kidney cell lines (9). In a previous study, we developed a novel PC-1 NF fusion protein that encodes part of the LRR and CWI&SRC domains of the PC-1 extracellular region. We demonstrated that this fusion protein may inhibit the proliferation of ADPKD cyst-lining epithelial cells (4,5). PC-1 may have a role in protecting against IgA nephropathy, which is characterized by mesangial proliferation. In the present study it was demonstrated *in vitro* that treatment with exogenous PC-1 NF fusion protein reduces the proliferation of RMCs in a concentration- and time-dependent manner. It was found that microgram concentrations of PC-1 NF fusion protein also markedly reduce PCNA mRNA expression. PCNA is a nuclear protein that is expressed specifically during cell proliferation. The expression of PCNA begins at the end of G_1 phase, reaches a peak during S phase and early G_2 phase and ceases during M and G_0 phases (10). These findings suggested that the PC-1 NF fusion protein inhibits mesangial cell proliferation in MsPGN.

The cell cycle is the final pathway for cell proliferation and has several checkpoints. The G_1-S checkpoint is key since this is the stage at which cells integrate and converge signals to determine if they should initiate cell division, enter into a resting state (G_0 phase) or undergo apoptosis. PC-1 NF fusion protein treatment increased the number of cells at the G_0/G_1 phase and correspondingly decreased the number of cells at the S phase, indicating that the PC-1 NF fusion protein inhibition of cell proliferation may be mediated through a regulatory effect at the G_1 phase.

Cyclin D1, encoded by the ClnD1 gene, is involved in G_1-phase regulation. Cyclin D1 controls cyclin-dependent kinase (CDK) activity by phosphorylation and enhances the expression of a number of genes that promote the passage of cells through the G_1-S checkpoint and, thus, the commencement of cell division (11). The first CDK inhibitor identified in mammalian cells, $p21^{Waf1}$, suppresses the activity of CDK or the cyclin D1-CDK complex through dephosphorylation and therefore inhibits cell proliferation (12). PKD1 activates the Janus kinase/ signal transducers and activators of transcription pathway, thereby upregulating $p21^{Waf1}$ and inducing cell cycle arrest in G_0/G_1. An increase in the expression of $p21^{Waf1}$ has been shown to be primarily responsible for mediating the growth-suppressing effects of PKD1 in an experimental system (13).

C-fos and c-jun are immediate-early genes that are associated with proliferation. The proteins encoded by these two genes, which are located in the nucleus, combine with specific DNA sequences and activate transcription (14). The expression of c-fos and c-jun is associated with cell proliferation (15). PKCα activates the transcription factor activator protein-1, leading to the upregulation of c-jun and c-fos expression to promote cell growth (16). It has been reported that, when increased PKCα expression is observed, there is an increase in cell proliferation (17).

PC-1 NF fusion protein treatment decreased cyclin D1 mRNA levels but increased $p21^{Waf1}$ mRNA levels in RMCs. Additionally, PC-1 NF fusion protein treatment resulted in decreased c-jun, c-fos and PKCα protein expression levels. These results suggest that the PC-1 NF fusion protein regulates the cell cycle in RMCs by inhibiting cyclin D1 expression and increasing $p21^{Waf1}$ expression to modulate the PKCα signaling pathway. Using this mechanism, the PC-1 NF fusion protein may prevent RMCs from entering into S phase and preserve the cells in a resting state.

Mesangial cell apoptosis and proliferation are dysregulated in MsPGN (18). Dysregulation of the balance between pro- and anti-apoptotic Bcl-2 family members correlates with increased apoptosis in MsPGN (19). The ratio of Bcl-2-Bax heterodimers to Bax-Bax homodimers is a critical factor for determining susceptibility to apoptosis (20). Therefore, the regulation of Bcl-2 and Bax expression may be a key mechanism underlying the PC-1 NF fusion protein-mediated induction of apoptosis in RMCs.

It is likely that decreased degradation of ECM is an important factor in the pathogenesis of MsPGN (21). MMPs are zinc-dependent metalloendopeptidases that belong to the collagenase supergene family. Primarily based on substrate specificity, they are classified into several groups (22). MMP2 has a crucial role in the degradation of collagen IV (23). MMP activity is regulated by natural inhibitors, predominantly the TIMPs (TIMP-1 to -4) (24). TIMP1 is the most important category of enzymes in ECM degradation and has an important role in the dynamic balance between the synthesis and degradation of the ECM, promoting tissue repair and remodeling following pathophysiological events (24,25). The regulation of ECM metabolism is likely the main function of these proteolytic enzymes and their inhibitors. Following PC-1 NF fusion protein treatment, the level of MMP2 was upregulated, whereas the TIMP1 level was downregulated. This indicates that the PC-1 NF fusion protein induces ECM degradation in RMCs by increasing the ratio of MMP2 to TIMP1 gene expression. In addition, the reduction of the ECM may contribute to the lower proliferation and higher apoptosis rate in RMCs.

In conclusion, PC-1 NF fusion protein has been shown to reduce proliferation and induce apoptosis in RMCs. The antiproliferative effect may be mediated by the modulation of the PKCα signal pathway, the inhibition of cyclin D1 expression and the stimulation of $p21^{Waf1}$ expression, thereby preventing the passage of cells through the G_1-S checkpoint. The PC-1 NF fusion protein may also induce the apoptosis of mesangial cells by decreasing the ratio of Bcl-2 to Bax gene expression. Furthermore, the PC-1 NF fusion protein induced ECM degradation by increasing the ratio of MMP2 to TIMP1 gene expression in RMCs. The strategy of inducing cell cycle arrest and subsequent apoptosis may contribute to the development of novel perspectives for the treatment of MsPGN. The PC-1 NF fusion protein may provide a protective effect and may lead to the development of a novel therapeutic drug for the treatment of MsPGN.

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References

1. Sethi S and Fervenza FC: Membranoproliferative glomerulonephritis: pathogenetic heterogeneity and proposal for a new classification. Semin Nephrol 31: 341-348, 2011.

2. Chapin HC and Caplan MJ: The cell biology of polycystic kidney disease. J Cell Biol 191: 701-710, 2010.
3. Harris PC and Torres VE: Polycystic kidney disease. Annu Rev Med 60: 321-337, 2009.
4. Zhao HD, Mei CL, Li L, *et al*: Preparation and characterization of monoclonal antibody against N-terminal domain of polycystin 1. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 20: 186-190, 2004 (In Chinese).
5. Zhao HD, Sun TM, Wang WJ, *et al*: Evaluation of polycystin-1 N-terminal peptide on the proliferation and apoptosis of cystic lining epithilial cells in human ADPKD. Chin J Nephrol 21: 664-668, 2005 (In Chinese).
6. Zheng R, Zhang Z, Lv X, *et al*: Polycystin-1 induced apoptosis and cell cycle arrest in G0/G1 phase in cancer cells. Cell Biol Int 32: 427-435, 2008.
7. Mei CL, Zhang LM and Chen L: The establishment of rat mesangial cell line. Chin J Nephrol Dial Transplant 5: 90-92, 1996 (In Chinese).
8. Suana AJ, Tuffin G, Frey BM, *et al*: Single application of low dose mycophenolate-mofetil-OX7-immunoliposomes ameliorates experimental mesangial proliferative glomerulonephritis. J Pharmacol Exp Ther 337: 411-422, 2011.
9. Boletta A, Qian F, Onuchic LF, *et al*: Polycystin-1, the gene product of PKD1, induces resistance to apoptosis and spontaneous tubulogenesis in MDCK cells. Mol Cell 6: 1267-1273, 2000.
10. Takahashi H, Oishi Y, Chuang SS and Morii S: Effects of tissue fixation and processing on proliferating cell nuclear antigen (PCNA) immunohistochemistry. Acta Pathol Jpn 42: 621-623, 1992.
11. Wang J, Wang Q, Cui Y, *et al*: Knockdown of cyclin D1 inhibits proliferation, induces apoptosis, and attenuates the invasive capacity of human glioblastoma cells. J Neurooncol 106: 473-484, 2012.
12. Wei J, Zhao J, Long M, *et al*: p21WAF1/CIP1 gene transcriptional activation exerts cell growth inhibition and enhances chemosensitivity to cisplatin in lung carcinoma cell. BMC Cancer 10: 632, 2010.
13. Bhunia AK, Piontek K, Boletta A, *et al*: PKD1 induces p21(waf1) and regulation of the cell cycle via direct activation of the JAK-STAT signaling pathway in a process requiring PKD2. Cell 109: 157-168, 2002.
14. Qiao X, Pham DN, Luo H and Wu J: Ran overexpression leads to diminished T cell responses and selectively modulates nuclear levels of c-Jun and c-Fos. J Biol Chem 285: 5488-5496, 2010.
15. Lee SY, Yoon J, Lee HS, *et al*: The function of heterodimeric AP-1 comprised of c-Jun and c-Fos in activin mediated Spemann organizer gene expression. PLoS One 6: e21796, 2011.
16. Hwang YP, Yun HJ, Kim HG, Han EH, Lee GW and Jeong HG: Suppression of PMA-induced tumor cell invasion by dihydroartemisinin via inhibition of PKCalpha/Raf/MAPKs and NF-kappaB/AP-1-dependent mechanisms. Biochem Pharmacol 79: 1714-1726, 2010.
17. Francis H, Onori P, Gaudio E, *et al*: H3 histamine receptor-mediated activation of protein kinase Calpha inhibits the growth of cholangiocarcinoma in vitro and in vivo. Mol Cancer Res 7: 1704-1713, 2009.
18. Uğuz A, Gönlüşen G, Ergin M and Tuncer I: Expression of Fas, Bcl-2 and p53 molecules in glomerulonephritis and their correlations with clinical and laboratory findings. Nephrology (Carlton) 10: 311-316, 2005.
19. Fuzio P, Ditonno P, Lucarelli G, *et al*: Androgen deprivation therapy affects BCL-2 expression in human prostate cancer. Int J Oncol 39: 1233-1242, 2011.
20. Shinagawa A, Yoshida Y, Kurokawa T, *et al*: The potent peptide antagonist to angiogenesis, C16Y, and cisplatin act synergistically in the down-regulation of the Bcl-2/Bax ratio and the induction of apoptosis in human ovarian cancer cells. Int J Oncol 39: 1359-1364, 2011.
21. Stamenkovic I: Extracellular matrix remodeling: the role of metalloproteinases. J Pathol 200: 448-464, 2003.
22. Selvais C, Gaide Chevronnay HP, Lemoine P, *et al*: Metalloproteinase-dependent shedding of low-density lipoprotein receptor-related protein-1 ectodomain decreases endocytic clearance of endometrial matrix metalloproteinase-2 and -9 at menstruation. Endocrinology 150: 3792-3799, 2009.
23. Bauvois B, Mothu N, Nguyen J, Nguyen-Khoa T, Nöel LH and Jungers P: Specific changes in plasma concentrations of matrix metalloproteinase-2 and -9, TIMP-1 and TGF-beta1 in patients with distinct types of primary glomerulonephritis. Nephrol Dial Transplant 22: 1115-1122, 2007.
24. Jacob-Ferreira AL, Lacchini R, Gerlach RF, Passos CJ, Barbosa F Jr and Tanus-Santos JE: A common matrix metalloproteinase (MMP)-2 polymorphism affects plasma MMP-2 levels in subjects environmentally exposed to mercury. Sci Total Environ 409: 4242-4246, 2011.
25. Davies M, Martin J, Thomas GJ and Lovett DH: Proteinases and glomerular matrix turnover. Kidney Int 41: 671-678, 1992.