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Characterization of SIS3, a Novel Specific Inhibitor of Smad3, and Its Effect on Transforming Growth Factor-β1-Induced Extracellular Matrix Expression[1]

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The abbreviations used are: TGF, transforming growth factor; ECM, extracellular matrix; PDGF, platelet derived growth factor; GST, glutathione S-transferase; ERK, Extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; MEM, modified Eagle's medium; CAT, chloramphenicol acetyltransferase; PBS, phosphate buffered saline; PCR, polymerase chain reaction

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Abstract

This is the first report that characterizes 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 TGF-β1-induced phosphorylation of Smad3 and interaction of Smad3 with Smad4.   On the other hand, the phosphorylation of Smad2 was not affected by this reagent.   Subsequently, 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.   Taken 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 wide 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, prevent 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 which 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 type 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 following recent findings; (i) Scleroderma fibroblasts

express elevated levels of TGF-β receptors, and this correlates with the elevated levels of α2(I) collagen mRNA (Kawakami et al., 1998), (ii) 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), (iii) 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 which 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).

Recently, several investigators 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 did not affect the phosphorylation of Smad3. Smad2D450E reduced hetero-oligomer formation of Smad2 with Smad4, but not of Smad3 with Smad4. Smad3D407E, that was not phosphorylated by the constitutively active form of the TGF-β type I receptor, and inhibited the phosphorylation of co-expressed 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 ALK5 with greater selectivity against other kinases including p38 MAPK, ALK2, 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 SIS3 (**S**pecific **I**nhibitor of **S**mad**3**), a new inhibitor of TGF-β signaling, expressed its effects via the selective suppression of

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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 (PDGF)-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 mitogen-activated protein kinase (MAPK) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antiphosphoserine specific antibody was from Zymed Laboratories (San Francisco, CA). Anti-Smad2/3 antibody (S66220) was from Transduction Laboratories (Lexington, KY). Anti-HA 3F10 antibody and FuGENE 6 were from Roche Diagnostics (Mannheim, Germany). Flag M2 antibody was from Kodak (New Haven, CT). The antibody for β-actin or α-smooth muscle actin (SMA) was from Sigma (St Louis, MO). Antibody for phospho-Smad2, phosphoinositide 3-kinase (PI3K) 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 SouthernBiotech (Birmingham, AL).

The synthesis of SIS3. Indole derivatives are regarded as structures which have high affinity to various receptors and then express important biological activities by binding

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with these receptors. SIS3 was indole derivatives with 2'-phenyl as hydrophobic group to induce translocation into the nucleus. We synthesized SIS3, on the assumption that they act as a ligand of nuclear receptors according to published methods of 2-(N-methylindolyl)acrylic acid, followed by condensation with the corresponding amine (Patent WO03/037862) (Inhoffen et al., 1963) (Fig. 1). SIS3 stored as a solution in DMSO, and this solution was used after diluting with medium for each assay.

Cell cultures. Normal human dermal fibroblasts were obtained by skin biopsies from 5 healthy donors. Scleroderma fibroblasts were obtained by skin biopsies from the affected areas (dorsal forearm) of 5 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), 2mM L-glutamine, and 50μg/ml gentamycin. 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, COS7 cell line and NIH3T3 cell line were purchased from American Type Culture Collection (Manassas, Virginia). 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 previously described (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 QuickChange site-directed mutagenesis kit (Stratagene) 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), Flag-tagged-Smad3 or 6Myc-tagged-Smad4 containing six tandem copies of the Myc-epitope tag, were kindly provided by Dr. Kohei Miyazono (Nakao et al., 1997; Yagi et al., 2002; Yagi et al., 1999). The p3TP-lux reporter plasmids and the pAR3-lux reporter plasmids were provided by Dr. Jeffrey Wrana (Carcamo et al., 1995; Hayashi et al., 1997). Xenopus fork-head activin signal transducer 1 (FAST1) cDNA were kindly provided by Dr. Malcolm Whitman (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, employing FuGENE6 as described previously (Ihn et

al., 2002).   In order to correct minor variations in transfection efficiency, pSV-β-galactosidase vector (Promega, Madison, WI) was included in all transfections. After 48 hours of incubation, cells were harvested in 0.25M Tris-HCl (pH8) and fractured by freeze-thawing for CAT assay or luciferase assay.   Extracts were normalized for protein content as measured by the Bio-Rad reagent.   Each experiment was performed in duplicate.

Cell lysis and immunoblotting.   Human dermal fibroblasts were cultured until they were confluent, then the media were collected.   Remaining cells were washed with cold phosphate buffered saline (PBS) twice and lysed in lysis buffer (Asano et al., 2004; Ihn et al., 2002).   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 $4\,^{\circ}C$, 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 antiphosphoserine specific antibodies or anti-p85 and antiphosphotyrosine 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.

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To examine the interaction between Smad3 and Smad4, COS7 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 ME et al. (Fernandez-Sanchez et al., 2003) with minor modification. The full-length Smad3 was cloned into pDEST27 (Invitrogen) and in vitro transcribed/translated using NHDF Nucleofector Kit (amaxa biosystems). Fibroblasts were then grown in MEM with 10% FCS for 24 hours, and the medium was replaced with serum-free medium. After 24 hours of incubation, cells were harvested in lysis buffer containing 20mM Tris-HCl, pH 7.5, 150mM NaCl, 2mM EDTA, 0.1mM EGTA, 1% TritonX-100, 50mM sodium fluoride, 25mM β-glycerophosphate hydrate, 1mM phenylmethylsulfonyl fluoride, 1mM sodium orthovanadate, 1μg/ml leupeptin, 1μg/ml aprotinin, and 1μg/ml pepstatin. Proteins were purified with glutathione Sepharose beads 4B (Amersham Biosciences, Uppsala, Sweden). 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.

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) 3xCAGA oligo, 5'-TCGAGAGCCAGACAAGGAGCCAGACAAGGAGCCAGACACTCGAG, which is trimer of CAGA motif; 2) 3xCAGA-M oligo, 5'-TCGAGAGCTACATAAAAAGCTACATATTTAGCTACATACTCGA, 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) 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 6-well culture plates and grown until subconfluency in MEM containing 10% FCS. Cells were quiesced by 24 hour incubation in serum-free MEM, followed by incubation in serum-free medium in the presence or absence of SIS3 prior to the collection of cells for 72 hour. Then, the cells were detached from the wells by trypsin treatment and counted using a Coulter Counter (Beckman Coulter, Jersey City, NJ) (Herbert et al., 1997).

Measurement of [^{3}H]proline incorporation. Cells (10^4/well) were plated into 96-well plates, and then the medium was changed to serum-free medium supplemented

with 50µg/ml ascorbic acid for the duration of the experiment. 0.05µCi/µl [^{3}H]proline (L-(2,3,4,5)-[^{3}H]proline) (Amersham Pharmacia) 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 (Isono et al., 2000; Ziyadeh et al., 1994). Proline incorporation was corrected for the cell viablility measured by MMT assay in additional cells plated in parallel wells (Mosmann, 1983).

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (PCR). 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 4 well LAK TEK chambers (Nunc, Naperville, Illinois) to subconfluence as described above. After 24 hours of serum starvation, the cells were fixed with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100 in PBS (PBST), and blocked with 10% FCS in PBST (Asano et al., 2004). The cells were stained with anti-α-SMA antibody as primary antibody, washed, and incubated with FITC-conjugated secondary antibodies. To visualize the fluorescence

a Zeiss microscope was used.

Statistical analysis.   Statistical analysis was carried out with the Mann-Whitney 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 co-transfection with constitutive 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 AP-1-binding sites of the collagenase 1 gene) (Carcamo et al., 1995; Hayashi et al., 1997) in Mv1Lu cells co-transfected 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 Xenopus) (Hayashi et al., 1997), constitutively active form of activin type I receptor (ALK-4QD), and an expression vector encoding the co-activator FAST1.   Taken together, these results indicated that SIS3 inhibits the signaling activity of TGF-β1 family.

SIS3 reduces the TGF-β1-dependent increase in the phosphorylation and the 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. 3A and B, the stimulation of TGF-β1 induced the marked phosphorylation of both endogenous and exogenous overexpressed Smad3 in human dermal fibroblasts. The pretreatment of 3μM SIS3 reduced them about 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 of 3μM SIS3 decreased the levels of the DNA-Smad3 binding about 50%.

When both Smad3 and Smad4 were simultaneously transfected into COS7 cells and stimulated by the co-transfection 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 phosphorylation of other signaling pathways, such as MAPK/p38 induced by NaCl (Arbabi et al., 2000), ERK induced by FBS (Thrane et al., 2001) or PI3K by PDGF-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.

The effect of SIS3 on the ECM expression induced by TGF-β1 in normal dermal fibroblasts. We investigated whether SIS3 can also affect 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 3μM of 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 3μM of SIS3 reduced TGF-β1 -mediated up-regulation of α2(I) collagen mRNA (Fig. 4C). To note, 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 of 3μM SIS3 reduced the promoter activity about 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 sites (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 -125bp (bp –108~+58 deletion construct). The TGF-β1-dependent increased promoter activity of the bp -264~+58 construct and the longer constructs significantly reduced by the treatment of 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 utilized 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 about 50%, the inhibitory effect of SIS3 was completely abolished in the mutated construct. Taken 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.

The effect of SIS3 on the TGF-β1-mediated myofibroblast differentiation of dermal fibroblasts. It is well-known that α-SMA expression is the classical marker of myofibroblast differentiation. As shown in Fig. 4A, immunoblotting revealed that α-SMA expressed little in normal cell lysates. Exogenous TGF-β1 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 immunocytochemistory. The stimulation of dermal fibroblasts with TGF-β1 increased the α-SMA expression, which was abolished by the pretreatment of 3μM SIS3 completely (Fig. 5). This result suggested that SIS3 can inhibit the myofibroblast differentiation induced by TGF-β1.

The effect of SIS3 on ECM expression in scleroderma fibroblasts. As described above, we demonstrated that scleroderma fibroblasts are regarded as a model of fibrosis which involves TGF-β signaling. There is a 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 Figure 6A and B, 3μM of SIS3 reduced the up-regulated expression of type I procollagen protein as well as α-SMA in scleroderma fibroblasts up to the same extents as those 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 of 3μM SIS3 completely 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 of 3μM SIS3 also reduced bp –353~+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 (3μM) decreased the expression of α-SMA up to the same extents as those in normal fibroblasts, which is consistent with the result of immunoblotting (Fig. 6A). These results indicated that SIS3 restores myofibroblast differentiation of scleroderma fibroblasts.

DISCUSSION

Our study is the first report which characterizes 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 express their effect via Smad2, Smad3 and Smad4 (Nakao et al., 1997).   Taken 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.

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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, neither the phosphorylation of Smad2, the expression of Smad4 or Smad7, nor the phosphorylation of other signaling pathways such as p38, p85 or ERK were affected by this reagent in the presence or absence of TGF-β1.   These results indicated 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 act as ligand of nuclear receptors.   Chou et al. reported that TGF-β-regulated Smads can have transcriptional cross talk with nuclear receptor hepatocyte nuclear factor-4 (Chou et al., 2003).   Interaction of some orphan nuclear receptors with Smad3 through SIS3 may inhibit the phosphorylation of Smad3 specifically via SSXS motif.

Subsequently, 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. To note, the 3μM 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. suggested that the myofibroblast phenotype might correspond to "activated fibroblast" phenotype found in systemic sclerosis (Kirk et al., 1995). 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 appears 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 pathologic 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.

Taken together, our study suggested 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

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and scleroderma fibroblasts, the model of cells with autocrine TGF-β signaling in vitro. Otherwise, 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 studies in vivo and in vitro are necessary.

ACKNOWLEDGMENTS

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FOOTNOTES

We have no conflinct of interest.

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Figure legends

Fig. 1. The structure of SIS3.

6,7-Dimethyl-2-[(2E)-3-(1-methyl-2-phenyl-1H-pyrrolo[2,3-b]pyridin-3-yl-prop-2-enoyl)-1,2,3,4tetrahydroisoquinoline hydrochloride.

Fig. 2. SIS3 suppressed Smad-responsive promoter activities induced by the co-transfection 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 hours, the indicated dose of SIS3 was added for additional 24 hours. 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 ± S.D. from five independent experiments is presented. * indicates significant results compared with the basal promoter activity ($p < 0.05$, Mann-Whitney U test).

Fig. 3. The effect of SIS3 on the TGF-β1-dependent Smad3 phosphorylation and the DNA-Smad3 binding.

A: Confluent quiescent human dermal fibroblasts were pretreated with the 3μM of SIS3 or the same amount of vehicle (DMSO) for 1 hour, and stimulated with 2ng/ml of TGF-β1 for 1 hour. Whole cell lysates were subjected to immunoprecipitation using anti-Smad3 antibody, and phospho-Smad3 was detected by immunoblotting analysis.

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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 (100AU). Data are expressed as the mean ± S.D. of five independent experiments (lower panel). * $p < 0.05$, as 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 hour, and stimulated with TGF-β1 for 1 hour. Phosphorylated levels of Smad3 were determined using anti-phospho-Smad2/3 antibody as described in 'MATERIALS and METHODS'.

C: Human dermal fibroblasts were pretreated with the indicated concentration of SIS3 or the same amount of vehicle (DMSO) for 1 hour, and stimulated with the 2ng/ml of TGF-β1 for 1 hour. 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: COS7 cells were transfected with the indicated combinations of cDNAs encoding Smads and ALK-5TD. After 24 hours, 3μM of SIS3 was added for additional 24 hours. 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 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

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membrane was subjected to immunoblotting using the anti-phosphoserine antibodies.

E: Human dermal fibroblasts were pretreated with indicated dose of SIS3 for 1 hour and then stimulated with exogenous 2ng/ml TGF-β1 for 1 hour prior to 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 (100AU). Data are expressed as the mean ± S.D. of five independent experiments (lower panel).

F: Human dermal fibroblasts were serum-starved for 24 hour and incubated in the presence or absence of SIS3 for 72 hour. Cell count was performed as described in Materials and Methods. Mean ± S.D. from three independent experiments are presented.

Fig. 4. Effects of SIS3 on the TGF-β1-induced ECMs in human dermal fibroblasts.

A: Human dermal fibroblasts were serum-starved for 24 hours and treated with the indicated dose of SIS3 for 1 hour, and then 2ng/ml TGF-β1 were added. After 72 hours, 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 (100AU). Data are expressed as the mean ± S.D. of five independent experiments (lower panel). * p < 0.05, as compared with the value in the

TGF-β1-treated cells without SIS3 .

B: Newly synthesized type I collagen in indicated cells were measured in a [^{3}H]proline incorporation assay after 1 hours of SIS3 treatment and 6 hours of 2ng/ml of TGF-β stimulation.   Summary of quantitative analysis of the collagenous proteins expressed by fibroblasts with TGF-β1 in five independent experiments were 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 2ng/ml of TGF-β for 24 hours in the presence or absence of SIS3 pretreatment. Cells were collected after 24 hours, 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 2ng/ml TGF-β1 for 24 hours.   In all experiments, cells were pretreated with the indicated concentration of SIS3 or the same amount of vehicle (DMSO) for 1 hour before the stimulation with TGF-β1.   Values represent the α2(I) collagen promoter activities relative to those of untreated cells, which was set at 100AU.   Mean ± S.D. from five independent experiments is presented.   *$p<0.05$ versus untreated cells.

E: Human dermal fibroblasts were transfected with 2μg 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 2ng/ml TGF-β1 for 24 hours. In all experiments, cells were pretreated with 3μM SIS3 or the same amount of vehicle (DMSO) for 1 hour 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. * indicates significant results compared with the basal promoter activities of each construct (p<0.05, Mann-Whitney U test).

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 hours and pretreated with 3μM SIS3 for 1 hour prior to addition of 2ng/ml of TGF-β1 for 24 hours.

Fig. 6. The effects of SIS3 on scleroderma fibroblasts.

A and B: Normal and scleroderma fibroblasts were serum-starved for 24 hours and treated with the indicated dose of SIS3 for 72 hours 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 5 normal and 5 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 (100AU). Data are expressed as the mean ± S.D. of independent experiments in each fibroblast (B). * p < 0.05, as compared with the value in untreated scleroderma fibroblasts. MEM, conditioned media; 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 hours under the same condition, and whole cell lysates were immunoprecipitation using anti-Smad3 antibody, and phospho-Smad3 was detected by immunoblotting analysis. One representative of experiments in 5 normal and 5 scleroderma fibroblasts is shown.

D: Normal and scleroderma fibroblasts were transfected with 2μg of the bp –353~+58 COL1A2/CAT construct or a site directed mutated construct -353m COL1A2/CAT, and incubated for 48 hours under the same condition. Cells were treated with 3μM SIS3 or DMSO for the last 24 hours. Values represent the promoter activities relative to those of scleroderma fibroblasts transfected with the bp –353~+58 COL1A2/CAT and treated with DMSO (100AU). Mean ± 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 hours and incubated for additional 24 hous in the presence or absence of indicated dose of SIS3 under the same condition.

Fig.1
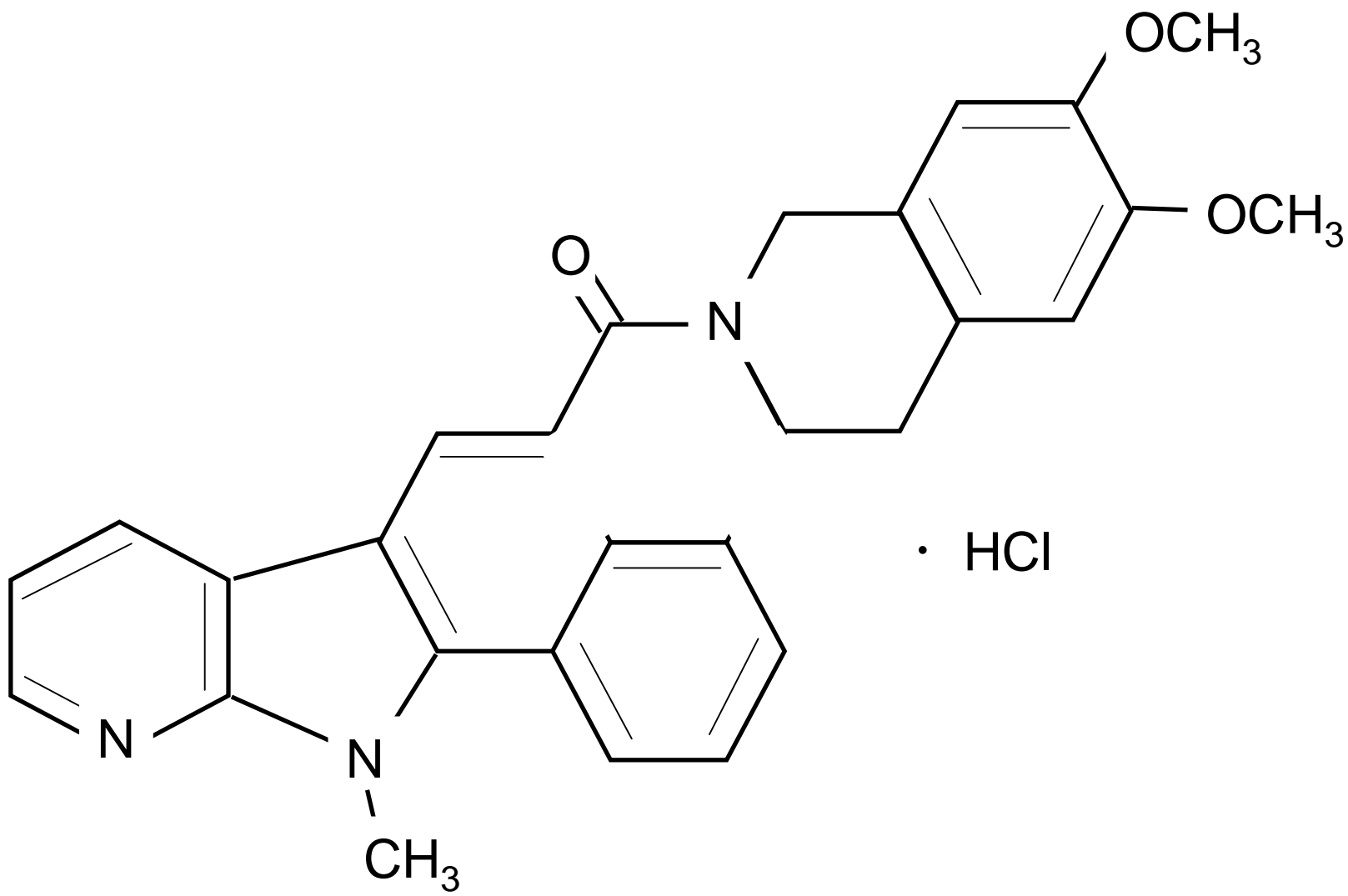
Fig.2
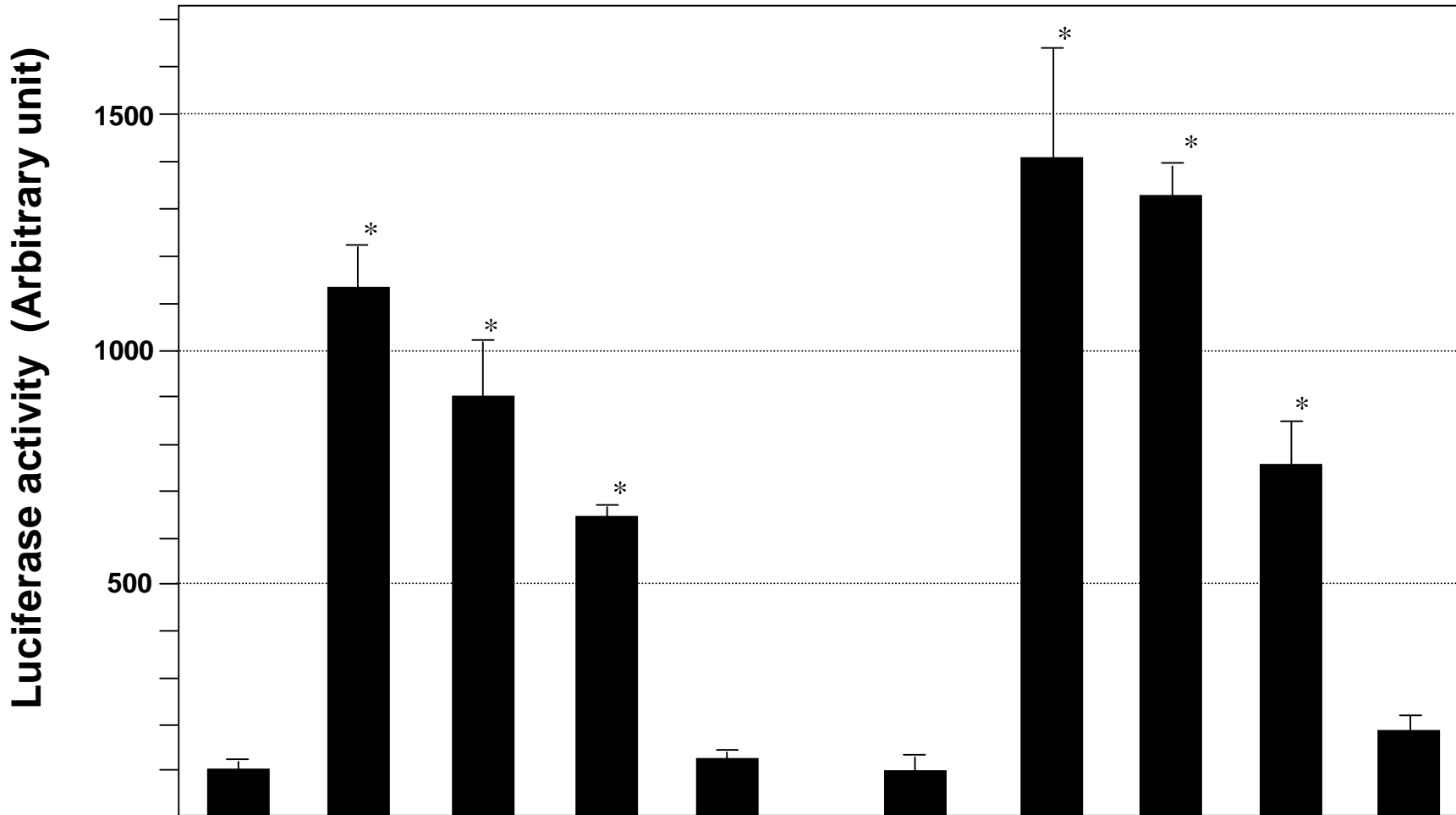
SIS3 (μM)	-	-	1	3	10		-	-	1	3	10
Receptor	-	ALK-5TD					-	ALK-4QD+FAST-1			
Vector	p3TP-Lux						pAR3-Lux				

Fig.3-A

Phospho-Smad3
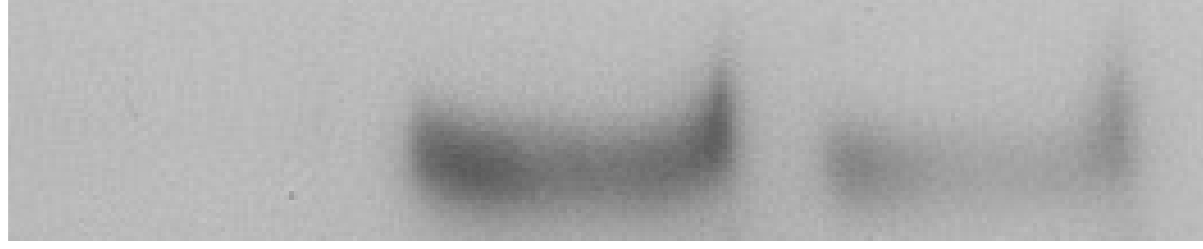
Total Smad3

| TGF-β | - | + | + |
| SIS3(μM) | 0 | 0 | 3 |

Fig.3-B

P-Smad3

GST-Smad3
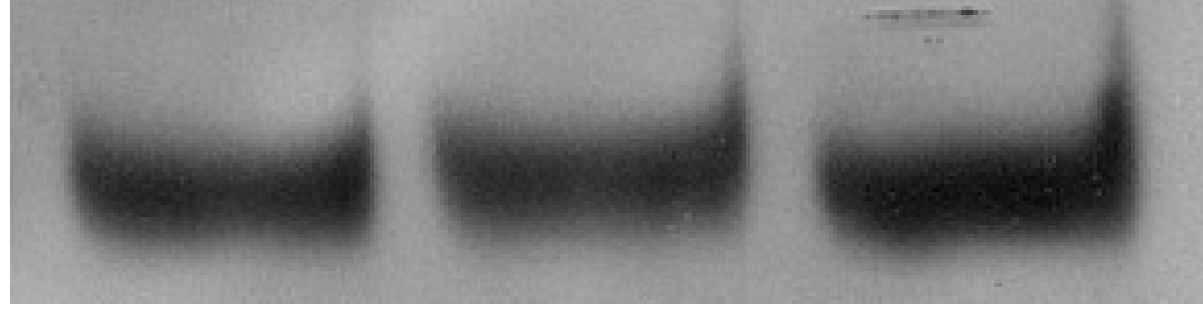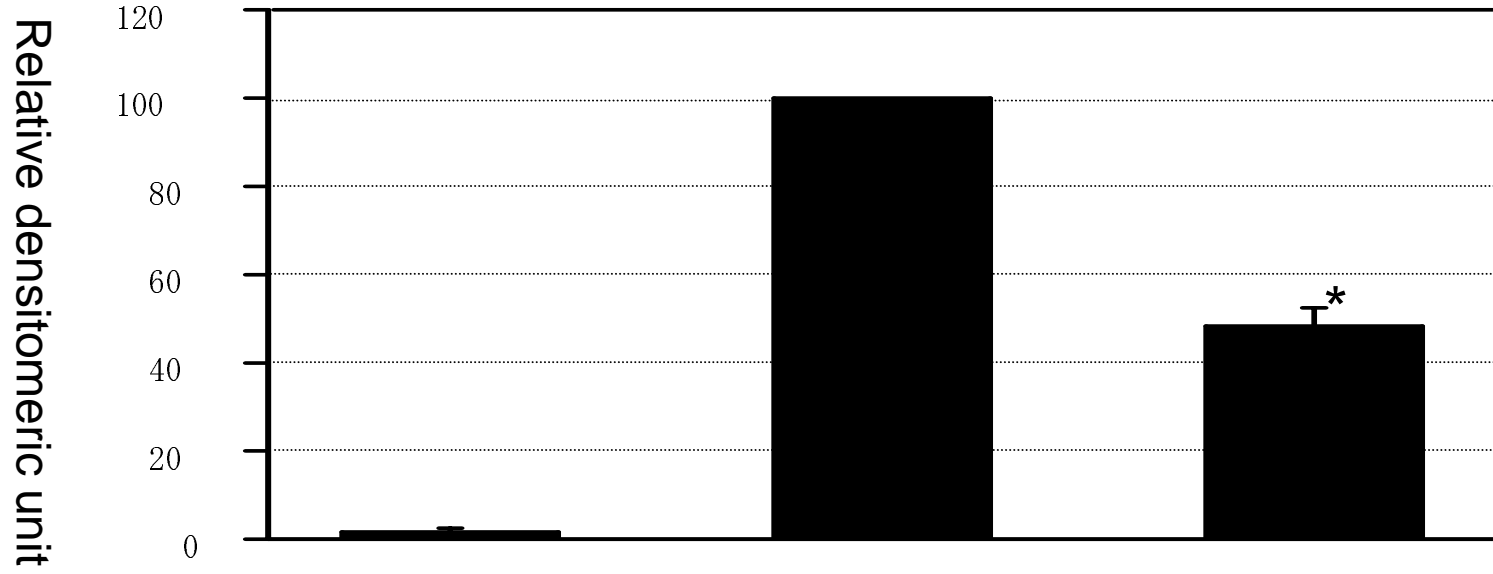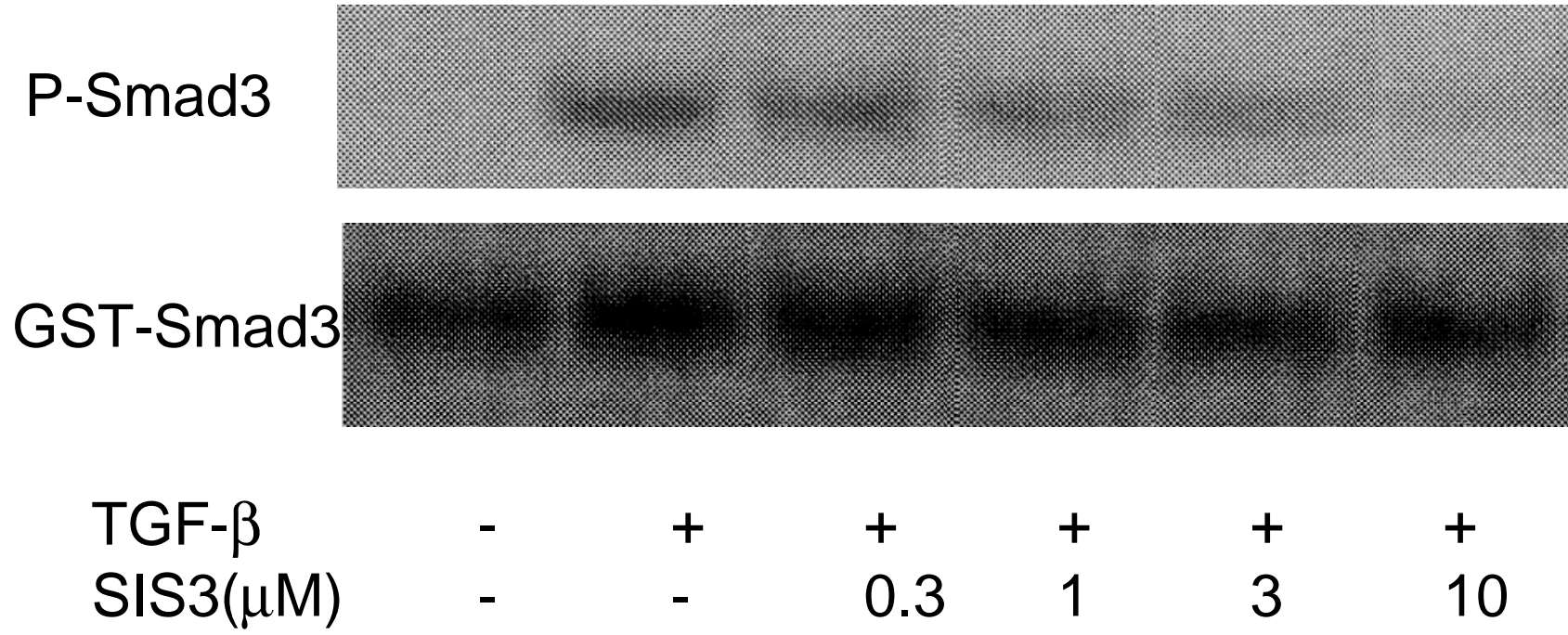
| TGF-β | - | + | + | + | + | + |
| SIS3(μM) | - | - | 0.3 | 1 | 3 | 10 |

Fig.3-C

Smad2 →
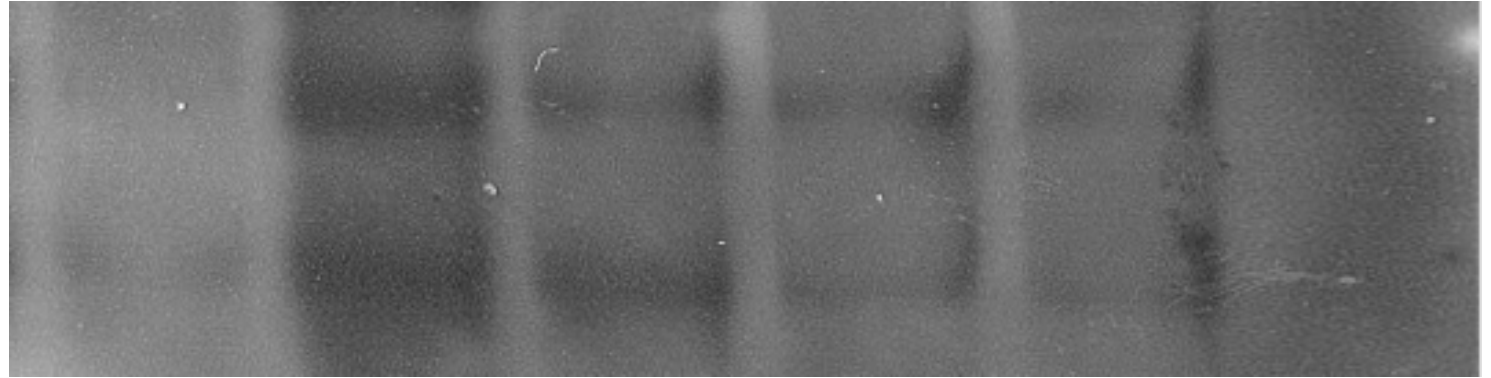
Smad3 →

TGF-β₁2ng/ml	-	+				
SIS3(μM)	-	-	1	3	10	-
Oligo		CAGA				CAGA-M

Fig.3-D

Fig.3-E

Phospho-Smad2

Total-Smad2
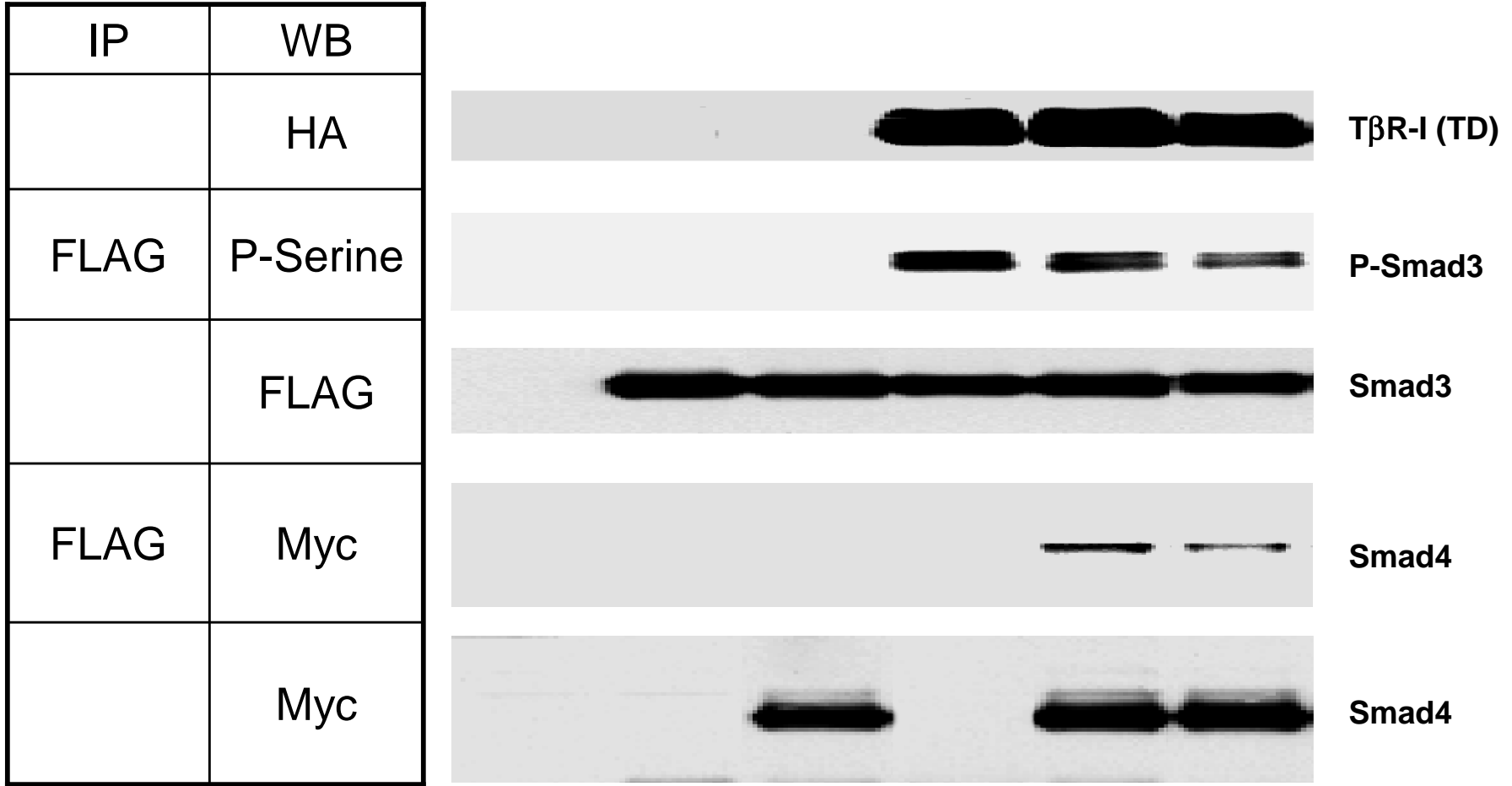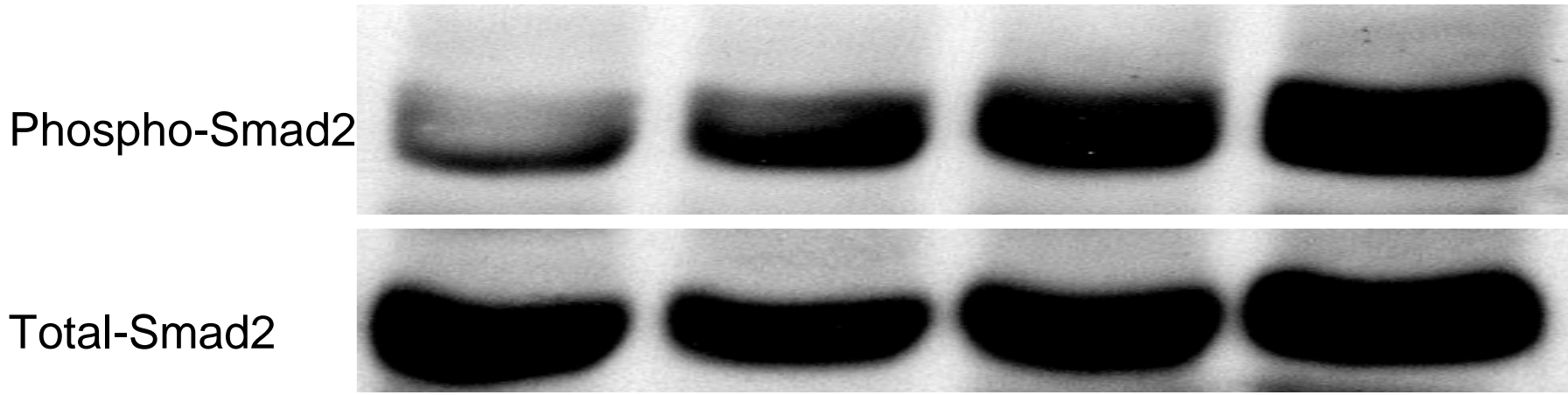
| TGF-β_1 | - | + | + | + |
| SIS3(μM) | - | - | 1 | 3 |
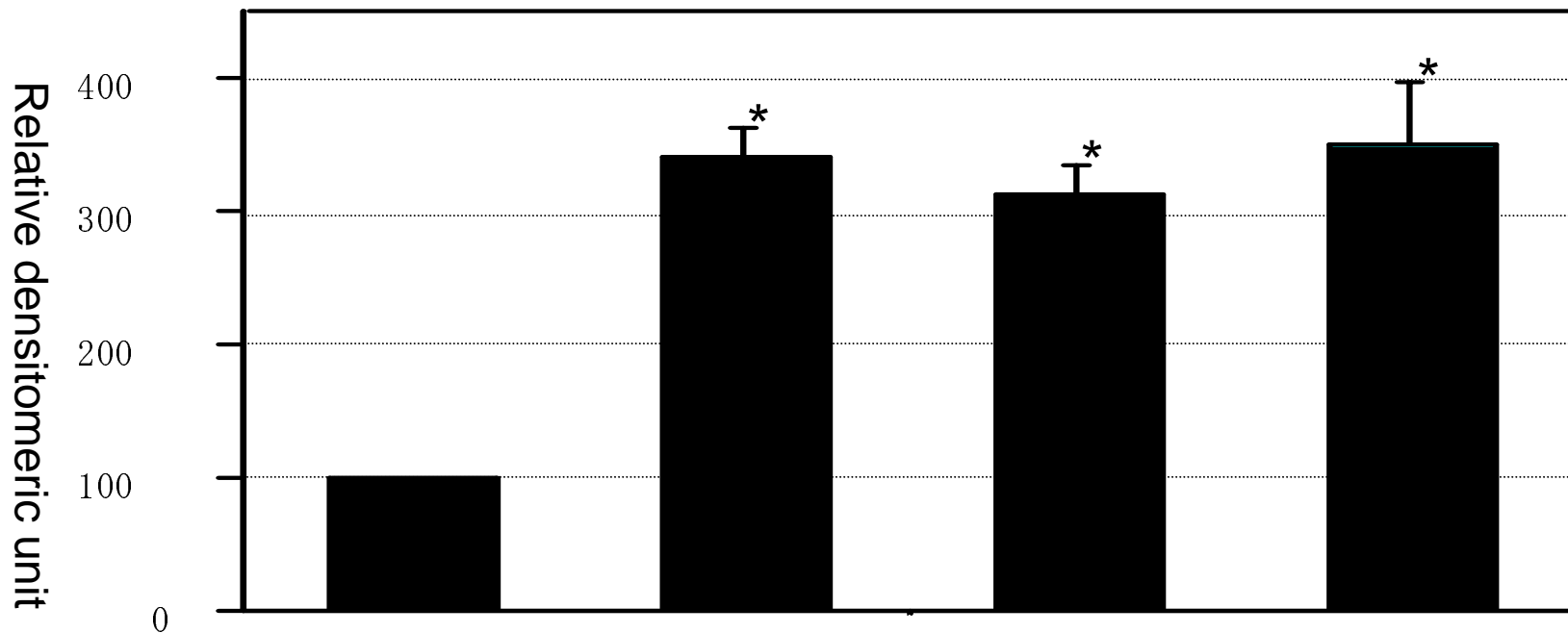
Fig.3-F

Relative increase of cell number (%)

SIS3(μM)   0   0.3   1   3
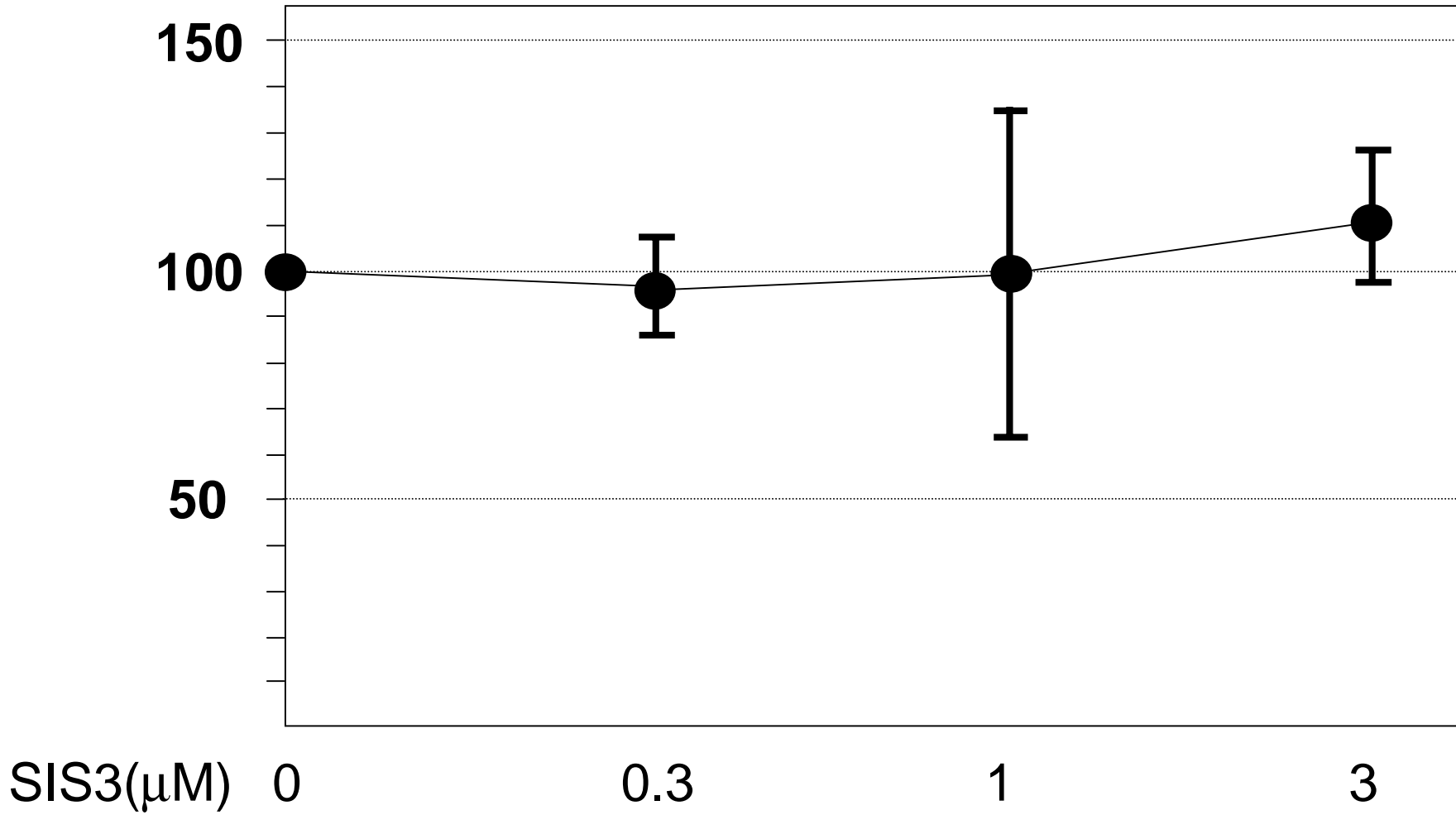
Fig.4-A

Type I procollagen

α-smooth muscle actin

β-actin
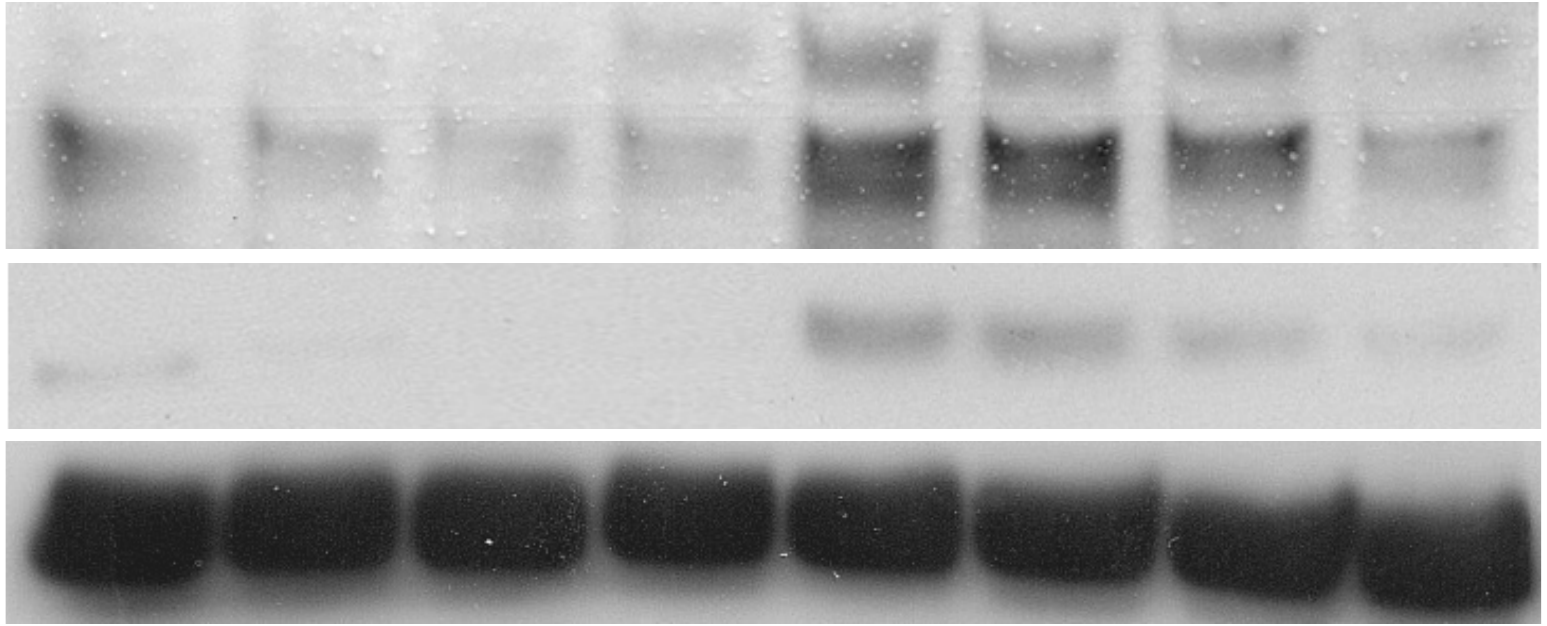
| TGF-β | - | - | - | - | + | + | + | + |
| SIS3(μM) | 0 | 0.3 | 1 | 3 | 0 | 0.3 | 1 | 3 |
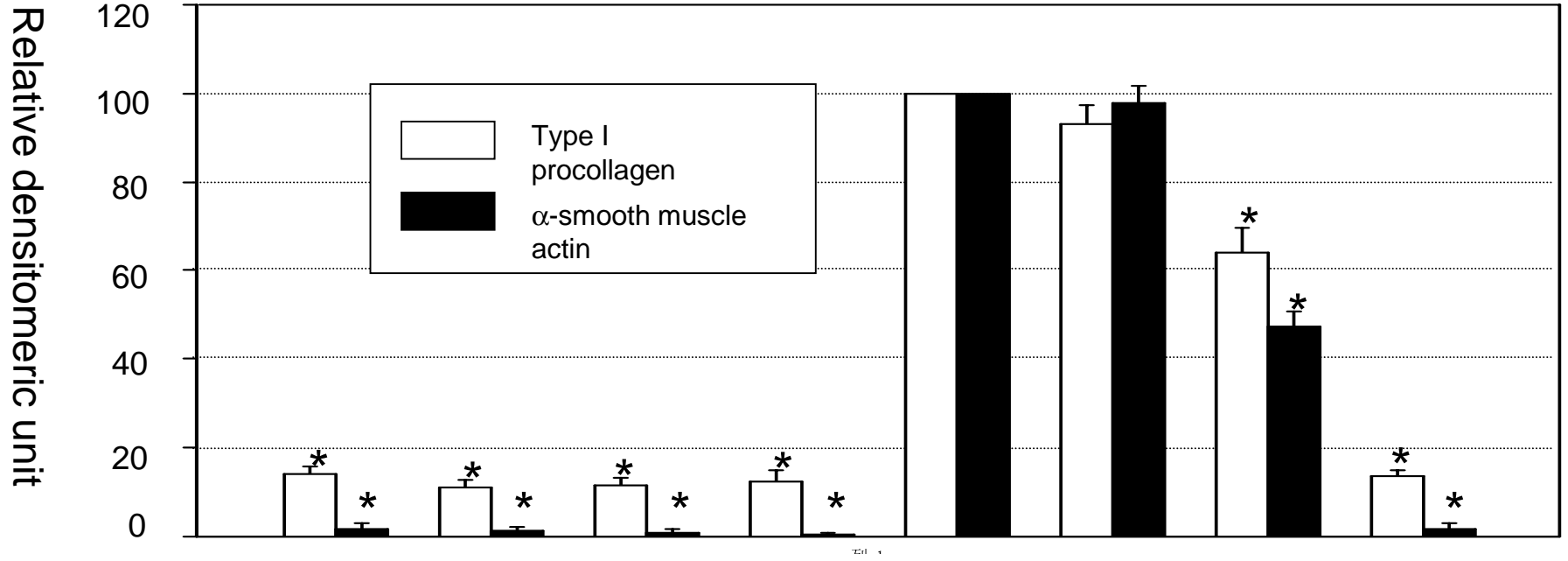
Fig.4-B

Relative increase of
[H3]Pro uptake (%)

Fig.4-C

TGF-β₁   -   +   +   +
SIS3(μM)   -   -   1   3
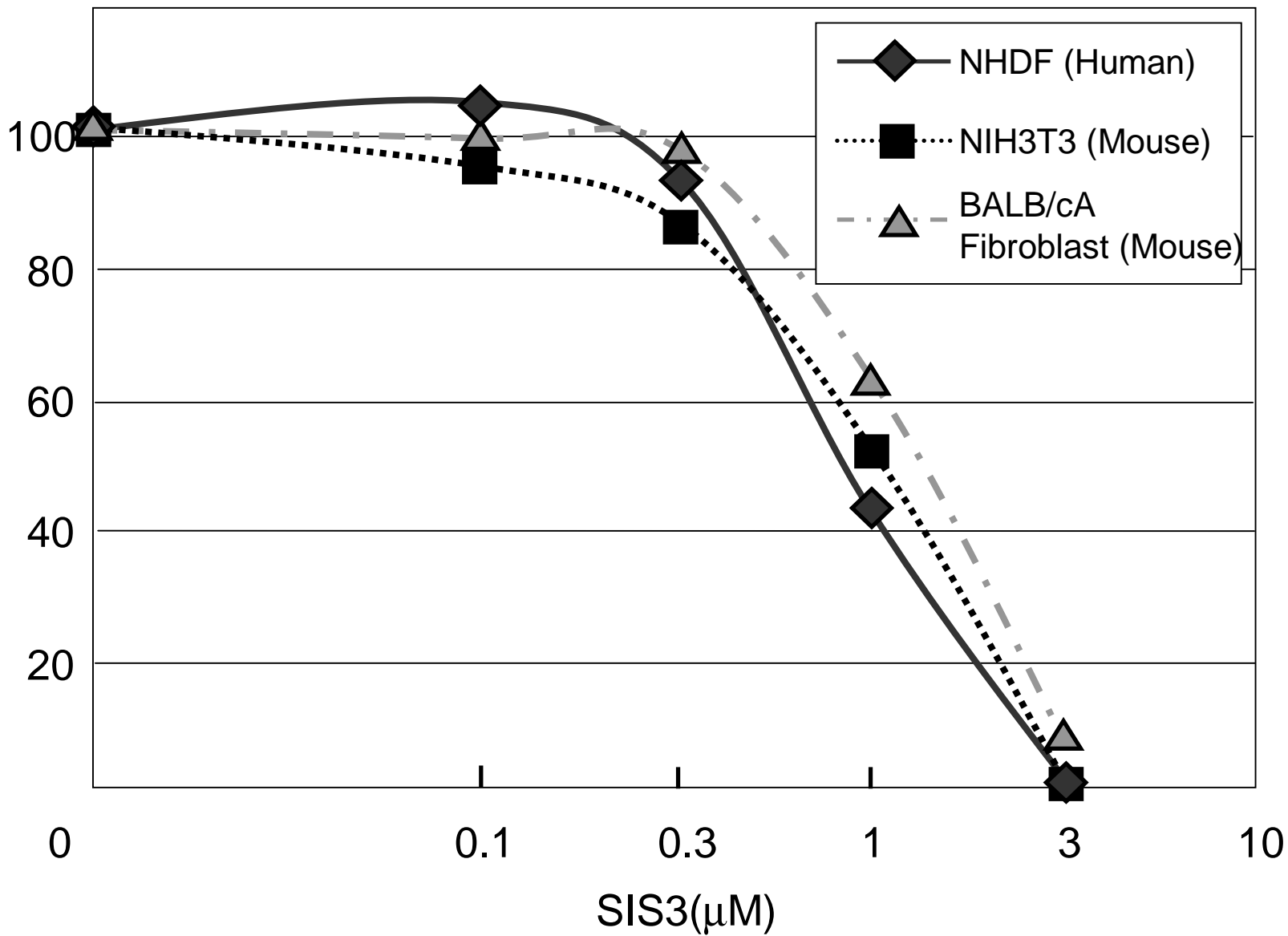
α2(I) collagen mRNA →
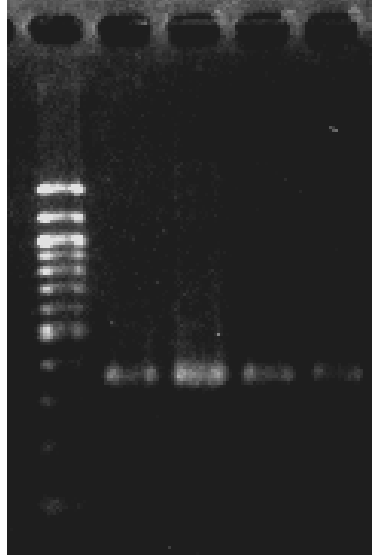
GAPDH mRNA →
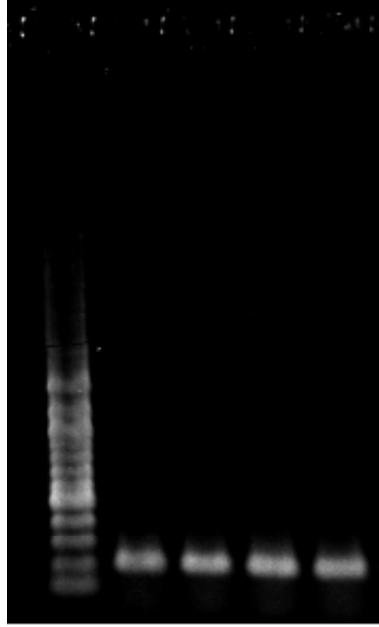
Fig.4-D

Fig.4-E

Fig.5

TGF-βι;-
SIS3;-

TGF-βι;2ng/ml
SIS3;-

TGF-βι;2ng/ml
SIS3;1μM
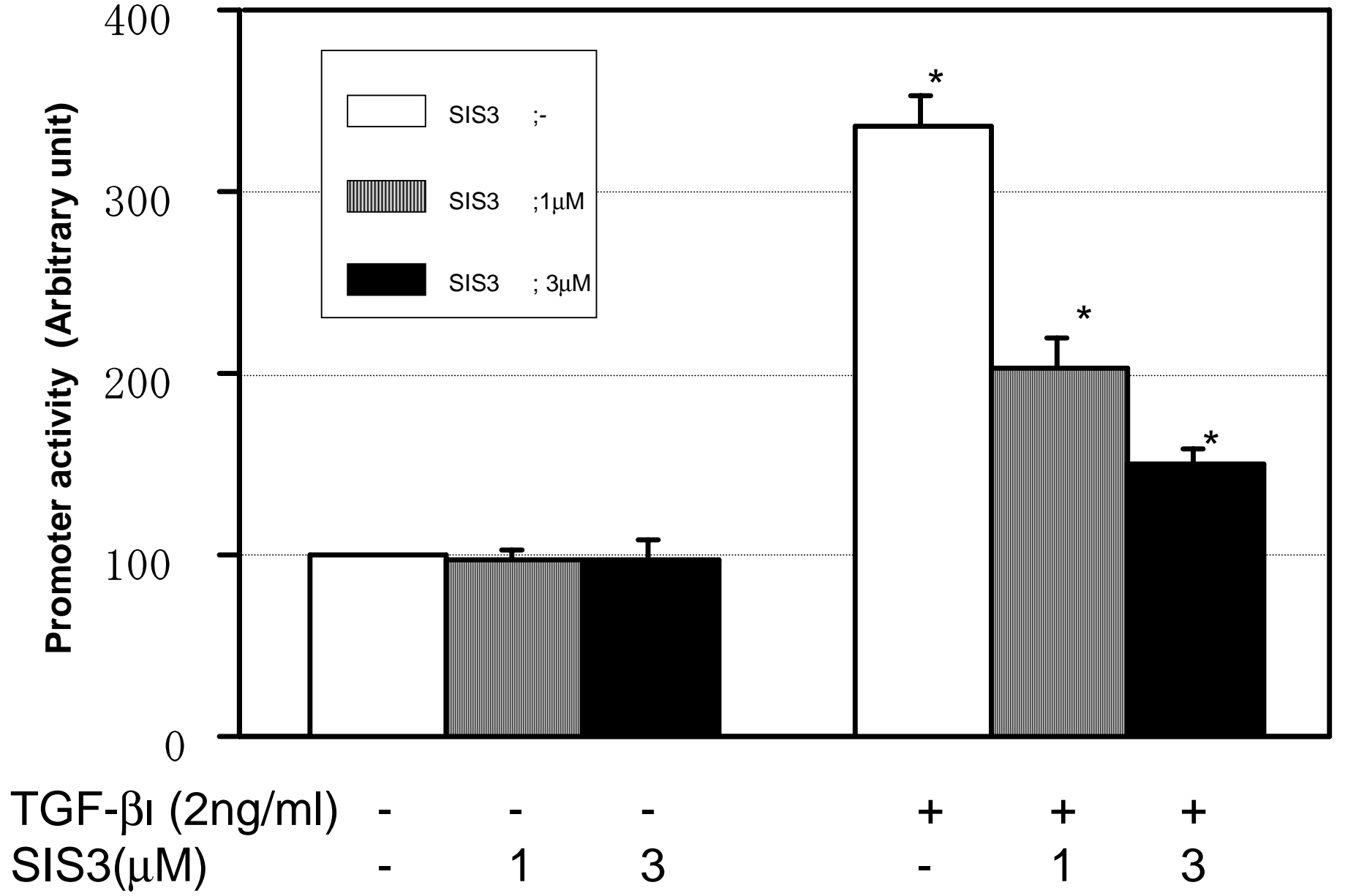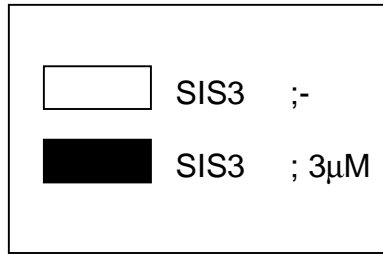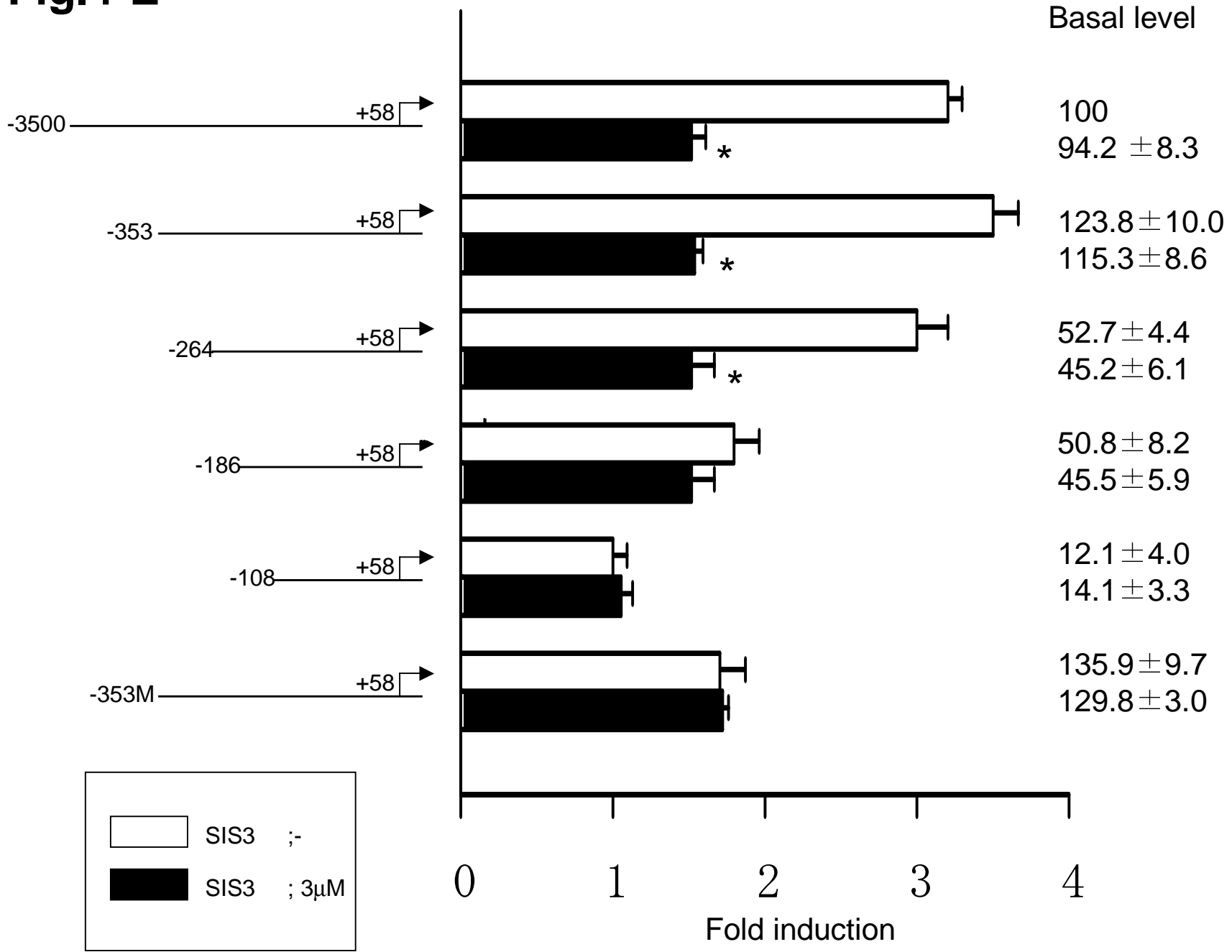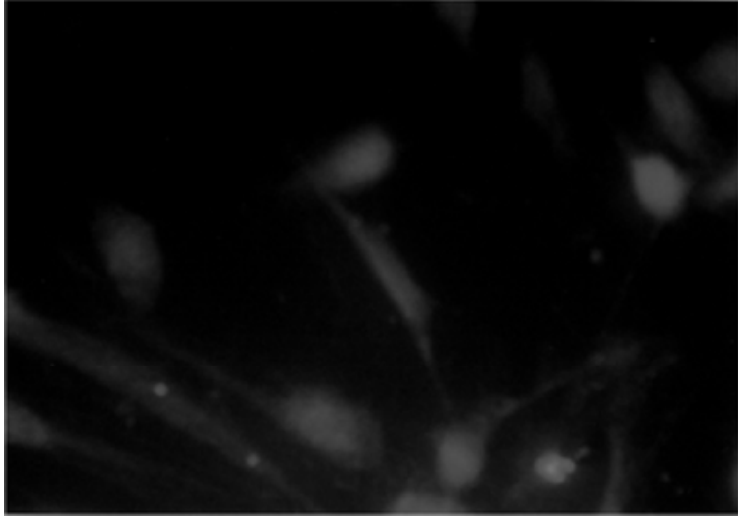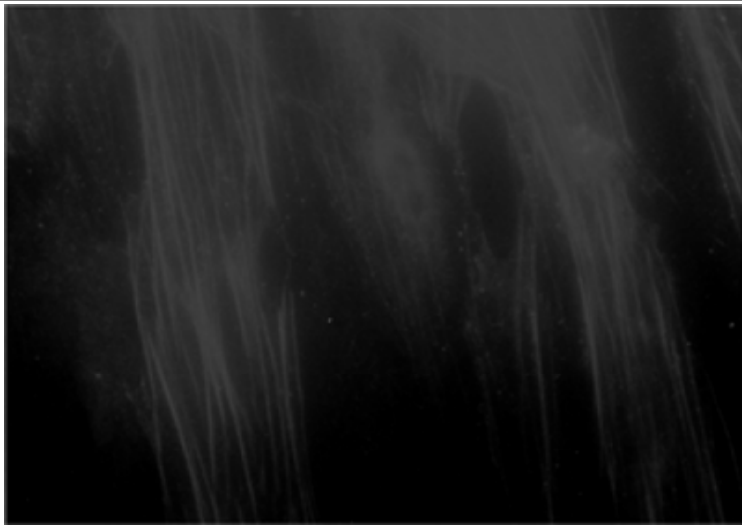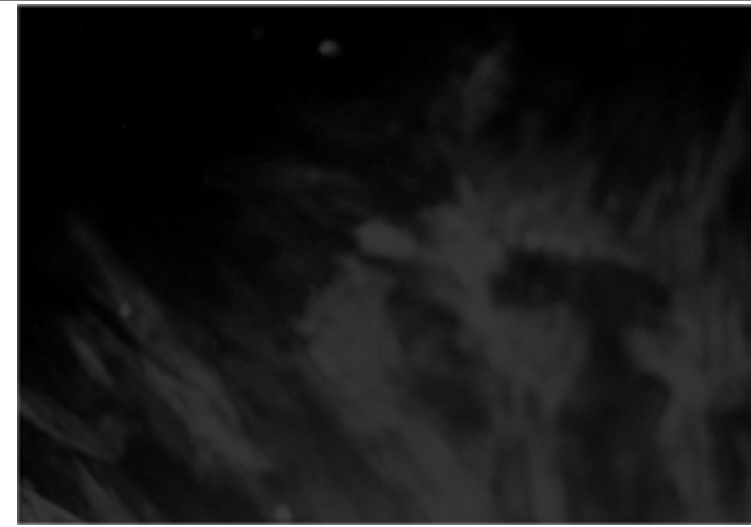
TGF-βι;2ng/ml
SIS3; 3μM

Fig.6-A

	Type I procollagen (MEM)				
Type I procollagen (CL)					
α-smooth muscle actin (CL)					
β-actin (CL)					
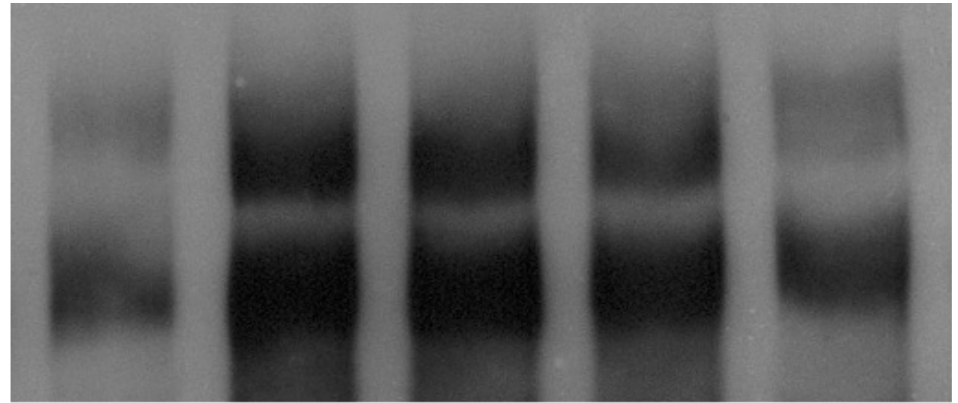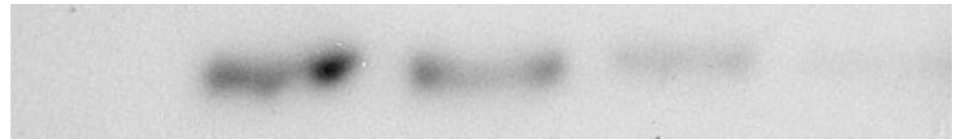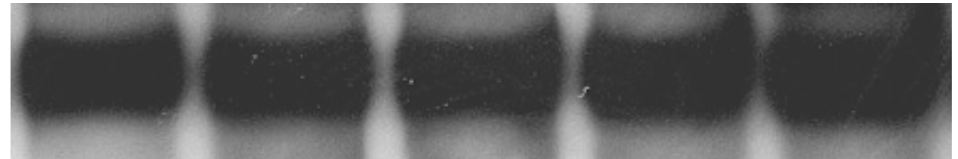
	Normal	Scleroderma			
SIS3(μM)	-	-	0.3	1	3

Fig.6-B
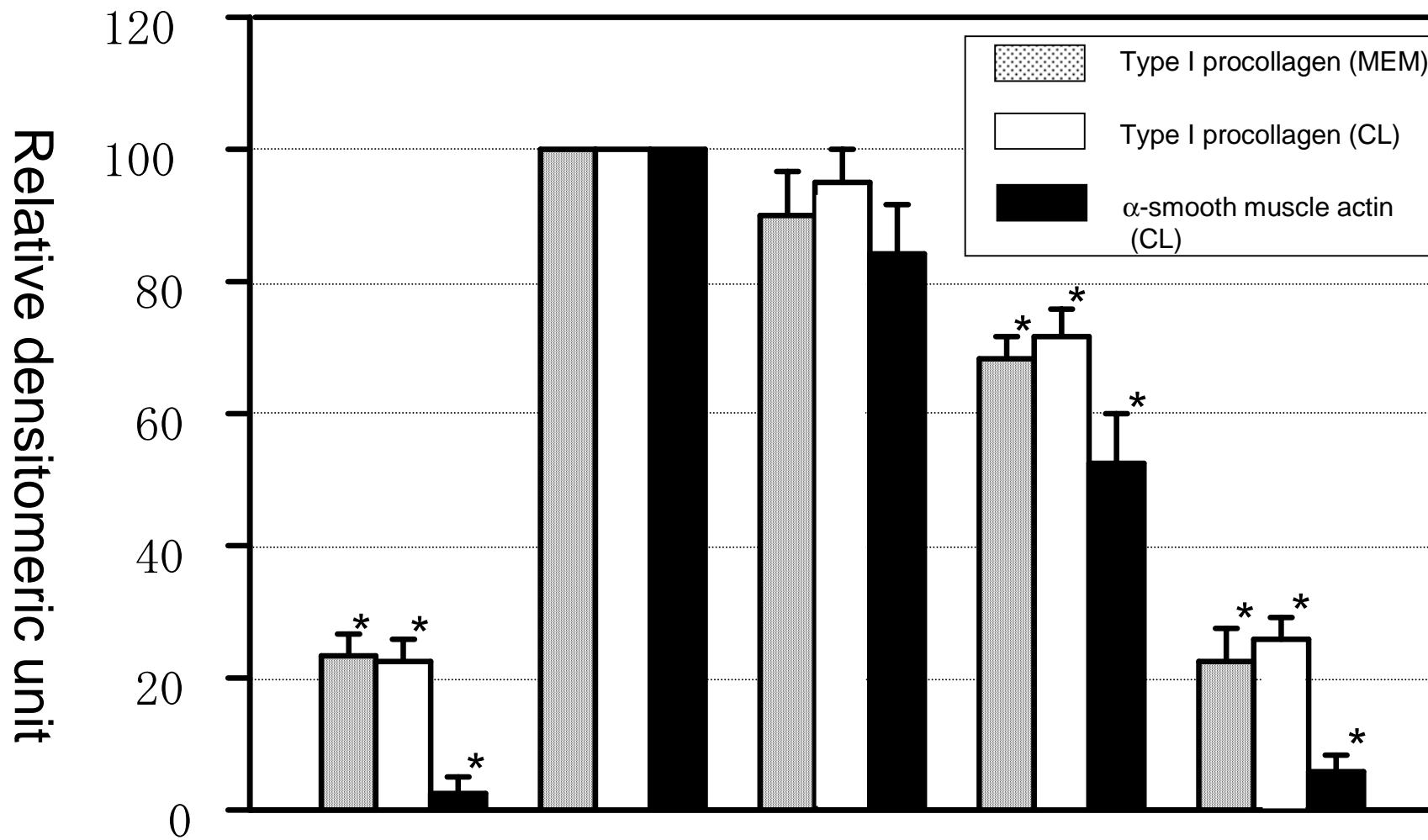
Fig.6-C

Phospho-Smad3

Total Smad3
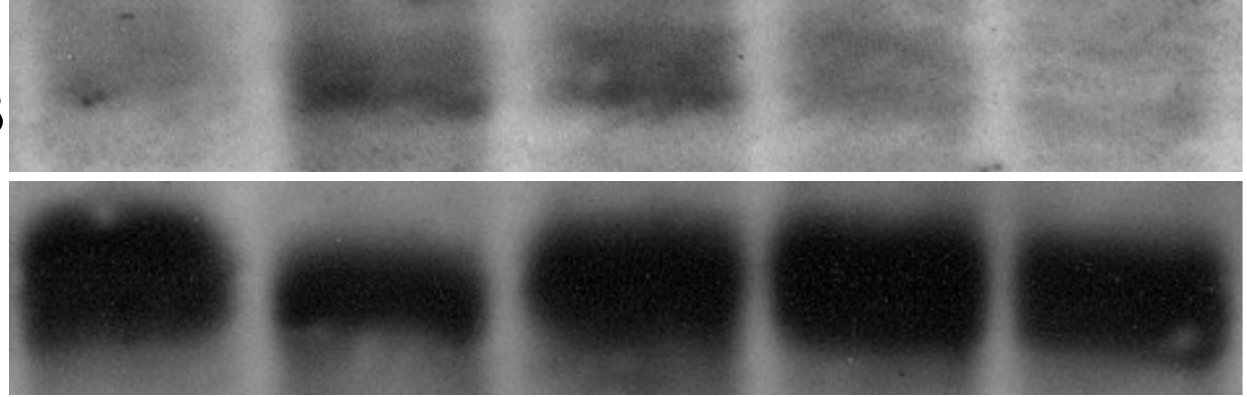
	Normal	Scleroderma		
SIS3(μM)	-	-	0.3	1	3
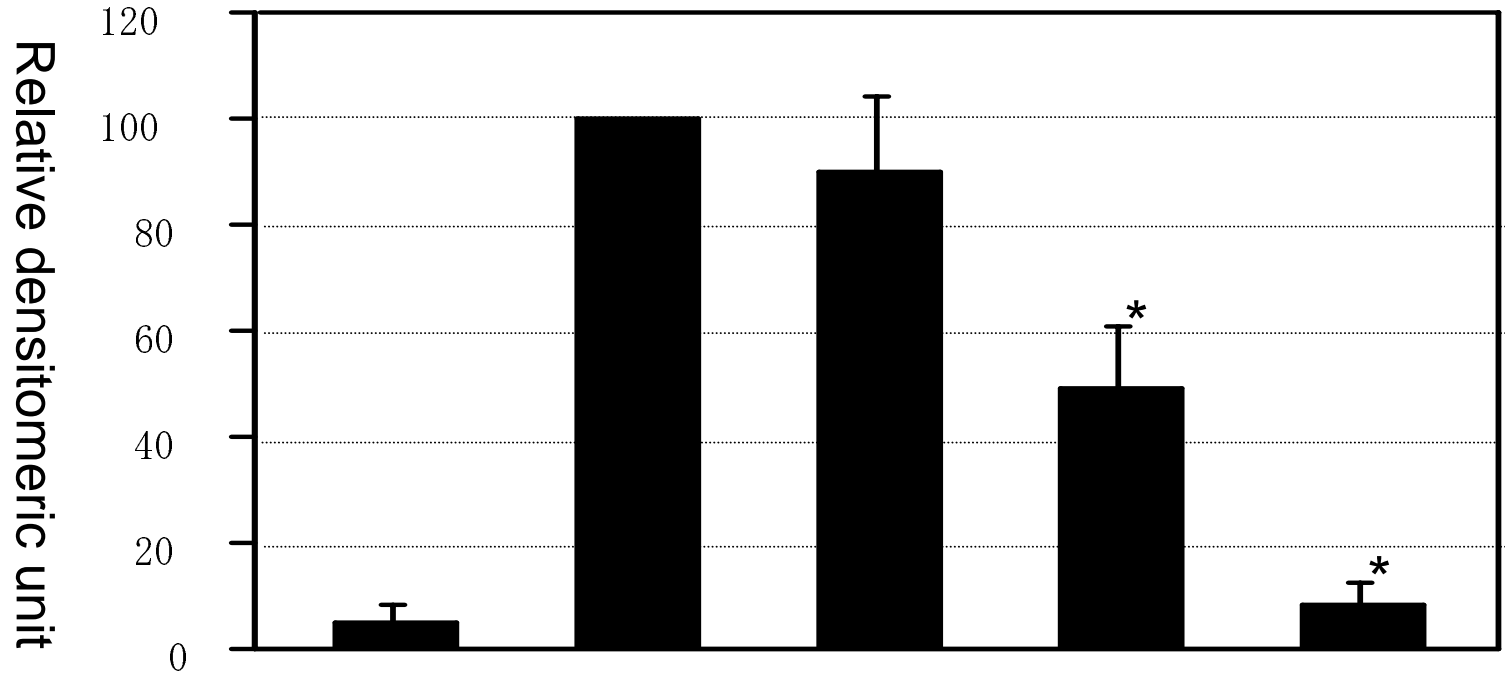
Fig.6-D
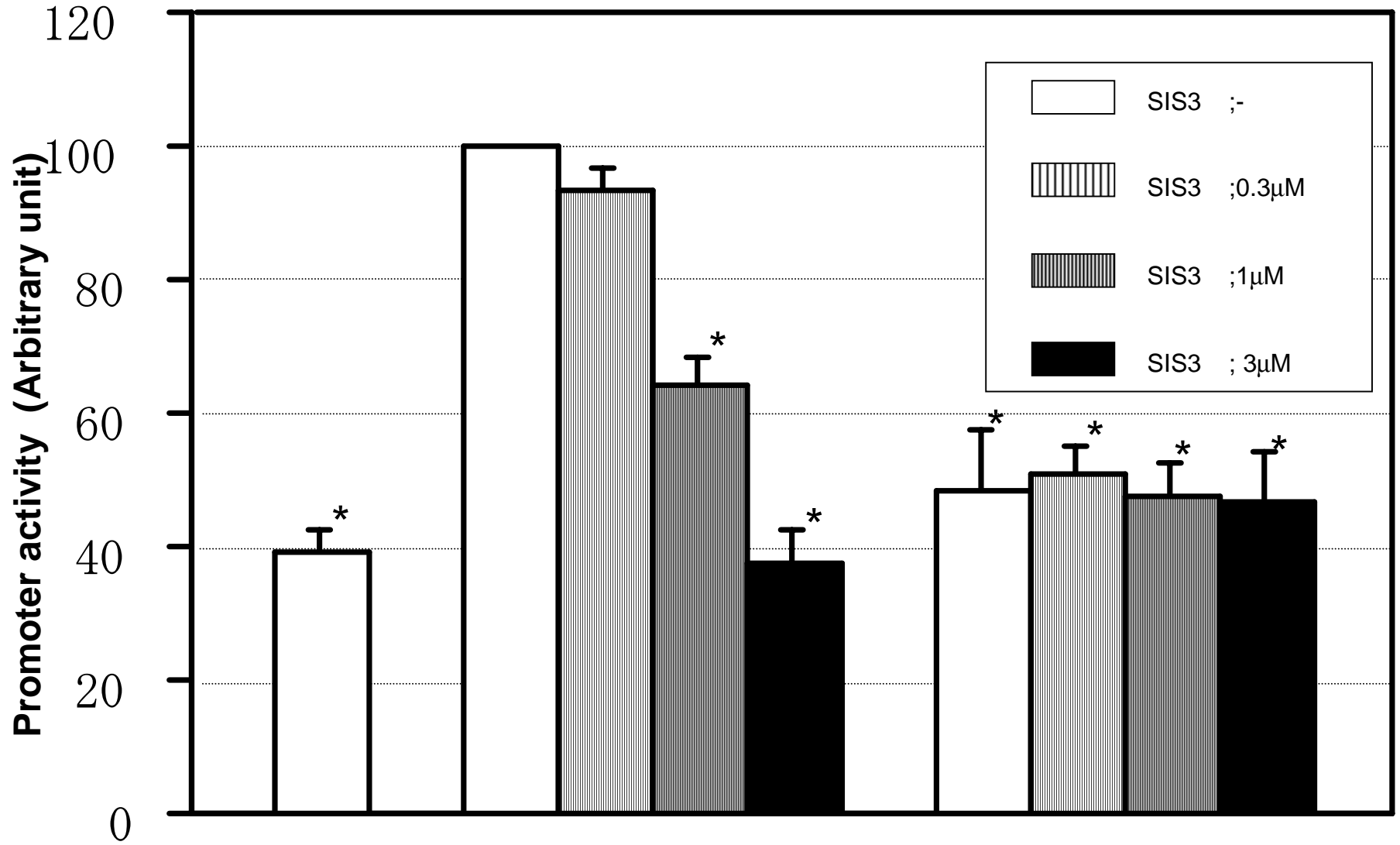
	Normal	Scleroderma						
SIS3(μM)	-	-	0.3	1	3	-	0.3	1	3
Vector	bp -353~+58 α2(I) collagen promoter					CAGA motif mutation construct		

Fig.7

Normal

SIS3(μM)

| 0 | 1 | 3 |

Scleroderma