0026-895X/06/6902-597–607$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2006 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 69:597–607, 2006

Vol. 69, No. 2
17483/3082057
Printed in U.S.A.

Characterization of SIS3, a Novel Specific Inhibitor of Smad3, and Its Effect on Transforming Growth Factor-β1-Induced Extracellular Matrix Expression

Masatoshi Jinnin, Hironobu Ihn, and Kunihiko Tamaki

Department of Dermatology, Faculty of Medicine, University of Tokyo, Tokyo, Japan (M.J., K.T.); and Department of Dermatology and Plastic and Reconstructive Surgery, Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan (H.I.)

Received July 30, 2005; accepted November 15, 2005

ABSTRACT

This is the first report that characterizes specific inhibitor of Smad3 (SIS3) as a potent and selective inhibitor of Smad3 function. In the reporter assay, the increased luciferase activity of p3TP-lux by the overexpression of constitutively active form of ALK-5 was abrogated by the treatment with SIS3 in a dose-dependent manner. Immunoprecipitation revealed that SIS3 attenuated the transforming growth factor (TGF)-β1-induced phosphorylation of Smad3 and interaction of Smad3 with Smad4. On the other hand, this reagent did not affect the phosphorylation of Smad2. Thereafter, we evaluated the ability of SIS3 in the suppression of the TGF-β1-induced type I procollagen up-regulation in human dermal fibroblasts. We found that the addition of SIS3 attenuated the effects of TGF-β1 by reducing the transcriptional activity. SIS3 also inhibited the myofibroblast differentiation of fibroblasts by TGF-β1. Moreover, we demonstrated that SIS3 completely diminished the constitutive phosphorylation of Smad3 as well as the up-regulated type I collagen expression in scleroderma fibroblasts. Together, our study suggested that SIS3 is a useful tool to evaluate the TGF-β-regulated cellular mechanisms via selective inhibition of Smad3.

Transforming growth factor (TGF)-β1 plays a critical role in a variety of biological processes, including proliferation, differentiation, extracellular matrix production, and apoptosis. The diverse cellular responses elicited by TGF-β1 are triggered by the activation of serine/threonine kinase TGF-β receptors. On activation by TGF-β1 or related ligands, signaling from the receptors to the nucleus is mediated by phosphorylation of cytoplasmic mediators called Smads. The receptor-associated Smads, such as Smad2 and Smad3, interact directly with, and are phosphorylated by, activated TGF-β receptor type I (Nakao et al., 1997). They are ligand-specific and form, on phosphorylation, heteromeric complexes with Smad4. The latter functions as a common mediator for all Smad pathways. These complexes then are translocated into the nucleus, where they function as transcription factors, possibly in association with other proteins, such as Sp1. The third group of Smad proteins, the inhibitory Smads such as Smad6 or Smad7, prevents phosphorylation and/or nuclear translocation of receptor-associated Smads.

TGF-β1 has been implicated in the development of fibrotic condition, including skin, lung, or liver. Systemic sclerosis or scleroderma is an acquired disorder that typically results in fibrosis of the skin and internal organs. Fibroblasts from affected scleroderma skin cultured in vitro produce excessive amounts of extracellular matrix (ECM), various collagens, mainly type I and III collagens, and display increased transcription of corresponding genes (Hitraya and Jimenez, 1996). Many of the characteristics of scleroderma fibroblasts resemble those of normal fibroblasts stimulated by TGF-β1 (LeRoy et al., 1989), suggesting that activation of dermal fibroblast in scleroderma may be a result of stimulation by autocrine TGF-β signaling. This notion is supported by our recent findings: 1) scleroderma fibroblasts express elevated

This study was supported in part by a grant for scientific research from the Japanese Ministry of Education, by project research for progressive systemic sclerosis from the Japanese Ministry of Health and Welfare.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.017483.

ABBREVIATIONS: TGF, transforming growth factor; ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; SIS3, specific inhibitor of Smad3; GST, glutathione *S*-transferase; ERK, extracellular signal-regulated kinase; SMA, smooth muscle actin; DMSO, dimethyl sulfoxide; MEM, modified Eagle's medium; FCS, fetal calf serum; CAT, chloramphenicol acetyltransferase; bp, base pair(s); HA, hemagglutinin; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; PCR, polymerase chain reaction; AU, arbitrary units; SB-431542, 4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1*H*-imidazol-2-yl]benzamide.

levels of TGF-β receptors, and this correlates with the elevated levels of α2(I) collagen mRNA (Kawakami et al., 1998); 2) the blockade of TGF-β signaling with anti-TGF-β antibodies or anti-TGF-β1 antisense oligonucleotides abolished the increased expression of human α2(I) collagen mRNA in scleroderma fibroblasts (Ihn et al., 2001b); and 3) Smad3 was constitutively phosphorylated in scleroderma fibroblasts (Asano et al., 2004). Thus, the blockade of autocrine TGF-β signaling is thought to be one of the most reliable approaches in the treatment of scleroderma, and there have been several reports that actually show that the blockade of autocrine TGF-β signaling can decrease collagen expression in vivo or in vitro (Yamamoto et al., 1999; Yamane et al., 2003a).

Several investigators have reported possible inhibitors of TGF-β signaling (Callahan et al., 2002; Kondo et al., 2004). A missense mutant of Smad2, Smad2D450E, that was not phosphorylated by TGF-β signaling suppressed the phosphorylation of Smad2, but it did not affect the phosphorylation of Smad3. Smad2D450E reduced hetero-oligomer formation of Smad2 with Smad4 but not of Smad3 with Smad4. Smad3D407E was not phosphorylated by the constitutively active form of the TGF-β type I receptor and inhibited the phosphorylation of coexpressed wild-type Smad2 and Smad3. Furthermore, Smad3D407E reduced hetero-oligomer formation of both Smad2 and Smad3 with Smad4. On the other hand, SB-431542 has been characterized as a potent inhibitor of ALK-5 with greater selectivity against other kinases, including p38 mitogen-activated protein kinase (MAPK) and ALK-2, -3, -4, -6, or -7, which can prevent the TGF-β1-induced elevation of fibronectin, plasminogen activator inhibitor-1, and α1(I)collagen mRNA (Laping et al., 2002).

In this study, we showed that specific inhibitor of Smad3 (SIS3), a new inhibitor of TGF-β signaling, expressed its effects via the selective suppression of Smad3 phosphorylation. Furthermore, we also evaluated whether this reagent can abolish the ECM overexpression in the TGF-β1-treated normal dermal fibroblasts and scleroderma fibroblasts in vitro.

Materials and Methods

Reagents. Recombinant human TGF-β1 and human platelet-derived growth factor-AA were obtained from R&D Systems (Minneapolis, MN). Antibodies for Smad2/3 (N-19), Smad3 (FL-425), phospho-Smad2/3, glutathione *S*-transferase (GST), c-Myc (9E10), Smad4, Smad7, phospho-extracellular signal-regulated kinase (ERK), ERK2, and p38 MAPK were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphoserine-specific antibody was from Zymed Laboratories (South San Francisco, CA). Anti-Smad2/3 antibody (S66220) was from BD Biosciences Transduction Laboratories (Lexington, KY). Anti-HA 3F10 antibody and FuGENE 6 were from Roche Diagnostics (Mannheim, Germany). FLAG M2 antibody was from Kodak IBI (New Haven, CT). The antibody for β-actin or α-smooth muscle actin (SMA) was from Sigma-Aldrich (St. Louis, MO). Antibody for phospho-Smad2, phosphoinositide 3-kinase p85, and phosphotyrosine (4G10) were from Upstate Biotechnology (Lake Placid, NY). The phospho-p38 MAPK (Thr180/Tyr182) rabbit polyclonal antibody was from New England Biolabs (Beverly, MA). Anti-type I collagen-UNLB was from Southern Biotechnology Associates (Birmingham, AL).

Synthesis of SIS3. Indole derivatives are regarded as structures that have high affinity to various receptors and then express important biological activities by binding with these receptors. SIS3 was composed of indole derivatives with 2′-phenyl as hydrophobic group to induce translocation into the nucleus. We synthesized SIS3, on the assumption that it acts as a ligand of nuclear receptors according to published methods of 2-(*N*-methylindolyl)acrylic acid, followed by condensation with the corresponding amine (Inhoffen et al., 1963; Yasufumi et al., 2003) (Fig. 1). SIS3 was stored as a solution in DMSO, and this solution was used after diluting it with medium for each assay.

Cell Cultures. Normal human dermal fibroblasts were obtained by skin biopsies from five healthy donors. Scleroderma fibroblasts were obtained by skin biopsies from the affected areas (dorsal forearm) of five patients with diffuse cutaneous systemic sclerosis and <2 years of skin thickening (Ihn et al., 2001b). Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki. Control donors were each matched with a scleroderma patient for age, sex, and biopsy site. Normal and patient samples were processed in parallel. Primary explant cultures were established in 75-cm^2 culture flasks in modified Eagle's medium (MEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 50 μg/ml gentamicin. Fibroblast cultures independently isolated from different individuals were maintained as monolayers at 37°C in 95% air, 5% CO_2 and studied between the third and sixth subpassages.

Mouse dermal fibroblasts were also obtained from BALB/cA Jcl mice (CLEA Japan Inc., Tokyo, Japan). Mv1Lu cell line, COS-7 cell line, and NIH3T3 cell line were purchased from American Type Culture Collection (Manassas, VA). These cells were also maintained as described above.

Plasmid Construction. Generation of a series of 5′-deletion constructs consisting of the human collagen α2(I) gene fragments linked to the chloramphenicol acetyltransferase (CAT) reporter gene (COL1A2/CAT) was done as described previously (Ihn et al., 1996). −353m COL1A2/CAT construct with point mutations introduced into the potential Smad3 recognition site (located between nucleotides −263 and −258) of the −353∼+58 base pair (bp) COL1A2/CAT deletion construct using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was as described previously (Asano et al., 2004). Mutation and deletion constructs were verified by sequencing.

Expression vectors for HA-tagged constitutively active form of TGF-β type I receptor, ALK-5 (ALK-5TD); constitutively active form of activin type I receptor, ALK-4 (ALK-4QD); and FLAG-tagged-Smad3 or 6Myc-tagged-Smad4 containing six tandem copies of the Myc-epitope tag were kindly provided by Dr. Kohei Miyazono (University of Tokyo, Tokyo, Japan) (Nakao et al., 1997; Yagi et al., 1999, 2002). The p3TP-lux reporter plasmids and the pAR3-lux reporter plasmids were provided by Dr. Jeffrey Wrana (University of Toronto, Toronto, ON, Canada) (Carcamo et al., 1995; Hayashi et al., 1997). *Xenopus laevis* forkhead activin signal transducer 1 cDNA were kindly provided by Dr. Malcolm Whitman (Harvard Medical School,

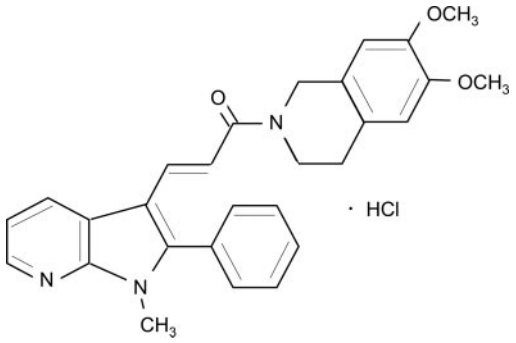


Fig. 1. Structure of SIS3, 6,7-dimethyl-2-[(2*E*)-3-(1-methyl-2-phenyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl-prop-2-enoyl)-1,2,3,4-tetrahydroisoquinoline hydrochloride.

Boston, MA) (Yagi et al., 1999). Plasmids used in transient transfection assays were purified twice on CsCl gradients. At least two different plasmid preparations were used for each experiment.

Transient Transfection. Fibroblasts were grown to 50% confluence in 100-mm dishes in MEM with 10% FCS. The medium was replaced with serum-free medium, and fibroblasts were transfected with promoter constructs, expression vectors, or corresponding empty constructs, using FuGENE6 as described previously (Ihn et al., 2002). To correct minor variations in transfection efficiency, pSV-β-galactosidase vector (Promega, Madison, WI) was included in all transfections. After 48 h of incubation, cells were harvested in 0.25 M Tris-HCl, pH 8, and fractured by freeze-thawing for CAT assay or luciferase assay. Extracts were normalized for protein content as measured by protein assay (Bio-Rad, Hercules, CA). Each experiment was performed in duplicate.

Cell Lysis and Immunoblotting. Human dermal fibroblasts were cultured until they were confluent, and then the media were collected. Remaining cells were washed with ice-cold phosphate-buffered saline twice and lysed in lysis buffer (Ihn et al., 2002; Asano et al., 2004). Aliquots of conditioned media (normalized for cell numbers) or cell lysates (normalized for protein concentrations as measured by the Bio-Rad reagent) were subjected to immunoblotting with antibodies for type I collagen, α-SMA, Smad4, Smad7, or β-actin.

For detection of phosphorylated levels of Smad2, p38 MAPK, or ERK, membranes were incubated with antibodies against phospho-Smad2, phospho-p38 MAPK, or phospho-ERK overnight at 4°C, respectively. As a loading control, the same membrane was then stripped and reprobed with antibodies against total Smad2/3 (N-19), p38 MAPK, or ERK2, respectively.

Immunoprecipitation. Phosphorylated levels of Smad3 or p85 were examined by immunoprecipitation using anti-Smad3 and anti-phosphoserine-specific antibodies or anti-p85 and anti-phosphotyrosine antibodies, respectively (Ihn et al., 2001a; Yamane et al., 2003b). The same membrane was then stripped and reprobed with anti-Smad2/3 (N-19) or p85 antibody to show the total amount of Smad3 or p85, respectively.

To examine the interaction between Smad3 and Smad4, COS-7 cells were transfected with expression constructs for ALK-5TD, FLAG-Smad3, or 6Myc-Smad4 (Nakao et al., 1997). Forty-eight hours after transfection, the cells were solubilized, and the cell lysates were incubated with the anti-FLAG M2 antibody, followed by incubation with protein G-Sepharose beads. The immunocomplexes were subjected to sodium dodecyl sulfate-polyacrylamide gels electrophoresis and transferred to nitrocellulose membranes, which were used for immunoblotting using antibody for phosphoserine or Myc 9E10.

GST Pull-Down Assay. A GST pull-down assay was performed according to the method of Fernandez-Sanchez et al. (2003) with minor modification. The full-length Smad3 was cloned into pDEST27 (Invitrogen, Carlsbad, CA) and in vitro transcribed/translated using NHDF Nucleofector Kit (amaxa GmbH, Cologne, Germany). Fibroblasts were then grown in MEM with 10% FCS for 24 h, and the medium was replaced with serum-free medium. After 24 h of incubation, cells were harvested in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1 mM EGTA, 1% Triton X-100, 50 mM sodium fluoride, 25 mM β-glycerophosphate hydrate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin. Proteins were purified with glutathione-Sepharose beads 4B (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Phosphorylated levels of Smad3 were analyzed by immunoblotting using phospho-Smad2/3 antibody. The same membrane was then stripped and reprobed with anti-GST antibody to show the total amount of Smad3.

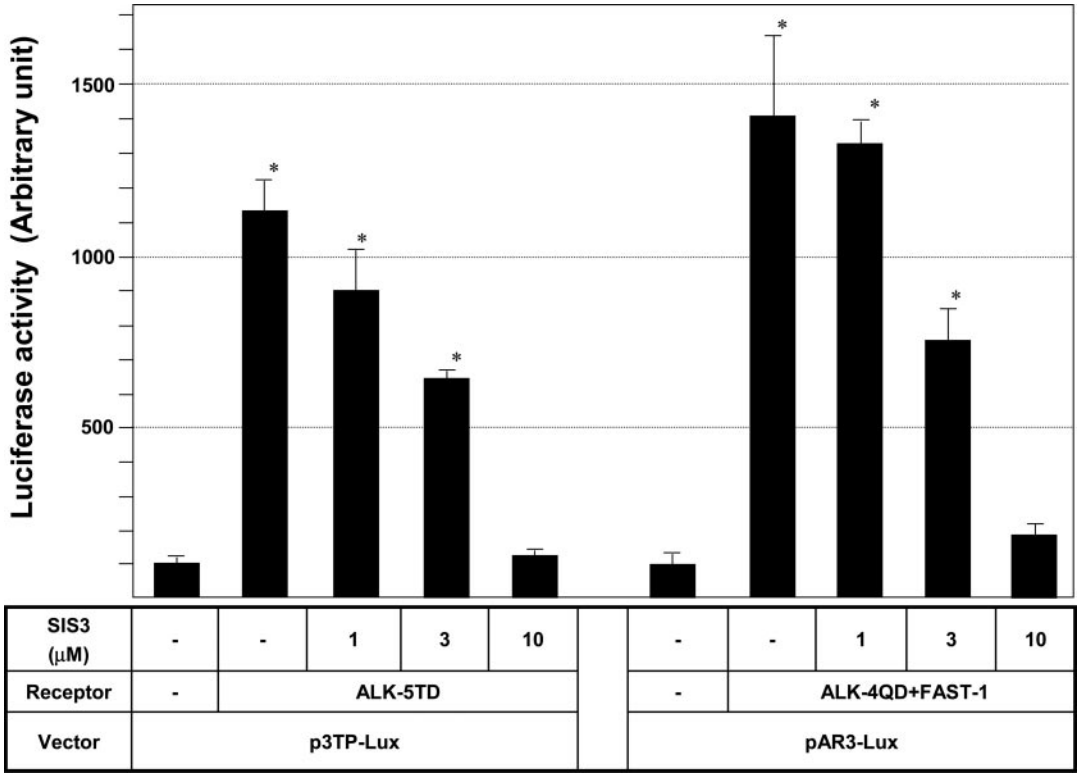


Fig. 2. SIS3 suppressed Smad-responsive promoter activities induced by the cotransfection with ALK-5TD or ALK-4QD. Mv1Lu cells transfected with p3TP-lux or pAR3-lux/Fast1 reporter constructs in the presence or absence of the constitutively active form of the TGF-β type I receptor (ALK-5TD) or the activin type I receptor (ALK-4QD), respectively. After 24 h, the indicated dose of SIS3 was added for additional 24 h. The luciferase activity was normalized to the relative β-galactosidase values. The bar graph represents fold stimulation of the luciferase activities relative to the basal promoter activity without TGF-β receptor construct or SIS3, which was arbitrarily set at 100 arbitrary units (AU). Mean $\pm$ S.D. from five independent experiments is presented. $*$, significant results compared with the basal promoter activity ($p < 0.05$; Mann-Whitney U test).

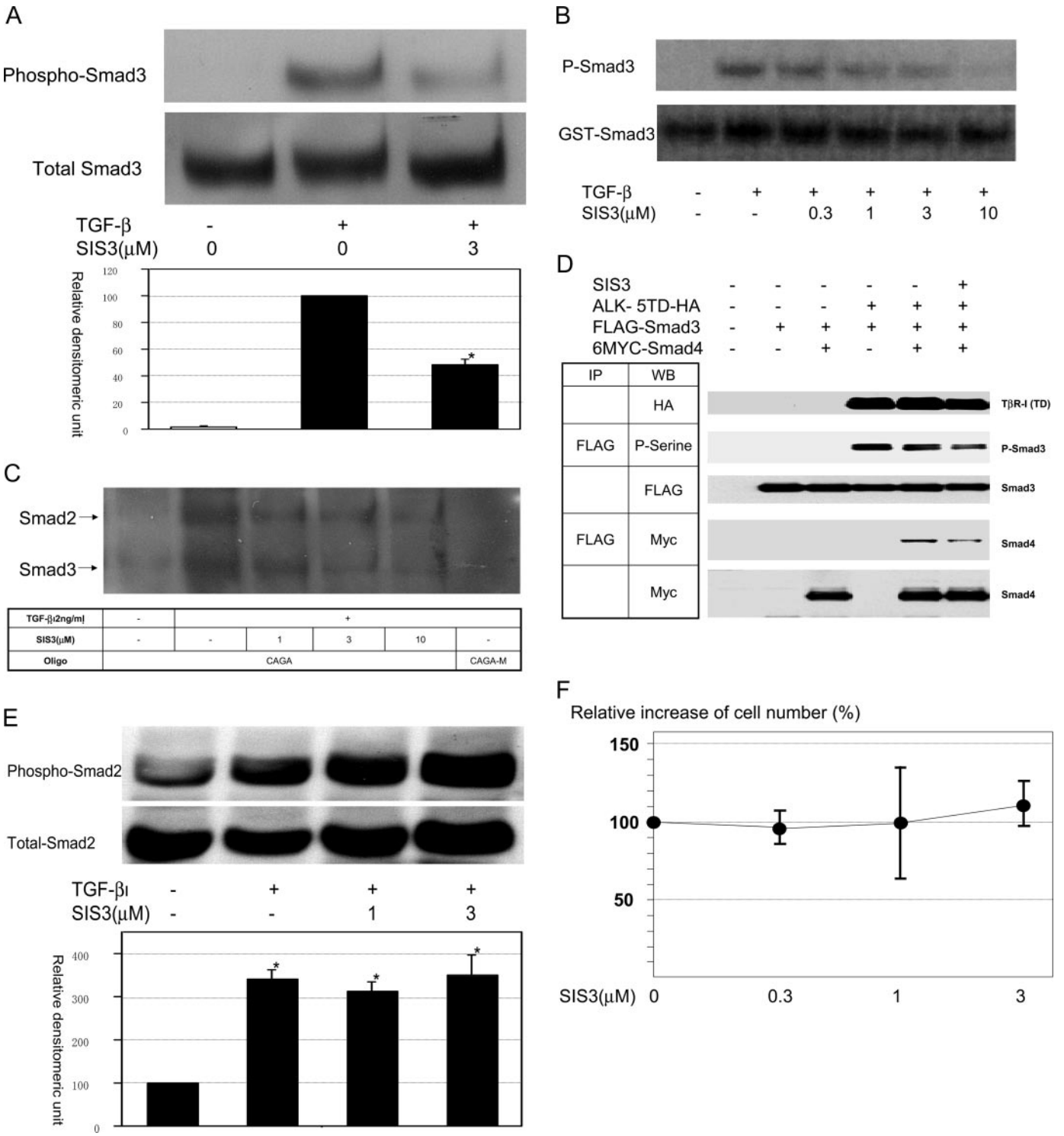


Fig. 3. Effect of SIS3 on the TGF-β1-dependent Smad3 phosphorylation and the DNA-Smad3 binding. A, confluent quiescent human dermal fibroblasts were pretreated with 3 μM SIS3 or the same amount of vehicle (DMSO) for 1 h and stimulated with 2 ng/ml TGF-β1 for 1 h. Whole cell lysates were subjected to immunoprecipitation using anti-Smad3 antibody, and phospho-Smad3 was detected by immunoblotting analysis. The same membrane was stripped and reprobed with anti-Smad2/3 antibody. One representative of five independent experiments is shown. Phospho-Smad3 levels quantitated by scanning densitometry and corrected for the levels of total-Smad3 are shown relative to those in the TGF-β1-treated cells without SIS3 (100 AU). Data are expressed as the mean $\pm$ S.D. of five independent experiments (bottom). $*$, $p < 0.05$ compared with the value in the TGF-β1-treated cells without SIS3. B, human dermal fibroblasts were transfected with the GST-Smad3. Cells were pretreated with the indicated dose of SIS3 or the same amount of vehicle (DMSO) for 1 h and stimulated with TGF-β1 for 1 h. Phosphorylated levels of Smad3 were determined using anti-phospho-Smad2/3 antibody as described under *Materials and Methods*. C, human dermal fibroblasts were pretreated with the indicated concentration of SIS3 or the same amount of vehicle (DMSO) for 1 h and stimulated with the 2 ng/ml TGF-β1 for 1 h. Cell lysates were incubated with biotin-labeled oligonucleotides. Proteins bound to these nucleotides were isolated with streptavidin-agarose beads, and Smad3 was detected by immunoblotting analysis. One representative of five independent experiments is shown. D, COS-7 cells were transfected with the indicated combinations of cDNAs encoding Smads and ALK-5TD. After 24 h, 3 μM SIS3 was added for an additional 24 h. Expression of ALK-5TD was detected by immunoblotting using the anti-HA antibody. Expression of total Smad3 or Smad4 was detected after stripping the membrane and immunoblotting

DNA Affinity Precipitation Assay. Two oligonucleotides containing biotin on the 5′ nucleotide of the sense strand were used. The sequences of these oligonucleotides are as follows: 1) 3×CAGA oligo, 5′-TCGAGAGCCAGACAAGGAGCCAGACAAGGAGCCAGACACT-CGAG, which is trimer of CAGA motif; and 2) 3×CAGA-M oligo, 5′-TCGAGAGCTACATAAAAAGCTACATATTTAGCTACATACT-CGA, which is trimer of CAGA motif mutated (Asano et al., 2004; Jinnin et al., 2004). These oligonucleotides were annealed to their respective complementary oligonucleotides, and double-stranded oligonucleotides were gel-purified and used. Cell lysates were obtained using lysis buffer (Yagi et al., 2002). Poly(dI-dC) competitor was incubated with the cell lysates, followed by incubation with each double-stranded oligonucleotide. After the incubation, streptavidin-agarose (Sigma-Aldrich) was added to the reaction and incubated. The protein-DNA-streptavidin-agarose complex was washed and loaded onto a sodium dodecyl sulfate-polyacrylamide gel. Detection of Smad3 was performed with anti-Smad2/3 antibody (S66220).

Cell Count. Normal human dermal fibroblasts were plated at a density of 10^5 cells/well in six-well culture plates and grown until subconfluence in MEM containing 10% FCS. Cells were quiesced by 24-h incubation in serum-free MEM, followed by incubation in serum-free medium in the presence or absence of SIS3 before the collection of cells for 72 h. Then, the cells were detached from the wells by trypsin treatment and counted using a Coulter counter (Beckman Coulter, Fullerton, CA) (Herbert et al., 1997).

Measurement of [^{3}H]Proline Incorporation. Cells (10^4/well) were plated into 96-well plates, and the medium was changed to serum-free medium supplemented with 50 μg/ml ascorbic acid for the duration of the experiment. Then, 0.05 μCi/μl [^{3}H]proline {L-(2,3,4,5)-[^{3}H]proline} (GE Healthcare) was added to the medium and incubated overnight. Medium was harvested from each well, and the incorporated radioactivity was counted in a liquid scintillation counter (Ziyadeh et al., 1994; Isono et al., 2000). Proline incorporation was corrected for the cell viability measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay in additional cells plated in parallel wells (Mosmann, 1983).

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction. Total RNA was extracted from the fibroblasts with Isogen (Nippongene, Tokyo, Japan). Complementary DNA was synthesized with ThermoScript reverse transcriptase (Invitrogen) (Li et al., 2003) and subjected to 25 cycles of PCR to amplify GAPDH, or 30 cycles of PCR to amplify human α2(I) collagen cDNA. The PCR cycle was 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. The products were fractionated by agarose gel electrophoresis and stained with ethidium bromide. The nucleic acid sequences of the specific PCR primers were as follows: α2(I) collagen, 5′-ACT CAG CCA CCC AGA GTG GA-3′ (sense) and 5′-TCT TGC AGT GGT AGG TGA TG-3′ (antisense); and GAPDH, 5′-AAG AAG GTG GTG AAG CAG GC-3′ (sense) and 5′-TCC ACC ACC CTG TTG CTG TA-3′ (antisense).

Immunofluorescence. Fibroblasts were grown in four-well Lab-Tek chambers (Nalge Nunc, Naperville, IL) to subconfluence as described above. After 24 h of serum starvation, the cells were fixed with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100 in phosphate-buffered saline, and blocked with 10% FCS in 0.5% Triton X-100 in phosphate-buffered saline (Asano et al., 2004). The cells were stained with anti-α-SMA antibody as primary antibody, washed, and incubated with fluorescein isothiocyanate-conjugated secondary antibodies. To visualize the fluorescence, a Zeiss microscope (Carl Zeiss GmbH, Jena, Germany) was used.

Statistical Analysis. Statistical analysis was carried out with the Mann-Whitney U test for comparison of means. p values less than 0.05 were considered significant.

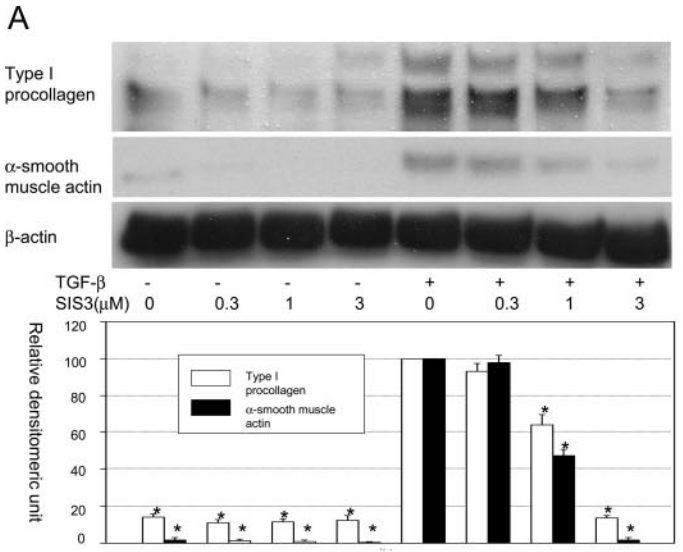
Results

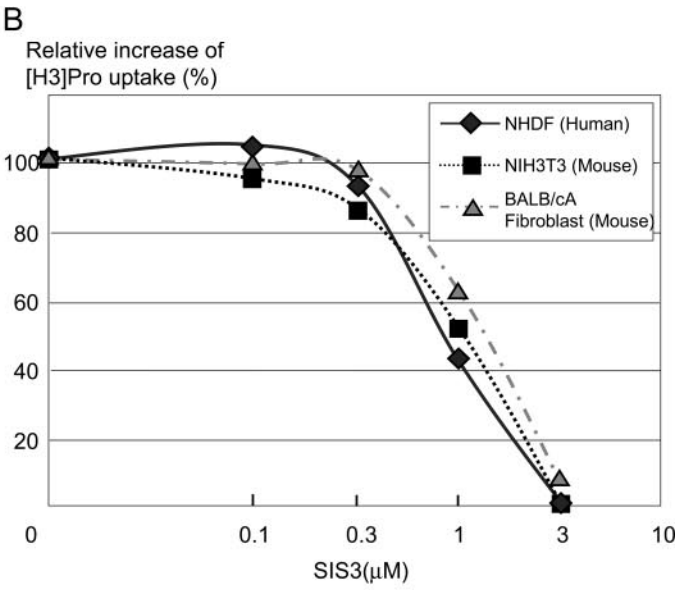
SIS3 Inhibited the Activation of p3TP Promoter Constructs Induced by the Cotransfection with Constitutively Active Form of TGF-β Type I Receptor. As an initial experiment, we compared the effects of SIS3 on the luciferase activity of p3TP-lux (which contains a part of the promoter region of the plasminogen activator inhibitor-1 gene and three tandem repeats of activator protein-1 binding sites of the collagenase 1 gene) (Carcamo et al., 1995; Hayashi et al., 1997) in Mv1Lu cells cotransfected with constitutively active form of TGF-β type I receptor (ALK-5TD). As shown in Fig. 2, the overexpression of ALK-5TD increased the luciferase activity significantly, and its effect was abrogated by the treatment with SIS3 in a dose-dependent manner. Similar results were obtained using pAR3-lux (which contains the activin-responsive element of Mix.2 promoter in *X. laevis*) (Hayashi et al., 1997), constitutively active form of activin type I receptor (ALK-4QD), and an expression vector encoding the coactivator *X. laevis* forkhead activin signal transducer 1. Together, these results indicated that SIS3 inhibits the signaling activity of TGF-β1 family.

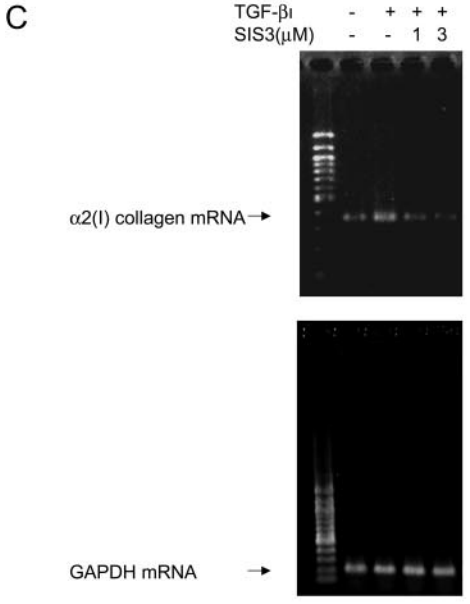
SIS3 Reduces the TGF-β1-Dependent Increase in Phosphorylation and DNA Binding of Smad3. Next, we investigated the TGF-β1-induced phosphorylation state of Smad3 in the presence or absence of SIS3. As shown in Fig. 3, A and B, the stimulation of TGF-β1 induced the marked phosphorylation of both endogenous and exogenous overexpressed Smad3 in human dermal fibroblasts. The pretreatment with 3 μM SIS3 reduced phosphorylation levels of Smad3 induced by TGF-β by approximately 50%. To further confirm this finding, we investigated the DNA binding ability of Smad3 by DNA affinity precipitation in human dermal fibroblasts. As shown in Fig. 3C, TGF-β1 also induced the strong binding of endogenous Smad3 with the 3xCAGA oligonucleotide, whereas the 3xCAGA-M oligonucleotide, which lacks the CAGA motif, did not bind with Smad3 even after treatment with TGF-β1. Consistent with the results of immunoprecipitation, the pretreatment with 3 μM SIS3 decreased the levels of the DNA-Smad3 binding by approximately 50%.

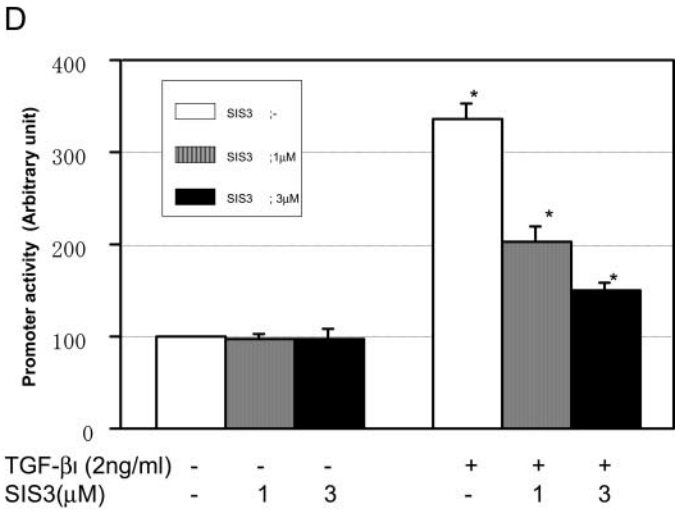
When both Smad3 and Smad4 were simultaneously transfected into COS-7 cells and stimulated by the cotransfection with ALK-5TD, SIS3 inhibited the increased interaction between Smad3 and Smad4 as well as increased phosphorylated level of Smad3 (Fig. 3D). However, the phosphorylated levels of Smad2 (Fig. 3E) and the protein expression levels of Smad4 and Smad7, the inhibitory Smad (data not shown), were not affected by this reagent in the presence or absence of TGF-β1. Furthermore, SIS3 did not affect the phosphory-

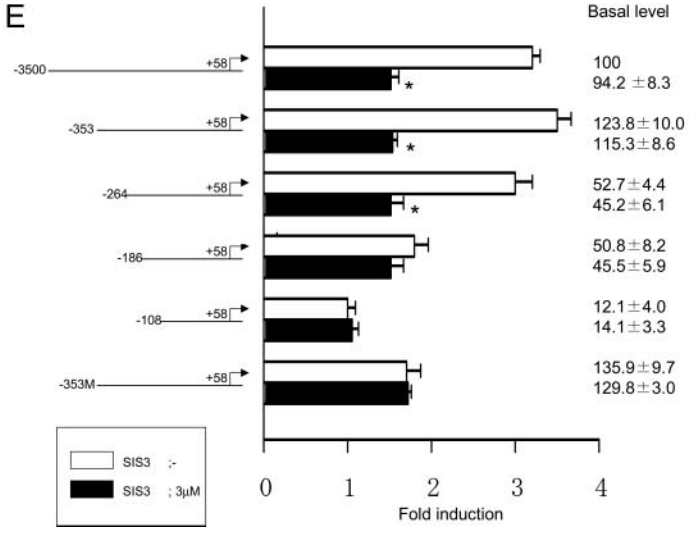
using the anti-FLAG antibody or anti-Myc antibody, respectively. Cell lysates were immunoprecipitated with the anti-FLAG M2 antibody. Smad4 levels interacting with Smad3 were detected by immunoblotting using the anti-Myc antibody. For the detection of the phosphorylated Smad3, the membrane was subjected to immunoblotting using the anti-phosphoserine antibodies. E, human dermal fibroblasts were pretreated with indicated dose of SIS3 for 1 h and then stimulated with exogenous 2 ng/ml TGF-β1 for 1 h before protein extraction. Whole cell lysate was subjected to immunoblotting with anti-phospho-Smad2 antibodies. After stripping, total levels of Smad2 were determined by anti-Smad2/3 antibody. One representative of five independent experiments is shown. The phospho-Smad2 levels quantitated by scanning densitometry and corrected for the levels of total Smad2 are shown relative to those in untreated cells (100 AU). Data are expressed as the mean ± S.D. of five independent experiments (bottom). F, human dermal fibroblasts were serum-starved for 24 h and incubated in the presence or absence of SIS3 for 72 h. Cell count was performed as described under *Materials and Methods*. Mean ± S.D. from three independent experiments are presented.











lation of other signaling pathways, such as MAPK/p38 induced by NaCl (Arbabi et al., 2000), ERK induced by fetal bovine serum (Thrane et al., 2001), or phosphoinositide 3-kinase by platelet-derived growth factor-AA (Wymann and Arcaro, 1994; data not shown). These results suggested that SIS3 attenuates the TGF-β1-dependent increased promoter activity of p3TP-lux by selectively reducing the Smad3 phosphorylation, the DNA-Smad3 binding, and the interaction of Smad3 with Smad4.

We subsequently evaluated cytotoxicity by SIS3. Because cell number was not affected by the addition of SIS3 (Fig. 3F), SIS3 did not have toxic effects.

Effect of SIS3 on the ECM Expression Induced by TGF-β1 in Normal Dermal Fibroblasts. We investigated whether SIS3 can also have an effect on the TGF-β1-mediated ECM up-regulation. TGF-β signaling contributes to the up-regulation of type I procollagen or α-SMA expression via Smad3 in human dermal fibroblasts (Chen et al., 1999; Hu et al., 2003). Pretreatment of cells with SIS3 did not alter the protein levels of type I procollagen as well as α-SMA in the absence of TGF-β1, but the effect of TGF-β1 was completely abrogated by 3 μM SIS3 (Fig. 4A). These results were confirmed by [^{3}H]proline incorporation assay, which showed the effect of SIS3 on newly synthesized type I procollagen in human dermal fibroblasts as well as NIH3T3 cell line or mouse dermal fibroblasts (Fig. 4B). RT-PCR analysis revealed that the treatment of cells with 3 μM SIS3 reduced TGF-β1-mediated up-regulation of α2(I) collagen mRNA (Fig. 4C). Note that the expression of GAPDH mRNA was not affected by SIS3, demonstrating that the indicated concentration of this reagent did not have generalized toxic effects. Thus, this inhibitory effect of SIS3 on the TGF-β1-induced type I procollagen protein up-regulation was paralleled with the levels of α2(I) collagen mRNA.

To determine whether SIS3 affects the basal and the TGF-β1-induced transcriptional activity of α2(I) collagen gene, we performed transient transfection assays using full-length COL1A2/CAT construct. As shown in Fig. 4D, SIS3 had no significant inhibitory effect on the basal α2(I) collagen promoter activity. In contrast, SIS3 reduced the TGF-β1-induced α2(I) collagen promoter activity significantly in a dose-dependent manner. The pretreatment with 3 μM SIS3 reduced the promoter activity by approximately 50%.

To identify potential regulatory elements of the human α2(I) collagen gene by SIS3, we performed transient transfection assays using a series of 5′ deletions of the COL1A2/CAT construct. As shown in Fig. 4E, the bp −353∼+58 construct responded at the highest level in the cells treated with TGF-β1. The TGF-β1-dependent increased promoter activity was decreased by the removal of a triple Sp1-binding site (bp −264∼+58 deletion construct) or a CAGA motif (bp −186∼+58 deletion construct) and was completely abrogated with the removal of another Sp1-binding site located at − 125 bp (bp −108∼+58 deletion construct). The TGF-β1-induced promoter activity of the bp −264∼+58 construct and the longer constructs was significantly reduced by treatment with SIS3. However, the inhibitory effect of SIS3 was completely diminished in the bp −186∼+58 construct and the subsequent deletion constructs. These data indicated that the responsive element of SIS3 in the α2(I) collagen promoter is located between bp −264 and −186. This region contains a CAGACA sequence (from bp −263 to −258) that was shown to be a functional Smad3-binding element (Chen et al., 1999). To further characterize the regulatory element of SIS3 in the α2(I) collagen promoter, we used the site-directed mutated construct −353m COL1A2/CAT, in which Smad3-binding site is mutated. Although mutating Smad3-binding sites resulted in the reduction of the response to TGF-β1 by approximately 50%, the inhibitory effect of SIS3 was abolished in the mutated construct. Together, the experiments with deletion and substitution promoter mutants suggested that SIS3 inhibited the TGF-β1 effect on type I procollagen expression at the transcriptional levels via the Smad3-binding site.

Effect of SIS3 on the TGF-β1-Mediated Myofibroblast Differentiation of Dermal Fibroblasts. It is well known that α-SMA expression is the established marker of myofibroblast differentiation. As shown in Fig. 4A, immunoblotting revealed that α-SMA expressed little in normal cell lysates. Exogenous TGF-β 1 induced the α-SMA expression in cultured normal fibroblasts, and SIS3 decreased the α-SMA expression in a dose-dependent manner. We further confirmed this result by immunocytochemistry. The stimulation of dermal fibroblasts with TGF-β 1 increased the α-SMA expression, which was abolished by the pretreatment with 3 μM SIS3 (Fig. 5). This result suggested that SIS3 can inhibit the myofibroblast differentiation induced by TGF-β1.

Fig. 4. Effects of SIS3 on the TGF-β1-induced ECMs in human dermal fibroblasts. A, human dermal fibroblasts were serum-starved for 24 h and treated with the indicated dose of SIS3 for 1 h and then 2 ng/ml TGF-β1 was added. After 72 h, the same ratio of conditioned media and aliquots of cell/matrix layer (normalized for protein concentrations as measured by the Bio-Rad reagent) were subjected to immunoblotting with anti-type I collagen antibody, or antibody for α-SMA or β-actin, respectively. One representative of five independent experiments is shown. Type I procollagen or α-SMA protein levels quantitated by scanning densitometry and corrected for the levels of β-actin are shown relative to those in the TGF-β1-treated cells without SIS3 (100 AU). Data are expressed as the mean ± S.D. of five independent experiments (bottom). ∗, $p < 0.05$ compared with the value in the TGF-β1-treated cells without SIS3. B, newly synthesized type I collagen in indicated cells was measured in a [^{3}H]proline incorporation assay after 1 h of SIS3 treatment and 6 h of 2 ng/ml of TGF-β stimulation. Summary of quantitative analysis of the collagenous proteins expressed by fibroblasts with TGF-β1 in five independent experiments is shown. The levels of type I collagen protein expressed by SIS3-untreated fibroblasts was arbitrarily set at 100%. C, effect of SIS3 on the expression of α2(I) collagen mRNA induced by TGF-β1 in human dermal fibroblasts. Cells were cultured in serum-free medium overnight and then treated with 2 ng/ml TGF-β for 24 h in the presence or absence of SIS3 pretreatment. Cells were collected after 24 h, and total RNAs were extracted and used for RT-PCR analysis. One experiment representative of five independent experiments is shown. D, full-length COL1A2/CAT promoter constructs were transfected in the presence or absence of 2 ng/ml TGF-β1 for 24 h. In all experiments, cells were pretreated with the indicated concentration of SIS3 or the same amount of vehicle (DMSO) for 1 h before the stimulation with TGF-β1. Values represent the α2(I) collagen promoter activities relative to those of untreated cells, which was set at 100 AU. Mean ± S.D. from five independent experiments is presented. ∗, $p < 0.05$ versus untreated cells. E, human dermal fibroblasts were transfected with 2 μg of the indicated 5′ deletion of the COL1A2/CAT construct or a site-directed mutated construct −353m COL1A2/CAT, in the presence or absence of 2 ng/ml TGF-β1 for 24 h. In all experiments, cells were pretreated with 3 μM SIS3 or the same amount of vehicle (DMSO) for 1 h before stimulation with TGF-β1. The bar graph on the right represents fold stimulation of the promoter activity stimulated by TGF-β1 relative to the promoter activity without TGF-β1, which was arbitrarily set at 1. The numbers on the right show the basal levels (i.e., without TGF-β1) of each construct relative to the full-length COL1A2/CAT, which was arbitrarily set at 100%. Mean ± S.D. from five independent experiments is presented. ∗, significant results compared with the basal promoter activities of each construct ($p < 0.05$; Mann-Whitney U test).

Effect of SIS3 on ECM Expression in Scleroderma Fibroblasts. As described above, we demonstrated that scleroderma fibroblasts are regarded as a model of fibrosis that involves TGF-β signaling. There is an expectation that SIS3 may reduce the up-regulated expression of α2(I) collagen gene through the inhibition of phosphorylated Smad3 in scleroderma fibroblasts. To confirm this, we investigated the effect of SIS3 on the up-regulated expression of α2(I) collagen gene in scleroderma fibroblasts. As shown in Fig. 6, A and B, 3 μM SIS3 reduced the up-regulated expression of type I procollagen protein as well as α-SMA in scleroderma fibroblasts to the same extent as that in normal fibroblasts. We next investigated the effect of SIS3 on the phosphorylation and the DNA binding ability of Smad3 in scleroderma fibroblasts. The DNA-Smad3 binding as well as Smad3 phosphorylation is reported to be increased in scleroderma fibroblasts (Asano et al., 2004). The treatment with 3 μM SIS3 abolished the phosphorylation (Fig. 6C) and DNA binding ability of Smad3 (data not shown) in scleroderma fibroblasts. These results are consistent with the results of normal dermal fibroblasts stimulated by exogenous TGF-β1. Furthermore, the treatment with 3 μM SIS3 also reduced bp $-353\sim+58$ COL1A2/CAT promoter activity in scleroderma fibroblasts completely, but -353m COL1A2/CAT was not affected by this reagent (Fig. 6D). This result suggested that the inhibitory effect of SIS3 on the up-regulated type I collagen expression in scleroderma fibroblasts involves Smad3.

SIS3 Restores Myofibroblast Differentiation of Scleroderma Fibroblasts. It is well established that most of cultured scleroderma fibroblasts express α-SMA (Jelaska and Korn, 2000), and Smad3 mediates the TGF-β-induced α-SMA expression (Hu et al., 2003). Therefore, there is a good possibility that the autocrine TGF-β signaling contributes to the maintenance of α-SMA expression in scleroderma fibroblasts. Thus, we evaluated the effect of SIS3 on the expression of α-SMA in scleroderma fibroblasts. As shown in Fig. 7, scleroderma fibroblasts, which looked like the TGF-β1-treated normal fibroblasts (Fig. 5), expressed α-SMA much more than normal fibroblasts. SIS3 (3 μM) decreased the expression of α-SMA to the same extent as that in normal fibroblasts, which is consistent with the result of immunoblotting (Fig. 6A). These results indicated that SIS3 restores myofibroblast differentiation of scleroderma fibroblasts.

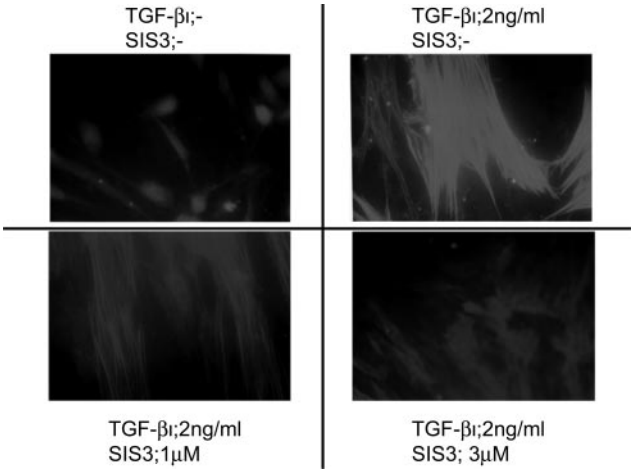


Fig. 5. Effect of SIS3 on the TGF-β1-mediated α-smooth muscle actin up-regulation in human dermal fibroblasts. The subcellular localizations of α-SMA were visualized by immunofluorescence. Human dermal fibroblasts were serum-starved for 24 h and pretreated with 3 μM SIS3 for 1 h before addition of 2 ng/ml TGF-β1 for 24 h.

Discussion

Our study is the first to characterize SIS3 as a potent and selective inhibitor of Smad3 function. In the reporter assay, SIS3 inhibited the signaling activity of activin as well as TGF-β1. Activin, a member of the TGF-β1 family, also expresses its effect via Smad2, Smad3, and Smad4 (Nakao et al., 1997). Together, because both TGF-β1 and activin expressed their effects via the common pathway, it is likely that SIS3 can inhibit the effects of TGF-β1 and activin.

Further study revealed that the effects of SIS3 were mediated by the suppression of the Smad3 phosphorylation, the DNA-Smad3 binding, and the interaction of Smad3 with Smad4. On the other hand, this reagent affected neither the phosphorylation of Smad2, the expression of Smad4 or Smad7, nor the phosphorylation of other signaling pathways, such as p38, p85, or ERK, in the presence or absence of TGF-β1. These results indicate that SIS3 can inhibit the function of Smad3 selectively in TGF-β family signaling pathway.

Propagation of TGF-β signals is mediated by the direct association of the receptor-associated Smads (Smad2 and Smad3) with the TGF-β receptor complex. The receptor-associated Smads are then directly phosphorylated by the type I TGF-β receptor kinase on the last two serines of a conserved specific phosphorylation motif, SSXS, located at the extreme carboxyl terminus of the MH2 domain (Attisano and Tuen Lee-Hoeflich, 2001). Thus, one of the mechanisms by which SIS3 specifically inhibited phosphorylation of Smad3 may involve this SSXS motif. However, SIS3 did not affect Smad2, which also contains SSXS motif. We assumed that SIS3 acts as ligand of nuclear receptors. Chou et al. (2003) reported that TGF-β-regulated Smads can have transcriptional crosstalk with nuclear receptor hepatocyte nuclear factor-4. Interaction of some orphan nuclear receptors with Smad3 through SIS3 may inhibit the phosphorylation of Smad3 specifically via SSXS motif.

Thereafter, we evaluated whether SIS3 suppresses the TGF-β1-induced type I procollagen protein or α2(I) collagen gene expression in human dermal fibroblasts. We found that SIS3 did not decrease the basal expression of type I procollagen protein or α2(I) collagen gene but that the addition of SIS3 attenuated the TGF-β1 effects on type I collagen expression by reducing its transcriptional activity.

Moreover, we demonstrated that SIS3 also decreased the excessive type I procollagen expression or the α2(I) collagen promoter activation, which may be because SIS3 abolished autocrine TGF-β signaling pathway in scleroderma fibroblasts. Note that 3 μM SIS3 completely diminished the constitutive phosphorylation and the DNA binding of Smad3 as well as the excessive type I procollagen expression or the α2(I) collagen promoter activation in scleroderma fibroblasts.

We also examined the effects of SIS3 on α-SMA expression in normal and scleroderma fibroblasts. SMA-positive fibroblasts, so-called myofibroblasts, are found in scleroderma and in a number of other fibrotic conditions (Sappino et al.,

1990). Kirk et al. (1995) suggested that the myofibroblast phenotype might correspond to "activated fibroblast" phenotype found in systemic sclerosis. These activated fibroblasts have a high synthetic capacity for ECM proteins, growth factors/cytokines, growth factor receptors, integrins, and oxidants (Thannickal and Fanburg, 1995). The presence/activation of myofibroblasts seems to be a consistent finding in the pathology of human fibrotic diseases involving diverse organ systems such as the lung, liver, and kidney (Border and Noble, 1994). Thus, persistent myofibroblast proliferation and/or survival represents a pathological repair process that can result in aberrant architectural remodeling of tissues associated with end-stage fibrosis and organ failure. On the other hand, TGF-β signaling contributes to the up-regulation of α-SMA expression in cultured fibroblasts via Smad3 (Hu et al., 2003). Our results suggested that SIS3, which inhibits TGF-β signaling, could be used as a therapeutic intervention for scleroderma by turning back scleroderma fibroblasts abnormally overexpressing α-SMA via the inhibition of Smad3.

Together, our data suggest that SIS3 is a useful reagent to evaluate TGF-β-regulated cellular mechanisms by the selective inhibition of Smad3 and that SIS3 can block excessive ECM production from the TGF-β1-treated normal fibroblasts

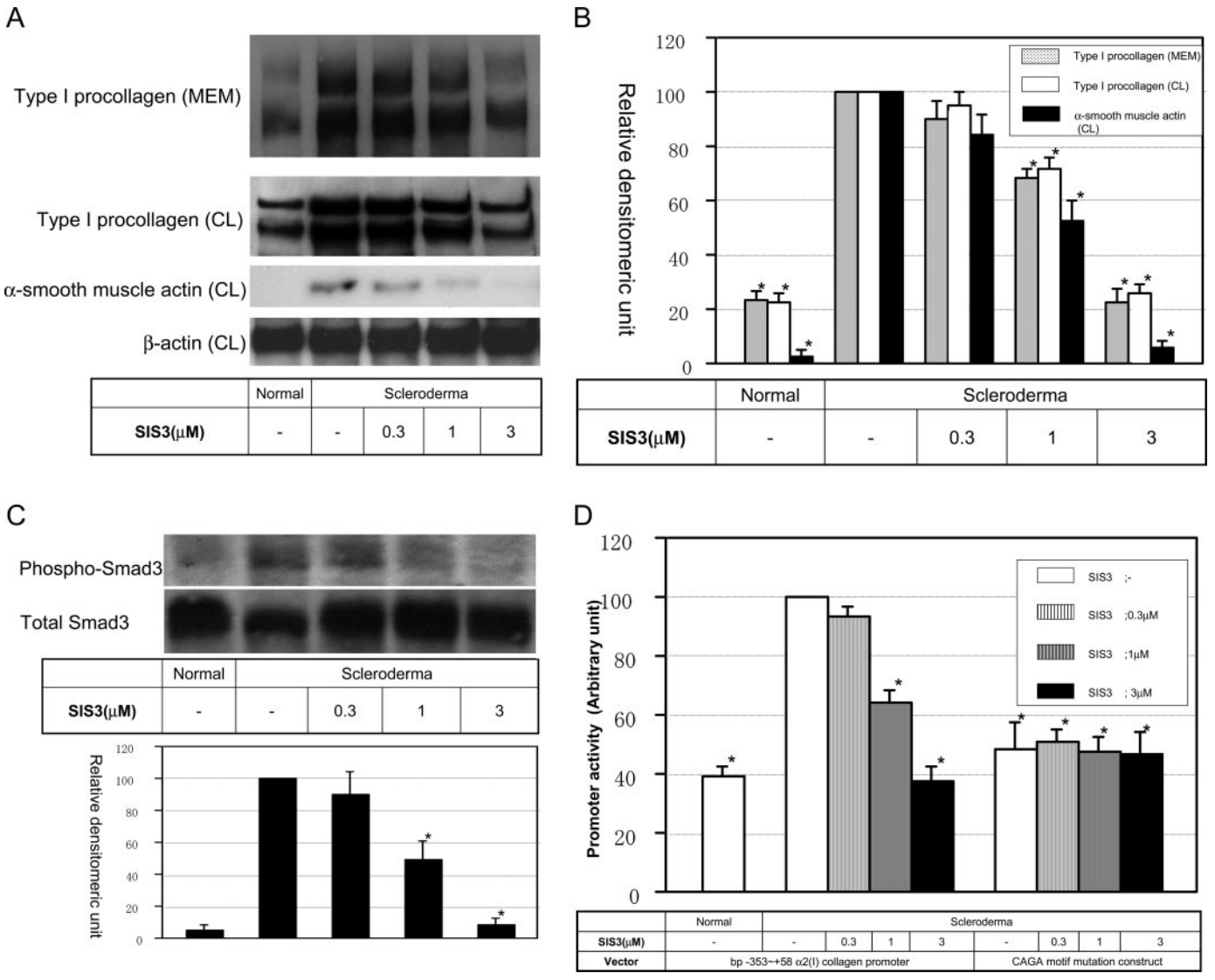


Fig. 6. Effects of SIS3 on scleroderma fibroblasts. A and B, normal and scleroderma fibroblasts were serum-starved for 24 h and treated with the indicated dose of SIS3 for 72 h under the same condition. The same ratio of conditioned media (MEM) and aliquots of cell/matrix layer (CL, normalized for protein concentrations as measured by the Bio-Rad reagent) were subjected to immunoblotting with antibody for type I collagen, α-SMA, or β-actin. One representative of experiments in five normal and five scleroderma fibroblasts is shown (A). Type I procollagen or α-SMA protein levels quantitated by scanning densitometry and corrected for the levels of β-actin are shown relative to those in untreated scleroderma fibroblasts (100 AU). Data are expressed as the mean $\pm$ S.D. of independent experiments in each fibroblast (B). $*$, $p < 0.05$ compared with the value in untreated scleroderma fibroblasts. CL, cell lysates. C, normal and scleroderma fibroblasts were treated with the indicated concentration of SIS3 or the same amount of vehicle (DMSO) for 24 h under the same conditions, whole cell lysates were immunoprecipitated using anti-Smad3 antibody, and phospho-Smad3 was detected by immunoblotting analysis. One representative of experiments in five normal and five scleroderma fibroblasts is shown. D, normal and scleroderma fibroblasts were transfected with 2 μg of the bp $-353\sim+58$ COL1A2/CAT construct or a site-directed mutated construct -353m COL1A2/CAT and incubated for 48 h under the same conditions. Cells were treated with 3 μM SIS3 or DMSO for the last 24 h. Values represent the promoter activities relative to those of scleroderma fibroblasts transfected with the bp $-353\sim+58$ COL1A2/CAT and treated with DMSO (100 AU). Mean $\pm$ S.D. from five independent experiments is presented.

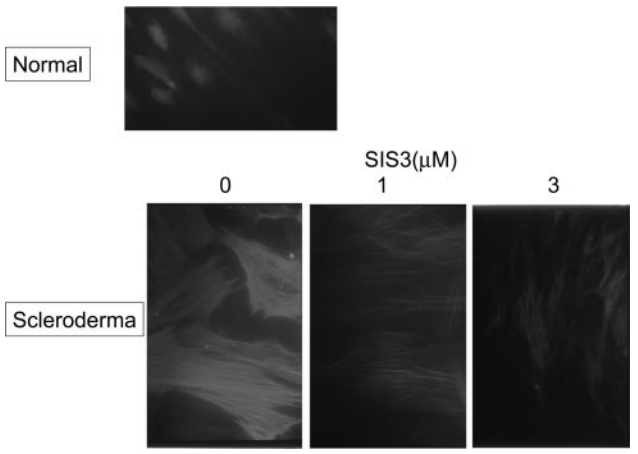


Fig. 7. Effect of SIS3 on α-smooth muscle actin overexpression in scleroderma fibroblasts. The subcellular localizations of α-SMA were visualized by immunofluorescence. Normal and scleroderma fibroblasts were serum-starved for 24 h and incubated for additional 24 h in the presence or absence of indicated dose of SIS3 under the same conditions.

and scleroderma fibroblasts, the model of cells with autocrine TGF-β signaling in vitro. Our study also indicated that the increased phosphorylation and the DNA binding ability of Smad3 in scleroderma fibroblasts is one of the causes of excessive ECM deposition in this disease, although the pathogenesis of this disease is still unclear. Because TGF-β is a potent stimulus for ECM synthesis, the inhibition of Smad3 may be beneficial in other fibrotic disorders. Therefore, further in vivo and in vitro studies are necessary.

Acknowledgments

We thank Dr. Kohei Miyazono for kindly providing expression vectors for ALK-5TD, ALK-4QD, FLAG-tagged-Smad3, and 6Myc-tagged-Smad4; Dr. Jeffrey Wrana for kindly providing the p3TP-lux reporter plasmids and the pAR3-lux reporter plasmids; and Dr. Malcolm Whitman for kindly providing *X. laevis* FAST1 cDNA.

References

Arbabi S, Rosengart MR, Garcia I, and Maier RV (2000) Hypertonic saline solution induces prostacyclin production by increasing cyclooxygenase-2 expression. *Surgery* **128:**198–205.

Asano Y, Ihn H, Yamane K, Kubo M, and Tamaki K (2004) Impaired Smad7-Smurf-mediated negative regulation of TGF-β signaling in scleroderma fibroblasts. *J Clin Investig* **113:**253–264.

Attisano L and Tuen Lee-Hoeflich S (2001) The Smads. *Genome Biol* **2:**1–8.

Border WA and Noble NA (1994) Transforming growth factor β in tissue fibrosis. *N Engl J Med* **331:**1286–1292.

Callahan JF, Burgess JL, Fornbald JA, Gaster LM, Harling JD, Harrington FP, Heer J, Kwon C, Lehr R, Mathur A, et al. (2002) Identification of novel inhibitors of the transforming growth factor-β1 (TGF-β1) type 1 receptor (ALK5). *J Med Chem* **45:**999–1001.

Carcamo J, Zentella A, and Massague J (1995) Disruption of transforming growth factor β signaling by a mutation that prevents transphosphorylation within the receptor complex. *Mol Cell Biol* **15:**1573–1581.

Chen SJ, Yuan W, Mori Y, Levenson A, Trojanowska M, and Varga J (1999) Stimulation of type I collagen transcription in human skin fibroblasts by TGF-β: involvement of Smad 3. *J Investig Dermatol* **112:**49–57.

Chou WC, Prokova V, Shiraishi K, Valcourt U, Moustakas A, Hadzopoulou-Cladaras M, Zannis VI, and Kardassis D (2003) Mechanism of a transcriptional cross talk between transforming growth factor-β-regulated Smad3 and Smad4 proteins and orphan nuclear receptor hepatocyte nuclear factor-4. *Mol Biol Cell* **14:**1279–1294.

Fernandez-Sanchez ME, Criado-Garcia O, Heath KE, Garcia-Fojeda B, Medrano-Fernandez I, Gomez-Garre P, Sanz P, Serratosa JM, and Rodriguez de Cordoba S (2003) Laforin, the dual-phosphatase responsible for Lafora disease, interacts with R5 (PTG), a regulatory subunit of protein phosphatase-1 that enhances glycogen accumulation. *Hum Mol Genet* **12:**3161–3171.

Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA Jr, Wrana JL, et al. (1997) The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. *Cell* **89:**1165–1173.

Herbert JM, Lamarche I, and Carmeliet P (1997) Urokinase and tissue-type plasminogen activator are required for the mitogenic and chemotactic effects of bovine fibroblast growth factor and platelet-derived growth factor-BB for vascular smooth muscle cells. *J Biol Chem* **272:**23585–23591.

Hitraya EG and Jimenez SA (1996) Transcriptional activation of the α(I) procollagen gene in systemic sclerosis dermal fibroblasts. Role of intronic sequences. *Arthritis Rheum* **39:**1347–1354.

Hu B, Wu Z, and Phan SH (2003) Smad3 mediates transforming growth factor-β-induced α-smooth muscle actin expression. *Am J Respir Cell Mol Biol* **29:**397–404.

Ihn H, Ihn Y, and Trojanowska M (2001a) Spl phosphorylation induced by serum stimulates the human α2(I) collagen gene expression. *J Investig Dermatol* **117:**301–308.

Ihn H, Ohnishi K, Tamaki T, LeRoy EC, and Trojanowska M (1996) Transcriptional regulation of the human α2(I) collagen gene. Combined action of upstream stimulatory and inhibitory cis-acting elements. *J Biol Chem* **271:**26717–26723.

Ihn H, Yamane K, Asano Y, Kubo M, and Tamaki K (2002) IL-4 up-regulates the expression of tissue inhibitor of metalloproteinase-2 in dermal fibroblasts via the p38 mitogen-activated protein kinase dependent pathway. *J Immunol* **168:**1895–1902.

Ihn H, Yamane K, Kubo M, and Tamaki K (2001b) Blockade of endogenous transforming growth factor β signaling prevents up-regulated collagen synthesis in scleroderma fibroblasts: association with increased expression of transforming growth factor β receptors. *Arthritis Rheum* **44:**474–480.

Inhoffen HH, Nordsiek KH, and Schafer H (1963) Konstitutionsermittlung eines Indol-Farbstoffs aus N-[β-indolyl-(3)-acryloyl]-glycin. *Justus Liebigs Ann Chem* **668:**104–120.

Isono M, Mogyorosi A, Han DC, Hoffman BB, and Ziyadeh FN (2000) Stimulation of TGF-β type II receptor by high glucose in mouse mesangial cells and in diabetic kidney. *Am J Physiol* **278:**F830–F838.

Jelaska A and Korn JH (2000) Role of apoptosis and transforming growth factor β1 in fibroblast selection and activation in systemic sclerosis. *Arthritis Rheum* **43:**2230–2239.

Jinnin M, Ihn H, Asano Y, Yamane K, Trojanowska M, and Tamaki K (2004) Tenascin-C upregulation by transforming growth factor-β in human dermal fibroblasts involves Smad3, Sp1 and Ets1. *Oncogene* **23:**1656–1667.

Kawakami T, Ihn H, Xu W, Smith E, LeRoy C, and Trojanowska M (1998) Increased expression of TGF-β receptors by scleroderma fibroblasts: evidence for contribution of autocrine TGF-β signaling to scleroderma phenotype. *J Investig Dermatol* **110:**47–51.

Kirk TZ, Mark ME, Chua CC, Chua BH, and Mayes MD (1995) Myofibroblasts from scleroderma skin synthesize elevated levels of collagen and tissue inhibitor of metalloproteinase (TIMP-1) with two forms of TIMP-1. *J Biol Chem* **270:**3423–3428.

Kondo M, Suzuki H, Takehara K, Miyazono K, and Kato M (2004) Transforming growth factor-β signaling is differentially inhibited by Smad2D450E and Smad3D407E. *Cancer Sci* **95:**12–17.

Laping NJ, Grygielko E, Mathur A, Butter S, Bomberger J, Tweed C, Martin W, Fornwald J, Lehr R, Harling J, et al. (2002) Inhibition of transforming growth factor (TGF)-β1-induced extracellular matrix with a novel inhibitor of the TGF-β type I receptor kinase activity: SB-431542. *Mol Pharmacol* **62:**58–64.

LeRoy EC, Smith EA, Kahaleh MB, Trojanowska M, and Silver RM (1989) A strategy for determining the pathogenesis of systemic sclerosis. Is transforming growth factor β the answer? *Arthritis Rheum* **32:**817–825.

Li Y, Xie M, Song X, Gragen S, Sachdeva K, Wan Y, and Yan B (2003) DEC1 negatively regulates the expression of DEC2 through binding to the E-box in the proximal promoter. *J Biol Chem* **278:**16899–16907.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65:**55–63.

Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, et al. (1997) TGF-β receptor-mediated signalling through Smad2, Smad3 and Smad4. *EMBO (Eur Mol Biol Organ) J* **16:**5353–5362.

Sappino AP, Schurch W, and Gabbiani G (1990) Differentiation repertoire of fibroblastic cells: expression of cytoskeletal proteins as marker of phenotypic modulations. *Lab Investig* **63:**144–161.

Thannickal VJ and Fanburg BL (1995) Activation of an H_2O_2-generating NADH oxidase in human lung fibroblasts by transforming growth factor β1. *J Biol Chem* **270:**30334–30338.

Thrane EV, Schwarze PE, Thoresen GH, Lag M, and Refsnes M (2001) Persistent versus transient map kinase (ERK) activation in the proliferation of lung epithelial type 2 cells. *Exp Lung Res* **27:**387–400.

Wymann M and Arcaro A (1994) Platelet-derived growth factor-induced phosphatidylinositol 3-kinase activation mediates actin rearrangements in fibroblasts. *Biochem J* **298:**517–520.

Yagi K, Furuhashi M, Aoki H, Goto D, Kuwano H, Sugamura K, Miyazono K, and Kato M (2002) c-myc is a downstream target of the Smad pathway. *J Biol Chem* **277:**854–861.

Yagi K, Goto D, Hamamoto T, Takenoshita S, Kato M, and Miyazono K (1999) Alternatively spliced variant of Smad2 lacking exon 3. Comparison with wild-type Smad2 and Smad3. *J Biol Chem* **274:**703–709.

Yamamoto T, Takagawa S, Katayama I, and Nishioka K (1999) Anti-sclerotic effect of transforming growth factor-β antibody in a mouse model of bleomycin-induced scleroderma. *Clin Immunol* **92:**6–13.

Yamane K, Ihn H, Asano Y, Jinnin M, and Tamaki K (2003a) Antagonistic effects of TNF-α on TGF-β signaling through down-regulation of TGF-β receptor type II in human dermal fibroblasts. *J Immunol* **171:**3855–3862.

Yamane K, Ihn H, and Tamaki K (2003b) Epidermal growth factor up-regulates expression of transforming growth factor β receptor type II in human dermal

fibroblasts by phosphoinositide 3-kinase/Akt signaling pathway: resistance to epidermal growth factor stimulation in scleroderma fibroblasts. *Arthritis Rheum* **48:**1652–1666.

Yasufumi M, Kazuko H, and Katsutoshi H (2003), inventors; Nippon Shinyaku Co., Ltd., Yasufumi M, Kazuko H, and Katsutoshi H, assignees. Amide derivatives and drugs. World patent number WO03037862. 2003 May 8.

Ziyadeh FN, Sharma K, Ericksen M, and Wolf G (1994) Stimulation of collagen gene expression and protein synthesis in murine mesangial cells by high glucose is mediated by autocrine activation of transforming growth factor-β. *J Clin Investig* **93:**536–542.

Address correspondence to: Dr. Hironobu Ihn, Department of Dermatology and Plastic and Reconstructive Surgery, Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan. E-mail: ihn-der@kaiju.medic.kumamoto-u.ac.jp