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SMAD and p38 MAPK Signaling Pathways Independently Regulate α1(I) Collagen Gene Expression in Unstimulated and Transforming Growth Factor-β-stimulated Hepatic Stellate Cells*

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Shigeki Tsukada‡§, John K. Westwick¶, Kenichi Ikejima§, Nobuhiro Sato§, and Richard A. Rippe‡‖

From the ‡Division of Gastroenterology and Hepatology, Department of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599, the §Department of Gastroenterology, Juntendo University School of Medicine, Tokyo 113-8421, Japan, and ¶Odessey Thera, Inc., San Ramon, California 94583

The hepatic stellate cell (HSC) is the predominant cell type responsible for excess collagen deposition during liver fibrosis. Both transforming growth factor-β (TGF-β), the most potent fibrogenic cytokine for HSCs, which classically activates Smad signaling, and p38 MAPK signaling have been shown to influence collagen gene expression; however, the relative contribution and mechanisms that these two signaling pathways have in regulating collagen gene expression have not been investigated. The aim of this study was to investigate the relative roles and mechanisms of both Smad and p38 MAPK signaling in α1(I) collagen gene expression in HSCs. Inhibiting either p38 MAPK or Smad signaling reduced α1(I) collagen mRNA expression in untreated or TGF-β-treated HSCs, and when both signaling pathways were simultaneously inhibited, α1(I) collagen gene expression was essentially blocked. Both signaling pathways were found to independently and additively increase α1(I) collagen gene expression by transcriptional mechanisms. TGF-β treatment increased α1(I) collagen mRNA half-life, mediated by increased stability of α1(I) collagen mRNA through p38 MAPK signaling but not through Smad signaling. In conclusion, both p38 MAPK and Smad signaling independently and additively regulate α1(I) collagen gene expression by transcriptional activation, whereas p38 MAPK and not Smad signaling increased α1(I) collagen mRNA stability.

Liver fibrosis represents a wound-healing process in response to a variety of chronic stimuli. Fibrosis is characterized by an excessive deposition of extracellular matrix proteins, of which type I collagen predominates. The activated hepatic stellate cell (HSC)[1] is the predominant cell type in the liver responsible for the increased synthesis and deposition of type I collagen during liver fibrosis. The HSCs normally reside in the liver in a quiescent state; however, following a fibrogenic stimulus, HSCs undergo a complex activation process in which the cell changes from a quiescent vitamin A-storing cell to an activated myofibroblast-like cell, which proliferates and becomes fibrogenic (1, 2). An increase in DNA synthesis and cell proliferation occurs with HSC activation. Altered collagen synthesis, at both mRNA and protein levels, is observed with a dramatic increase in type I collagen along with smaller but significant increases in type III collagen (3–5).

Transforming growth factor-β (TGF-β), the most potent profibrogenic cytokine for activated HSCs (6), classically transmits intracellular signaling via Smad proteins (7). TGF-β binds to the constitutively active type II receptor, which then recruits and phosphorylates the type I receptor. Smad2 and Smad3 are recruited to the activated type I receptor where Smad2 and Smad3 are phosphorylated (8, 9). These then form a heteroligomeric complex with Smad4, and the complex translocates into the nucleus and regulates transcription of target genes. Smad7, an inhibitor of Smad signaling, associates with the activated type I receptor and interferes with Smad2 and Smad3 interaction with the receptor effectively inhibiting Smad2 and Smad3 phosphorylation and subsequent downstream signaling events (10–12). It has been shown that overexpression of Smad7 could effectively inhibit fibrogenesis. Gene transfer of Smad7 prevented bleomycin-induced lung fibrosis in mice considerably by intratracheal injection of a recombinant adenovirus containing Smad7 cDNA (13). In addition, gene transfer of Smad7 into unilateral ureteral obstruction-induced renal fibrosis in rats significantly prevented the accumulation of extracellular matrix proteins (14). Finally, inhibition of TGF-β signaling by Smad7 overexpression inhibited rat HSCs transdifferentiation and block fibrosis induced by bile duct ligation in rat (15).

Members of the mitogen-activated protein kinase (MAPK) family, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase-1, and p38 MAPK represent central kinases that typically transduce signals generated by growth factors and stress (16). TGF-β also can induce the activation of the p38 MAPK pathway mediated by the upstream TGF-β-activated kinase 1 (TAK1), a member of the MAPK kinase kinase family (17, 18). MKK3 and MKK6, known as the MAPK kinases, are thought to be activators of p38 MAPK, but the expression and the roles of MKK3 and MKK6 differ in cell types. For example, MKK3 is required for p38 MAPK activation in murine embryonic fibroblasts (19), whereas MKK6 is an important activator of p38 MAPK in cells

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‖ To whom correspondence should be addressed: CB 7032, Medical Biomolecular Research Bldg., Rm. 7340B, Division of Gastroenterology and Hepatology, Dept. of Medicine, University of North Carolina, Chapel Hill, NC 27599-7032. Tel.: 919-966-7469; Fax: 919-966-7468; E-mail: rarippe@med.unc.edu.

[1] The abbreviations used are: HSC, hepatic stellate cell; TGF-β, transforming growth factor-β; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; FBS, fetal bovine serum; m.o.i., multiplicity of infection; SMA, smooth muscle α-actin; MEK, MAPK/ERK kinase.

exposed to osmotic stress (20). p38 MAPK signaling has been implicated in TGF-β-mediated signaling and collagen gene expression (21, 22). In HSCs isolated from Smad3 knock-out mice α1(I) collagen, mRNA levels were reduced ~30% than in HSCs from wild-type mice (23). HSCs transduced with Ad5Smad7 also showed reduced α1(I) collagen mRNA expression in activated, TGF-β-untreated, HSCs (15). Therefore, although both Smad and p38 MAPK signaling pathways have been shown to influence collagen gene expression, the relative contribution and mechanisms these two signaling pathways have in regulating collagen gene expression are unknown. Furthermore, the role of both MKK3 and MKK6 in the HSC remain to be elucidated.

We undertook the present study to assess the role of Smad and p38 MAPK signaling on α1(I) collagen gene expression in primary rat HSCs. We found that inhibition of either p38 MAPK or Smad signaling pathways decreased culture-induced and TGF-β-stimulated expression of the α1(I) collagen mRNA steady state levels to similar degrees. However, when both signaling pathways were inhibited, α1(I) collagen expression was essentially blocked. In addition, both MKK3 and MKK6 were shown to activate p38 MAPK, which is coupled with enhanced collagen gene expression in HSCs. By using HSCs isolated from transgenic pCol9GFP-HS4,5 mice blocking either p38 MAPK or Smad, signaling pathways inhibited GFP expression to similar degrees, indicating that both p38 MAPK and Smad signaling increased transcriptional activity of the α1(I) collagen gene. Post-transcriptional regulation was found to be mediated by p38 MAPK signaling, but not Smad signaling, where p38 MAPK signaling increased the stability of α1(I) collagen mRNA in untreated activated HSCs. Moreover, TGF-β treatment increased α1(I) collagen mRNA stabilization that was mediated by p38 MAPK signaling and not by Smad signaling. Together, our results show that expression of the α1(I) collagen gene in HSCs is independently and positively regulated by both p38 MAPK and Smad signaling in both untreated and TGF-β-treated HSCs by both transcriptional and post-transcriptional mechanisms.

MATERIALS AND METHODS

Hepatic Stellate Cell Isolation and Culture—HSCs were isolated by *in situ* perfusion of the liver with collagenase and Pronase, followed by arabinogalactan gradient ultracentrifugation from adult male Sprague-Dawley rats (>400 g) or transgenic mice as described previously (24, 25). HSC purity, assessed by the autofluorescence of the cells by ultraviolet-excited fluorescence microscopy, was typically between 90 and 95%. Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and cultured in a 95% air, 5% CO_2 humidified atmosphere at 37 °C. Growth medium was changed every other day. HSCs were treated with 5 ng/ml recombinant human TGF-β1 (R&D Systems, Minneapolis, MN), 1–10 μM SB203580, a specific p38 MAPK inhibitor (Calbiochem), 1–10 μM PD98059, a selective inhibitor of MEK (Calbiochem), 1–10 μM SB431542, a potent inhibitor of TGF-β type I receptor (Tocris, Ellisville, MO), or 5 ng/ml actinomycin D (Sigma) following a 24-h serum-free period. All animal procedures were performed under the guidelines set by the University of North Carolina Institutional Animal Care and Use Committee and are in accordance with those set by the National Institutes of Health.

Adenovirus Transduction of HSCs—Ad5LacZ, which contains the β-galactosidase gene driven by the cytomegalovirus promoter, was used as a control virus throughout this study. The Ad5Smad7 virus expresses a C-terminal hemagglutinin (HA)-tagged human Smad7 protein. The human Smad7 cDNA was obtained from Dr. Wrana (Hospital for Sick Children, Toronto, Canada) and cloned into the pGI-AdCMV5 transfer vector (Qbiogene, Carlsbad, CA). FLAG-tagged adenovirus dominant negative form of MKK3 (Ad5dnMKK3) and MKK6 (Ad5dnMKK6) were obtained from Dr. Brydon Bennett (Celgene, San Diego, CA). Viral amplification was performed in 293 cells and cesium chloride purified by standard methodology. Viral titer estimates were performed by optical density measurements. For single adenovirus transductions, HSCs were transduced with Ad5Smad7, Ad5dnMKK3, Ad5dnMKK6, or Ad5LacZ at a multiplicity of infection (m.o.i.) of 250 2 days after isolation (day 2) for 16 h in Dulbecco's modified Eagle's medium containing 2% FBS. When transducing cells with both Ad5dnMKK3 and Ad5dnMKK6, the cells were simultaneously treated using an m.o.i. of 150 for each adenovirus. After 16 h, the transduction medium was changed to fresh growth medium containing 10% FBS.

Western Blot Analysis—Cultured HSCs were washed with phosphate-buffered saline, and the cells were lysed with protein sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, and 20% glycerol). Protein concentrations were measured using the Bradford method (Bio-Rad). Protein samples were heated at 95 °C for 5 min and then applied to a 10% SDS-polyacrylamide gel. Following electrophoresis, the proteins were electrophoretically transferred onto a nitrocellulose membrane. Membranes were stained with 0.5% Ponceau S to ensure equal protein loading and transfer. The membranes were blocked for 1 h with 5% milk in TBS-T (25 mM Tris-HCl, pH 8.0, 144 mM NaCl, 0.1% Tween 20). Afterward, membranes were incubated with the following antibodies, each diluted 1:1000 in 5% milk in TBS-T: primary anti-phospho-ERK, anti-phospho-p38, anti-p38, anti-phospho-JNK, or anti-phospho-Smad2 antibody (Cell Signaling, Beverly, MA) incubated for 16 h at 4 °C followed by the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA), incubated for 1 h at room temperature; primary anti-ERK2 antibody (Santa Cruz Biotechnology) or anti-JNK1 antibody (Cell Signaling, Beverly, MA) incubated for 1 h at room temperature followed by the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology), incubated for 1 h at room temperature; primary anti-actin antibody (ICN Biomedicals, Costa Mesa, CA) incubated for 1 h at room temperature followed by the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology), incubated for 1 h at room temperature; primary anti-human smooth muscle actin antibody (Dako, Carpinteria, CA) incubated for 1 h at room temperature followed by the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology) incubated for 1 h at room temperature; primary anti-α-tubulin antibody (Oncogene Research Products, Boston, MA), incubated for 1 h at room temperature followed by the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology) incubated for 1 h at room temperature. The membranes were washed four times with TBS-T at room temperature for 15 min each time after incubation with the primary and secondary antibody, respectively. Immunodetected proteins were visualized using ECL assay kit (Amersham Biosciences) following the manufacturer's recommended protocol.

RNase Protection Assay—Total RNA was isolated from rat HSCs cultured for 3 and 10 days, and RNase protection assays were performed as described previously (24). Radiolabeled probes were prepared for rat α1(I) collagen (26) and glyceraldehyde-3-phosphate dehydrogenase (pTRI-GAPDH-Rat, Ambion Inc., Austin, TX) and mixed with 5 μg of total HSC RNA. Protected fragments were analyzed on standard 6% sequencing gels. Following electrophoresis, bands were visualized by autoradiography and quantitated by PhosphorImager analysis (Amersham Biosciences).

GFP Expression—To analyze GFP transgene expression in HSCs, HSCs were isolated from GFP transgenic mice and cultured. GFP fluorescence was assessed and quantitated by PhosphorImager analysis (Amersham Biosciences) daily.

Statistical Analysis—The results were analyzed for statistical significance according to the Student's t test. Statistical values of $p < 0.05$ were considered to be significant. Data are presented as means ± S.D.

RESULTS

TGF-β Treatment Leads to Phosphorylation of p38 MAPK and ERK but Not JNK—To evaluate the effect of TGF-β on activating MAPK signaling, HSCs, cultured for 2 days, were serum-starved for 24 h and then treated with 5 ng/ml TGF-β for 24 h. Cell lysates were examined by Western blot analysis for p38 MAPK, ERK, and JNK activation status using phospho-specific antibodies. Following TGF-β treatment, activation of both ERK and p38 MAPK was observed 60 and 120 min, respectively, following TGF-β treatment in 3-day cultured HSCs (Fig. 1). On the other hand, JNK activation was not observed following treatment of the cells with TGF-β.

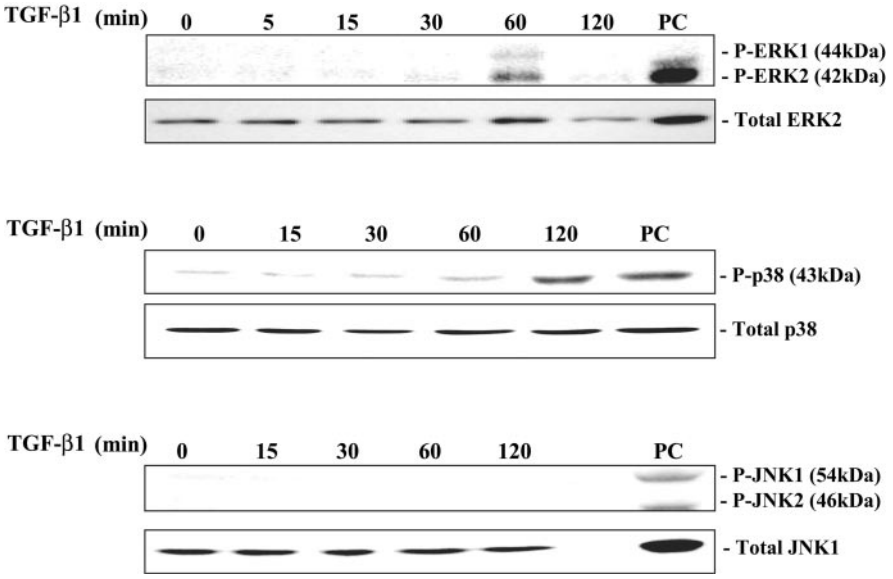

FIG. 1. **TGF-β activates ERK 1/2 and p38 MAPK signaling pathways but not JNK.** To evaluate the effect of TGF-β on activation of MAPK, three members of MAPK signaling, ERK, p38, and JNK, were examined. HSCs, 2 days in culture, were serum-starved for 24 h followed by treatment with 5 ng/ml TGF-β for the indicated times. Cell extracts (40 μg for phospho-ERK and 80 μg for phospho-p38 and phospho-JNK) were subjected to Western blot analysis using phospho-specific antibodies. Equal loading of the gels were confirmed using total ERK2, p38, and JNK1 protein levels. Results are representative of at least three independent experiments.

Inhibition of p38 MAPK and ERK Activities Prevents TGF-β-induced Kinase Activation—To demonstrate the inhibitory effect of SB203580, a specific inhibitor of p38 MAPK activity, on TGF-β-induced p38 MAPK activation, HSCs were cultured for 3 days and then incubated for 1 h with increasing concentrations of SB203580 and subsequently stimulated with 5 ng/ml TGF-β for 2 h. Activation of p38 MAPK was assessed by Western blot analysis using a phospho-specific antibody recognizing activated phospho-p38. Blocking p38 MAPK activity with SB203580 inhibited TGF-β-induced p38 phosphorylation at concentrations of 3 and 10 μM but not at a concentration of 1 μM (Fig. 2*A*). To assess the effect of inhibiting MEK activity, an upstream kinase that activates ERK with PD98059, a specific MEK inhibitor, on TGF-β-induced ERK activity, 3-day cultured HSCs were pretreated with increasing concentrations of PD98059 for 1 h and subsequently stimulated with 5 ng/ml TGF-β for 1 h. Inhibiting ERK activity with 10 μM PD98059 completely blocked TGF-β-induced ERK activation; treatment with 3 μM PD98059 only partially inhibited ERK activity, and incubation with 1 μM did not inhibit ERK activity (Fig. 2*B*).

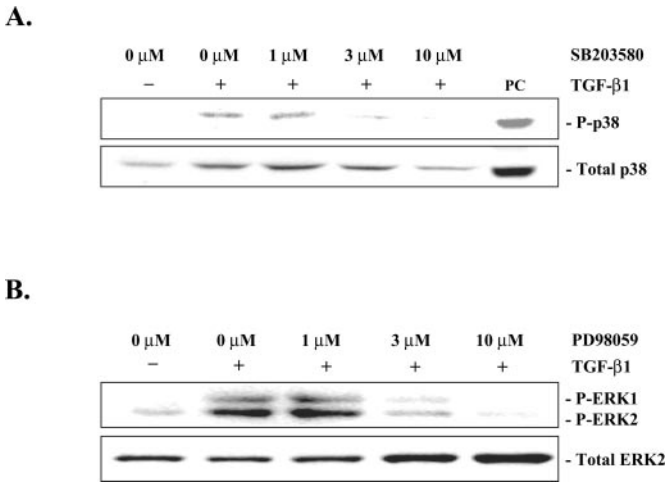

FIG. 2. **Concentration-dependent inhibition of TGF-β-induced p38 MAPK and ERK activities with SB203580 and PD98059, respectively.** To show concentration-dependent inhibitory effects of SB203580 and PD98059, kinase-specific chemical inhibitors of p38 MAPK and ERK activities, respectively, on TGF-β-induced kinase activation, Western blot analyses were performed using phospho-p38 and phospho-ERK antibodies, respectively. HSCs, cultured for 3 days, were preincubated 1 h with increasing concentrations of the kinase inhibitors and subsequently stimulated with 5 ng/ml TGF-β for 2 h (*A*) and 1 h (*B*). Equal loading of the gels was confirmed by using total ERK2 and p38 MAPK protein levels. Results are representative of at least three independent experiments.

TGF-β Independently Activates Smad and p38 MAPK Signaling in HSCs—Because both Smad and p38 MAPK signaling pathways are activated following TGF-β treatment, we wanted to determine whether TGF-β induces the activation of these signaling pathways independently of each other or if they are activated by a common mechanism. Because Smad proteins act as mediators for TGF-β signaling, the effect of inhibiting Smad signaling using Ad5Smad7 on basal Smad activity and Smad activity in response to TGF-β stimulation in HSCs was investigated. To inhibit TGF-β-mediated cell signaling, HSCs were transduced with Ad5Smad7 (m.o.i. of 250). HSCs, 2 days in culture, were transduced with Ad5Smad7 and on the following day cells were harvested. Transduction of HSCs with Ad5Smad7 showed high levels of Smad7 protein expression as assessed by Western blot analysis using the HA tag (Fig. 3*A*). Treatment of day 3 cultured HSCs with 5 ng/ml TGF-β-induced Smad2 phosphorylation within 15 min was maximal 30 min after treatment and persisted for at least 120 min (Fig. 3*B*). To assess the effect of inhibiting Smad signaling followed by TGF-β treatment, HSCs, cultured for 2 days, were transduced with Ad5Smad7, and the following day Smad2 phosphorylation was assessed after stimulation with TGF-β for 30 min. TGF-β stimulation increased Smad2 phosphorylation 3.5-fold compared with both untransduced and Ad5LacZ control virus-transduced HSCs (Fig. 3*C*). Transduction with Ad5Smad7 significantly inhibited TGF-β-induced Smad2 phosphorylation compared with Ad5LacZ-transduced HSCs. To investigate the effect of blocking Smad signaling on the activation of p38 MAPK, HSCs were transduced with Ad5Smad7 and treated with TGF-β for 2 h, and the phosphorylation of p38 MAPK was assessed by Western blot analysis using a phospho-p38-specific antibody. Phosphorylation of p38 MAPK was found to be increased 7.5-fold following TGF-β treatment, compared with both untransduced and Ad5LacZ-transduced control HSCs (Fig. 3*D*). Blocking Smad signaling with Ad5Smad7 did not affect basal or TGF-β-stimulated phosphorylation of p38 MAPK, indicating that these two signaling pathways are independent from each other (Fig. 3*D*).

Similarly, HSCs, cultured for 3 days, were preincubated with 10 μM SB203580 for 1 h and then stimulated with 5 ng/ml TGF-β for 2 h. TGF-β-induced p38 MAPK phosphorylation was

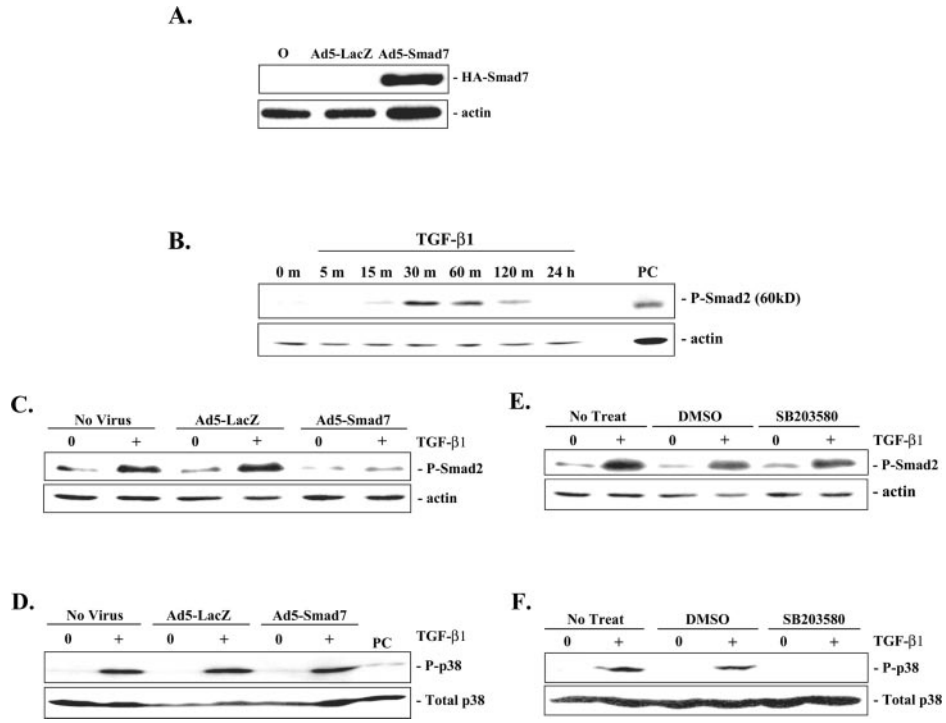

FIG. 3. **TGF-β-induced p38 MAPK and Smad signaling pathways are independently activated in HSCs.** The effect of inhibiting TGF-β signaling with Ad5Smad7 transduction or treatment of SB203580 on basal and TGF-β-induced Smad and p38 MAPK activity in HSCs was investigated. To confirm Smad7 expression, HSCs, 2 days in culture, were transduced with Ad5Smad7 (m.o.i. of 250), and on the following day cell extracts were prepared, and Western blot analysis was performed by using anti-HA antibody (*A*). HSCs, cultured for 3 days, were treated with TGF-β (5 ng/ml) for the indicated times as shown. Levels of Smad2 phosphorylation following TGF-β treatment were assessed by Western blot analysis using phospho-specific Smad2 antibody (*B*). HSCs, cultured for 2 days, were transduced with Ad5Smad7 (m.o.i. of 250), and the following day (day 3 of culture) Smad2 activity was assessed after TGF-β (5 ng/ml) stimulation for 30 min. *C,* TGF-β-induced phosphorylation of p38 MAPK was assessed by Western blot analysis using phospho-p38-specific antibody following transduction with Ad5Smad7 (*D*). HSCs, cultured for 3 days, were pretreated with 10 μM SB203580 for 1 h followed by 5 ng/ml TGF-β treatment for 30 min, and Smad2 activity was assessed (*E*). TGF-β-induced phosphorylation of p38 MAPK was assessed by Western blot analysis using phospho-p38-specific antibody following SB203580 treatment for 1 h (*F*). Equal loading of the gels were confirmed using actin and total p38 MAPK protein levels. Results are representative of at least three independent experiments.

completely inhibited by SB203580 (Fig. 3*F*). On the other hand, blocking p38 MAPK activity did not inhibit TGF-β-induced phosphorylation of Smad2 (Fig. 3*E*). Together these results show that these two TGF-β-induced signaling pathways are independent from each other.

Blocking Either p38 MAPK or Smad Signaling Inhibits TGF-β-induced α1(I) Collagen mRNA Expression—To assess the role of p38 MAPK and ERK activities on TGF-β-induced α1(I) collagen mRNA steady state expression, HSCs were cultured for 3 days and then pretreated for 1 h with 10 μM SB203580 or 10 μM PD98059, respectively. Afterward, the cells were treated with 5 ng/ml TGF-β for 24 h, and total RNA was isolated, and then α1(I) collagen mRNA steady state expression was assessed. TGF-β increased α1(I) collagen mRNA steady state levels 2.1-fold in quiescent HSCs (3-day cultures; Fig. 4, *A* and *B*); however, TGF-β-induced α1(I) collagen mRNA expression was not observed in HSCs cultured for 10 days (Fig. 4, *C* and *D*). Inhibiting p38 MAPK activity with 10 μM SB203580 blocked total α1(I) collagen mRNA expression by 38%, whereas inhibiting ERK activity with 10 μM PD98059 had no effect on α1(I) collagen mRNA expression (Fig. 4, *A* and *B*). Inhibiting Smad signaling with Ad5Smad7 reduced total α1(I) collagen mRNA expression 59% compare with HSCs treated with TGF-β alone (Fig. 5*A*, B). Inhibition of both p38 MAPK and Smad signaling reduced total expression of α1(I) collagen mRNA by 84% to that of TGF-β-treated cells.

Inhibition of p38 MAPK or Smad Signaling Suppresses Culture-induced α1(I) Collagen mRNA Expression—To assess the effect of inhibiting Smad and p38 MAPK signaling on culture-induced α1(I) collagen gene expression, HSCs were transduced with Ad5Smad7 on day 2 and/or treated with 10 μM SB203580 continuously, from day 2 to day 5. To keep a continuous inhibition of kinase activity, the culture medium containing SB203580 was replaced daily until the cells were harvested. Expression of α1(I) collagen mRNA, which was up-regulated in culture-induced activated HSCs (5 days in culture), remained unchanged after Ad5LacZ transduction (Fig. 6). Inhibiting Smad signaling with Ad5Smad7 reduced steady state levels of α1(I) collagen mRNA by 84%, whereas inhibition of p38 MAPK activity inhibited α1(I) collagen mRNA expression by 80%. When both signaling pathways were simultaneously inhibited, α1(I) collagen mRNA expression was reduced 96% compared with control cells, indicating that these two signaling pathways are primarily responsible for α1(I) collagen expression in the HSC.

Both MKK3 and MKK6 Locate Upstream of p38 MAPK and Regulate Collagen Gene Expression through p38 MAPK in HSCs—Although MKK3 and MKK6 are generally considered to be upstream kinases and activators of p38 MAPK, their role in HSCs has not been investigated previously. To determine their roles in TGF-β/p38 MAPK signaling, HSCs were transduced with Ad5dnMKK3 and/or Ad5dnMKK6 on day 2, and subsequently treated with 5 ng/ml TGF-β for 2 h. Cell extracts were harvested, and activation of p38 MAPK was assessed by Western blot analysis using a phospho-specific p38 MAPK antibody. Viral transduction was confirmed by Western blot analysis for expression of the FLAG tag present on the dnMKK3 and MKK6 proteins (data not shown). Blocking either MKK3 or MKK6 activity partially inhibited TGF-β-induced p38 phosphorylation, whereas transduction with both Ad5dnMKK3 and

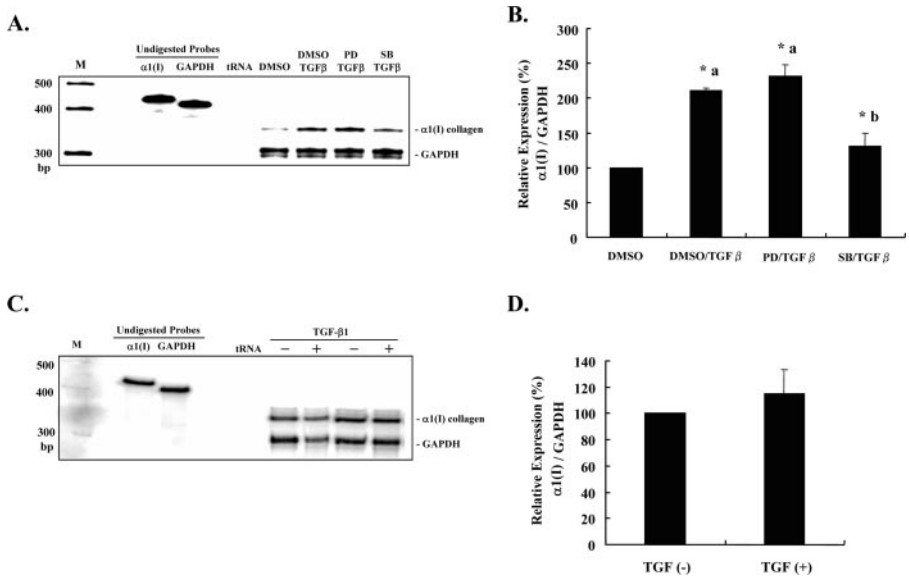

FIG. 4. **TGF-β induces α1(I) collagen mRNA expression in 3-day cultured HSCs but not in fully activated HSCs, which is partially regulated by p38 MAPK signaling.** To study the role of p38 MAPK and ERK on TGF-β-induced α1(I) collagen mRNA, quiescent HSCs (3 day cultures; *A* and *B*) and activated HSCs (10 day cultures; *C* and *D*) were treated with SB203580 (10 μM) or PD98059 (10 μM), respectively, for 1 h prior to treatment with 5 ng/ml TGF-β for 24 h. Total RNA was harvested, and RNase protection assays were performed to assess the levels of α1(I) collagen mRNA steady state levels, normalized to that of GAPDH. Representative RNase protection assay results are shown. As a negative control, tRNA was used (*A* and *C*). Graphical analysis of the RNase protection assay data is shown (*B* and *D*). Data represent results from three individual RNase protection assays. *Error bars* represent S.D. *DMSO*, Me_2SO; *M*, size marker. *, $p < 0.05$; *a*, *versus* control; *b*, *versus* TGF-β treated HSC.

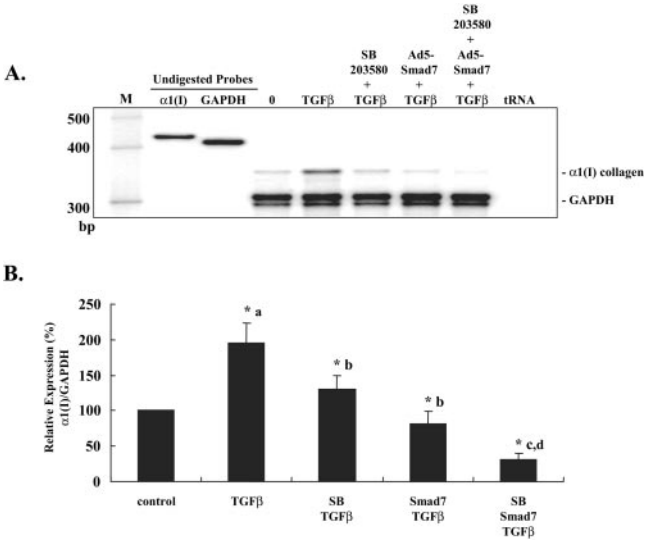

FIG. 5. **Inhibition of Smad signaling with Ad5Smad7 inhibits TGF-β-induced α1(I) collagen mRNA in HSCs.** HSCs, cultured for 2 days, were transduced with Ad5Smad7 (m.o.i. of 250) to inhibit Smad signaling. On the following day (day 3), the cells were treated for 24 h with 5 ng/ml TGF-β alone or in combination with 10 μM SB203580 added 1 h prior to the addition of 5 ng/ml TGF-β. Total RNA was isolated and used (5 μg) to measure α1(I) collagen mRNA expression, normalized to that of GAPDH. A representative RNase protection assay is shown. tRNA was used as a negative control RNA sample (*A*). Graphical analysis from three individual RNase protection assays is shown (*B*). *Error bars* represent S.D. *, $p < 0.05$; *a*, *versus* control; *b*, *versus* TGF-β treated HSC; *c*, *versus* SB203580 (*SB*)-treated HSC; *d*, *versus* Ad5Smad7-transduced HSC. *M*, size marker.

Ad5dnMKK6 completely blocked p38 phosphorylation (Fig. 7*A*). These data show that both MKK3 and MKK6 are positioned upstream of p38 MAPK and positively transmit TGF-β/p38 MAPK signaling. To confirm the role of Smad signaling in HSCs, SB431542, a potent and selective inhibitor of TGF-β type I receptor kinase, was used to block TGF-β/Smad signaling (30). To show the inhibitory effect of SB431542 on TGF-β-induced Smad2 activation, HSCs were cultured for 3 days, then incubated for 1 h with increasing concentrations of SB431542, and subsequently stimulated with 5 ng/ml TGF-β for 30 min. Activation of Smad2 was assessed by Western blot analysis using a phospho-specific antibody recognizing activated phospho-Smad2. Blocking TGF-β type I receptor activity with SB431542 completely abolished TGF-β-induced Smad2 phosphorylation at concentration of 10 μM without affecting phosphorylation of p38 MAPK (Fig. 7, *B* and *C*). To examine the role of MKK3/6 on TGF-β-induced α1(I) collagen mRNA expression, HSCs, day 2 in culture, were transduced with Ad5dnMKK3 and/or Ad5dnMKK6 and treated with 5 ng/ml TGF-β for 24 h. RNA was harvested, and RNase protection assays were performed. Blocking MKK3 reduced steady state levels of α1(I) collagen mRNA by 46% (Fig. 7, *D* and *E*). Most interestingly, inhibiting MKK6 alone decreased α1(I) collagen mRNA expression by 62% which was nearly at the same level as when both MKK3 and MKK6 activities were blocked (64%). Therefore, both MKK3 and MKK6 play important roles in regulating α1(I) collagen gene expression through p38 MAPK; however, MKK6 signaling appears to have a stronger effect than MKK3 activity. Blocking Smad signaling with SB431542 reduced α1(I) collagen mRNA expression by 63%, and inhibiting both Smad and MKK3/6 (as a result of blocking both Smad and p38 MAPK) decreased α1(I) collagen mRNA expression by 88% (Fig. 7, *D* and *E*). Furthermore, transduction of HSCs with Ad5dnMKK3 reduced culture-induced steady state levels of α1(I) collagen mRNA by 48%, and inhibiting MKK6 alone with Ad5dnMKK6 decreased α1(I) collagen mRNA expression by 75%, nearly the same level as blocking both MKK3 and MKK6 by 73% (Fig. 7, *F* and *G*). Blocking Smad signaling with SB431542 reduced culture-induced α1(I) collagen mRNA expression by 81%, and inhibiting both Smad and MKKs decreased by 97% (Fig. 7, *F* and *G*).

Both p38 MAPK and Smad Signaling Contribute to the Expression of Smooth Muscle α-Actin in HSCs—Because Smad and p38 MAPK signaling both regulate α1(I) collagen expression in HSCs, we wanted to determine whether these signaling pathways also regulate smooth muscle α-actin (α-SMA) expression, a classical marker for HSC activation in the HSC. To evaluate the roles of p38 MAPK and Smad signaling on α-SMA expression in HSCs, day 2 cultured HSCs were transduced with Ad5Smad7 and/or treated with 10 μM SB203580. Cells were cultured for 5 days with media containing the p38 MAPK inhibitor changed daily. Inhibition of Smad signaling with Ad5Smad7 significantly reduced the level of α-SMA expression by 50% compared with Ad5LacZ-transduced control cells, whereas inhibition of p38 MAPK activity reduced α-SMA expression by 52% compared with Me_2SO-treated HSCs (Fig. 8). Moreover, α-SMA expression was significantly reduced by 69% compared with control cells when both p38 MAPK and Smad signaling pathways were inhibited

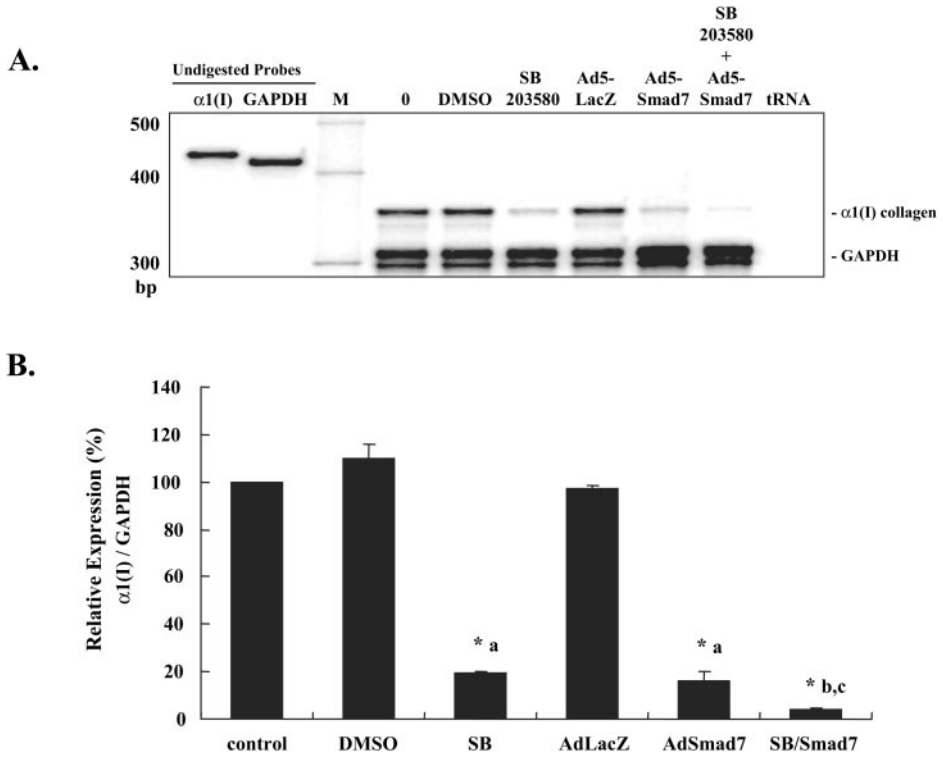

FIG. 6. **Inhibition of Smad and p38 MAPK signaling reduces culture-induced α1(I) collagen gene mRNA steady state levels.** To determine the effect of inhibiting Smad and p38 MAPK signaling on the culture-induced α1(I) collagen mRNA expression, cultured HSCs were transduced with Ad5Smad7 (m.o.i. of 250) on day 2 of culture. Transduced cells and untransduced cells (as controls) were treated with 10 μM SB203580 (*SB*), from day 2 to day 5. Media were replaced daily. After 5 days, total RNA was harvested and subjected to RNase protection assays (5 μg/reaction) to assess α1(I) collagen mRNA steady state levels, which were normalized to GAPDH. A representative RNase protection assay is shown (*A*). tRNA was used as a negative RNA control. Graphical analysis from three independent RNase protection assays data is shown (*B*). *Error bars* represent S.D. *, $p < 0.05$; *a*, *versus* control; *b*, *versus* SB203580 treated HSC; *c*, *versus* Ad5Smad7 transduced HSCs. *DMSO*, Me_2SO; *M*, size marker.

(Fig. 8). These data implicate a role for both Smad and p38 MAPK signaling in HSC activation.

Both p38 MAPK and Smad Signaling Stimulate Transcriptional Regulation of the α1(I) Collagen Gene Following Culture-induced HSC Activation—Because p38 MAPK and Smad signaling both regulate α1(I) collagen gene expression (Figs. 4–7), we wanted to investigate the mechanism by which these signaling pathways mediate this effect. To assess a potential transcriptional role for these signaling pathways, HSCs were isolated from transgenic mice harboring the pCol9GFP-HS4,5 transgene (25, 27). This α1(I) collagen reporter gene has been shown previously to be regulated similarly to that of the endogenous α1(I) collagen gene following HSC activation (25). Isolated HSCs were cultured for 5 days, and the cells were treated with 10 μM SB203580 from day 2 to day 5 and/or transduced with Ad5Smad7 on day 2. GFP expression was monitored daily and quantitated by PhosphorImager analysis. Inhibiting either p38 MAPK or Smad signaling decreased GFP expression (Fig. 9). After 5 days in culture, GFP expression in HSCs treated with SB203580 was reduced 54% compared with untreated control cells, whereas Ad5Smad7 transduction reduced GFP expression 67% compared with untreated control cells. Together, the data show that p38 MAPK and Smad signaling both positively influence transcriptional regulation of the α1(I) collagen gene.

p38 MAPK but Not Smad Signaling Increases α1(I) Collagen mRNA Stability in Culture-activated HSCs—Stabilization of the α1(I) collagen mRNA has been shown to be important following HSC activation (28). To determine the contribution that both p38 MAPK and Smad signaling have in stabilizing α1(I) collagen mRNA, p38 MAPK activity was blocked with 10 μM SB203580 for 48 h, from day 3 to day 5, or Smad signaling was inhibited with Ad5Smad7 transduction on day 2 in culture before transcription was blocked with 5 μg/ml actinomycin D on day 5. Total RNA was harvested from the cells after 6, 12, or 18 h following actinomycin D treatment and was subjected to RNase protection assays. The levels of α1(I) collagen mRNA were quantitated and normalized to that of GAPDH. In culture-activated HSCs, the half-life of α1(I) collagen mRNA has been estimated previously to be 24 h, while in quiescent HSCs, the half-life is reported to be 1.5 h (28). In HSCs grown for 5 days, we found the half-life of α1(I) collagen mRNA to be ~27 h (Fig. 10, *A* and *B*). When p38 MAPK activity was blocked with SB203580, the half-life of α1(I) collagen mRNA was reduced to 13 h, indicating that p38 MAPK activity stabilizes α1(I) collagen mRNA (Fig. 10, *A* and *B*). On the other hand, inhibition of Smad signaling did not affect the half-life of α1(I) collagen mRNA (Fig. 10, *C* and *D*). The decrease in α1(I) collagen mRNA stability following inhibition of p38 MAPK activity was not caused by cell toxicity, because cell viability was not adversely affected by treatment of the cells with SB203580 (data not shown). These data indicate that p38 MAPK signaling stabilizes α1(I) collagen mRNA, whereas Smad signaling does not influence α1(I) collagen mRNA stabilization.

TGF-β Increases Stability of the α1(I) Collagen mRNA That Is Mediated by p38 MAPK but Not Smad Signaling—TGF-β has been demonstrated to increase α1(I) collagen mRNA expression, at least in part by influencing gene transcription (29). However, a potential role for TGF-β in stabilizing α1(I) collagen mRNA in the HSC has not been investigated. Therefore, we examined the effect of TGF-β on α1(I) collagen mRNA stability by transducing HSCs, day 2 in culture, with Ad5Smad7, or on the following day HSCs, 3 days in culture, were pretreated 1 h with SB203580 prior to a 24-h treatment with 5 ng/ml TGF-β. The half-life of α1(I) collagen mRNA in control cells (Me_2SO-treated) was ~11 h (Fig. 11*A*). Inhibition of p38 MAPK signaling decreased the stability of α1(I) collagen mRNA to 7 h (Fig. 11*A*). Treatment of the cells with TGF-β significantly increased the half-life of α1(I) collagen mRNA to 20 h compared with control (Me_2SO-treated) cells (Fig. 11*A*). Most interestingly, TGF-β-induced stability of α1(I) collagen mRNA was decreased to the same level of the control cells following treatment with SB203580, suggesting that p38 MAPK signaling is completely responsible for the TGF-β-induced increased stability of the α1(I) collagen mRNA (Fig. 11*A*). On the other hand, inhibition of Smad signaling did not change the half-life of α1(I) collagen

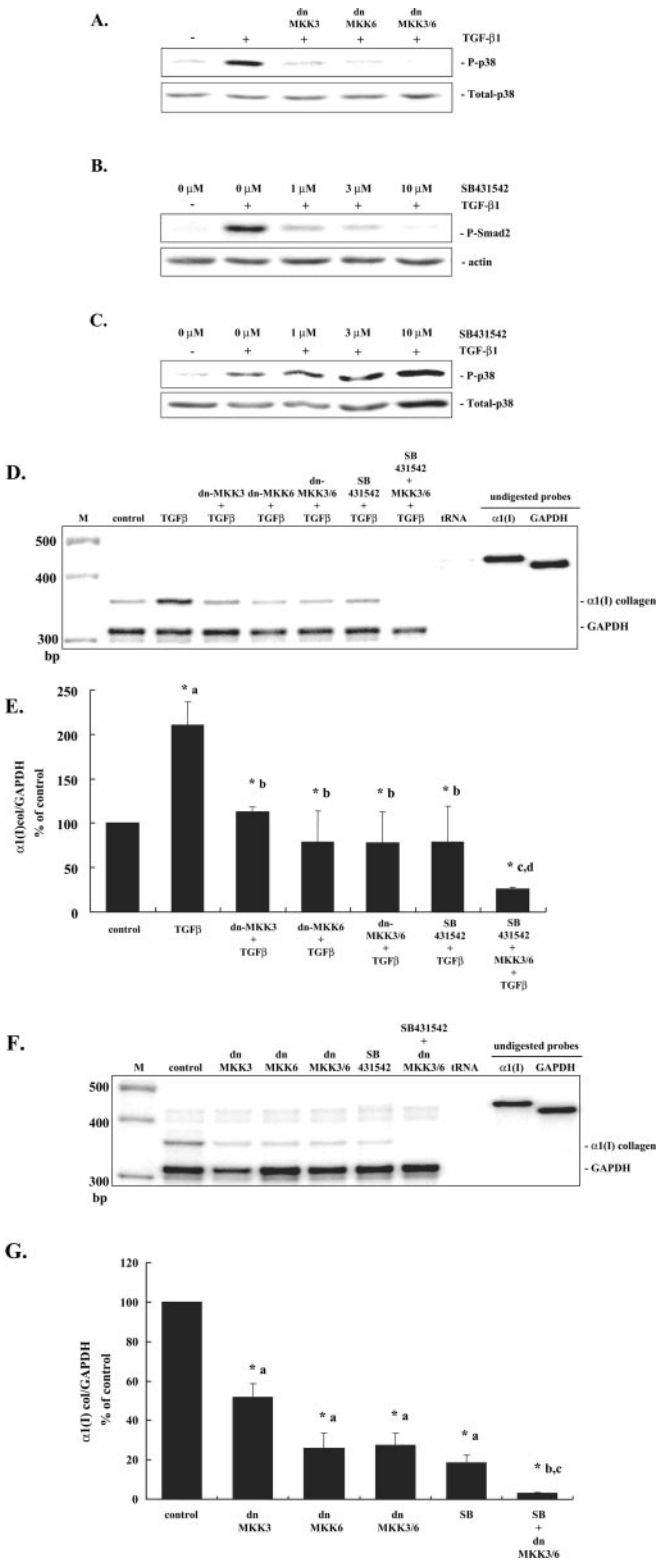

FIG. 7. **Both MKK3 and MKK6 activate p38 MAPK and regulate α1(I) collagen gene expression through p38 MAPK in HSCs.** To determine the roles of MKK3 and MKK6 on TGF-β-induced phosphorylation of p38 MAPK, HSCs were transduced with Ad5dnMKK3 and/or Ad5dnMKK6 on day 2 and subsequently treated with 5 ng/ml TGF-β for 2 h on the following day. Cell extracts were harvested, and activation of p38 MAPK was assessed by Western blot analysis using a phospho-specific antibody (*A*). To demonstrate the effect of SB431542, a selective inhibitor of TGF-β type I receptor kinase, on TGF-β-induced Smad2 and p38 MAPK activation, HSCs were cultured for 3 days and then incubated 1 h with increasing concentrations of SB431542 and subsequently stimulated with 5 ng/ml TGF-β for 30 min (for phospho-Smad2) or 2 h (for phospho-p38 MAPK). Activations of Smad2 and p38 MAPK were assessed by Western blot analysis using a phospho-specific antibody (*B* and *C*). To examine the role of MKK3/6 on TGF-β-induced α1(I) collagen mRNA expression, HSCs, day 2 in culture, were transduced with Ad5dnMKK3 or/and Ad5dnMKK6. The following day (day 3), cells were treated for 24 h with 5 ng/ml TGF-β alone or in combination with 10 μM SB431542 added 1 h prior to the addition of 5 ng/ml TGF-β for 24 h. RNA was harvested, and RNase protection assays were performed. A representative RNase protection assay is shown. tRNA was used as a negative control (*D*). Graphical analysis from three individual RNase protection assays is shown (*E*). *Error bars* represent S.D. *, $p < 0.05$; *a*, *versus* control; *b*, *versus* TGF-β-treated HSC; *c*, *versus* SB421543-treated HSC; *d*, *versus* Ad5dnMKK3- and Ad5dnMKK6-transduced HSC. To assess the effect of inhibiting both Smad and MKK3/6 signaling in culture-induced α1(I) collagen mRNA expression, cultured HSCs were transduced with Ad5dnMKK3 and/or Ad5dnMKK6 on day 2 of culture. Transduced cells and untransduced cells (as controls) were treated with 10 μM SB421543 from day 2 to day 5. Media were replaced daily. After 5 days in culture, total RNA was harvested and subjected to RNase protection assays (5 μg/reaction) to assess α1(I) collagen mRNA steady state levels and normalized to GAPDH. A representative RNase protection assay is shown (*F*). Graphical analysis from three independent RNase protection assays data is shown (*G*). *Error bars* represent S.D. *, $p < 0.05$; *a*, *versus* control; *b*, *versus* SB421543-treated HSC; *c*, *versus* Ad5dnMKK3- and Ad5dnMKK6-transduced HSCs. *M*, size marker.

mRNA in untreated or TGF-treated HSCs (Fig. 11*B*). Together, these data show that TGF-β increases the stability of α1(I) collagen mRNA that is mediated by p38 MAPK and not by Smad signaling.

DISCUSSION

TGF-β is the most potent profibrogenic cytokine known for activated HSCs. TGF-β classically induces intracellular signaling via Smad proteins. Signaling is initiated by ligand binding to the constitutively active type II receptor, which then recruits and phosphorylates the type I receptor. The activated type I receptor rapidly associates with and phosphorylates Smad2 and Smad3 (8, 9), which then forms an oligomeric complex with Smad4. This complex translocates into the nucleus and binds to specific nucleotide motifs regulating transcription of target genes. Smad7 forms a stable complex with activated type I receptor and effectively prevents Smad2 and Smad3 interaction with the receptor and subsequent phosphorylation, thus efficiently preventing downstream signaling (10–12). Smad7 is an effective inhibitor for TGF-β signaling, and recent studies (13–15) have implicated Smad7 as a key molecule for regulating TGF-β-induced fibrogenesis in experimental models of fibrosis. Expression of α1(I) collagen mRNA is reduced, but not eliminated, in HSCs isolated from both Smad3 knock-out mice (21) and in HSCs transduced with Ad5Smad7 (15), suggesting additional signaling pathways contribute to collagen gene expression in basal and TGF-β-induced states. p38 MAPK signaling has been implicated recently in mediating TGF-β signal transduction and in α1(I) collagen gene expression (21, 22). However, the relative contribution of these two signaling pathways and the mechanisms by which they may regulate collagen gene expression in HSCs have not been investigated.

In this study, we investigated the relative contribution of p38 MAPK and Smad signaling in regulating α1(I) collagen gene expression in cultured HSCs. We show that inhibiting either p38 MAPK or Smad signaling decreased α1(I) collagen mRNA steady state levels in TGF-β-stimulated and culture-activated HSCs (Figs. 4–6). It is possible that inhibition of p38 MAPK signaling with SB203580 could inhibit phosphorylation of the TGF-β type I receptor and subsequent phosphorylation of Smad2 and Smad3, as there appear to be similarities in the ATP binding pocket between TGF-β type I receptor and p38 MAPK (30). However, we demonstrated that 10 μM SB203580 did not inhibit TGF-β-induced Smad2 phosphorylation (Fig. 3*E*), indicating that this concentration does not affect Smad signaling and is thus an appropriate concentration to investi-

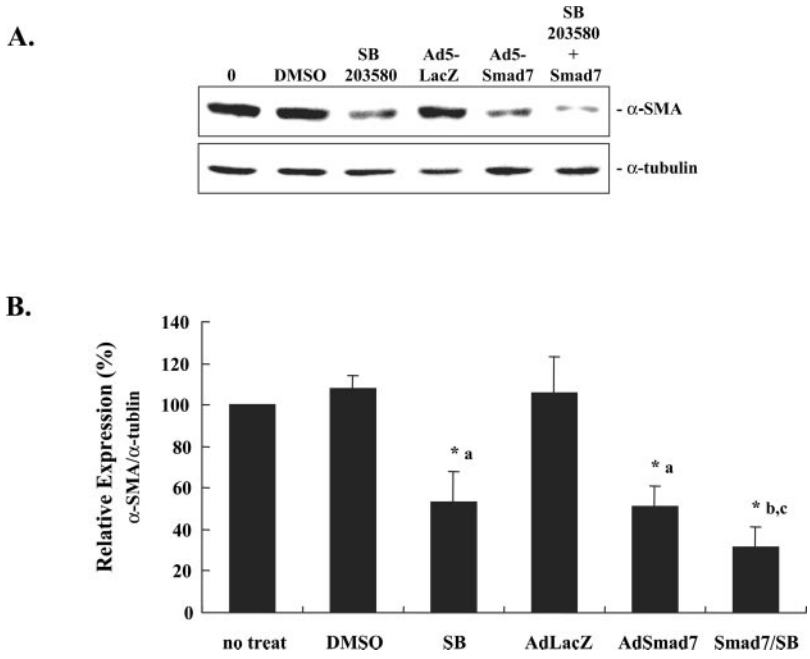

FIG. 8. **Inhibiting both p38 MAPK and Smad signaling reduces smooth muscle α-actin gene expression in HSCs.** To evaluate the effect of inhibiting p38 MAPK and Smad signaling on SMA expression in HSCs, day 2 culture HSCs were transduced with Ad5Smad7 (m.o.i. of 250) and/or HSCs were treated with 10 μM SB203580 from day 2 to day 5. Total cellular proteins were harvested after 5 days, and Western blot analyses were performed (10 μg of protein) using an anti-α-SMA or α-tubulin (*A*) antibody. The Western blot shown is representative of three independent experiments where the average data are graphically presented (*B*). *Error bars* represent S.D. *, $p < 0.05$; *a*, *versus* control; *b*, *versus* SB203580-treated HSC; *c*, *versus* Ad5Smad7-transduced HSC. *DMSO*, Me_2SO.

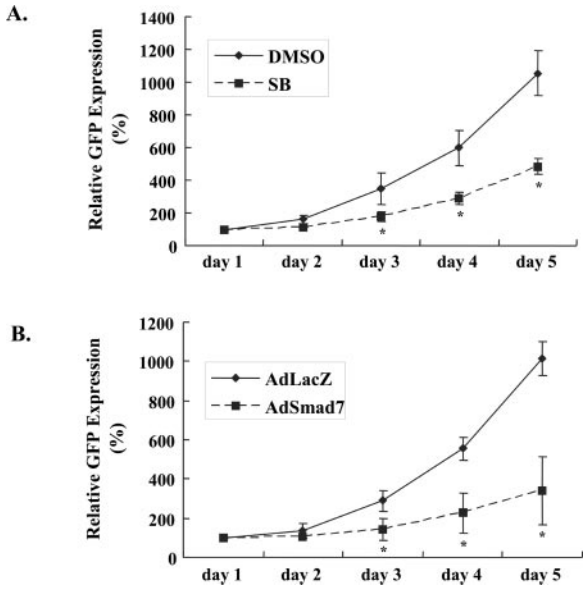

FIG. 9. **Both p38 MAPK and Smad signaling regulate α1(I) collagen gene transcription.** HSCs, isolated from transgenic mice harboring the pCol9GFP-HS4,5 transgene, were culture-activated for 5 days. HSCs were treated with 10 μM SB203580 from day 2 to day 5 or transduced with Ad5Smad7 on day 2. GFP expression was monitored and quantitated daily by PhosphorImager analysis. Graphical analysis of the PhosphorImager data is shown (*A* and *B*). Data represent results from three individual assays. *Error bars* represent S.D. *, $p < 0.05$; *versus* control. *DMSO*, Me_2SO.

gate the role of p38 MAPK in TGF-β-mediated cell signaling. We suggest that overexpressing Smad7 in HSCs and treatment of HSCs with SB203580 might exert their antifibrotic effects by inhibiting Smad2 and p38 MAPK phosphorylation, respectively (Fig. 3, *C* and *F*). In addition, we found that expression of α1(I) collagen mRNA was nearly completely blocked when both Smad and p38 MAPK signaling pathways were inhibited (Figs. 5 and 6). Our data also show that both p38 MAPK and Smad signaling independently and additively regulate the α1(I) collagen gene in both untreated and TGF-β-treated HSCs, and that these two signaling pathways represent the only two signaling pathways primarily responsible for regulating α1(I) collagen gene expression in the HSC.

Multiple MAPK pathways can be simultaneously regulated by the upstream kinase cascades known as MAPK kinases (31). Although MKK3 and MKK6 are considered to be important mediators for p38 MAPK signaling, their roles are different depending on the cell type. For example, MKK6 is the key upstream kinase for p38 MAPK in the mice thymus (32), whereas activation of MKK3 is required for TGF-β1-induced p38 MAPK activation in murine mesangial cells (33). The roles of MKK3 and MKK6 in HSCs have not been investigated previously. In this study, MKK3 and MKK6 activities were inhibited by transduction with Ad5dnMKK3 and/or Ad5dnMKK6 to investigate whether MKK3 and MKK6 are the upstream activators of p38 MAPK in HSCs. Inhibiting both MKK3 and MKK6 activities effectively blocked TGF-β-induced phosphorylation of p38 MAPK and reduced α1(I) collagen gene expression in both untreated and TGF-β-treated HSCs (Fig. 7). The level at which α1(I) collagen gene expression was reduced was similar to that when p38 MAPK signaling was blocked using SB203580. These data indicate that both MKK3 and MKK6 are positioned upstream and play important roles for activating p38 MAPK in HSCs. To emphasize the roles of Smad in HSCs, TGF-β/Smad signaling was blocked using SB431542, a potent and selective inhibitor of TGF-β type I receptor (30). SB431542 was found to inhibit α1(I) collagen mRNA expression in both untreated and TGF-β-treated HSCs, and when both Smad and MKK3/6 signaling pathways were simultaneously inhibited, α1(I) collagen mRNA expression was almost completely blocked (Fig. 7), further supporting our contention that both unstimulated and TGF-β-stimulated collagen gene expression are essentially controlled by both p38 MAPK and Smad signaling.

Smooth muscle α-actin is well known as a marker of HSC activation. We demonstrated that both p38 MAPK and Smad signaling independently regulate culture-induced α-SMA expression in untreated 5-day cultured HSCs (Fig. 8). Our data show that both p38 MAPK and Smad signaling contribute to HSC activation; however, because ~30% of α-SMA expression still remains after inhibiting both p38 MAPK and Smad signaling, other signaling pathways are probably also involved. Expression of α-SMA is also correlated with morphological changes in HSCs. On day 2 in culture, HSCs are small and contain retinoid droplets in the cytoplasm. Control HSCs, both transduced with Ad5LacZ and treated with Me_2SO, 5 days in

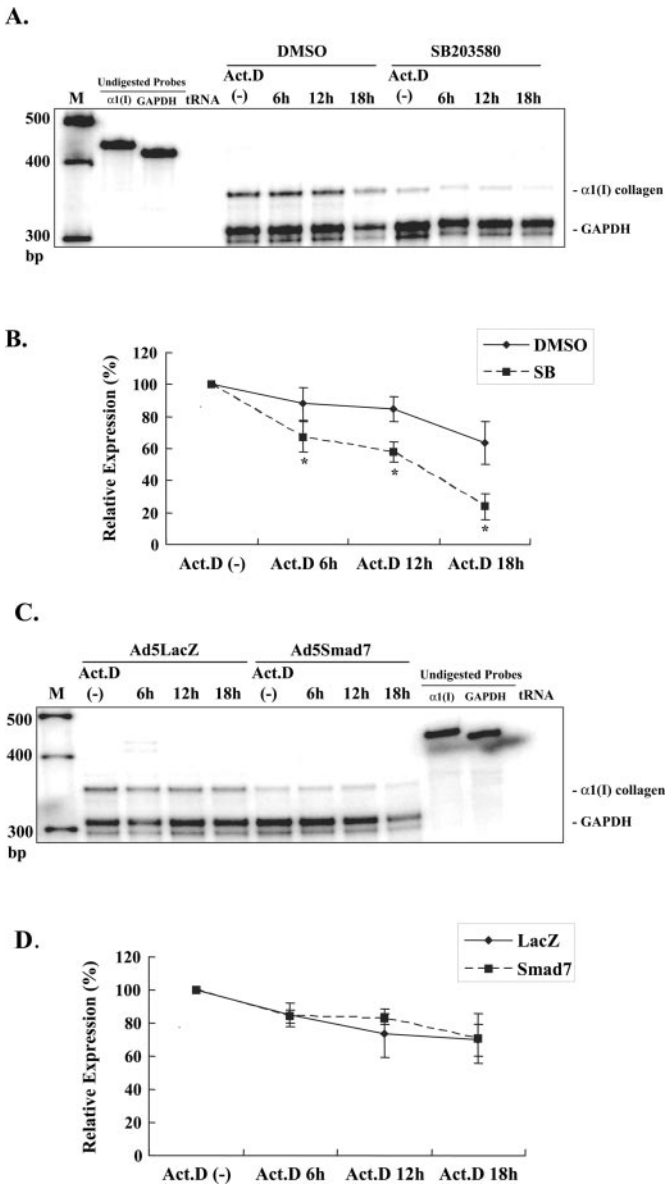

FIG. 10. **Stability of α(I) collagen mRNA is increased by p38 MAPK signaling but not by Smad signaling in culture-activated HSCs.** To determine the contribution of p38 MAPK and Smad signaling in stabilization of α1(I) collagen mRNA, HSCs cultured for 3 days were treated with 10 μM SB203580 and incubated for an additional 48 h, or HSCs cultured for 2 days were transduced with Ad5Smad7 and cultured for an additional 3 days. Afterward, the cells were treated with 5 ng/ml actinomycin D (*ActD*) for 6, 12, and 18 h. Total RNA was harvested and subjected to RNase protection assays (5 μg/reaction) to assess α1(I) collagen mRNA steady state levels, which were normalized to GAPDH as an internal control. A representative RNase protection assay is shown (*A* and *C*). tRNA was used as a negative control (*A* and *C*). Graphical analysis of data obtained from three individual RNase protection assays is shown (*B* and *D*). *Error bars* represent S.D. *, $p < 0.05$; *versus* control. *DMSO*, Me_2SO; *M*, size marker.

culture lost their retinoid droplets and changed into myofibroblast-like cells. In contrast, HSCs transduced with Ad5Smad7 or treated with SB203580 are smaller and still retain retinoid droplets, even after 5 days in culture (data not shown). Our data are, however, in contrast to one report showing that blocking TGF-β signaling with Smad7 did not decrease α-SMA expression in activated cells (5, 7, and 10 days in culture) (15). On the other hand, our data are consistent with another report showing that inhibition of TGF-β signaling in HSCs (8 days in culture), when transduced with an adenovirus expressing a soluble TGF-β type II receptor on day 5, reduced α-SMA expression (34).

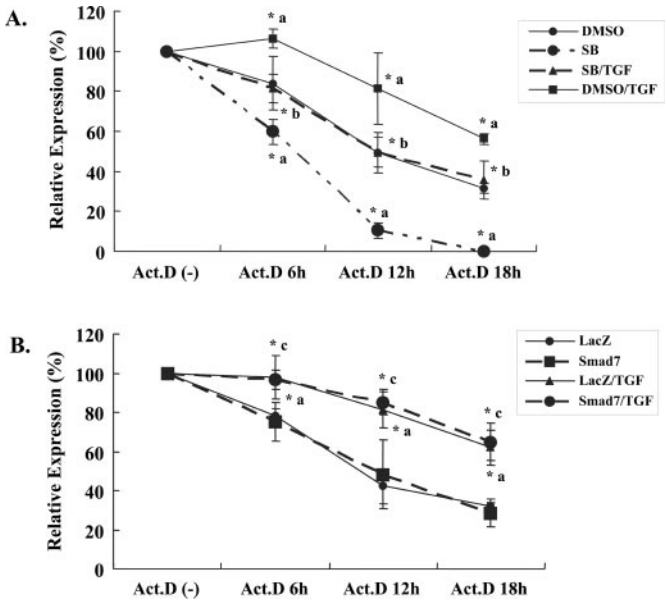

FIG. 11. **TGF-β increases α1(I) collagen mRNA stability mediated by p38 MAPK but not Smad signaling.** To determine the contribution of p38 MAPK and Smad signaling in TGF-β-induced α1(I) collagen mRNA stability, day 3 culture HSCs were treated with 10 μM SB203580 for 1 h, or day 2 culture HSCs were transduced with Ad5Smad7 followed by the treatment with 5 ng/ml TGF-β. On the following day (4 days in culture) transcription was blocked with 5 ng/ml actinomycin D (*ActD*) for the times indicated. HSCs were harvested, and total RNA (5 μg) was subjected to RNase protection assays to assess α1(I) collagen mRNA steady state levels, which were normalized to GAPDH as an internal control. Graphical analysis of data obtained from three independent RNase protection assays data is shown (*A* and *B*). *Error bars* represent S.D. *, $p < 0.05$; *a*, *versus* control HSCs; *b*, *versus* TGF-β-treated HSCs; *c, versus* Ad5Smad7-transduced HSCs. *DMSO*, Me_2SO.

It is well established that the activated HSC is primarily responsible for the increase in collagen gene expression. The molecular mechanisms responsible for regulating the α1(I) collagen gene in HSCs are only partially understood. Increased collagen expression following HSC activation has been shown to be mediated by both an increase in gene transcription and an increase in the stability of the α1(I) collagen mRNA (23, 25, 27). To investigate the mechanisms of how p38 MAPK and Smad signaling regulate the α1(I) collagen gene, their effects on the transcriptional and post-transcriptional regulation were examined. In order to assess a potential role of p38 MAPK and Smad signaling on collagen gene transcription, HSCs were isolated from transgenic mice harboring the pCol9GFP-HS4,5 transgene (27). This reporter gene has been shown previously to be regulated similarly to that of the endogenous α1(I) collagen gene (25). It is well known that the Smad proteins transmit TGF-β signals from TGF-β receptors directly to nuclear transcriptional targets. We showed that inhibiting p38 MAPK or Smad signaling reduced GFP expression in HSCs isolated from pCol9GFP-HS4,5 transgenic mice (Fig. 9), thereby demonstrating that both p38 MAPK and Smad signaling pathways are both involved in transcriptional regulation of the α1(I) collagen gene. TGF-β has been recognized as a potent inducer of both the α1(I) and α2(I) collagen genes. A TGF-β-response element in the α2(I) collagen gene has been located between nucleotides −330 and −255 bp within the gene promoter (35). In contrast, the location of the TGF-β-response element in the α1(I) collagen gene has not been clearly mapped. A TGF-β regulatory element has been reported ~1600 bp from the transcriptional start site (36), and another report (37) indicates a TGF-β-response element located between nucleotide −370 and −344 bp; however, these putative TGF-β-response elements have not

been independently confirmed, and it is still unknown whether these TGF-β-response elements function in the HSC.

In addition to transcriptional regulation, another mechanism by which gene expression can be effectively controlled is through mRNA stabilization. Therefore, we investigated the effects of p38 MAPK and Smad signaling on stabilizing the α1(I) collagen mRNA in early activated HSCs. TGF-β is known to affect stabilization of several mRNAs, including α1(I) collagen (38) and elastin (39). We found that TGF-β increased the stability of α1(I) collagen mRNA from 12 to 20 h in HSCs cultured for 4 days (Fig. 11). Inhibiting p38 MAPK activity decreased both culture-induced and TGF-β-induced stability of α1(I) collagen mRNA (Figs. 10, *A* and *B,* and 11*A*), indicating that p38 MAPK plays an important role in post-transcriptional regulation of α1(I) collagen gene expression in HSCs. In contrast, inhibition of Smad signaling did not affect either culture-induced or TGF-β-induced stability of α1(I) collagen mRNA (Figs. 10, *C* and *D,* and 11*B*). p38 MAPK activity has been shown to be involved in stabilizing unstable mRNAs of several cytokine genes (40, 41). Culture-induced activation of HSCs is accompanied by a dramatic stabilization of the α1(I) collagen mRNA, where the half-life of α1(I) collagen mRNA increases from an estimated 1.5 h in quiescent HSCs to ~24 h in activated HSCs (28). This increase in mRNA stabilization correlates with increased binding activity of αCP to the α1(I) collagen 3′-untranslated region following HSC activation (28). It is also reported that p38 MAPK activity positively influences cell activation in both quiescent HSCs (42) and in pancreatic stellate cells (43), and this effect of p38 MAPK might affect on α1(I) collagen mRNA stability. Together, our data demonstrate that in HSCs TGF-β increases α1(I) collagen mRNA stability through p38 MAPK signaling and not by a Smad-mediated pathway.

In summary, both p38 MAPK and Smad signaling independently regulate α1(I) collagen gene in short term cultured HSCs in an additive manner. Collagen transcription is activated by both Smad and p38 MAPK signaling pathways, whereas the stability of α1(I) collagen mRNA is mediated by p38 MAPK signaling, but not by Smad signaling. In addition, we found that TGFβ increases α1(I) collagen mRNA stability, which was mediated by p38 MAPK activity.

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