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Smad3 mediates TGF-β_1-induced collagen gel contraction by human lung fibroblasts

Tetsu Kobayashi [a], Xiangde Liu [a], Fu-Qiang Wen [b], Tadashi Kohyama [c], Lei Shen [a], Xing Qi Wang [a], Mitsuyoshi Hashimoto [d], Lijun Mao [a], Shinsaku Togo [a], Shin Kawasaki [a], Hisatoshi Sugiura [a], Koichiro Kamio [a], Stephen I. Rennard [a,*]

[a] *Pulmonary and Critical Care Medicine, University of Nebraska Medical Center, Omaha, NE, USA*
[b] *Department of Respiratory Medicine, West China Hospital, Chengdu, Sichuan, PR China*
[c] *Department of Respiratory Medicine, University of Tokyo, Tokyo, Japan*
[d] *University of Nagoya, Nagoya, Japan*

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Abstract

Transforming growth factor-β_1 (TGF-β_1) is a key mediator in tissue repair and fibrosis. Using small interference RNA (siRNA), the role of Smad2 and Smad3 in TGF-β stimulation of human lung fibroblast contraction of collagenous matrix and induction of α-SMA and the role of α-SMA in contraction were assessed. HFL-1 cells were transfected with Smad2, Smad3 or control-siRNA, and cultured in floating Type I collagen gels ±−TGF-β_1. TGF-β_1 augmented gel contraction in Smad2-siRNA- and control-siRNA-treated cells, but had no effect in Smad3-siRNA-treated cells. Similarly, TGF-β_1 upregulated α-SMA in Smad2-siRNA- and control-siRNA-treated cells, but had no effect on Smad3-siRNA-treated cells. α-SMA-siRNA-treated cells did not contact the collagen gels with or without TGF-β_1, suggesting α-SMA is required for gel contraction. Thus, Smad3 mediates TGF-β_1-induced contraction and α-SMA induction in human lung fibroblasts. Smad3, therefore, could be a target for blocking contraction of human fibrotic tissue induced by TGF-β_1.

Keywords: Small interference RNA; Smad3; Transforming growth factor-β_1; Fibroblast

Many chronic human disorders are characterized by fibrotic distortion of tissue structure. This often leads to compromise of tissue function. As a result, therapies that could prevent the development of fibrosis have great potential for the treatment of a large number of disorders.

TGF-β is believed to be a major mediator driving both normal wound healing and tissue fibrosis [1–3]. TGF-β also mediates a large number of other effects, including regulation of inflammation and the immune system. Identification of the pathways by which TGF-β contributes to fibrosis, therefore, is important to develop therapeutic strategies. In this context, previous results using murine cells derived from genetically deficient animals have suggested that Smad3, one of the signaling molecules activated by TGF-β, is key to mediating TGF-β-induced contraction of three-dimensional collagen gels [4], which is widely used as an in vitro model of the tissue contraction that characterizes fibrosis [5,6]. The current study was designed to determine if Smad3 plays a similar role in human cells and to explore the mechanisms by which Smad3 augments collagen gel contraction.

To accomplish these ends, RNAi was used to suppress Smad2 and Smad3 in normal human lung fibroblasts, and the ability of these cells to respond to TGF-β was subsequently monitored. The results demonstrate that Smad3 is key for TGF-β-augmented contraction of human lung

* Corresponding author. Fax: +1 402 559 4878.
E-mail address: srennard@unmc.edu (S.I. Rennard).

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fibroblasts and that α-SMA expression, which is also induced by TGF-β through Smad3 signaling, is essential for contraction.

Materials and methods

Cell culture. Human fetal lung fibroblasts (HFL-1) were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in 100-mm tissue culture dishes (Falcon; Becton–Dickinson Labware, Lincoln Park, NJ) in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin G sodium, 50 μg/ml streptomycin sulfate (penicillin–streptomycin; Invitrogen, Life Technologies, Grand Island, NY), and 1 μg/ml amphotericin B (pharma-Tek, Elvira, NY). The fibroblasts were refed three times weekly and cells between passages 15 and 18 were used.

Small interfering RNA and transfection. Small interfering RNA (siRNA) for Smad3 was designed to target the coding sequence of human Smad3 as described previously [7]. To control for off-target effects, a second siRNA sequence targeting Smad3 was also used. For this purpose, the sequences (sense 5′-CUGUGUGAGUUCGCCUUCAUU, antisense 5′-UGAAGGCGAACUCACACAGUU) were selected as described [7].

siRNA for Smad2, α-SMA, and non-specific siRNA for control were purchased from Dharmacon (SMARTpool). Transfection of siRNA was performed as described previously [7]. Briefly, HFL-1 cells were seeded in 6-well plates at 1×10^4 cells/cm^2 in DMEM with 10% FCS. At 70% confluence, transfection of siRNA was performed. In one tube, 10 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was mixed gently with 250 μl of Opti-MEM medium (Invitrogen) and incubated 5 min at room temperature. In another tube, 200 pmol of each siRNA was mixed gently with 250 μl of Opti-MEM medium. These two tubes were combined, gently mixed, and incubated for 20 min at room temperature. After incubation, DMEM without FCS and antibiotics was added to obtain a final volume of 2 ml (final concentration of siRNAs = 100nM) for each well. Cells were washed with sterile PBS twice and incubated with siRNA transfection solution for 6 h at 37 °C. After that, media were changed to DMEM with 10% FCS and cultured until assay.

Reporter gene assay. After HLF-1 cells were treated with Smad3-siRNA for 24 h, cells were transfected with a total of 2 μg/well CAGA12-Luc (kindly provided by A. Roberts, NIH) together with pRL-TK (*Renilla* for normalization, Promega, Madison, WI) plasmid DNA again. Forty-eight hours after transfection, media were changed to serum-free DMEM and cells were treated with or without 100 pM TGF-β_1 for 16 h, after which luciferase and *Renilla* activities were determined by Dual-Luciferase Reporter Assay kit (Promega) and luminometer (MicroLumat Plus-LB96V, EG&G Berthold, Bad Wildbad, Germany). The luciferase values were normalized against *Renilla* values and presented as fold induction compared with control.

Three-dimensional collagen gel culture. Type I collagen (rat-tail tendon collagen [RTTC]) was extracted from rat-tail tendons by a previously published method [6,8]. Protein concentration was determined by weighing a lyophilized aliquot from each batch of collagen. The RTTC was stored at 4 °C until use. Twenty-four hours after siRNA transfection, HFL-1 cells were harvested and used for the gel contraction assay.

Prior to preparing collagen gels as described below, fibroblasts were detached by 0.05% trypsin in 0.53 mM EDTA and suspended in 10 ml serum-free DMEM containing soybean trypsin inhibitor. The cell number was then counted with a Coulter Counter. Collagen gels were prepared as previously described [6,9] by mixing RTTC, distilled water, 4× DMEM, and cells. The final concentration was 1× DMEM, 0.75 mg/ml collagen, and fibroblasts were present at 3×10^5 cells/ml. Following this, 500 μl of the mixture was cast into each well of a 24-well culture plate (Falcon). The solution was then allowed to polymerize at room temperature, generally completed in 20 min. After polymerization, the gels were either allowed to remain attached to the plates in which they were cast or, for the gel contraction assay, the gels were gently released from the plates in which they were cast and transferred into 60-mm tissue culture dishes (three gels in each dish), which contained 5 ml SF-DMEM with or without 100 pM TGF-β_1 (R&D Systems, Minneapolis, MN). The gels were then incubated at 37 °C in a 5% CO_2 atmosphere. The area of each gel was measured daily with an image analyzer (Optomax, Burlington, MA). Data are expressed as the percentage of area compared with the initial gel area.

Western blot analysis. Cells were washed with sterile PBS twice and then 100 μl cell lysis buffer (35 mM Tris–HCl, pH 7.4, 0.4 mM EGTA, 10 mM $MgCl_2$, 100 μg/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 0.1% Triton X-100) was added. After scraping the cells, lysates were briefly sonicated on ice and centrifuged at 10,000*g* for 3 min. The protein concentration in the cell lysates was measured using the Bio-Rad Protein Assay Kit. 10% SDS–polyacrylamide gel electrophoresis was performed under reducing conditions. To accomplish this, cell lysate proteins were diluted with 2× concentrated sample buffer (250 mM Tris–HCl, pH 6.9, 4% SDS, 10% glycerol, 0.006% bromphenol blue, and 2% β-mercaptoethanol) and heated at 95 °C for 5 min before loading (10 μg/lane). After SDS–PAGE, proteins were transferred onto a PVDF membrane (Bio-Rad). The membrane was blocked for 1 h at room temperature with 5% skim milk in PBS-Tween and incubated overnight at 4 °C with anti-α-SMA, anti-β-actin monoclonal antibody (Sigma, St. Louis, MO) or anti-Smad3 polyclonal antibody (Zymed Laboratories, South San Francisco, CA). After incubation with HRP-conjugated anti-mouse-IgG or anti-Rabbit-IgG, an ECL Western blot detection system was used according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

Immunofluorescence staining for F-actin and α-smooth muscle actin. HFL-1 cells were seeded on single chamber glass slides (Nunc, Rochester, NY) at 4×10^5 cells/slide in DMEM with 10% FCS. At 70% confluence, transfection of siRNA was performed. After silencing, cells were incubated with or without 300 pM TGF-β_1 in 0.1% FCS-DMEM for 48 h. The cells were then fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min and incubated with Texas Red-conjugated phalloidin (1 μg/ml in PBS, Sigma) or FITC-conjugated anti-α-SMA Ab (1:500 in PBS, Sigma) for 30 min, washed with PBS, and then mounted using VectaShield mounting medium.

Statistical analysis. Results are presented as means ± SEM. Data were analyzed by analysis of variance (ANOVA), with $p < 0.05$ considered significant using Statview software (Abacus Concepts, Cary, NC).

Results

Smad3 mediates the TGF-β_1-induced collagen gel contraction by human lung fibroblasts

Inhibition of HFL-1 Smad2 and Smad3 protein expression after treating with the siRNA used has been assessed by Western blot and previously reported [7]. To evaluate transcriptional activation of Smad3, we used reporter constructs specific for Smad3 (CAGA12) [10,11]. As expected, transfection with Smad3-siRNA resulted in almost 100% reduction in TGF-β_1-stimulated CAGA12 reporter activity. This extends the previous results obtained by Western blot and demonstrates inhibition of TGF-β-induced Smad3-mediated transcriptional activation (Fig. 1).

Using cells transfected with siRNA, gel contraction assays were performed in the presence and absence of TGF-β_1. Smad2-siRNA- and control-siRNA-treated cells significantly augmented contraction of collagen gels in the presence of TGF-β_1, while, in contrast, Smad3-siRNA-treated

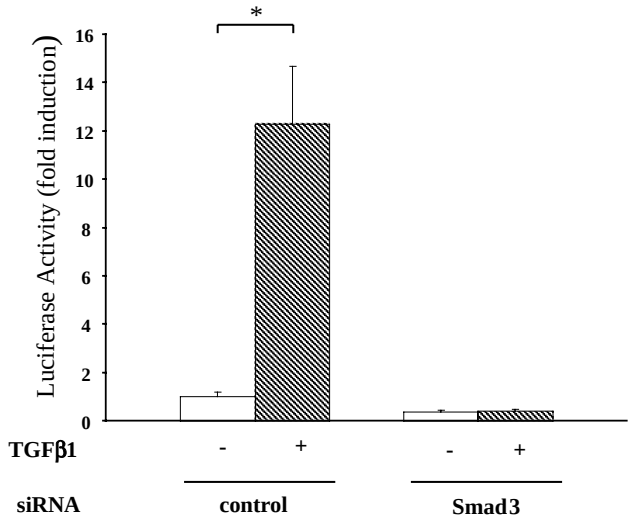

Fig. 1. Effect of siRNA targeting Smad3 on transcriptional activity of Smad3 in human lung fibroblasts. HFL-1 cells treated with Smad3-siRNA were co-transfected with CAGA12-Luc reporter plasmid together with pRL-TK. Forty-eight hours after transfection, cells were treated with or without TGF-β_1. Vertical axis: luciferase activity estimated after 16 h of TGF-β_1 treatment, normalized against *Renilla* values and presented as fold induction compared with control. Horizontal axis: treatment group. CAGA12 reporter activity was stimulated by TGF-β in control cells but not in Smad3-siRNA-treated cells, suggesting loss of transcriptional activation of Smad3. Data are shown as means ± SEM. *$p < 0.05$ vs. control.

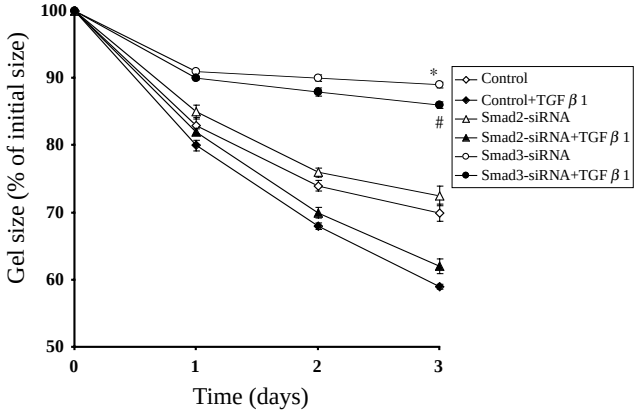

Fig. 2. Role of Smads in TGF-β-induced collagen gel contraction. siRNA targeting Smad2 or Smad3 were added to HFL-1 cells, after which cells were cast into collagen gels, released, and cultured with or without TGF-β_1. Gel size was measured daily with an image analyzer. Vertical axis: gel size expressed as % of initial size. Horizontal axis: time (days of culture). Smad2-siRNA- and control-siRNA-treated cells significantly augmented contraction of collagen gels in the presence of TGF-β_1, while Smad3-siRNA-treated cells did not. Data are shown as means ± SEM of three experiments performed in triplicate on separate occasions. *$p < 0.05$ as compared with control. $^{\#}p < 0.05$ as compared with control + TGF-β_1.

cells did not (Fig. 2). To determine if the inhibition of gel contraction was associated with altered expression of α-SMA, Western blot for α-SMA was performed. Smad2-siRNA- and control-siRNA-treated cells upregulated α-SMA after TGF-β_1 exposure. In contrast, Smad3-siRNA-treated cells did not (Fig. 3).

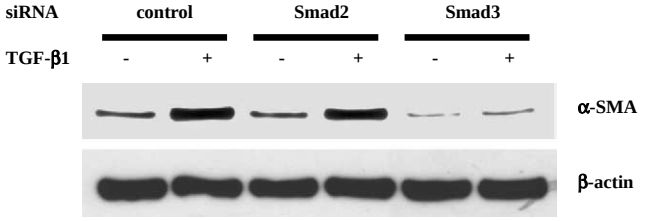

Fig. 3. Expression of α-SMA protein in the cells after Smad2- or Smad3-siRNA. After being transfected with Smad2- or Smad3-siRNA, respectively, cells were cultured in the presence or absence of TGF-β_1. Smad2-siRNA and control cells upregulated α-SMA after TGF-β_1 exposure, but Smad3-siRNA cells did not. β-Actin antibody was used to demonstrate equal protein loading. Data presented are one of three separate experiments with similar results.

Morphologic effects

Rhodamine–phalloidin staining was performed to visualize actin filaments. After TGF-β_1 stimulation, control-siRNA- and Smad2-siRNA-treated cells showed cell retraction. In contrast, Smad3-siRNA-treated cells have no significant morphologic changes (Fig. 4). α-SMA staining showed similar results. After TGF-β_1 stimulation, control-siRNA- and Smad2-siRNA-treated cells showed significantly higher α-SMA expression and clearly increased the expression of stress fibers. In contrast, Smad3-siRNA cells had no significant change in α-SMA expression following TGF-β_1 exposure (Fig. 4).

Role of α-SMA in contraction

To determine the role of α-SMA in TGF-β_1-augmented gel contraction, α-SMA-siRNA was used to suppress α-SMA expression (Fig. 5A). Following α-SMA-siRNA of treatment, gel contraction assay was performed.

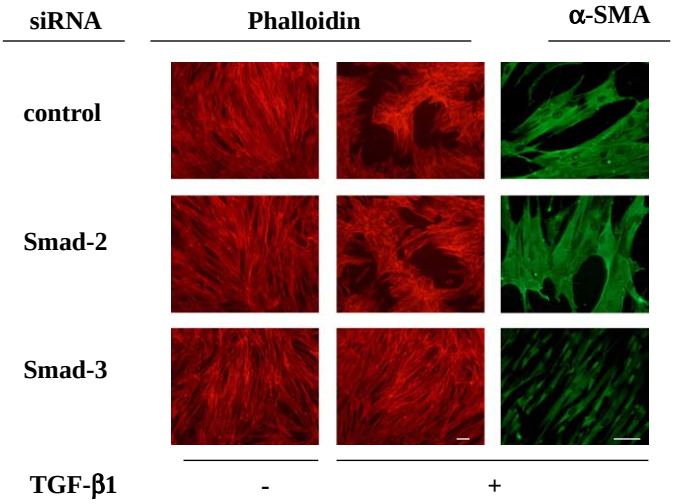

Fig. 4. F-actin and α-SMA expression. After silencing, cells were incubated with or without 300 pM TGF-β_1 in 0.1% FCS-DMEM for 48 h followed by staining with TRITC-conjugated phalloidin or FITC-conjugated α-SMA. Scale bar = 20 μm.

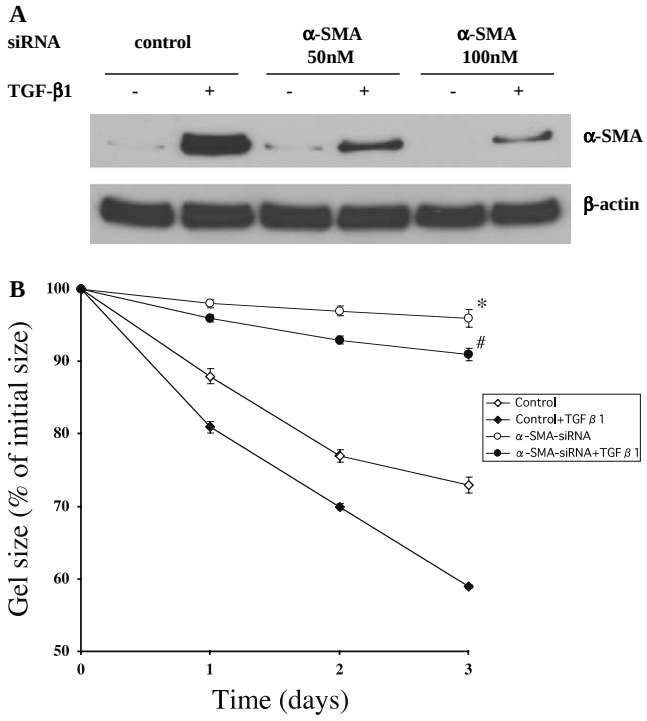

Fig. 5. Effect of α-SMA-siRNA on HFL-1 cells. (A) Expression of α-SMA protein in the cells after α-SMA-siRNA. After being transfected with α-SMA-siRNA, cells were cultured in the presence or absence of TGF-β_1. Control-siRNA cells upregulated α-SMA after TGF-β_1 exposure, but α-SMA-siRNA cells did not. β-Actin antibody was used to demonstrate equal protein loading. Data presented are from one of three similar experiments with similar results. (B) Effect of α-SMA-siRNA on collagen gel contraction. After being transfected with α-SMA, siRNA, HFL-1 cells were populated into collagen gels and released into 60 mm tissue culture dishes with or without TGF-β_1. Gel size was measured daily with an image analyzer. Vertical axis: gel size expressed as % of initial size. Horizontal axis: time (days of culture). Control-siRNA cells significantly augmented contraction of collagen gels in the presence or absence of TGF-β_1, while α-SMA-siRNA cells did not even in the presence of TGF-β_1. Data are shown as means ± SEM. $^{*}p < 0.05$ as compared with control. $^{\#}p < 0.05$ as compared with control + TGF-β_1. Data presented are from means of triplicate from one experiment that was replicated on three separate occasions.

Control-siRNA cells significantly augmented contraction of collagen gels in the presence of TGF-β_1, while α-SMA-siRNA-treated cells contracted poorly under control conditions and exhibited minimal induction of contraction in the presence of TGF-β_1 (Fig. 5B).

Confirmation of Smad3-siRNA effect

Because siRNAs may have off-target effects, we also evaluated a second siRNA targeting Smad3 for the same effects as noted above. Importantly, this second siRNA, which had a different sequence, effectively suppressed TGF-β-induced Smad3-dependent luciferase activity (Fig. 6B), markedly inhibited TGF-β induction of α-SMA (Fig. 6C), and suppressed TGF-β-induced augmentation of fibroblast contraction of three-dimensional collagen gels (Fig. 6D).

Discussion

Fibroblasts cultured in a three-dimensional collagen matrix attach to the collagen fibers and generate mechanical tension [5,6]. If maintained in floating culture, fibroblasts will cause the surrounding matrix to contract [12]. This in vitro effect is believed to be a model of the contraction that characterizes both wound healing and the development of fibrosis. Transforming growth factor beta (TGF-β) stimulates the process [13]. The current study demonstrates that, in human fibroblasts, the ability of TGF-β_1 to stimulate fibroblast-mediated collagen gel contraction is dependent on Smad3 signaling. In addition, the study demonstrates that α-SMA, which is stimulated by TGF-β_1 through a Smad3-dependent effect, is required for this effect.

The TGF-βs are a family of three cytokines believed to modulate many biological processes, including tissue repair and remodeling [1–3]. TGF-βs act on activin-type receptors, which are serine/threonine kinases that phosphorylate Smads [14]. TGF-β specifically results in phosphorylation of Smad2 and Smad3. These molecules, in turn, bind Smad4 after which they migrate into the nucleus and regulate gene transcription [15]. TGF-β can also activate the TAK1 kinase leading to Smad-independent signaling. The various pathways activated by TGF-β have differing functional consequences. Definitive identification of the signaling pathways responsible for TGF-β-mediated fibrosis is important in both understanding disease pathogenesis and, potentially, in developing pathway-specific therapies to mitigate disease. In this context, defining that Smad3 is essential for TGF-β-mediated fibrosis could contribute to the development of therapeutic strategies.

Previous studies have suggested that TGF-β_1-augmented contraction of three-dimensional collagen gels is mediated through Smad3 [4]. These studies utilized cells cultured from mice that were genetically deficient in either Smad2 or Smad3. The current study both confirms and extends these previous results. Importantly, the current study demonstrates that Smad3 mediates TGF-β_1-augmented contraction in human cells. In addition, the current study used a novel approach, namely inhibition of Smad expression through the use of RNAi.

There are important differences between protein knockdown consequent to RNAi and genetically deficient animals [16,17]. In contrast to the essentially complete lack of function observed in genetically deficient animals, reduction in protein expression following RNAi is both incomplete and transient. The abrogation of TGF-β-mediated contraction observed in the current study using RNAi suggests that even partial inhibition of this signaling pathway, which may be accomplished by a variety of pharmacologic approaches, could have important therapeutic significance.

There are a number of potential artifacts with the use of RNAi. In the current study, Smad2-RNAi was observed to be without effect. This both demonstrates the specificity of Smad3 signaling in mediating TGF-β_1-augmented collagen gel contraction and serves as a control for non-specific

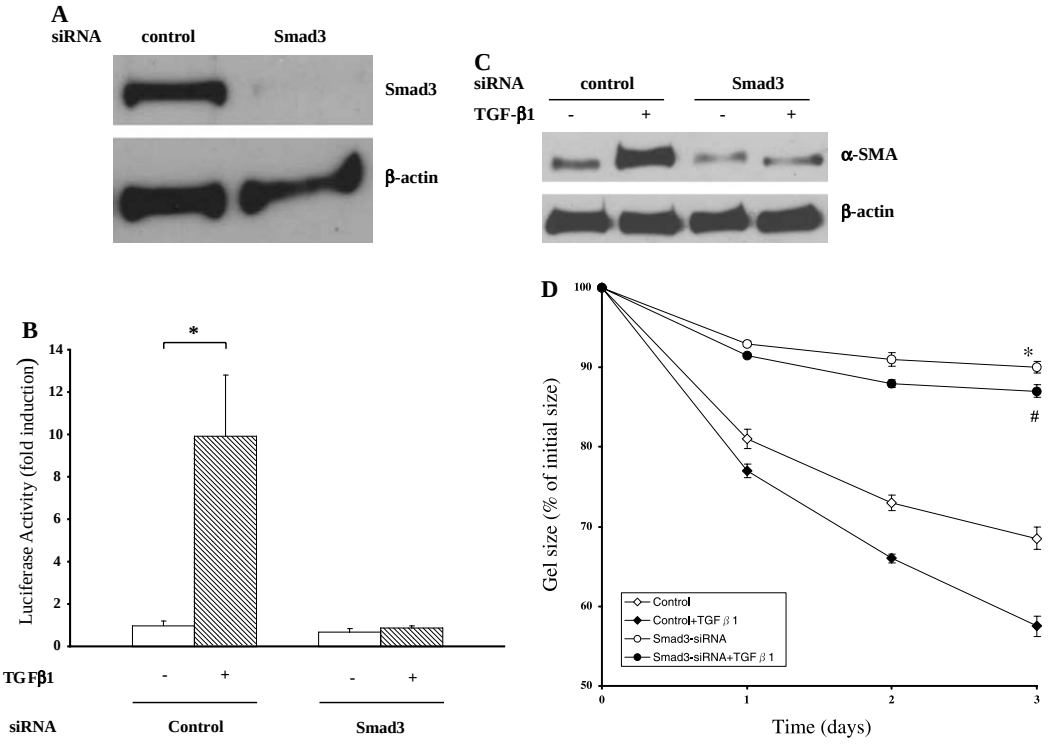

Fig. 6. Effect of a second siRNA targeting Smad3 in human lung fibroblasts. (A) Effect of siRNA targeting Smad3 on HFL-1 cell expression of Smad3 protein. Western blot representative of three similar experiments. Smad3 RNA interference suppressed Smad3 protein significantly. (B) Effect of siRNA targeting Smad3 on transcriptional activity of Smad3. HFL-1 cells treated with Smad3-siRNA were co-transfected with CAGA12-Luc reporter plasmid together with pRL-TK. Forty-eight hours after transfection, cells were treated with or without TGF-β_1. Vertical axis: luciferase activity estimated after 16 h of TGF-β_1 treatment, normalized against *Renilla* values, and presented as fold induction compared with control. Horizontal axis: treatment group. CAGA12 reporter activity was stimulated by TGF-β in control cells but not in Smad3-siRNA-treated cells, suggesting loss of transcriptional activation of Smad3. Data are shown as means ± SEM. $^*p < 0.05$ vs. control. (C) TGF-β inhibition of α-SMA protein in HFL-1 cells after Smad3-siRNA. After being transfected with Smad3-siRNA or control-siRNA, cells were cultured in the presence or absence of TGF-β_1. Control-siRNA cells upregulated α-SMA after TGF-β_1 exposure, but Smad3-siRNA cells did not. β-Actin was used to control for protein loading. Data presented are one of three separate experiments with similar results. (D) Effect of Smad3-siRNA on TGF-β-induced collagen gel contraction. SiRNA targeting Smad3 or control-siRNA was added to HFL-1 cells, after which cells were cast into collagen gels, released, and cultured with or without TGF-β_1. Gel size was measured daily with an image analyzer. Vertical axis: gel size expressed as % of initial size. Horizontal axis: time (days of culture). Control-siRNA-treated cells significantly augmented contraction of collagen gels in the presence of TGF-β_1, while Smad3-siRNA-treated cells did not. Data are shown as means ± SEM of three experiments performed in triplicate on separate occasions. $^*p < 0.05$ as compared with control. $^{\#}p < 0.05$ as compared with control + TGF-β_1.

effects. It is possible, of course, that the siRNAs used to suppress Smad3 could have interacted with other targets. This is, however, a problem inherent in siRNA technology in general. To help address this issue, we repeated the evaluation of suppression of Smad3 with a second siRNA. This second sequence yielded essentially identical results suppressing Smad3 protein as well as TGF-β-induced Smad3-dependent gene activation, α-SMA expression and gel contraction. The use of this second siRNA makes it extremely unlikely that the effect of the siRNAs is due to an off-target effect. The conclusion that Smad3 mediates these effects is strengthened by the fact that cells derived from genetically deficient mice behave similarly to human cells treated with siRNAs.

Smad3 modulates the expression of a large number of genes in response to TGF-β activation. Included among these is the expression of α-SMA. The expression of this cell cytoskeletal and contractile protein may be particularly relevant for the function of collagen gel contraction. Myofibroblasts, which are characterized by high levels of α-SMA expression, are believed to be particularly effective in mediating contraction of three-dimensional collagen gels and the tissue contraction that characterizes fibrosis and wound healing [6,12,18]. Because of this, the role of α-SMA was explored in the current study. Blockade of Smad3 signaling prevented the increase in α-SMA expression that is observed with TGF-β stimulation. Moreover, use of siRNA to block α-SMA expression blocked the ability of fibroblasts to contract three-dimensional collagen gels. This result demonstrates the importance of α-SMA expression for collagen gel contraction and suggests that TGF-β acting through Smad3 to increase α-SMA expression could contribute to the augmented contraction induced by TGF-β.

Many chronic disorders of adults are characterized by alterations in tissue structure that include architectural changes due to fibrosis and the associated tissue contraction. TGF-β is believed to play a key role in many of these diverse disorders [19–21]. Preventing the

architectural changes that compromise tissue function disorders could represent an important therapeutic option. The current study, by identifying Smad3 signaling as a key pathway for TGF-β-induced fibroblast-mediated contraction in human cells, will help define novel therapeutic strategies for disorders characterized by fibrotic tissue disruption.

Acknowledgments

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References

[1] G.R. Grotendorst, Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts, Cytokine Growth Factor Rev. 3 (1997) 171–179.
[2] A. Leask, D.J. Abraham, TGF-beta signaling and the fibrotic response, FASEB J. 7 (2004) 816–827.
[3] G.C. Blobe, W.P. Schiemann, H.F. Lodish, Role of transforming growth factor beta in human disease, N. Engl. J. Med. 18 (2000) 1350–1358.
[4] X. Liu, F.Q. Wen, T. Kobayashi, S. Abe, Q.H. Fang, E. Piek, E.P. Bottinger, A.B. Roberts, S.I. Rennard, Smad3 mediates the TGF-beta induced contraction of type 1 collagen gels by mouse embryo fibroblasts, Cell Motil. Cytoskeleton (2003) 248–253.
[5] F. Grinnell, Fibroblasts, myofibroblasts and wound contraction, J. Cell Biol. (1994) 401–404.
[6] T. Mio, Y. Adachi, D.J. Romberger, R.F. Ertl, S.I. Rennard, Regulation of fibroblast proliferation in three dimensional collagen gel matrix, In Vitro Cell. Dev. Biol. (1996) 427–433.
[7] T. Kobayashi, X. Liu, F.Q. Wen, Q. Fang, S. Abe, X. Wang, M. Hashimoto, L. Shen, S. Kawasaki, H. Kim, T. Kohyama, S. Rennard, Smad3 mediates TGF-beta1 induction of VEGF production in lung fibroblasts, Biochem. Biophys. Res. Commun. 11 (2005) 393–398.
[8] T. Elsdale, J. Bard, Collagen substrata for studies on cell behavior, J. Cell Biol. (1972) 626–637.
[9] T. Mio, X. Liu, Y. Adachi, I. Striz, C.M. Skold, D.J. Romberger, J.R. Spurzem, M.G. Illig, R. Ertl, S.I. Rennard, Human bronchial epithelial cells modulate collagen gel contraction by fibroblasts, Am. J. Phys. (1998) L119–L126.
[10] S. Dennler, S. Itoh, D. Vivien, P. ten Dijke, S. Huet, J.M. Gauthier, Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene, EMBO J. 11 (1998) 3091–3100.
[11] A.K. Kamaraju, A.B. Roberts, Role of Rho/ROCK and p38 MAP kinase pathways in transforming growth factor-beta-mediated Smad-dependent growth inhibition of human breast carcinoma cells in vivo, J. Biol. Chem. 2 (2005) 1024–1036.
[12] F. Grinnell, C.H. Ho, Transforming growth factor beta stimulates fibroblast-collagen matrix contraction by different mechanisms in mechanically loaded and unloaded matrices, Exp. Cell Res. 2 (2002) 248–255.
[13] X.D. Liu, T. Umino, R. Ertl, T. Veys, C.M. Skold, K. Takigawa, D.J. Romberger, J.R. Spurzem, Y.K. Zhu, T. Kohyama, H. Wang, S.I. Rennard, Persistence of TGF-beta1 induction of increased fibroblast contractility, In Vitro Cell. Dev. Biol. Anim. 3 (2001) 193–201.
[14] J. Massague, TGF-beta signal transduction, Annu. Rev. Biochem. (1998) 753–791.
[15] J. Massague, D. Wotton, Transcriptional control by the TGF-beta/Smad signaling system, EMBO J. 8 (2000) 1745–1754.
[16] A. Fire, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, C.C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, Nature 6669 (1998) 806–811.
[17] P.D. Zamore, RNA interference: listening to the sound of silence, Nat. Struct. Biol. 9 (2001) 746–750.
[18] M.B. Vaughan, E.W. Howard, J.J. Tomasek, Transforming growth factor-beta1 promotes the morphological and functional differentiation of the myofibroblast, Exp. Cell Res. 1 (2000) 180–189.
[19] W.R. Roche, R. Beasley, J.H. Williams, S.T. Holgate, Subepithelial fibrosis in the bronchi of asthmatics, Lancet (1989) 520–524.
[20] C. Chatziantoniou, J.C. Dussaule, Insights into the mechanisms of renal fibrosis: is it possible to achieve regression? Am. J. Physiol. Renal Physiol. 2 (2005) F227–F234.
[21] R. Bataller, D.A. Brenner, Liver fibrosis, J. Clin. Invest. 2 (2005) 209–218.