Postnatal Deletion of the Type II Transforming Growth Factor-β Receptor in Smooth Muscle Cells Causes Severe Aortopathy in Mice

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Objective—Prenatal deletion of the type II transforming growth factor-β (TGF-β) receptor (TBRII) prevents normal vascular morphogenesis and smooth muscle cell (SMC) differentiation, causing embryonic death. The role of TBRII in adult SMC is less well studied. Clarification of this role has important clinical implications because TBRII deletion should ablate TGF-β signaling, and blockade of TGF-β signaling is envisioned as a treatment for human aortopathies. We hypothesized that postnatal loss of SMC TBRII would cause aortopathy.

Approach and Results—We generated mice with either of 2 tamoxifen-inducible SMC-specific Cre (SMC-CreER<sup>T2</sup>) alleles and homozygous floxed Tgfr2 alleles. Mice were injected with tamoxifen, and their aortas examined 4 and 14 weeks later. Both SMC-CreER<sup>T2</sup> alleles efficiently and specifically rearranged a floxed reporter gene and efficiently rearranged a floxed Tgfr2 allele, resulting in loss of aortic medial TBRII protein. Loss of SMC TBRII caused severe aortopathy, including hemorrhage, ulceration, dissection, dilation, accumulation of macrophage markers, elastolysis, abnormal proteoglycan accumulation, and aberrant SMC gene expression. All areas of the aorta were affected, with the most severe pathology in the ascending aorta. Cre-mediated loss of SMC TBRII in vitro ablated both canonical and noncanonical TGF-β signaling and reproduced some of the gene expression abnormalities detected in vivo.

Conclusions—SMC TBRII plays a critical role in maintaining postnatal aortic homeostasis. Loss of SMC TBRII disrupts TGF-β signaling, acutely alters SMC gene expression, and rapidly results in severe and durable aortopathy. These results suggest that pharmacological blockade of TGF-β signaling in humans could cause aortic disease rather than prevent it.

Key Words: alleles ■ aortic aneurysm ■ homeostasis ■ tamoxifen ■ transforming growth factors

Transforming growth factor-β (TGF-β) signaling in cultured smooth muscle cells (SMCs) or SMC precursors regulates cell growth and migration. TGF-β signaling also promotes synthesis of SMC differentiation markers and extracellular matrix proteins. Loss of SMC TGF-β signaling during development prevents SMC differentiation and matrix synthesis, causing severe vascular abnormalities and embryonic lethality. Without question, SMC TGF-β signaling is essential for normal embryogenesis. However, the role of TGF-β signaling in adult aortic SMC is less well established, and the critical question of whether SMC TGF-β signaling is salutary or pathogenic remains controversial. Salutary roles for adult SMC TGF-β signaling are suggested by animal studies showing that cardiovascular overexpression of TGF-β increases matrix synthesis and prevents aneurysm growth and that systemic blockade of TGF-β exacerbates angiotensin II–induced aortic aneurysms. In contrast, pathogenic effects of adult SMC TGF-β signaling are suggested by apparent increases in aortic SMC TGF-β signaling in association with human aortopathies (Marfan, Loeys–Dietz, Shprintzen–Goldberg syndromes, familial thoracic aortic aneurysms, and dissections associated with mutations in TGFBR2) and in mouse models of these diseases. These reports, along with evidence that TGF-β antagonism achieved by injection of a neutralizing anti-TGF-β antibody could prevent aortopathy in Fbn1-deficient mice, spawned the hypothesis that TGF-β signaling in aortic SMC is pathogenic, and prompted clinical trials aimed at preventing aortopathy with a drug (Losartan) that is hypothesized to block TGF-β signaling.

See cover image

We used genetically modified mice to test whether disruption of physiological TGF-β signaling by SMC-targeted deletion

Received on: March 8, 2014; final version accepted on: October 14, 2015.
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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.115.306573/-/DC1.
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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.115.306573
expression from both SMC-Cre alleles appeared SMC-restricted both with and without tamoxifen: no nonvascular blue cells were seen grossly or microscopically in SMC poor tissues, including heart and skeletal muscle. There was no Cre activity (no blue cells) in tamoxifen-injected SMC-CreER\textsuperscript{2}\textsuperscript{+/0} R26R\textsuperscript{+} mice of either line.

To more precisely compare SMC-Cre activity in the 2 lines, we measured LacZ mRNA in the aortic media (see below) of tamoxifen-treated Cre\textsuperscript{+/-} R26R\textsuperscript{+/-} mice of both lines. Expression of LacZ mRNA in the 2 lines was equivalent and far above the background levels in tamoxifen-injected Cre\textsuperscript{0/0} R26R\textsuperscript{+/-} controls (Figure III in the online-only Data Supplement). For the remainder of our experiments, we compared tamoxifen-treated Cre\textsuperscript{+/-} R26R\textsuperscript{+/-} Tgfbr2\textsuperscript{floxed/flox} mice with tamoxifen-treated Cre\textsuperscript{0/0} R26R\textsuperscript{+/-} Tgfbr2\textsuperscript{floxed/flox} mice. This design compares Tgfbr2\textsuperscript{floxed/flox} SMC to Tgfbr2\textsuperscript{floxed/flox} SMC, eliminates concerns about background Cre activity, and controls for exposure to tamoxifen, an agent that affects the vasculature directly.\textsuperscript{26-32}

Measurement of TBIIR Protein and Tgfbr2 mRNA in Aortic SMC
Because vascular Cre activity is limited to medial SMC (Figure I in the online-only Data Supplement)—and because our primary goal is to determine the role of Tgfbr2 in SMC—we focused our biochemical analyses on aortic medial tissue. We accomplished this by removing the aortic luminal endothelium, separating the media from the adventitia (Figure IV in the online-only Data Supplement), and extracting protein and RNA from the entire aortic media. Measurement of SMC markers in RNA extracted from either media or adventitia confirmed significant enrichment of SMC in medial tissue (Figure V in the online-only Data Supplement). Injection of tamoxifen in Cre\textsuperscript{+/-} mice induced substantial reduction of TBIIR protein in the aortic media of both lines (Figure I). Surprisingly, however, when we measured Tgfbr2 mRNA using primers internal to the floxed Tgfbr2 exon 4 region, we found that aortic medial Tgfbr2 mRNA was either increased or unchanged in tamoxifen-injected Cre\textsuperscript{+/-} mice of both lines (Figure VI in the online-only Data Supplement). To confirm that Cre had excised the expected region, we sequenced null allele amplicons produced by polymerase chain reaction of tail DNA. The amplicons contained discontinuous sequences of Tgfbr2 separated by a loxP site (Figure II in the online-only Data Supplement), confirming Cre-mediated deletion of Tgfbr2. As an additional control, we used exon 2–specific primers to measure Tgfbr2 mRNA. Again, we found increased Tgfbr2 mRNA after tamoxifen injection, this time in Cre\textsuperscript{0/0} mice of both lines (Figure VI in the online-only Data Supplement). These results suggest compensatory increases in Tgfbr2 mRNA, but loss of TBIIR protein in SMC of tamoxifen-injected Cre\textsuperscript{+/-} mice.

Loss of TBIIR in Aortic SMC Disrupts Aortic Structure
Six-week-old Cre\textsuperscript{+/-} and Cre\textsuperscript{0/0} mice of both lines were injected with tamoxifen daily for 5 days, euthanized 4 or 14 weeks after the last injection, and their aortas examined grossly and

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Similar Cre Recombinase Activity in 2 Lines of SMC-CreER\textsuperscript{2} Mice
We compared the inducibility, efficiency, and tissue specificity of Cre recombinase activity in 2 lines of mice with SMC-targeted inducible Cre alleles (Acta2-CreER\textsuperscript{2} and Myh11-CreER\textsuperscript{2}).\textsuperscript{26,27} We also report in vitro data suggesting that cell-autonomous alterations in SMC TGF-\beta alterations in SMC TGF-\beta signaling are the primary cause of aortopathy in mice with SMC-specific loss of TBRII. Our results reveal a critical role for adult aortic SMC TGF-\beta signaling and suggest a cautious approach toward pharmacological strategies that block SMC TGF-\beta signaling in humans.
Aortic wall morphology, evaluated on transverse sections, was abnormal in Cre<sup>0/0</sup> mice of both lines. Four weeks after tamoxifen injection, the lengths of the internal elastic lamina microscopically. Therefore, mice were either 11 weeks or 21 weeks of age when euthanized. At 4 weeks, gross aortic hemorrhage (defined as focal reddish-brown discoloration of the aortic wall) was present in ascending aorta (AscA)/aortic arch, descending thoracic aorta (DTA), or abdominal aorta (AA) of 32 of 84 (38%) Cre<sup>0/0</sup> mice (19/58 Acta2-Cre<sup>+/0</sup> and 13/26 Myh11-Cre<sup>+/0</sup>) and in none of the Cre<sup>0/0</sup> mice (Figure 2; Figure VII in the online-only Data Supplement). Hemorrhage was more common in the AscA and arch in both lines (31% of all Cre<sup>0/0</sup> mice) than in the DTA or AA (7% in each area and P<0.02). Therefore, aortic hemorrhage begins shortly after deletion of Tgfb2, resolves, and does not seem to recur.

To determine whether aortic disease progressed over time, we examined aortas from mice euthanized 14 weeks after tamoxifen injection. At this time point, PAUs were present in 13 of 14 (93%) Cre<sup>0/0</sup> mice of both lines (almost exclusively in the AscA) and in 0 of 14 Cre<sup>0/0</sup> mice (P<0.0001). On gross examination, aortic discoloration suggestive of hemorrhage was noted in 10 of 18 Cre<sup>0/0</sup> mice (56%) of both lines (most commonly in the AscA and arch; Table II in the online only Data Supplement) and in 0 of 14 Cre<sup>0/0</sup> controls (P<0.002). Surprisingly, microscopical examination of aortic sections of these mice (including all 10 aortas judged on gross examination to have aortic hemorrhage) revealed no free red blood cells in the aortic wall. To determine whether the absence of red cells in aortic media of Cre<sup>0/0</sup> mice 14 weeks after tamoxifen injection was because of partial resolution of IMH, we stained AscA sections with Prussian blue. Sections of AscA from 9 of 14 (64%) of Cre<sup>0/0</sup> mice of both lines stained with Prussian blue compared with 0 of 14 AscA of Cre<sup>0/0</sup> controls (P=0.0006; Table II and Figure VIIIC and VIIIF in the online-only Data Supplement). Four weeks after tamoxifen injection, Prussian blue staining was also present in 6 of 15 (40%) Cre<sup>0/0</sup> mice of both lines and 0 of 13 controls (Table II in the online only Data Supplement; P<0.02). Therefore, aortic hemorrhage begins shortly after deletion of Tgfb2, resolves, and does not seem to recur.

Aortic wall morphology, evaluated on transverse sections, was abnormal in Cre<sup>0/0</sup> mice of both lines. Four weeks after tamoxifen injection, the lengths of the internal elastic lamina

**Figure 1.** Knockdown of type II transforming growth factor-β (TGF-β) receptor (TBRII) protein in aortic medial smooth muscle cell. Four weeks after treatment with tamoxifen, protein was extracted from the aortic media of Tgfb2<sup>Cre<sup>ERT2</sup>lox/lox</sup> mice that either lacked (Acta2-Cre<sup>0/0</sup> and Myh11-Cre<sup>0/0</sup>) or expressed a CreER<sup>ERT2</sup> transgene (Acta2-Cre<sup>+/0</sup> and Myh11-Cre<sup>+/0</sup>). Western blots were probed with an antibody to TBRII or β-actin. Each lane is from a single mouse.

**Figure 2.** Knockdown of Tgfb2 in aortic smooth muscle cells causes aortopathy. Four weeks after treatment with tamoxifen, aortas were harvested from Tgfb2<sup>Cre<sup>ERT2</sup>lox/lox</sup> mice that either lacked (Acta2-Cre<sup>0/0</sup>; A, C, E, G, I, and K) or expressed an Acta2-CreER<sup>ERT2</sup> transgene (Acta2-Cre<sup>+/0</sup>; B, D, F, H, J, and L). A-F, Representative pictures of ascending aorta (AscA) and arch, descending thoracic aorta (DTA), and abdominal aorta (AA). G-L, Representative transverse sections. H, Arrowhead: penetrating aortic ulcer. H, J, and L, Arrows: aortic intramural hematoma or dissection (J). A-F, Ruler is in mm. G-L, Scale bar, 100 μm.
and external elastic lamina of Cre+/0 aortas were increased marginally, if at all, compared with Cre0/0 controls of both lines. However, aortic medial thickness and medial cross-sectional area were increased in AscA, DTA, and AA of both lines (14%–39% and 14%–52%, respectively; Figure 3). All of these increases were significant for the Acta2-Cre line; increases of similar magnitude in the Myh11-Cre line were less uniformly significant, probably because of smaller group sizes (6 versus 7–9). Fourteen weeks after tamoxifen injection, the internal elastic lamina and external elastic lamina lengths of Cre+/0 aortas were significantly increased compared with Cre0/0 controls (6%–32% and 6%–31%, respectively; Figure 4), and lumens seemed larger (Figure 5; Figure IX in the online-only Data Supplement). However, increased medial thickness in Cre+/0 versus Cre0/0 mice at 14 weeks (8%–21%) was less pronounced than at 4 weeks, and it was confined to the AscA and AA (Figure 4). In contrast, increases in medial area of Cre+/0 aortas were relatively larger at 14 versus 4 weeks (40%–60% versus Cre0/0 controls) and they were also confined to the AscA and AA (Figure 4). Therefore, SMC Tgfbr2 deletion causes early medial thickening followed by vessel dilation and relative medial thinning. That is, Cre+/0 aortas dilate between 4 and 14 weeks, whereas their medial thickness changes relatively little (Cre+ medias thicken less from 4 to 14 weeks than Cre0 medias). Medial area in 14-week Cre+/0 aortas is increased primarily because of a larger circumference.
not increased thickness. Overall, morphological changes are far greater in the AscA and AA than in the DTA, especially at 14 weeks. Abnormal aortic morphology (eg, significantly increased internal elastic lamina and external elastic lamina lengths) was present even in the most caudal AA sections, taken at the level of the left renal artery. The infrarenal AA was not analyzed histologically.

Loss of TBRII in Aortic SMC Results in Elastin Damage, Increased Macrophage Markers, Cell Proliferation, and Matrix Accumulation

To further characterize aortic damage and potential healing responses after loss of SMC TBRII, we stained sections to detect elastin fibers, macrophage markers, cell proliferation, and extracellular matrix proteins. We measured elastin damage by counting elastic lamina breaks in AscA sections from both lines. Elastin breaks were 2- to 5-fold more frequent in Cre<sup>0/0</sup> mice of both lines, at both time points (Figure X in the online-only Data Supplement; P<0.01). In both lines, elastin breaks increased over time (P<0.01).

We assessed the presence of macrophage markers by staining aortic sections for Mac-2. For both lines, Mac-2 staining was rare in sections from Cre<sup>0/0</sup> mice but common in sections from Cre<sup>0/0</sup> mice. The percentage of Mac-2-positive medial area was increased significantly in all 3 regions (3- to 8-fold; P<0.05 except for 14-week DTA and AA in the Myh11-Cre line; Figure XI in the online-only Data Supplement). Mac-2 staining was typically located near an area of medial damage/hemorrhage, identified by the presence of PAU, IMH, or Prussian blue stain on adjacent slides. An independent measure of macrophage marker accumulation, CD68 mRNA, was 4- to 5-fold more abundant in extracts from Cre<sup>0/0</sup> versus Cre<sup>0/0</sup> aortic media (P<0.01 for both lines; Figure 6).

We measured cell proliferation in the AscA by pulse labeling with BrdU just before the 4-week time point. For both lines, BrdU-positive nuclei were rare in Cre<sup>0/0</sup> aortas and significantly increased in Cre<sup>0/0</sup> aortas (5- to 13-fold increases in total BrdU-positive nuclei; P≤0.01). BrdU-positive cells were most common in the adventitia, with fewer along the luminal surface and even fewer in the media (Figure XII in the online-only Data Supplement).

Movat staining showed increased proteoglycan and collagen deposition in aortas of both lines of Cre<sup>0/0</sup> mice at 4 and 14 weeks. Proteoglycan and collagen accumulation were predominantly in the adventitia and most evident in AscA sections (Figure XIII in the online-only Data Supplement). An observer was blinded to genotype, given Movat-stained AscA sections from Cre<sup>0/0</sup> and Cre<sup>0/0</sup> mice of both lines, and asked to assign Cre genotype based only on extent of blue-green staining indicative of proteoglycan accumulation. The observer correctly genotyped 21 of 28 of the mice euthanized 4 weeks after tamoxifen and 27 of 28 of the mice euthanized 14 weeks after tamoxifen (P=0.006 and P<0.0001, respectively).

We also stained sections from aortas of Acta2-Cre<sup>0/0</sup> and Acta2-Cre<sup>0/0</sup> mice for versican, a proteoglycan that is regulated by TGF-β<sup>33,34</sup> and affects both SMC phenotype and tissue inflammation.<sup>34,35</sup> Versican staining was significantly increased in AscA and AA at both 4 and 14 weeks (4 – 47-fold, P<0.001). Versican staining was typically highest in regions with PAU and adjacent Mac-2 staining (Figure XIV in the online-only Data Supplement).

Loss of TBRII Alters Aortic SMC Gene Expression

To begin to identify the mechanisms through which loss of SMC TBRII affects the aorta, 4 weeks after tamoxifen treatment we isolated total aortic medial RNA and measured mRNA of several classes of genes: (1) genes involved in TGF-β signaling (Tgfb1, Tgfb3, Tgfb1, Tgfb3, and Smad7); (2) genes involved in maintaining the differentiated SMC phenotype (Acta2, Myh11, Tgln, Smtn, and Cnn1); (3) genes involved in extracellular matrix metabolism (Lox, Loxl1, Colla1, Mmp2, Mmp9, and Mmp12); (4) Canonical targets of TGF-β signaling (Ctgf and Serpine1); and (5) Other genes involved in aneurysm formation (Igf1 and Ace).<sup>36</sup>

mRNA encoding Tgfb3, Tgfb2, Tgfb3, and Smad7 were significantly increased after loss of TBRII in SMC from both lines (2- to 4-fold; P≤0.01 for all; Figure 6). Tgfb1 and Tgfb1 mRNA were increased in Acta2-Cre<sup>0/0</sup> mice (P<0.03) and unchanged in Myh11-Cre<sup>0/0</sup> mice. Genes encoding SMC lineage markers (Acta2, Myh11, Tgln, Smtn, and Cnn1) were upregulated in both lines (2- to 4-fold; P<0.05 for all). Among genes involved in extracellular matrix metabolism, Loxl1 and Mmp2 were significantly upregulated in both lines (2- to 4-fold; P≤0.005 and P<0.04, respectively). Lox, Colla1, Mmp9, and Mmp12 were either unchanged or divergent between the lines. Ctgf mRNA decreased significantly in Acta2-Cre<sup>0/0</sup> mice.

Figure 5. Knockdown of Tgfb2 in aortic smooth muscle cell causes aortic dilation. Fourteen weeks after treatment with tamoxifen, aortas were harvested from Tgfb2<sup>lox/lox</sup> mice that either lacked (Acta2-Cre<sup>0/0</sup>, A, C, and E) or expressed an Acta2-CreER<sup>12</sup> transgene (Acta2-Cre<sup>0/0</sup>; B, D, and F). Representative sections of ascending aorta (AscA), descending thoracic aorta (DTA), and abdominal aorta (AA) are shown. Arrowheads: penetrating aortic ulcers. A–F, Scale bar, 200 μm.
and trended lower (P=0.06) in Myh11-Cre0/- mice. Serpine1 mRNA trended lower in both lines (P=0.1). Neither Igf1 nor Ace was significantly increased after loss of TBRII.

TBRII Knockdown in Cultured Aortic SMC Abrogates Canonical and Noncanonical TGF-β Signaling

Aortic SMC from Acta2-Cre0/0Tgbr22lox/2lox mice were established in culture and treated with either AdCMVCre or AdCMVNull. Treatment with AdCMVCre reduced TBRII protein by 95% (P=0.006; Figure 7A and 7B). After treatment of SMC with either AdCMVCre or AdCMVNull, we added recombinant mouse TGF-β1 (1 ng/mL) to the cells. Cre-mediated deletion of Tgbr2 significantly decreased TGF-β1 activation of both canonical (p-Smad2) and noncanonical (p-ERK and p-P38) TGF-β signaling pathways (60%–90% reduction in peak levels; P<0.05 for all; Figure 7A, 7C–7E).

Loss of TBRII Alters Gene Expression in Cultured Aortic SMC

Cre-mediated loss of TBRII altered gene expression in cultured SMC in a pattern similar—although not identical—to that found in medial SMC of tamoxifen-treated SMC-CreERT22/2 mice (Figure XV in the online-only Data Supplement). mRNA encoding Tgfbr1 was significantly increased (2-fold; P<0.001), with trends toward increased expression of Tgfb1, Tgfb2, and Tgfb3. mRNA encoding 3 of 4 SMC lineage markers were also increased (Myh11, Cnn1, and Tagln; 2- to 5-fold; P≤0.01). Levels of Mmp2 and Mmp9 mRNA were far higher in cells lacking TBRII (8- to 10-fold; P≤0.002).

Discussion

We used mice with inducible SMC-targeted Cre alleles and conditional Tgbr2 alleles to delete TBRII in SMC and discover the consequences of loss of physiological SMC TGF-β signaling. Our major findings are (1) loss of TBRII in aortic SMC causes a major disruption of aortic wall structure within 4 weeks, including intramural hemorrhage, wall thickening, ulceration, elastolysis, matrix accumulation, evidence of macrophage infiltration, and increased cell proliferation; (2) at a later time point (14 weeks) aortas that lack SMC TBRII are dilated with relative medial thinning, progressive elastolysis, and evidence of persistent macrophage accumulation; (3) loss of TBRII in aortic SMC in vivo dramatically alters SMC expression of genes encoding proteins involved in TGF-β signaling, contractile function, and extracellular matrix metabolism; and (4) deletion of Tgbr2 in cultured SMC rapidly alters expression of many of the same genes. Taken together, our results suggest that physiological TGF-β signaling plays a critical role in maintaining aortic homeostasis and that loss of physiological TGF-β signaling in SMC causes aortopathy that largely results from cell-autonomous effects on SMC.

We began this study by comparing 2 lines that express SMC-targeted inducible Cre recombinase.26,27 Lines of transgenic mice can lose both transgene activity and tissue specificity over time;7,37 therefore, we characterized both lines

Figure 6. Loss of aortic SMC Tgbr2 alters expression of other genes. 4 weeks after treatment with tamoxifen, aortas were harvested from Tgbr22lox/2lox mice that either lacked (Acta2-Cre0/-; white bars in A; Myh11-Cre0/-; dark gray bars in B) or expressed a CreERT2 transgene (Acta2-Cre0/+; light gray bars in A; Myh11-Cre0/+; black bars in B). Gene expression was measured by quantitative reverse transcriptase-mediated polymerase chain reaction, using RNA isolated from the aortic media. *P<0.05; **P<0.01; ***P<0.001 compared to Cre0/0 controls. n=9 to 10 for Acta2-Cre groups; n=6 for Myh11-Cre groups.
extensively. Both lines efficiently and specifically rearranged the R26R-reporter allele in SMC, with no detectable differences between them. Both lines also produced essentially identical phenotypes when crossed into Tgfb2<sup>flox/flox</sup> mice. Therefore, the theoretical concern that the Myh11-Cre allele must be used to avoid confounding Cre recombinase activity that could be present in non-SMC that express Acta2<sup>Cre</sup>-<sup>allele</sup> must be used to avoid confounding Cre recombinase activity. Therefore, the theoretical concern that the Myh11-Cre allele must be used to avoid confounding Cre recombinase activity that could be present in non-SMC that express Acta2<sup>Cre</sup>-<sup>allele</sup> was not supported by our results. Others have also reported equivocal results with these 2 lines. Importantly, in this other study as well as ours, Cre activity was induced by tamoxifen administration to mice without pre-existing vascular disease. The Acta2-Cre allele should probably be used cautiously in disease states because Acta2 is expressed by non-SMC in diseased vessels. A new aspect of our study, as concerns characterization of these SMC-Cre alleles, is our finding of background (tamoxifen independent) Cre activity in both vascular and nonvascular SMC. This has not been reported by others and should be accounted for in experimental designs, especially when clonal gene deletion could yield an important phenotype.

Our results address the question of whether SMC TGF-β signaling is pathogenic or protective of aortic disease. This question is important because current clinical trials are based on the premise that TGF-β signaling is pathogenic. If this premise is incorrect, these trials are unlikely to have positive outcomes. Arguments that TGF-β signaling in SMC is pathogenic are based on finding elevated pSMAD-2 in human aneurysmal tissue and in mouse models of human aortopathy; however, it is uncertain whether this finding is a cause or an effect of aortopathy, and pSMAD-2 can be increased via pathways independent of TGF-β. Moreover, elevated pSMAD-2 in diseased human aortas is not physically associated with TGF-β ligand accumulation, arguing that TGF-β is not the cause of pSMAD-2 upregulation. Proponents of the TGF-β-driven aortopathy hypothesis bolster their arguments by pointing to a tissue signature of increased TGF-β signaling in diseased human and mouse aortas: elevated expression of connective tissue growth factor, collagen, and PAI-1 (plasminogen activator inhibitor type 1). However, the absence of this signature in diseased aortas is not accepted as evidence that TGF-β activity is not elevated, and therefore not pathogenic. Our results support a protective role for SMC TGF-β signaling by showing conclusively that physiological SMC TGF-β signaling causes severe aortic pathology: IMH, PAU, dilation, and dissection. These results are consistent with other reports of salutary roles for TGF-β signaling in the aortic wall, and decrease enthusiasm for human therapies that target aortic TGF-β signaling.

Our experiments were performed in young mice, raising concern that they may not apply to adult mice or humans. This concern is consistent with a recent report that systemic neutralization of TGF-β in Fbn1-deficient mice worsens aortopathy in young mice but prevents aortopathy in older mice. This report and the observation that deletion of SMC Tgfb2 causes aortic dissection in younger but not in older mice suggests that TGF-β inhibition may be dangerous in youth but protective later on. In a smaller study, however, we found serious aortic pathology (IMH and PAU) in a majority (70%) of mice (n=10) in which SMC Tgfb2 deletion was induced at...
11 to 19 weeks of age (ie, well into adult life; Figure VIII A and VIIID in the online-only Data Supplement and data not shown). This result (in which the prevalence of aortic pathology is likely underestimated because the aortas were sectioned less extensively than in this study) suggests that inhibition of physiological SMC TGF-β signaling may be unsafe at any age.

Our study does not identify the precise pathways through which loss of TBRII in aortic SMC causes aortopathy; however, it does provide some clues. Our in vitro studies show that SMC loss of TBRII causes rapid loss of both canonical and noncanonical TGF-β signaling pathways, and significant alterations in SMC gene expression. Analyses of aortic medial RNA reveal that altered SMC gene expression persists for at least 4 weeks after Tgfb2 deletion, and it is accompanied by gross and microscopic evidence of medial and endothelial damage: loss of endothelial barrier function leading to entry of blood into the media, loss of medial cell cohesiveness leading to ulceration and dissection, and medial elastolysis. Loss of barrier function, medial cell cohesiveness, and elastolysis are all plausible sequelae of the alterations in SMC gene expression that we describe: increased matrix metalloprotease expression and dysregulation of SMC contractile proteins. Medial thickening at 4 weeks could be because of SMC hypertrophy, edema because of increased permeability, macrophage infiltration, IMH that have partially resolved, or a combination of these processes. Because cell proliferation affects only a small minority of medial cells, medial cell proliferation seems to play a minor role. Aortic dilation and PAU 14 weeks after SMC loss of TBRII are likely because of persistent medial SMC dysfunction. Increased Mac-2 abundance throughout the vessel wall and enhanced matrix deposition and cell proliferation in the adventitia probably reflect compensatory healing responses to medial SMC dysfunction. According to this model (Figure XVI in the online-only Data Supplement), aortopathy in mice with SMC-specific loss of TBRII is driven by cell-autonomous events in medial SMC. This model is supported by our in vitro data showing significant changes in aortic SMC gene expression after loss of TBRII, in the absence of exposure to other cell types.

In both the in vitro and in vivo studies presented here, loss of SMC TBRII caused upregulation of SMC expression of TGF-β signaling components: ligands, receptors, and a regulator of canonical TGF-β signaling (Smad7). These findings raise the possibility that compensatory, cell-autonomous upregulation of TGF-β signaling occurs after loss of TBRII, as reported in murine palatal mesenchymal cells with Tgfb2 deletion. 49 Upregulation of TGF-β signaling in TBRII-null SMC—if present—would be congruent with reports of paradoxical upregulation of TGF-β signaling in humans with loss-of-function mutations in TGF-β signaling components. 20,42,50 This paradoxical upregulation of TGF-β signaling is thought to drive these human aortopathies and could potentially explain aortopathy in this study. SMC upregulation of Myh11, Tagln, and Acta2 mRNA would be consistent with increased TGF-β signaling. However, our data are inconsistent with simple upregulation of either canonical or noncanonical TGF-β signaling. In vitro, TBRII-null SMC seems to lose both canonical and noncanonical signaling in response to TGF-β ligand. In vivo, we found no evidence that loss of TBRII leads to upregulated expression of well-established TGF-β-responsive genes (Col1a1, Ctgf, and Serpine1). In summary, although TGF-β signaling components are upregulated after SMC-specific loss of TBRII, it is unclear whether SMC TGF-β signaling increases, and if so, whether this drives the aortopathy or is an epiphenomenon.

While this study was in progress, another group reported the use of the Myh11-CreERT2 allele to delete a different Tgfb2 exon (exon 2) in postnatal mice. 24 Both studies found that SMC loss of TBRII in young mice causes aortopathy with early-medial thickening, elastin fragmentation, adventitial fibrosis and proliferation, as well as later medial atrophy and aortic dilation. Both studies also found elevated Tgfb2 and Tgfb3 mRNA after Tgfb2 deletion in vivo as well as loss of both canonical and noncanonical TGF-β signaling in vitro in SMC lacking TBRII. Our study adds new information by including a more detailed histological characterization of the aortopathy (including a small study in older mice), by characterizing aberrant SMC gene expression that occurs early after loss of TBRII (in vitro), by focusing our in vivo gene expression studies on aortic media, and by including females (≈50% of Acta2-CreERT2 mice). Our novel findings include (1) the aortopathy includes the AA and is least pronounced in the DTA (regional variability of aortic pathology could potentially be because of variations in the embryonic origin of SMC, with AscA SMC derived from neural crest, DTA SMC from somites, and abdominal aortic SMC from mesoderm); (2) aortopathy is common after SMC Tgfb2 deletion in older mice; (3) mRNA encoding SMC contractile proteins are upregulated in the aortic media (and in cultured SMC) after Tgfb2 deletion, whereas Col1a1 is not upregulated; (4) evidence of leukocytic infiltration is not limited to the adventitia; (5) cell proliferation after loss of SMC Tgfb2 occurs predominantly in the adventitia; and (6) aortic hematomas and dissections seem confined to the early period after Tgfb2 deletion and resolve over time. Moreover, our in vitro gene expression data suggest that SMC dysfunction and upregulated matrix metalloprotease expression occur early after SMC loss of TBRII. When combined with the modest effect of TBRII loss on medial proliferation, our data support SMC dysfunction as a primary cause of aortopathy after loss of TBRII, and tend to discount a critical role for adventitial growth factor–driven proliferative medial disease. 24

Loss-of-function mutations in TGFB2 cause aortopathy in humans, 42,52 raising the question of whether our data provide mechanistic insight into this human disease. We think our data to provide insight, but with a caveat. Specifically, our finding that disruption of SMC TGF-β signaling causes SMC dysfunction and aortopathy identifies SMC dysfunction as a probable cause of aortic disease in humans with TGFB2 mutations. However, we are hesitant to derive mechanistic insights beyond this, because the TGFB2 mutants that cause human aortic disease are not null alleles 42,52,53 and it is uncertain to what extent acute homozygous receptor knockout can model a disease that is caused by the lifelong presence of heterozygous mutant receptors. The most important application
of this study to human health is the unambiguous lesson that physiological TGF-β signaling is vital for postnatal aortic health. Strategies aimed at blocking TGF-β signaling in humans should take this into account.

Acknowledgments

We thank Dr Jude Alssarraj for technical assistance, members of the laboratory of Dr Cecilia Giachelli for teaching us the aortic medial isolation protocol, Dr Pamela Johnson at the Histology Core of the Benaroya Research Institute for performing Movat staining and ver-  

Source of Funding

This study was supported by grants (to D.A. Dichek) from the American Heart Association (12GRNT932002), the National Heart, Lung, and Blood Institute (HL116612) and the John L. Locke Jr. Charitable Trust and by grants to T.N. Wight from the National Institutes of Health (EB012558 and HL098067). S.N. Angelov was supported by T32HL007828.

Disclosures

None.

References

Intracellular signals initiated by transforming growth factor-β (TGF-β) ligands and transduced by the type II TGF-β receptor in smooth muscle cell (SMC) play essential roles in SMC differentiation and vascular development. However, the contribution of these signals to postnatal aortic homeostasis is less well defined. By deleting the type II TGF-β receptor specifically in SMC of adult mice, we determined that SMC TGF-β signaling plays a critical role in maintaining postnatal aortic health. Specifically, mice that lack physiological SMC TGF-β signaling develop severe aortic pathology, including hemorrhage, ulceration, elastolysis, and aneurysmal dilatation. Aortic pathology extends along the full length of the aorta and worsens over time. These results are clinically relevant because several human aortopathies are currently attributed to excess SMC TGF-β signaling and therapies that aim to block SMC TGF-β signaling are proposed for human trials. Our results suggest that blocking SMC TGF-β signaling may worsen—not prevent—aortic disease.
Postnatal Deletion of the Type II Transforming Growth Factor-β Receptor in Smooth Muscle Cells Causes Severe Aortopathy in Mice

Jie Hong Hu, Hao Wei, Mia Jaffe, Nathan Airhart, Liang Du, Stoyan N. Angelov, James Yan, Julie K. Allen, Inkyung Kang, Thomas N. Wight, Kate Fox, Alexandra Smith, Rachel Enstrom and David A. Dichek

Arterioscler Thromb Vasc Biol. 2015;35:2647-2656; originally published online October 22, 2015;
doi: 10.1161/ATVBAHA.115.306573
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Methods and Material

Animals and genotyping
Mice with a floxed Tgfr2 allele (exon 4) were from Per Levéen and Stefan Karlsson,1 mice with a Myh11-CreER<sup>T2</sup> allele were from Dr. Stefan Offermanns,2 Acta2-CreER<sup>T2</sup> transgenic mice were from Daniel Metzer and Pierre Chambon,3 and mice transgenic for the R26R allele were from Philippe Soriano.4 All lines were extensively backcrossed (> 10 generations) into the C57BL/6 background. Mice were maintained in specific-pathogen-free housing and fed normal chow. Genotyping was performed on tail-tip DNA using primers we reported previously for the Tgfr2<sup>lox</sup>, Tgfr2<sup>null</sup>, and R26R alleles.5,6 Acta2-CreER<sup>T2</sup> mice were genotyped with primers that amplify Cre.5 The Myh11-CreER<sup>T2</sup> allele was detected using a mixture of 3 primers: SMWT1 (5’-TGACCCCATCTCTTCACCTCC-3’); SMWT2 (5’-AACTCCACGCCACCTCATC-3’); and phCREAS1: (5’-AGTCCCTCACATCCTCAGGTT-3’).2 The amplicon generated by PCR amplification of the Tgfr2<sup/null</sup> allele was column-purified then sequenced commercially (Eurofins MWG Operon; Huntsville, Alabama). For experiments with the Acta2-CreER<sup>T2</sup> allele, Acta2-CreER<sup>T2</sup><sup>/0</sup> mice were compared to Acta2-CreER<sup>T2</sup>]<sup>0/0</sup> littermate controls. Because the Myh11-CreER<sup>T2</sup> allele is on the Y chromosome, littermate controls are not possible. Therefore, experimental Myh11-CreER<sup>T2</sup><sup>/0</sup> Tgfr2<sup>lox/lox</sup> R26R<sup>+/+</sup> mice were compared to congeneric male controls (genotype Myh11-CreER<sup>T2</sup>]<sup>0/0</sup> Tgfr2<sup>lox/lox</sup> R26R<sup>+/+</sup>). All animal protocols were approved by the University of Washington Office of Animal Welfare.

Tamoxifen injections
0.04 g of tamoxifen free base (Sigma-Aldrich Corp., St. Louis, MO; #T5648) was dissolved in 0.5 mL of 100% ethanol, and 9.5 mL autoclaved olive oil was added. The mixture was vortexed, then sonicated until completely dissolved and aliquots stored at −20 ºC. 0.25 mL of this solution (1 mg tamoxifen)—or of a control solution (autoclaved olive oil or olive oil/ethanol (20:1 v/v)—was injected intraperitoneally into experimental mice each day for 5 days.

Isolation of aortic medial layer and extraction of protein and RNA
Mice were deeply anesthetized then saline perfused and exsanguinated via cardiac puncture. The full-length aorta was excised, placed in cold PBS on a clear dissection Petri dish (Living Systems Instrumentation, St. Albans, VT, #DD-90-S), cut open longitudinally, and pinned with the luminal side up. Endothelium was removed by abrasion with a cotton swab and a scalpel was used to cut through the medial layer, stopping short of the adventitia. The medial layer was then peeled off of the adventitia with a fine-point forceps and snap-frozen in liquid N<sub>2</sub> and later stored at −80 ºC. For protein extraction, the medial layer was ground in liquid N<sub>2</sub> using mortar and pestle, and resuspended in 100 µl of complete Lysis-M with protease inhibitors (Roche, #4719956001). The suspension was then vortexed twice, incubated on ice for 30 minutes, centrifuged at 14,000 g for 15 minutes at 4 ºC, and supernatant was collected for use in Western blotting. For RNA extraction, the medial layer was ground as described above, and resuspended in 1 ml of Trizol (Invitrogen). The Trizol-tissue suspension was passed through a 20-gauge needle 10 times, and 200 µl of chloroform was added and mixed vigorously. The mixture was incubated at room temperature for 5 minutes, then centrifuged 14,000 g for 15 minutes at 4 ºC. Supernatant was collected, mixed with an equal volume of 70% ethanol, then loaded onto an RNeasy column (Qiagen, RNeasy Mini Kit #74104). The column was washed and RNA eluted according to the manufacturer’s protocol.

Western blot detection of TBRII protein in aortic SMC
For each sample, 12 µg of aortic SMC protein were separated by SDS/PAGE and transferred to PVDF membranes.7 TBRII was detected with a rabbit antibody to mouse TBRII (1:1000, Santa Cruz Antibodies, Santa Cruz, CA; #SC-400) and peroxidase-conjugated anti-rabbit IgG (1:2,000; Bio-Rad, Hercules, CA; #170-6515). To control for protein loading, membranes were stripped and re-probed with a mouse monoclonal antibody to β-actin (1:6000; Sigma #A5316). Bound
antibody was detected with peroxidase-conjugated anti-mouse IgG (1:3,000; Bio-Rad, Hercules, CA; #170-6516).

**Measurement of Tgfbr2 and other mRNA in aortic SMC**
Expression levels of mRNAs were measured with the Verso 1-step RT-qPCR Kit with SYBR Green and low ROX (Life Technologies, #AB-4106), using the ΔΔCt method, with normalization to 18S RNA. Tgfbr2 mRNA was measured with 2 approaches, aimed at detecting either the floxed exon 4 or the intact exon 2. To detect Tgfbr2 transcripts containing exon 4 (which is deleted by Cre-mediated recombination), we used primers internal to exon 4, as before. For most reactions we used the SYBR Green detection method; however the same results were obtained using an internal fluorogenic probe. To detect Tgfbr2 transcripts that contain exon 2, we used primers internal to exon 2. We also used qRT-PCR and SYBR Green to measure aortic SMC mRNA for LacZ (expressed from the R26R transgene after Cre-mediated recombination), 6 molecules integral to TGF-β signaling (Tgfb1, Tgfb2, Tgfb3, Tgfbr1, Tgfbr3, and Smad7), 5 SMC marker genes (Acta2, Myh11, Tagln, Smtn, Cnn1), 6 genes involved in extracellular matrix metabolism (Col1a1, Lox, Loxl1, Mmp2, Mmp9, and Mmp12), 2 canonical TGF-β-responsive genes (Ctgf and Serpine1), and 2 genes involved in aneurysm formation (Igf1, Ace) (Table I).

**Tissue harvest, processing and detection of β-galactosidase activity**
We detected in vivo recombination of the R26R allele by measuring activity of β-galactosidase protein expressed by the recombined allele. Mice were first deeply anesthetized and perfused with saline. Aorta, carotid arteries, bladder, ileum, and several other organs were dissected free and stained with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal). Briefly, tissue was fixed in 2% paraformaldehyde for 10–20 minutes at 4 °C, washed in detergent, transferred to X-Gal staining mixture and incubated at 34 °C for 2–4 hours. Whole-mount images were taken and tissues were dehydrated then embedded in paraffin. 5-micron-thick sections were counterstained with nuclear fast red (Vector Laboratories, Burlington, CA).

**BrdU (5-Bromo-2’-deoxyuridine) injection**
Mice were injected subcutaneously with 250 μl of 3 mg/ml BrdU solution (30 mg/kg; B5002, Sigma-Aldrich, St. Louis, MO) at 17, 9, and 1 hour before euthanasia. A segment of the ascending aorta was removed, processed and sectioned (as described below). 5 step sections per aorta (96-μm spacing) were stained with antibody to BrdU (1:200 Abcam, Cambridge, MA #ab6326). Bound antibody was detected with the ABC kit (Vector Labs #PK4-000) and VECTOR NovaRED Peroxidase Substrate Kit (SK-4800). No staining was obtained if the primary antibody was omitted. Two observers blinded to treatment counted the number of stained nuclei in each section, including nuclei along the luminal surface, in the media, and in the adventitia. Any disagreements between the observers were resolved by reviewing the sections and agreeing on the numbers of stained nuclei in each section. Means of the 5 values per mouse were used as a result for that mouse.

**Tissue harvest, processing, and analysis**
Mice were exsanguinated by saline perfusion. Hearts and aortas were fixed in situ by perfusion at physiologic pressure with 10% formalin, dissected free, and removed. The tissues were then incubated in 10% formalin overnight, followed by storage in 70% ethanol at 4 °C. Perivascular fat surrounding heart and aorta was then removed with the aid of a dissecting microscope. Three regions of interest along the length of the aorta (ascending aorta and arch, descending thoracic aorta, and abdominal aorta) were then photographed using a Leica S6D microscope and digital camera (model DFC295; Leica Microsystems, Buffalo Grove IL).

A randomly selected subset of aortas was processed further for sectioning and staining. These aortas were selected without consideration as to whether gross pathology was present or absent. A segment of the ascending aorta was obtained by transverse sectioning of the aorta
above the aortic root and at the takeoff of the innominate artery. This segment was embedded in OCT compound (4583, Sakura Finetek USA, Torrance, CA) with the cranial end positioned at the OCT block edge. A total of 60 serial 8-µm-thick sections were cut, covering 480 µm along the ascending aorta. For each stain, 5 sections per ascending aorta at 96-µm steps were stained for each mouse. Measurements from each of the 5 sections were used to calculate a single mean value for that mouse. A segment of descending thoracic aorta was obtained by transverse sectioning of the aorta just caudal to the left subclavian artery takeoff and then approximately 5 mm distally. A segment of the abdominal aorta was obtained by transverse sectioning of the aorta just caudal to the celiac artery takeoff and then approximately 5 mm distally. This segment contained takeoffs of the superior mesenteric artery, right renal artery, and left renal artery. The descending thoracic and abdominal aorta segments from each mouse were embedded in OCT compound alongside each other, with the cranial edge of each segment positioned at the OCT block edge. For each of the 2 segments, 12 8-µm-thick serial sections were cut at each of 5 592-µm steps, covering a total of 2,464 µm of aortic length. For each stain, measurements from each of 5 step sections were used to calculate a single mean value for each mouse.

Sections of ascending aorta were stained with hematoxylin and eosin, Prussian blue, Mac-2 antibody, versican antibody, and Movat stain. Antibodies and controls were: Rat anti-mouse Mac-2 (Cedarlane labs #CL8942AP) and control rat IgG2b (Serotec #MCA1125); rabbit anti-versican (Millipore #AB1033; directed at GAG beta domain) and control rabbit IgG (Jackson ImmunoResearch Laboratories, Inc. #011-000-003). Bound Mac-2 antibody was detected as described above for anti-BrdU. For versican immunostaining, sections were pretreated with 0.2 U/ml chondroitinase ABC (Sigma #C3667) in 18 mM Tris, 1mM sodium acetate, 1 mg/ml BSA pH 8.0 for 1 hour at 37 °C. The sections were then incubated for 1 hour with 2.5 µg/ml primary antibody in Bond Primary Antibody Diluent (Leica Biosystems, Buffalo Grove IL). Bound antibody was detected with the Bond Polymer Refine Detection Kit containing a peroxidase block, a ready-to-use secondary goat anti-rabbit conjugated to polymeric HRP, DAB chromagen, and hematoxylin counterstain (Leica Microsystems #DS9800).

Sections of descending thoracic and abdominal aorta were stained with all of these stains/antibodies except Prussian blue and anti-BrdU. Stained sections were photographed using a Leica DM4000B microscope and digital camera (model DFC295; Leica Microsystems). Planimetry (measurement of IEL and EEL length, calculation of medial thickness and area) were performed on H & E-stained sections, using ImagePro Plus (Media Cybernetics; Rockville, MD). Mean medial thickness was calculated by assuming circular geometry of the IEL and EEL and calculating the difference between the radii of these circles. Medial area was calculated by subtracting the area within the IEL from the area within the EEL. BrdU-positive cells per section were counted individually. We used color thresholding to measure area of medial Mac-2 positivity, and divided this value by total medial area (measured on an adjacent H&E-stained section) to determine % medial Mac-2-stained area.

Aortic medial elastin damage was quantified by counting elastin breaks. Hematoxylin and eosin-stained sections of AscA were illuminated with fluorescein filters, causing autofluorescence of elastic laminae. Images were acquired with a Leica DM4000B microscope and a digital camera (model DFC295; Leica Microsystems). An observer blinded to genotype counted the number of elastin breaks per section. A break was defined as presence of two free ends of what seemed otherwise to be a continuous elastin fiber. Mean numbers of breaks per section were calculated from 5 step sections per mouse and were used as a value for that mouse.

**Prussian blue staining**
To detect hemosiderin, sections from the ascending aorta were stained with Prussian blue. Briefly, working solution was prepared by mixing equal parts of 10% hydrochloric acid and 10%
potassium ferrocyanide solution (P3289, Sigma-Aldrich, St. Louis, MO) just before use. Sections were dried at room temperature for 1 hour and then incubated in working solution for 20 minutes followed by wash with distilled water 3 times. Sections were then counterstained with nuclear fast red (R5463200-500A, Ricca Chemical, Arlington, TX) for 5 minutes and washed 3 times with distilled water, followed by dehydration through 70% and 95% ethanol solution for 2 minutes each. Sections were then air dried and coverslipped.

**Primary culture of aortic SMC**

Mice were deeply anesthetized then saline perfused and exsanguinated via cardiac puncture. Aortas were removed from 4 – 8 week old Cre^{0/0} Tgfb2^{floxtfloxt} mice and washed in cold PBS. The adventitial layer was carefully stripped from the media and discarded. The endothelium was then removed by scraping the luminal surface with a scalpel. The media was digested in type I collagenase (Worthington Biochemical, Lakewood, NJ; 0.5 mg/ml in DMEM) at 37 °C for 8 minutes, with gentle agitation. The large pieces of tissue were allowed to settle and the supernatant (containing dissociated cells) was decanted into a vial containing 100% FBS. Additional digestion media was added to the undigested medial tissue and the process was repeated every 8 minutes until there was nearly complete dissolution of the media (typically 12 – 15 repetitions). The pooled cell suspension was then pelleted by centrifugation at approximately 180 g and the supernatant was discarded. The cell pellet was resuspended in DMEM containing 15% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin (Life Technologies, Grand Island, NY). The cells were plated into 24-well plates (~ 2 x 10^6 cells per well) and incubated at 37 °C with 5% CO_2. Cells were passaged at a 1:5 ratio, at 80% confluence. Experiments were performed using cells at passages 3 to 5.

**Characterization of aortic SMC**

SMC were plated onto glass coverslips and incubated in the above growth medium until they were approximately 80% confluent. The cells were fixed with acetone for 20 minutes at –20 °C, blocked for 30 minutes at room temperature with 3% BSA in PBS, then incubated with an antibody to alpha smooth muscle actin (Abcam Cambridge, MA #5694; 1:250) overnight at 4 °C. The cells were washed 3 times with cold PBS and incubated with a FITC-conjugated secondary antibody (Abcam #6717; 1:500). After washing 3 times with cold PBS, the coverslips were incubated in 5 ng/mL Hoescht 33258 (Sigma # 94403) and mounted on slides using mounting media (Vectashield; Vector Laboratories). The cells were visualized using a Leica DM4000B fluorescence microscope. To estimate the relative population of SMCs, the number of cells with positive staining for alpha smooth muscle actin and the total number of nuclei (Hoescht nuclear stain) were counted in several random high-power fields. Results were compared to a negative control in which the primary antibody was omitted. Over 90% of cells stained positively for alpha smooth muscle actin.

**Viral transduction of SMC**

SMC were plated in six-well culture plates (Corning) at a density of 0.5 x 10^6 cells/well in DMEM containing 15% FBS. Approximately 24 hours after plating, the cells were washed twice with DMEM then incubated in DMEM containing adenovirus (AdCMVCre or AdCMVNull), 1 x 10^3 viral particles/mL for 6 hours. AdCMVCre (Microbix Biosystems, Toronto, ON, CA) is an adenoviral vector containing the Cre-recombinase cDNA. AdCMVNull is an essentially identical vector without Cre. After incubation with the vectors, the cells were washed twice with PBS and incubated for an additional 48 hours in DMEM with 15% FBS. Cells were then washed with PBS and incubated with X-gal for 6 hours at 37 °C. Approximately 90% of SMC exposed to AdCMVCre stained blue with X-Gal.

**Detection of signaling in response to TGF-β1 ligand**

SMC were plated in 6-well plates in DMEM with 15% FBS, as above. At confluence, cells were washed 2 times with PBS then incubated with either FGAdCMVCre or FGAdNull, also as above. The virus was removed, cells were washed x 3 with PBS and cultured in DMEM with 15% FBS.
for 2 days. Medium was then removed, cells were washed 2 times with PBS then fed with DMEM with penicillin and streptomycin for 24 hours.

TGF-β1 (R & D Systems #7666-MB; 1 ng/ml final concentration) was then added to the culture medium. Wells of cells were harvested at \( t = 0 \) (no exposure to TGF-β1) and at 15, 60, and 180 minutes after addition of TGF-β1. Whole cell lysates were prepared by replacing the medium with 250 µl of “Complete Lysis M” buffer with protease inhibitors (Roche, Indianapolis, IN), according to the manufacturer’s protocol. The lysates were centrifuged at 14,000g x 10 minutes, supernatant was collected and stored at –80 °C. After thawing, total protein was quantified with the BCA protein assay (Pierce #23225). 20 µg of protein from each sample was mixed in 4X LDS sample buffer (Novex, Carlsbad, CA), incubated at 95 °C for 5 minutes, resolved by SDS-PAGE (10% gel; Novex, Carlsbad, CA) and transferred to PVDF membranes. The membranes were blocked for 1 hour at room temperature with 5% powdered skim milk in TBS with 0.05% Tween (TBST) and incubated with antibodies to TGFBR2 (Santa Cruz # SC400; 1:1000), phospho-SMAD2 (S465-467, Cell Signaling #3101S; 1:1000), SMAD2 (Cell Signaling #3122S; 1:1000), phospho-p44/42 (T202-Y204, Cell Signaling #9101S; 1:1000), p44/42 (Cell Signaling #9102S; 1:1000), phospho-p38 (T180-Y182, Cell Signaling #4511P; 1:2000), p38 (Cell Signaling #9212S; 1:2000) or β-actin (Sigma #A5316; 1:5000). Antibodies were diluted in TBST with 5% milk and incubated overnight at 4 °C. Blots were then washed 3 times (5 minutes each) in TBST and incubated with HRP-conjugated secondary antibody (goat anti-rabbit or anti-mouse; Biorad, Hercules, CA; #170-6515 or #170-6516; 1:3000 – 1:5000 in TBST with 5% milk) for 1 hour at room temperature. Bound antibodies were detected using ECL (Thermo, Rockford, IL). Band density was analyzed using densitometry (Image J, Version 1.48, NIH, Bethesda, MD). Normalization of band density, to allow for comparison among experiments, was performed by dividing the density of each band by the total density of that specific band in all lanes.

**Quantitative RT-PCR**

To determine the acute effects of deletion of Tgfbr2 on SMC gene expression, we measured several mRNA 72 hours after transduction with either FGAdCMVCre or FGAdNull. Total RNA was extracted from SMC using the RNAEasy kit (Qiagen) and quantified using a Nanodrop spectrophotometer (Thermo Scientific). 20 ng of RNA was used as a substrate for all qRT-PCR reactions except 18S, for which 0.2 ng was used (Verso SYBR Green 1-step qRT-PCR kit, Thermo Scientific). Expression levels of each transcript were normalized to 18S and relative gene expression was calculated using the 2^ΔΔCT method. For gene-specific primer sequences see Table I.

**Statistics**

Categorical data were compared by Fisher exact test. For experiments with continuous data and only one control group the t test was applied after verifying normal distribution and equal variances. For continuous data that were not normally distributed, or if variances were unequal, we used the Mann-Whitney rank-sum test. Group data are shown as mean ± SEM. Most comparisons are made between littermate genotype controls (i.e., Cre^+/+^ versus Cre^-/-^) of the same line. However, to gain statistical power and avoid falsely negative results, we sometimes pooled Cre^+/+^ and Cre^-/-^ genotypes of the 2 lines (i.e., results from Acta2-Cre^+/+^ and Myh11-Cre^+/+^ mice were combined and compared to results obtained with Acta2-Cre^-/-^ and Myh11-Cre^-/-^ mice). We did this only for categorical end points and in all of these cases we also present the line-specific primary data (Table 2). Protein levels on western blots performed at different time points were compared by 2-way ANOVA. All tests were carried out with the SigmaStat program (Systat Software, Chicago, IL).
References


### Table I. Primers used for qRT-PCR.

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Table II. Gross and microscopic aortic pathology after SMC loss of TBRII

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<td>Intramural Hematoma</td>
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<td>Ascending Aorta†</td>
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<tr>
<td>Abdominal Aorta</td>
<td>2/58</td>
<td>0/56</td>
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</table>

|                      |         |          |               |                |         |          |               |                |
|                      | Myh11-Cre |         |          |               |                |         |          |               |                |
| Cre Genotype         | +/0     | 0/0      | +/0           | 0/0            | +/0     | 0/0      | +/0           | 0/0            |
| Ascending Aorta†     | 9/26    | 0/27     | 0/6           | 0/6            | 6/6     | 0/6      | 2/6           | 0/6            |
| Descending Thoracic Aorta | 4/26   | 0/27     | 2/6           | 0/6            | 0/6     | 0/6      | 2/6           | 0/6            |
| Abdominal Aorta      | 4/26    | 0/27     | 1/6           | 0/6            | 0/6     | 0/6      | 1/6           | 0/6            |

*All mice were examined for gross aortic hemorrhage. Of these mice, random subsets were used for the histological analyses shown. Most of the other aortas were used for mRNA and protein studies; a few were damaged during removal.

†Gross hemorrhage was either in the ascending aorta or arch. Other pathologies were evaluated only in the ascending aorta. A few mice had one of the pathologies (e.g., gross hemorrhage, penetrating ulcer) in more than one aortic segment.
Figure I. Cre recombinase activity in both Acta2-CreER*T2/+0 and Myh11-CreER*T2/+0 lines is efficient and SMC-specific. Tissues are from Acta2-CreER*T2/+0 R26R+ mice (A – J, S) or Myh11-CreER*T2/+0 R26R+ mice (K – R, T) that were treated with Tamoxifen (Tm) or vehicle (V). Tissues include: aorta (A, B, K, L, S, T); carotid artery (C, D, M, N); bladder (E, F, I, J, O, P); and ileum (G, H, Q, R). All tissues were stained with X-gal. Arrows (D, S, T) indicate blue cells in arteries of vehicle-treated mice. A, B, E, G, I, K, O, Q, S are of whole organs/tissues; all others are tissue sections, counterstained with nuclear fast red. Scale bars (C, D, M, N, T) are 100 microns.
Figure II. Detection and sequencing of Tgfr2 null allele. A, Tail DNA was used as a substrate for allele-specific PCR for Tgfr2<sup>+</sup>. DNA was from Acta2-CreER<sup>T2</sup> <sup>+/+</sup> or Acta2-CreER<sup>T2</sup> <sup>−/−</sup> mice (all Tgfr2<sup>Lox/Lox</sup>) treated with tamoxifen or vehicle. Positive control (+) is template DNA from a Tgfr2<sup>Lox/Lox</sup> mouse (Frutkin et al., 2006). Negative control (−) is template DNA from an Acta2-Cre<sup>−/−</sup> Tgfr2<sup>Lox/Lox</sup> mouse. NTC = no-template control (no DNA). B, Schematic of floxed Tgfr2 allele before and after Cre-mediated rearrangement that yields null allele (Leveen et al., 2002). Primers P3 and P5 were used to detect the null allele. C, Tail DNA from a tamoxifen-treated Acta2-Cre<sup>+/+</sup> Tgfr2<sup>Lox/Lox</sup> mouse was amplified with primers P3 and P5, then sequenced. The amplicon included mouse Tgfr2 gene sequences (black, underlined), and a single 34-bp LoxP site (red, the core sequence is underlined) that is flanked by sequences likely introduced during construction of the conditional allele (black, no underlining).
Figure III. LacZ mRNA expression in aortic media. 4 weeks after treatment with tamoxifen, RNA was extracted from the aortic media of *Tgfb2*<sup>cre<sub>lox/lox</sub></sup> mice that either lacked (*Cre<sup>0/0</sup>*) or expressed a CreER<sup>T2</sup> transgene (*Acta2-Cre<sup>+/+</sup>* and *Myh11-Cre<sup>+/-</sup>*); all mice are R26R<sup>cre<sup>+</sup></sup>+. LacZ mRNA was measured by qRT-PCR and normalized to 18S rRNA in each sample. The background “expression” level of LacZ in *Cre<sup>0/0</sup>* mice of both lines (broken line) indicates the highest LacZ mRNA signal detected in aortic media of 17 *Cre<sup>0/0</sup>* controls of both lines.
Figure IV. Technique for isolation of arterial media. **A**, A carotid artery from a tamoxifen-treated Acta2-Cre\textsuperscript{ERT2} R26R\textsuperscript{LoxP} mouse was removed, opened longitudinally, and stained with X-Gal. The luminal surface was abraded to remove the endothelium. **B**, Transverse incisions extending from the luminal surface to the medial/adventitial border allow the media to be peeled free, leaving behind the adventitia and some adherent medial cells (blue dots). The translucent adventitia is visible above the forceps tips. **C**, The peeled medial SMC layer. In the present study this technique was used to isolate the aortic media.
Figure V. Expression of SMC lineage markers in aortic media and adventitia. The technique illustrated in Figure IV was used to obtain aortic medial and adventitial tissue from 11–12-week-old Acta2-CreERT2+/0 (n = 4–5) and Acta2-CreERT20/0 mice (n = 5). All mice were Tgibr2lox/lox, without tamoxifen treatment. RNA was extracted and used for qRT-PCR measurement of SMC lineage markers: A, Tagln; B, Myh11; C, Acta2 (all normalized to 18S rRNA in same samples). Data points are individual mice. Genotypes indicate no effect of the Acta2-CreERT2 transgene on expression of these 3 genes.
Figure VI. Expression of Tgfb2 mRNA in aortic media. 4 weeks after treatment with tamoxifen, RNA was extracted from the aortic media of Tgfb2^flox/flox mice that either lacked (Acta2-Cre^0/0 and Myh11-Cre^0/0) or expressed a CreER<sup>T2</sup> transgene (Acta2-Cre<sup>ex</sup>/0 and Myh11-Cre<sup>ex</sup>). Tgfb2 mRNA was measured by qRT-PCR using primers internal to either exon 4 (A) or exon 2 (B) of the Tgfb2 gene.
Figure VII. Loss of Tgfbr2 in aortic SMC causes aortopathy. 4 weeks after treatment with tamoxifen, aortas were harvested from Tgfbr2\textsuperscript{floxflo} mice that either lacked (Myh11-Cre\textsuperscript{0/0}: A, C, E, G, I, K) or expressed a Myh11-CreER\textsuperscript{T2} transgene (Myh11-Cre\textsuperscript{+/-}: B, D, F, H, J, L). A – F, Ascending aorta (AscA) and arch, descending thoracic aorta (DTA), and abdominal aorta (AA). G – L, hematoxylin and eosin-stained transverse sections of AscA, DTA, and AA. B, arrow: aortic hemorrhage; H, arrows: penetrating aortic ulcers; J, arrowhead: aortic dissection. A – F, Ruler is in mm. G – L, Scale bar: 100 μm.
Figure VIII. Aortic pathology in mice with SMC-specific loss of  Tgfr2. Sections are from ascending aortas of Acta2-CreERT2+/- Tgfr2flx/flox mice and were obtained 8 (A and D), 4 (B and E), or 14 (C and F) weeks after treatment with tamoxifen. Mice were 26 (A and D), 11 (B and E), or 21 (C and F) weeks old when euthanized. A and D, penetrating aortic ulcer; B and E, aortic dissection with large false lumen; C and F, medial Prussian blue stain in an aorta with no other evidence of hemorrhage. Scale bars: (A – F): 100 μm. A and D, Movat stain; B and E, hematoxylin and eosin stain; C and F, Prussian blue stain. B and E, L = true lumen; FL = false lumen.
Figure IX. Loss of Tgfb1 in aortic SMC causes aortic dilation. 14 weeks after treatment with tamoxifen, aortae were harvested from Tgfb1lox/lox mice that either lacked (Myh11-Cre0/0: A, C, E) or expressed a Myh11-CreER<sup>T2</sup> transgene (Myh11-Cre<sup>+/-</sup>: B, D, F). A – F, Representative sections of hematoxylin and eosin-stained transverse sections of ascending aorta (AscA), descending thoracic aorta (DTA), and abdominal aorta (AA). A – F, Scale bar: 100 μm.
Figure X. Aortic elastin damage in mice with SMC-specific loss of Tgfr2. Aortas were obtained 4 (G, H) or 14 (A – H) weeks after tamoxifen treatment of Tgfr2<sup>flx/flx</sup> mice that either lacked (Acta2-Cre<sup>0/0</sup> and Myh11-Cre<sup>0/0</sup>) or expressed a CreER<sup>T2</sup> transgene (Acta2-Cre<sup>+/0</sup> and Myh11-Cre<sup>+/0</sup>). A – F, Sections of hematoxylin and eosin-stained ascending aortas illuminated with fluorescein wavelengths; boxed areas in A and C are enlarged in B and D, and further enlarged in E and F. G and H, Elastin damage in ascending aortas (AscA) was quantified by counting elastin breaks on images such as in A – F. A break was considered present when 2 free ends of an elastin fiber were visible in proximity (arrow pairs in D and F). Mean numbers of elastin breaks per section were calculated from examining 5 step-sections per mouse. ***P < 0.001 compared to Cre<sup>0/0</sup> controls; two-way ANOVA. †P < 0.001 compared to aortas harvested 4 weeks after tamoxifen treatment; two-way ANOVA. n = 7 – 9 for Acta2-Cre groups; n = 6 for Myh11-Cre groups. A – F, Scale bar: 100 μm.
Figure XI. Loss of Tgfr2 in aortic SMC increases aortic medial macrophage markers. Aortas were obtained from Tgfr2lox/lox mice that either lacked (Acta2-Cre0/0 and Myh11-Cre0/0) or expressed a CreER<sup>T2</sup> transgene (Acta2-Cre<sup>T2</sup>/ and Myh11-Cre<sup>T2</sup>). Mice were killed 4 weeks (A, B, C, D, I, K) or 14 weeks (E, F, G, H, J, L) after tamoxifen treatment. Sections from ascending aorta (AsCA), descending aorta (DTA), and abdominal aorta (AA) were stained for the Mac-2 antigen. A – H, Mac-2-stained transverse sections of AsCA. Boxes in A, C, E, and G are enlarged in B, D, F, and H. I – L, medial Mac-2-positive area (%) was calculated by dividing medial Mac-2-stained area by total medial area. *P < 0.05, **P < 0.01, ***P < 0.001 compared to Cre0/0 controls. Acta2-Cre0/0: n = 7 – 9; Myh11-Cre0/0: n = 6. A – H, Scale bar: 100 μm.
Figure XII. Loss of Tgfr2 in aortic SMC causes elevated cell proliferation. Mice that either lacked (Acta2-Cre<sup>0/0</sup> and Myh11-Cre<sup>0/0</sup>) or expressed a CreERT2 transgene (Acta2-Cre<sup>+/0</sup> and Myh11-Cre<sup>+/0</sup>) were pulse labeled with BrdU 4 weeks after tamoxifen treatment. A segment of ascending aorta (AscA) of each mouse was sectioned and stained with BrdU antibody (5 step-sections per mouse). A and B, BrdU antibody-stained transverse sections of AscA are from Myh11-Cre<sup>0/0</sup> (A) and Myh11-Cre<sup>+/0</sup> (B) mice. Arrows: BrdU-positive nuclei. Dashed lines designate the external elastic lamina, near the media/adventitial border. Scale bar: 100 μm. C and D, number of BrdU-positive nuclei per section, including those in the adventitia, media, and along the luminal surface. All = sum of BrdU+ cells in all 3 areas. *P < 0.05, **P < 0.01, ***P < 0.001 compared to Cre<sup>0/0</sup> controls; Acta2-Cre<sup>0/0</sup>: n = 6 per group; Myh11-Cre<sup>0/0</sup>: n = 5 per group.
Figure XIII. Loss of Tgfbr2 in aortic SMC causes proteoglycan and collagen accumulation. A – D. Transverse sections of ascending aorta (AscA) obtained 4 or 14 weeks after tamoxifen treatment. Aortas were harvested from Tgfbr2<sup>fl/flox</sup> mice that either lacked (Myh11-Cre<sup>0/0</sup>) or expressed a Myh11-CreER<sup>T2</sup> transgene (Myh11-Cre<sup>+/+</sup>). Movat stain; scale bar: 100 μm.
Figure XIV. Loss of Tgfbr2 in aortic SMC causes versican accumulation. Aortas were obtained 4 (A) or 14 (B) weeks after tamoxifen treatment of Tgfbr2\textsuperscript{lox/lox} mice that either lacked (Acta2-Cre\textsuperscript{0/0}) or expressed an Acta2-Cre\textsuperscript{ERT\textsuperscript{2}} transgene (Acta2-Cre\textsuperscript{+/0}). Transverse sections of ascending aorta (AscA), descending thoracic aorta (DTA), or abdominal aorta (AA) from Acta2-Cre\textsuperscript{0/0} mice or Acta2-Cre\textsuperscript{+/0} mice were stained with antibody to versican. Versican-positive (+) area was calculated by dividing versican-stained area by total tissue area on the same section. C – N, Transverse sections of AscA were stained with antibody to versican, Mac-2, or Movat stain. Boxes in C, E, G, I, K, and M are enlarged in D, F, H, J, L, and N. E and F, Arrow: penetrating aortic ulcer. C – N, Scale bar: 100 μm. A and B, **P < 0.01, ***P < 0.001 compared to Acta2-Cre\textsuperscript{0/0} controls (n = 7 – 9 per group).
Figure XV. Loss of Tgfb2 alters gene expression in cultured aortic SMC. Aortic SMC from Acta2-Cre^{0/0} Tgfb2^{flx/flx} mice were established in culture and treated at passages 3 – 5 with either AdCMVNull or AdCMVCre. 72 hours after viral transduction, total RNA was extracted and gene expression measured with qRT-PCR. Data are pooled from 3 independent experiments, each from a separate SMC harvest. *P < 0.05, **P < 0.01, ***P < 0.001 compared to AdCMVNull-treated cells; n = 3 for both groups.
Figure XVI. Development and progression of aortopathy after deletion of SMC TBRII in 6-week-old mice. Four weeks after loss of TBRII, a normal aorta (A) is compared to an aorta with SMC-specific loss of TBRII (B). Loss of TBRII results in medial thickening, elastolysis, intramural hematoma (arrow), penetrating ulcer (arrowhead), macrophage marker accumulation (not shown), adventitial cell proliferation and matrix accumulation. Ten weeks later (C), hematomas have resolved; however, the aorta is now dilated with continued ulceration (arrowhead), progressive elastolysis, and increased adventitial matrix accumulation. In order to emphasize the aortic wall features, the ratio of wall thickness to lumen diameter is exaggerated.