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Gene transfer of Smad7 modulates injury-induced conjunctival wound healing in mice

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Purpose: Smad7 is a molecule that blocks the Smad2/3 signal. Herein, we examined the effects of Smad7 gene introduction on post-injury conjunctival wound healing in mice. Its effects on the cultured human subconjunctival fibroblasts (SCFs) were also investigated.

Methods: A circumferential incision was made in the equatorial conjunctiva by using scissors in the right eye of fully anesthetized adult C57BL/6 mice (n=72). Smad7 cDNA-expressing adenoviral vector was topically applied. The control eye received nonfunctioning adenoviral vector. After 2, 5, 7, and 30 days the eyes were processed for histological or immunohistochemical examination to evaluate wound healing of conjunctiva. The expressions of type-I collagen and growth factors were evaluated by real time-reverse transcriptase-polymerase chain reaction. The effects of Smad7 gene introduction on the cultured human SCFs were also studied.

Results: Marked Smad7 protein expression was detected in the vector-treated conjunctival epithelium and fibroblasts that coincided with green fluorescein protein expression, whereas faint endogenous Smad7 expression was observed in the control tissue. In vivo Smad7 gene introduction blocked Smad2/3 nuclear translocation with suppression of α-smooth muscle actin (αSMA) and vascular endothelial growth factor (VEGF) in fibroblasts and invasion of macrophages. Smad7 gene transfer suppressed mRNA expressions of connective tissue growth factor (CTGF), VEGF, and monocyte chemoattractant protein-1 in vivo and those of type-I collagen, αSMA, and CTGF in vitro.

Conclusions: Smad7 gene transfer modulates injury-induced wound healing of conjunctival tissue in mice, suggesting that this strategy may be effective in preventing excessive scarring following filtration surgery. The mechanism might include suppression of activation of fibroblasts and reduction of macrophage invasion.

Excess scarring of the conjunctiva potentially causes reduction in the filtration efficacy following glaucoma filtering surgery. It is well established that transforming growth factorβ (TGFβ) is closely involved in scarring of the conjunctiva [1-4].

The TGFβ family has three isoforms: namely, β1, β2, and β3. Each isoform of TGFβ has many abilities to regulate numerous cell functions, such as proliferation, differentiation, apoptosis, epithelial-mesenchymal transition, and production of extracellular matrix [5,6]. The aqueous humor contains abundant TGFβ2, while TGFβ1 and TGFβ2 are expressed in the local cells in the filtering bleb [2,7]. Thus, it might be more effective to block all TGFβ family members instead of targeting each TGFβ isoform. Each isoform of TGFβ propagates its signal via a signal transduction network, such as mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK), p38MAPK, C-Jun-N-terminal kinase (JNK), and Smad, involving receptor serine/threonine kinases at the cell surface and their substrates [5,6,8,9]. Among these pathways, we especially focused on Smad because of its close correlation with fibrosis induced by TGFβ. We previously described blocking of Smad signaling via deletion of Smad3 by gene targeting or adenoviral gene introduction of Smad7, an inhibitory Smad that is capable of blocking Smad2/3 signal [10,11]. Regardless of the ligand isoform, blocking Smad2/3 signal is considered to suppress TGFβ/Smad signal, allowing us to pay no attention to the tissue-specific distribution of each TGFβ isoform. Moreover, another signaling pathway that may potentially activate TGFβ is the p38MAPK pathway, which is required for epithelial cell migration [12]. Thus blocking TGFβ stimuli at the ligand or receptor level may potentially impair epithelial healing on the ocular surface, thereby supporting the potential advantage of blocking TGFβ signal at the Smad signaling level.

We hypothesized that introduction of the Smad7 gene might modulate injury-induced conjunctival wound healing and provide a potential therapy to inhibit excessive bleb scarring in the conjunctiva following glaucoma surgery. In the present study, to evaluate the effects of Smad7 gene transfer on tissue fibrogenic reaction during conjunctival wound healing, we used a mouse model of injury-induced conjunctival wound healing and cultured human subconjunctival fibroblasts (SCFs).

METHODS

All experimental procedures were approved by the DNA Recombinant Experiment Committee as well as the Animal Care and Use Committee of Wakayama Medical University, Wakayama, Japan, and conducted in accordance with the

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guidelines of the Association for Research in Vision and Ophthalmology for the Use of Animals in Ophthalmic and Vision Research and Declaration of Helsinki.

Adenoviral vector construction and virus purification: We used the Adenoviral Cre/LoxP-regulated Expression Vector Set (catalog number 6151; Takara, Tokyo, Japan) to generate recombinant adenovirus as previously reported [10,11]. In brief, cosmid pAxCALNLmSmad7 was constructed by insertion of mouse *Smad7* cDNA (Smad7-Ad). Using the COS-TPC method, we generated the recombinant adenovirus of AxCALNLmsmad7 by transfecting 293 cells with pAxCALNLSmad7. AxCANCre (Cre-Ad) was generated by transfecting 293 cells with Ax-CALNLCreDNA-TPC as described in the manufacturer's protocol. Each adenovector was used at a concentration of $2x10^7$ PFU/μl. When these two viral vectors co-infect cells, Cre recombinase expressed under the CAG promoter (cytomegalovirus enhancer, chicken β-actin promoter plus a part of the 3' untranslated region of rabbit β-globin) activates the stuffer PoliA through the Cre/LoxP system.

Primary cell culture of human subconjunctival fibroblasts and Smad7 gene introduction to the cells in vitro: Primary culture of human SCFs was conducted by following previously reported directions [13]. In brief, redundant subconjunctival connective tissue was obtained from patients aged 5 to 8 years during strabismus surgery, after informed consent was obtained from the parents of each patient. This tissue was exoplanted for cell outgrowth in a 25 ml culture bottle (Falcon, Becton Dickinson, Lincoln Park, NJ) and incubated until confluent in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, antibiotics and an antimycotic (MEM-10). After 2 or 3 passages, the cells were trypsinized for seeding for the following experiments.

The efficacy of gene transfer was evaluated by co-infection of Cre-Ad and green fluorescent protein (GFP) under control of the Cre/LoxP system (GFP-Ad). The expressions of GFP in the cells were evaluated in unfixed cultured cells using fluorescent microscopy.

The cells ($5.0x10^5$/ml in 60x15 mm culture dishes, Becton Dickinson Labware, Franklin Lakes, NJ), or $7.4x10^5$/ml, 16 well chamber slides (Nalge Nunc International, Naperville, IL) were cultured in MEM-10 until confluence. The cells were then incubated for 2 h in a serum-free medium containing Cre-Ad or both Cre-Ad and Smad7-Ad at a concentration of $4x10^3$ PFU/ml, and then incubated for another 48 h in MEM-10. The cells were then exposed to 10 ng/ml of recombinant human TGFβ1 (R&D systems, Minneapolis, MN) for 48 h with serum-free MEM, and were processed for immunohistochemistry, immunoassay, and western blotting as will be described.

Effects of exogenous Smad7 on the expression of type-I collagen, α smooth muscle actin (αSMA), and connective tissue growth factor (CTGF) by cultured human SCFs: Evaluation of mRNA for type-I collagen alpha-1 chain (COL1A1) and connective tissue growth factor (CTGF) was conducted by real-time reverse transcription-polymerase chain reaction (RT-PCR) following guidelines previously reported in the literature [10,11]. The total RNA from the cultured cells was extracted using ISOgene (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol and processed for semiquantitative RT-PCR for mRNA of the human COL1A1 and CTGF.

RT-PCR was performed by using the Taqman One-Step RT-PCR Master Mix Reagents Kit and the Applied Biosystems Prism 7700 (PE Applied Biosystems, Foster City, CA) as previously reported [11,12]. Primers and oligonucleotide probes were designed according to the cDNA sequences in the GeneBank database, using Primers Express software (PE Applied Biosystems) and listed in Table 1. RT-PCR conditions were as follows: 20 min at 50 °C (stage 1, reverse transcription), 10 min at 95 °C (stage 2, reverse transcription inactivation and AmpliTaq Gold activation), and then 40 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C (stage 3, polymerase chain reaction).

The concentrations of type-I collagen protein were immuno-assayed by employing a commercially available immunoassay kit (Takara) as previously reported [13]. In brief, cells were seeded into the wells of a 24 well cell culture plate

Table 1.

Transcript	Sequence
hCTGF	
	F: 5'-GCCCAAGGACCAAACCGT-3'
	R: 5'-GGACCAGGCAGTTGGCTCTA-3'
	P: 5'-AAGACACGTTTGGCCCAGACCCAACT-3'
hCOL 1A1	
	F: 5'-GCCGATGTGGCCATCCAG-3'
	R: 5'-TGCAGTGGTAGGTGATGTTC-3'
	P: 5'-CTGCGCCTGATGTCCACCGAGG-3'

Transcript and sequence of each primer and probe used in real time RT-PCR. In the table, F indicates forward primer, R indicates reverse primer, and P indicates probe.

Table 2.

Transcript	Sequence
mCTGF	
	F: 5'-AAGGGCCTCTTCTGCGATTT-3'
	R: 5'-TTTGGAAGGACTCACCGCTG-3'
	P: 5'-CCTGTGTCTTCGGTGGGTCGGTGTAC-3'
mTGFβ1	
	F: 5'-GCAACATGTGGAACTCTACCAGAA-3'
	R: 5'-GACGTCAAAAGACAGCCACTC-3'
	P: 5'-ACCTTGGTAACCGGCTGCTGACCC-3'
mMCP-1	
	F: 5'-TGGCTCAGCCAGATGCAGT-3'
	R: 5'-CCAGCCTACTCATTGGGATCA-3'
	P: 5'-CCCCACTCACCTGCTGCTACTCATTCA-3'
mVEGF	
	F: 5'-AGCGGAGAAAGCATTTGTTTG-3'
	R: 5'-CAACGCGAGTCTGTGTTTTTG-3'
	P: 5'-CCAAGATCCGCAGACGTGTAAATGTTCC-3'

Transcript and sequence of each primer and probe used in real time RT-PCR. In the table, F indicates forward primer, R indicates reverse primer, and P indicates probe.

(Becton Dickinson) and incubated until confluence. After infection of each adenoviral vector, the cells in each well were then treated with Eagle's MEM or TGFβ1, and the concentration of type-I collagen was measured. The medium was supplemented with β-aminopropionitrile fumarate, a lysyl oxidase inhibitor, to prevent the collagen peptides from being deposited in the cell layer.

αSMA expression was evaluated by western blotting and immunohistochemistry following previously reported guidelines [10,11]. After infection of each adenoviral vector, the cells in each 60 mm dish were then treated with Eagle's MEM or TGFβ1 and evaluated. For western blotting, the cells in each 60 mm dish were homogenized in a lysis buffer (100 μl; CelLytic™-M Mammalian Cell lysis/Extraction Reagent, Sigma, St. Louis, MO) supplemented with a cocktail of proteinase inhibitors (complete protease inhibitor cocktail tablet, Rosch, Mannheim, Germany). The cell lysate was centrifuged, mixed with 3X sample buffer, run on SDS-polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride (PVDF) membrane, and western blotted for αSMA with an antibody purchased from Sigma. Immunoreactive bands were visualized using a Lumino Analyzer LAS 1000 (Fuji Film, Tokyo, Japan) and ECL western blotting detection reagents (Amersham, UK).

For immunohistochemistry, cells were fixed in cold acetone for 5 min. Indirect immunostaining was carried out following previously reported directions [13]. The primary antibodies were mouse monoclonal anti-human type-I collagen (1:100 in phosphate-buffered saline [PBS], Fuji Chemical, Toyama, Japan), goat polyclonal anti-CTGF (1:100 in PBS, Santa Cruz Biotechnology, Santa Cruz, CA), and mouse polyclonal anti αSMA (1:100 in PBS, Sigma). Fluorescein isothiocyanate (FITC)-conjugated specific secondary antibodies (1:100 in PBS, Cappel, Aurora, OH) were used for detection of the primary antibodies, and 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc. Burlingame, CA) was used for nuclear counterstaining.

Conjunctival injury in C57BL/6 mice and Smad7 gene transfer to injured conjunctival tissue in vivo: The efficacy of gene transfer was evaluated by coinfection of Cre-Ad and GFP-Ad. The expression of GFP in the uninjured mouse conjunctiva was evaluated in unfixed cryosections using fluorescence microscopy.

A circumferential incision was made in the conjunctiva at the equator using scissors in the right eye of adult C57BL/6 mice (n=72) under general anesthesia. A mixture of Cre-Ad and Smad7-Ad was administered (3 μl) once after incision (Smad7-Ad group). Preliminary experiments showed that there were no obvious differences in the histological features or in healing at the microscopic level in the mechanically injured mouse eye with CAG/Cre virus (Cre-Ad group) or without application of adenovirus carrying Cre (no vector group). Thus, the eyes of the Cre-Ad group were used as controls in our current study. On days 2, 5, 7, and 30 (each n=24), the eyes were enucleated and processed for histological or immunohistochemical examination to evaluate the conjunctival wound healing.

Histology and immunohistochemistry in vivo: Deparaffinized sections (5 μm thick) were processed for indirect immunofluorescence microscopy using previously reported directions [11,12]. The primary antibodies used were

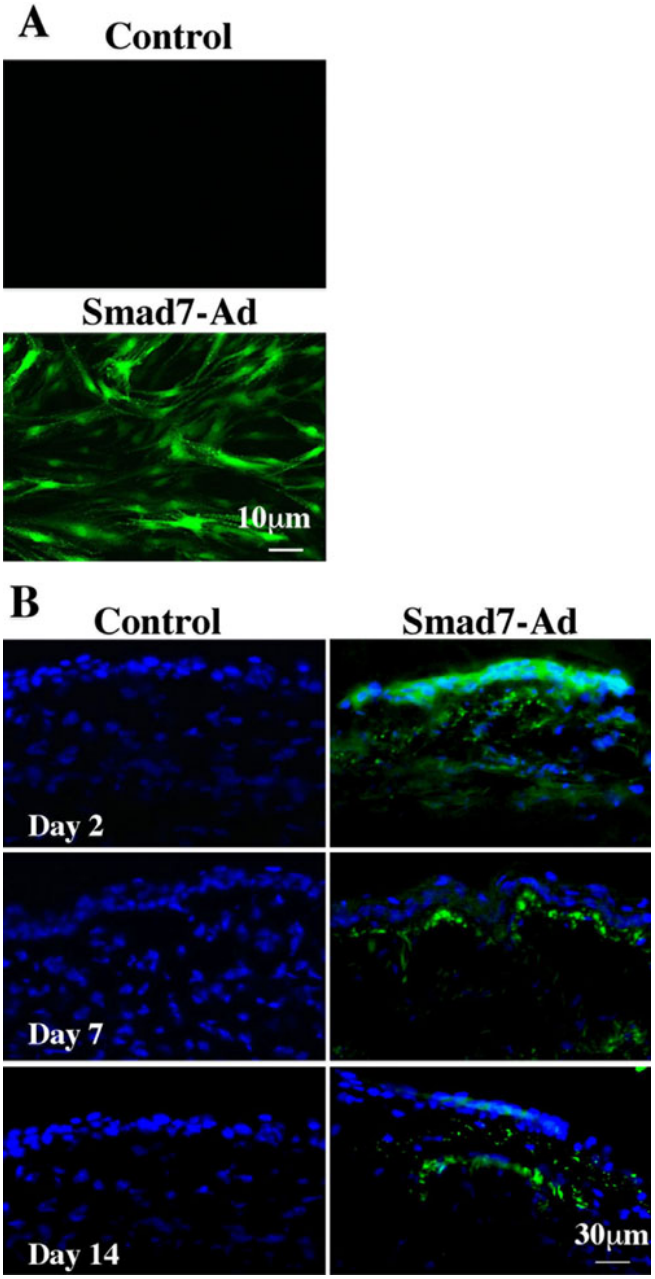


Figure 1. GFP expression of normal conjunctiva in vitro and in vivo. When the cultured human subconjunctival fibroblasts (SCFs) were coinfected with adenoviral vectors encoding Cre under control of the CAG promoter and GFP under control of Cre-LoxP system, GFP was observed on day 2 and later in cytoplasm and nucleous in human SCFs. No fluorescence was detected in cultured human SCFs with CAG-Cre vector only (**A**). As for gene introduction in vivo, GFP was detected in conjunctival fibroblasts and epithelium of normal mice without mechanical injury at day 2 and later (until 14 days). No fluorescence was detected in normal eyes with CAG-Cre vector only (**B**).

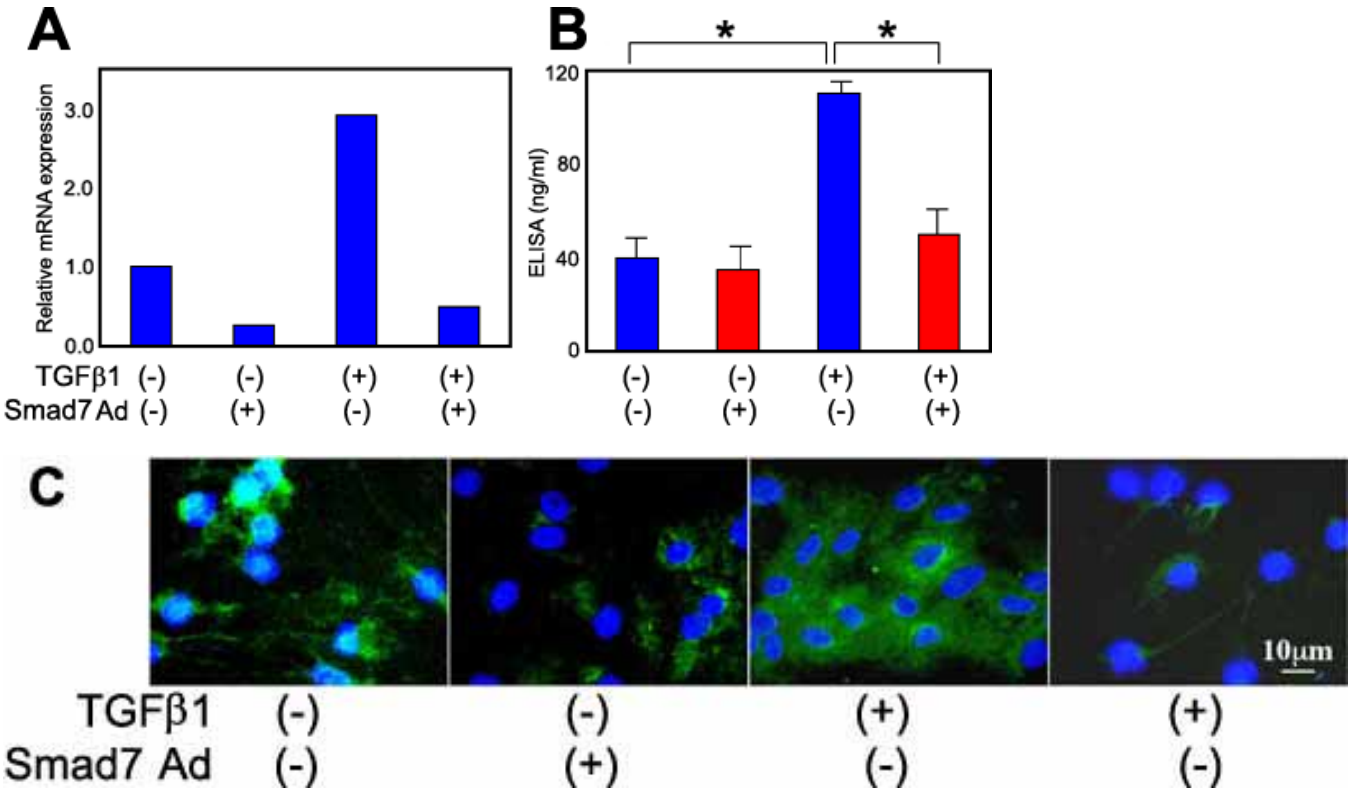


Figure 2. Effects of Smad7 gene introduction on expression of type-I collagen by cultured SCFs. Real-time RT-PCR (**A**) or an ELISA (**B**) showwd an increment of expression of mRNA or protein of type-I collagen in the presence of exogenous TGFβ1 and its reversal by Smad7 gene introduction. The asterisk indicates a $p<0.01$. **C**: Immunoreactivity for type-I collagen was detected in the majority of cells in control cultures (negative for both TGFβ1 and Smad7) and more intense reactivity was seen in the cells treated with TGFβ1 only (TGFβ1 positive, Smad7 negative). Smad7 gene introduction decreased immunoreactivity for type-I collagen in the cells in the absence (TGFβ1 negative, Smad7 positive) and presence (TGFβ1 positive, Smad7 positive) of TGFβ1. The scale bar represents 10 μm.

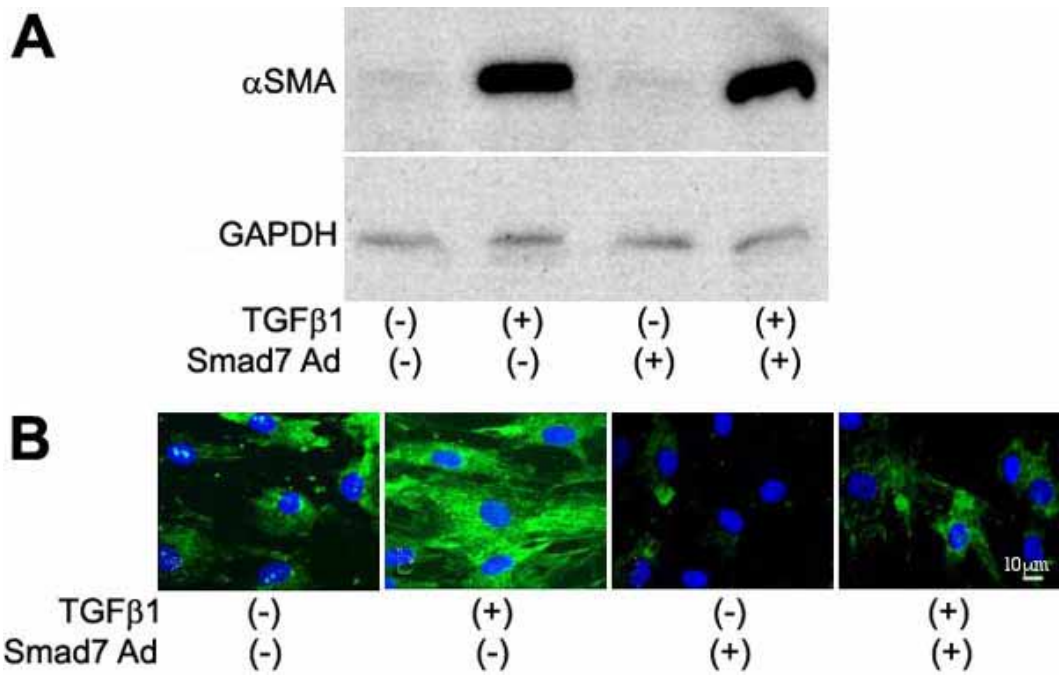


Figure 3. Effects of Smad7 gene introduction on expression of αSMA by cultured human SCFs. Whereas western blotting did not show a reduction of protein expression of αSMA (**A**), immunocytochemical expression of αSMA-labeled cytoplasmic fibers was detected in the majority of cells in control cultures (**B**, TGFβ1 and Smad7-Ad negative) and more intense reactivity is seen in the cells treated with TGFβ1 (**B**, TGFβ1 positive and Smad7-Ad negative). Smad7 gene transfer decreased immunoreactivity for αSMA in the cells in the absence (**B**, TGFβ1 negative and Smad7-Ad positive) and presence (**B**, TGFβ1 positive and Smad7-Ad positive) of TGFβ1. The scale bar represents 10 μm.

antibodies against rabbit polyclonal antiphosphorylated Smad2 antibody (1:50 dilution; Chemicon, Temecula, CA), rabbit polyclonal anti-Smad3 antibody (1:100 dilution in PBS; Zymed, San Francisco, CA), goat polyclonal anti-Smad7 antibody (1:200 in PBS; Santa Cruz Biotechnology), rat monoclonal F4/80 anti-macrophage antigen antibody (Clone A3-1, 1:200 dilution in PBS; BMA Biomedicals, Augst, Switzerland), goat polyclonal anti-VEGF antibody (1:100 dilution in PBS; Santa Cruz), goat polyclonal anti-CTGF antibody (1:100 dilution in PBS; Santa Cruz), and mouse monoclonal anti-αSMA antibody (1:100 dilution in PBS; Neomarker, Fremont, CA). The reaction with FITC-conjugated secondary antibodies and DAPI nuclear staining were performed as described above. The histological features were observed after staining the tissues with hematoxyline and eosin (H&E).

mRNA expression: For RNA extraction and real-time RT-PCR, mechanically injured eyes from eight mice were obtained from each treatment group. The animals were killed on days 2, 5, 7, and 14 using both CO_2 asphyxia and cervical dislocation, and the treated eyes were enucleated. The lens, uveal tissue, and retina were removed from the enucleated eye and processed for total RNA extraction. The untreated eyes of C57BL/6 mice were also enucleated to determine baseline mRNA expression. Total RNA extraction and real-time RT-PCR for mRNAs of the mouse CTGF, TGFβ1, MCP-1, and VEGF were performed as already described. The primers and oligonucleotide probes were designed according to the cDNA sequences in the GeneBank database using Primers Express software (P-E Applied Biosystems) and are listed in Table 2.

RESULTS

Gene introduction efficacy in vitro and in vivo as examined by GFP expression: The efficiency of gene transfer was evaluated by coinfection of adenoviruses carrying Cre under control of the CAG promoter and GFP under control of the Cre/LoxP system.

When the cultured human SCFs were co-infected with Cre-Ad and GFP-Ad, GFP was readily observed in the cells on day 2 and later (Figure 1A).

As for gene introduction in vivo, GFP was detected in the conjunctival fibroblasts and epithelium of normal mice without mechanical injury until day 14 (Figure 1B). No fluorescence was detected in the normal eyes with Cre-Ad only.

Effects of Smad7 gene introduction on the expressions of type-I collagen, αSMA, and CTGF by cultured human SCFs: We employed TGFβ1-treated fibroblast culture to mimic scarring tissue fibroblasts in vitro. Real-time RT-PCR (Figure 2A) and ELISA (Figure 2B) also showed an increment of the expression of protein or mRNA of type-I collagen in the presence of exogenous TGFβ1 and its reversal by Smad7 gene introduction. TGFβ1 enhanced the secretion of type-I collagen into the culture medium. The concentration of type-I collagen in the TGFβ1-treated culture was 275% as compared to that component in the control culture. Smad7 gene transfer reduced the production of type-I collagen in the TGFβ1-treated cells to near the control level (Figure 2B).

Immunoreactivity for type-I collagen was detected in the cytoplasm pattern in the majority of cells in the control culture (Figure 2C, TGFβ1 and Smad7-Ad negative), and more intense reactivity was seen in the cells treated with TGFβ1 (Figure 2C, TGFβ1 positive and Smad7-Ad negative). Smad7 gene introduction decreased the immunoreactivity for type-I collagen in the cells in the absence (Figure 2C, TGFβ1 negative and Smad7-Ad positive) and presence (Figure 2C, TGFβ1 positive and Smad7-Ad positive) of TGFβ1.

While western blotting did not show any reduction of protein expression of αSMA (Figure 3A), immunocytochemical expression of αSMA-labeled cytoplasmic fibers was detected

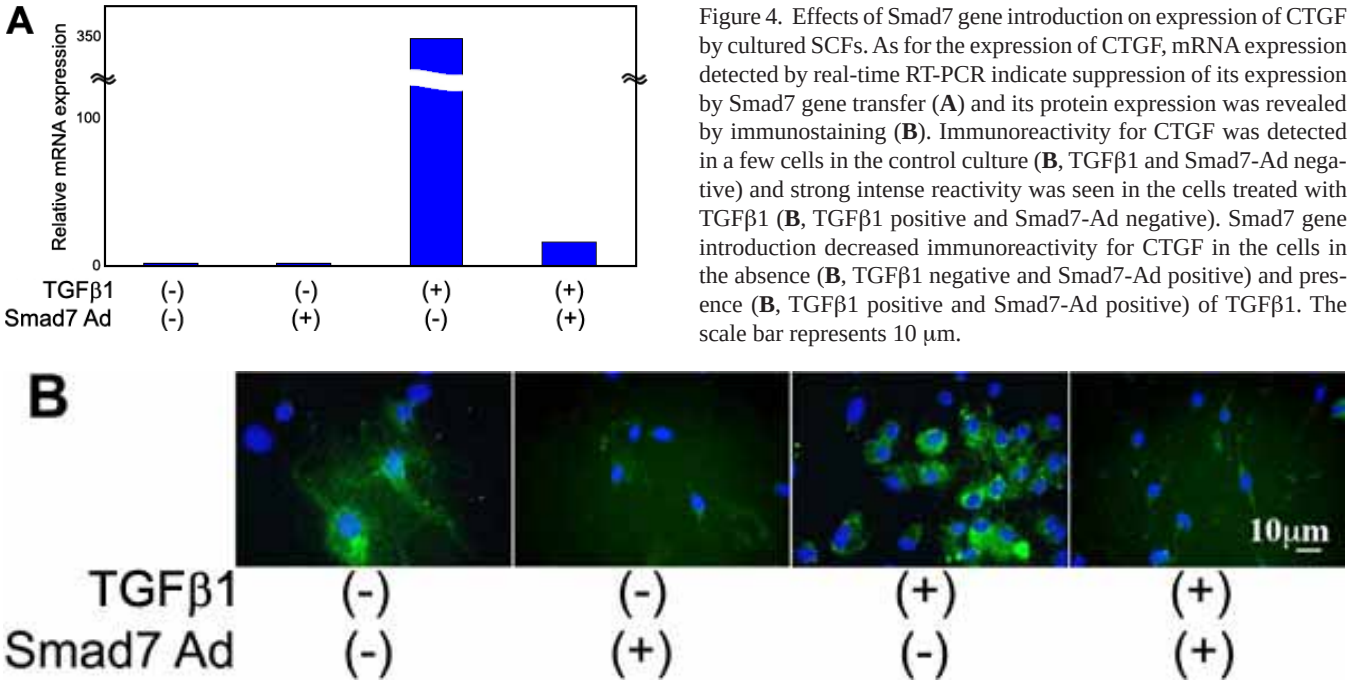


Figure 4. Effects of Smad7 gene introduction on expression of CTGF by cultured SCFs. As for the expression of CTGF, mRNA expression detected by real-time RT-PCR indicate suppression of its expression by Smad7 gene transfer (**A**) and its protein expression was revealed by immunostaining (**B**). Immunoreactivity for CTGF was detected in a few cells in the control culture (**B**, TGFβ1 and Smad7-Ad negative) and strong intense reactivity was seen in the cells treated with TGFβ1 (**B**, TGFβ1 positive and Smad7-Ad negative). Smad7 gene introduction decreased immunoreactivity for CTGF in the cells in the absence (**B**, TGFβ1 negative and Smad7-Ad positive) and presence (**B**, TGFβ1 positive and Smad7-Ad positive) of TGFβ1. The scale bar represents 10 μm.

in the majority of cells in the control culture (Figure 3B, TGFβ1 and Smad7-Ad negative), and more intense reactivity was seen in the cells treated with TGFβ1 (Figure 3B, TGFβ1 positive and Smad7-Ad negative). Smad7 gene transfer decreased the immunoreactivity for αSMA in the cells in the absence (Figure 3B, TGFβ1 negative and Smad7-Ad positive) and presence (Figure 3B, TGFβ1 and Smad7-Ad positive) of TGFβ1.

Alterations of mRNA expression and immunoreactivity for CTGF were similar to those of type-I collagen. mRNA expression, as detected by real-time RT-PCR, and protein expression, as revealed by immunostaining, were both suppressed by Smad7 gene transfer (Figure 4).

Histology and immunohistochemistry in vivo: H&E staining showed that Smad7 gene introduction also apparently suppressed the degree of conjunctival edema and inflammatory cell infiltration as compared to the Cre-Ad group (Figure 5A-P). Whereas the epithelial defect was not closed on day 5 in the Cre-Ad group (Figure 5E,F), the conjunctival epithelial defect was sealed as early as day 5 in the Smad7-Ad group (Figure 5G,H). On day 7, the epithelial defect was closed in both groups (Figure 5I-L). On day 30, however, H&E histology of both groups of specimens exhibited similar findings: reduced cell population and reduction of the thickness of subconjunctival matrix (Figure 5M-P). Polymorphonuclear leu-

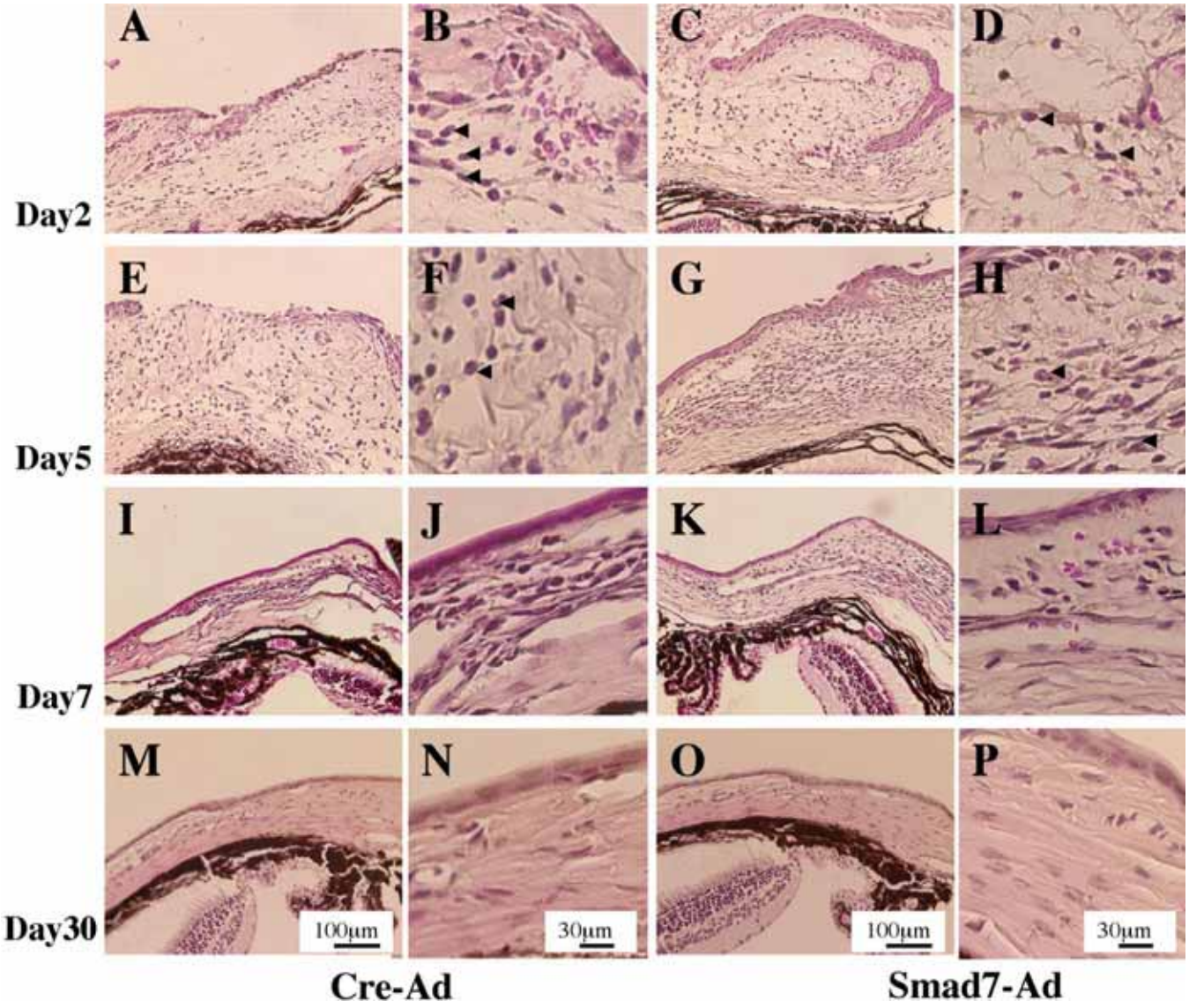
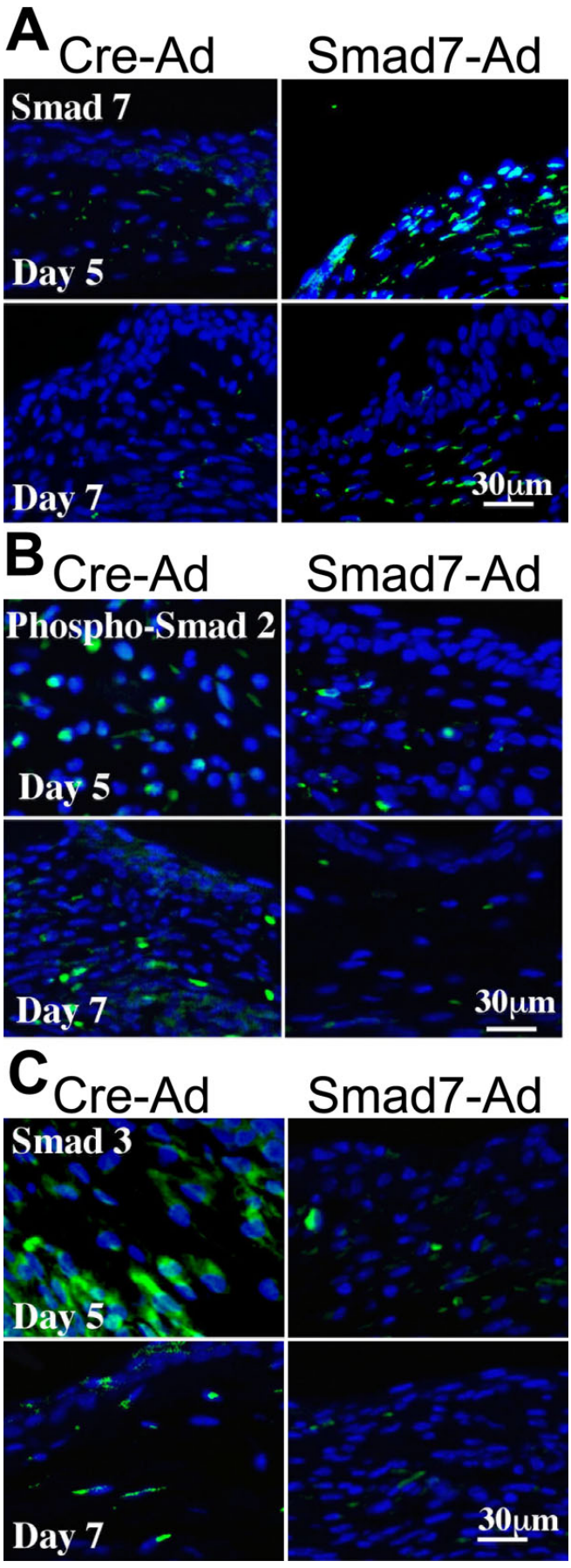


Figure 5. H&E histology in a mechanically injured mice conjunctiva. Smad7 gene introduction seemed to suppress the degree of conjunctival edema and cell population compared to control (**A**-**P**). The conjunctival epithelium defect sealed as early as day 5 in the Smad7-Ad group (**G**,**H**), whereas the epithelial defect was not closed at day 5 in the Control group (**E**,**F**). On day 7, the epithelial defect was closed in both groups (**I**-**L**). On day 30, however, H&E histology of both groups of specimens exhibited similar findings: reduced cell population and reduction of the thickness of subconjunctival matrix (**M**-**P**). Polymorphonuclear leukocytes were seen in subconjunctival tissue at day 2-5, but there seemed to be no difference of the distribution of this cell type. The scale bar represents 100 μm for **A**,**C**,**E**,**G**,**I**,**K**,**M**,**O** and 30 μm for **B**,**D**,**F**,**H**,**J**,**L**,**N**,**P**. Arrowheads point to polymorphonuclear leukocytes.



kocytes were seen in subconjunctival tissue on days 2-5, but there seemed to be no difference of the distribution of this cell type. To further confirm the effect of Smad7 overexpression on the healing process of conjunctival tissue, immunohistochemical analysis was carried out. The presumed exogenous Smad7 protein was strongly expressed in the healing conjunctival epithelium and fibroblasts from day 2, while faint immunoreactivity for endogenous Smad7 was seen in healing conjunctiva in the control group up to day 7 (Figure 6A). On day 5, phosphorylated Smad2 (Figure 6B) and Smad3 (Figure 6C) were both detected in the nuclei of the conjunctival fibroblasts and epithelial cells in the control Cre-Ad treated eyes, whereas in the Smad7-Ad treated eyes phospho-Smad2 was not detected and Smad3 was seen only faintly in the cytoplasm. On day 7 and after, a few nuclei were positive for both Smad 2 and Smad 3 in the control, but negative in the Smad7-Ad treated specimens. The number of cells with phospho-Smad2 labeling was determined in one area (150 μmx150 μm) adjacent to the incision-induced injury site. The numbers of phospho-Smad2-positive fibroblasts were significantly less in the Smad7-Ad treated group than in the Cre-Ad control group on day 5 and day 7, while there was no significant difference on day 30 (Figure 6D). Invasion of F4/80-labeled macrophages, appearance of αSMA-positive fibroblasts, and protein expression of VEGF were all less in the Smad7-Ad group than in the Cre-Ad group (Figure 7A-C). The number of F4/80-labeled

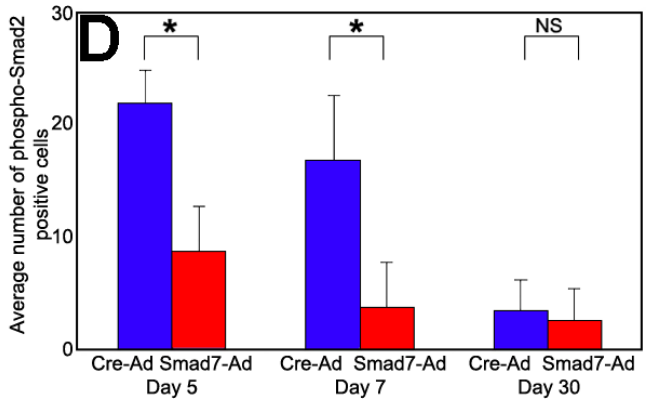


Figure 6. Immunolocalization of Smad in mechanically-injured mice conjunctiva. Smad7 protein, presumably the translated produce derived from exogenously introduced Smad7 cDNA, was strongly detected in the healing conjunctival epithelium and fibroblasts from day 2 onward, while faint immunoreactivity for endogenous Smad7 was seen in the healing conjunctiva in the control group for up to 10 days (**A**). At day 5, phosphorylated Smad2 and Smad3 were both detected in the nuclei of conjunctival fibroblasts and epithelial cells in control eyes, whereas in Smad7-Ad treated eyes phospho-Smad2 was not detected and Smad3 was seen only faintly in the cytoplasm (**B**,**C**). At and after day 10, a few of the nuclei were positive for both Smad2 and Smad3 in the control, but negative in Smad7-Ad treated specimens. The numbers of phospho-Smad2-positive fibroblasts were significantly lower in Smad7-Ad treated group than in Cre-Ad control group, while there was no significant difference on day 30 (**D**). The asterisks in Panel **D** indicates a $p<0.01$; NS represents nonsignificant.

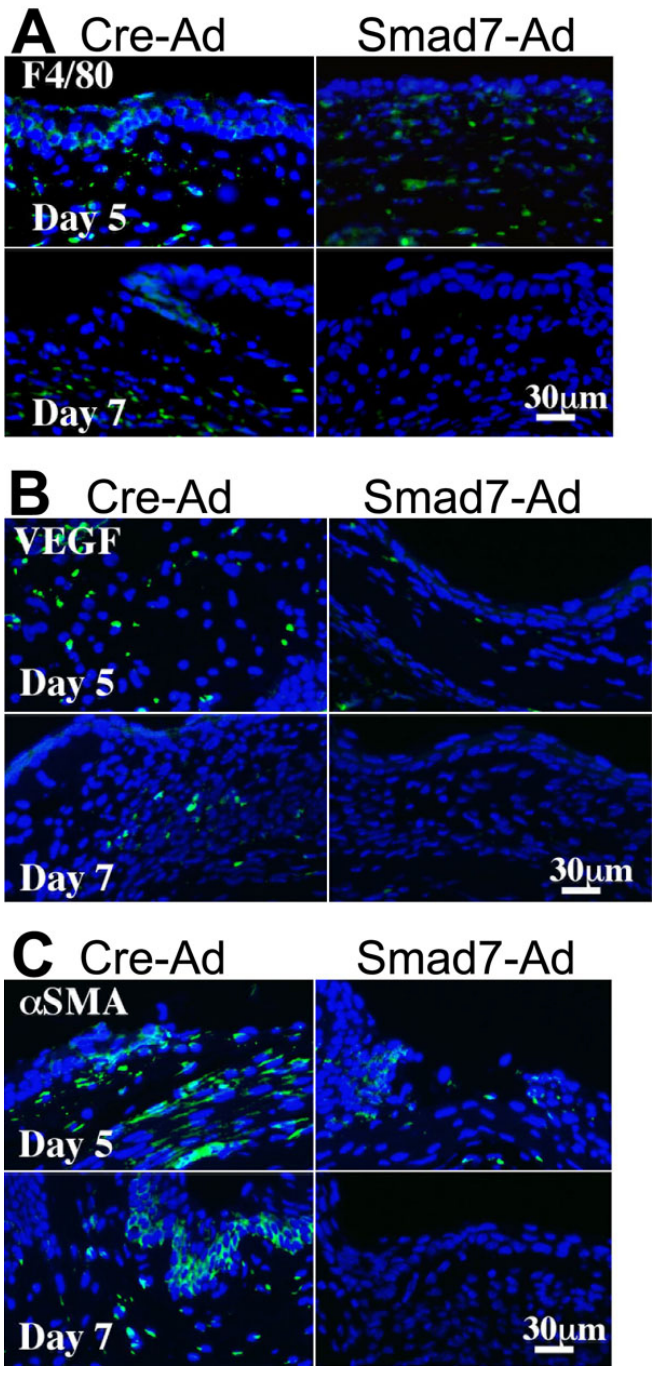
macrophages was determined in one area (150 μmx150 μm) adjacent to the incision-induced injury site. The numbers of F4/80-labeled macrophages were significantly lower in the Smad7-Ad treated group than in the Cre-Ad control group on day 5 and day 7, while there was no significant difference on day 30 (Figure 7D).

mRNA expression: The expressions of mRNA of CTGF, TGFβ1, MCP-1, and VEGF were much higher in the Cre-Ad group than in the Smad7-Ad group on day 2. Subsequently, the expression of each protein mRNA decreased, and there were no significant differences between the Cre-Ad group and the Smad7-Ad group on day 14 (Figure 8).

DISCUSSION

In this study, we first showed, in an in vitro experiment, that gene introduction of Smad7 cDNA suppressed the expression of type-I collagen and CTGF and also attenuated formation by the αSMA-labeled contractile cytoskeletal fibers in cultured fibroblasts derived from human eyes. Although Smad2 is reportedly involved in the expression of αSMA [14], our present study showed that Smad7 gene introduction did not reduce the protein expression level of αSMA and suppressed cytoplasmic fiber formation. Blocking Smad2 and Smad3 signal might inhibit production of a molecule that is essential for αSMA fiber formation. A similar finding was noticed in cultured hepatic stellate cells, the main component of mesenchymal cells involved in liver fibrosis [15]. The expression of molecules that affect αSMA fiber formation (e.g., coffilin, LIM kinase, and slingshot) might be changed upon Smad7 overexpression.

These finding prompted us to hypothesize that Smad7 overexpression might suppress excess fibrosis in subconjunctival tissue following injury. Smad7 gene introduction suppressed fibrogenic reaction, as revealed by H&E histology and immunohistochemistry for αSMA and CTGF, and reduced injury-related upregulation of CTGF, TGFβ1, MCP-1, and VEGF in the injured tissue until day 7. Local inflammation, as examined by the distribution of monocytes/macrophages, was also suppressed. These results indicated that Smad7 gene introduction effectively inhibited inflammation and modulated injured conjunctival wound healing during the relatively earlier phase from healing of mechanical injury. The in vitro ef-



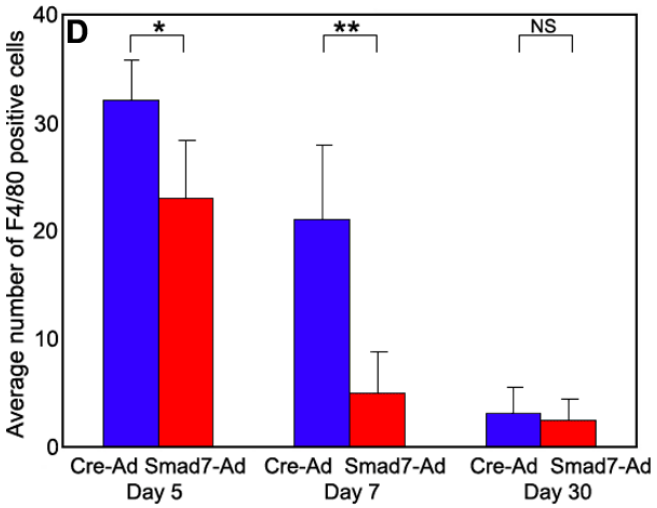


Figure 7. Immunohistochemistry in mechanically-injured mice conjunctiva. Invasion of F4/80-labeled macrophages (**A**) and VEGF (**B**) and αSMA (**C**) expression by fibroblasts were both lower in the Smad7-Ad treated group as compared with those in control group. The numbers of F4/80-positive macrophages were significantly lower in Smad7-Ad treated group than in Cre-Ad control group, while there was no significant difference on day 30 (**D**). In Panel **D**, the asterisk indicates a $p<0.05$, the double asterisk indicates a $p<0.01$, and NS means nonsignificant.

fects of TGFβ1 and TGFβ2 on fibroblast behavior were quite similar. In our study, we used TGFβ1 in SCF culture to mimic the activated condition of in vivo cell post-injury/surgery, although TGFβ1 and TGFβ2 are both believed to be involved in conjunctival scarring following trabeculectomy, while TGFβ2 predominates in the aqueous humor that is drained to the subconjunctival filtering bleb following trabeculectomy.

It remains to be determined whether the present model of conjunctival injury could represent the local healing process after filtering surgery. Although the present model lacked local effects by TGFβ2 derived from aqueous humor, we consider it to represent the component of tissue reaction upon conjunctival surgical intervention. We did not use the model of conjunctival scarring reported by Reichel et al. [16] (induced by topical injection of phosphate buffer saline), because our purpose was to test the effect of Smad7 overexpression on the process of conjunctival fibrogenic reaction upon injury that might be orchestrated by various growth factors. Indeed, we detected upregulation of VEGF in a mouse eye with conjunctival injury. In their model of conjunctival fibrosis, activation of fibroblasts was mostly observed on days 3-7. In our model of mechanical injury also wound healing-related molecules were upregulated on days 2-7, and therefore, the kinetics of fibroblast activation in wound healing procession in both models are considered to be similar. The efficacy of Smad7 overexpression in the present model suggests that TGFβ/Smad signal has a significant role in fibrogenic process. However, our present study failed to detect the effects of Smad7 gene

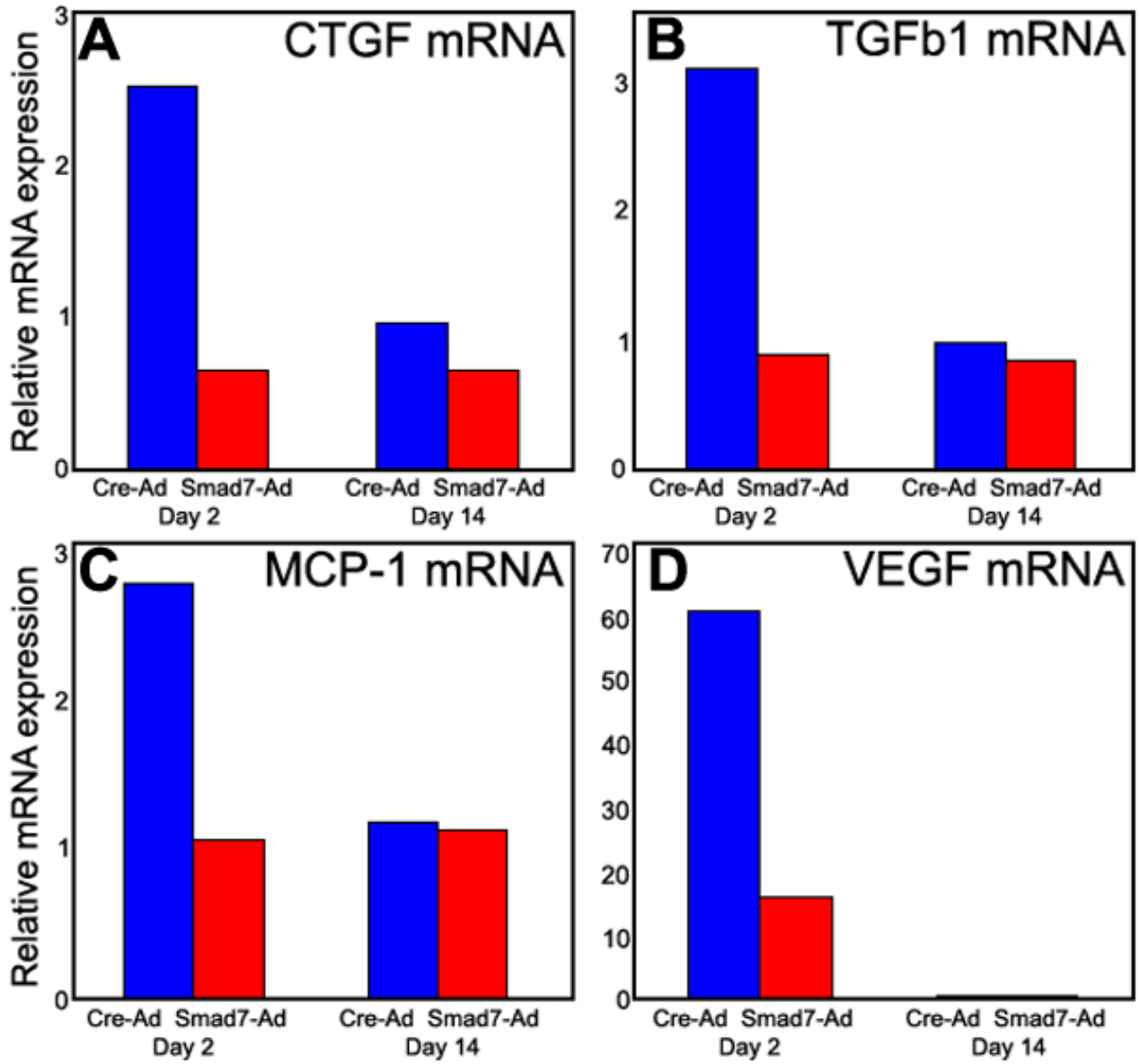


Figure 8. Expression of mRNA of CTGF, TGFβ1, MCP-1, and VEGF in mechanically-injured mice conjunctivas. Expressions of mRNA of CTGF (**A**), TGFβ1 (**B**), MCP-1 (**C**), and VEGF (**D**) are much higher in control group at two days than Smad7-Ad treated group. On day 14, these mRNA expressions did not show a significant difference.

transfer on either day 14 or 30 as observed by real-time RT-PCR or histology. Smad7 overexpression was not recognized on day 14. Therefore, the effects of Smad7 overexpression is considered to mainly interfere in early tissue reaction. Although we detected Smad7's antifibrogenic effect in healing of conjunctival injury, we need to study this further, using a different injury model that is associated with aqueous filteration.

It has been reported that blocking TGFβ2 by applying a neutralizing antibody in vivo is effective in suppressing conjunctival scarring [17,18]. Blocking TGFβ2 at the ligand level might result in suppression of all signaling lims derived from TGFβ2 binding to the receptor. Such signaling lims include pathways of MAPK, JNK, and p38MAPK besides Smad signaling. Smad2 or Smad3 is phosphorylated at the COOH-terminal region by TGFβ receptor upon binding of TGFβ to the receptor. However, it has recently been reported that MAPK or JNK phosphorylated the linker region of Smad2 or Smad3, which is required for full function of Smad signaling [19,20]. On the other hand, Smad7 blocks phosphorylation of Smad2/3 by the receptor, thus blocking COOH-terminal phosphorylation. Consequently, the effects of blocking TGFβ2 at the ligand level by applying a neutralizing antibody, which may lead to suppression of Smad phosphorylation at both of the linker and COOH-terminal regions, must differ from those resulting from suppressing Smad COOH-terminal phosphorylation by Smad7 gene introduction at the cellular level. The difference in action by each approach has to be investigated.

Many studies revealed that TGFβ is closely involved in scarring of various regions, such as the skin, cornea, lung, liver, and kidney [21-25]. Although classically TGFβ has been believed to accelerate wound healing, this may be dependent on promotion of granulation tissue formation. On the other hand, Aschcroft et al. [26] reported that an absence of Smad3 accelerates epidermal healing in association with suppression of scarring and macrophage invasion. We also previously reported that Smad7 gene introduction and the absence of Smad3 by gene targeting both suppressed excessive scarring in the mouse cornea following exposure to alkali [10]. Similarly, lacking Smad3 also attenuated scarring and macrophage invasion in the injured conjunctiva (unpublished data). All these findings are in consensus with the notion presented here that blocking TGFβ/Smad signal is beneficial to suppress excessive conjunctival scarring, which is favorable in terms of maintenance of the post-trabeculectomy filtering bleb. Moreover, this strategy can be applied to conjunctival scarring disorders caused by other diseases (e.g., vernal conjunctivitis or Stevens-Johnson syndrome).

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