Transduction of PTEN Proteins Using the Tat Domain Modulates TGF-β1–Mediated Signaling Pathways and Transdifferentiation in Subconjunctival Fibroblasts

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Purpose. This study investigated the effects of the tumor suppressor protein PTEN (phosphatase and tensin homolog) on transforming growth factor (TGF)-β1–mediated signaling pathways and the transdifferentiation of human subconjunctival fibroblasts (SCFs) after the transduction of this protein containing a transactivator of transcription (Tat) domain.

Methods. The Tat-PTEN expression vector was constructed to express the Tat domain of HIV-1 fused to PTEN. After transduction of the fusion protein and TGF-β1 stimulation, the dose-dependent effect of the transduced Tat-PTEN fusion protein on Akt phosphorylation and the stability of the Tat-PTEN fusion protein in SCF cells were evaluated by Western blot analysis. The effect of the Tat-PTEN fusion protein on the TGF-β1–stimulated expression of α-SMA and fibronectin was also evaluated by Western blot analysis and immunocytochemistry.

Results. To increase the efficiency of enzyme activity and to successfully deliver this protein to cells, the authors used a PTEN fusion protein that contained the transduction domain of the Tat protein from HIV-1. By Western blot analysis, the transduced Tat-PTEN fusion protein was found to modulate TGF-β1 signaling in SCF cells and result in the suppression of Akt phosphorylation. Furthermore, the transduction of the Tat-PTEN fusion protein was found to suppress the TGF-β1–stimulated expression of α-SMA and fibronectin by Western blot analysis and immunocytochemical staining, and the effects of the transduced fusion protein could be controlled in a dose-dependent manner.

Conclusions. The Tat-PTEN fusion proteins were successfully transduced into the SCF cells and induced the suppression of transdifferentiation and fibrosis through the regulation of TGF-β–mediated signaling. The ability of the Tat-PTEN fusion protein to regulate cell survival could potentially be applied to protein therapy to counteract postoperative scarring in glaucoma surgery. (*Invest Ophthalmol Vis Sci.* 2012;53:379–386) DOI:10.1167/iovs.11-8491

In the eye, failed glaucoma filtration surgery arises from the development of subconjunctival fibrosis, which results in conjunctival scarring from the wound healing process.[1–3]

Transforming growth factor beta (TGF-β) is a key mediator of wound healing and is critically involved in postoperative scarring.[4,5] At the cellular level, TGF-β drives the conversion of fibroblasts into myofibroblasts, which is a key step for all fibrotic processes.[6] This transdifferentiation is characterized by the de novo synthesis of alpha-smooth muscle actin (α-SMA).[7] The persistence of the myofibroblasts is associated with an increased deposition of extracellular matrix (ECM) proteins and leads to tissue fibrosis.[8,9] Therefore, TGF-β is a major target in the development of antifibrotic treatment strategies, and intracellular signaling pathways downstream of TGF-β may provide additional therapeutic targets. Importantly, the inhibition of p38- or Rho-dependent signaling has been shown to inhibit myofibroblast transdifferentiation in vitro.[10–12]

Among the signaling pathways triggered by TGF-β, the phosphoinositide 3-kinase (PI3K)/Akt pathway that regulates a variety of cellular processes, including cell proliferation, metabolism, migration, and survival, is involved in the establishment of fibrotic fibroblasts by opposing the functions of phosphatase and tensin homolog (PTEN).[13–15] PTEN is a tumor suppressor gene located at chromosome 10q23.3, and deletions or mutations within the PTEN gene have been reported in various forms of human cancer.[16–18] The PTEN protein is a protein phosphatase and a lipid phosphatase and is therefore considered a dual specificity phosphatase.[19] As a lipid phosphatase, PTEN acts as a tumor suppressor and a phosphatidylinositol-3 phosphatase to downregulate the activity of phosphoinositol-3-kinase, which is important for the regulation of cell proliferation, growth, and survival.[20–22]

Previous studies have reported that the transactivator of transcription (Tat) from HIV-1 has the capacity to cross cell membranes.[23,24] Recently, HIV-1 Tat proteins have been shown capable of transducing many heterologous proteins, such as p16, p27, E2F, Cdk2, and SOD, across the cell membrane, although the exact delivery mechanisms are not known.[25–30] In this study, subconjuctival fibroblast cells stimulated with TGF-β served as a model of the antagonistic scarring process, and Tat-PTEN fusion proteins were developed to investigate the role of PTEN in this system.

Materials and Methods

Cell Cultures and Chemicals

Human subconjunctival fibroblasts were obtained from the Tenon capsule as previously described.[31] This research followed the tenets of

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the Declaration of Helsinki. Written informed consent was obtained before the operative excision of these cells, and approval from the institutional human experimentation committee was also granted. The subconjunctival fibroblast cells were cultured at 37°C in a 5% humidified CO_2 environment and in a culture medium containing Dulbecco's modified Eagle's medium that was supplemented with 10% fetal bovine serum, 50 μg/mL penicillin, and 50 μg/mL streptomycin. The cells used for these studies had been passaged between three and six times. TGF-β1 was purchased from a commercial supplier (R&D Systems, Minneapolis, MN), and the concentration of TGF-β1 that was used to directly treat the culture medium was 10 ng/mL.

Expression and Purification of the Tat-PTEN Fusion Protein

The Tat-PTEN expression vector was designed to express the Tat domain of HIV-1 fused to PTEN, as described by Nagahara et al.[30] Two oligonucleotides were synthesized to generate 11 amino acids of the Tat domain flanked by glycine residues. The sequences of top strands were 5′-(ggatcc)GGT TAC GGT CGT AAG AAA CGT AGA CAG CGC AGA CGT GGT(c)-3′, and the sequences of bottom strands were 5′-(ctcgag)ACC ACG TCT GCG CTG TCT ACG TTT CTT ACG ACC GTA CCA-3′. The nucleotides in parentheses were included to form *Bam*HI and *Xho*I restriction sites. The synthesized double-stranded oligonucleotide was inserted into a *Bam*HI- and *Xho*I-digested pRSET vector (Invitrogen, San Diego, CA), which had an N-terminal polyhistidine (6xHis) tag for purification and epitope identification (Xpress using the anti-Xpress antibody). Next, the human PTEN gene was inserted into the pRSET vector and the Tat domain was inserted in-frame to create the Tat-PTEN expression vector.

The open reading frame of human PTEN was amplified using a human cDNA library (Stratagene, La Jolla, CA) as the template. The PCR primers were synthesized using the GenBank database of PTEN (u93051) as a reference. The sequence of the top strand of PTEN was 5′-(aaa-ctc-gag)ATG ACA GCC ATC ATC AAA GA-3′, and the sequence of the bottom strand was 5′-(aaa-aag-ctt)TCA GAC TTT TGT AAT TTG TGT ATG CTG-3′. Both the top-strand and the bottom-strand sequences had *Xho*I and *Hin*dIII restriction sites, which were indicated in parentheses, for subcloning into the pRSET vector that contained the Tat domain. The PCR amplifications were performed using a precision *Taq* polymerase (TaqPlus; Clontech, Palo Alto, CA), and the PCR consisted of 25 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 68°C for 1 minute. The PCR products were purified and cloned into the TA cloning vector (Promega). The sequences of the inserts from the selected colonies were confirmed by sequence analysis with a DNA analyzer system (ABI 3100; PerkinElmer, Waltham, MA). Once more, the selected TA cloning vector was digested with *Xho*I and *Hin*dIII and was then relegated into the Tat-containing pRSET expression vector that had been previously digested with *Xho*I and *Hin*dIII. The Tat-PTEN construct and the PTEN pRSET construct were then transformed into *E. coli* BL21 (DE3). The fusion proteins were purified according to the slightly modified protocols from Becker-Hapak et al.[32] The selected BL21 colony was cultured in 3 mL of L-Broth (LB) medium overnight. The next day, the BL21 culture was inoculated into 1 L of LB medium and cultured until an OD600 was attained. Next, 1 mM of IPTG (isoprophyl-1-thio-β-D-galactopyranoide) was added and incubated for 8 hours at 37°C for the overexpression of the fusion protein. After the culture medium was centrifuged at 10,000*g* for 10 minutes, the bacterial pellets that contained the PTEN or Tat-PTEN proteins were suspended in 20 mL of binding buffer without urea (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and were sonicated. The disrupted cells were centrifuged at 14,000*g* for 20 minutes, and then the pellets were resuspended in buffer A (8 M urea, 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) for further purification. The resuspended sonicates were applied to a preequilibrated histidine binding resin (Novagen) with distilled water, charge buffer (50 mM $NiSO_4$), and binding buffer and were allowed to flow by gravity. Next, the column was washed with 10 volume equivalents of binding buffer and with 6 volume equivalents of wash buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and elution buffer (100 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The fusion proteins were eluted using elution buffer with increasing concentrations of imidazole that ranged from 250 to 500 mM. After purification, preequilibrated PD-10 columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and serum-free media were used to change the media. The concentration of the purified fusion protein was determined by a protein assay procedure (Bradford) using bovine serum albumin as the standard. The purified fusion proteins were stored at −80°C in serum-free media that contained 3% glycerol.

Transduction of the Tat-PTEN Fusion Protein and TGF-β1 Treatment

For the transduction of the Tat-PTEN fusion protein, subconjuctival fibroblast cells were grown to confluence. Before treatment with the fusion proteins, the cells were serum-starved in serum-free media for 2 hours. Next, the media was replaced with serum-free media that contained the fusion proteins, and the transduction period lasted for 2 hours. After the transduction of the fusion proteins, the samples were washed twice with serum-free media to eliminate any remaining fusion protein. For the stimulation with TGF-β1, 10 ng/mL TGF-β1 was directly added to the cells in serum-free media, and this treatment lasted for 48 hours. After the transduction and TGF-β1 stimulation, the cells were harvested for Western blot analysis.

Next, the effects of the Tat-PTEN fusion protein on fibronectin and α-SMA activity after TGF-β1 stimulation were evaluated. After 2 hours of transduction with Tat-PTEN, the cells were either treated with TGF-β1 or left untreated, and then the samples were analyzed by Western blot and immunocytochemistry using antibodies against fibronectin and α-SMA.

Western Blot Analysis

The cells were washed twice with ice-cold PBS and were then lysed in cold 1 × cell lysis buffer, which consisted of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM Na_2EDTA, 1 mM EGTA, 1 mM PMSF, 1 μg/mL leupeptin, 1 mM Na_3VO_4, 2.5 mM sodium pyrophosphate, and 1 mM β-glycerophosphate (Cell Signaling Technology, Danvers, MA), by sonication for 10 minutes on ice. The protein concentration was determined using a protein assay procedure (Bradford reagent; Sigma). The protein was loaded onto the appropriate percentage of SDS-PAGE gel and was then transferred onto a nitrocellulose membrane. The membrane was incubated with a primary antibody at 4°C overnight. The primary antibodies used in these experiments included anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-JNK antibody, anti-JNK antibody, anti-phospho-P38 antibody, anti-P38 antibody (Cell Signaling Technology), anti-phospho-ERK antibody, anti-ERK antibody (Santa Cruz Biotechnology), anti-phospho-Smad2 antibody (Millipore), anti-Smad2/3 (BD Transduction Laboratory), epitope identification antibody (Anti-Xpress; Invitrogen), anti-PTEN (Santa Cruz Biotechnology), and anti-cellular fibronectin (Sigma-Aldrich). After a washing step, the membranes were incubated with HRP-conjugated sheep anti-mouse IgG or anti-rabbit IgG (Amersham Pharmacia Biotech) secondary antibodies for 1 hour at room temperature. The protein signals were detected using a Western blot detection reagent (ECL Reagent; Amersham Pharmacia Biotech).

Immunocytochemistry

Subconjunctival fibroblasts that had attached to the chamber slides were washed and then fixed in 4% (v/v) formaldehyde for 5 minutes at room temperature. The cells were permeabilized by a 5-minute treatment with 0.05% (v/v) Triton X-100 in PBS. After the cells were washed with cold PBS, the Tat-PTEN staining, which consisted of the addition of the anti-epitope identification antibody (Xpress) followed by the FITC-conjugated antibody, was performed overnight at 4°C. The nuclei were stained with DAPI and incubated for 15 minutes at room temperature. The cells were then washed with binding buffer (10 mM

HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) and mounted. The cells were imaged using a confocal microscope and a laser scanning microscope (Zeiss LSM-510) with an optical slice of 0.8 μm. The cells were incubated overnight at 4°C with the appropriate fibronectin (Santa Cruz Biotechnology) and α-SMA (DAKO) primary antibodies that were diluted 1:100 in PBS. The cells were subsequently incubated with FITC-conjugated anti-mouse IgG (Vector Laboratories) secondary antibodies, which were diluted 1:100 in PBS for 2 hours at room temperature in the dark. The cells were then washed three times with PBS and viewed using immunofluorescence microscopy (IX71 instrument; Olympus). Negative controls were routinely included and consisted of cells that were incubated in normal buffered serum without the primary antibody.

Results

Cloning of the Human PTEN Gene and the Construction of the Expression Vector

As an initial step in the production of cell-permeable PTEN fusion proteins, the PTEN gene was amplified from the human cDNA library by PCR using the *Pfu* DNA polymerase. For the overexpression and purification of the Tat fusion proteins, we constructed a Tat-PTEN expression vector that contained the Tat protein transduction domain and the cDNA sequence encoding human PTEN (Tat-PTEN). In addition, we constructed a PTEN expression vector without the Tat protein transduction domain to test the permeability of intact PTEN. Each expression vector had six histidine residues as well as epitope identification residues (Xpress) at the amino terminus for protein purification and detection by Western blot analysis.

Expression and Purification of the Tat-PTEN and PTEN Proteins

After overexpression of the fusion proteins in *E. coli* BL21 (DE3), the Tat-PTEN, PTEN, and Tat-SOD fusion proteins were purified by affinity chromatography using a metal chelating matrix under urea-denaturing conditions. The Tat-PTEN and PTEN proteins were purified from the inclusion bodies of the bacterial cells. The Tat-PTEN and PTEN purified products were determined by SDS-PAGE analysis with Coomassie brilliant blue staining (Fig. 1A), which was further confirmed by Western blot (using an anti-Xpress antibody) (Fig. 1B). The Tat-PTEN fusion proteins migrated at an approximately 5-kDa increased molecular weight than the PTEN protein due to the presence of the Tat transduction domain.

Transduction of Tat-PTEN into Cultured Human Subconjunctival Fibroblasts

To analyze the ability of the Tat-PTEN fusion protein to transduce into human conjunctival fibroblast cells, 1 μM of the purified Tat-PTEN fusion proteins were added to the human SCF culture media for 2 hours, and the levels of the transduced proteins in the cells were determined by Western blot. In Figure 2A, the control cells are shown in lane 1, and the cells transduced with the Tat-PTEN fusion protein and immunoblotted with the anti-PTEN antibody are shown in lane 2. The results indicated that the Tat-PTEN fusion proteins were efficiently transduced into the SCF cells and that human SCF cells appear to have low baseline PTEN expression levels. The cell transduction capacity of the Tat domain was confirmed using an epitope identification antibody (Anti-Xpress), as shown in Figure 2B. The Tat-PTEN protein was efficiently delivered to subconjuctival fibroblasts, whereas no bands were detected from cells that received the control or the PTEN without the Tat domain. To determine the transduction efficiency of Tat-PTEN, the Tat-PTEN fusion proteins were added to the cells at increasing concentrations for a 2-hour transduction period. As a result, the Tat-PTEN protein was transduced into the cells in a dose-dependent manner, and the transduction was most effective at the 2-μM protein concentration (Fig. 2C). The nuclei were stained with DAPI, and the TAT-PTEN fusion proteins were stained (using the Anti-Xpress antibody) for immunofluorescence analysis (Fig. 2D).

Next, we sought to determine the extent to which the Tat-PTEN fusion proteins were maintained in the transduced cells. To do this, the Tat-PTEN fusion proteins were added to cells in a time-dependent manner. After serum starvation for 2 hours, 1 μM of the Tat-PTEN fusion protein was added to the cells for a 2-hour transfection period. Then, the cells were consecutively harvested after another 30 minutes, 5 hours, 24 hours, 48 hours, or 72 hours in culture. The stability of the transduced proteins was analyzed by Western blot (using the

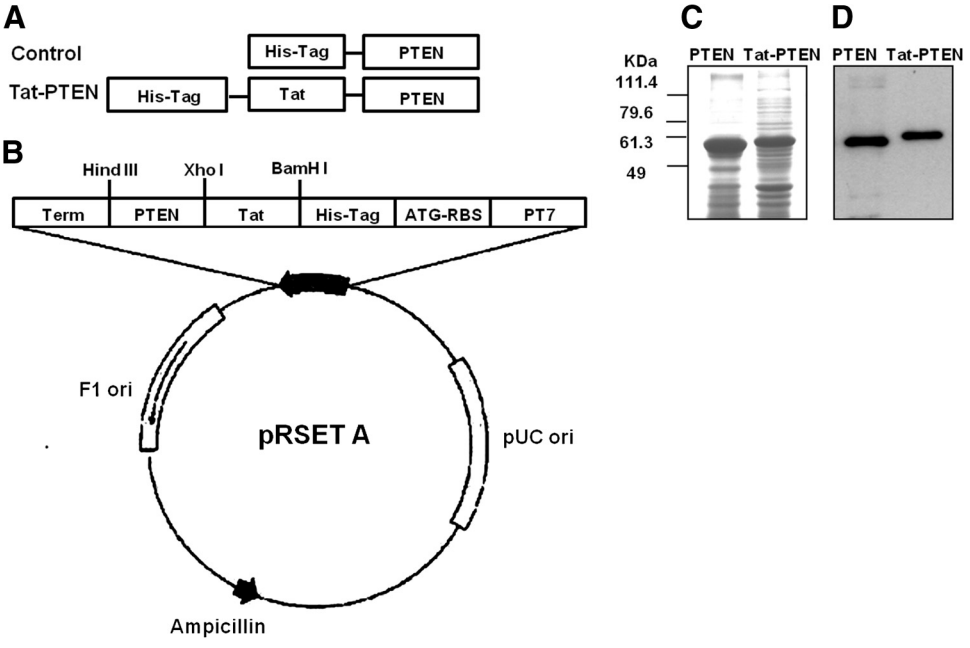

Figure 1. Cloning and purification of the Tat-PTEN fusion proteins in *E. coli* and their transduction into the SCF cells. A diagram for the controlled expression of PTEN and Tat-PTEN fusion proteins. The coding frame of human PTEN is represented by an open box and the 6His and the HIV-1 Tat basic domains (RKKRRQRRR) are shown (**A**). Synthetic oligomers that corresponded to Tat were cloned into the *Bam*HI and *Xho*I sites of the pRSET A vector to make Tat, and then human PTEN cDNA was cloned into the *Xho*I and *Hin*dIII sites of Tat. The resulting vector was named Tat-PTEN. The expression vector was under the control of the T7 promoter and ATG-RBS. Expression was induced by the addition of IPTG. Ampicillin, selection of the plasmid; F1 Ori, single-strand rescue of DNA; pUC ori, replication and growth (**B**). Purified fusion proteins were detected by Coomassie brilliant blue staining on a 10% SDS-PAGE gel (**C**) and were immunoblotted with a mouse monoclonal IgG1 antibody (**D**).

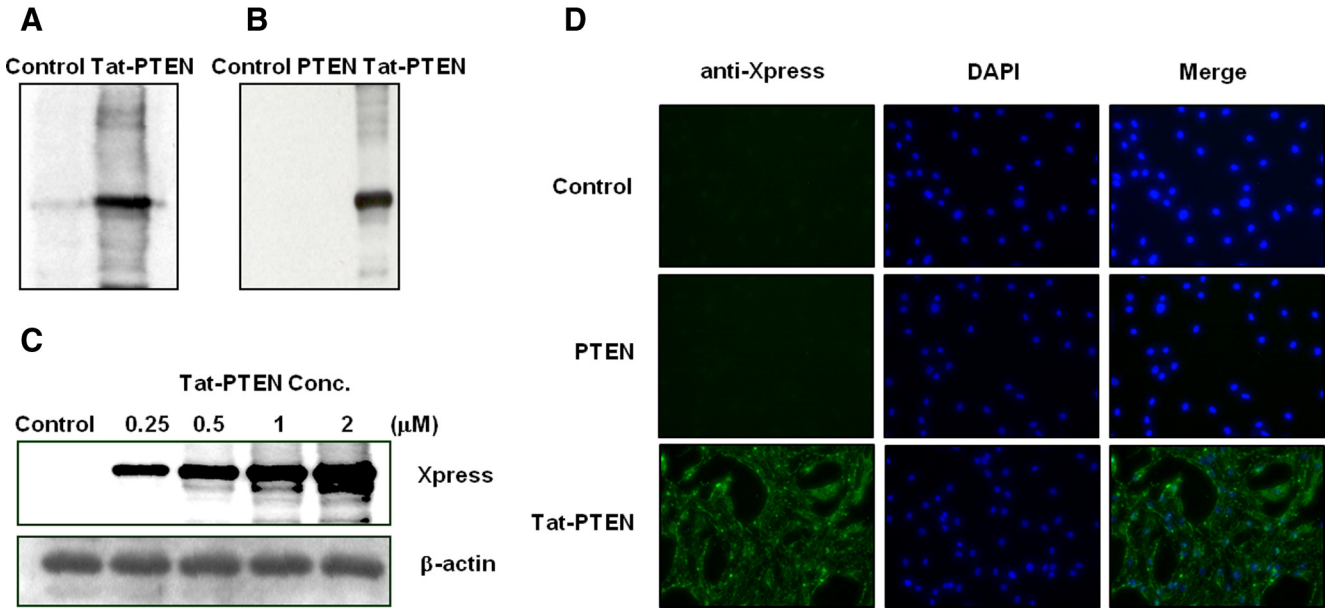

FIGURE 2. Transduction of Tat-PTEN into the SCF cell. After 2 hours of transduction with 1 μM of Tat-PTEN or PTEN, the transduction of the SCF cells was analyzed by Western blot using an anti-PTEN antibody (**A**) and a mouse monoclonal IgG1 antibody (**B**). The SCF cells were treated with 0.25, 0.5, 1, and 2 μM of the Tat-PTEN fusion proteins for 2 hours and were then immunoblotted using the mouse monoclonal IgG1 antibody. The levels of the proteins increased depending on the concentration of Tat-PTEN (**C**). Immunofluorescence staining of the TAT-PTEN transduced subconjunctival fibroblasts. The nuclei were stained with DAPI, and TAT-PTEN was stained with monoclonal IgG1 antibody (**D**).

Anti-Xpress antibody). After the initial transduction period of 2 hours, the transduced proteins were found at the highest level after another 30 minutes in culture, and their levels decreased gradually thereafter. However, the Tat-PTEN proteins persisted in the SCF cells for at least an additional 72 hours (Fig. 3).

Effect of Dose-Dependent Transduction of Tat-PTEN Fusion Proteins on Akt Phosphorylation and Tat-PTEN Fusion Protein Stability in SCF Cells

We previously demonstrated TGF-β1–induced Akt phosphorylation in SCF cells.[33] Therefore, in this experiment, we investigated the effect of TGF-β1 stimulation on the PI3K/Akt signaling pathway in Tat-PTEN–transduced SCF cells. The cells were harvested for Western blot analysis after the Tat-PTEN transduction and TGF-β1 stimulation. Treatment with TGF-β1 resulted in Akt phosphorylation, and the Tat-PTEN proteins that were transduced into the cells downregulated these TGF-β1–mediated changes (Fig. 4A). The total levels of these proteins were not affected by PTEN transduction or TGF-β1 stimulation, which indicated that PTEN fusion proteins modulate TGF-β–mediated signaling in SCF cells. The phosphorylation of Akt was downregulated by transduction of the Tat-PTEN fusion proteins in a dose-dependent fashion (Fig. 4B). The phosphorylation states of ERK1/2 and Smad2/3 were not definitely altered after the transduction of the fusion proteins (Fig. 4C).

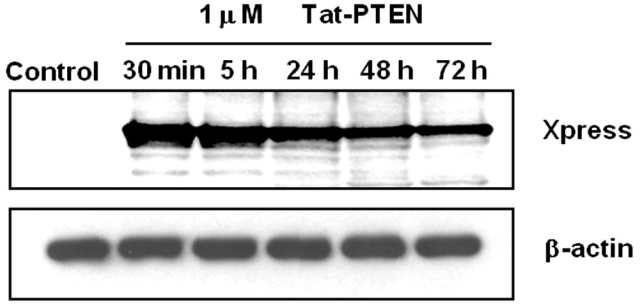

FIGURE 3. Stability of the Tat-PTEN fusion proteins in the SCF cells. After a transfection with 1 μM of Tat-PTEN protein, the level of transduced protein peaked at 30 minutes and persisted for >72 hours.

Effect of the Tat-PTEN Fusion Proteins on TGF-β1–Stimulated Expression of α-SMA and Fibronectin in Human SCF Cells

TGF-β is known to drive the conversion of fibroblasts into myofibroblasts, which is a key step for all fibrotic processes.[6] To analyze the effect of the Tat-PTEN fusion protein on TGF-β1–stimulated expression of α-SMA and fibronectin in human SCF cells, 1 μM of the fusion protein was added to the human SCF cell culture media. After 2 hours of fusion protein transduction, the cells were treated with or without TGF-β1, and expressions of α-SMA and fibronectin were analyzed. The Western blot analysis and immunocytochemical staining demonstrated that the transduction of the Tat-PTEN fusion protein reduced the expression of both α-SMA and fibronectin in the SCF cells (Figs. 5A, 5B).

DISCUSSION

Excess scarring of the conjunctiva after glaucoma filtration surgery is a major cause of the failure of surgical treatments.[3,34] Treatment with antimetabolites, such as mitomycin C and 5-fluorouracil, could decrease the frequency of scar formation and improve the surgical outcome. However, these drugs could be associated with serious complications such as bleb leakage, hypotonus maculopathy, and infective endophthalmitis.[35,36] Moreover, the response of individual patients to these agents can be idiosyncratic, making dose titration difficult.[37] These findings prompted us to consider the need for novel agents that can efficiently increase the surgical outcome with less toxicity and fewer complications.

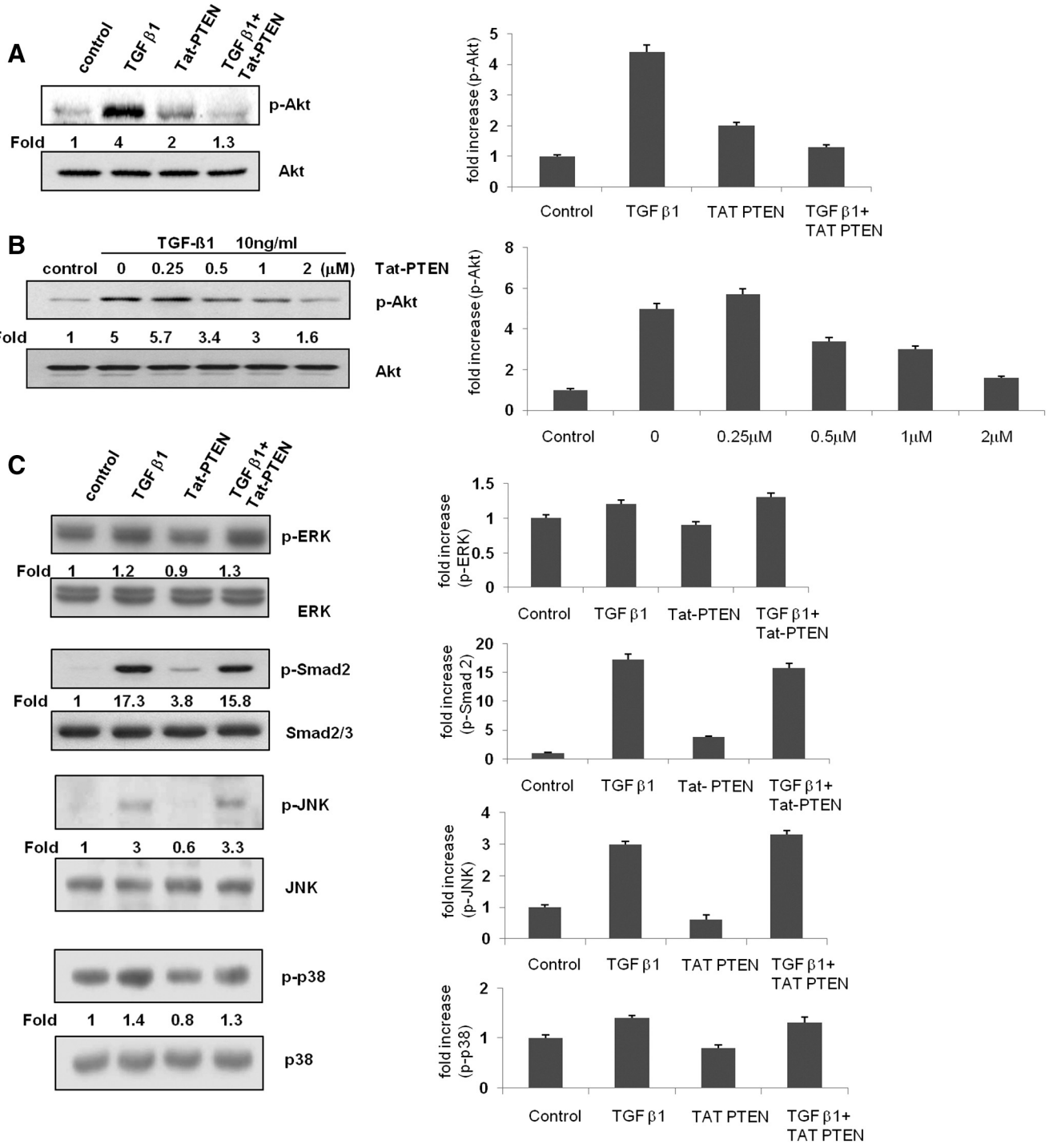

FIGURE 4. The effects of Tat-PTEN transduction on the phosphorylation of Akt in SCF cells stimulated by TGF-β1. After 2 hours of Tat-PTEN transduction, the cells were treated with or without TGF-β1 and were then immunoblotted using an anti-phospho-Akt antibody. The phospho-Akt that was stimulated by TGF-β1 was downregulated after the transduction of the Tat-PTEN fusion proteins into the SCF cells. (**A**) The dose-dependent effect of Tat-PTEN fusion protein transduction on the phosphorylation of Akt by the SCF cells. The phospho-Akt (Ser473) that was stimulated by TGF-β1 was downregulated in a dose-dependent manner by the transduction of the Tat-PTEN fusion proteins, irrespective of Akt expression (**B**). TGF-β1 also elevated the phosphorylation states of ERK1/2, p38, JNK, and Smad 2/3, which were not altered after the transduction of the fusion proteins (**C**). Results are representative of those in three repeated experiments.

The conversion of fibroblasts into myofibroblasts, which are the cellular protagonists of fibrosis, requires the cytokine TGF-β.[12] TGF-β is a major target of current antifibrotic treatment strategies, as approaches using scavenging antibodies, endogenous antagonists, and nucleotide-based methods have been demonstrated.[5,38–40] The PI3K/Akt pathway is a downstream mediator of TGF-β signaling that is involved in the establishment of fibrotic fibroblasts, and its activity is antagonized directly by PTEN, which dephosphorylates PIP3 at the D3 position of the inositol ring.[13–15,21] In light of these findings, we assessed the effect of Tat-PTEN fusion protein transduction on TGF-β–induced myofibroblast transdifferentiation. Our data

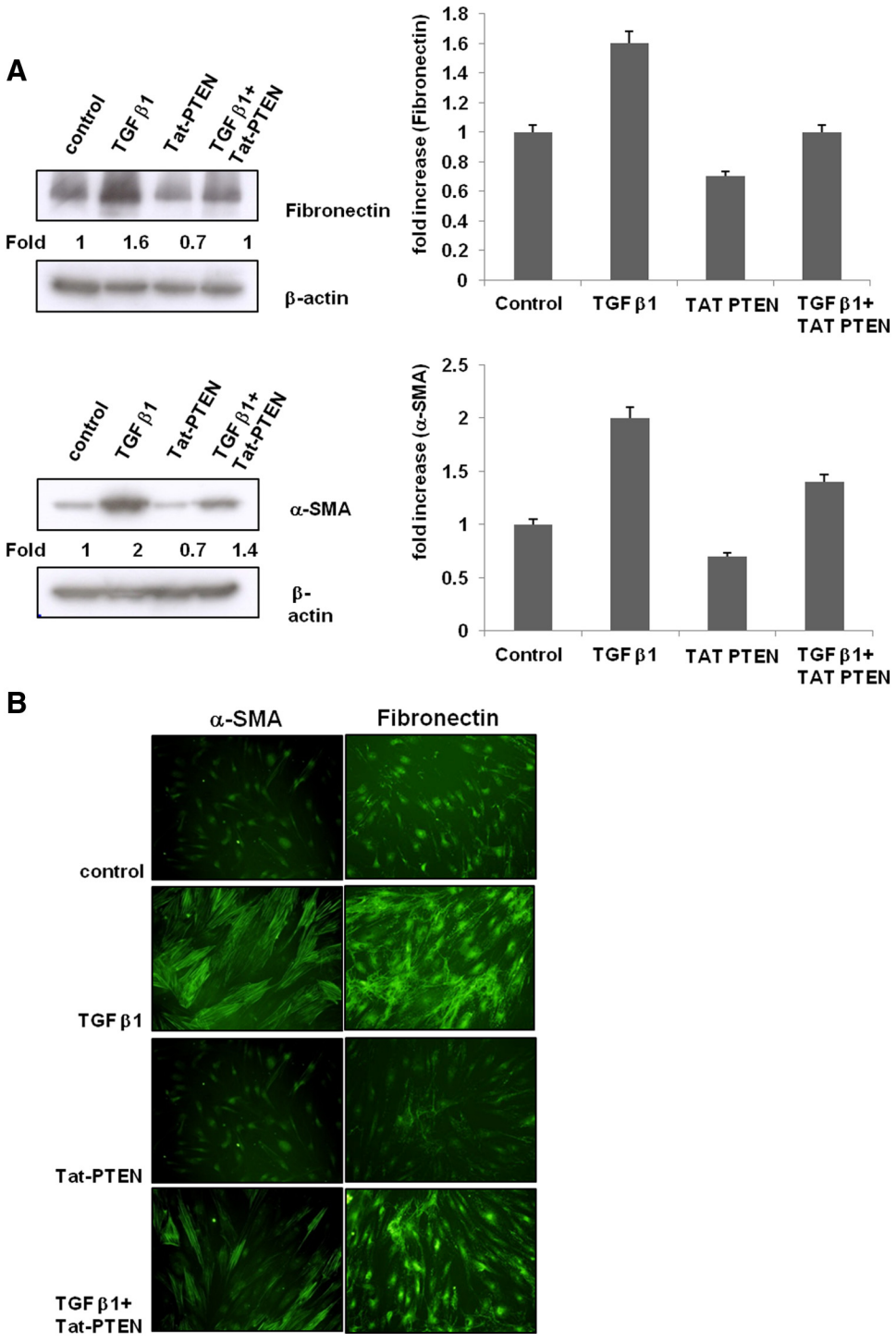

FIGURE 5. Effect of Tat-PTEN fusion protein on the TGF-β1–stimulated expression of α-SMA and fibronectin in human SCF cells. After 2 hours of fusion protein transduction (1 μM), the cells were treated with or without TGF-β1, and expressions of α-SMA and fibronectin were analyzed. The transduction of the Tat-PTEN fusion proteins significantly reduced the expression of both α-SMA and fibronectin in the SCF cells as assessed by immunoblotting (**A**) and immunocytochemical staining (**B**). Results are representative of those in three repeated experiments.

indicate that transduction with the Tat-PTEN fusion protein blocks this process in human SCF cells through the inhibition of the PI3K/Akt signaling pathway.

For this study, we used TGF-β1–stimulated SCF cells that had been transduced with Tat-PTEN fusion proteins in vitro as a model for the antiscarring process.[41–43] To transfer and control the enzymatic activity of PTEN in cells, we developed the Tat domain transduction approach for protein delivery that has been previously described.[30] The purified Tat-PTEN fusion protein efficiently entered the SCF cell within the 2-hour initial treatment period, although the PTEN protein without the Tat domain could not. The level of fusion protein transduction into the SCF cells could be increased in a dose-dependent manner, and the peak transduction was determined to occur at a protein concentration of 2 μM.

Of the signaling pathways that are triggered by TGF-β1, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2) activation has been shown to be involved in the TGF-β1–induced phenotypic transformation of human lung fibroblasts into myofibroblasts.[44,45] Furthermore, the activity of the phosphoinositide 3-kinase/Akt pathway, which regulates a wide variety of cellular processes, including cell proliferation, metabolism, migration, and survival, is also involved in the establishment of fibrotic fibroblasts by inhibit-

ing the functions of PTEN.[13–15] To investigate the signals that are involved in the modulation of TGF-β1 signaling by the Tat-PTEN fusion protein, we examined the phosphorylation status of key molecules that are involved in proliferation signaling pathways by Western blot. The addition of TGF-β1 (10 ng/mL) elevated the phosphorylation status of Akt, ERK1/2, and p38 MAPK. These results confirm previous findings that have demonstrated that the mitogenic activity of TGF-β results in fibroblast-mediated collagen contraction, proliferation, and migration.[46,47] The phosphorylation states of ERK1/2, JNK, p38, and Smad2/3 were not definitely altered after the transduction of the fusion proteins. However, the transduction of the Tat-PTEN fusion proteins decreased the TGF-β1–stimulated expression of phospho-Akt. The phosphorylation status of Akt was lower in the absence of TGF-β1 than for the control, and this was regardless of whether the levels of these proteins were constant. These results imply that exogenous PTEN protein can effectively block the TGF-β1 signal.

As shown here and in previous reports, the activity of PI3K/Akt is antagonized directly by PTEN. PTEN has been implicated in myofibroblast transdifferentiation because distinct inhibition or loss of PTEN expression in fibroblasts has been correlated with α-SMA expression from lung biopsy samples from patients with idiopathic pulmonary fibrosis or usual interstitial pneumonia. In vitro, myofibroblast differentiation has also been shown to be inhibited by PTEN, and the in vivo inhibition of PTEN promotes fibrosis.[13–15,48] To evaluate the influence of an exogenous Tat-PTEN fusion protein on TGF-β1–stimulated structural changes in SCF cells, we assessed the expression of the myofibroblast markers α-SMA and fibronectin. Tat-PTEN fusion protein transduction inhibited TGF-β1–stimulated myofibroblast differentiation and ECM production, and this was determined by Western blotting and by immunocytochemical staining for α-SMA and fibronectin. White et al.[14] also demonstrated that myofibroblasts have diminished PTEN expression, that inhibition of PTEN in vivo promotes fibrosis, and that PTEN inhibits myofibroblast differentiation in vitro.

As a model system representing the antiscarring process in subconjunctival fibroblasts, we developed a fusion protein that consisted of the PTEN tumor suppressor gene fused to the Tat transduction domain of HIV-1. Our results indicated that the Tat-PTEN fusion protein was successfully delivered into the cells, whereby it suppressed the transdifferentiation of human SCF cells by modulating TGF-β1 signaling in a relatively short time period. Nagahara et al.[30] reported the ability of TAT-p27Kip1 proteins to be transduced directly into almost 100% of primary or transformed cells, and this resulted in induced cell migration and represented arbitrary control over the intracellular processes. The potent capacity of the Tat-PTEN fusion proteins to suppress myofibroblastic transdifferentiation could potentially be very useful for protein therapy. Although gene therapy relies on the transfer of a viral or nonviral vector into cells, this method using a Tat-PTEN fusion protein does not rely on a vector and could be easily adapted for use with a variety of cell types and for applications such as cancer therapy. The exact mechanism behind the inhibition of TGF-β1–mediated transdifferentiation in SCF cells remains to be elucidated by further investigations. However, our results should encourage further in vivo studies regarding efficacy, toxicity, and the potential role of Tat-PTEN fusion protein as a means to prevent bleb failure in glaucoma filtration surgery.

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