RhoA Modulates Smad Signaling during Transforming Growth Factor-β-induced Smooth Muscle Differentiation*

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We recently reported that transforming growth factor (TGF)-β induced the neural crest stem cell line Monc-1 to differentiate into a spindle-like contractile smooth muscle cell (SMC) phenotype and that Smad signaling played an important role in this phenomenon. In addition to Smad signaling, other pathways such as mitogen-activated protein kinase (MAPK), phosphoinositide-3 kinase, and RhoA have also been shown to mediate TGF-β actions. The objectives of this study were to examine whether these signaling pathways contribute to TGF-β-induced SMC development and to test whether Smad signaling cross-talks with other pathway(s) during SMC differentiation induced by TGF-β. We demonstrate here that RhoA signaling is critical to TGF-β-induced SMC differentiation. RhoA kinase (ROCK) inhibitor Y27632 significantly blocks the expression of multiple SMC markers such as smooth muscle α-actin, SM22α, and calponin in TGF-β-treated Monc-1 cells. In addition, Y27632 reversed the cell morphology and abolished the contractility of TGF-β-treated cells. RhoA signaling was activated as early as 5 min following TGF-β addition. Dominant negative RhoA blocked nuclear translocation of Smad2 and Smad3 because of the inhibition of phosphorylation of both Smads and inhibited Smad-dependent SBE promoter activity, whereas constitutively active RhoA significantly enhanced SBE promoter activity. Consistent with these results, C3 exotoxin, an inhibitor of RhoA activation, significantly attenuated SBE promoter activity and inhibited Smad nuclear translocation. Taken together, these data point to a new role for RhoA as a modulator of Smad activation while regulating TGF-β-induced SMC differentiation.

Vascular smooth muscle cell (VSMC) differentiation is an important process during vasculogenesis and angiogenesis. It is well known that alterations of VSMC phenotype play a role in the progression of several cardiovascular disorders including atherosclerosis, hypertension, and restenosis (1–3). The developmental origins of VSMC are diverse, including both embryonic mesoderm precursors and the neural crest, an ectodermal cell population (4, 5). However, the molecular mechanisms controlling the differentiation and development of VSMC, including the intracellular signaling pathways and transcriptional regulation of VSMC-specific gene expression, are largely unknown. It is clear that the identification of these mechanisms may enhance our understanding of VSMC phenotypic regulation and further provide potential therapeutic or preventive targets for cardiovascular diseases.

In vivo studies have shown that TGF-β signaling plays a vital role in VSMC function. TGF-β1, TGF-β receptors (including endoglin, TβR-II, TβR-I and Alk1), and signaling intermediates have all been identified by homologous recombination in the mouse as critical for embryonic angiogenesis. Loss of any of these components leads to defects in smooth muscle cell recruitment and/or differentiation during angiogenesis in the developing embryo and extraembryonic structures (6). In vitro studies have shown that TGF-β1 can induce differentiation of C3H10T1/2 mouse embryonic fibroblasts to a smooth muscle phenotype directly (7). We recently reported that TGF-β induced a contractile VSMC phenotype from neural crest stem cell line Monc-1 and that Smad proteins appeared to be critical in TGF-β-induced VSMC differentiation (8).

TGF-β signaling is initiated by ligand binding to the transmembrane receptors, TβR-I and TβR-II, and transduced predominantly by phosphorylation of receptor-associated Smad proteins (R-Smads) (9, 10). R-Smads combine with the common Smad, Smad4, and translocate into the nucleus where they function as transcription factors alone or in association with other DNA binding factors (10). We have demonstrated that Smads play a critical role in TGF-β-induced VSMC differentiation from neural crest cells in vitro (8). Much work clearly demonstrates, however, that other pathways including those activated by MAP and JNK kinases, PI3 kinase and RhoA also mediate TGF-β signaling (11, 12). The small GTP-binding protein RhoA is expressed in VSMC. RhoA activation was observed in cultured VSMC cells with a contractile phenotype (13, 14). Other studies have shown that signaling through RhoA controls VSMC function, at least in part, through modulating myocardin/SRF-dependent transcription of SMC marker genes (15, 16). Recent studies suggest that RhoA downstream target PKN interacts with p38 MAPK to regulate the SMC marker promoter activity in rat pulmonary arterial smooth muscle cell line (17). It has been suggested that the loss of RhoA expression observed in pulmonary arteries from rats with chronic pulmonary hypertension could be involved in pulmonary artery VSMC dedifferentiation and pulmonary artery remodeling (18, 19). Similarly, coronary VSMC differentiation from proepicardial cells also requires RhoA-mediated actin reorganization and Rho kinase activity (19). However, it is not known whether RhoA cross-talks with Smad signaling in TGF-β-induced VSMC differentiation.

In the present studies, we have evaluated the function of non-canonical pathways in the differentiation of TGF-β-treated Monc-1 cells. We demonstrate that RhoA signaling plays a critical role in TGF-β-induced VSMC differentiation. RhoA kinase (ROCK) inhibitor significantly

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3 The abbreviations used are: VSMC, vascular smooth muscle cell; TGF, transforming growth factor; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; DNRhoA, dominant negative RhoA; DAPI, 4′,6-diamidino-2-phenylindole.
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blocked VSMC maker gene expression and abolished the contractility of VSMC induced by TGF-β. In addition, we demonstrate for the first time that RhoA signaling modulates Smad function during VSMC differentiation through enhancing Smad phosphorylation and regulating Smad-mediated gene transcription.

MATERIALS AND METHODS

Reagents—α-SMA, α-tubulin monoclonal antibodies, and secondary antibodies were purchased from Sigma. Affinity-purified rabbit polyclonal SM22α antiserum was generously provided by Dr. Mario Gimona (Salzburg, Austria). Smad2 monoclonal antibody was obtained from BD Bioscience. Rabbit anti-smad3 antibody was purchased from Zymed Laboratories Inc. Phospho-Smad2 antibody was from Upstate Biotechnology. Phospho-Smad3 antibody was a generous gift from Dr. Ed Leof (Mayo Clinic). Monoclonal RhoA antibody was purchased from Pierce. Clostridium botulinum C3 exotoxin was from List Biological Laboratories, Inc. Dominant negative mutant Rho A T19N adenovirus (DN/RhoA) was a gift from Dr. Tetsuaki Hirase (Kobe University Graduate School of Medicine, Kobe, Japan) (20). Constitutively activated RhoA G14V adenovirus (AC/RhoA) was generously provided by Dr. Avi Hassid (University of Tennessee Health Science Center, Memphis, Tennessee) (21). SBE promoter construct was a gift from Dr. Peter ten Dijke (Leiden University Medical Center, Leiden, The Netherlands).

Cell Culture, Adenovirus Infection, and Transfection—Monc-1 and C3H10T1/2 cell culture were performed as previously described (8, 22). Ligand-induced contractility of TGF-β-induced Monc-1 cells was monitored as follows: Monc-1 cells were treated under indicated conditions for 3 days, and then washed with phosphate-buffered saline, followed by stimulation with DM containing 1 mM carbachol for 1 min. Contractility of SMCs was observed with Nikon Microscope, and the same fields before and after carbachol treatment were imaged (8).

DN/RhoA, AC/RhoA, or green fluorescent protein adenovirus infection was performed following published procedures (20, 21). Monc-1 cells were infected at 200 plaque-forming units/cell (23), and C3H10T1/2 cells were infected at 100 plaque-forming units/cell. The infection was maintained for 48 h prior to TGF-β treatment. C3H10T1/2 cell transfection of SBE plasmid and luciferase assay were performed as previously described (22).

RhoA Activation Assay—RhoA activation was determined by affinity precipitation of the active GTP-bound RhoA using a glutathione S-transferase (GST) fusion protein of the Rho-binding domain of the Rho effector rhoetokin (GST-RBD) following the manufacturer’s instruction as described in RhoA activation kit (Pierce). Detection of RhoA was performed by Western blot using anti-RhoA antibody.

Immunocytofluorescence, Western Blot Analysis, and Reverse Transcription-PCR—All analyses were performed as previously described (8). PCR primer sequences for α-SMA, SM22a, and calponin have been previously published (8).

Statistics—All values are expressed as mean ± S.E. Data were analyzed using analysis of variance with pairwise comparisons between relevant groups. A level of p values < 0.05 was considered statistically significant.

RESULTS

To study the molecular mechanism of VSMC differentiation, we previously developed an in vitro model showing that TGF-β induced the neural crest stem cell line Monc-1 to differentiate into VSMC (8). Further experiments demonstrated that Smad signaling was essential for smooth muscle differentiation. In addition to Smads, other signaling pathways such as p38 MAPK, p42/44 MAPK, PI3 kinase, and RhoA have also been shown to mediate TGF-β function (11, 12). We sought to determine whether these pathways were important for TGF-β-induced VSMC differentiation. We used kinase inhibitors to individually block each signaling pathway in Monc-1 cells treated by TGF-β, and then examined the expression of VSMC-specific markers. p38 MAPK, p44/42 MAPK, and PI3 kinase inhibitors did not inhibit TGF-β induction of smooth muscle α-actin (α-SMA), suggesting that p38 MAPK, p44/42 MAPK (data not shown), or PI3 kinase signaling (Fig. 1A) are not important for VSMC differentiation from Monc-1 cells. The ROCK inhibitor Y27632, however, significantly blocked TGF-β-induced α-SMA expression (Fig. 1A). To determine the extent of this effect, reverse transcription-PCR analysis of additional markers was performed. We found that Y27632 inhibited the expression of VSMC markers α-SMA, SM22a, and calponin (Fig. 1B). These results suggest that RhoA signaling plays an important role in TGF-β-induced VSMC differentiation.

We and others previously reported that treatment of Monc-1 cells with serum also induced VSMC markers (8, 24), although a contractile phenotype was not observed with serum treatment (8). Similar to results obtained with TGF-β, VSMC marker expression induced by serum was also blocked following Y27632 treatment (Fig. 1A), suggesting that RhoA signaling plays a role in serum-induced VSMC differentiation also. To detect whether RhoA and TGF-β have a synergistic role in serum-induced SMC differentiation, we used Y27632 and TGF-β type I receptor Alk5 inhibitor, SB431542, to treat serum-induced Monc-1 cells individually or in combination. We found that the maker gene expression was significantly inhibited by Y27632 or SB431542. SB431542 had a much greater effect than Y27632 (Fig. 1C). In addition, the combinatorial use of two inhibitors completely blocked the marker gene expres-
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Our previous studies showed that TGF-β induced a spindle-like contractile VSMC phenotype (8). To determine whether RhoA signaling is important in this phenotypic conversion, we performed carbachol-induced contraction assay in Monc-1 cells treated with or without TGF-β and Y27632. In this assay, cells were treated with carbachol, a muscarinic agonist, and imaged before and after treatment. Cells responsive to carbachol contract during the course of the assay (8). We found that RhoA inhibitor completely reversed TGF-β-induced spindle-like morphology of Monc-1 cells (Fig. 2E). Carbachol induced a contraction in cells treated with TGF-β (Fig. 2, compare C with D). The addition of Y27632, however, abolished the contractility (Fig. 2, compare E with F). These results suggest that RhoA signaling is necessary for TGF-β-induced contractility of the VSMC phenotype.

To further investigate how RhoA signaling regulates TGF-β-induced VSMC differentiation, we first determined whether TGF-β indeed activates RhoA in Monc-1 cells. We performed RhoA activation assay by treating Monc-1 cells with TGF-β for various times, and measuring GTP-bound RhoA. As shown in Fig. 3A, GTP-RhoA can be detected as early as 5 min following TGF-β treatment. The activation can last up to 16 h, the longest time point measured. There was little change in the level of RhoA expression in TGF-β-treated cells (Fig. 3A). C3H10T1/2 cells have been used as an in vitro cell model to study VSMC differentiation (7, 25). Our results showed that TGF-β also induced RhoA activation in C3H10T1/2 cells (Fig. 3B).

Because both Smad and RhoA signals play important roles in TGF-β-induced VSMC differentiation, we sought to determine whether RhoA could cross-talk with Smad signals. We used ROCK inhibitor Y27632 to treat Monc-1 cells before, after, or at same time of TGF-β addition. We did not detect the alteration of Smad activation induced by TGF-β, although the expression of the SMC marker α-SMA was blocked (Fig. 4A). These results suggest that the ROCK may not affect Smad activation in our system.

To test whether RhoA affects Smad signaling directly, we infected Monc-1 cells with an adenoviral vector expressing DNRhoA and treated the cells with TGF-β for short (30 min and 1 h) or longer (24 h) times and analyzed C-terminal phosphorylation by Western blot. We found that DNRhoA was expressed at a high level in adenovirus-infected cells (Fig. 4B). TGF-β induced phosphorylation of both Smad2 and Smad3 at 30 min after TGF-β addition (Fig. 4B). In DNRhoA-expressing cells, however, the phosphorylation of Smad2 and Smad3 was reduced significantly at 30 min or 1 h after TGF-β induction (Fig. 4B). These results suggested that the ROCK may not affect Smad activation in our system.
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infected Monc-1 cells with DNRhoA adenovirus followed by TGF-β treatment. Immunocytofluorescence staining showed that both Smad2 and Smad3 were then treated without (DM) or with TGF-β (5 ng/ml) for 30 min. Immunostaining was performed using Smad2 (A) or Smad3 (B) antibody. 4',6-Diamidino-2-phenylindole (DAPI) stains nuclei of cells. Colocalization of Smads with DAPI (Merge) indicates the nuclear translocation of Smads. Bar = 100 μm.

Following receptor association, Smad proteins are phosphorylated on C-terminal serine residues, and this phosphorylation is essential for nuclear translocation and transcriptional activity (12). To determine whether RhoA regulates Smad functional activity, we used transient transfection assays of a Smad-specific reporter SBE along with adenovirus expression of dominant negative and constitutively activated RhoA proteins in C3H10T1/2 cells. We could not use Monc-1 cells in this functional assay because we found that combination of transfection and virus infection in Monc-1 caused significant cell death. C3H10T1/2 cells have been shown to differentiate to SMCs with TGF-β stimulation as do Monc-1 cells (Fig. 3B), and to have a higher transfection efficiency than Monc-1 cells. C3H10T1/2 cells were transfected with the reporter, infected with adenovirus expressing green fluorescent protein (GFP), DNRhoA, or ACRhoA for 48 h. Cells were then treated without (control) or with TGF-β (5 ng/ml) for 16 h, and luciferase assay was performed. Data shown is one of the three independent experiments. *, p < 0.01 for comparison to green fluorescent protein expressing cells. B, effect of C3 exotoxin on SBE promoter activity. C3H10T1/2 cells were transfected with SBE luciferase reporter construct followed by addition of C3 exotoxin (5 μg/ml) for 16 h. Cells were then left untreated (control) or treated with TGF-β (5 ng/ml) for 8 h, and luciferase assay was performed. Data shown is a representative of two independent experiments. *, p < 0.05 for comparison to C3 untreated group.

To test C3 exotoxin function on Smad activation, we treated Monc-1 cells with C3 exotoxin followed by TGF-β addition and performed immunostaining with Smad antibodies. We found that TGF-β induced Smad3 nuclear translocation, indicating Smad phosphorylation. C3 exotoxin treatment, however, significantly decreased the nuclear accumulation of Smad3 (Fig. 7), indicating that C3 exotoxin inhibited Smad3 phosphorylation. Similarly, C3 exotoxin also inhibited Smad2 phosphorylation (data not shown). Taken together, these results demonstrate that RhoA modulates Smad-mediated gene transcription through modifying Smad phosphorylation.

Previous studies have shown that RhoA signaling is important for the down-regulation of c-Myc protein and up-regulation of p21 protein in TGF-β-mediated growth inhibition of breast cancer cell MCF10CA1h (32). To determine whether these cell cycle regulators are involved in TGF-β-induced SMC differentiation and whether they are regulated in a RhoA-dependent manner in our system, we overexpressed DNRhoA in Monc-1 cells with adenovirus infection and then treated the cells with TGF-β. Similar to the previous report (32), we found that TGF-β significantly up-regulated p21 but down-regulated c-Myc expression in Monc-1 cells. Overexpression of DNRhoA, however, blocked p21 but increased c-Myc protein expression (Fig. 8). These data suggest that as in other cell types (32), TGF-β regulates p21 and c-Myc and that these factors may play a role in TGF-β-induced SMC differentiation from neural crest cells. RhoA signaling plays an important role in regulating the expression of these cell cycle regulators.

DISCUSSION

We previously developed an in vitro VSMC differentiation model for the study of TGF-β induction of a contractile VSMC phenotype (8). In
then treated with (C3) or without C3 exotoxin (5 μg/ml) for 16 h. Cells were then treated without (DM) or with TGF-β (5 ng/ml) for 30 min. Immunostaining was performed using Smad3 antibody. Colocalization of Smad3 with DAPI (Merge) indicates the nuclear translocation of Smad3. Bar = 100 μm.

FIGURE 7. Effect of RhoA inhibitor C3 exotoxin on Smad3 activation. Monc-1 cells were plated on coverslips and treated with (C3) or without C3 exotoxin (5 μg/ml) for 16 h. Cells were then treated without (DM) or with TGF-β (5 ng/ml) for various times (hours) as indicated. Western blotting was performed using antibodies as indicated. α-Tubulin serves as an internal control for equal loading.

![Image](image1.png)

DABCYL-H11001

TGF-β

C3

TGF-β +

DABCYL-H11002

p21

c-Myc

α-Tubulin

FIGURE 8. Expression of cell cycle regulators p21 and c-Myc in Monc-1 cells. Monc-1 cells were infected with (+) DNRhoA adenovirus for 48 h or left uninfected (−). Cells were then treated with (+) or without (−) TGF-β (5 ng/ml) for various times (hours) as indicated. Western blotting was performed using antibodies as indicated. α-Tubulin serves as an internal control for equal loading.

this model, Monc-1 neural crest stem cells are treated with TGF-β, and a contractile SMC phenotype results. We further demonstrated that Smad signaling played an important role in this process (8). Because recent studies suggest that other signaling pathways are important for TGF-β function also, the goal of this study was to identify other pathway(s) important for TGF-β-induced VSMC differentiation. To this end, our data demonstrated that in addition to Smads, RhoA signaling is another pathway critical to TGF-β induction of VSMC differentiation. The blockade of RhoA signaling with ROCK inhibitor Y27632 significantly inhibited expression of the VSMC markers α-SMA, SM22α, and calponin. ROCK inhibitor Y27632 also blocked serum-induced VSMC marker expression. We previously reported that TGF-β played a critical role in serum-induced VSMC differentiation, which was demonstrated by the blockade of serum effect with type I receptor Alk5-specific inhibitors (8). In the present studies, we further demonstrated that RhoA and TGF-β have a synergistic effect on serum-induced SMC differentiation.

Contraction is a primary function of VSMC. VSMC induced from Monc-1 cells by TGF-β displayed contraction upon treatment with the muscarinic agonist carbachol. ROCK inhibitor Y27632 completely reversed the morphological change and abolished the carbachol-induced contraction, suggesting that RhoA plays an important role in the contractility of VSMC induced by TGF-β. This finding is consistent with the previous studies of RhoA activity on SMC function (14, 15). RhoA has been shown to regulate coronary or cultured SMC differentiation via cytoskeleton reorganization or actin polymerization through SRF-dependent transcription (19). RhoA is also critical in the regulation of contractility of cultured SMC cells (14). Our results provide direct evidence for the role of RhoA signaling in differentiation and contraction of VSMC developed from neural crest stem cells.

The Smad signaling pathway is the canonical transduction route downstream of TGF-β receptors. However, understanding cross-talk between Smad and other signaling pathways is necessary for determining how TGF-β regulates cellular function. Many studies have tried to link Smad signaling with RhoA activity, especially in TGF-β-induced cytoskeletal organization and epithelial mesenchymal transdifferentiation (EMT) (27–33). Although most of the studies showed that actin reorganization and EMT were mediated through Smad-independent RhoA signaling, several evidences showed that Smad was involved in RhoA function. Shen et al. (33) reported that dominant negative Smad3 inhibited the induction of guanine exchange factor NET and the activation of Rho activity. Edlund et al. (29) found that Smad7 was required for activation of Cdc42, a small GTPase that plays a role in cytoskeleton rearrangement. It is possible that in our system Smad signaling also plays a role in regulating RhoA function. The rapid production of GTP-bound RhoA by TGF-β, however, suggests that the initial activation of RhoA is likely to be Smad-independent. Whether later RhoA-specific effects are dependent on Smad signaling remains to be determined.

Other evidence suggests that the Rho pathway may also regulate Smad activation. Recent studies by Kamaraju et al. (32) revealed that the Rho/ROCK pathway affects the linker region phosphorylation of Smad2/3 in human breast carcinoma cells. The linker region of Smad2/3 was shown to contain phosphorylation sites for MAPK or Cdk2/4. However, C-terminal phosphorylation of Smad2 and Smad3 and Smad-mediated promoter activation were not altered by ROCK inhibitor in these cells (32). Smads are known to be activated by receptor kinases through C-terminal phosphorylation, followed by nuclear translocation and regulation of gene transcription (12). Our studies demonstrate for the first time that RhoA directly modulates Smad activity during TGF-β-induced VSMC differentiation. Dominant negative RhoA as well as RhoA inhibitor C3 exotoxin inhibited Smad2 and Smad3 C-terminal phosphorylation or nuclear translocation. DNRhoA and C3 exotoxin also significantly attenuated Smad-mediated gene activation. Furthermore, constitutively activated RhoA enhanced Smad-mediated promoter activity. Most importantly, VSMC marker expression was regulated simultaneously with the modulation of Smad activity, indicating that RhoA may regulate VSMC differentiation through altering Smad activation. Together, these data demonstrate that RhoA can influence Smad signaling either by modulating phosphorylation in the linker region (32) or in the C terminus. The effects may be
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cell type-specific and may serve to help RhoA signaling modulate TGF-β function. The fact that Smad and RhoA signals are both necessary for VSMC marker induction further suggests that both pathways must be activated for smooth muscle differentiation from neural crest cells (Fig. 9).

RhoA function in VSMC contractility appears to be independent of its role in modulating Smad activity. This hypothesis is supported by our data showing that ROCK inhibitor Y27632 blocked SMC marker gene activation (Fig. 1) and abolished SMC contraction (Fig. 2) but did not affect Smad phosphorylation (Fig. 4A). In addition to Rho kinase ROCK, another key downstream target of RhoA is a member of the protein kinase PKN family (17). Because the affinity of Y27632 for ROCK is at least 20–30 times higher than that for PKN (34), Y27632 likely has little effect on PKN while sufficiently blocking ROCK activity. ROCK appears to be important for SMC contraction (14, 15), whereas PKN could mediate RhoA function in modulating Smad activation. PKN has been found to play a role in TGF-β-induced promoter activities of SMC marker genes (17). An alternative pathway for RhoA function is myocardin/SRF-dependent mechanism (15). How RhoA, TGF-β, and myocardin pathways converge to regulate VSMC differentiation and contractility, and whether RhoA modulates Smad activity through PKN remains to be determined. Future work will be directed toward understanding the mechanism by which RhoA induces VSMC contraction and the role of RhoA in modulating Smad activity, especially how RhoA interacts with other components in the Smad signaling pathway.

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