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Antagonism of transforming growth factor-β signaling inhibits fibrosis-related genes

Xing-Jun Liu[1,2], Cheng-Mai Ruan[3], Xian-Feng Gong[2], Xing-Zhou Li[3], Huai-Liang Wang[4], Min-Wei Wang[2] & James Q. Yin[1,*]
[1]*Protein & Peptide Pharmaceutical Laboratory, Institute of Biophysics, Chinese Academy of Sciences, 100101, Beijing, China*
[2]*School of Pharmacy, Shengyang Pharmaceutical University, 110016, Shengyang, China*
[3]*Institute of Pharmacology Toxicology, The Academy of Military Medical Sciences, 100850, Beijing, China*
[4]*School of Basic Medicine, China Medical University, 110001, Shenyang, China*
**Author for correspondence (Fax: +86-10-64871293; E-mail: jqwyin@sun5.ibp.ac.cn)*

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Abstract

In the fibrotic process, the transforming growth factor-β1 (TGF-β1)/Smad3 (Sma- and Mad-related protein 3) signaling plays a central role. To screen for antagonists of TGF-β1/Smad3 signaling and to investigate their effects on the genes related to fibrosis, we construct a molecular model with a luciferase reporter gene. Results showed that both SB-431542 [4-(5-benzo[1,3]dioxol-5-yl-4-pyridin-2-yl-1*H*-imidazol-2-yl)-benzamide] and small interference RNA (siRNA) against Smad3 could dose-dependently suppress the reporter gene. More importantly, they both significantly inhibited the expression of plasminogen activator inhibitor-type 1 (PAI-1) and type I collagenα1 (Col Iα1) genes in rat hepatic stellate cells. Thus, SB-431542 and Smad3/siRNA may be potential therapeutics for fibrosis.

Introduction

Transforming growth factor-β (TGF-β), a 112 amino acid homodimeric protein, is the most potent profibrogenic mediator in liver fibrosis, and a key cytokine of the fibrotic response to wounding (Hui *et al.* 2003). The multiple biological effects of TGF-β contribute to its critical role in many fibrotic diseases, including cirrhosis, chronic hepatitis, glomerulonephritis, scleroderma and pulmonary fibrosis (Border *et al.* 1994). As TGF-β not only enhances synthesis of matrix proteins but also increases secretion of protease inhibitors while reducing secretion of proteases, it is a potent stimulator of matrix accumulation (Roberts *et al.* 1993). TGF-β is composed of three highly homologous isoforms (TGF-β 1, 2 and 3) that often have similar biological activities *in vitro*, while eliciting distinct biological responses *in vivo*. Briefly, signaling from TGF-β1 is mediated by a series of highly conserved transmembrane receptors and Smads proteins in mammalian cells. After TGF-β1 binds to and phosphorylates the type II receptor (TβRII), the activated TβRII recruits, phosphorylates and activates the type I receptor (TβRI). The activation of TβRI is one of the critical events in TGF-β1 signaling and the initiation point for downstream events. Smads 2 and 3 are recruited to the activated type I receptor by SARA (Smad anchor for receptor activation) and are directly phosphorylated by the type I TGF-β receptor kinase. Subsequently, the activated Smad3 binds to the common mediator Smad4, and forms a Smad3–Smad4 complex

(ten Dijke *et al.* 2004). After the complex shuttles into the nucleus, it interacts with various transcription factors and regulates transcription of target genes, including plasminogen activator inhibitor-type 1 (PAI-1), type I collagenα1 (Col Iα1), fibronectin and other extracellular matrix (ECM) genes (Dennler *et al.* 1998, Verrecchia *et al.* 2001, Ota *et al.* 2002).

Hepatic stellate cells (HSCs) play a central role in the pathogenesis of liver fibrosis. Numerous growth factors and cytokines are involved in HSC activation, including transforming growth factor, among others. Activation of HSCs is responsible for increased deposition of ECM and reduced matrix degradation (Friedman *et al.* 2000). The process of HSCs activation and transition into myofibroblasts is orchestrated by TGF-β1. TGF-β1 is considered a potent mediator in the accumulation of extracellular matrix, because it induces matrix gene expression and inhibits its degradation via induction of tissue inhibitor metalloproteinase (TIMP). HSCs respond to TGF-β1 with increased production of type I collagen, the predominant ECM protein in liver fibrosis, via multiple pathways, including the Smad proteins, extracellular signal-regulated kinase (ERK) signaling and oxidative stress. Therefore, the development of TGF-β1signaling antagonists is one of the most potent therapeutic strategies for hepatic fibrosis and cirrhosis. For example, antibodies against TGF-β ligands, small molecule inhibitors of the TβRI, and soluble TβRII:Fc fusion proteins are anti-signaling approaches currently under development (Border *et al.* 1994, George *et al.* 1999, Nakamura *et al.* 2000, Inman *et al.* 2002, Laping *et al.* 2002).

In this study, we have developed a molecular model with the luciferase reporter gene, based on a previous report (Dennler *et al.* 1998), by which antagonists of the TGF-β1/Smad3 pathway could be screened *in vitro*. In the stable transfection of mink lung epithelial cells (Mv1Lu) or rat HSCs, we examined the biological effects of the specific small molecular inhibitor of TβRI, SB-4315412 (Callahan *et al.* 2002, Inman *et al.* 2002, Laping *et al.* 2002), and siRNA (siRNA) against Smad3. To determine if Smad3 mediated TGF-β induction of hepatic fibrosis, SB-4315412 was employed to inhibit the phosphorylation of Smad3 protein, and the RNA interference technique (Yin *et al.* 2002, Tan *et al.* 2005) was utilized to silence the Smad3 gene. In this context, we also investigated the relationship between Smad3 and other genes involving lung and hepatic fibrosis.

Materials and methods

Expression and reporter plasmids

CAGA reporter vectors were generated using a B_4-vector (Promega, USA). The $(CAGA)_{12}$ and MLP (Promega, USA) fragments were PCR-amplified and ligated with ligase, under standard conditions, before the $(CAGA)_{12}$-MLP fragments were subcloned into a T-vector for amplification in *E. coli.*

After being cut from the T-vector with both *Nde*I and *Xho*I sites, the $(CAGA)_{12}$-pMLP fragments were ligated into a B4-vector. Finally, a luciferase reporter gene (Promega, USA) was subcloned into a B4-$(CAGA)_{12}$-pMLP plasmid at the *Nde*I and *Xba*I sites. All constructs were sequence-checked.

Cell culture and transfection

The Mv1Lu mink lung epithelial cell line (CCL 64) was from ATCC. Samples of the hepatic stellate cell line (HSC-T6), derived from immortalized and activated rat HSCs transfected by SV40, were kindly donated by Professor S.L. Friedman of Mount Sinai Medical Center. Mv1Lu and rat HSC-T6 cells were maintained at 37 °C in DMEM (Gibco, USA), containing 100 ml fetal calf serum (FCS, Gibco, USA)/l, 100 units penicillin/ml and 100 μg streptomycin/ml in a 50 ml CO_2 atmosphere/l. Mv1Lu and rat HSC-T6 cells were transiently transfected with the indicated construct and the Renilla luciferase internal control plasmids using Lipofectamine 2000, according to the manufacturer's instructions (Life Technologies, New England). The stable transfection of Mv1Lu was selected with 500 μg geneticin/ml. For Mv1Lu cells with stable expression of the reporter gene, 2×10^4 cells were seeded in 48-well plates and then 50 ng Renilla luciferase internal control plasmid was transiently transfected into cells at approximately 50% confluence. Similarly, 10^5 HSC-T6 cells/well were

seeded in 12-well plates for the introduction of 500 ng B4-$(CAGA)_{12}$-MLP-Luc plasmid and 50 ng Renilla luciferase plasmid. For RT-PCR assay, HSC-T6 cells were seeded 5×10^5 cells/well in 6-well plates for the transfection of synthesized Smad3/siRNA.

Mv1Lu cells with the luciferase reporter gene were transiently transfected with the Renilla luciferase plasmids by Lipofectamine 2000, as described above. After 12 h transfection, cells were incubated for 30 min with the indicated concentrations of SB-431542 before adding 10 ng human recombinant TGF-β1 (R&D, USA)/ml DMEM, containing 10 ml FCS/l. In the other experiments, the siRNA against Smad3 was simultaneously transfected with Renilla luciferase. After 12 h stimulation with 10 ng TGF-β1/ml, the cell lysates were harvested as indicated by the manufacture's protocol. Luciferase and Renilla luciferase activities were measured 24 h after transfection. Total light emission was measured during the initial 30 s of the reaction using a microplate luminometer (EG&G, Australia). Luciferase activity was normalized to Renilla luciferase activity (Dennler *et al.* 1998). Luciferase values shown in the figures are representative of transfection experiments performed in duplicate for at least three independent experiments.

RNA extraction and RT-PCR assays

Total RNA was prepared with Trizol reagents (Molecular Research Center) and cDNAs were reverse transcribed from 1 μg total RNA using a RT kit (MBI, Lithuan). The resulting cDNA was amplified with the matching primers: rat Smad3: forward, 5-CTGGCTACCTGAGTGAAGATG-3′; reverse, 5′-TGTGAAGCGTGGAATGTCTC-3′ (product size: 211 bp); rat PAI-1: forward, 5′-GGCTTCATGCCCCACTTCTTC-3′; reverse, 5′-TACTCGTGCCCATCCGGAGT-3′ (product size: 341 bp); rat Col Iα1: forward, 5′-ACTTTG-CTTCCCAGATGTCC-3′; reverse, 5′-CCATCC-AAACCACTGAAACC-3′ (product size: 349 bp); and rat β-actin (internal control), forward, 5′-TGGCGCTTTTGACTCAGGAT-3′; reverse, 5′-AGCCCTGGCTGCCTCAAC-3′ (product size: 452 bp). PCR was performed for 40 cycles at 94 °C for 45 s, 57 °C for 45 s and extension at 72 °C for 20 s.

Results

B4-$(CAGA)_{12}$-MLP-Luc reporter gene plasmid

In accordance with previous reports (Dennler *et al.* 1998, Inman *et al.* 2002, DaCosta *et al.* 2004, Yeom *et al.* 2004), the B4-$(CAGA)_{12}$-MLP-Luc reporter gene system was developed (Figure 1).

The CAGA reporters contain between 9 and 12 tandem copies of the Smad-binding element from the PAI-1 promoter (Dennler *et al.* 1998), but the 12 tandem copies of CAGACA have the most precise characteristics to bind to the Smad3/Smad4 complex. When extracellular cytokine TGF-β1 triggers signal cascades, TβRII is activated in mammalian cells by the reporter gene system. After the Smad3–Smad4 complex shuttles into the nucleus, the complex potently and specifically binds to the $(CAGA)_{12}$ box. Subsequently, the MLP, an adenovirus major late promoter, begins to induce the expression of the luciferase protein. The interference of any mediate site will lead to a halt in the expression of the reporter gene. Through numerous tests, we have established a mink lung epithelial cell line that can continuously and stably express the luciferase protein. Moreover, significant indications of TGF-β1/Smad3 signaling can be obtained though anti-G418 screening. Thus, it has been successfully established as a highly efficacious model for screening antagonists of the TGF-β1/Smad3 signaling pathway.

SB-431542 and Smad3 siRNA inhibit the reporter gene

Based on the B4-$(CAGA)_{12}$-MLP-Luc screening system, antagonists of the TGF-β1/Smad3 signaling pathway were evaluated. SB-431542 and Smad3/siRNA were introduced into Mv1Lu cells. The inhibitory effect of SB-431542 and Smad3/siRNA on Smad3 by the B4-$(CAGA)_{12}$-MLP-Luc screening system was assessed. Light emission analysis indicated that the administration of different amounts of SB-431542 decreased luciferase activity (Figure 2a).

Similarly, the transfection of siRNAs directed against Smad3 mRNA resulted in a decrease in luciferase activity in a dose-dependent manner (Figure 2b). The results were consistent with

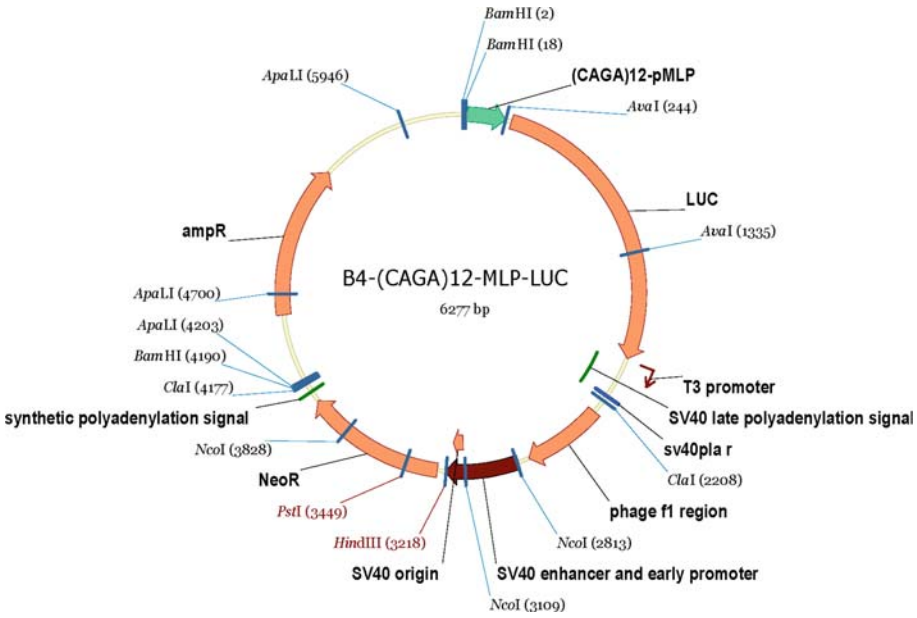

Fig. 1. B4-$(CAGA)_{12}$-MLP-Luc reporter gene plasmid. The $(CAGA)_{12}$-pMLP-Luc sequences were inserted into the B4-vector. The resulting plasmid was screened by the Neo R gene with G418 antibiotics. The ampR gene was used in amplification of the plasmid in *E. coli*. This reporter system was employed to evaluate the regulatory activity of potential antagonists on the TGF-β1/Smad3 signaling pathway.

other findings that SB-431542 selectively inhibited TβRI (ALK5) through competitively inhibiting ATP-binding sites and potently halting phosphorylation of Smad3. Meanwhile, our findings also supported the fact that Smad3/siRNA could induce the direct knockdowns Smad3 gene, and decrease the level of Smad3 and the Smad3/Smad4 complex. Finally, the expression of the reporter gene was reduced by treatment with SB-431542 and Smad3/siRNA. A similar effect of SB-431542 and siRNA was observed in transient transfection HSCs (data not shown). Thus, the reporter gene system is highly susceptible to SB-431542 and the Smad3/siRNA.

SB-431542 inhibits transcription of PAI-1 and Col Iα1*, but not Smad3*

To examine whether SB-431542 has the ability to suppress fibrosis, different doses of SB-431542 were applied to rat HSC-T6 cells and the changes in the PAI-1 and Col Iα1 gene levels assayed. RT-PCR analysis indicated that SB-431542 inhibited the expression of PAI-1 and Col Iα1 genes in a dose-dependent manner, compared with no inhibitory roles of DMSO in the control group (Figure 3a).

Densitometric analysis of related bands showed that PAI-1 and Col Iα1 levels decreased by $\sim$10-fold at 10 μM SB-431542 (Figure 3b). Furthermore, in an observation of whether SB-431542 had any effects on the Smad3 mRNA, there was no evidence that the compound could monitor the levels of Smad3 mRNA (Figure 3a). This suggested that SB-431542 could inhibit the Smad3 phosphorylation process and formation of the Smad3/Smad4 complex (Laping *et al.* 2002) at protein level, but had no effect on Smad3 mRNA.

siRNA silences Smad3 gene and inhibits PAI-1 and Col Iα1 *transcription*

To confirm if Smad3/siRNA can suppress its cognate mRNA target and lessen the degree of hepatic fibrosis, a Smad3/siRNA was designed to silence the Smad3 gene. Different doses of Smad3/siRNA were transfected into rat HSCs and, 24 h after transfection, Smad3/siRNAs could dose-dependently down-regulate the expression of Smad3 mRNA (Figure 4a).

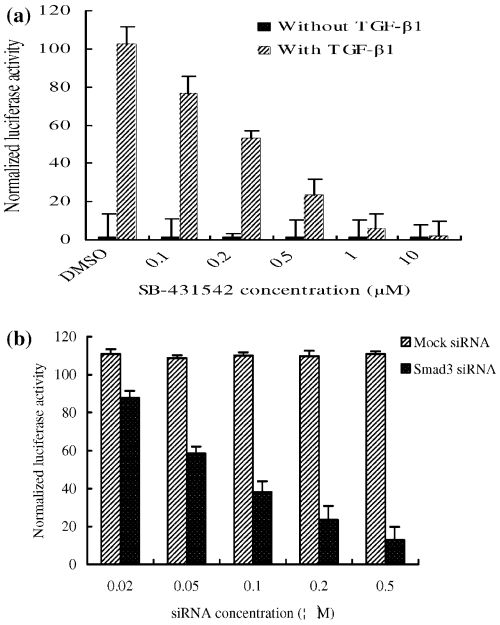

Fig. 2. SB-431542 and Smad3 siRNA inhibit expression of the reporter gene. (a) After transfection, cells were cultured in media containing DMSO alone or 0.1, 0.2, 0.5, 1.0 or 10 μM SB-431542, for a further 24 h. Based on results of the measurement, expression levels of the luciferase were determined under conditions with or without TGF-β1. (b) The Smad3/siRNA or mock siRNA were simultaneously transfected with the internal control plasmid. The biological effects of Smad3/siRNA at concentrations of 20, 50, 100, 200 and 500 nM were measured. A scrambled siRNA was used as the control (mock siRNA). Data are representative experiments performed in triplicate and displayed as mean and SD.

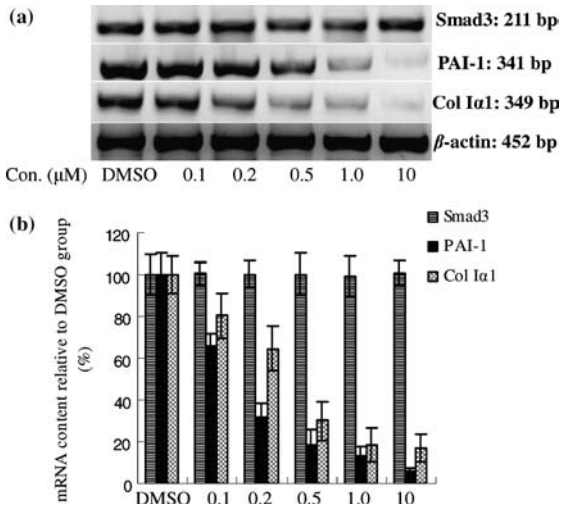

Fig. 3. SB-431542 inhibits transcription of PAI-1 and Col Iα1, but not Smad3. (a) When grown to near confluence in 6-well plates, hepatic stellate cells were serum-starved for 24 h. Subsequently, cells were pretreated with SB-431542 at concentrations of 0.1, 0.2, 0.5, 1.0 and 10 μM for 12 h followed by the addition of TGF-β1 for another 12 h. Twenty-four hours after incubation, the cells were subjected to Trizol treatment. RT-PCR was performed as described in Section 2. (b) Relative levels of the expressed Smad3, PAI-1 and Col Iα1 mRNAs under various conditions were determined and normalized to their levels in the DMSO control. Data are representative experiments in triplicate and are displayed as mean and SD.

More interestingly, the results from RT-PCR showed that Smad3/siRNA not only decreased the transcriptional level of Smad3 in HSCs, but also reduced the levels of PAI-1 and Col Iα1 mRNAs (Figure 4a). In further quantitative analysis, shown in Figure 4b, Smad3/siRNA significantly decreased the levels of PAI-1 and Col Iα1 mRNA 2- to 10-fold at concentrations of 50–500 nM. These results demonstrated that Smad3/siRNA directly inhibited Smad3 expression and indirectly led to the synthesis of ECM components, indicating that Smad3/siRNA may be a potent and promising antagonist of hepatic fibrosis.

Discussion

A large number of studies on fibrosis at cellular and animal levels have confirmed the fibrotic nature of TGF-β1, which is an increasingly investigated cytokine with multiple biological functions (Massague *et al.* 2000). Initiated by TGF-β1, the extracellular signal induces the formation of Smad3/Smad4 complex, stepwise, through TβRII, TβRI and Smad3. When the complex translocates into the nucleus, the transcription of PAI-1 gene would be induced, in which the promoter contains 12 tandem copies of the Smad-binding element $(CAGA)_{12}$ box. In the majority of reports (Johnsen *et al.* 2002, Suganuma *et al.* 2002, Blokzijl *et al.,* 2003), the $p(CAGA)_{12}$-MLP-Luc or $p(CAGA)_9$-MLP-Luc reporter gene system was used to verify the responses of TGF-β1 or the translocation of the Smad3/Smad4 complex into the nucleus. We were the first to clone a stable expression reporter containing the reporter gene (Figure 1) and the first to employ it for screening specific antagonists of the TGF-β1/Smad3 signal cascade, although similar reporter genes have been used to test the inhibitory effect of some small molecular

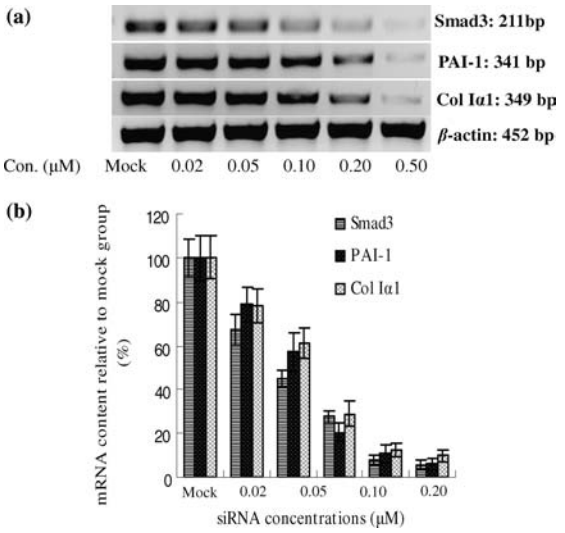

Fig. 4. Smad3 siRNA inhibits the transcription of Smad3, PAI-1 and Col Iα1. (a) When HSCs were at 70–80% confluence, cells were transfected by Smad3/siRNA at 20, 50, 100, 200 and 500 nM. Then, 500 nM mock siRNA was introduced into cells as control. After a 12-h transfection, the cells were serum-starved and induced with TGF-β1 for 12 h. RT-PCR was performed at 24 h after transfection as described in Section 2. (b) Relative levels of the expressed Smad3, PAI-1 and Col Iα1 mRNAs under various conditions were determined and normalized to their levels in the mock control. Data are representative experiments in triplicate and are displayed as mean and SD.

compounds and siRNAs against Smad3 (Inman *et al.* 2002, DaCosta *et al.* 2004, Yeom *et al.* 2004). Because TGF-β1/Smad3 signaling was highly conserved, the stable clone from Mv1Lu was almost identical when compared with mammalian cells, which was supported by the results from transiently transfected HSCs. The RT-PCR results, especially the decrease in PAI-1 and Col Iα1 mRNA, were in agreement with those of the reporter gene, suggesting that the molecular screening model is feasible and suitable.

SB-431542 is a potent and specific inhibitor of TβRI. In cultured renal epithelial carcinoma A498 cells, it also inhibits TGF-β1-induced transcription of ECM mRNA (Laping *et al.* 2002). HSCs are the primary cell type responsible for matrix deposition in liver fibrosis, undergoing a process of trans-differentiation into myofibroblasts. SB-431542 significantly decreased the level of PAI-1 and Col Iα1 mRNA, but did not affect Smad3 mRNA (Figure 3), suggesting that the compound could block the TGF-β1/Smad3 signaling pathway by suppressing phosphorylation of Smad3 and/or the formation of Smad3/Smad4 complex and its translocation. Furthermore, the compound was not found to be toxic at 10 μM in this and other reports (Inman *et al.* 2002, Laping *et al.* 2002). All these traits contribute to the potential therapeutic benefits for the development of SB-431542 as a potent anti-fibrotic agent.

Due to the pleiotropic biological actions of TGF-β1 through multiple signaling pathways, antagonists against TGF-β1 or its receptors may potentially induce a number of unwanted side-effects. As an intermediary in the TGF-β1 signal pathway in the nucleus, Smad3 protein is a central mediator of the fibrotic response (Schnabl *et al.* 2001, Flanders, 2004). Because Smad3 mediates the fibrotic signals, not only from the TGF-β1 pathway but also other pathways, inhibition of Smad3 may have tremendous clinical potential in the treatment of pathological fibrotic diseases and could be a prime target for intervention in fibrotic conditions. RNA interference has become a powerful tool for silencing gene expression and may be employed as a new approach to gene therapy for fibrotic and other diseases (Yin *et al.* 2002, Tan *et al.* 2005). In this study, Smad3/siRNAs were designed and synthesized in order to effectively silence the Smad3 gene. After selection with the luciferase reporter gene in Mv1Lu cells (Figure 2), this siRNA not only suppressed Smad3 gene, but also notably reduced mRNA transcription of PAI-1 and Col Iα1 in cultured rat HSC-T6 (Figure4).

Through experiments on screening antagonists of the TGF-β1/Smad3 pathway, SB-431542 and Smad3/siRNA were established to be effective in the modulation of fibrosis-related genes. The anti-fibrotic roles of SB-431542 and Smad3/siRNA need further and detailed investigation *in vivo*. In addition, the screening method established in this study may be used for the application of other antagonists from extracellular ligands and type I or II receptors to Smad3 and Smad4, including antibodies, small molecule inhibitors, soluble TβRII:Fc fusion proteins, traditional Chinese herbal extracts, or gene therapeutic strategies targeting the TGF-β1/Smad3 pathway. Taken together, the screening model and antagonists might contribute to the treatment and prevention of lung and hepatic fibrosis in the future.

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