Smad2 and Myocardin-Related Transcription Factor B Cooperatively Regulate Vascular Smooth Muscle Differentiation From Neural Crest Cells

Wei-Bing Xie,* Zuguo Li,* Ning Shi, Xia Guo, Junning Tang, Wenjun Ju, Jun Han, Tengfei Liu, Erwin P. Bottinger, Yang Chai, Pedro A. Jose, Shi-You Chen

Rationale: Vascular smooth muscle cell (VSMC) differentiation from neural crest cells (NCCs) is critical for cardiovascular development, but the mechanisms remain largely unknown.

Objective: Transforming growth factor-β (TGF-β) function in VSMC differentiation from NCCs is controversial. Therefore, we determined the role and mechanism of a TGF-β downstream signaling intermediate Smad2 in NCC differentiation to VSMCs.

Methods and Results: By using Cre/loxP system, we generated a NCC tissue–specific Smad2 knockout mouse model and found that Smad2 deletion resulted in defective NCC differentiation to VSMCs in aortic arch arteries during embryonic development and caused vessel wall abnormality in adult carotid arteries where the VSMCs are derived from NCCs. The abnormalities included 1 layer of VSMCs missing in the media of the arteries with distorted and thinner elastic lamina, leading to a thinner vessel wall compared with wild-type vessel. Mechanistically, Smad2 interacted with myocardin-related transcription factor B (MRTFB) to regulate VSMC marker gene expression. Smad2 was required for TGF-β–induced MRTFB nuclear translocation, whereas MRTFB enhanced Smad2 binding to VSMC marker promoter. Furthermore, we found that Smad2, but not Smad3, was a progenitor-specific transcription factor mediating TGF-β–induced VSMC differentiation from NCCs. Smad2 also seemed to be involved in determining the physiological differences between NCC-derived and mesoderm-derived VSMCs.

Conclusions: Smad2 is an important factor in regulating progenitor-specific VSMC development and physiological differences between NCC-derived and mesoderm-derived VSMCs. (Circ Res. 2013;113:e76-e86.)

Key Words: myocardin-related transcription factor B ■ neural crest ■ Smad2 ■ smooth muscle differentiation

Vascular smooth muscle cells (VSMCs) play a pivotal role in angiogenesis and vasculogenesis during embryonic development. Abnormal VSMC differentiation or modulation leads to the development of several prominent cardiovascular diseases, including congenital heart diseases, atherosclerosis, hypertension, and restenosis, after angioplasty.1–3 During embryonic development, VSMCs originate from ≥8 progenitors, including neural crest cells (NCC), secondary heart field, somites, mesoangioblasts, proepicardium, splanchnic mesoderm, mesothelium, and various stem cells.4 Interestingly, VSMCs from different progenitors display distinct functional properties. For instance, neural crest–derived VSMCs and mesoderm-derived VSMCs show dramatically different responses to the stimulation of morphogenetic factors such as transforming growth factor-β (TGF-β).2 In fact, these 2 VSMC subtypes exhibit opposite responses to TGF-β regarding cell growth, extracellular protein expression, and gene promoter activation.5 Furthermore, mesoderm-derived VSMCs were unable to rescue the outflow tract defects observed in neural crest–ablated chicken embryos.6 Studies from several laboratories demonstrate that embryonically distinct subpopulations of VSMCs are not functionally equivalent and that VSMCs within different vascular beds use distinct cis-elements and control regions to regulate VSMC marker gene activation.7–11 These studies suggest that VSMC differentiation is controlled by different intracellular mechanisms among distinct VSMC subtypes. The molecular mechanisms governing the progenitor-specific regulation of VSMC differentiation, however, remain largely unknown.

Original received May 30, 2013; revision received June 27, 2013; accepted July 1, 2013. In June 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.67 days.

From the Department of Physiology and Pharmacology, University of Georgia, Athens, GA (W.-B.X., Z.L., N.S., X.G., J.T., T.L., S.-Y.C.); School of Basic Medical Sciences, Southern Medical University, Guangzhou, China (W.-B.X., Z.L., T.L.); Division of Nephrology, Department of Internal Medicine, University of Michigan, Ann Arbor, MI (W.J.); Center for Craniofacial Molecular Biology, University of Southern California Ostrow School of Dentistry, Los Angeles, CA (J.H., Y.C.); Division of Nephrology, Department of Medicine, Mount Sinai School of Medicine, New York, NY (E.P.B.); and Department of Medicine, University of Maryland School of Medicine, Baltimore, MD (P.A.J.).

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.113.301921/-/DC1.

*These authors contributed equally to this study.

Correspondence to Shi-You Chen, PhD, Department of Physiology and Pharmacology, University of Georgia, 501 D.W. Brooks Dr, Athens, GA 30602. E-mail sc229@uga.edu

© 2013 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.113.301921
Xie et al  

Sma2 in VSMC Differentiation of Neural Crest  
e77

In This Issue, see p 945  
Editorial, see p 946

The NCC is a multipotent cell population that develops in the dorsal neural tube and then migrates throughout the embryo and differentiates into numerous tissue types. In response to poorly understood developmental cues, a subpopulation of NCCs called cardiac NCCs migrate ventrally and populate aortic arch arteries and cardiac outflow tract. The NCC derivatives then differentiate into VSMCs forming the media layers of the aortic arch, ascending aorta, and carotid arteries. Notch signaling and myocardin-related transcription factor (MRTF) B have been shown to be important for VSMC differentiation of NCC. Although TGF-β induces smooth muscle cell (SMC) differentiation from NCC in vitro, the in vivo findings are controversial. Wurdak et al reported that knockout of TGF-β type II receptor in NCCs abolishes the development of VSMC at embryonic day 10.5. However, a subsequent study using the same strategy to mutate TGF-β type II receptor in NCCs failed to identify VSMC defects, which is likely because of an in vivo compensatory mechanism or the use of a different TGF-β type II receptor–floxed mouse line, as discussed by the authors. Endoglin, a TGF-β accessory protein, also seems to be required for VSMC differentiation from NCCs, but endoglin function is independent of TGF-β/Smad signaling.

Knockout of a type I receptor of TGF-β family Alk2 leads to outflow tract and VSMC defects. However, Alk2 is a receptor of bone morphogenetic proteins that signal through Smad1, Smad3, and Smad5. Therefore, the role of TGF-β/Smad2 signaling in NCC differentiation and the progenitor-specific regulator for VSMC differentiation from NCC have not been established.

In the present study, we identified Smad2, one of the TGF-β downstream signaling molecules, as a critical regulator of VSMC differentiation from NCCs. NCC-specific knockout of Smad2 leads to a defective VSMC differentiation in aortic arch arteries and reduced layers of VSMCs and distortion of elastic lamina in the media of adult mouse carotid arteries. Smad2 interacts with MRTFB to activate VSMC marker promoter activity. Mechanistically, Smad2 mediates MRTFB nuclear translocation, whereas MRTFB enhances Smad2 binding to VSMC marker promoter. Although both Smad2 and Smad3 are important intermediates for TGF-β signaling, Smad2 plays a more important role than Smad3 in VSMC differentiation from NCCs, whereas Smad3 is more important than Smad2 in VSMC differentiation from mesenchymal progenitors. Furthermore, Smad2 also seems to be involved in determining the physiological differences between NCC-derived and mesoderm-derived VSMCs.

### Methods

#### Cell Culture and Reagents

Neural crest Monc-1 cells and mesenchymal C3H10T1/2 (10T1/2) cells were cultured as previously described. Smad3 expression plasmids were previously described. Smad2 expression plasmid was a generous gift from Dr Ying Zhang. MRFB expression plasmid was provided by Dr Joseph Miano. Smooth muscle α-actin (α-SMA) and SM22α promoter–luciferase constructs were previously described.

#### Generation of Smad2 NCC-Specific Knockout Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Georgia. Smad2-floxed (Smad2fl/fl) and Wnt1-Cre mice were previously described. To generate Smad2 deletion in NCCs, Wnt1-Cre male mice were crossed with Smad2fl/fl mice to produce Wnt1-Cre;Smad2fl/+ mice. The male and female Wnt1-Cre;Smad2fl/+ mice were then crossed to produce Wnt1-Cre;Smad2fl/fl mice. Wnt1-Cre;Smad2fl/fl littermates serve as controls. For in vivo fate mapping of NCCs, Wnt1-Cre mice heterozygous for Smad2-floxed allele (Wnt1-Cre;Smad2fl/+ mice) were mated with Smad2fl/fl mice carrying a ROSA26 Cre reporter allele, which expresses β-galactosidase on Cre-mediated recombination. Genotyping and X-gal staining for NCC fate mapping were performed as described.

#### Histomorphometric Analysis and Immunohistochemistry Staining

Mouse embryos on embryonic day 11.5 or carotid arteries from adult mice were fixed with 4% paraformaldehyde and paraffin-embedded. Embryos or vessels were cut by serial sectioning (5 μm). The sections were stained with hematoxylin and eosin for structural observation or Elastica van Gieson for elastin. For immunohistochemistry, sections were rehydrated, blocked with 5% goat serum, permeabilized with 0.01% Triton X-100 in phosphate-buffered saline, and incubated with rabbit anti-Smad2 or α-SMA antibody overnight at 4°C, followed by incubation with hors eradish peroxidase–conjugated secondary antibody. α-SMA staining was visualized using the Vectastain method using NovaRed as a substrate. Smad2 staining was visualized using Vectastain ABC-AP kit by following the manufacturer’s protocol (Vector Laboratories). The vessel sections were counterstained with hematoxylin.

#### Preparation of Small Hairpin RNA

**Adenoviral Vector**

Adenoviral small hairpin RNA (shRNA) target sequences were ATG GAG CTG GTG GAG AA for MRTFB and TGG TGT TCA ATG GCA TAC TAT for Smad2. Double-stranded DNAs coding MRTFB or Smad2 shRNAs were cloned into pRNA-H1.1/Adeno shuttle vector (Genscript). Adenovirus expressing Flag-tagged MRTFB was constructed by cloning human MRTFB cDNA into the XhoI site of pShuttle-IRESCMV-HRGFP-1 (Agilent) and was confirmed by sequencing. Adenovirus was packaged in 293 cells (Agilent) and purified by CsCl gradient ultracentrifugation as previously described. Viral particle titer was determined by plaque assay. Adenovirus expressing Smad3 shRNA was previously described. For adenoviral transduction, Monc-1 cells were transduced with 100 multiplicity of infection of adenovirus expressing control, MRTFB, or Smad2 shRNA for 24 to 48 hours.

#### Reverse-Transcription Polymerase Chain Reaction and Quantitative Polymerase Chain Reaction

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer’s instruction. cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad). Reverse-transcription polymerase chain reaction (PCR) was performed using Bio-Rad C1000 thermal cycler. Quantitative PCR (qPCR) was performed in MX3000P qPCR machine using SYBR Green qPCR Mastermix (Agilent). The primer sequences were as follows: Smad2, 5′-CCG GCC GAT GCA TTC CCT CTT GTT GCT-3′ (forward) and 5′-GCA GAA CTT GCT CGT GCT-3′ (reverse); and Smad3, 5′-CTG GAG CTA GAT GCT CAA ATG TTT TG-3′ (forward) and 5′-GCA GAA AAT TCC TGG TTG TT-3′ (reverse). The primers used for VSMC markers were described previously.
Western Blotting
Monc-1 cells were lysed in radio-immunoprecipitation assay lysis buffer. Carotid arteries were homogenized using a tissue homogenizer in radio-immunoprecipitation assay buffer containing protease inhibitor mix (Sigma). Samples were separated on SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were incubated at 4°C for 16 hours with antibodies against α-SMA (Millipore), SM22α (Abcam), Smad2 (Cell Signaling), or α-tubulin (Sigma) in blocking buffer containing 5% milk, followed by incubation with horseradish peroxidase–conjugated secondary antibody (Sigma).

Transient Transfection and Luciferase Assay
Monc-1 and 10T1/2 cells were plated at 2×10⁵ cells/well in 12-well plates and incubated at 37°C in a 5% CO₂ incubator until 80% confluence. Cells were then transiently transfected (in triplicate) with LipofectAMINE LTX Plus according to the manufacturer’s recommendation (Invitrogen, Carlsbad, CA). Luciferase assay was performed as described previously.21,36

Coimmunoprecipitation Assay and Immunoblotting Analysis
Monc-1 cells were transduced with adenovirus expressing Flag-tagged MRTFB followed by vehicle or TGF-β treatment for 2 hours. Cells were then lysed with ice-cold lysis buffer containing protease inhibitor mix. The lysates were incubated with IgG or Flag antibody for 1 hour and then protein-A/G agarose at 4°C for 12 hours. The immunoprecipitates were pelleted, washed, and subjected to immunoblotting using Flag or Smad2 antibody as described previously.34,37

Immunofluorescent Staining
Adenovirus expressing Flag-tagged MRTFB was transduced with adenovirus expressing green fluorescent protein or Smad2 shRNA into Monc-1 cells for 2 days, followed by vehicle or 5 ng/mL of TGF-β treatment for 2 hours. The cells were then fixed and incubated with rabbit anti-Flag antibody (Sigma), followed by incubation with tetramethylrhodamine isothiocyanate-conjugated secondary goat anti-rabbit IgG as described previously.5,36 MRTFB nuclear translocation was observed with fluorescent microscopy (Nikon). The nuclei were stained with 4',6-diamidino-2-phenylindole.

Chromatin Immunoprecipitation Assay
Chromatin immunoprecipitation assays were performed as described previously.14 Monc-1 cells were transduced with adenovirus expressing green fluorescent protein, MRTFB, or MRTFB shRNA, followed by TGF-β treatment for 2 hours. Chromatin complexes were immunoprecipitated with 3 µg Smad2 antibody or IgG (negative control). Semi-qPCR and qPCR were performed to amplify the SM22α promoter region containing functional Smad-binding element using the following primer set: 5′-TCT GCC CCA GCC CAG ACA CC-3′ (forward) and 5′-CCC ACA GCC CTT CTG CTC CC-3′ (reverse).

Statistical Analysis
All values are expressed as mean±SEM. Data were analyzed using ANOVA with pairwise comparisons between groups. P<0.05 was considered statistically significant.

Figure 1. Smad2 neural crest cell (NCC)–specific deletion blocked vascular smooth muscle cell (VSMC) differentiation in aortic arch arteries. A, Smad2 deletion in NCCs blocked VSMC marker gene expression in aortic arch arteries. Sagittal sections of wild-type (Smad2+/+, left) or Smad2 NCC–specific deleted embryonic day 11.5 mouse embryos (Smad2−/−, right) were stained with hematoxylin and eosin (H&E) or were immunostained with smooth muscle α-actin (α-SMA) or Smad2 antibody as indicated. α-SMA staining in dorsal aorta (DA) and in aortic arch arteries III, IV, and VI is indicated by arrows, respectively. Smad2 deletion in NCCs blocked α-SMA expression in aortic arch arteries but not in dorsal aorta (×40). B, Higher magnification of α-SMA expression in wild-type or Smad2 NCC–deleted mouse aorta and the fourth aortic arch artery (AAA). α-SMA was significantly blocked in the AAA with Smad2 deletion in NCC but not in the aorta (×400). C, Smad2 deletion in NCCs did not alter NCC migration. Embryonic day 11.5 wild-type (top) and Smad2 NCC–specific deleted (bottom) mouse embryos carrying R26R reporter were stained with X-gal as described in Methods (left). The embryos were then paraffin-embedded and sectioned to observe LacZ staining in DA and aortic arch arteries III, IV, and VI as indicated (right). Smad2 deletion did not affect NCC migration to aortic arch arteries.
Results

NCC-Specific Deletion of Smad2 Blocked VSMC Differentiation in Aortic Arch Arteries but Did Not Affect NCC Migration

To address the role of Smad2 in VSMC differentiation from NCCs in vivo, we used the Cre/loxp system to conditionally delete Smad2 gene in NCCs. Crossing Wnt1-Cre mouse with Smad2-floxed mouse generated Smad2 knockout in NCCs and NCC-derived tissues, including VSMCs (Online Figure 1). At embryonic day 11.5, Smad2 mutant embryos did not display obvious malformations. However, Smad2 NCC-specific deletion blocked VSMC differentiation in aortic arch arteries III, IV, and VI (Figure 1A). In the wild-type mouse embryos (Figure 1A, left), expression of VSMC marker α-actin (α-SMA) was detected in both dorsal aorta and aortic arch arteries (Figure 1A, left middle). Smad2 deletion diminished α-SMA expression in aortic arch arteries but not in dorsal aorta (Figure 1A, right middle). It is known that aortic arch VSMCs are derived from NCCs, whereas dorsal aortic VSMCs are derived from mesoderm. Higher magnification of dorsal aorta and the fourth aortic arch artery images showed that the numbers of cells expressing α-SMA were significantly reduced in Smad2-deleted fourth aortic arch artery (Figure 1B), suggesting a defective VSMC differentiation. This differentiation impairment was not because of a defect in NCC migration because Smad2-deficient NCCs migrated correctly to the cardiac outflow tract, as evaluated by in vivo fate mapping using the ROSA26 Cre reporter mouse line (Figure 1C). These data demonstrate that Smad2 is important for VSMC differentiation from NCCs in vivo.

Smad2 Deficiency in NCCs Caused Reduced VSMC Layers and Distorted and Thinner Elastic Lamina, Resulting in a Thinner Vessel Wall in Adult Mouse Carotid Arteries

Although NCC-specific deletion of Smad2 impaired VSMC differentiation in aortic arch arteries, most Smad2 mutant mice were born normally and can survive until adulthood. We then examined whether Smad2 deletion causes defects in adult blood vessels in which VSMCs are derived from NCCs. We found that the vessel walls of Smad2-deficient carotid arteries were thinner than those in the wild-type mice (Figure 2A–2D). A detailed examination revealed that 1 layer of VSMCs in the media disappeared in Smad2-deleted arteries, which led to a significant reduction of VSMC numbers in the tunica media of Smad2-deleted vessels (Figure 2B and 2D). Furthermore, Smad2-deficient vessels exhibited abnormality in elastic lamina, including reduced layer and content and distorted structure, compared with wild-type vessels (Figure 2B). These data demonstrate that defective VSMC differentiation because of Smad2 deletion significantly alters the normal vessel wall structure.

To determine whether the cells in Smad2-deleted artery are...
SMCs, we stained the expression of α-SMA in the artery sections and found that Smad2−/− cells in the arteries displayed a reduced level of α-SMA (Figure 2E), suggesting that they retained SMC phenotype. To determine whether these SMCs were derived from NCCs or other origins, we detected Cre protein expression because Cre was driven by Wnt1 promoter and thus only was expressed in NCCs or NCC-derived cells in our system. We found that Smad2−/− SMCs expressed Cre, indicating that SMCs in the Smad2−/− artery were derived from NCCs.

**Smad2 and MRTFB Cooperatively Regulate VSMC Marker Gene Expression in NCCs**

Previous studies have shown that MRTFB is specifically important for VSMC differentiation from NCCs.12,13 To determine the mechanisms underlying Smad2 function in VSMC differentiation from NCCs, we explored whether Smad2 and MRTFB coordinately regulate NCC differentiation to VSMC. MRTFB is induced in Monc-1 cells 2 hours after TGF-β treatment (Online Figure II). We did not explore the function of myocardin or MRTFA in NCC differentiation because MRTFA and myocardin were not induced in Monc-1 cells until 48 hours of TGF-β induction (Online Figure II), a time after the activation of SMC marker genes, including smooth muscle myosin heavy chain.23

![Image](http://circres.ahajournals.org/)

**Figure 3.** Smad2 and myocardin-related transcription factor B (MRTFB) cooperatively regulated vascular smooth muscle cell (VSMC) marker gene expression in neural crest cells (NCCs). A and B, Smad2 and MRTFB synergistically enhanced transforming growth factor (TGF)-β-induced VSMC marker gene promoter activity; pcDNA, Smad2, Smad2 and MRTFB cDNA was cotransfected individually or in combination with smooth muscle α-actin (α-SMA; A) or SM22α promoter (B) as indicated followed by vehicle (Ctrl) or TGF-β treatment (5 ng/mL) for 16 hours. Luciferase assays were performed. Luciferase activity was normalized to renilla activity. *P<0.01 compared with Smad2 or MRTFB alone–transfected group with TGF-β treatment in (A) and (B), respectively. C and D, Smad2 and MRTFB synergistically enhanced TGF-β-induced mRNA expression of VSMC markers. Monc-1 cells were transfected with pcDNA, Smad2, Smad3, or MRTFB cDNA individually or in combination, followed by vehicle (Ctrl) or TGF-β treatment (5 ng/mL) for 8 hours. α-SMA (C) and SM22α (D) mRNA expression was detected by quantitative polymerase chain reaction and normalized to GAPDH expression. *P<0.01 compared with Smad2 or MRTFB alone–transfected group treated with TGF-β in both (C) and (D), respectively.

By coexpressing MRTFB with Smad2 or Smad3 in Monc-1 cells, we found that MRTFB and Smad2, but not Smad3, cooperatively upregulated TGF-β–induced α-SMA and SM22α promoter activities compared with the effect of individual proteins (Figure 3A and 3B). MRTFB and Smad2 also synergistically enhanced the endogenous mRNA expression of both α-SMA and SM22α genes (Figure 3C and 3D). MRTFB also seems to increase VSMC marker promoter activity in the absence of TGF-β (Figure 3A and 3B), probably because the transfected promoters were located in the cytoplasm where a certain level of MRTFB was present when the cells were in the basal status.

To determine whether MRTFB is required for Smad2 function in VSMC differentiation, we used adenovirus-expressing shRNA to knock down MRTFB expression in Monc-1 cells. These cells were then cotransfected with Smad2 and SMC marker gene

![Image](http://circres.ahajournals.org/)

**Figure 4.** Myocardin-related transcription factor B (MRTFB) was essential for Smad2-mediated vascular smooth muscle cell (VSMC) marker expression in neural crest cells. A and B, MRTFB was required for Smad2-mediated VSMC marker gene transcription. Monc-1 cells were transduced with adenovirus expressing green fluorescent protein (Ad-GFP) or MRTFB small hairpin RNA (Ad-shMRTFB) and cotransfected with smooth muscle α-actin (α-SMA; A) or SM22α (B) promoter construct and pcDNA or Smad2 cDNA as indicated, followed by vehicle (Ctrl) or transforming growth factor (TGF)-β treatment (5 ng/mL) for 16 hours. Luciferase assays were performed. Luciferase activity was normalized to renilla activity. *P<0.01 compared with Ad-GFP groups with TGF-β. C–E, MRTFB was required for Smad2-mediated VSMC marker mRNA and protein expression. Monc-1 cells were treated as in (A) and (B) but without promoter transfection. α-SMA (C) and SM22α (D) mRNA expression were detected by quantitative polymerase chain reaction and normalized to GAPDH expression. Their protein expression was detected by Western blot (E). *P<0.01 compared with Ad-GFP group treated with TGF-β for both α-SMA (C) and SM22α (D), respectively. F, Quantitative analysis of protein expression in (E) by normalization to α-tubulin. *P<0.01 compared with Ad-GFP group for both α-SMA and SM22α, respectively.
Smad2 Is Essential for MRTFB Nuclear Translocation in NCCs

Consistent with a previous report, MRTFB is predominantly localized in the cytoplasm of Monc-1 cells (Figure 6A). However, MRTFB nuclear translocation is essential for the activation of VSMC endogenous gene transcription. We found that TGF-β-induced MRTFB nuclear translocation. To determine whether Smad2 and Smad3 affect MRTFB nuclear translocation, we knocked down Smad2 and Smad3 in Monc-1 cells using shRNA, respectively. As shown in Figure 6A, deficiency of Smad2, but not Smad3, blocked TGF-β-induced MRTFB nuclear translocation. To quantify the nuclear translocation of MRTFB, we separated cytoplasmic and nuclear proteins of vehicle-treated and TGF-β-treated cells (Figure 6B and 6C). TGF-β induction increased the nuclear portion to 80% of the total MRTFB (Figure 6B and 6C). Smad2 knockdown by shRNA, however, significantly reduced the nuclear MRTFB level to 50% of the total in TGF-β-treated cells (Figure 6B and 6C). Previous studies have shown that MRTF nuclear translocation was mediated by cytoskeleton reorganization. Therefore, we sought to determine whether Smad2 mediates MRTFB nuclear translocation through facilitating F-actin formation. As shown in Figure 6D, knockdown of Smad2 blocked TGF-β-induced F-actin formation, suggesting that Smad2 mediated MRTFB nuclear translocation by regulating F-actin formation. These data demonstrate that Smad2 regulates MRTFB function by mediating its nuclear translocation.

MRTFB Physically Interacts With Smad2 and Facilitates Smad2 Binding to VSMC Marker Gene Promoters

In addition to being a TGF-β signaling molecule, Smad2 is also a transcription factor regulating TGF-β target genes. To further determine the mechanism by which Smad2 regulates VSMC differentiation from NCCs, we examined whether Smad2 physically interacts with MRTFB. Because high-quality MRTFB antibody is not available, we used adenoviral vector to express Flag-tagged MRTFB in Monc-1 cells and performed coimmunoprecipitation of MRTFB with endogenous Smad2. To determine whether Smad2 or Smad3 is required for MRTFB function in VSMC differentiation from NCCs, we used adenovirus-expressing shRNA to knock down Smad2 or Smad3 in Monc-1 cells. The cells were then cotransfected with MRTFB and VSMC marker gene promoters. We found that knockdown of Smad3 weakly inhibited, whereas knockdown of Smad2 strongly inhibited, MRTFB-induced α-SMA and SM22α promoter activities in both vehicle-treated and TGF-β-treated cells (Figure 4A and 4B). Importantly, knockdown of MRTFB also significantly blocked Smad2-induced mRNA (Figure 4C and 4D) and protein expression (Figure 4E and 4F) of both α-SMA and SM22α genes. These data demonstrate that MRTFB is essential for Smad2 function in VSMC differentiation from NCCs.

To determine whether Smad2 or Smad3 is required for MRTFB function in VSMC differentiation from NCCs, we used adenovirus-expressing shRNA to knock down Smad2 or Smad3 in Monc-1 cells. The cells were then cotransfected with MRTFB and VSMC marker gene promoters. We found that knockdown of Smad3 weakly inhibited, whereas knockdown of Smad2 strongly inhibited, MRTFB-induced α-SMA and SM22α promoter activities in both vehicle-treated and TGF-β-treated cells (Figure 5A and 5B). However, knockdown of Smad2, but not Smad3, significantly blocked MRTFB-induced mRNA (Figure 5C and 5D) and protein expression (Figure 5E and 5F) of both α-SMA and SM22α genes. Similar to the results in Figure 3A and 3B, MRTFB induced the activity of exogenously introduced VSMC marker gene promoters in the absence of TGF-β, because transfected promoters were located in the cytoplasm where MRTFB was present in the basal status (see Figure 6 for MRTFB cellular location). These data demonstrate that Smad proteins are essential for the full function of MRTFB in VSMC differentiation from NCCs. Smad2 seems to be more important than Smad3 in mediating MRTFB function in NCCs.

Figure 5. Smad2 was essential for myocardin-related transcription factor B (MRTFB)-mediated vascular smooth muscle cell (VSMC) marker expression in neural crest cells (NCCs). A and B, Smad2 was required for MRTFB-mediated VSMC marker gene transcription. Monc-1 cells were transduced with adenovirus expressing green fluorescent protein (Ad-GFP), Smad2 (Ad-shS2), or Smad3 small hairpin RNA (Ad-shS3) and cotransfected with smooth muscle α-actin (α-SMA; A) or SM22α (B) promoter construct and pcDNA or MRTFB cDNA as indicated, followed by vehicle (Ctrl) or transforming growth factor (TGF)-β treatment (5 ng/mL) for 16 hours. Luciferase assays were performed. Luciferase activity was normalized to renilla activity. *P<0.01, #P=0.043, &P=0.048 compared with Ad-GFP groups with TGF-β for both (A) and (B), respectively. C and D, Smad2 was required for MRTFB-mediated VSMC marker mRNA expression. Monc-1 cells were treated as in (A) and (B) but without promoter transfection, α-SMA (C) and SM22α (D) mRNA expression were detected by quantitative polymerase chain reaction and normalized to GAPDH expression. **P<0.05 and @P<0.05 compared with Ad-GFP group treated with TGF-β for both (C) and (D). E, Smad2 was required for MRTFB-mediated VSMC marker protein expression. Monc-1 cells were transduced with Ad-GFP or Ad-shS2 as indicated and treated with TGF-β. α-SMA and SM22α protein expression were detected by Western blot. F, Quantitative analysis of the protein expression in (E) by normalization to α-tubulin. *P<0.05 compared with Ad-GFP group for both α-SMA and SM22α, respectively.
proteins. As shown in Figure 7A, endogenous Smad2 formed a complex with Flag-tagged MRTFB, indicating a physical interaction between Smad2 and MRTFB. TGF-β induction seemed to enhance Smad2–MRTFB interaction. To confirm MRTFB–Smad2 interaction, we reversed the order of antibodies used for coimmunoprecipitation assay and immunoblotting by using Smad2 antibody to coimmunoprecipitate MRTFB. We found that MRTFB was in the complex immunoprecipitated by Smad2 antibody as shown in the blotting with Flag antibody (Figure 7B). TGF-β treatment clearly enhanced their interaction (Figure 7B). These data demonstrate that Smad2 physically interacts with MRTFB in Monc-1 cells.

Smad2 regulates VSMC marker gene transcription by binding to VSMC marker promoters. To determine how MRTFB regulated Smad2 activity, we tested whether MRTFB alters Smad2 interaction with SM22α promoter in Monc-1 cells. We found that in TGF-β–treated Monc-1 cells, MRTFB overexpression significantly increased Smad2 binding to SM22α promoter, whereas shRNA knockdown of MRTFB completely blocked Smad2 binding to the promoter (Figure 7C and 7D). These data demonstrate that MRTFB serves as a Smad2 coactivator to mediate and enhance Smad2 binding to VSMC marker promoters, leading to activation of VSMC marker gene transcription.

**Smad2 Is More Important Than Smad3 in Mediating VSMC Differentiation From NCCs, Whereas Smad3 Is Important in the Differentiation From Mesenchymal Progenitors**

TGF-β signaling is transduced predominantly by both Smad2 and Smad3. Previous studies from our and other laboratories have shown that Smad3 plays an important role in TGF-β–induced VSMC differentiation from mesenchymal progenitor cells.21,41,42 Our present study demonstrates that Smad2 is essential for VSMC
differentiation from NCCs. These results led us to hypothesize that Smad2 and Smad3 are involved in progenitor-specific regulation of VSMC differentiation. To test this hypothesis, we knocked down the expression of endogenous Smad2 and Smad3 individually in Monc-1 cells (Online Figure III) and then transected the cells with SM22α promoter. Luciferase assay showed that knockdown of Smad3 slightly inhibits both the basal and TGF-β-induced SM22α promoter activity but did not alter TGF-β-induced fold induction of the promoter activity. However, knockdown of Smad2 completely abolishes the promoter function (Figure 8A). Furthermore, knockdown of Smad2 blocked TGF-β-induced protein expression of endogenous α-SMA and SM22α, whereas knockdown of Smad3 had no effect (Figure 8B and 8C). These data demonstrate that Smad2, but not Smad3, plays a major role in TGF-β-induced VSMC differentiation from NCCs. Interestingly, when we overexpressed Smad2 and Smad3 individually in mesenchymal progenitor C3H10T1/2 cells, Smad3, but not Smad2, enhanced TGF-β-induced SM22α promoter activity (Figure 8D). These data, together with the previously published results, suggest that Smad proteins serve as progenitor-specific regulators in VSMC fate determination or VSMC differentiation from at least some of the progenitors, that is, Smad2 regulates VSMC differentiation from NCCs, whereas Smad3 controls VSMC differentiation from mesenchymal progenitors.

Because VSMCs from different progenitors display distinct functional properties and because our results suggest that Smad2 is important for NCC, but not mesenchymal progenitor, differentiation to VSMC, we sought to determine whether Smad2 is involved in the physiological differences between NCC-derived and mesoderm-derived VSMCs. To this end, we crossed SM22α-Cre mice with Smad2fl/fl mice to knock out Smad2 in VSMCs derived from NCC and the mesoderm. To test whether Smad2 affects the properties of these VSMCs, we isolated the media layers of carotid arteries (SMCs originate from mesoderm) that contain mainly SMCs, prepared the total RNA and proteins, and detected the expression of matrix metalloproteinase-2 and proliferating cell nuclear antigen, a well-known cell proliferation marker. It seemed that the expression levels of these 2 proteins were different in NCC-derived and mesoderm-derived VSMCs (Figure 8E–8I). Importantly, Smad2 was essential for MMP2 mRNA and protein expression in NCC-derived VSMCs but not in mesoderm-derived VSMCs (Figure 8E, 8G, and 8H). However, Smad2 seemed not to affect proliferating cell nuclear antigen expression in NCC-derived VSMCs, but was required for proliferating cell nuclear antigen expression in mesoderm-derived VSMCs (Figure 8F, 8G, and 8I), which is consistent with the critical role of Smad2 in VSMC differentiation of NCCs but not the mesenchymal progenitor. Collectively, our data suggest that Smad2 is involved in defining the physiological difference between NCC-derived and mesoderm-derived VSMCs.

**Discussion**

By using tissue-specific knockout mouse model, we demonstrate for the first time that Smad2 is critical for VSMC differentiation from NCCs in vivo. The results are very specific because Smad2 deletion in NCCs only blocks VSMC marker gene expression in aortic arch arteries but not in the dorsal aorta. Although previous studies have tried to link Smad2 function with NCC differentiation to VSMCs or endothelial cells, the results are limited to the detection of Smad2 expression or use of in vitro systems. Our results represent the first direct evidence showing the critical role of Smad2 in VSMC differentiation from NCCs in vivo. Interestingly, Smad proteins seem to play a role in the progenitor-specific regulation of VSMC differentiation. Although both Smad2 and Smad3 mediate TGF-β function, Smad3 is important for the differentiation of mesenchymal-originated progenitors, whereas Smad2 plays a
were treated similarly as described in (A), with shGFP/TGF-β treatment. Luciferase assays were performed. Luciferase activity was normalized to renilla activity. α small hairpin RNA for 2 days and transfected with SM22α cells (NCCs). Monc-1 cells were transduced with adenovirus expressing green fluorescent protein (shGFP), Smad2 (shS2), or Smad3 (shS3) and protein expression. A, Smad2 was more important than Smad3 in regulating VSMC marker gene promoter activity in neural crest cells (NCCs). Monc-1 cells were cotransfected with SM22α-SMA and SM22α-α-SMA promoter and pcDNA, Smad2, or Smad3 cDNA, followed by vehicle (Ctrl) or TGF-β treatment. Luciferase assays were performed. Luciferase activity was normalized to renilla activity. *P<0.001 and **P<0.05 compared with shGFP/TGF-β-treated groups. B, Smad2, but not Smad3, was essential for VSMC marker protein expression in NCCs. Monc-1 cells were treated similarly as described in (A) without promoter transfection. Smooth muscle α-actin (α-SMA) and SM22α protein expression was detected by Western blot. C, Quantification of protein expression shown in (B) by normalization to α-tubulin. *P<0.01 compared with adenovirus expressing GFP (Ad-GFP) group for α-SMA and SM22α, respectively. D, Smad3 was more important than Smad2 in VSMC differentiation from mesenchymal progenitors. 10T1/2 cells were cotransfected with SM22α promoter and pcDNA, Smad2, or Smad3 cDNA, followed by vehicle (Ctrl) or TGF-β treatment. Luciferase assays were performed. Luciferase activity was normalized to renilla activity. *P<0.01 compared with pcDNA/TGF-β-treated group. E and F, Smad2 was deleted in smooth muscle cells (SMCs) by crossing Smad2-floxed mice with SM22α-cre mice. Carotid (CA) or thoracic arterial (TA) media layers from wild-type (WT) littermates or SMC-specific Smad2 knockout (KO) mice were homogenized, and total RNA was extracted. Quantitative polymerase chain reaction was performed to detect matrix metalloproteinase-2 (MMP2) and proliferating cell nuclear antigen (PCNA) expression (F), and fold inductions are shown. *P<0.01 compared with the corresponding WT SMCs. G, MMP2 and PCNA protein expression in WT and Smad2 KO SMCs were examined by Western blot. H and I, MMP2 (H) and PCNA expression (I) were normalized to α-tubulin. *P<0.01 compared with the corresponding WT SMCs.

Figure 8. Progenitor-specific roles of Smad2 and Smad3 in regulating vascular smooth muscle cell (VSMC) marker gene transcription and protein expression. A, Smad2 was more important than Smad3 in regulating VSMC marker gene promoter activity in neural crest cells (NCCs). Monc-1 cells were transduced with adenovirus expressing green fluorescent protein (shGFP), Smad2 (shS2), or Smad3 (shS3) small hairpin RNA for 2 days and transfected with SM22α promoter, followed by vehicle (Ctrl) or transforming growth factor (TGF)-β (5 ng/mL) treatment. Luciferase assays were performed. Luciferase activity was normalized to renilla activity. *P<0.001 and **P<0.05 compared with shGFP/TGF-β-treated groups. B, Smad2, but not Smad3, was essential for VSMC marker protein expression in NCCs. Monc-1 cells were treated similarly as described in (A) without promoter transfection. Smooth muscle α-actin (α-SMA) and SM22α protein expression was detected by Western blot. C, Quantification of protein expression shown in (B) by normalization to α-tubulin. *P<0.01 compared with adenovirus expressing GFP (Ad-GFP) group for α-SMA and SM22α, respectively. D, Smad3 was more important than Smad2 in VSMC differentiation from mesenchymal progenitors. 10T1/2 cells were cotransfected with SM22α-SMA and SM22α-α-SMA promoter and pcDNA, Smad2, or Smad3 cDNA, followed by vehicle (Ctrl) or TGF-β treatment. Luciferase assays were performed. Luciferase activity was normalized to renilla activity. *P<0.01 compared with pcDNA/TGF-β-treated group. E and F, Smad2 was deleted in smooth muscle cells (SMCs) by crossing Smad2-floxed mice with SM22α-Cre mice. Carotid (CA) or thoracic arterial (TA) media layers from wild-type (WT) littermates or SMC-specific Smad2 knockout (KO) mice were homogenized, and total RNA was extracted. Quantitative polymerase chain reaction was performed to detect matrix metalloproteinase-2 (MMP2) and proliferating cell nuclear antigen (PCNA) expression (F), and fold inductions are shown. *P<0.01 compared with the corresponding WT SMCs. G, MMP2 and PCNA protein expression in WT and Smad2 KO SMCs were examined by Western blot. H and I, MMP2 (H) and PCNA expression (I) were normalized to α-tubulin. *P<0.01 compared with the corresponding WT SMCs.

major role in VSMC differentiation from NCCs. Smad3 knockout mice are viable and show no abnormality in cardiovascular or VSMC development, indicating that loss of Smad3 function in these mice may be compensated by Smad2. However, Smad3 is unable to compensate for the loss of Smad2 function because Smad2 knockout mice die during gastrulation before the cardiovascular system starts to develop. Our study further demonstrates that Smad3 cannot compensate for the loss of Smad2 function in NCC differentiation to VSMCs in vivo because deletion of Smad2 in NCCs resulted in VSMC defects.

Both TGF-β signaling and MRTFB play critical roles in regulating VSMC differentiation from NCCs. It is unclear, however, how these 2 independent pathways contribute to the same biological process. Our study has provided a novel mechanism underlying the functions of TGF-β signaling and MRTFB in VSMC differentiation from NCCs. It seems that MRTFB and TGF-β downstream signaling protein Smad2 cooperatively regulate VSMC marker gene transcription. In fact, Smad2 and MRTFB are interdependent in regulating VSMC marker gene expression. Knockdown of Smad2 impairs MRTFB regulation of VSMC gene expression, whereas knockdown of MRTFB blocks Smad2 activity in this process. More importantly, we have identified a novel function of Smad2 in promoting MRTFB nuclear translocation. Smad2 likely binds to MRTFB on TGF-β induction to facilitate MRTFB nuclear translocation. Smad2 physically interacts with MRTFB, and this interaction is enhanced by TGF-β. Importantly, MRTFB–Smad2 interaction not only results in transporting MRTFB into nuclei but also facilitates Smad2 binding to VSMC marker promoter, which is essential for TGF-β induction of VSMC differentiation.

Previous studies have shown that a muscle-specific actin-binding protein named striated muscle activator of Rho signaling activates serum response factor by inducing the nuclear translocation of MRTFA and MRTFB, and the striated muscle activator of Rho signaling–dependent nuclear import of MRTFs requires RhoA and actin polymerization. Smad2 seems to mediate MRTFB nuclear translocation through regulating actin polymerization. Because TGF-β activates RhoA and RhoA crosstalks with Smad...
signaling in Monc-1 cells, it is likely that RhoA, STARS, and Smad2 work together to coordinate MRTFB nuclear translocation. Interestingly, TGF-β increases MRTFA nuclear localization but has no effect on MRTFB nuclear location in 10T1/2 cells. The present study shows that TGF-β induces MRTFB nuclear translocation in Monc-1 cells, which is likely because of a progenitor-specific effect. 10T1/2 cells are derived from mesoderm and are considered mesenchymal progenitors, in which Smad2 is not important for their differentiation to VSMCs. However, in NCCs, Smad2 plays a major role in differentiation and mediates MRTFB nuclear translocation. These results further support the novel concept that Smad proteins may be important mediators for progenitor-specific regulation of VSMC differentiation.

Smad2 knockout in NCCs not only causes defective VSMC differentiation during embryonic development but also leads to abnormal structure in the wall of the adult vasculature where VSMCs are derived from NCCs. These abnormalities, that is, less VSMCs, distorted and thinner elastic lamina, and thinner vessel walls, are likely because of the defective VSMC differentiation, leading to reduced numbers of VSMCs in the tunica media. VSMCs have been shown to produce elastin, the major component of the elastic fibers. It is unknown, however, how Smad2 deletion causes abnormal elastin expression in VSMCs and distorts the structure of the elastic lamina in the tunica media of the carotid artery, which requires an extensive future study. Although it is well-recognized that SMCs derived from different origins display distinct physiological properties, the molecular mechanisms controlling these differences or regulatory factors involved in defining these variances remain largely unknown. Smad2 seems to be one of these factors because Smad2 not only plays distinct roles in regulating SMC gene expression in different progenitors but also discretely regulates the properties of NCC-derived and mesoderm-derived VSMCs, including the production of extracellular matrix protein regulator MMP2 and proliferation-related gene proliferating cell nuclear antigen. Although the physiological properties are not extensively studied, our results provide initial evidence that Smad2 is involved in defining the different properties of VSMCs derived from NCC and mesoderm.

In summary, we have found that Smad2 is essential for VSMC differentiation during embryonic development and also leads to abnormal structure in the wall of the adult vasculature where VSMCs are derived from NCCs. These abnormalities, that is, less VSMCs, distorted and thinner elastic lamina, and thinner vessel walls, are likely because of the defective VSMC differentiation, leading to reduced numbers of VSMCs in the tunica media. VSMCs have been shown to produce elastin, the major component of the elastic fibers. It is unknown, however, how Smad2 deletion causes abnormal elastin expression in VSMCs and distorts the structure of the elastic lamina in the tunica media of the carotid artery, which requires an extensive future study.

Sources of Funding
This study was supported by grants from National Institutes of Health (HL093429 and HL107526 to S. Y. Chen).

Disclosures
None.

References
Myocardin-related transcription factor B (MRTFB) regulates VSMC


What New Information Does This Article Contribute?

- Transforming growth factor (TGF)-β signaling plays an important role in vascular smooth muscle cell (VSMC) differentiation. TGF-β receptors and its accessory protein, endoglin, are implicated in VSMC differentiation from neural crest cells (NCCs).
- During embryonic development, VSMCs originate from >8 different progenitors. VSMCs from different progenitors display distinct functional properties and use distinct cis-elements and control regions to regulate their marker gene activation.
- Myocardin-related transcription factor B (MRTFB) regulates VSMC differentiation from NCCs. MRTFB is normally located in cytoplasm and translocates into the nucleus to exert its function as a coactivator.

What Is Known?

Novelty and Significance

- TGF-β downstream signaling protein Smad2 is essential for VSMC differentiation from NCCs. Targeted deletion of Smad2 in NCC results in VSMC defects in aortic arch arteries and abnormal carotid arterial structure. Am J Physiol Cell Physiol. 2007;292:C1031–C1037.
- Previous studies have shown that both TGF-β signaling and MRTFB play essential roles in VSMC differentiation from NCC. Here, we used tissue-specific Smad2 knockout mouse model, as well as molecular and cellular analyses, to identify novel mechanisms by which TGF-β regulates NCC differentiation to VSMC. Our results show that TGF-β downstream signaling intermediate Smad2 is essential for VSMC differentiation from NCCs. We found that Smad2 deletion in NCC results in defective VSMC differentiation in aortic arch arteries during embryonic development and blood vessel wall abnormalities in adult arteries where VSMCs are derived from NCCs. The abnormalities include a missing layer of VSMCs and distorted elastic fibers, resulting in a thinner vessel wall compared with the wild-type vessel. It seems that Smad2 interacts with MRTFB to regulate VSMC marker gene expression. Smad2 mediates TGF-β-induced MRTFB nuclear translocation, whereas MRTFB enhances Smad2 binding to VSMC marker promoter. Importantly, our studies indicate that Smad2 is a progenitor-specific regulator for VSMC differentiation from NCCs, whereas Smad3 is more important than Smad2 in mediating VSMC differentiation from mesenchymal progenitors. These findings suggest that TGF-β signaling is involved in the determination of VSMC diversity.
Smad2 and Myocardin-Related Transcription Factor B Cooperatively Regulate Vascular Smooth Muscle Differentiation From Neural Crest Cells
Wei-Bing Xie, Zuguo Li, Ning Shi, Xia Guo, Junming Tang, Wenjun Ju, Jun Han, Tengfei Liu, Erwin P. Bottinger, Yang Chai, Pedro A. Jose and Shi-You Chen

Circ Res. 2013;113:e76-e86; originally published online July 1, 2013; doi: 10.1161/CIRCRESAHA.113.301921

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/113/8/e76

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/07/01/CIRCRESAHA.113.301921.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental Materials

Smad2 and MRTFB cooperatively regulate vascular smooth muscle differentiation from neural crest cells

Wei-Bing Xie1, Zuguo Li1, Ning Shi1, Xia Guo1, Junming Tang1, Wenjun Ju2, Jun Han3, Tengfei Liu1, Erwin P Bottinger4, Yang Chai3, Pedro A Jose5, Shi-You Chen1

1Department of Physiology & Pharmacology, University of Georgia, Athens, GA 30602; 2Division of Nephrology, Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109; 3Center for Craniofacial Molecular Biology, University of Southern California Ostrow School of Dentistry; 4Division of Nephrology, Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029; 5Department of Medicine, University of Maryland School of Medicine, Baltimore, MD 21201

I. Detailed Methods

Cell Culture and reagents
Monc-1 and C3H10T1/2 (10T1/2) cells were cultured as previously described.1,2 Smad3 expression plasmids were previously described.3,4 Smad2 expression plasmid was a generous gift from Dr. Ying Zhang.5 MRTFB expression plasmid was provided by Dr. Joseph Miano. Smooth muscle α-actin (α-SMA) and SM22α promoter-luciferase constructs were previously described.1,6

Generation of Smad2 NCC-specific knockout mice
All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Georgia. Smad2-floxed (Smad2fl/fl), Wnt1-Cre, and SM22α-Cre mice were previously described.7,8 To generate Smad2 deletion in NCCs or SMC, Wnt1-Cre or SM22α-Cre male mice were cross-breed with Smad2fl/fl mice to produce Wnt1-Cre;Smad2fl/+ or SM22α-Cre;Smad2fl/+ mice. The male and female Wnt1-Cre;Smad2fl/+ or SM22α-Cre;Smad2fl/+ mice were then cross-bred to produce Wnt1-Cre;Smad2fl/fl or SM22α-Cre;Smad2fl/fl mice. SM22α-Cre or Wnt1-Cre;Smad2+/+ littermates serve as control. For in vivo fate mapping of NCCs, Wnt1-Cre mice heterozygous for Smad2-floxed allele (Wnt1-Cre;Smad2fl/+ or SM22α-Cre;Smad2fl/) were mated with Smad2fl/fl mice carrying a ROSA26 Cre reporter (R26R) allele, which expresses β-galactosidase (β-Gal) upon Cre-mediated recombination.9 Genotyping and X-gal staining for NCC fate mapping were performed as described.7,10-12 X-gal staining was performed as follows: 11.5 days mouse embryos of Wnt1-Cre;Smad2+/+;R26R and Wnt1-Cre;Smad2fl/fl;R26R were stained whole mount for X-gal using LacZ Tissue Staining kit (InvivoGen) according to the manufacturer’s instruction. Briefly, the embryos were fixed in fixative solution on ice for 2-3 h and washed in PBS at 4°C overnight, and then incubated with staining solution at 37°C for 2-4 hours followed by rinsing with PBS for three times. The development of blue color was observed using a microscope. To observe the inside staining, the embryos were dehydrated and embedded in paraffin. 5 μm sections were cut and counterstained with 0.1% nuclear fast red.

Histomorphometric analysis and immunohistochemistry (IHC) staining
11.5 days of mouse embryos or carotid arteries from adult mice were fixed with 4% paraformaldehyde and paraffin-embedded. Embryos or vessels were cut by serial sectioning (5μm). The sections were stained with hematoxylin and eosin for structural observation or Elastica van Gieson for elastin. For IHC, sections were rehydrated, blocked with 5% goat serum and permeabilized with 0.01% Triton X-100 in PBS, and incubated with rabbit anti-Smad2 or α-SMA antibody overnight at 4°C followed by incubation
with HRP-conjugated secondary antibody. α-SMA staining was visualized by the Vectastain method using NovaRed as a substrate. Smad2 staining was visualized by using Vectastain ABC-AP kit by following the manufacturer’s protocol (Vector Laboratories). The vessel sections were counterstained with hematoxylin.

**Preparation of shRNA adenoviral vector**

Adenoviral short hairpin RNA (shRNA) target sequences were ATG GAG CTG GTG GAG AAG AA for MRTFB and TGG TGT TCA ATC GCA TAC TAT for Smad2. Double-stranded DNAs coding MRTFB shRNAs were cloned into pRNAT-H1.1/Adeno shuttle vector (Genscript). Adenovirus expressing Flag-tagged MRTFB was constructed by cloning human MRTFB cDNA into the Xho I site of pShuttle-IRES-hrGFP-1 (Agilent) and was confirmed by sequencing. Adenovirus was packaged in 293 cells (Agilent) and purified by CsCl2 gradient ultracentrifugation as previously described.13,14 Viral particle titer was determined by plaque assay. Adenovirus expressing Smad3 shRNA was previously described.15 For adenoviral transduction, Monc-1 cells were transduced with 100 moi of adenovirus expressing control, MRTFB, or shRNA for 24 to 48 hours.

**Reverse Transcription-PCR (RT-PCR) and quantitative PCR (qPCR)**

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer’s instruction. cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad). RT-PCR was performed using Bio-Rad C1000 thermal cycler. qPCR was performed in MX3000P qPCR machine using SYBR Green qPCR Mastermix (Agilent). The primer sequences were as follows: Smad2: 5’-CCG GCT GAA CTG TCT CCT AC-3’ (forward) and 5’-GCA GAA CCT CTC CGA GTT TG-3’ (reverse); Smad3: 5’-CTG GGC CTA CTG TCC AAT GT-3’ (forward) and 5’-GCA GCA AAT TCC TGG TTG TT-3’ (reverse). The primers used for VSMC markers were described previously.2,16

**Western blotting**

Neural crest Monc-1 cells were lysed in RIPA lysis buffer. Carotid arteries were homogenized using a tissue homogenizer in RIPA buffer containing protease inhibitor mix (Sigma). Samples were separated on SDS-polyacrylamide gels, and electrotransferred onto PVDF membranes (Bio-Rad). The membranes were incubated for 16 h at 4°C with antibodies against α-SMA (Millipore), SM22α (Abcam), Smad2 (Cell Signaling), or α-tubulin (Sigma) in blocking buffer containing 5% milk followed by incubation with HRP-conjugated secondary antibody (Sigma).

**Transient transfection and luciferase assay**

Monc-1 and 10T1/2 cells were plated at 2 x 10^5 cells/well in 12-well plates and incubated at 37°C in a 5% CO2 incubator until 80% confluence. Cells were then transiently transfected (in triplicate) with LipofectAMINE LTX Plus according to the manufacturer's recommendation (Invitrogen, Carlsbad, CA). Luciferase assay was performed as described previously.1,17

**Co-immunoprecipitation assay (Co-IP) and immunoblotting analysis**

Monc-1 cells were transduced with adenovirus expressing Flag-tagged MRTFB followed by vehicle or TGF-β treatment. Cells were then lysed with ice-cold lysis buffer containing protease inhibitor mix. The lysates were incubated with IgG or Flag antibody for one hour and then protein-A/G agarose at 4°C for 12 hours. The immunoprecipitates were pelleted, washed and subjected to immunoblotting using Flag or Smad2 antibody as described previously.15,18

**Immunofluorescent staining and confocal microscopy**

Adenovirus expressing Flag-tagged MRTFB was transduced with adenovirus expressing GFP or Smad2 shRNA into Monc-1 cells for two days followed by vehicle or 5 ng/ml of TGF-β treatment for 2 hours. The cells were then fixed and incubated with rabbit anti-Flag antibody (Sigma), followed by incubation
with TRITC-conjugated secondary goat anti-rabbit IgG as described previously. MRTFB nuclear translocation was observed with confocal microscopy. DAPI stains nuclei.

**Chromatin immunoprecipitation assay (ChIP)**

ChIP assays were performed as described previously (34). Monc-1 cells were transduced with adenovirus expressing GFP, MRTFB, or MRTFB shRNA followed by TGF-β treatment for 2 hours. Chromatin complexes were immunoprecipitated with 3 µg Smad2 antibody or IgG (negative control). Semiquantitative PCR and qPCR were performed to amplify the SM22α promoter region containing functional Smad binding element (SBE) using the following primer set: 5’- TCT GCC CCA GCC CAG ACA CC -3’ (forward) and 5’- CCC ACA GCC CTT CTG CTC CC -3’ (reverse).

**Statistical analysis**

All values are expressed as mean ± SEM. Data were analyzed using ANOVA with pairwise comparisons between groups. P values < 0.05 were considered statistically significant.

**References:**

II. Supplemental Data

**Online Figure I.** (A) Genotyping of Smad2-floxed mice mated with Wnt1-Cre mice was detected by RT-PCR using mouse tail biopsy. 471bp band indicates homozygous Smad2-floxed mice. 276bp band indicates wild type mice. Appearance of two bands indicate heterozygous Smad2-floxed mice. (B) Western blot confirmed that Smad2 protein was expressed in wild type (WT) but not in the Smad2 KO carotid arteries.

**Online Figure II.** Myocardin (Myocd), MRTFA, and MRTFB protein expression in TGF-β-treated Monc-1 cells. Monc-1 cells were treated with TGF-β (5 ng/ml) for the times indicated. Western blot were performed. Myocd and MRTFA were induced 48 h after TGF-β treatment while MRTFB was induced 2 h after the treatment.
Online Figure III. shRNA knockdown efficiency of Smad2 and Smad3. Smad2 (A) and Smad3 (B) were knocked down by Smad2 (shS2) and Smad3 shRNA (shS3), respectively. The knockdown efficiency was detected by qPCR.