Transforming Growth Factor-β–Induced Differentiation of Smooth Muscle From a Neural Crest Stem Cell Line

Shiyou Chen, Robert J. Lechleider

Abstract—During vascular development, nascent endothelial networks are invested with a layer of supporting cells called pericytes in capillaries or smooth muscle in larger vessels. The cellular lineage of smooth muscle precursors and factors responsible for regulating their differentiation remain uncertain. In vivo, cells derived from the multipotent neural crest can give rise to vascular smooth muscle in parts of the head and also the cardiac outflow tract. Although transforming growth factor-β (TGF-β) has previously been shown to induce some smooth muscle markers from primary cultures of neural crest cells, the extent of the differentiation induced was not clear. In this study, we demonstrate that TGF-β can induce many of the markers and characteristics of vascular smooth muscle from a neural crest stem cell line, Monc-1. Within 3 days of in vitro treatment, TGF-β induces multiple smooth muscle–specific markers, while downregulating epithelial markers present on the parent cells. Treatment with TGF-β also induces a contractile phenotype that responds to the muscarinic agonist carbachol and is not immediately reversed on TGF-β withdrawal. Examination of the signaling pathways involved revealed that TGF-β activation of Smad2 and Smad3 appear to be essential for the observed differentiation. Taken together, this system provides a novel model of smooth muscle differentiation that reliably recapitulates the process observed in vivo and allows for dissection of the pathways and processes involved in this process. (Circ Res. 2004;94:1195-1202.)

Key Words: smooth muscle  ■  development  ■  transforming growth factor-β  ■  Smad

During vascular development, endothelial cells form a plexus that is subsequently remodeled into a mature vasculature during a process called angiogenesis.¹ During angiogenic remodeling, the endothelium is invested with a layer of specialized mesenchymal cells called pericytes in capillaries or smooth muscle in larger vessels.¹ Vascular smooth muscle cells (VSMCs) contribute to vessel development by inducing endothelial cell quiescence, providing physical support, and aiding in maintenance of endothelial viability.¹ Development of smooth muscle is required for proper vascular development, as demonstrated by genetic studies in the mouse.² In mice that have defects in smooth muscle development, embryonic lethality inevitably results. Similarly in humans, several vascular diseases can be traced to a defect in smooth muscle proliferation or development.³⁻⁵

VSMCs come from multiple sources during vascular development. The most common precursor for vascular smooth muscle (VSM) are poorly defined mesenchymal cells derived from mesoderm.⁶ During in vitro culture, endothelial cells can be induced to form smooth muscle–like cells, although whether and to what extent this occurs in vivo is uncertain.⁶ A third source of smooth muscle both in vivo and in vitro is the neural crest. Neural crest cells are a population of migratory cells derived from delamination of the lateral neural tube beginning about day 8.5 of murine development.⁷

These multipotent cells can give rise to neurons, cartilage, bone, melanocytes, and in the branchial arch distribution and cardiac outflow tract, vascular smooth muscle.⁸ Demonstration of a neural crest origin for vascular smooth muscle in vivo comes from both genetic studies in the mouse, where failure to form neural crest produces a phenotype reminiscent of DiGeorge syndrome with outflow tract abnormalities and reduced smooth muscle in the proximal great vessels,⁹ and developmental studies in the chick, which showed that the neural crest is important for developing vasculature in the cardiac outflow tract and head.¹⁰ In vitro, primary neural crest stem cells (NCSCs) have been shown to be capable of producing the smooth muscle marker α-smooth muscle actin (α-SMA) after treatment with transforming growth factor (TGF)-β, although a more extensive evaluation has not been performed.¹¹ Factors that induce smooth muscle have not been well studied. In vivo, loss of either Pdgfαa or Pdgfrα has been shown to result in a loss of pericytes surrounding smaller vessels, but larger vessels appear to be unaffected.¹² The loss of the lipid sphingosine 1-phosphate by homologous recombination resulted in a defect in migration of VSM precursors, but differentiation to a smooth muscle phenotype appeared not to be impaired.¹³ TGF-β and signaling intermediates that mediate its effects are critical for VSM formation during

Original received December 2, 2003; revision received March 18, 2004; accepted March 19, 2004.
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Circulation Research is available at http://www.circresaha.org
DOI: 10.1161/01.RES.0000126897.41658.81

1195
development. Mouse knockouts of the cell surface accessory protein Endoglin, the type II receptor, the type I receptors Alk1 and Alk5, and the signaling intermediate Smad5 all result in defects in angiogenesis due, at least in part, to defects in smooth muscle proliferation, recruitment, or differentiation. 14 Despite the advances that genetic studies have produced in understanding smooth muscle development, little is known about the process of VSMC differentiation. In vitro, C3H10T1/2 fibroblasts have been induced to express markers of smooth muscle by both coculture with endothelial cells and treatment with TGF-β. 15 Implantation of these cells in a collagen matrix under the skin of syngeneic mice resulted in incorporation into vessels invading the gel. Similarly, the neural crest stem cell line Monc-1 has been used as a model of smooth muscle differentiation by serum, although marker analysis after differentiation was not extensively pursued in that study. 16 Other in vitro systems have been used to study smooth muscle differentiation, notably the isolation of small round cells from developing murine lung, 17 but the applicability of these systems to the study of VSM is unclear.

In the present study, we have used the immortalized neural crest cell line Monc-1 18 as a model of in vitro smooth muscle differentiation by TGF-β. Treatment of Monc-1 cells with TGF-β results in a rapid, reproducible induction of multiple smooth muscle markers. Unlike the previously demonstrated differentiation using serum, 16 treatment with TGF-β results in a spindle shaped morphology and contraction in response to the muscarinic agonist carbachol. Examination of the signaling pathways responsible for smooth muscle differentiation reveal that activation of the Smad pathway is necessary for smooth muscle differentiation and that both Smad2 and Smad3 contribute to this effect.

Materials and Methods

Antibodies
Smooth muscle α-actin (α-SMA), Cy3-conjugated α-SMA, and β-tubulin monoclonal antibodies and secondary antibodies were purchased from Sigma. Affinity-purified rabbit polyclonal SM22α antisera was generously provided by Dr Mario Gimona (Salzburg, Austria). Rabbit anti-Smad2 and Smad3 antibodies were obtained from Zymed or Pharmingen. Phospho-Smad2 antibody was purchased from Upstate Biotechnology. Phospho-Smad3 antibody was a generous gift from Dr Ed Leof (Mayo Clinic).

Cell Culture
The Monc-1 cell line was provided by David Anderson (California Institute of Technology). Monc-1 cells were cultured in the undifferentiated state in standard medium (SM), as described by Stemple and Anderson. 19 SMC differentiation was induced by TGF-β (R&D Systems) in defined medium (DM), which contains all the other components in SM except chicken embryo extract and reduced amount (10%) of FGF as compared with SM. The Alk5 inhibitor SB431542 (Tocris) was used at 10 µmol/L as described. 20 Ligand-induced contractility of TGF-β-induced SMCs was monitored as follows: Monc-1 cells were treated under indicated conditions for 3 days, and then washed with PBS, followed by stimulation with DM containing 1 mmol/L carbachol for 1 minute. Contractility of newly differentiated SMCs was observed with a Nikon microscope, and the same fields before and after carbachol treatment were imaged. Silicone wrinkle assays were performed as described. 21

Western Blot Analysis
Monc-1 cells were cultured in DM alone or treated with TGF-β or other indicated factors as indicated. Cells were washed two times with PBS, followed by protein extraction using RIPA buffer (50 mmol/L Tris-HCl, pH 7.4, 1% Triton X-100, 0.25% wt/vol sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA, 0.1% SDS, protease inhibitors). Protein concentration was measured using BCA Protein Assay Reagent (Pierce). Five or 10 µg of the lysates was resolved by SDS-PAGE and transferred to PVDF or nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk (Carnation) for regular antibodies or 3% BSA for anti-phospho antibodies, and then incubated for 1 to 2 hours with primary antibodies in blocking buffer. Goat anti-rabbit or rabbit anti-mouse IgG were used as secondary antibodies and diluted 1:5000. Detection was by enhanced chemiluminescence (Pierce), and images were analyzed with a Fuji image system.

Immunocytofluorescence
Monc-1 cells were cultured on fibronectin-coated coverslips and treated as indicated. Cells were fixed with 4% paraformaldehyde (PFA), or 1% PFA for 1 minute followed by methanol for 10 minutes at −20°C for SM22α staining. Anti-α-SMA was used at 1:100 in blocking buffer consisting of 3% BSA in PBS. Anti-SM22α was used at 1:50.

For siRNA assay, anti-Smad2 was used at 1:50 and anti-Smad3 used at 1:15. Cells were then incubated with FITC-conjugated secondary antibodies (1:100) followed by incubation with Cy3-conjugated anti-α-SMA (1:400). Stained cells were imaged with a Zeiss Axioplan microscope.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction (RT-PCR)
Total RNA from cultured cells was prepared using Trizol (Invitrogen) and Rneasy (Qiagen) according to the manufacturer’s instructions. Reverse transcription was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Primers for amplifying SMC markers, TGF-β family receptors and Smads are shown in the online Table (available in the online data supplement at http://circres.ahajournals.org). Semiquantitative RT-PCR was performed as described. 22

Smad Knockdowns
Smad2 and Smad3 siRNAs were from Dharmacon based on sequences of mouse Smad2 (Accession no. U60530) and Smad3 (Accession no. AB008192). Monc-1 cells (2.0×105) were transfected with 400 pmol siRNA using Lipofectamine 2000 in a 6-well plate. After 24 hours of incubation, Monc-1 cells were transfected again under the same conditions. Six hours later, cells were treated with or without TGF-β for 48 to 48 hours.

Results
In order to understand the mechanism of TGF-β induction of VSM, we developed an in vitro model of smooth muscle differentiation. In our hands, C3H10T1/2 cells expressed a high level of the smooth muscle markers α-SMA and SM22α in the absence of TGF-β (data not shown), and we thus felt that this was not a good model for investigating the role of growth factors in inducing smooth muscle differentiation. We therefore investigated the ability of a neural crest stem cell line, Monc-1, to differentiate to smooth muscle. We grew Monc-1 cells in standard medium (SM) and then replated in differentiation medium (DM) without or with the addition of growth factors. As a positive control, we also plated Monc-1 cells in M199 medium with 10% FBS to induce markers as previously described. 16 As shown in Figure 1, addition of
TGF-β for 2 days induced differentiation of Monc-1 cells toward a smooth muscle phenotype, as demonstrated by induction of the markers α-SMA and SM22α. No other growth factors tested were able to induce similar expression of these markers, although treatment with serum induced both markers. As expected, neither BMP2 nor BMP7 induced VSM marker expression, as these ligands are known to induce a neuronal phenotype from neural crest cells. Activin, another TGF-β superfamily ligand that activates signaling pathways similar to those induced by TGF-β, also failed to induce marker expression.

In order to investigate the role of TGF-β in inducing VSM marker expression, we examined a time course of marker induction after TGF-β treatment. We again examined expression of α-SMA and SM22α by Western blotting after treatment of Monc-1 cells with TGF-β. As seen in Figure 1B, 1 day after TGF-β addition, both markers were induced and maintained through 3 days, the longest time examined. Treatment with serum yielded a similar result. To examine the role of TGF-β in the serum induction of VSM markers, we evaluated serum induction of α-SMA in the absence and presence of an inhibitor of the Alk5 TGF-β type I receptor. As seen in Figure 1C, blockade of TGF-β, either alone or when found in serum or induced by serum treatment, is necessary and sufficient for induction of smooth muscle marker expression in Monc-1 cells.

Examination of marker expression by Western blotting analysis does not indicate whether the induction of marker expression is global or is localized to a few cells or clusters. In order to address this, we examined expression of α-SMA and SM22α by indirect immunofluorescence before and after treatment with TGF-β or serum. As shown in Figure 2, both α-SMA and SM22α were induced in nearly all of the...
observed cells after 3 days of TGF-β treatment. Expression was roughly uniform between different cells, and more cells appeared to be recruited to express either marker with increasing treatment time. These data indicate that the changes observed are universal and not due to the induction of markers in a few susceptible cells. This suggests that the role of TGF-β and serum in inducing these markers is not to further induce a few preconditioned cells, but rather as a global differentiating agent.

Although induced expression of α-SMA and SM22α suggested that Monc-1 cells treated with TGF-β were differentiating along a VSM lineage, two markers do not sufficiently define the VSM phenotype. In order to more fully characterize the Monc-1 cells treated with TGF-β, we examined several other markers whose expression is largely limited to the smooth muscle lineage. We treated Monc-1 cells with TGF-β or serum and examined expression of markers each day for 3 days by semiquantitative RT-PCR. As seen in Figure 3, TGF-β–induced expression of Calponin, SM γ-actin, smooth muscle myosin heavy chain (SMMHC), and Smoothelin within 1 day of treatment. Similarly, treatment with FBS induced these same markers, although the induction of SMMHC was delayed compared with TGF-β treatment. TGF-β treatment downregulated expression of the epithelial cell–specific E-cadherin, whereas treatment with FBS reduced, but did not eliminate E-cadherin gene expression. These data suggest that the induction of the markers α-SMA and SM22α is not an isolated phenomenon, but is part of a differentiation process induced by TGF-β. Similarly, the loss of expression of E-cadherin suggests that the cells have lost epithelial characteristics when treated with TGF-β, a process that is incomplete with serum treatment.

A salient characteristic of smooth muscle is the ability to contract in response to stimulus. Although both C3H10T1/2 cells treated with TGF-β and Monc-1 cells grown in serum express markers of smooth muscle, including contractile proteins, neither cell has been shown to contract in response to a stimulus after differentiation. We sought to determine if Monc-1 cells treated with TGF-β would contract in response to a ligand. To do this, we used carbachol, a muscarinic agonist that can induce contraction of cultured smooth muscle.23 Cells were grown for 3 days either in DM, DM with TGF-β, or M199 medium with 10% FBS. Cells grown in DM did not change their morphology when compared with those grown in SM, which is the primary medium used for passage and maintenance of the cells (not shown). As seen in Figures 4C and 4F and as previously published,16 cells grown in serum developed a flattened morphology consistent with a
synthetic smooth muscle phenotype. Cells grown in TGF-β, however, developed an elongated spindle-shaped morphology that differs from cells grown either in DM, those grown in FBS, or the FBS-treated cells (Figure 4, left panels). After imaging, cells were treated for 1 minute with carbachol, and then the identical area was reimaged. As seen in Figure 4 (compare A and D), treatment of DM grown cells with carbachol had no obvious effect on cell shape. Similarly, cells grown in FBS did not appear to change shape in response to carbachol treatment (Figure 4, compare C and F). Cells treated with TGF-β, however, responded to carbachol treatment by appearing to contract (Figure 4, compare B and E). Although not all cells were responsive, a large proportion of cells either shortened or rounded up with carbachol treatment.

We performed similar experiments using silicone as a substrate,21,24 (Figures 4G through 4L), with similar results. Treatment of the Monc-1 cells with carbachol did not lead to cell death as determined by trypan blue exclusion assays before and after treatment (data not shown). These data indicate that although they express proteins that are part of the contractile machinery, Monc-1 cells treated with serum may not represent a contractile smooth muscle phenotype, but may instead represent a synthetic phenotype. Cells treated with TGF-β, however, can contract in response to extracellular stimulus, suggesting that they represent an alternative differentiated phenotype and may be a useful model for the study of smooth muscle development and function.

Unlike many other differentiated cells, smooth muscle cells display a high degree of plasticity in culture. We sought to determine if TGF-β treatment of Monc-1 cells to induce a smooth muscle phenotype was reversible, or whether TGF-β treatment caused a terminal differentiation of Monc-1 cells. To test this, we performed two experiments. In the first, Monc-1 cells were treated with TGF-β for 2 days to induce differentiation, and then the TGF-β was withdrawn and neutralizing antibodies that recognize all three isoforms of TGF-β were added at a concentration sufficient to block the effect of exogenous TGF-β. Cells were collected 1 and 2 days after addition of blocking antibodies. As seen in Figure 5A, initial treatment of Monc-1 cells induced the markers α-SMA and SM22α and addition of blocking antibodies did not induce a loss of expression of these two markers. This suggests that once TGF-β has induced the smooth muscle phenotype from Monc-1 cells, it is not required further for maintenance of that phenotype, at least under the conditions examined. One caveat is that TGF-β may be sequestered in the tissue culture plastic or any extracellular matrix the Monc-1 cells have produced. To test this, we performed a second experiment that should effectively eliminate any residual TGF-β. Monc-1 cells were grown in the absence or presence of TGF-β for 3 days and then trypsinized and replated in DM lacking TGF-β. Cells were grown in DM for 1 to 3 days and analyzed by Western blotting for the presence of smooth muscle markers. As seen in Figure 5B, cells treated with TGF-β continued to express α-SMA and SM22α even after trypsinization and replating for two passages in medium lacking TGF-β. To further examine the permanence of the changes induced by TGF-β, we treated cells with TGF-β for 3 days, then withdrew TGF-β, split the cells, and added PDGF BB, which represses expression of smooth muscle markers.25,26 As seen in Figure 5C, PDGF had little effect on α-SMA expression immediately after TGF-β withdrawal, but suppressed α-SMA after treatment for four passages. Taken together, these results indicate that TGF-β can induce a long lasting and perhaps heritable differentiation of Monc-1 cells into a smooth muscle phenotype.

Having established Monc-1 cells as a model of TGF-β-induced smooth muscle differentiation, we sought to identify the signaling pathways involved. TGF-β superfamily members signal through ligand binding to the type II receptor, recruitment, and transphosphorylation of a type I receptor, and downstream signal propagation predominantly through the phosphorylation, activation, and nuclear translocation of Smad proteins. TGF-β signals exclusively through the type II receptor TβR-II, and uses two type I receptors, Alk5 (TβR-I) and Alk1, although Alk1 expression is largely restricted to endothelial cells. We performed RT-PCR using RNA isolated from Monc-1 cells either untreated or treated with TGF-β for...
3 days in order to examine expression of the receptors in the TGF-β superfamily. As seen in Figure 6A, four known type II receptors were expressed at the mRNA level in untreated and treated Monc-1 cells. Of the known type I receptors, Alk1 through Alk7, all except Alk7 and Alk1 were expressed in Monc-1 cells. There was no major change of receptor expression with TGF-β treatment.

Downstream signaling by Smad proteins is largely restricted according to the receptors activated. The type I TGF-β receptor Alk5 signals through Smad2 and Smad3. BMP receptors and the TGF-β type I receptor Alk1 signal through Smad1, 5, and 8. Smad4 is required for signaling from all Smads except in exceptional circumstances. We examined expression of the various Smad genes in Monc-1 cells both before and after TGF-β treatment to determine the possible pathways involved. As shown in Figure 6B, all of the R-Smads examined and Smad4 were expressed by untreated Monc-1 cells. There was no significant difference in expression of the different Smads after TGF-β treatment except for a modest increase in Smad2 mRNA. Thus, it appears that Monc-1 cells contain, before differentiation, all of the components necessary for transducing a signal from the cell surface receptors to the nucleus, and the predominant pathway activated is the canonical TGF-β signaling pathway involving Alk5 and Smad2 and Smad3.

In order to determine whether TGF-β signals through Smad2 and Smad3, we performed Western blotting experiments using antibodies specific to phosphorylated Smad2 or Smad3. Monc-1 cells were treated with TGF-β for various times and cell lysates prepared for Western blotting. As seen in Figure 6C, TGF-β induced a rapid phosphorylation of both Smad2 and Smad3, but phosphorylation of Smad2 appeared to be sustained at a somewhat higher level than that of Smad3. Interestingly, a second band is present in the Smad3 blot at early time points. It is not known if this is an alternatively phosphorylated form of Smad3, or perhaps cross reactivity with Smad1 or Smad5, which have a highly similar C-terminal tail and are recognized by the antibody. These data suggest that TGF-β signals predominantly, if not exclu-
sively, through the type I receptor Alk5. The rapid induction of phosphorylation of Smad2 and Smad3 after treatment with TGF-β is sustained more clearly for Smad2 at longer times, whereas Smad3 phosphorylation appears to decrease after a fast initial induction. Treatment of Monc-1 cells with Activin revealed a similar pattern, with rapid induction of both Smad2 and Smad3 phosphorylation. Smad2 phosphorylation appeared to be sustained at a slightly lower level with Activin treatment compared with treatment with TGF-β.

We next sought to determine if blocking expression of Smad2 or Smad3 proteins would inhibit marker expression after TGF-β treatment. To do this, we used siRNA to knockdown expression of Smad2 or Smad3 in Monc-1 cells. We transfected pools of oligoribonucleotides into Monc-1 cells and then determined Smad2 or Smad3 expression by indirect immunofluorescence either with or without TGF-β treatment. As seen in Figure 7, Smad-specific siRNA transfection significantly decreased Smad protein expression and nuclear accumulation, whereas transfection with control siRNAs did not. We then tested whether knockdown of Smad protein levels would affect smooth muscle differentiation. Cells were transfected twice in 2 days with siRNA to Smad2, Smad3, or control GFP siRNAs. Six hours after the second transfection, cells were treated with TGF-β or left untreated and then levels of α-SMA determined as a marker of smooth muscle differentiation. Cells transfected with control siRNA demonstrated induction with TGF-β treatment. Cells transfected with siRNA to either Smad2 or Smad3 showed a decrease in α-SMA expression, especially in cells that did not have Smad nuclear localization. This suggests that both Smad2 and Smad3 are necessary for complete induction of at least one SM marker, α-SMA, and may cooperate to induce a smooth muscle phenotype. Expression levels of the appropriate Smad proteins were decreased by siRNA treatment as shown in each panel.

**Discussion**

TGF-β plays an important role in regulating smooth muscle phenotype. In vitro studies have shown that TGF-β can cause proliferation or growth inhibition of cultured smooth muscle cells, depending on the origin of the cells and the conditions of culture. The TGF-β signaling components Endoglin, Alk1, Alk5, and TβR-II are all necessary for proper smooth muscle development during murine embryogenesis. Previous in vitro studies have shown that TGF-β is sufficient to induce certain smooth muscle markers from embryonic fibroblasts and primary neural crest cultures. In this study, we show that TGF-β induces a functional smooth muscle phenotype from the neural crest stem cell line Monc-1. In addition, we demonstrate that expression of Smad2 and Smad3 is necessary for induction of this phenotype. This study thus provides a basis for understanding the mechanism of smooth muscle induction from the neural crest and may provide insight into the mechanism of smooth muscle induction in general.

The vertebrate neural crest is a migratory population of cells derived from the lateral neural tube. NCSCs differentiate into neurons, glia, cartilage, bone, and melanocytes as well as smooth muscle. NCSCs contribute to VSM in the cardiac outflow tract and derivatives of the branchial arch arteries. Although the majority of VSM is derived from mesoderm, the in vivo functions and properties of VSM derived from the neural crest do not differ significantly from that derived from mesoderm. Thus, analysis of an in vitro model system of smooth muscle differentiation from the neural crest is likely to provide significant insight into the mechanisms of VSM differentiation in general and from the neural crest in particular.

Previous studies have shown that TGF-β can induce smooth muscle markers from primary neural crest stem cell cultures, and that serum can induce smooth muscle differentiation from Monc-1 cells. We have extended these observations significantly. In our hands, TGF-β treatment of Monc-1 cells induces numerous markers of smooth muscle, including SMMHC and Smoothelin. Previous studies of primary neural crest or Monc-1 cells did not analyze these markers. The morphological appearance of Monc-1 cells treated with TGF-β also differs. Cells treated with TGF-β develop an elongated shape, and can contract by treatment with carbachol. Serum differentiated cells, in contrast, develop a flattened phenotype and do not contract after carbachol treatment. This demonstrates that cells treated with TGF-β have developed into a more differentiated state than those treated with serum. Other in vitro differentiation systems have not, to our knowledge, demonstrated a functionally contractile cell type after differentiation. The model we have developed thus provides an excellent system with which to understand the molecular mechanisms that produce a functionally active smooth muscle cell.

The role of TGF-β in inducing smooth muscle can now be dissected at the molecular level. Knockdown of Smad2 or Smad3 expression leads to a loss of α-SMA expression in affected cells. Further studies will be necessary to determine which Smads mediate which responses, and to determine how coordination of Smad2 and Smad3 signaling regulate specific responses. The finding that Smad2 and Smad3 are phosphorylated with similar kinetics by both TGF-β and activin, and the failure of activin to induce smooth muscle markers suggests that other pathways from the TGF-β receptor are necessary for smooth muscle induction. Our results also suggest that the differentiated phenotype induced by TGF-β is long lasting, at least at the level of expression of marker genes. Whether the contractile phenotype will remain after withdrawal of TGF-β is not yet known.

**Acknowledgments**

This work was supported by National Institutes of Health Grant HL65681 to R.J.L., an American Heart Association Scientist Development Grant to S.C., and funds from Georgetown University. The authors thank Dr Mahendra Rao for helpful advice in culturing the Monc-1 cells, Dr Ed Leof for generously providing phospho-Smad3 antibody, and those indicated in the text for sharing reagents.

**References**


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_Circ Res._ 2004;94:1195-1202; originally published online April 1, 2004; doi: 10.1161/01.RES.0000126897.41658.81

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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