Dimorphic Effects of Transforming Growth Factor-β Signaling During Aortic Aneurysm Progression in Mice Suggest a Combinatorial Therapy for Marfan Syndrome

Jason R. Cook, Nicholas P. Clayton, Luca Carta, Josephine Galatìo, Emily Chiu, Silvia Smaldone, Carol A. Nelson, Seng H. Cheng, Bruce M. Wentworth, Francesco Ramirez

**Objective**—Studies of mice with mild Marfan syndrome (MFS) have correlated the development of thoracic aortic aneurysm (TAA) with improper stimulation of noncanonical (Erk-mediated) TGFβ signaling by the angiotensin type I receptor (AT1r). This correlation was largely based on comparable TAA modifications by either systemic TGFβ neutralization or AT1r antagonism. However, subsequent investigations have called into question some key aspects of this mechanism of arterial disease in MFS. To resolve these controversial points, here we made a head-to-head comparison of the therapeutic benefits of TGFβ neutralization and AT1r antagonism in mice with progressively severe MFS (Fbn1mgR/mgR mice).

**Approach and Results**—Aneurysm growth, media degeneration, aortic levels of phosphorylated Erk and Smad proteins and the average survival of Fbn1mgR/mgR mice were compared after a ≈3-month-long treatment with placebo and either the AT1r antagonist losartan or the TGFβ-neutralizing antibody 1D11. In contrast to the beneficial effect of losartan, TGFβ neutralization either exacerbated or mitigated TAA formation depending on whether treatment was initiated before (postnatal day 16; P16) or after (P45) aneurysm formation, respectively. Biochemical evidence-related aneurysm growth with Erk-mediated AT1r signaling, and medial degeneration with TGFβ hyperactivity that was in part AT1r dependent. Importantly, P16-initiated treatment with losartan combined with P45-initiated administration of 1D11 prevented death of Fbn1mgR/mgR mice from ruptured TAA.

**Conclusions**—By demonstrating that promiscuous AT1r and TGFβ drive partially overlapping processes of arterial disease in MFS mice, our study argues for a therapeutic strategy against TAA that targets both signaling pathways although sparing the early protective role of TGFβ. (Arterioscler Thromb Vasc Biol. 2015;35:911-917. DOI: 10.1161/ATVBAHA.114.305150.)

**Key Words:** aortic aneurysm ■ losartan ■ Marfan syndrome ■ receptor, angiotensin, type 1 ■ transforming growth factor β

Aortic aneurysms are life-threatening pathologies characterized by progressive vessel dilation associated with smooth muscle cell (SMC) dysfunction, localized inflammatory infiltrates and destructive (maladaptive) extracellular matrix (ECM) remodeling that together predispose the aortic wall to tear (dissection) and rupture.1,2 In contrast to the strong association of abdominal aortic aneurysm (AAA) with advanced age and a collection of environmental risk factors, thoracic aortic aneurysm (TAA) shows high heritability often accounted for by mutations in components of the ECM, cytoskeleton and transforming growth factor beta (TGFβ) signaling cascade.1,2 Studies of mouse models of TAA and AAA have implicated the angiotensin II type I receptor (AT1r) in the cause of both types of aneurysm, in addition to raising a controversy about TGFβ’s role in arterial disease.3 On the one hand, inhibition of TGFβ signaling with a pan-TGFβ-neutralizing antibody (TGFβ-Nab) prevented TAA formation in a mouse model of the mild Marfan syndrome (MFS).4 However, the TGFβ-Nab administration exacerbated the pathology of angiotensin-induced AAA and TAA in mice and conversely, TGFβ overexpression stabilized the expansion of experimental AAA in rats.5,6

MFS is a relatively common disease of connective tissue caused by mutations that alter the structure or impair the expression of the ECM component and TGFβ modulator fibrillin-1.1 Two mouse models representing distinct degrees of MFS severity have been instrumental in delineating the pathogenic mechanisms responsible for cardiovascular and musculoskeletal abnormalities. The first mouse model (Fbn1C1039G/+ mouse) produces equal amounts of normal and abnormal...
fibrillin-1 and replicates the less commonly observed form of mild MFS. Chung et al. have reported that by 6 months of age >90% of Fbn1C1039G/+ mice developed TAA of variable severity, but only ≈5% of them died of ruptured aortic aneurysm by 8 months of age. The second mouse model (Fbn1mgR/mgR mice) produces ≈20% of the normal amount of fibrillin-1 and replicates the most frequently diagnosed form of early onset, progressively severe MFS. In contrast to Fbn1 C1039G/+ mice, ruptured TAA is a fully penetrant manifestation that leads to death of nearly all Fbn1mgR/mgR mice within the first year of life (average survival, 2.5 months). Prior analyses of Fbn1 C1039G/+ mice have shown that either systemic AT1r antagonism or TGFβ neutralization normalize aneurysm growth along with the levels of phosphorylated (p)-Smad2 and p-Erk1/2. Even though AT1r and TGFβ can both activate Smad2 and Erk1/2 proteins, this finding was interpreted as indirect evidence of AT1r-dependent stimulation of canonical (Smad mediated) and noncanonical (Erk mediated) TGFβ signaling. Subsequent experiments have suggested a prominent role of the noncanonical Erk1/2 pathway in TGFβ-promoted arterial disease in Fbn1 C1039G/+ mice. By contrast, studies of Fbn1mgR/mgR mice have implied that mechanisms other than improper AT1r activation stimulate promiscuous TGFβ signaling, as losartan administration mitigated but did not prevent ruptured TAA in this animal model of progressively severe MFS. Although our study was being completed, Li et al. has reported that genetic disruption of TGFβ receptor II (Tgfbr2) in postnatal SMCs of Fbn1 C1039G/+ mice at 4 weeks increased the rate and degree of TAA and aortic dissection.

In the original study of Fbn1 C1039G/+ mice, losartan and TGFβ-Nab dosing occurred in vastly different periods of time and treatment efficacy was assessed at different ages. To correct these disparities, here we used the same treatment protocol to compare and contrast the impact of TGFβ versus AT1r inhibition of TAA progression and survival of Fbn1mgR/mgR mice. Similar to prior studies with Fbn1 C1039G/+ mice, we also examined the relative levels of p-Erk1/2 and p-Smad2 as surrogate molecular readouts of treatment efficacy. The results of our experiments expose the complexity associated with TGFβ inhibition in the diseased aorta, reconcile the existing controversy concerning TGFβ’s role in aortic aneurysms, exclude a strict dependence of TGFβ overactivation on AT1r signaling, and correlate promiscuous AT1r and TGFβ activity with partially overlapping processes of arterial disease. Together, our findings substantially revise the current view of TAA pathogenesis in MFS, in addition to suggesting that targeting both AT1r and TGFβ signaling is a more effective therapeutic strategy than solely blocking AT1r activity.

**Materials and Methods**

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

**Results**

**Mutant Aortas Exhibit Distinct Temporal Profiles of p-Erk1/2 and p-Smad2 Accumulation**

We first established the natural history of TAA formation in Fbn1mgR/mgR mice as the baseline for subsequently comparing

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AT1r</td>
<td>angiotensin II type I receptor</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>MFS</td>
<td>Marfan syndrome</td>
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<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
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<tr>
<td>TAA</td>
<td>thoracic aortic aneurysm</td>
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<tr>
<td>TGFβ-Nab</td>
<td>TGFβ-neutralizing antibody</td>
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<td>Tgfbr2</td>
<td>TGFβ receptor II</td>
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<td>WT</td>
<td>wild-type</td>
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**Figure 1.** TAA progression in mice with severe MFS. **A,** Quantified diameters of the aortic root (AoR) and proximal ascending aorta (AsA), and **B** combined scores of wall thickness and elastic lamella integrity (MD) in WT (black) Fbn1mgR/mgR (gray) mice of the indicated ages (n≥5 per time-point and genotype); asterisks indicate statistically significant differences between individual sets of WT and MFS samples (P<0.05).
the efficacy of different drug treatments. To this end, aneurysm growth, p-Erk1/2 and p-Smad2 accumulation and media degeneration (ie, elastic fiber fragmentation and aortic wall thickening, histopathologic markers of TGFβ-driven tissue proteolysis and fibrosis, respectively) were monitored at selected time intervals during the first 3 months of life, when about half of Fbn1^mgR/mgR mice die from complications of arterial disease.9 Echocardiographic measurements revealed a statistically significant enlargement of both the root and proximal ascending segment of the mutant aorta beginning at P45 that steadily increased thereafter relative to the wild-type (WT) counterparts (Figure 1A). Histomorphometric analyses of aortic tissue sections identified P45 at the first time point in which media degeneration was statistically significant (Figure 1B). Immunoblot assays of protein extracts from the aortic arches of mutant and WT mice showed substantially higher than normal levels of p-Erk1/2 as early as P16 followed by a steep decline at P60, a stage when abnormal p-Smad2 accumulation in the dilating mutant aorta was first noted to be statistically significant (Figure 2A). Abnormal activation of Erk and Smad proteins at discrete stages of TAA progression in Fbn1^mgR/mgR mice was in line with our previous observation that overactivation of the stress response p-38 MAPK protein preceded abnormal p-Smad2 accumulation in the aortas of Fbn1^−/− mice (a validated animal model of neonatal lethal MFS).14 On the basis of these findings, we selected P60 and P90 as the most informative ages in which to evaluate the effects of the different drug treatments on p-Erk1/2 and p-Smad2 accumulation and on aneurysm growth and media degeneration, respectively.

**AT1r and TGFβ Inhibition Exert Distinct Stage-Related Effects on TAA Progression**

Fbn1^mgR/mgR mice were treated with either losartan or TGFβ-Nab for a period of 74 days starting at P16. The mouse monoclonal TGFβ-Nab 1D11 was used in place of the rabbit polyclonal antibody used in the original study of Fbn1^C1039G/+ mice4 so as to avoid that immunoreactivity to the neutralizing antibody might reduce its efficacy. The dose and dosing regimen of 1D11 was chosen to maximal efficacy for the neutralization of circulating TGFβ in mice, a value previously estimated to be ≈115 ng/mL in Fbn1^C1039G/+ mice.15 Studies conducted in murine models of TGFβ-mediated disease have demonstrated that a subcutaneous dose of 5 mg/kg administered 3× per week is highly efficacious.16,17 We therefore used a 2-fold higher dose (10 mg/kg) thrice per week so as to assure maximal efficacy. At such a dose and dosing regimen, the steady-state plasma levels of 1D11 were estimated to range between 400 and 200 μg/mL.16 Because the Ki for 1D11 neutralization

![Figure 2. TAA markers in mice with severe MFS at baseline and after drug treatment.](image-url)
of TGFβ1 in vitro is 0.3 μg/mL, the circulating levels of 1D11 in the current study were estimated to range between 1200- and 800-fold over the Ki for 1D11. In accordance with these considerations and the reported 1D11-induced modifications of osteoblast and osteoclast activities, our treatment protocol led to a statistically significant increase of bone mass and quality in both WT and Fbn1mgR/mgR mice (Figure 3A and data not shown). To ensure that modifications of TAA pathology in 1D11-treated Fbn1mgR/mgR mice were not a consequence of vasopressor qualities of TGFβ inhibition, we also measured blood pressure in WT mice treated with 1D11 and observed no detectable effects (Figure 3B). Survival curves of Fbn1mgR/mgR mice in the various placebo treatments were grouped together (total n=54) because the distinct placebo arms behaved in nearly identical manners (Figure 4A). As reported previously, systemic administration of losartan from P16 onward significantly improved the survival of Fbn1mgR/mgR mice by attenuating aneurysm growth and to a lesser extent, media degeneration (Figure 4). Additionally, losartan treatment resulted in greater reduction of p-Erk1/2 than p-Smad2 levels (Figure 2B). Together, these findings suggested a causal relationship between aneurysm growth and AT1r-induced p-Erk1/2 accumulation, as well as a partial contribution of AT1r to Smad2 overactivation.

In contrast to losartan, administration of 1D11 from P16 onward exacerbated arterial disease in Fbn1mgR/mgR mice, thereby causing death from ruptured TAA significantly earlier than in placebo-treated mutant mice (Figure 4A). Echocardiographic and histomorphometric analyses of 1D11-treated Fbn1mgR/mgR mice that survived to P45 suggested an accelerated TAA progression, as evidenced by the greater aneurysm growth and medial degeneration relative to placebo-treated mutant animals (Figure 5). Because TGFβ neutralization had no negative effects on either aortic tissue morphology or fitness of WT littermates, we concluded that baseline TGFβ signaling is necessary to stabilize the fibrillin-1 deficient vessel under recurring hemodynamic load during the early stages of aneurysm formation. Furthermore, the beneficial effect of losartan treatment on TAA formation implied that the protective function of TGFβ is exerted independently of AT1r activity.
β is predominantly dependent on Erk-mediated AT1r signaling—these differences as additional evidence that aneurysm growth to placebo-treated mutant animals (Figure 4A). This result implied that elevated TGFβ signaling plays a detrimental role at later stages of TAA formation. Two important differences were noted between P16-initiated treatment with losartan and P45-initiated treatment with 1D11. Whereas AT1r antagonism mostly restricted aneurysm growth, TGFβ neutralization largely delayed media degeneration (Figure 4B and 4C); and whereas losartan normalized p-Erk1/2 levels, 1D11 reduced them by only ~25% relative to placebo-treated mutant animals (Figure 2B). We interpreted these differences as additional evidence that aneurysm growth is predominantly dependent on Erk-mediated AT1r signaling (as opposed to Erk-mediated TGFβ signaling) 10 and that AT1r activity is partially (as opposed to solely) responsible for TGFβ-driven media degeneration in MFS mice.

Combined AT1r and TGFβ Antagonism Prevents TAA Formation and Death of MFS Mice

In light of the above findings, we decided to treat Fbn1mgR/mgR mice with losartan from P16 onward together with 1D11 from P45 onward. This combined drug treatment normalized both aneurysm growth and medial degeneration (Figure 4B and C), thereby dramatically improving the survival of Fbn1mgR/mgR mice relative to either the P16-initiated losartan treatment or the P45-initiated 1D11 treatment (Figure 4A). Indeed, only one of the sixteen Fbn1mgR/mgR mice subjected to the double drug treatment died prematurely and from TAA-unrelated complications. The greater therapeutic benefit of combining losartan and 1D11 versus administering losartan alone indicated that additional AT1r-independent signaling pathways participate in an augmenting TGFβ activity (Figure 4). Along the same lines, paradoxical Erk1/2 hyperactivity in the aorta of Fbn1mgR/mgR mice treated with both agents suggested compensatory activation of other signaling pathways (either irrelevant or supportive of normal function) in the absence of both AT1r and TGFβ signaling (Figure 2B). The finding that p-Erk1/2 levels were also significantly elevated in WT mice treated with the two drugs indirectly supported this conclusion (Figure 3C).

Irrespective of this last point, the remarkable therapeutic benefit of the combined drug treatment validated the notion that promiscuous AT1r and TGFβ signaling drive partially overlapping disease processes in the aorta of mice with severe MFS.

Discussion

Although there is an emerging consensus that altered aortic mechanobiology represents the most common trigger of inherited TAAs, 22 the identity of and interactions among downstream signaling pathways that connect different mutant genes to tissue dysfunction remain poorly defined. In the case of MFS, it is widely believed that TGFβ hyperactivity is the main driver of TAA progression and that AT1r is the principle stimulator of pathogenic noncanonical (Erk-mediated) TGFβ signaling. 1 This belief rests on the original observation that either AT1r antagonism or TGFβ neutralization prevented aneurysm growth and medial degeneration in MFS mice that seldom manifest dissection and rupture of the dilating vessel. 4,10 As subsequent investigations have raised several questions about different key aspects of this disease model, 3 we evaluated how inhibition of either AT1r or TGFβ activity influences TAA progression in mice with lethal MFS. This first head-to-head comparison of the two drug regimens was performed by monitoring the same disease markers as in the original study of Fbn1C1039G/+ mice, 4 and with the additional advantage of including mouse survival as a more informative clinical end point of TAA progression. Our major finding is that, contrary to the current view, promiscuous AT1r and TGFβ activity in the MFS aorta are principally responsible for driving aneurysm growth and media degeneration through p-Erk1/2 and p-Smad2/3 signaling, respectively. We also found that TGFβ exerts opposite contextual effects on TAA pathology that broadly correlate with the early and late stages of disease progression. Both of these findings have important implications for TAA treatment at MFS.

Possible explanations for the discrepancy between our and earlier results of systemic TGFβ neutralization with Fbn1C1039G/+ mice include the potentially greater neutralizing activity of monoclonal antibody 1D11 and the different experimental designs and MFS models used in the two studies. Ongoing dosing experiments with Fbn1C1039G/+ mice seem to exclude the possibility that differences in the amount and frequency of TGFβ-Nab administration played a major role in causing such dramatically diverse vascular phenotypes. Similarly, the alternative explanation of differences between the two mouse models of MFS is not supported by ongoing investigations indicating that chronic (7-month long) treatment of Fbn1C1039G/+ mice with 1D11 (administered at a dose comparable with that of the present study and to mutant mice of the same genetic background as in the original report) 8 is associated with an appreciable trend toward disrupting (rather than preserving) aortic tissue architecture (data not shown). We therefore argue that the observed discrepancy is more likely to reflect the stratification of additional TAA-related events in mice with progressively severe MFS relative to those afflicted with a milder form of the disease. In support of this postulate, we note that losartan treatment was recently shown to decrease...
the rate of aneurysm growth in a large cohort of adult patients with MFS without modifying critical end points of disease, such as vessel wall dissection.23 Along the same lines, a randomiz ed clinical trial of MFS children and young adults has subsequently demonstrated no significant differences in the rate of aortic root dilation between patient groups subjected to either losartan or the β-blocker atenolol monotherapy.24

The dimorphic effects of TGFβ activity during TAA formation are yet another example of the contextual activity of these multifunctional signaling molecules in several physiological and disease processes.25 We think that the beneficial action of TGFβ signaling during the early stage of TAA formation in MFS mice reflects the physiological response of a structurally compromised aortic wall to increasing hemodynamic stress. We rest our belief on the finding that P16-initiated TGFβ neutralization had a negative impact on the TAA formation in Fbn1<sup>m<sup>mutant</sup></sup> but not in WT mice. Accordingly, we propose that the initial consequence of fibrillin-1 deficiency is to impair rather than to enhance TGFβ signaling, as currently thought.1,4,10 The pathogenic action of TGFβ hyperactivity in advanced stages of TAA is conceivably accounted for by the establishment of a negative TGFβ-centered feedback loop that is likely to involve, among others, the immunoinflammatory response to locally generated signals of ECM remodeling/repair.13 By documenting the greater therapeutic efficacy of the combined losartan/1D11 treatment versus losartan alone, our analyses indicate that deleterious TGFβ signaling late in TAA formation is only partially dependent on AT1r action. Overall, our data demonstrate that TAA pathogenesis in MFS is significantly more complex than currently thought.1,4,10 Indeed, the paradoxical finding of Erk1/2 overactivation in doubly treated MFS mice exemplifies the complex dynamics of molecular interactions among pathogenic, productive, and correlative signaling events in response to chronic wall stress and pharmacological interventions.

In conclusion, we propose a revised model of TAA pathogenesis in MFS that is based on the data presented here and related findings in the literature (Figure 6).2,3,23 A fibrillin-1Deficient ECM triggers and communicates to resident cells signals of mechanical stress that are translated into biochemical responses aimed at maintaining physiological tone. Among others, the early vessel response to mechanical stress includes constitutive AT1r overactivation and protective TGFβ signaling. As arterial dysfunction exacerbates under recurring mechanical stress, promiscuous TGFβ signaling becomes the major driver of medial degeneration stimulated in part by the constitutive AT1r activity, inflammatory cells and tissue remodeling enzymes. Together with TGFβ-induced collagen overaccumulation, this unopposed degenerative process further impairs aortic integrity and mechanics thereby leading to dissection and rupture of the vessel wall. Our model provides a unifying understanding of how genetically distinct lesions may promote TAA formation as mutations in cytoskeletal proteins impair SMC contractility in response to hemodynamic load and mutations in components of the TGFβ signaling cascade are expected to perturb the development of a mechanically compliant aortic wall.1,2,22 In this view, TGFβ hyperactivity in the MFS aorta represents a marker of unproductive tissue repair rather than the initial trigger of arterial disease.22 Our model also argues for a combinatorial treatment strategy against TAA that would target both promiscuous AT1r and TGFβ signaling without interfering with the early protective role of TGFβ activity in the structurally compromised aorta of MFS patients. However, a major obstacle against implementing such a promising treatment strategy is the lack of informative biomarkers of TAA severity that could rigorously determine the appropriate timing of TGFβ neutralization. Ongoing experiments are investigating new drug treatments that may solely target the detrimental action of TGFβ hyperactivity in the MFS aorta.

Acknowledgments
We thank Y. Lu, L. Phillips, and W. Zhang for assisting in statistical and histopathologic analyses and K. Johnson for organizing the article.

Sources of Funding
This work was supported by the National Institutes of Health grants P01-AR49698 (F. Ramirez) and T32GM007280 (J. Cook) and funds from the National Marfan Foundation and the Elster Family Research Endowment (F. Ramirez).

Disclosures
Genzyme Corporation manufactured the monoclonal antibodies 1D11 and 13C4.

References
β signaling exerts stage-specific dimorphic effects on arterial disease comparing the impact of AT1r and TGFβ activity on aneurysm progression and its strict dependence on AT1r activity. Our report addresses these two key aspects of TAA pathogenesis by models of TAA and AAA has identified AT1r as a common primary trigger of arterial disease, in addition to raising a controversy on TGFβ progression that are largely independent of AT1r action. Consistent with these observations, we documented the superior benefit of combin-
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Arterioscler Thromb Vasc Biol. 2015;35:911-917; originally published online January 22, 2015; doi: 10.1161/ATVBAHA.114.305150

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

Animals and drug treatments. Fbn1<sup>mgR/mgR</sup> experiments were performed using male mutant mice or sex-matched wild-type (WT) littermates maintained on the mixed 129T2/SvEmsJ;C57Blk/6J genetic background. Mice received 0.6 g/L losartan <i>ad libitum</i> in drinking water and the 1D11 antibody by intraperitoneal injection 3 times per week at a dose of 10 mg/kg of body weight; the antibody 13C4 was used as placebo treatment. TAA progression in Fbn1<sup>mgR/mgR</sup> mice was monitored bi-weekly by echocardiography performed using a VisualSonics Vevo 2100 imaging system and a 40-MHz transducer. The tail-cuff method was used to measure blood pressure on conscious WT animals treated with placebo or 1D11; bone mass and quality were evaluated in the same experimental groups according to standard ex vivo protocols. The Institutional Animal Care and Use Committees of the Icahn School of Medicine at Mount Sinai and Genzyme Corporation reviewed and approved all animal studies.

Histomorphometry. Proximal ascending aortas harvested from WT and mutant mice were processed, stained and evaluated for the extent of media degeneration based on the combined assessment of wall thickness and wall architecture. Average aortic thickness of 3 representative areas was independently measured and averaged by two observers blinded to genotype and treatment. Likewise, two individuals blinded to genotype and treatment independently counted and averaged the number of free ends along elastin-stained lamellae at 3 equally distant rings along the vessel’s length. Aortic wall architecture was graded giving thickness and number of free ends equal weight according to a severity scale for a combined score ranging from 1 (normal elastic fiber morphology) to 5 (diffuse elastic fiber disruption).

Biochemistry. Protein extracts were prepared from frozen aortic tissues and processed for immunoblots as previously described. Antibodies against phosphorylated and non-phosphorylated Smad2/3 and Erk1/2 proteins (Cell signaling) were diluted 1:1000 in Tris-buffered saline, pH 7.4, and 0.1% (v/v) Tween 20 in the presence of 5% BSA and incubated with the membrane for 12 h at 4 °C. In all cases, Ponceau S Solution staining (Sigma) of the transfer membrane was used as a protein loading control. Immunoreactive products were visualized by chemiluminescence using Clarity Western ECL substrate (Bio-Rad) and their relative intensity was evaluated using Photoshop (Adobe Systems Inc.).

Statistics. Equality of group variances was examined by F-test and Brown-Forsythe test (GraphPad Prism Statistical Software Package). Un-paired two-tailed t-tests were used to determine the statistical significance between two groups assuming significance at p < 0.05 with Welch’s correction applied when necessary and further confirmed with the non-parametric Mann-Whitney Test. Analyses between multiple groups employed one-way ANOVA with p < 0.05 considered statistically significant. Tukey’s Multiple Comparison test was used for post-hoc 2-sample comparisons. All values are expressed as mean ± S.D. Overall survival was calculated from weaning through death or collection at the intended time points. Overall survival was evaluated with Mantle-Cox (log-rank) statistical tests (GraphPad Prism); Kaplan-Meier curves were constructed for each treatment.

References:
