Targeted Disruption of TGF-β–Smad3 Signaling Leads to Enhanced Neointimal Hyperplasia With Diminished Matrix Deposition in Response to Vascular Injury

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Abstract—The role of transforming growth factor (TGF)-β and its signal in atherogenesis is not fully understood. Here, we examined mice lacking Smad3, a major downstream mediator of TGF-β, to clarify the precise role of Smad3-dependent signaling in vascular response to injury. Femoral arteries were injured in wild-type and Smad3-null (null) male mice on C57Bl/6 background. Histopathological evaluation of the arteries 1 to 3 weeks after the injury revealed significant enhancement of neointimal hyperplasia in null compared with wild-type mice. Transplantation of null bone marrow to wild-type mice did not enhance neointimal thickening, suggesting that vascular cells in situ play a major role in the response. Null intima contained more proliferating smooth muscle cells (SMC) with less amount of collagen compared with wild-type intima. TGF-β caused significant inhibition of cellular proliferation in wild-type aortic SMC, whereas the growth of null SMC was only weakly inhibited by TGF-β in vitro, indicating a crucial role of Smad3 in the growth inhibitory function. On the other hand, Smad3-deficiency did not attenuate chemotaxis of SMC toward TGF-β. TGF-β increased transcript level of α2 type I collagen and tissue inhibitor of metalloproteinases-1, and suppressed expression and activity of matrix metalloproteinases in wild-type SMC. However, these effects of TGF-β were diminished in null SMC. Our findings altogether show that the loss of Smad3 pathway causes enhanced neointimal hyperplasia on injury through modulation of growth and matrix regulation in vascular SMC. These results indicate a vasculoprotective role of endogenous Smad3 in response to injury. (Circ Res. 2005;96:904-912.)

Key Words: transforming growth factor-β ■ Smad3 ■ atherosclerosis ■ neointimal hyperplasia ■ smooth muscle cells

Transforming growth factor (TGF)-β is a prototypic member of the TGF-β superfamily that exerts a wide range of biological effects on various cell types.1 Well described functions of TGF-β including growth inhibition, cell migration, differentiation, extracellular matrix production, and immunomodulation. Abnormality in TGF-β signaling may cause pathological conditions such as tumorigenesis, fibrotic disorders, and vascular diseases.2 At present, however, the role of TGF-β and its signaling molecules in atherosclerosis is not fully understood.

TGF-β is often regarded to have proatherosclerotic effect on arteries. For example, TGF-β expression is increased in human restenotic lesions as well as in neointimal hyperplasia after balloon injury in animals.3 TGF-β facilitates extracellular matrix deposition by stimulating production of procollagen and fibronectin, downregulating the expression of proteases, and upregulating protease inhibitors, such as plasminogen activator inhibitor type 1 (PAI-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1).4–8 TGF-β transgene into vascular wall causes fibroproliferative intimal thickening in animal models in the presence or absence of vascular injury.9,10 Moreover, TGF-β antagonism by antibody, soluble receptor, or ribozyme reduces constrictive remodeling after balloon injury in animals.11–13

On the other hand, considerable evidence implies antithrombolytic effects of TGF-β. TGF-β has been shown to inhibit proliferation and migration of vascular smooth muscle cells (SMCs) in vitro.14,15 Inhibition of TGF-β signal systemically by use of neutralizing antibody and soluble TGF-β receptor type (TβR)-II or in T-cells by expressing a dominant-negative TβR-II results in an unstable plaque phenotype in mouse models of atherosclerosis.16–18 SMCs
obtained from human atherosclerotic plaques were shown to be defective in the TGF-β signal pathway and were resistant to TGF-β-mediated growth suppression and apoptosis. Furthermore, low blood levels of active TGF-β were associated with severity of vascular disease in a manner consistent with an antiatherosclerotic effect of TGF-β.

TGF-β elicits its effects via signaling through tetramerization of two different receptor serine/threonine kinases, TβR-I and TβR-II. Activation of the receptors leads to phosphorylation of cytoplasmic signal transducers Smad2 and Smad3, classified as so-called receptor-activated Smads (R-Smads). The activated R-Smad heterooligomerizes with Smad4, a common mediator Smad, and the complex is transported to the nucleus where it regulates gene expression. In addition, pathways independent of Smads, which involve MAP kinases have also been described. In mice lacking TGF-β signaling molecules, ie, TβR-I and TβR-II, Smad2 and Smad4 turned out to be embryonic lethal. However, it was recently found that the mice null for Smad3 survive into adulthood.

We undertook the present study examining Smad3-null mice in vivo and in vitro to elucidate the precise role of Smad3-dependent TGF-β signaling in the vascular response to injury.

**Materials and Methods**

**Reagents**
Reagents are described in an expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org.

**Mice**
The generation of Smad3−/−null mice by homologous recombination was described previously. See expanded Materials and Methods section for details.

**Femoral Artery Injury**
Mice femoral arteries were injured by use of photochemically-induced thrombosis method. See expanded Materials and Methods section for details.

**Histological Evaluation**
Fixed femoral artery segments were embedded in paraffin and cut into 5-μm-thick serial sections. Six sections per one irradiated segment at 1-mm intervals were stained with hematoxylin and eosin. Neointima was defined as the region between the lumen and the internal elastic lamina. The media was defined as the region between the internal and external elastic lamina. The cross-sectional areas of the internal elastic lamina and media were measured using NIH image version 1.62I (National Institutes of Health, USA). The intima-to-media (I/M) ratio was then calculated, and the mean I/M of all 6 sections per one irradiated segments was determined. The sections with intimal hyperplasia were also subjected to Masson’s trichrome staining and immunohistochemistry. Masson’s trichrome-positive intimal area was analyzed using Photoshop version 7.0 (Adobe). All the measurements were made in blinded manner.

**Immunohistochemistry**
Immunohistochemistry is described in the expanded Materials and Methods section.

**Bone Marrow Transplantation**
Bone marrow transplantation (BMT) was performed principally as described previously. Briefly, bone marrow cell suspensions obtained from either Smad3-null or wild-type mice thigh bone were treated with ACK lysis buffer (0.155 mol/L ammonium chloride, 0.1 mol/L disodium EDTA, and 0.01 mol/L potassium bicarbonate) to lyse erythrocytes. The cells were intravenously injected to recipient Smad3-null or wild-type mice (1×10⁶ per body) between the age of 6 and 9 weeks, 3 hours after lethal irradiation (8.5 Gy). Engraftment of the transferred bone marrow was confirmed by polymerase chain reaction (PCR) on peripheral blood DNA according to the protocol by Yang et al. Femoral artery injury was performed 6 weeks after the bone marrow transfer.

**Cell Culture**
Mouse aortic SMCs were obtained and cultured as described by Ohmi et al (see expanded Materials and Methods section). Experiments were performed on cells after 5 to 10 passages from the primary culture.

**Growth Inhibition Assay**
Growth inhibition assay was performed as described by Datto et al (see expanded Materials and Methods section).

**Real-Time Quantitative PCR**
Real-time quantitative PCR is described in expanded Materials and Methods section.

**Gelatin Zymography**
Gelatin zymography is described in the expanded Materials and Methods section.

**Statistical Analysis**
Results were presented as mean±SEM. Statistical analyses used two-tailed, unpaired student t test.

**Results**

**Mice Lacking Smad3 Show Enhanced Neointimal Hyperplasia in Response to Injury**
To evaluate a role of Smad3 in the pathogenesis of neointimal hyperplasia, femoral arteries of wild-type (n=12) and Smad3-null (n=10) male mice were injured by use of the photochemically-induced thrombosis method. Histopathological examination of the arteries 1 to 3 weeks after the injury revealed markedly enhanced neointimal thickening in Smad3-null mice compared with wild-type mice (Figure 1A and 1B). As shown in Figure 1C, mean I/M ratios evaluated at 1 and 3 weeks after the injury were significantly higher in Smad3-null arteries (0.193±0.034 at 1 week and 0.541±0.093 at 3 weeks) than those of wild-type arteries (0.059±0.018 at 1 week and 0.115±0.060 at 3 weeks, P<0.01 at each time point).

Immunohistochemical examination showed that both neointimal and medial cells were positive for α-SMA (Figure 2A and 2B) but negative for pan-leukocyte marker CD45 (Figure...
2C and 2D), indicating that the intima was exclusively composed of SMCs. The same anti-CD45 antibody recognized leukocytes in vasa vasorum (Figure 2D) as well as lymphocytes in the mouse spleen (Figure 2E).

TGF-β is well known for its antiinflammatory effect.1,2 To determine whether systemic inflammation due to Smad3 deficiency contributes to enhanced neointimal formation, we injured femoral artery of wild-type and Smad3-null mice after bone marrow transplantation (BMT). Lethally irradiated Smad3-null mice received \( 1 \times 10^6 \) bone marrow cells from a wild-type mouse (BMT wild → null mice). At the same time, irradiated wild-type mice were given bone marrow either from Smad3-null or wild-type mice (BMTnull → wild and BMTwild → wild mice). Photochemical injury was performed 6 weeks after the bone marrow transfer, and the arterial cross section was analyzed 3 weeks later. As shown in Figure 1D, mean I/M ratio was significantly higher in BMTwild → null arteries \( (0.353 \pm 0.091) \) than those of BMTnull → wild \( (0.067 \pm 0.031, P=0.011) \) or BMTwild → wild \( (0.073 \pm 0.018, P=0.039) \) arteries. I/M ratios in BMTnull → wild and BMTwild → wild mice tended to be lower than those of Smad3-null and wild-type mice, respectively, presumably due to the effect of vascular irradiation.35,36 Representative cross sections of BMTnull → wild and BMTwild → null femoral arteries are shown in Figure 1E and 1F.

**Smad3-Null Intima Is Rich in Proliferating Cells but Contains Low Amounts of Collagen Fibers**

Intimal cell proliferation was assessed by immunohistochemical detection of proliferating cell nuclear antigen (PCNA) in the femoral artery sections 1 week after the injury (Figure 3A and 3B). The ratio of the PCNA-positive nuclei to total cell nuclei was higher by 1.8-fold in Smad3-null intima compared with wild-type intima (Figure 3C). The result shows an increased proliferative activity of SMCs in Smad3-null artery at the early stage after injury.

We next evaluated intimal cell density in hematoxylin and eosin–stained arterial sections 3 weeks after the injury. As shown in Figure 4A, the ratio of intimal cell number to total intimal area was 1.6-fold higher in Smad3-null artery...
(133±8.6) compared with wild-type artery (85.3±7.7, P<0.01), indicating higher cell density relative to extracellular area in Smad3-null intima. Because TGF-β/Smad3 signal is implicated in extracellular matrix (ECM) deposition, Masson’s trichrome staining was also performed on a 3-week artery specimen to evaluate the amount of extracellular collagen fibers (Figures 4C and 4D). As summarized in Figure 4B, Smad3-null neointima showed 60% reduction in the ratio of Masson’s trichrome-positive area to total intimal area compared with that of wild-type intima. These results suggest that Smad3 deficiency caused increased SMC number with less collagen deposition in neointima.

Growth Inhibition by TGF-β Is Attenuated in SMCs Lacking Smad3

To identify the mechanisms by which Smad3 deficiency caused exaggerated intimal hyperplasia, biological responses of the aortic SMCs obtained from wild-type and Smad3-null mice were examined in vitro. The cells were positive for both α-SMA and SMM (Figure 5A and 5B) as examined by immunocytochemistry. They also exhibited the classic “hills and valley” appearance, a feature characteristic of confluent cultured vascular SMCs. No morphological differences were observed between wild-type and Smad3-null SMCs (data not shown). It was confirmed by immunoblotting that SMCs derived from Smad3-null mice lacked expression of Smad3, whereas Smad2 level was similar in both cells (Figure 5C).

The SMCs were first tested for proliferation. As shown in Figure 6A, TGF-β dose-dependently inhibited FBS-stimulated DNA synthesis in wild-type SMCs with the maximal inhibition of 70% at 1 ng/mL and higher doses. In contrast, growth of Smad3-null SMCs was only weakly (<30%) inhibited by TGF-β. In addition, the basal growth rate of the null cells was ≈1.4-fold higher than that of the wild-type. Similar results were obtained for two additional cell lines of each genotype. The results firmly establish an essential role for Smad3 in TGF-β-mediated inhibition of cellular proliferation in vascular SMCs.

Smad3 Deficiency Does Not Attenuate TGF-β-Mediated Migratory Response in SMCs

The cells were next examined for migration, another function crucial to neointimal formation. Aschcroft et al previously reported that Smad3-null monocytes and neutrophils were unable to migrate toward TGF-β, suggesting Smad3 is required for migration signal downstream of TGF-β. As shown in Figure 6B, Smad3-null SMCs dose-dependently migrated toward TGF-β at least to a similar extent as wild-type SMCs in a modified Boyden chamber assay. Moreover, Smad3-null cells showed a higher migratory capacity (P<0.05) than wild-type cells at 10 ng/mL TGF-β. The result suggests that Smad3-dependent signal is not essential for TGF-β-induced chemotaxis in murine vascular SMCs.

SMCs Require Smad3 for the Regulation of Type I Collagen, Matrix Metalloproteinases, and TIMP-1 by TGF-β

Previous studies suggested that migration of medial SMCs to intima involves extracellular matrix degradation.38,39 Because TGF-β is implicated in extracellular matrix metabolism through transcriptional regulation of collagens, matrix metal-
loproteinases (MMPs), and TIMP-1,7,8 we examined the ability of TGF-β to regulate mRNA expression of these components in wild-type and Smad3-null SMC. Transcript levels of COL1A2, membrane-type matrix metalloproteinase 1 (MT1-MMP), and TIMP-1 were evaluated by real-time quantitative PCR. As shown in Figure 7A, TGF-β time-dependently upregulated mRNA level of COL1A2 in wild-type cells with a maximal increase of 3-fold. Induction of COL1A2 by TGF-β was significantly less in Smad3-null SMCs compared with wild-type cells at all time points. TGF-β suppressed mRNA expression of MT1-MMP, an activator of pro-MMP-2,40 to 64% of the basal level in wild-type SMCs (Figure 7B). However, MT1-MMP level was not affected by TGF-β in Smad3-null SMCs. Moreover, TGF-β increased TIMP-1 expression by 5-fold over the basal level in wild-type SMCs (Figure 7C), whereas no significant induction was observed in Smad3-null SMCs. Finally, the effect of TGF-β on MMP activity in SMC culture media was examined by gelatin zymography (Figure 7D). The basal gelatinolytic activity of MMP-2 in a serum-free conditioned media was similar for wild-type and Smad3-null SMCs. TGF-β time-dependently suppressed MMP-2 activity in wild-type cells with the maximal suppression of 29% at 24 hours, but it did not show significant effect in Smad3-null SMCs. These results suggest that Smad3 plays an essential role in TGF-β-mediated regulation of type I collagen, MMPs, and TIMP-1 in vascular SMCs.

Discussion

We report six novel findings in this article. First, mice lacking Smad3 showed a significant enhancement of neointimal hyperplasia on endothelial injury compared with corresponding wild-type mice. Second, neointima of Smad3-null mouse after injury contained a larger number of PCNA-positive cells compared with wild-type, indicating an increased proliferative activity of Smad3-null SMCs in vivo. Third, Smad3-null neointima showed higher cell density with reduced collagen area. Fourth, TGF-β-induced growth inhibition was diminished in Smad3-null SMCs in vitro. Fifth, Smad3-null SMCs retained migratory activity toward TGF-β. And finally, Smad3-null SMCs were impaired in induction of type I collagen and TIMP-1 as well as in suppression of MMPs by TGF-β. These results confirm a regulatory role of endogenous Smad3 in vascular remodeling in response to injury.

Enhanced neointimal hyperplasia in Smad3-null mice (Figure 1) lend support to previous reports describing the association of low TGF-β activity either at the ligand or receptor levels with intimal lesion formation. Grainger et al41 showed that transgenic expression of apolipoprotein(a) promoted SMC proliferation and subsequent development of early vascular lesions by inhibiting proteolytic activation of TGF-β. Conversely, treatment with the antiestrogen tamoxifen increased serum TGF-β levels and suppressed the formation of aortic lesions in mice42; a similar effect was also observed in human subjects.43 McCaffrey et al19 reported that reduced TβR-II activity due to genomic mutations led to SMC expansion in human atherosclerosis. Moreover, inhibition of TGF-β by use of a soluble type II receptor or a neutralizing antibody accelerated atherosclerosis and induced an unstable plaque phenotype in apoE-deficient mice.17,18 And our present findings, for the first time, demonstrate a direct evidence that attenuation of TGF-β signal at the postreceptor level results in enhanced neointimal formation on injury.
TGF-β. It is to be noted that lack of Smad3 did not eliminate TGF-β-induced growth suppression in SMCs (Figure 6A). The residual growth inhibitory activity is likely to depend on another mediator downstream of TGF-β receptors, possibly Smad2.

Ashcroft et al. reported that Smad3 is required for TGF-β-induced migration of monocytes, leukocytes, and keratinocytes. Unexpectedly, Smad3-null SMCs were able to migrate toward TGF-β (Figure 6B). The finding suggests that, in contrast to the growth inhibitory function, Smad3-dependent signal is not essential for chemotaxis by TGF-β in murine vascular SMCs. It is therefore likely that the ability of medial SMCs to migrate into intima is preserved in Smad3-null arteries. The signaling pathway responsible for TGF-β-induced SMC motility remains to be elucidated.

TGF-β is known as a potent inducer of ECM deposition. It has been demonstrated that overexpression and intravenous administration of TGF-β caused arterial intimal thickening largely consisted of increased ECM. TGF-β exerts fibrogenic activity through enhancement of ECM synthesis as well as inhibition of ECM degradation by downregulating MMP expression and upregulating MMP inhibitors. Previous studies, mainly performed on dermal fibroblasts, showed that TGF-β-mediated regulation of many ECM-related genes, such as type I, III, V, and VI collagens, TIMP-1 and MMP-1 was Smad3-dependent. In this study, we reported that Smad3-null neointima was rich in SMCs with relatively less matrix-deposition compared with wild-type intima, as evaluated by intimal cell density and Masson’s trichrome staining (Figure 4), confirming a crucial role of Smad3-dependent signals in vascular ECM regulation. Moreover, TGF-β was unable to enhance mRNA expression of COL1A2 and TIMP-1 or suppress MT1-MMP expression in Smad3-null SMCs (Figure 7), establishing Smad3-dependency of these genes in vascular SMCs. Regulation of MMP-2 or gelatinase also seems to depend on Smad3-pathway in SMCs, because TGF-β attenuated MMP-2 activity in the culture media of wild-type but not in Smad3-null SMCs. Because degradation of matrix scaffold by MMPs enables cell movement and general tissue reorganization, inability of TGF-β to suppress MMPs in Smad3-null SMCs may facilitate cell migration from media to intima in vivo. Our in vitro finding that Smad3-null SMCs show a higher migration than wild-type at 10 ng/mL TGF-β (Figure 6B) may support this idea. MMP activity uninhibited by TGF-β as well as decreased matrix deposition might also have contributed to enhancement of intimal thickening in Smad3-null mice.

There have been reports on injury models suggesting that TGF-β promotes intimal thickening. The present result that Smad3 deficiency accelerates intimal response to injury appears inconsistent with these results. However, we do not think that our findings contradict other reports on TGF-β transgene or antagonism. Our model differs from any other previous models in the point it specifically lacks Smad3 signal but not other TGF-β signal components, eg, Smad2 and MAP kinases. Smad3 not only transduces signal but not other TGF-β signal components, eg, activin A is expressed in atherosclerotic lesion and promotes the contractile or nonproliferative phenotype of SMCs, playing a role in stabilization of atherosclerotic plaque. Adenovirus-mediated overexpression of activin A suppresses neointimal formation. Although we have not examined the involvement of activin A in the present study, it is assumed that the defect in activin A signal in addition to TGF-β accounts for the drastic neointimal hyperplasia in Smad3-null mice. It is of interest to determine whether specific activation of Smad3 in arterial SMCs in vivo attenuates neointimal hyperplasia. As another possibility, proinflammatory status caused by systemic Smad3 deficiency might have influenced neointimal response. Although our BMT results (Figure 2D through 2F) show that the degree of intimal hyper-
plasia mainly depends on the origin of blood vessels and not of bone marrow cells, further investigation is needed to elucidate the entire role of inflammation in Smad3-null vascular response.

Finally, overactivation of TGF-β–Smad3 pathway is implicated in various fibrotic diseases involving organs such as skin, lung, liver, and kidney. Molecular agents that block Smad3-dependent TGF-β signal are anticipated as an ideal therapeutic option for these disorders. However, our present results lead us to surmise that systemic suppression of Smad3 signaling can cause undesirable effects in the arteries by facilitating proliferative intimal lesions. Therefore, selective drug-delivery to the affected organs as well as careful monitoring of possible vascular lesions should be considered on clinical application of Smad3 inhibitors for fibrotic diseases.

In conclusion, mice lacking Smad3 developed marked neointimal hyperplasia on injury accompanying modulation of growth and matrix regulation in vascular SMCs. This study documents direct evidence and novel information on the functional significance: a vasculoprotective role of Smad3-dependent TGF-β signaling in response to injury.

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degradation of gelatin by MMP was visualized as a translucent band on the dark background. Graph shows the gelatinolytic activity, evaluated by densitometrical scanning of the bands, relative to those of wild-type SMCs at 0 hour. Data were expressed as the means of 4 separate experiments. + P<0.05, compared with the value of 0 hour.

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Materials and Methods

Reagents
Recombinant human TGF-β1 and PDGF (platelet-derived growth factor)-BB was purchased from R&D Systems (USA). [methyl-3H]thymidine was from Amersham Pharmacia Biotech (UK). Mouse monoclonal anti-α-smooth muscle actin (SMA) antibody (clone 1A4) and 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) were from Sigma (USA). Rabbit polyclonal antibody against smooth muscle myosin (SMM) II heavy chain was from Biomedical Technologies Inc. (USA). Mouse monoclonal anti-CD45 antibody (clone 35Z6) and anti-proliferating cell nuclear antigen (PCNA) antibody (clone PC10) were from Santa Cruz Biotechnology (USA). Mouse monoclonal anti-Smad2/3 antibody was from Transduction Laboratories (USA). FITC-conjugated rabbit anti-mouse and TRITC-conjugated swine anti-rabbit immunoglobulins were from DAKO (Denmark). Horseradish peroxidase (HRP)-conjugated sheep polyclonal anti-mouse immunoglobulins were from Amersham Pharmacia Biotech.

Mice
Mice heterozygous for the targeted disruption were mated with C57Bl/6J (The Jackson Laboratory, USA) for at least 6 generations, and maintained as C57Bl/6J Smad3 heterozygous mice. The heterozygous mice were inter-crossed to produce homozygous offsprings. Smad3-null and wild type male mice between the age of 12 and 15 weeks were used for the experiments. All experimental
procedures were in accordance with institutional guidelines for animal research.

**Femoral Artery Injury**

Mice were anesthetized with intraperitoneal administration of pentobarbital (50 µg/g body weight). Rose-Bengal dye [10 mg/mL in phosphate-buffered saline (PBS, Sigma)] was then injected into orbital venous plexus (25 µg/g body weight). Meanwhile, the left femoral artery was carefully exposed and transilluminated with green light (wavelength 540 nm) using a xenon lamp equipped with a heat-absorbing filter (Hamamatsu photonics, Japan) for 10 minutes. The procedure results in local intraluminal thrombus formation accompanied by endothelial injury. Either 7 or 21 days later, the mice were anesthetized and perfusion fixed with 2% formaldehyde in PBS. The irradiated femoral artery segments were removed and fixed further by immersion overnight in 10% formaldehyde in PBS.

**Immunohistochemistry**

Deparaffinized sections were incubated with the endogeneous peroxidase-blocking reagent supplied in ENVISION kit/HRP (DAKO) and a 1:10 dilution of normal goat serum. After incubation with the primary antibody for overnight at 4 °C, the slides were washed in PBS and incubated with polymer reagent containing HRP-conjugated anti-mouse for 30 minutes at room temperature. The slides were then washed in PBS, visualized the antibody binding with diaminobenzidine and counter-stained with hematoxylin. For the primary antibody, anti-α-SMA, CD45 and PCNA antibodies were used at a
dilution of 1:100.

**Cell Culture**

Twelve-week-old mice were anesthetized by intraperitoneal pentobarbital injection (50 µg/g body weight), and the whole aorta was taken out. After removal of adventitial connective tissue and the luminal endothelial cells, the aorta was cut into pieces of approximately 1 mm². The tissue pieces were digested through incubation with 1 mg/mL collagenase (Nitta gelatin Co., Ltd., Japan) and 20 U/mL elastase (ELASTIN PRODUCTS Co., Inc., USA) in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) for 40 minutes at 37 °C. The incubate was then centrifuged at 1500 rpm for 10 minutes at 4 °C, and the precipitate was resuspended in a growth media [DMEM supplemented with 10% fetal bovine serum (FBS, GIBCO™, Invitrogen Corporation, USA) and 40 µg/mL gentamycin (Schering-Plough, USA)] at the concentration of 300 cells/100 µL. A 100 µL of cell suspension was then applied to each well of type I collagen-coated 96-well cell culture dish (IWAKI, Japan). The cells were cultured at 37 °C in a humidified incubator containing 5% CO₂. Cells positive for α-SMA and SMM staining were dispersed upon subconfluency by trypsinization, and subjected to further experiments.

**Immunocytochemistry**

Cells cultured on Lab-Tek II chamber slides (Nalge Nunc International, USA) were fixed in 2% paraformaldehyde for 2 minutes, washed in phosphate-buffered saline (PBS), permeabilized in 0.1% Triton X-100 for 5 minutes, washed once in PBS, and incubated with 10% bovine serum albumin
(BSA, Sigma) in PBS for 30 minutes at room temperature. The slides were then incubated with a the primary antibody (a 1:200 dilution of anti-α-SMA or anti-SMM antibodies) for 1 hour at 37 °C, washed in PBS, followed by incubation with a 1:40 dilution of FITC-conjugated (for α-SMA) or TRITC-conjugated (for SMM) immunoglobulins for 45 minutes at 37 °C. The slides were then washed in PBS, counter-stained with 1 µg/mL DAPI in PBS, mounted and examined under a fluorescent microscope.

**Immunoblotting**

Total cell lysates were analyzed by 7.5 % SDS-polyacrylamide gel electrophoresis (PAGE) and electrically transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). The membrane was blocked in PBS containing 3% bovine serum albumin (BSA) and 0.1% Tween 20, incubated with anti-Smad2/3 antibody (1:500 dilution) overnight at 4 °C, incubated with a 1:2500 dilution of HRP-conjugated anti-mouse immunoglobulins for 1 hour at room temperature, washed in a buffer (0.1% Tween-20 in PBS), and the sites of antibody binding were detected with ECL western blotting detection reagents (Amersham Pharmacia Biotech).

**Growth Inhibition Assay**

SMC were platted at a density of 20,000 cells/well in six-well tissue culture plates in growth media containing 10% FBS, incubated with various concentrations of TGF-β at 37 °C in a humidified incubator containing 5% CO₂ for 24 hours. Five µCi of ³H-thymidine was added for the last 4 hours of incubation. The cells were fixed in 10% trichloroacetic acid, lysed in 1 N NaOH,
and ^3^H-thymidine incorporation into DNA was measured with a scintillation counter. Experiments were performed in quadruplicate for every concentration of TGF-β and were repeated at least 3 times.

**Cell Migration Assay**

Cell migration ability of SMC toward TGF-β was examined by use of 96-well chemotaxis chamber (AB96, Neuro Probe, USA). A polyvinylpyrrolidone-free polycarbonate membrane of 8.0 µm pore size (PFD8, Neuro Probe) was coated with 100 mg/mL type I collagen (Vitrogen 100, Collagen Corp, USA) before use. For each set of experiments, migration in the absence of TGF-β (considered as random migration) served as control and is referred to as 100% migration. Cell counts were made in blinded manner. Experiments were performed in triplicate for every concentration of TGF-β and were repeated five times.

**Real-time quantitative PCR**

The mRNA expression of α2 type I collagen (COL1A2), membrane-type matrix metalloproteinases 1 (MT1-MMP) and TIMP-1 in cultured SMC was quantified by real-time quantitative PCR using the specific primers. Subconfluent SMC on 100 mm tissue culture dishes (IWAKI) were serum-starved for 24 hours, incubated with 10 ng/mL TGF-β for various periods, and total RNA was extracted from the SMC using RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer’s instruction. The samples were then treated with RNase-free Dnase I (Roche Diagnostics, Germany), to avoid contamination of genomic DNA. Approximately 100 µg of RNA was obtained from a 100 mm dish of the SMC. The extracted RNA was reverse transcribed using
Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, UK) including buffer, dNTP and murine reverse transcriptase. Real-time quantitative PCR was performed using the SYBR Green Master Mix (Perkin-Elmer Applied Biosystems, Foster City, USA) and analyzed on an ABI PRISM 7000 Sequence Detector System (Perkin-Elmer Applied Biosystems). Primers were designed with the assistance of the computer program Primer Express version 1.5 (Applied Biosystems, USA). The forward and reverse primers for each molecule were 5’-CTACTGGTGAAACCTGCATCCA-3’ and 5’-GGGCCGCGCTGTATGAG-3’ for COL1A2 (Gene Bank accession No. K01832), 5’-CGGCCCTCTGTCCCAGATA-3’ and 5’-GTGTCAAAGTTCCCGTCACAGAT-3’ for MT1-MMP (Gene Bank accession No. U54984), 5’-GTGGGAAATGCCGCAGAT-3’ and 5’-GGGCATATCCACAGAGGCTTT-3’ for TIMP-1 (Gene Bank accession No. NM011593), 5’-TGTGTCCGTCGTGGATCTGA-3’ and 5’-CCTGCTTCACCACCTTCTTGA-3’ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Gene Bank accession No. M32599), respectively. The thermal cycling comprised an initial step at 50 °C for 2 minutes followed by denaturation step at 95 °C for 10 minutes, 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minutes. Varying lengths of oligonucleonucleotides produce dissociation peaks at different melting temperatures. Consequently, at the end of the PCR cycles, the PCR products were analyzed using the heat dissociation protocol to confirm that one single PCR product was detected by SYBR dye. Quantitative values were obtained from the threshold PCR cycle number at which the increase in signal associated with an exponential growth of PCR product starts to be detected. The relative
mRNA levels in each sample were normalized to its GAPDH content. All PCR reactions were run in triplicate and were repeated at least 3 times.

**Gelatin zymography**

SMC grown to about 70 % confluence in 60 mm tissue culture dishes (IWAKI) were serum-starved for 3 hours and incubated with DMEM containing 10 ng/ml TGF-β and 0.1 % BSA for 0, 12 and 24 hours. Culture media were then collected, centrifuged to pellet detached cells, and the supernatant was concentrated using the Centricon Y-10 system (Amicon-Millipore, USA). MMP activity in the concentrated media normalized to its cell count was measured by gelatin zymography using Gelatin Zymo Electrophoresis kit (Yagai Research Center, Japan) according to manufacturer’s instruction. Briefly, Twenty microliters of concentrated culture media were electrophoresed on a polyacrylamid gel containing gelatin. The gel was then incubated in MMP-activation buffer provided in the kit for 30 hours at 37 °C, stained in protein-staining solution for 30 minutes, followed by destaining for 3 hours in 65 % methanol: 30 % acetic acid at room temperature. Signal intensity was measured by densitometric scanning and analyzed using NIH image version 1.62f. Experiments were repeated 4 times.
Supplementary figure

PCR results of tail vein genomic DNA using the primer pairs described in the on-line data supplement.
“Online Data Supplements”

Materials and Methods

Reagents

Recombinant human TGF-β1 and PDGF (platelet-derived growth factor)-BB was purchased from R&D Systems (USA). \([\text{methyl}^3\text{H}]\)thymidine was from Amersham Pharmacia Biotech (UK). Mouse monoclonal anti-α-smooth muscle actin (SMA) antibody (clone 1A4) and 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) were from Sigma (USA). Rabbit polyclonal antibody against smooth muscle myosin (SMM) II heavy chain was from Biomedical Technologies Inc. (USA). Mouse monoclonal anti-CD45 antibody (clone 35Z6) and anti-proliferating cell nuclear antigen (PCNA) antibody (clone PC10) were from Santa Cruz Biotechnology (USA). Mouse monoclonal anti-Smad2/3 antibody was from Transduction Laboratories (USA). FITC-conjugated rabbit anti-mouse and TRITC-conjugated swine anti-rabbit immunoglobulins were from DAKO (Denmark). Horseradish peroxidase (HRP)-conjugated sheep polyclonal anti-mouse immunoglobulins were from Amersham Pharmacia Biotech.

Mice

Mice heterozygous for the targeted disruption were mated with C57Bl/6J (The Jackson Laboratory, USA) for at least 6 generations, and maintained as C57Bl/6J Smad3 heterozygous mice. The heterozygous mice were inter-crossed to produce homozygous offsprings. Smad3-null and wild type male mice between the age of 12 and 15 weeks were used for the experiments. All experimental
procedures were in accordance with institutional guidelines for animal research.

**Femoral Artery Injury**

Mice were anesthetized with intraperitoneal administration of pentobarbital (50 µg/g body weight). Rose-Bengal dye [10 mg/mL in phosphate-buffered saline (PBS, Sigma)] was then injected into orbital venous plexus (25 µg/g body weight). Meanwhile, the left femoral artery was carefully exposed and transilluminated with green light (wavelength 540 nm) using a xenon lamp equipped with a heat-absorbing filter (Hamamatsu photonics, Japan) for 10 minutes. The procedure results in local intraluminal thrombus formation accompanied by endothelial injury. Either 7 or 21 days later, the mice were anesthetized and perfusion fixed with 2% formaldehyde in PBS. The irradiated femoral artery segments were removed and fixed further by immersion overnight in 10% formaldehyde in PBS.

**Immunohistochemistry**

Deparaffinized sections were incubated with the endogeneous peroxidase-blocking reagent supplied in ENVISION kit/HRP (DAKO) and a 1:10 dilution of normal goat serum. After incubation with the primary antibody for overnight at 4 °C, the slides were washed in PBS and incubated with polymer reagent containing HRP-conjugated anti-mouse for 30 minutes at room temperature. The slides were then washed in PBS, visualized the antibody binding with diaminobenzidine and counter-stained with hematoxylin. For the primary antibody, anti-α-SMA, CD45 and PCNA antibodies were used at a
dilution of 1:100.

Cell Culture
Twelve-week-old mice were anesthetized by intraperitoneal pentobarbital injection (50 µg/g body weight), and the whole aorta was taken out. After removal of adventitial connective tissue and the luminal endothelial cells, the aorta was cut into pieces of approximately 1 mm². The tissue pieces were digested through incubation with 1 mg/mL collagenase (Nitta gelatin Co., Ltd., Japan) and 20 U/mL elastase (ELASTIN PRODUCTS Co., Inc., USA) in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) for 40 minutes at 37 °C. The incubate was then centrifuged at 1500 rpm for 10 minutes at 4 °C, and the precipitate was resuspended in a growth media [DMEM supplemented with 10% fetal bovine serum (FBS, GIBCO™, Invitrogen Corporation, USA) and 40 µg/mL gentamycin (Schering-Plough, USA)] at the concentration of 300 cells/100 µL. A 100 µL of cell suspension was then applied to each well of type I collagen-coated 96-well cell culture dish (IWAKI, Japan). The cells were cultured at 37 °C in a humidified incubator containing 5% CO₂. Cells positive for α-SMA and SMM staining were dispersed upon subconfluency by trypsinization, and subjected to further experiments.

Immunocytochemistry
Cells cultured on Lab-Tek II chamber slides (Nalge Nunc International, USA) were fixed in 2% paraformaldehyde for 2 minutes, washed in phosphate-buffered saline (PBS), permeabilized in 0.1% Triton X-100 for 5 minutes, washed once in PBS, and incubated with 10% bovine serum albumin
(BSA, Sigma) in PBS for 30 minutes at room temperature. The slides were then incubated with a the primary antibody (a 1:200 dilution of anti-α-SMA or anti-SMM antibodies) for 1 hour at 37 °C, washed in PBS, followed by incubation with a 1:40 dilution of FITC-conjugated (for α-SMA) or TRITC-conjugated (for SMM) immunoglobulins for 45 minutes at 37 °C. The slides were then washed in PBS, counter-stained with 1 µg/mL DAPI in PBS, mounted and examined under a fluorescent microscope.

**Immunoblotting**

Total cell lysates were analyzed by 7.5 % SDS-polyacrylamide gel electrophoresis (PAGE) and electrically transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). The membrane was blocked in PBS containing 3% bovine serum albumin (BSA) and 0.1% Tween 20, incubated with anti-Smad2/3 antibody (1:500 dilution) overnight at 4 °C, incubated with a 1:2500 dilution of HRP-conjugated anti-mouse immunoglobulins for 1 hour at room temperature, washed in a buffer (0.1% Tween-20 in PBS), and the sites of antibody binding were detected with ECL western blotting detection reagents (Amersham Pharmacia Biotech).

**Growth Inhibition Assay**

SMC were plated at a density of 20,000 cells/well in six-well tissue culture plates in growth media containing 10% FBS, incubated with various concentrations of TGF-β at 37 °C in a humidified incubator containing 5% CO₂ for 24 hours. Five µCi of ³H-thymidine was added for the last 4 hours of incubation. The cells were fixed in 10% trichloroacetic acid, lysed in 1 N NaOH,
and 3H-thymidine incorporation into DNA was measured with a scintillation counter. Experiments were performed in quadruplicate for every concentration of TGF-β and were repeated at least 3 times.

**Cell Migration Assay**

Cell migration ability of SMC toward TGF-β was examined by use of 96-well chemotaxis chamber (AB96, Neuro Probe, USA). A polyvinylpyrrolidone-free polycarbonate membrane of 8.0 µm pore size (PFD8, Neuro Probe) was coated with 100 mg/mL type I collagen (Vitrogen 100, Collagen Corp, USA) before use. For each set of experiments, migration in the absence of TGF-β (considered as random migration) served as control and is referred to as 100% migration. Cell counts were made in blinded manner. Experiments were performed in triplicate for every concentration of TGF-β and were repeated five times.

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