Basic Fibroblast Growth Factor Antagonizes Transforming Growth Factor-β1–Induced Smooth Muscle Gene Expression Through Extracellular Signal–Regulated Kinase 1/2 Signaling Pathway Activation

Keiko Kawai-Kowase, Hiroko Sato, Yuko Oyama, Hiroyoshi Kanai, Mahito Sato, Hiroshi Doi, Masahiko Kurabayashi

Objective—Transforming growth factor-β1 (TGFβ1) and fibroblast growth factor (FGF) families play a pivotal role during vascular development and in the pathogenesis of vascular disease. However, the interaction of intracellular signaling evoked by each of these growth factors is not well understood. The present study was undertaken to examine the molecular mechanisms that mediate the effects of TGFβ1 and basic FGF (bFGF) on smooth muscle cell (SMC) gene expression.

Methods and Results—TGFβ1 induction of SMC gene expression, including smooth muscle protein 22-α (SM22α) and smooth muscle α-actin, was examined in the pluripotent 10T1/2 cells. Marked increase in these mRNA levels by TGFβ1 was inhibited by c-Src-tyrosine kinase inhibitors and protein synthesis inhibitor cycloheximide. Functional studies with deletion and site-directed mutation analysis of the SM22α promoter demonstrated that TGFβ1 activated the SM22α promoter through a CArG box, which serves as a serum response factor (SRF)–binding site. TGFβ1 increased SRF expression through an increase in transcription of the SRF gene. In the presence of bFGF, TGFβ1 induction of SMC marker gene expression was significantly attenuated. Transient transfection assays showed that bFGF significantly suppressed induction of the SM22α promoter–derived luciferase activity by TGFβ1, whereas bFGF had no effects on the TGFβ1-mediated increase in SRF expression and SRF:DNA binding activity. Mitogen-activated protein kinase kinase-1 (MEK1) inhibitor PD98059 abrogated the bFGF-mediated suppression of TGFβ1-induced SMC gene expression.

Conclusion—Our data suggest that bFGF-induced MEK/extracellular signal–regulated kinase signaling plays an antagonistic role in TGFβ1-induced SMC gene expression through suppression of the SRF function. These data indicate that opposing effects of bFGF and TGFβ1 on SMC gene expression control the phenotypic plasticity of SMCs. (Arterioscler Thromb Vasc Biol. 2004;24:1-7.)

Key Words: basic fibroblast growth factor ■ transforming growth factor-β1 ■ serum response factor ■ SM22α ■ smooth muscle cells

Phenotypic modulation of smooth muscle cells (SMCs) contributes to development of atherosclerotic and restenotic lesions. There is considerable interest in identifying the various extracellular signals that regulate SMC phenotype and the molecular mechanisms underlying such SMC plasticity. Transforming growth factor-β1 (TGFβ1) is one of the primary differentiation factors for SMCs. TGFβ1 upregulates several SMC differentiation markers, such as smooth muscle α-actin (SMα-act), smooth muscle myosin heavy chain, smooth muscle protein 22-α (SM22α), and h1 calponin in vitro. Moreover, TGFβ1 induces expression of these SMC differentiation maker genes in a variety of nonsmooth muscle precursor cell types in culture, including multipotent embryonic fibroblast (10T1/2 cells) and neural crest cells. These results suggest that TGFβ1 evokes an important signal that induces SMC differentiation.

In contrast, basic fibroblast growth factor (bFGF) is one of the most important mitogenic growth factors for SMCs and plays an important role in the onset and development of vascular disease. Several studies indicated that experimental reduction in bFGF expression inhibits SMC proliferation after intimal injury. Lindner et al suggested that in injured arteries, bFGF and FGF receptor-type 1 may be involved in the continued proliferative response of SMCs leading to neointima formation. SMCs respond to bFGF stimulation with proliferation, migration, and cytokine secretion occurring after vascular lesion. The promoter of almost all examined SMC-specific genes contain CArG (CC[A/T]6GG) box, which serves as a binding site for serum response factor (SRF). An increasing number of studies provide direct evidence that the CArG
boxes are required for promoter activation of SMC genes in vitro and in vivo.9-11 The smooth muscle calponin gene contains CArG box within the first intron, which mediates SMC-specific enhancer activity.12 However, the signaling pathways that regulate SRF expression and function have been poorly understood.

In the present study, we investigated the effects of TGFβ1 and bFGF on SMC gene expression in the pluripotent 10T1/2 cells and in vascular SMCs. The results demonstrated that TGFβ1 induces CArG-dependent SM22α gene expression via an increase in SRF expression, and Src family of tyrosine kinase is important for this effect. Furthermore, bFGF inhibits TGFβ1-induced SM22α gene expression via an increase in SRF expression, and Src family of tyrosine kinase pathways act as antagonistic growth factors that regulate the CArG-dependent SMC marker gene expression by modulating SRF expression and function, respectively. These findings shed light on the role of TGFβ1 and bFGF for modulating SMC gene expression during development and in vascular disease, in which SMC phenotypic change plays a crucial role.

Materials and Methods

The Materials and Methods section can be found in an online supplement available at http://atvb.ahajournals.org.

Results

TGFβ1 Induces Expression of SMC Marker Genes in 10T1/2 Cells

Northern blot analyses revealed that the levels of mRNAs for SM22α and SMα-actin were significantly induced by TGFβ1 in 10T1/2 cells. SMemb and KLF5/BTEB2, a transcription factor implicated in the regulation of SMemb and others, were also induced. Calponin mRNA was scarcely detected by Northern blot, but RT-PCR analysis revealed its significant induction by TGFβ1 in 10T1/2 cells. Analysis of temporal expression showed that SMα-actin and SM22α mRNA levels were induced by TGFβ1×2 hours and remained elevated for 12 hours after TGFβ1 stimulation. SRF mRNA levels were peaked at 6 hours after TGFβ1 stimulation (Figure 1A through IC, available online at http://atvb.ahajournals.org).

As shown in Figure ID, cycloheximide (4 μg/mL, a nonspecific inhibitor for protein synthesis) alone modestly increased the expression of SM22α and SMα-actin mRNAs. TGFβ1-induced expression of SM22α and SMα-actin mRNAs was rather attenuated by cycloheximide. These results suggest that de novo protein synthesis is partly required for the TGFβ1-induced expression of SM22α and SMα-actin genes.

Src Family of Tyrosine Kinase Mediates TGFβ1-Induced Expression of SM22α and SMα-Actin Genes

As shown in Figure 1A, TGFβ1-induced SM22α mRNA expression in 10T1/2 cells was blocked by genistein (10 μmol/L; a tyrosine kinase inhibitor) but not by other protein kinase inhibitors, such as PD98059 (50 μmol/L; a specific inhibitor for MEK1, a mitogen-activated protein [MAP] kinase kinase for extracellular signal-regulated kinase [ERK]), SB203580 (10 μmol/L; a specific inhibitor for p38MAP kinase), calphostin C (1 μmol/L; protein kinase C inhibitor), and wortmannin (1 μmol/L; a PI3 kinase inhibitor). We then examined the effects of daidzein (10 μmol/L; an inactive analog of genistein) and other tyrosine kinase inhibitors, such as herbimycin A (1 μmol/L; and other tyrosine kinase inhibitors, such as herbimycin A (1 μmol/L; an inactive analog of genistein) and other tyrosine kinase inhibitors, such as herbimycin A (1 μmol/L; an inactive analog of genistein) and other tyrosine kinase inhibitors, such as herbimycin A (1 μmol/L; an inactive analog of genistein) and other tyrosine kinase inhibitors, such as herbimycin A (1 μmol/L; an inactive analog of genistein) and other tyrosine kinase inhibitors, such as herbimycin A (1 μmol/L; an inactive analog of genistein) and other tyrosine kinase inhibitors, such as herbimycin A (1 μmol/L; an inactive analog of genistein) and other tyrosine kinase inhibitors, such as herbimycin A (1 μmol/L; an inactive analog of genistein) and other tyrosine kinase inhibitors, such as herbimycin A (1 μmol/L; an inactive analog of genistein) and other tyrosine kinase inhibitors, such as herbimycin A (1 μmol/L; 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CARG Box Is Required for TGFβ1-Mediated Increase in SM22α Promoter Activity

To determine the effects of TGFβ1 on SM22α promoter activity, a series of 5′-deletion constructs of SM22α promoter was transfected into 10T1/2 cells and cultured RASMCs...
Luciferase activity in unstimulated cells transfected with indicated reporter genes was evaluated by transfecting mouse arterial smooth muscle cells and RASMCs. As shown in Figure IVC, TGFβ1 stimulated SRF gene expression at least partly at the transcriptional level through Src family of tyrosine kinase pathway.

bFGF Inhibits TGFβ1-Induced SM22α and Smα-Actin Gene Via Activation of MEK1

To investigate the effects of mitogenic stimulation on TGFβ1-induced SM22α and Smα-Actin gene expression, we used various growth factors, including platelet-derived growth factor (PDGF)-BB, vascular endothelial growth factor (VEGF), interleukin (IL)-6, epidermal growth factor (EGF), transforming growth factor (TGF)-β1 (1 ng/mL) for 24 hours as described in Materials and Methods. *P<0.05 vs control.

Figure 3. Effect of TGFβ1 on SRF gene expression and binding to CArG box. A, EMSA. Nuclear extracts from 10T1/2 cells grown in absence or presence of TGFβ1 were incubated with wild-type probe, which corresponds to SM22α (from –158 bp to –133 bp sequence), and were subjected to electrophoresis. Nuclear extract from 10T1/2 cells in absence or presence of TGFβ1 or PP1 were incubated with the probe using the antibody against SRF. Positions of the sequence-specific DNA protein complexes (C) and supershifted complexes (S) are indicated. B, Northern blot analysis. 10T1/2 cells were pretreated with either PP1 or herbimycin A for 1 hour, exposed to TGFβ1 for 12 hours, and analyzed by Northern blotting for SRF.
and bFGF in 10T1/2 cells (Figure VA, available online at http://atvb.ahajournals.org). Among these, bFGF markedly inhibited TGFβ1 induction of mRNA levels for SM22α, SMα-actin, SMemb, and KLF5/BTEB2 mRNAs. Methylene blue–stained 28S ribosomal RNA indicates that comparable amounts of total RNA actually blotted onto a membrane. Bottom, Southern blot analysis of RT-PCR products for calponin and GAPDH. B, Top, 10T1/2 cells were pretreated with PD98059, SB203580, genistein, and wortmannin for 1 hour and were exposed to TGFβ1 or bFGF for 12 hours. Total cellular RNA was analyzed by Northern blot for SM22α. PD indicates PD98059; SB, SB203580; Genist, genistein; Wort, wortmannin. Bottom, Statistical analysis of the effect of protein kinase inhibitors on reduction of TGFβ1-induced SM22α expression by bFGF. *P<0.05 vs bFGF(−). C, Phosphorylation of ERK1/2 by TGFβ1 or bFGF for 5 minutes in 10T1/2 cells. 10T1/2 cells were treated with or without TGFβ1 or bFGF, and total cellular lysates were prepared for Western blotting using either anti-phospho-ERK1/2 or anti-ERK1/2.

To examine whether bFGF affects TGFβ1-induced SM22α promoter activity, we performed luciferase assays. bFGF significantly attenuated TGFβ1 effects on the SM22α promoter, and PD98059 inhibited such an effect of bFGF (Figure VC).

To evaluate TGFβ1 or bFGF pathway for ERK1/2 activation, 10T1/2 cells were treated with TGFβ1 or bFGF. Figure 4C and Figure VD show ERK phosphorylation was detected at 5 minutes and remained 60 minutes after bFGF stimulation. Although ERK phosphorylation was detected 5 minutes after TGFβ1 stimulation, this phosphorylation significantly attenuated thereafter.

Next, we performed Western blot analyses to test whether activation of MEK1 pathways by bFGF inhibits TGFβ1-induced c-Src activation. As shown in Figure VI (available online at http://atvb.ahajournals.org), bFGF as well as TGFβ1 induced c-Src activation, and simultaneous stimulation with TGFβ1 and bFGF exerts the additive effect on c-Src phosphorylation. PD98059 by itself induced c-Src phosphorylation, and thus an induction of c-Src phosphorylation by bFGF
was not blocked by PD98059. However, these data imply that the inhibitory effects of bFGF on TGFβ1-induced SM22α expression are not mediated through inhibition of c-Src phosphorylation.

**MEK1 Inhibits SRF Function Independent of Expression and DNA Binding Activity of SRF**

To determine whether MEK1 activation inhibits SM22α expression, 10T1/2 cells and cultured RASMCs were transfected with expression vector for MEK1 or empty vector pcDNA3 along with the SM22α-luciferase reporter gene. Overexpression of MEK1 reduced TGFβ1-stimulated luciferase activity of −305Luc (Figure 5 and Figure VIIA, available online at http://atvb.ahajournals.org). Furthermore, induction of the SM22α promoter activity by SRF was prevented by MEK1 overexpression in 10T1/2 cells (Figure 5).

The observation that bFGF inhibited the TGFβ1-induced SM22α promoter activity led us to test whether bFGF inhibits SRF expression. Interestingly, TGFβ1 had no measurable effects on SRF expression as assessed by Northern blot and Western blot analyses. Furthermore, EMSA showed that bFGF did not decrease and rather increased the binding of SRF to the CArG box (Figure VIIIB through VIID). Collectively, these results suggest that bFGF inhibits SM22α expression not through inhibition of the SRF protein synthesis or inhibition of DNA binding but possibly through repression of SRF function (Figure 6).

**Discussion**

The current study shows TGFβ1 actions on SMC gene expression in 10T1/2 cells and RASMCs. TGFβ1-directed SMC gene expression is mediated through Src-tyrosine kinase activation and is associated with the increases in SRF gene expression at the transcriptional level. We also demonstrated that TGFβ1 induction on SMC gene expression is significantly inhibited by bFGF. ERK1/2 activation mediates this effect of bFGF on TGFβ1 induction of SMC gene expression; in fact, inducible expression of SM22α was attenuated in the presence of PD98059. In addition, phosphorylation of ERK1/2 by transfecting MEK1 expression plasmid inhibited TGFβ1 action on SM22α promoter. Furthermore, bFGF did not inhibit TGFβ1-induced SRF expression and DNA binding activity. Finally, when several growth factors were tested for their capacity to inhibit TGFβ1 action on SMC gene expression, we observed that PDGF-BB, VEGF, PGI2, IL-6, and EGF did not possess the potent capacity of inhibiting TGFβ1-driven SM22α expression in 10T1/2 cells. Thus, our data highlight the critical role of bFGF–MEK1 pathway in inhibition of TGFβ1-driven SMC differentiation.

It is worth stressing that TGFβ1 signaling was inhibited by Src family of tyrosine kinase inhibitor. This finding is somewhat surprising because it has been described that TGFβ1 decreased Src kinase activity or induced degradation of activated Src kinase. Very few precedent reports showed the activation of Src tyrosine kinases by TGFβ1. Su et al have shown that in bovine articular chondrocyte, TGFβ1-mediated induction of tissue inhibitor of metalloproteinases-3 expression was inhibited by Src tyrosine kinase inhibitor as well as by serine/threonine protein kinase inhibitor. We confirmed the activation of Src family of tyrosine kinase by Western blot analysis using the anti-c-Src antibody. Although the
precise mechanisms remain to be determined, it is possible that TGFβ1 signaling varies depending on cell types.

Despite the fact that bFGF did not decrease but rather increased SRF mRNA and protein levels, bFGF significantly attenuated induction of TGFβ1-mediated SMC-specific genes. Then how can bFGF inhibit the effect of TGFβ1? We examined whether bFGF inhibits the binding of SRF to CarG box and found that bFGF had essentially no effect on the binding activity of SRF to the CarG box. These data led us to speculate that bFGF represses SRF function through: (1) post-transcriptional SRF modification; (2) an induction of repressors of SRF including SRFδ5,15 Id2,16 and SMART;17 (3) a repression of SRF coactivators such as ternary complex factor, p300/calcium-binding protein, SRC-1, and myocardin;18,19 and (4) an alteration of chromatin structure regulated by histone acetyltransferase or histone deacetylase activity.20,21 Studies to examine these possibilities are currently in progress.

Our finding that MEK1 inhibitor PD98059 attenuated bFGF-mediated repression of TGFβ1-induced SMα-actin and SM22α expression deserves further attention. Previous studies have provided ample evidence indicating that MEK1 activation leads to the increase in SRF-dependent transcription through activation of accessory factors that bind to the SRF–CarG box complex, including members of the ets family of transcription factors, Elk-1, SAP-1a, b, SAP-2, and ERP-1.22 Thus, a critical question is how SRF function could selectively be upregulated or downregulated by MEK1. This issue appears to be analogous to the question regarding the differential effects of mitogenic stimulation on the expression of SMC-specific genes and c-fos gene, both of which contain functional CARG box.23 The most plausible explanation is that modulation of SRF function by MEK1 signaling is dependent on sequence flanking CARG box. However, our data indicate that PDGF and EGF, both of which are known to activate MEK1 signaling, are less potent than bFGF in inhibiting TGFβ1-induced SM22α expression in 10T1/2 cells. Thus, it is tempting to speculate that bFGF-specific events other than MEK1 activation are necessary to inhibit TGFβ1-induced SMC gene expression.

It has been shown that the nuclear factors involved in the TGFβ1 control element–mediated transcription belong to the zinc finger family of KLFs, such as GKLF and KLF5/BTEB2.24 MacLellan et al have described previously that TGFβ1-induced activation of skeletal α-actin promoter required cooperation of SRF, YY1, and transcriptional enhancer factor-1.25 However, our data suggest that GkLF, BTEB2/IKLF, or YY1 do not play a major role in repressing the SM22α promoter by bFGF. The reasons for such an assumption are: (1) mRNA for BTEB2/IKLF, which positively regulates SM22α promoter, was induced by bFGF; (2) mRNA for GkLF, which functions as a negative regulator of SM22α expression, was not changed by bFGF; and (3) anti-YY1 antibody did not affect the DNA:protein complex as assessed by EMSA (data not shown).

What is the in vivo relevance of our observation? It has been shown previously that in early, simple, and advanced atherosclerotic lesions, both TGFβ1 and bFGF were expressed in intimal SMCs. Although TGFβ1 acts as a bifunctional regulator for SMC differentiation depending on growth status and the presence of other growth factors, an increased expression of bFGF was associated consistently with SMC proliferation of the atheromatous lesions.26 However, the role of bFGF in SMC differentiation has been described poorly. Our data indicate that SMC or embryonic fibroblasts exposed simultaneously to TGFβ1 and bFGF express SMC marker genes significantly less than in response to TGFβ1 alone. This suggests that SMCs in atherosclerotic lesions that contain abundant bFGF are less differentiated than those expressing predominant TGFβ1. Given that undifferentiated SMCs that highly express the genes for proteases that degrade matrix proteins and bFGF may potentially be involved in plaque neovascularization, bFGF-driven events may contribute to formation of unstable plaques and to life-threatening complications of atheroma and provide new options for therapeutic intervention.

In summary, we demonstrated that 2 growth factors (TGFβ1 and bFGF), which have been shown independently to play critical roles in regulation of smooth muscle development, antagonistically affect SRF-dependent SMC gene expression.
expression. In addition, we demonstrate that bFGF-induced MEK1 signaling attenuates SRF function but not its DNA binding activity and expression. These findings provide novel insight into SRF function regulation during SMC differentiation, which is influenced profoundly by growth factors.

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Basic FGF antagonizes the TGFβ1-Induced Smooth Muscle Gene Expression through the activation of ERK1/2 Signaling Pathway

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On-line Supplement

Materials and Methods

RNA isolation and Northern blot analysis

Murine embryonic fibroblasts (10T1/2 cells) and cultured rat aortic smooth muscle cells (RASMC) have been previously described\textsuperscript{1,2}. Total RNA was isolated from 10T1/2 cells or cultured rat VSMC using the ISOGEN reagent (Nippon gene) in accordance with the manufacturer's instruction. Total RNA from 10T1/2 cells or cultured RASMC was subjected to Northern blot analysis as described\textsuperscript{3}. Hybridization was carried out with \textsuperscript{32}P-labeled SM22α, SMα-actin, SMemb, KLF5/BTEB2, and SRF cDNA probes.

Reverse transcriptase (RT) -PCR

Total RNA was reverse-transcribed with oligo (dT)-primer with the use of AMV reverse transcriptase and amplified with Taq DNA polymerase (Takara). The PCR products were electrophoresed on 2 % agarose gels, transferred to a nylon membrane (Pall Biodyne), and then hybridized with calponin and GAPDH cDNA. Primer sequences were as follows: GAPDH, 5\textsuperscript{'}-GCCAAAAGGGTCATCATCTC-3\textsuperscript{'} and 5\textsuperscript{'}-GTAGAGGCAGGGATGATG-TTC-3\textsuperscript{'};
calponin, 5'-AAGACAAAAGGAAACAAAGTCAAT-3' and 5'-CATGGCAGAGTTGTAGTAGTTGTG-3'.

**Real-time RT-PCR**

To quantify the expression level of mRNA in cultured RASMC, real-time RT-PCR analysis (iCycler, Bio-Rad Laboratories, Inc. Hercules, CA) was performed using dual fluorescence labeled probe. Primer and probe sequences of SM22\(\alpha\) were as follows: primer, 5'-GCATAAGAGGGAGTTCACAGACA-3' and 5'-GCCTTCCCTTTCTAACTGATGATC-3', probe, 5'-TCGCCCATAGCCTGTCATGCCAGC-3'. Primer and probe sequences of SM\(\alpha\)-actin and 18S rRNA have been previously described\(^4\).

**Western blot analysis**

Western blot analyses were performed essentially as previously described\(^3\). SRF proteins were detected according to the ECL protocol (Amersham corp.) using 1:1000 dilution of rabbit anti-SRF antiserum (Santa Cruz Biotechnology, Inc. 100 \(\mu\)g IgG/mL).

**Analysis of ERK and Src kinase activity in 10T1/2 cells**

The activities of ERK and Src kinase were examined by Western blot analyses of 10T1/2 cell extracts using antibodies against their phosphorylated forms. 10T1/2 cells were pretreated with PD98059 and/or bFGF for 1 hour and were exposed to TGF\(\beta\)1 for 1 minute for the analysis of Src kinase activity. For the analysis of ERK activity, 10T1/2 cells were treated with TGF\(\beta\)1 (1 ng/mL) and/or bFGF (10 ng/mL). And to confirm the expression into 10T1/2 cells, cells were transfected with MEK1/pcDNA for 48 hours. Western blot analyses were performed to detect level of phosphorylated ERK and phosphorylated c-Src using anti-phospho-ERK (Cell Signaling Technology) or anti-phospho-c-Src (PY418, BioSource International Inc) antibodies. The same blots were used to detect total protein using anti-ERK (Cell Signaling Technology) or anti-c-Src antibodies (BioSource International Inc).

**Promoter-Luciferase vector chimeric constructs**

SM22\(\alpha\) promoter-luciferase reporter gene construct was made by inserting a fragment of the mouse SM22\(\alpha\) promoter, corresponding to –305 bp to +41 from the transcriptional start site, into
pGL3BV (Promega). Serial deletion constructs were prepared as follows; The forward and reverse primers used for generation of –215Luc were

5’-CCCAGGCTCTCTCAGGAAGCATGCAGAAGAATG-3’ and 5’-GGGAAGCTTGAAGGAGAGTAGCTTCGGTGT-3’, with SacI and HindIII site (underlined), respectively. The forward and reverse primers used for generation of –158Luc were

5’-CCCAGGCTCTTTCCCCAAATATGGAGCCTG-3’ and 5’-GGGAAGCTTGAAGGAGAGTAGCTTCGGTGT-3’, with SacI and HindIII site (underlined), respectively.

For generation of mutants in the SM22α promoter, recombinant PCR was performed. The PCR primers (mutations of wild-type sequence appear in boldface) for -158mut1Luc, -158m2Luc, and -158m3Luc, were as follows; -158mut1Luc were 5’-CCCAGGCTCTGGAACCCAAATATGGAGCCTG-3’, -158m2Luc were 5’-CCCAGGCTCTTCCCAGCGTATGGAGCCTG-3’, -158m3Luc were 5’-CCCAGGCTCTTTCCCAAGCGTAGCCTG-3’, with SacI site (underlined).

SRF promoter-luciferase reporter gene construct, designated as -2052SRF/Luc, was made by inserting a –2052 to +114 fragment of the mouse SRF promoter into pGL3BV (kindly provided by Dr. Takeshi Miwa, Osaka University, Osaka, Japan).

Expression plasmids

The expression plasmid SRF/pME18S, the coding region of mouse SRF, (kindly provided by Dr. Takeshi Miwa, Osaka University, Japan) has been described5. The expression plasmid MAP/ERK kinase 1 (MEK1)/CMV (kindly provided by Dr. Roger J. Davis, Howard Hughes Medical Institute, Worcester, MA) has been described6.

Transfection and luciferase assay

10T1/2 cells were transfected with plasmid according to a modified calcium phosphate precipitation method as previously described3. Cultured rat VSMC were transfected with plasmid using Fugene (Roche) in accordance with the manufacturer's instruction. The total amount of DNA per well was kept constant by adding the corresponding amount of expression vector without a
cDNA insert. All luciferase activities are expressed relative to that of promoterless plasmid pGL3BV. The data reflect means ±SEM of at least three independent experiments in duplicate.

**Preparation of Nuclear Extracts and Electrophoretic gel mobility shift assay (EMSA)**

Preparation of nuclear extracts and EMSA were performed as previously described\(^3\). For experiments using antibodies, anti-SRF, anti-STAT3, and anti-Smad2/3 polyclonal antibodies (Santa Cruz Biotechnology, Inc. 100 µg IgG/0.1mL) were added to the protein. Nucleotide sequences of the oligonucleotides used for EMSA were 5’-GTCTTTCCCAAAATATGGAGCTGTGT-3’ (CArG box is *underlined*).
Figure Legends

**Supplemental figure I.** Effect of TGFβ1 on the mRNA expression of SMC marker gene in 10T1/2 cells. A, Northern blot analysis. Total RNA (10 µg) was extracted from 10T1/2 cells at 12 hours after stimulation with TGFβ1 (1 ng/mL) and analyzed by Northern blotting for SM22α, SMα-actin, SMemb, and KLF5/BTEB2 mRNAs. 28S ribosomal RNA stained by methylene blue indicates that comparable amounts of total RNA actually blotted onto a membrane. B, Southern blot analysis of reverse transcription (RT)-PCR products for expression of calponin and GAPDH in 10T1/2 cells. C, Time course of the SM22α, SMα-actin and SRF expression. D, Total RNA was prepared from 10T1/2 cells treated with TGFβ1 for 12 hours in the presence or absence of cycloheximide, and analyzed by Northern blotting for SM22α, SMα-actin mRNAs.

**Supplemental figure II.** Effect of TGFβ1 and src-kinase inhibitor in cultured RASMC. Cultured RASMC were pretreated with herbimycin A and were exposed to TGFβ1 (1 ng/mL) for 6 hours. SM22α and SMα-actin mRNA expression was measured by real-time RT-PCR. An arbitrary value of 1.0 was assigned to control. *P<0.05 vs. control.

**Supplemental figure III.** Effects of TGFβ1 on the SM22α promoter activity. A, A series of constructs with the SM22α promoter of various lengths was transfected into 10T1/2 cells. Transfected cells were incubated with vehicle or TGFβ1 (1 ng/mL) for 24 hours as described in Material and Methods. B, cultured RASMC transfected with the –305Luc were incubated with vehicle or TGFβ1. Cells transfected with indicated reporter genes were incubated with vehicle or TGFβ1 (1 ng/mL) for 24 hours as described in Materials and Methods. *P<0.05 vs. control.

**Supplemental figure IV.** A, EMSA with the SRF binding sites from the SM22α promoter. Nuclear extracts from 10T1/2 cells grown in absence or presence of TGFβ1 were incubated with wild type probe, which corresponds to SM22α (from -158 bp to -133 bp) sequence, and were subjected to electrophoresis. Nuclear extracts were incubated with specific antibodies against SRF, STAT3, and Smad2/3. Positions of the sequence-specific DNA-protein complexes (C) and supershifted complexes (S) are indicated. B, Western blot analysis of SRF protein in 10T1/2 cells. Total cellular lysates were prepared from 10T1/2 cells treated with or without TGFβ1 for 12 hours,
and immunoblotted with anti-SRF antibody. Total cellular lysate from 10T1/2 cells transfected with SRF/pME18S, SRF expression vector, was used as a positive control. C, Luciferase activity of the SRF promoter. 10T1/2 cells and cultured RASMC transfected with the -2052SRF/Luc, a luciferase reporter gene containing SRF promoter region between -2052 and +114, were incubated with vehicle or TGFβ1 as described in Material and Methods. *P<0.05 vs. control.

Supplemental figure V. Effects of bFGF on the TGFβ1-stimulated SMC marker gene expression. A, Northern blot analysis. 10T1/2 cells were treated with either PDGF-BB (10 ng/mL), VEGF (10 ng/mL), IL-6 (5μ/mL), EGF (10 ng/mL) or bFGF (10 ng/mL) for 12 hours. 28S ribosomal RNA stained by methylene blue indicates that comparable amounts of total RNA actually blotted onto a membrane. B, Cultured RASMC were pretreated with PD98059 and were exposed to TGFβ1 or/and bFGF for 6 hours. SM22α mRNA expression was measured by real-time RT-PCR. An arbitrary value of 1.0 was assigned to control. *P<0.05 vs. bFGF(-). C, Repression of TGFβ1-incuced SM22α-luciferase activity by bFGF. 10T1/2 cells transfected with the SM22α-305Luc were incubated with or without TGFβ1, bFGF and PD98059 as described in Material and Methods. *P<0.05 vs. control. D, Temporal changes of ERK1/2 phosphorylation induced by TGFβ1 and/or bFGF in 10T1/2 cells. 10T1/2 cells were treated with TGFβ1 and/or bFGF for designated times. Whole-cell lysate (10µg protein per lane) were used in Western blot analysis.

Supplemental figure VI. Western blot analysis of c-Src kinase activity in 10T1/2 cells. 10T1/2 cells were pretreated with 50 μmol/L PD98059 or/and 10 ng/mL bFGF for 1 hour and were exposed to TGFβ1 for 1 minutes.

Supplemental figure VII. Effects of bFGF on the TGFβ1-stimulated SRF expression and function. A, Luciferase assay. Cultured RASMC were transfected with the SM22α-305Luc along with either pcDNA3 or MEK1/pcDNA3 and stimulated with TGFβ1. B, Northern blot analysis of SRF in 10T1/2 cells. Total RNA (10 μg) was extracted from 10T1/2 cells at 12 hours after stimulation with or without TGFβ1 or bFGF and analyzed by Northern blotting for SRF mRNA. 28S ribosomal RNA stained by methylene blue indicates that comparable amounts of total RNA actually blotted onto a membrane. C, Western blot analysis of SRF protein in 10T1/2 cells treated with or without
TGFβ1 or bFGF. Total cellular lysate from 10T1/2 cells transfected with SRF/pME18S, SRF expression vector, was used as a positive control. D, EMSA. Nuclear extracts from 10T1/2 cells treated with or without TGFβ1 or bFGF were incubated with the probe, correspond to the sequence from -158 to –133 of the SM22α promoter. DNA:SRF complex is indicated by arrow.
References


A  control TGFβ
SM22α
SM α-actin
SMemb
KLF5/BTEB2
28S

B  control  TGFβ
calponin
GAPDH

C  hrs after TGFβ stimulation

SM22α  SM22α
SM α-actin  SM α-actin
SRF  SRF
28S  28S

D  TGFβ  cycloheximide
-  -  +  +  -  -  +  +
SM22α  SM22α
SM α-actin  SM α-actin
28S  28S
RASMC

![Graph showing SM22α mRNA/18S mRNA (Fold of control) and SMα-actin mRNA/18S mRNA (Fold of control) with TGFβ and Herb. conditions.]

* indicates a significant difference.
A 10T1/2 cells

SM22α promoter

-305Luc -
-215Luc -
-158Luc -
-115Luc -

| CArG-box | GC-box | CAGA motif |

pGL3

Relative luciferase activities

control

TGFβ

B RASMC

SM22α promoter

-305Luc -

Relative luciferase activities

*
A

TGFβ - +

antibody

- SRF Smad2/3
- STAT3

Free probe

10T1/2 cells

B

overexpression of SRF

TGFβ - +

10T1/2 cells
C

SRF promoter activities in 10T1/2 cells

SRF promoter activities in RASMC
A 10T1/2 cells

<table>
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B RASMC

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</table>
C

10T1/2 cells

Relative luciferase activities

TGFβ
bFGF
PD

TGFβ(-) TGFβ(+) Vehicle TGFβ bFGF TGFβ+bFGF

D

10T1/2 cells

Phospho-ERK1/2

Time (min) 0 5 10 30 60

- + - + - + - + + + - - + - + - + + +

*
10T1/2 cells

TGFβ

PD bFGF PD+bFGF bFGF (1min) Hypoxia (15min)

Phosho-c-Src

Total c-Src
A

RASMC

Relative 
luciferase 
activities
(-305Luc)

TGFβ
- + - + pcDNA3 MEK1/pcDNA3

B

10T1/2 cells

TGFβ
bFGF - + - +

SRFmRNA →

28S

C

bFGF - + - +

TGFβ
SRF over expression

SRF protein →

D

bFGF - + - +

TGFβ

DNA-SRF complex →