



Effect of TIMP1 transfection on PTEN expression in human kidney proximal tubular cells

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ABSTRACT. To explore the role of metalloproteinase-1 (TIMP-1) tissue inhibitor in the mechanisms of kidney aging, we observed the effects of sense and antisense transfection of *TIMP-1* and of metalloproteinase (MMP) inhibitors on phosphatase and tensin homolog (PTEN), vascular endothelial growth factor (VEGF), and Flk-1 expression in *TIMP-1* transgenic human proximal tubular epithelial cells (HKCs). Transfected HKCs were co-incubated with 100 μ M MMP-2 and MMP-9 inhibitor III for 24 h to affect enzyme inhibition. *TIMP-1*, *MMP-2*, *MMP-9*, *PTEN*, *VEGF*, and *Flk-1* mRNA expression was detected by reverse transcription-polymerase chain reaction. PTEN, VEGF, and Flk-1 protein expression in cells of each experimental group was measured by indirect immunofluorescence. We found that PTEN expression was up-regulated ($P < 0.05$) in the sense *TIMP-1*-transfected group ($P < 0.05$) compared with the non-transfected and empty vector groups, and that expression of VEGF and Flk-1 was down-regulated ($P < 0.05$). In contrast, the antisense *TIMP-1* transgenic group showed the opposite results ($P < 0.05$). No significant differences

in expression of PTEN, VEGF, or Flk-1 were observed among the MMP-2/MMP-9 inhibitor III, non-transfected, and empty vector groups ($P > 0.05$). These results suggest that in the progression of renal aging, high expression of TIMP-1 up-regulates PTEN expression through an MMP-independent pathway, and subsequently down-regulates the expression of VEGF and Flk-1, indicating that PTEN and TIMP-1 are involved in the aging-associated impairment of renal angiogenesis. Our study provides a theoretical basis for further exploration of the mechanism underlying TIMP-1 participation in renal aging progression.

Key words: TIMP-1; Kidney aging; Angiogenesis; PTEN

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a crucial pro-angiogenic factor that plays an important role in angiogenesis. It mainly acts by interacting with the receptor Flk-1, further initiating intracellular signal transduction, and thereby promoting endothelial cell proliferation, migration, and angiogenesis (Kang et al., 2002; Huang et al., 2012). Research has revealed that highly expressed sense and antisense *TIMP-1* might be involved in age-related impairment of angiogenesis via up-regulating phosphatase and tensin homolog (PTEN) in the aging kidney. However, whether highly expressed sense and antisense *TIMP-1* can also exert an impact on disordered age-related angiogenesis by down-regulating VEGF/Flk-1 has not yet been elucidated. In this study, sense and antisense *TIMP-1* recombinant expression vectors were transfected into human kidney proximal tubular cells (HKCs) followed by simultaneous treatment of matrix metalloproteinase (MMP)-2/MMP-9-specific enzyme inhibitors. The impact of sense and antisense *TIMP-1* transfection and enzyme inhibitor interference on HKCs were observed to explore the underlying mechanisms of TIMP-1 on the aging kidney.

MATERIAL AND METHODS

Reagents

MMP-2/MMP-9 inhibitor III was obtained from Merck Pharmaceuticals (Kenilworth, NJ, USA); goat MMP-9/MMP-2 polyclonal antibody, rabbit TIMP-1 polyclonal antibody, rabbit VEGF polyclonal antibody, rabbit β -actin polyclonal antibody, mouse anti-human PTEN monoclonal antibody, FITC-labeled goat anti-mouse IgG, and goat anti-rabbit IgG were from Santa Cruz Biotechnology (Dallas, TX, USA); horseradish peroxidase-labeled goat anti-rabbit IgG, rabbit anti-goat IgG, and goat rabbit anti-mouse IgG were also from Santa Cruz Biotechnology; M-MLV reverse transcriptase was from Invitrogen Life Technologies (Carlsbad, CA, USA); human and mouse *TIMP-1*, mouse *MMP2*, mouse *MMP9*, human *PTEN*, and *VEGF* primers were synthesized by SBS Genetech Co., Ltd. (Beijing, China).

Cell culture and establishment of a cell line with stable expression of TIMP-1

HKCs were stored in the Department of Nephrology, Chinese PLA General Hospital. Three HKC cell lines stably transfected with pcDNA 3.0, TIMP-1S-pcDNA 3.0, and TIMP-1AS-pcDNA 3.0 (pcDNA 3.0 recombinant plasmid containing full-length sense and antisense human TIMP-

1 cDNA) were previously constructed in the Molecular Biology Research Center of the Second Xiangya Hospital of Central South University as described (Lin Hongli et al., 2001). The three established cell lines were referred to as 1) normal non-transfected cells (parental/HKCs); 2) empty vector transfected cells (vector/HKCs); 3) sense TIMP-1 transfected cells (TIMP-1S/HKCs); and 4) antisense TIMP-1 transfected cells (TIMP-1AS/HKCs). The HKCs were cultured and passaged in DMEM medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C, with 5% CO₂ in a humid atmosphere.

Construction and identification of plasmids

The *PTEN* siRNA plasmid and control siRNA plasmid were donated by Professor Huang Boqu of Northeastern University of China. The RNAi target sequences of human *PTEN* (5' - GAC TTG AAG GCG TAT ACA G - 3') were designed according to the full-length human *PTEN* gene according to the GenBank database. The oligonucleotide sequences that encoded the chosen siRNA sequence were designed and inserted into the pSilencer 2.0-U6 vector. The plasmid (pSilencer 2.0-U6 Vector) was sequenced and confirmed by Santa Cruz Biotechnology, China (Figure 1).

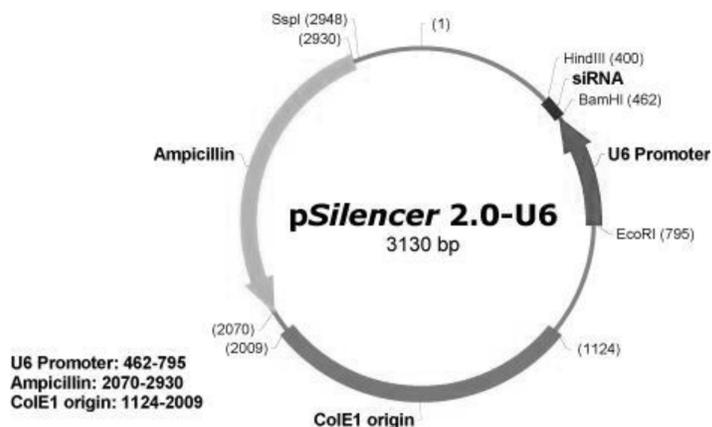


Figure 1. pSilencer 2.0-U6 vector map.

Experimental design, grouping, and liposome-mediated transfection of plasmid DNA

Experimental groups

In order to observe the impact of the sense and antisense *TIMP-1* transfection and enzyme inhibitor interference on the expression of PTEN and VEGF in HKCs, the cells were divided into five groups: 1. normal non-transfected group (parental); 2. empty vector of the transfected group (vector); 3. sense *TIMP-1* transfected group (TIMP-1S); 4. antisense *TIMP-1* transfected group (TIMP-1AS); 5. normal non-transfected MMP inhibitor group (inhibitor III 100 μm) (Akahane et al., 2004).

Cells in each group were seeded in cell culture flasks (surface area 25 cm²; 5 flasks per sample) at a density of 5 × 10⁴/mL, and were cultured in DMEM medium containing 10% FBS and 100 U/mL penicillin and streptomycin in a humid atmosphere of 95% air and 5% CO₂ at 37°C. Upon reaching 60 to 80% confluence, the cells were washed with phosphate-buffered saline and sub-cultured with serum-free DMEM for 16 h. Subsequently, the cells were incubated with DMEM

plus 10% FBS or RPMI1640 medium with or without 100 μ M MMP-2/MMP-9 inhibitor III for 24 h. The cells and the culture supernatant was harvested and analyzed for the presence of cytokines.

The *PTEN* siRNA was transfected into HKCs stably expressing human TIMP-1S for further observation. There were three experimental groups: A. sense *TIMP-1* transfected group (TIMP-1S); B. *PTEN* siRNA group (*PTEN* siRNA plasmid transfected HKCs stably expressing human TIMP-1S); and C. control siRNA group (control siRNA plasmid transfected HKCs stably expressing human TIMP-1S). Three replicate wells were used for each group.

Liposome-mediated transfection of plasmid DNA

Twenty-four hours prior to transfection, cells in the TIMP-1S group in logarithmic growth phase were seeded into 6-well plates and cultured in DMEM containing 10% FBS, or RPMI1640 medium consisting of 100 U/mL penicillin and streptomycin in a humid atmosphere of 95% air and 5% CO₂ at 37°C until the cells were grown to 40-60% confluence. The culture medium was replaced 1 h before transfection with 2 mL fresh medium per well and culturing was continued. For transfection, 5 μ g *PTEN* siRNA or control siRNA plasmid was added into normal saline (total volume 100 μ L), mixed thoroughly, and incubated for 5 min at room temperature. Simultaneously, 2 μ g Vigofect was added into normal saline (total volume 100 μ L) followed by thorough mixing and 5 min incubation at room temperature. Then the diluted Vigofect mixture was added drop by drop into the diluted DNA solution accompanied with gentle vortexing. After 15 min incubation, the reaction mixture was evenly added into 2 mL cell culture medium and incubated for 6 h. The medium was replaced with fresh medium and incubated for another 48 h before harvesting.

PTEN and VEGF expression detection by indirect immunofluorescence

The cells in the five study groups: parental, vector, TIMP-1S, TIMP-1AS, and inhibitor III 100 μ M groups, were treated according to kit instructions and were observed under a confocal laser scanning microscope. In each sample, 20 cells were randomly selected and the mean fluorescence intensities of PTEN and VEGF positive cells were measured using the Image-Pro Plus 4.5 software (Media Cybernetics, Rockville, MD, USA), and the mean value of the expression levels of PTEN and VEGF were used to evaluate their overall expression.

TIMP-1, MMP-2, MMP-9, PTEN, and VEGF mRNA expression evaluated using reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the TRIzol reagent. RT-PCR primer sequences are shown in Table 1. *GAPDH* was used as the internal control. Agarose gel electrophoresis was used to detect the PCR product: 8 μ L each PCR product was electrophoresed on a 1.5% agarose gel and the images were captured and semi-quantitatively analyzed using an imaging system (AlphaImager, 2200, Alpha Innotech, Santa Clara, CA, USA).

Statistical analyses

Statistical analysis was performed using the SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Data are reported as means \pm standard deviation. Univariate analysis of variance was used to compare mean differences in the study groups. P values less than 0.05 were considered to be statistically significant.

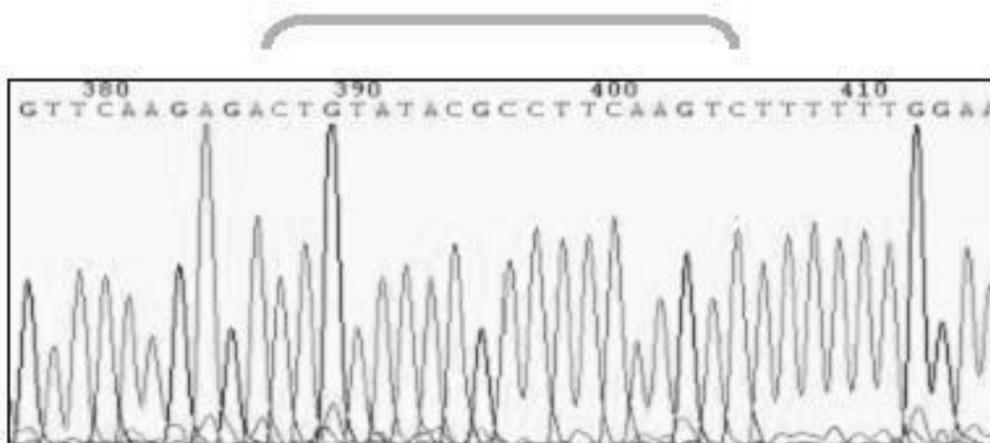
Table 1. Primer sequences used for reverse transcription-polymerase chain reaction.

Protein	Primer sequence	Fragment length (bp)	Annealing temperature (°C)
TIMP-1	Upstream: 5' - CAG ACC ACC TTA TAC CAG CGT - 3' Downstream: 5' - GAT AAA CAG GGA AAC ACT GTG C - 3'	311	60.0°
MMP-2	Upstream: 5' - AGA CCC CTG GCT TTT CAC TGC TGG - 3' Downstream: 5' - GTT TCC GCT TCT GGC TGG GTC TGT - 3'	412	59.0°
MMP-9	Upstream: 5' - CGC GCT GGG CTT AGA TCA TTC CTC - 3' Downstream: 5' - GCA GCG CGG GCC ACT TGT C - 3'	476	62.0°
PTEN	Upstream: 5' - GAT GGC ACT TTC CCG TTT TA - 3' Downstream: 5' - TCT GAG CAT TCC CTC CAT TC - 3'	254	55.0°
VEGF	Upstream: 5' - TCC GGA TGG GCA CGA GTA - 3' Downstream: 5' - TGG CGG AGA GGG GCA CAT - 3'	121	61.0°
Flk-1	Upstream: 5' - GGA ACG CTA CCC CGC ACG AAT GAT - 3' Downstream: 5' - GGT CCT GGC CCG CAA AGA AGT C - 3'	186	66.0°
GAPDH	Upstream: 5' - TGC ACC ACC AAC TGC TTA GC - 3' Downstream: 5' - GGC ATG GAC TGT GGT CAT GAG - 3'	191	58.0°

RESULTS

Identification of *PTEN* siRNA plasmid DNA

A gene fragment for insertion of 62 bp was generated based on literature review and plasmid map creation. The sequencing result showed that the oligonucleotide inserted had the sequence of 5' - GAC TTG AAG GCG TAT ACA G - 3', which was consistent with the target DNA sequence (Figure 2).

**Figure 2.** Target DNA sequence insert.

Identification of exogenous DNA in HKCs

Specific primers for exogenous gene identification were designed according to the vectors with exogenous DNA sequence. PCR results showed that the exogenous DNA was integrated into the genome of HKCs. In addition, a neo gene was successfully included in vector/HKC, TIMP-1S/HKC, and TIMP-1AS/HKC groups, and no inclusion was found in parental/HKCs. Inclusion of the antisense *TIMP-1* gene was observed in TIMP-1AS/HKCs, which was not identified in other cells (Figure 3).

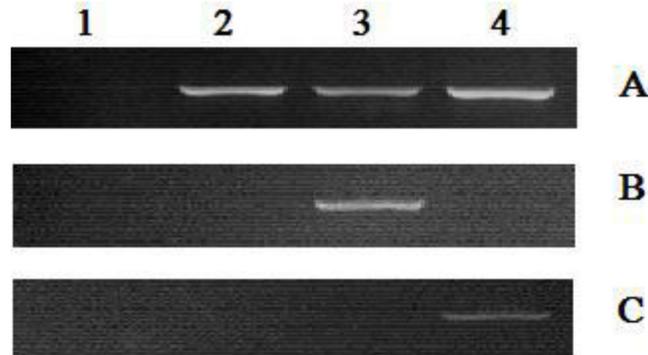


Figure 3. Identification of exogenous DNA in HKCs. Lanes 1-4 indicate parental/HKCs, vector/HKCs, TIMP-1S/HKCs, and TIMP-1AS/HKCs, respectively. Row A: stable transfected pcDNA3.0; B: TIMP-1S-pcDNA3.0; and C: TIMP-1AS-pcDNA3.0. HKCs, human kidney cells.

Detection of PTEN, VEGF, and Flk-1 expression with indirect immunofluorescence

Effect on PTEN expression in HKCs

The results showed the strongest expression of PTEN in the TIMP-1S/HKCs ($P < 0.05$), and the weakest expression in the TIMP-1AS/HKCs ($P < 0.05$). Nevertheless, no significant difference in PTEN expression was found between parental/HKCs, vector/HKCs, and MMP-2/MMP-9 inhibitor III (100 μ M) HKCs ($P > 0.05$). Detailed information is shown in Figure 4.

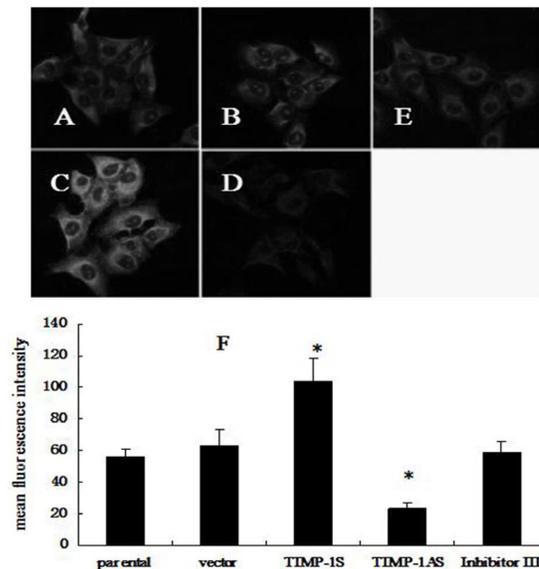


Figure 4. PTEN expression as measured by indirect immunofluorescence in each group. **A.** Parental/HKCs; **B.** vector/HKCs; **C.** TIMP-1S/HKCs; **D.** TIMP-1AS/HKCs; **E.** MMP-2/MMP-9 inhibitor III (100 μ M); and **F.** histogram of the mean fluorescence intensity of PTEN in each group. * $P < 0.05$ compared with groups of parental/HKCs, vector/HKCs, and inhibitor III/HKCs (100 μ M); 600X. HKCs, human kidney cells.

Effect on VEGF expression in HKCs

The results showed that the weakest signal of VEGF expression was detected in the TIMP-1S/HKCs ($P < 0.05$), and the strongest in the TIMP-1AS/HKCs ($P < 0.05$). However, no significant difference in VEGF expression was observed between parental/HKCs, vector/HKCs, and MMP-2/MMP-9 inhibitor III (100 μM) HKCs ($P > 0.05$). The results are shown in Figure 5.

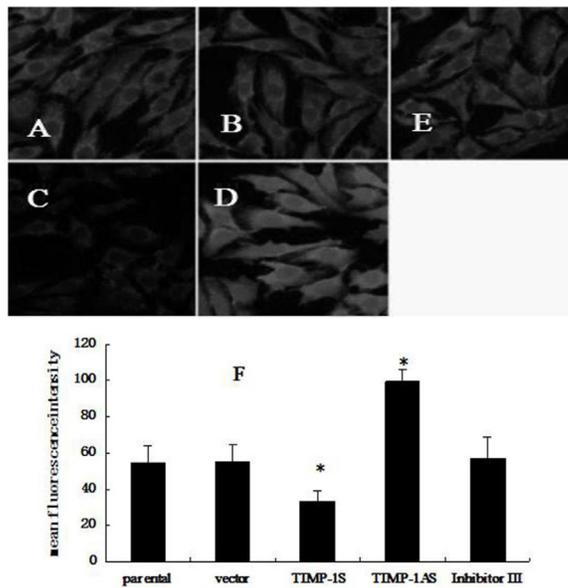


Figure 5. VEGF expression as measured by indirect immunofluorescence in each group. **A.** Parental/HKCs; **B.** vector/HKCs; **C.** TIMP-1S/HKCs; **D.** TIMP-1AS/HKCs; **E.** MMP-2/MMP-9 inhibitor III (100 μM); and **F.** histogram of the mean fluorescence intensity of VEGF in each group. * $P < 0.05$ compared with groups of parental/HKCs, vector/HKCs, and inhibitor III/HKCs (100 μM); 600X. HKCs, human kidney cells.

RT-PCR test results

Expression of TIMP-1, MMP-2, and MMP-9 mRNA in HKCs

The study revealed higher expression of *TIMP-1*, *MMP-2*, and *MMP-9* mRNA in TIMP-1S/HKCs and TIMP-1AS/HKCs than in parental/HKCs, vector/HKCs, and MMP-2/MMP-9 inhibitor III (100 μM) HKCs ($P < 0.05$). Nevertheless, the difference in expression of *TIMP-1*, *MMP-2*, and *MMP-9* mRNA was not significantly different between parental/HKCs, vector/HKCs, and MMP-2/MMP-9 inhibitor III (100 μM) HKCs ($P > 0.05$), which is displayed in Figure 6.

VEGF mRNA expression in HKCs

Among the five groups, TIMP-1S/HKCs presented a minimal expression of *VEGF* mRNA ($P < 0.05$), and TIMP-1AS/HKCs showed the maximum value ($P < 0.05$). Nevertheless, there was no significant difference in expression of *VEGF* mRNA between parental/HKCs, vector/HKCs, and MMP-2/MMP-9 inhibitor III (100 μM) HKCs ($P > 0.05$). The findings are shown in Figure 7.

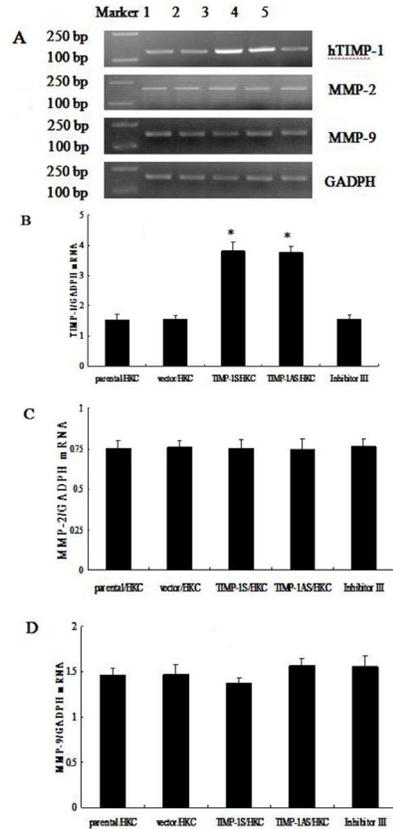


Figure 6. Expression of TIMP-1, MMP-2, and MMP-9 mRNA. **A.** Lane 1: parental/HKCs, lane 2: vector/HKCs, lane 3: TIMP-1S/HKCs, lane 4: TIMP-1AS/HKCs, and lane 5: MMP-2/MMP-9 inhibitor III (100 μ M). **B.-D.** Histograms for image analysis results; *P < 0.05, compared with parental/HKCs, vector/HKCs, and inhibitor III/HKCs (100 μ M). HKCs, human kidney cells.

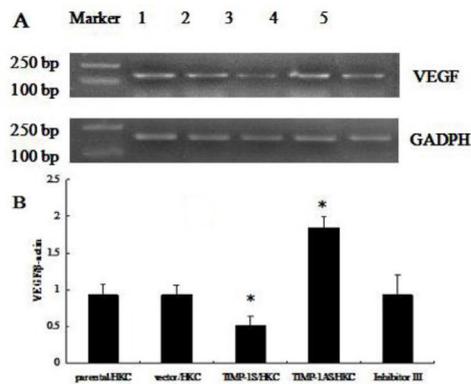


Figure 7. VEGF mRNA expression in HKCs. **A.** Lane 1: parental/HKCs, lane 2: vector/HKCs, lane 3: TIMP-1S/HKCs, lane 4: TIMP-1AS/HKCs, lane 5: MMP-2/MMP-9 inhibitor III (100 μ M). **B.** Histogram of image analysis results. *P < 0.05, compared with parental/HKCs, vector/HKCs, and inhibitor III/HKCs (100 μ M). HKCs, human kidney cells.

Expression of *TIMP-1*, *PTEN*, and *VEGF* mRNA in *PTEN* siRNA and control siRNA transfected *TIMP-1S/HKCs*

The results showed significantly lower *PTEN* mRNA expression in the *PTEN* siRNA group than the *TIMP-1S/HKCs* and control siRNA group. The latter two groups, however, had no significant difference in *PTEN* mRNA expression ($P > 0.05$). Furthermore, *VEGF* mRNA expression was significantly higher in the *PTEN* siRNA group compared with the *TIMP-1S/HKCs* and control siRNA group. However, the *VEGF* mRNA expression demonstrated no significant difference in the latter two groups ($P > 0.05$). There was no significant difference in the expression of *TIMP-1* mRNA among the three groups (Figure 8).

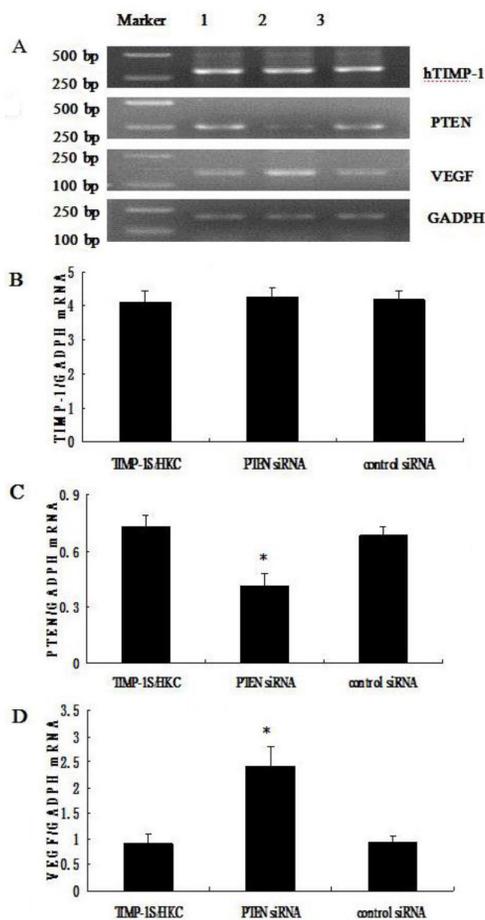


Figure 8. Expression of *TIMP-1*, *PTEN*, and *VEGF* mRNA. **A.** Lane 1: *TIMP-1S/HKCs*, lane 2: *PTEN* siRNA, and lane 3: control siRNA. **B.-D.** Histograms of image analysis results. * $P < 0.05$, compared with the *TIMP-1S/HKCs* and control siRNA group. HKCs, human kidney cells.

DISCUSSION

Impaired microangiogenesis occurs in the development of chronic progressive nephropathy (Otrock, et al., 2007; Gao, et al., 2011) and kidney aging (Kang et al., 2001; Zhang et al., 2003; Long et al., 2004). In an age-dependent nephropathy model, decreased expression of VEGF was proven to be related to impaired angiogenesis. Previous research has revealed that the expression level of TIMP-1 was considerably elevated in the aging process in human *TIMP-1* transgenic mice as compared with wild-type mice. In addition, the glomerular and peritubular capillary density decreased significantly. At the same time, PTEN expression was greatly upregulated and VEGF expression was down-regulated in the *TIMP-1* transgenic mice. The results suggested that highly expressed TIMP-1 might contribute to aging-related impairment of angiogenesis via up-regulating PTEN expression during renal senescence (Zhang et al., 2003).

VEGF is an important pro-angiogenic factor. Vascular endothelial growth factor receptors (VEGFRs) include Flt-1, Flk-1, and Flt-4. Flt-1 and Flk-1 can be produced by most vascular endothelial cells except that Flt-4 is only expressed on lymphatic endothelial cells. Studies have indicated that Flk-1 is the main mediator to promote endothelial cell division, proliferation, and survival, as well as microvascular permeability. It has been proven that Flt-1 has a dual function in angiogenesis, acting in a positive or negative manner in different circumstances. Especially during early stages of embryonic development, Flt-1 exerts its negative regulation on angiogenesis by preventing VEGF from binding to Flk-1. Recent studies have shown that the expression of VEGF can be induced by hypoxia and its transcription can be directly activated by hypoxia-inducible transcription factor-1 (HIF-1). Nevertheless, up-regulation of VEGF is not only mediated by HIF-1, but also by the SP-1 and ras oncogenes (Lee et al., 2012; Zhou and Tu, 2012). Furthermore, transcription of *VEGF* was found to be blocked by the tumor suppressor genes p53 and p57. *PTEN* is a new tumor suppressor gene that encodes a dual-specificity phosphatase with both protein and lipid phosphatase activities. PTEN lipid phosphatase dephosphorylates membrane-bound PIP3, blocking Akt kinase activity and its downstream kinase activity, thereby exerting its negative regulation on the cell cycle and proliferation. PTEN plays an important role in endothelial and epithelial cells. In endothelial cells, overexpressed PTEN can inhibit cell survival and angiogenesis, activating caspase-3 and thereby inducing apoptosis (Huang and Kontos, 2002). In epithelial cells, overexpressed PTEN suppresses cell proliferation and induces apoptosis via up-regulating apoptosis-related proteins (Wu et al., 2007).

In this study, after the HKCs were transfected with sense and antisense *TIMP-1* and subjected to enzyme inhibitors, significantly higher PTEN expression was observed in the sense *TIMP-1* transfected group than in the normal non-transfected and empty vector transfected groups ($P < 0.05$). However, PTEN expression decreased in the antisense *TIMP-1* transfected group ($P < 0.05$). In this experiment, *PTEN* siRNA was transfected into HKCs stably expressing human TIMP-1S, so as to further explore the effect of sense *TIMP-1* transfection on PTEN expression in HKCs. The results suggested that PTEN expression was significantly down-regulated and VEGF expression was upregulated in the *PTEN* siRNA transfection group compared with the normal non-transfected and control siRNA groups ($P < 0.05$). The findings indicated that TIMP-1 might exert its anti-angiogenic function by upregulating PTEN expression as well as down-regulating VEGF expression. Further studies have identified that the anti-angiogenic function of TIMP-1 is completely independent of MMP inhibitory activity, which was consistent with the report by Akahane et al. (2004).

Angiogenesis is a pathophysiological process in which endothelial cell proliferation and migration occur first, and new blood vessels form at the end stage. Furthermore, in angiogenesis,

MMPs might promote angiogenesis by degrading the extracellular matrix, promoting migration of endothelial cells as well as releasing matrix debris. Both MMP-2 and MMP-9 can degrade capillary basement membranes and promote the migration of endothelial cells, thereby being involved in angiogenesis (Nguyen et al., 2001). In a multi-stage carcinogenesis mouse model study, Bergers et al. (2000) confirmed that MMP-9 initiated angiogenesis promoted VEGF bioavailability and VEGF receptor activation in endothelial cells. Additionally, Hashimoto et al. (2002) claimed that MMPs might activate VEGF through degrading connective tissue growth factor. TIMP-1 is an inhibitor of MMPs. Therefore, we speculated that the anti-angiogenic function of TIMP-1 could also be induced by MMP inhibitory activity.

In conclusion, this study showed that highly expressed TIMP-1 could inhibit angiogenesis by upregulating PTEN expression through an MMP-independent pathway and by down-regulating VEGF expression. Furthermore, as an endogenous inhibitor of MMPs, TIMP-1 might modulate angiogenesis via MMP inhibitory activity. We also observed that TIMP-1 was possibly involved in the MMP pathway to regulate angiogenesis in this study. Therefore, the role of the anti-angiogenic function of TIMP-1 in the progression of kidney disease still needs to be further explored and elucidated.

Conflicts of interest

The authors declare no conflict of interest.

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