Smad2 and Smad3 as mediators of the response of adventitial fibroblasts induced by transforming growth factor β1

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Abstract. Transforming growth factor β1 (TGF-β1) is a known pleiotropic growth factor in cell proliferation, migration and phenotypic transition. Fibroblasts are considered the most reactive in the vascular wall. We aimed to explore the effect and mechanism of TGF-β1 on aortal adventitial fibroblasts (AFs) induced by TGF-β1. AFs were cultured *in vitro* by tissue explants. After stimulation by TGF-β1 and treatment with small interfering RNA (siRNA)-Smad2 and siRNA-Smad3, MTT and the transwell chamber techniques were used for assessing AF proliferation and migration. The expression of phospho (pho)-Smad2, pho-Smad3, Smad7, α-smooth muscle actin (SMA) and collagen I and III were evaluated by Western blot analysis and real-time RT-PCR. After stimulation by TGF-β1 for 24 h, the proliferation and migration of treated AFs were higher compared to those of untreated AFs, as was the expression of Smad2, Smad3, pho-Smad2, pho-Smad3, SMA and collagen I and III (P<0.05), but not Smad7 (P>0.05). Knockdown of Smad2 and Smad3 inhibited proliferation and migration of AFs and down-regulated the expression of SMA and collagen I and III (P<0.05). TGF-β1 plays a key role in remodeling processes by contributing to the proliferation, migration and phenotypic modulation of AFs and collagen composition. The mechanism may be related to both the Smad2 and Smad3 signaling pathways.

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Introduction

Adventitia, traditionally considered supporting tissue that provides nourishment and support to the blood vessels, is emerging as a prominent factor in the pathogenesis of cardiovascular diseases. Adventitia plays an important role in controlling vascular remodeling and vessel tone through regulatory systems. It is the most sensitive layer responding to blood pressure (1). One of the earliest signs found in hypertensive remodeling is the change in adventitial cells. Adventitial fibroblasts (AFs), the main adventitial cell type, drive remodeling and may initiate other changes, such as alterations in the arrangement of neointimal proliferation, smooth muscle cells and extracellular matrix (ECM), which are expected to lead to vascular remodeling (2-4). Therefore, determining the mechanism responsible for adventitial activation provides a potential therapeutic strategy for vascular diseases.

Transforming growth factor β1 (TGF-β1), up-regulated at the site of vascular injury, is a multifunctional cytokine involved in various functions, such as promoting proliferation, adhesion, migration, synthesis of ECM and myofibroblast formation in fibrosis, among many cell types (5,6). TGF-β1 is an important perivascular growth factor in the adventitia (7), and adventitial cells play a significant role in vascular remodelling processes under the influence of TGF-β1 (8). The classical signal transduction pathway of the TGF-β family is through Smad (9). The decrease of the transcription of TGF-β1 response genes is a consequence of inhibiting Smad3 phosphorylation in human dermal fibroblasts (10). Treating cells with SIS3, a potent and selective inhibitor of Smad3, may reduce TGF-β1-mediated up-regulation of α2 (I) collagen mRNA (11). Smad2, 3 and 4 participate in AF proliferation stimulated by TGF-β1 (12). However, whether the TGF-β1/Smad signaling pathway takes part in stimulating AFs and remains unclear, as does its precise effect.

In the present study, we hypothesized that the Smad2 and 3 signaling pathways are simultaneously involved in the proliferation, migration, transdifferentiation and ECM deposition of AFs stimulated by TGF-β1. AF induction by TGF-β1

Table I. Primers for quantitative real-time RT-PCR.

Genes	Forward and reverse primers (5'-3')	Amplicon size (bp)	Temperature (˚C)
β-actin	F: GACAGGATGCAGAAGGAGATTACT	142	57.9
(NM-031144)	R: TGATCCACATCTGCTGGAAGGT		57.7
Smad2	F: GCCGAGTGCCTAAGTGAT	153	57.3
(NM-019191)	R: AGACTGAGCCAGAAGAGC		57.3
Smad3	F: TGTCATCTACTGCCGCTTGTG	163	57.6
(NM-013095)	R: CAACACTGGAGGTAGCACTGG		59.5
SMA	F: CTGGTATTGTGCTGGACTC	289	55.2
(NM-031004)	R: CATCAGGCAGTTCGTAGC		54.9
Collagen I	F: GGCATAAAGGGTCATCGTG	714	55.2
(NM-053304)	R: GAACCTTCGCTTCCATACTC		55.4
Collagen III	F: CCCAGAACATTACATACCACT	190	53.7
(NM-032085)	R: GTCTTGCTCCATTCACCAG		55.2

and then interference by small interfering RNAs (siRNAs) was used to examine the effect of TGF-β1 through Smad2 and 3 in the remodeling processes.

Materials and methods

Cell culture. AFs were obtained from the thoracic aorta adventitia of Wistar-Kyoto rats. Adventitia was carefully decorticated, cut into small pieces (1 mm³) and distributed equally into culture flasks, then cultured in DMEM supplemented with heat-inactivated fetal calf serum (FCS) (10%, v/v) and 10 mmol/l HEPES in a humidified atmosphere with 5% (v/v) CO_2 at 37˚C. Three to seven days later, AFs were observed to grow out from the tissues, and cells at passages 3-8 were used in assays. Immunocytochemistry with vimentin (Wuhan Boster Biological Technology, LDT, China) and α-smooth muscle actin (SMA; Abcam, Cambridge, UK) was used to identify AFs and their purity.

Experimental groups. AFs were divided into two groups: AFs treated with TGF-β1 (20 ng/ml; recombinant human TGF-β1) (PeproTech, Rocky Hill, NJ, USA) and control (untreated) AFs. Other AFs were divided into five groups: TGF-β1-treated AFs, negative (non-targeting siRNA duplexes) + TGF-β1-treated AFs, siRNA-Smad2/3 + TGF-β1-treated AFs and control (untreated) AFs.

SiRNA method. Target siRNAs were identified by a siRNA design tool (Dharmacon Inc., Lafayette, CO, USA) and the transfection was performed according to the manufacturer's instructions. The duplexes of siRNAs targeting Smad2 (GenBank no. NM-019191) were 5'-GCC AGUUACUUAUUCAGAATT-3' and 5'-UUCUGAAUAAGU AACUGGCTG-3' and those targeting Smad3 (GenBank no. NM-013095) were 5'-GCUGUUCCAGCGUGUCUUATT-3' and 5'-UAAGACACGCUGGAACAGCGG-3'. All siRNAs were chemically synthesized by Shanghai GenePharma Co. AFs were seeded at a density of 50% the day before transfection, then treated with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and 100 pmol/ml of siRNA mixed in

Opti-MEM. Twenty-four hours after transfection, the cells were stimulated by TGF-β1 and incubated for another 24 h. Western blot analysis was used to confirm the effect of knockdown.

MTT assay. AFs were seeded in 96-well plates at 2.0x10⁴ cells/well. After incubation in complete medium containing 10% FCS overnight, the cells were serum-starved and synchronized for 4 h. AFs in the TGF-β1 experiment were treated with TGF-β1 for 24 h. An amount of 20 μl MTT (5 mg/ml) was added, followed by incubation for 4 h at 37˚C. Finally, the medium was removed and the cells were lysed with DMSO (150 μl) for 15 min. The absorbance of the samples was recorded at 490 nm using a microplate reader. Six wells per treatment were used. Wells with only MTT and DMSO served as blank controls.

Transwell migration assay. AFs were grouped and treated as described above. Cells were cultured in serum-free DMEM for 4 h prior to the migration assays. The migration of the AFs was measured using a transwell chamber apparatus (Greiner Bio One Ltd., Germany) (8-μm pore size, 24.85-mm diameter). Briefly, the cells were trypsinized and counted, a 600 μl cell suspension at 5x10⁵/ml in DMEM was added to the upper compartment of the chamber, and 1.5 ml DMEM containing 10% FCS was added to the lower compartment. After incubation at 37˚C for 6 h, cells on the upper face of the membrane were removed with a cotton-tipped applicator. Then, the membranes were fixed in methanol and stained with H&E. The number of migrated cells was counted in 5 random fields in each membrane. Each experiment was performed in triplicate.

Real-time RT-PCR. After treatment, total RNA was isolated from the AFs using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA quality and concentration were determined with a spectrophotometer (DU®800; Beckman, Palo Alto, CA, USA). RNA (1 μg) was treated with DNase, then reverse transcribed by a standard protocol (Fermentas, Glen Burnie, MD, USA). Quantitative real-time

RT-PCR was performed with a Light Cycler apparatus (Roche Diagnostics, Mannheim, Germany). Negative control samples (no template) were run concurrently and β-actin was used as an internal control. Primers are shown in Table I. Experiments were performed in triplicate. The relative mRNA expression of the genes was determined by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. Cells were treated as described and then harvested. Protein was extracted and detected, and 30 μg protein samples were used for SDS-PAGE. After the protein was transferred onto nitrocellulose membranes, the membrane was blocked and incubated with antibodies: anti-Smad2 (1:1,000), anti-phospho (pho)-Smad2 (1:300), anti-Smad3 (1:300), anti-pho-Smad3 (1:500; all Cell Signaling Technology, Inc., Danvers, MA, USA), anti-Smad7 (1:500; R&D Systems, Minneapolis, MN, USA), anti-SMA (1:200) and anti-pro-COL1A1 (1:300, A-17; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The bands were quantified by densitometry and normalized to the levels of β-actin.

Statistical analysis. Data are presented as the mean ± SD and were analyzed using SPSS v16.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to analyze intergroup differences. When only two groups were compared, the independent samples t-test was used. Data with abnormal distribution were analyzed by non-parametric statistics. P<0.05 was considered statistically significant. All experiments were repeated at least three times.

Results

AF identification and Smad protein expression. Three to seven days after culture, AFs were observed to grow out from the tissues. Cells appeared in a single layer and were irregularly shaped with a large body. Immunocytochemistry demonstrated negative staining for SMA and positive staining for vimentin, which suggested the AFs had 100% purity. After treatment with 100 pmol/ml siRNA, Smad2 and 3 expression was knocked down to <20% (Fig. 1A and B). Moreover, the knockdown was selective, because Smad3 protein levels were decreased by Smad3 siRNA only, as were Smad2 protein levels (Fig. 1C and D).

AF proliferation induced by TGF-β1 inhibited by siRNA-Smad2 and siRNA-Smad3. The MTT assay was used to quantify cell proliferation. TGF-β1 treatment for 24 h resulted in a significant induction of cell proliferation as compared to the control group (Fig. 2). However, the proliferation decreased when Smad2 or 3 expression was knocked down by siRNA (P<0.01), with no significant difference between Smad2 and 3 knockdown.

AF migration induced by TGF-β1 inhibited by siRNA-Smad2 and siRNA-Smad3. After stimulation by TGF-β1 for 24 h, the migration of the AFs was significantly increased (P<0.01) (Fig. 3). However, the blockade of Smad2 and 3 expression by siRNA reduced the TGF-β1-induced migration of the AFs (P<0.01). Smad3 was reported to play a crucial role in the proliferation and migration of fibroblasts induced by TGF-β1 (13). Thus, the up-regulation of migration in response to TGF-β1 in AFs may depend on both Smad2 and Smad3.
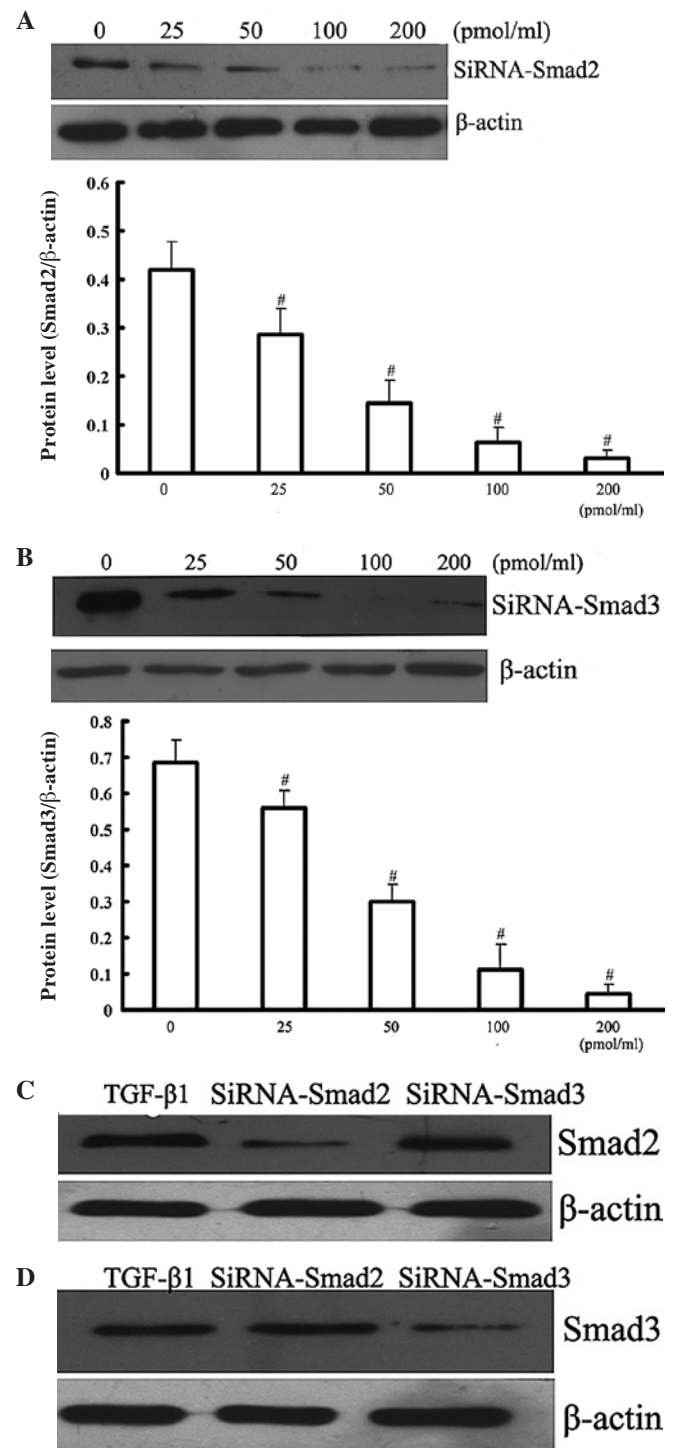

Figure 1. Efficiency of Smad knockdown. (A and B) Efficiency of Smad knockdown with different concentrations of siRNA oligomer. Knockdown of protein levels of (C) Smad2 and (D) Smad3 by Smad2 and Smad3 siRNA.

AF transdifferentiation and ECM deposition induced by TGF-β1 requires Smad2 and Smad3. Under the influence of TGF-β1, the expression of Smad2, SMA and collagen I and III was increased by 3.55-, 4.96-, 12.9- and 3.52-fold, respectively, compared to the control (Fig. 4A and B). The expression of Smad3 was also increased after stimulation (14). However, Smad7 expression did not change compared to the control (P>0.05), which agrees with findings that Smad7 is an immediate-early gene target of TGF-β1 in fibroblasts (15,16).
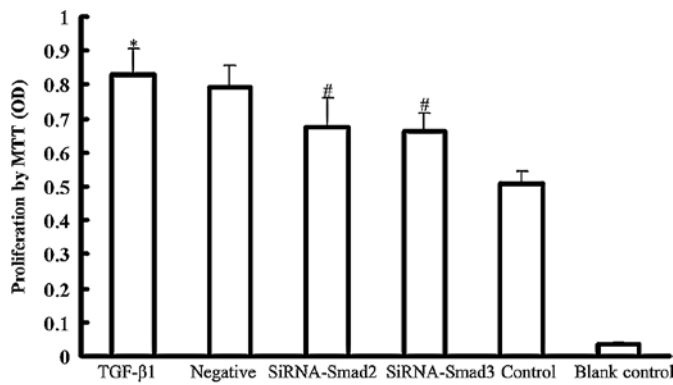
Figure 2. Effect of TGF-β1 and siRNA on the proliferation of AFs by MTT. Data are expressed as the mean ± SD (n=6). Stimulation by TGF-β1 for 24 h, *P<0.01 compared to the control. Twenty-four hours after transfection, followed by another 24-h stimulation by TGF-β1, #P<0.01 compared to TGF-β1 alone.
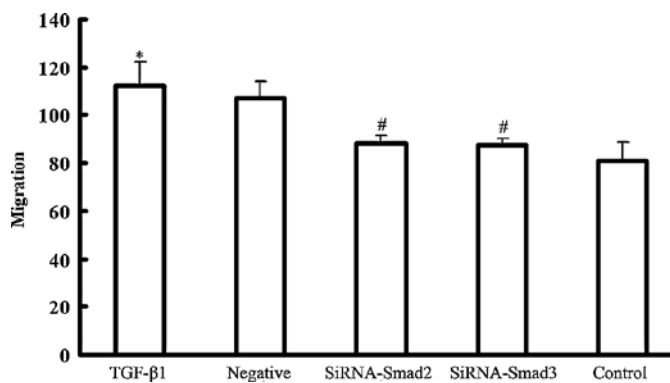

Figure 3. Effect of TGF-β1 and siRNA on the migration of AFs measured by transwell chamber analysis. The number of migrated cells was counted in 5 random fields. Results are the mean ± SD from at least three independent experiments. Stimulation by TGF-β1 for 24 h, *P<0.01 compared to the control. Twenty-four hours after transfection, followed by another 24-h stimulation by TGF-β1, #P<0.01 compared to TGF-β1 alone.

Figure 4. RT-PCR analysis of mRNA expression with TGF-β1 and siRNA treatment. (A) mRNA expression of Smad2 and 7. Data are expressed as the mean ± SD. β-actin served as the internal standard. *P<0.01 compared to the control. (B) Effect of siRNA on the mRNA expression of SMA and collagen I and III. Data are the mean ± SD. β-actin served as the internal standard. *P<0.01; **P<0.05; #P<0.01; ##P<0.05 compared to the control or the TGF-β1 group.

The phosphorylation of Smad2 and Smad3 was enhanced upon challenge with TGF-β1 for 24 h (P<0.01) (Fig. 5B and C). When Smad2 and Smad3 expression was silenced with siRNA, the expression of pho-Smad2 and pho-Smad3 was attenuated (P<0.05) (Fig. 5B and C). Unfortunately, in the present study, the phosphorylation of Smad2 and Smad3 was not completely arrested by siRNA knockdown. This result may be associated with the time we selected to detect the protein levels or with the delayed degradation of Smad2 and 3.

We investigated the effect of Smad2 and 3 knockdown on TGF-β1-induced SMA expression by RT-PCR and Western blotting. TGF-β1-induced SMA expression was decreased by Smad2 or 3 knockdown to an equal extent (Figs. 4B and 5A). Thus, TGF-β1-induced SMA expression depends on both the Smad2 and 3 signaling pathways in AFs. An increase in collagen I and III expression was observed after 24 h of TGF-β1 treatment (Figs. 4B and 5A), which suggests that collagen I and III are involved in the synthesis of ECM induced by TGF-β1. This induction was inhibited by Smad2 and 3 knockdown with siRNA, with no significant difference between their efficacy (P>0.05). Thus, the induction of collagen I and III by TGF-β1 in AFs is both Smad2- and Smad3-dependent.
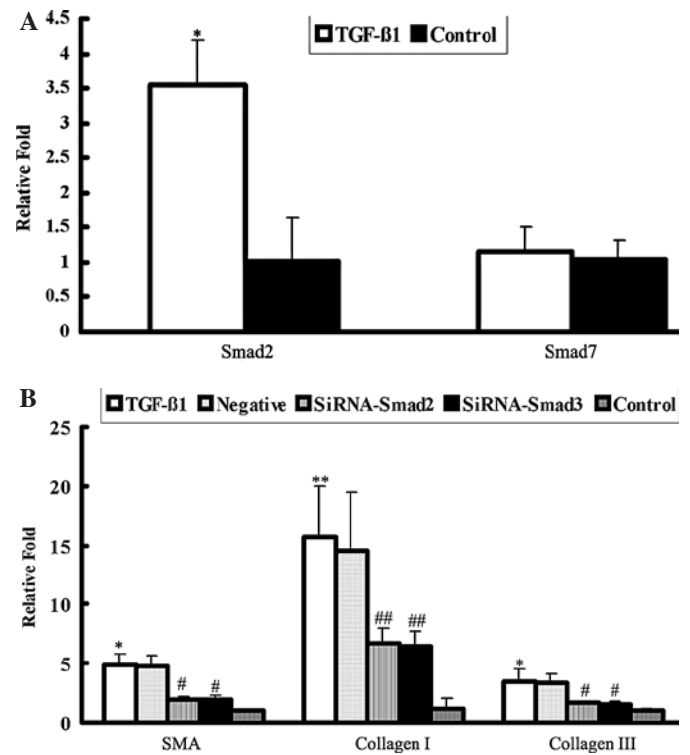
Discussion

Increasing evidence suggests that TGF-β1 plays a key role in arterial remodeling processes. In the present study, we investigated the effect of TGF-β1 on AFs in cell proliferation, migration, differentiation and the synthesis of ECM. Our results suggest that TGF-β1 is a risk factor for vascular remodeling. Moreover, the roles of receptor-associated Smads (Smad2 and 3) were shown in the regulation of TGF-β1-driven pro-fibrotic events in AFs. Our results demonstrate that Smad2 and 3 are both required for inducing the proliferation and migration of AFs, and for increasing SMA and collagen I and III content in AFs, in response to TGF-β1.

The origin of the cells that contribute to neointimal formation after endoluminal vascular injury has been widely debated. Currently, the adventitia is viewed as a key player during vascular growth and repair. Fibroblasts are the most abundant cell type of the adventitia. The migratory and proliferative responses of myofibroblasts, the specialized phenotype of activated fibroblasts, in addition to the synthesis of ECM, play essential roles during vascular remodeling. Nevertheless, the role of AFs in vascular remodeling remains disputed. The contribution of coronary AFs to neointimal formation was found to be negligible (17), but the research contained some limitations, such as the time, age of the animals and the recapitulation of the diseased vessels in humans after percutaneous transluminal coronary angioplasty. We found that the
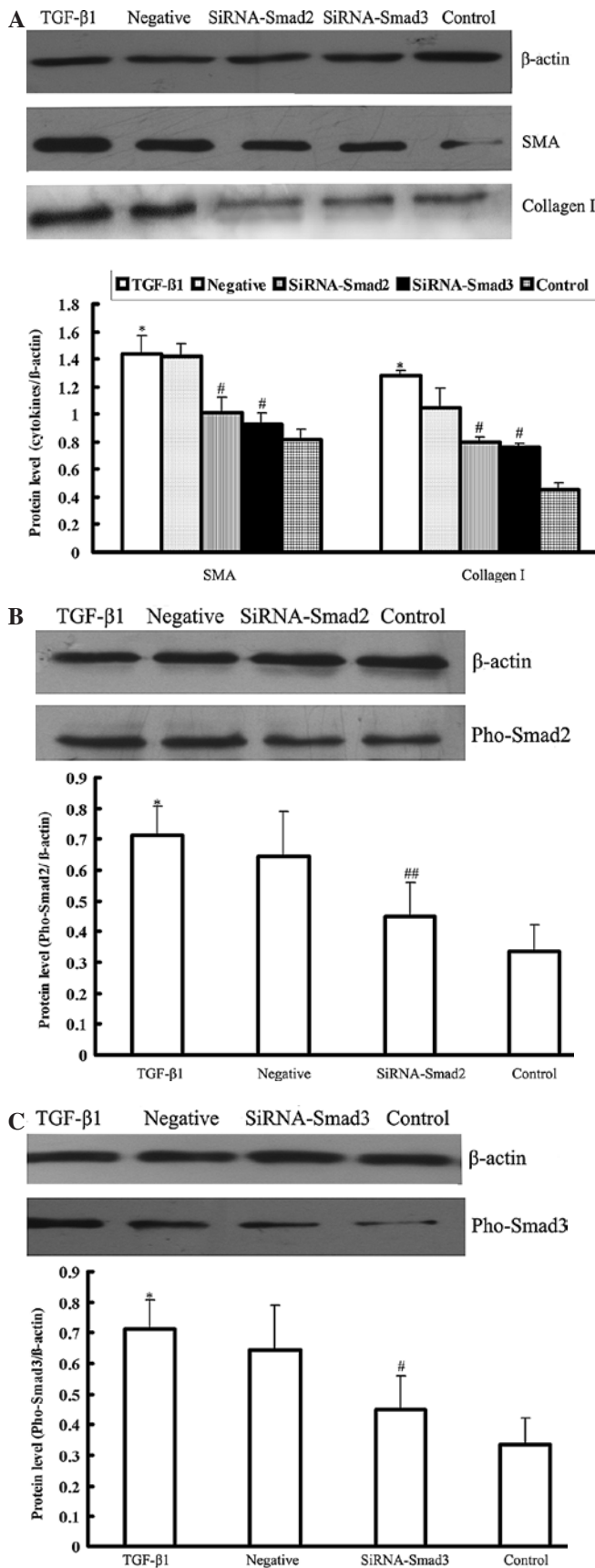
Figure 5. Western blot analysis of protein expression with TGF-β1 and siRNA treatment. (A) Expression of SMA and Pro-COL1A1 protein. Results are the mean ± SD. *P<0.01 compared to the control; #P<0.01 compared to the TGF-β1 group. (B) Expression of phosphorylated Smad2. Results are the mean ± SD. *P<0.01 compared to the control; ##P<0.05 compared to the TGF-β1 group. (C) Expression of phosphorylated Smad3. Results are the mean ± SD. *P<0.01 compared to the control; #P<0.01 compared to the TGF-β1 group.

proliferation, migration, differentiation and collagen composition of AFs increased under the influence of TGF-β1.

TGF-β1, the most potent mediator of myofibroblast differentiation, induces SMA expression in fibroblastic cells (18,19) and affects the migration, proliferation and synthesis of collagens and other matrix components of fibroblasts (20,21). Myofibroblasts originating from adventitia play a crucial role in neointimal formation and remodeling after coronary injury (22). TGF-β1 signals through transmembrane receptor serine/threonine kinases that activate the cytoplasmic proteins Smads to regulate cell proliferation, differentiation and death (23). Our results showed that TGF-β1-induced SMA expression depended on both Smad2 and Smad3 in AFs. Moreover, Smad7 expression is induced by TGF-β1 and may act via an autoregulatory negative feedback loop (24). We found no significant change in Smad7 gene expression after TGF-β1 stimulation for 24 h, which was consistent with previous reports (15,16).

Cross-talk among Smad, mitogen-activated protein kinase (MAPK) and integrin signaling pathways may account for the mechanisms of AF function (12). SMC expression is regulated by p38 MAPK, extracellular-regulated signal kinase 1/2 and Smad signaling in renal proximal tubular cells induced by TGF-β1 (25). TGF-β1 inducing fibroblast transdifferentiation to myofibroblasts is involved in integrin-mediated signaling and focal adhesion kinase (26). The Smad signaling pathway is the most classical pathway induced by TGF-β1. The signaling molecules Smad2 and 3 play a key role in the progression of renal fibrosis (27). Smad3 mediates TGF-β1-induced collagen I expression in human mesangial cells (28). As well, Smad3 and pho-Smad3 contribute to liver fibrosis (29). Hu et al have shown that TGF-β1 may contribute to the up-regulation of α-SMA via Smad3 in rat lung fibroblasts (30). Any adventitial effect of Smad3 overexpression was found to be indirect, mediated by infected medial or neointimal cells (31), but this research did not have a relevant control group to exclude other factors that up-regulated Smad3 overexpression directly in adventitia. In our study, we found that after direct induction by TGF-β1, Smad3 and pho-Smad3 were increased. Although many profibrotic genes principally mediated by Smad3, Smad2 and the phosphorylation of Smad2 play critical roles in human tenon fibroblast stimulation by TGF-β1 and scar formation (32,33), our finding suggested that Smad2 and 3 both work as critical factors in AF proliferation and migration induced by TGF-β1. Both Smad2 and 3 knockdown attenuated proliferation, migration and TGF-β1-based SMA gene activation in myofibroblasts. Rhubarb has been found to attenuate the TGF-β1-mediated migration of hepatic stellate cells by interfering with Smad2 and 3 phosphorylation (34).

Collagen types I and III, the major components of the ECM, play a role in the remodeling of the aortic wall in response to hypertension (35). Our study showed that the mRNA and protein levels of collagen I and III were down-regulated after intervention by siRNA-Smad2 and siRNA-Smad3 with TGF-β1 stimulation for 24 h as compared to TGF-β1 alone, with no significant difference between Smad2 and Smad3 knockdown. Smad3 is usually considered the key mediator of fibrotic response, whereas Smad2 is more limited. Smad3 has been shown to play a role in the TGF-β1-mediated induction of collagen expression (36). However, the knockdown

of Smad2 also prevents the effect of TGF-β1 in enhancing α1 (I) collagen promoter (37). There is evidence to suggest that tetrandrine suppresses Smad2 signaling and fibrogenic responses in association with Smad7 up-regulation in human subconjunctival fibroblasts (38). We conclude that the TGF-β1-induced ECM synthesis of AFs may be involved in both the Smad2 and Smad3 signaling pathways.

In conclusion, we found that TGF-β1 initiated the proliferation, migration, differentiation and collagen synthesis of AFs, and that the mechanism is critically dependent on Smad2 and Smad3. Our study supports that both Smad2 and Smad3 are key mediators of TGF-β1-induced AF activity.

Acknowledgements

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