Pharmacologically Improved Contractility Protects Against Aortic Dissection in Mice With Disrupted Transforming Growth Factor-β Signaling Despite Compromised Extracellular Matrix Properties

Jacopo Ferruzzi,* Sae-Il Murtada,* Guangxin Li,* Yang Jiao, Selen Uman, Magdalene Y.L. Ting, George Tellides, Jay D. Humphrey

Objective—Transforming growth factor-beta is a pleiotropic cytokine having diverse roles in vascular morphogenesis, homeostasis, and pathogenesis. Altered activity of and signaling through transforming growth factor-beta has been implicated in thoracic aortic aneurysms and dissections, conditions characterized by a reduced structural integrity of the wall that associates with altered biomechanics and mechanobiology. We quantify and contrast the passive and active biaxial biomechanical properties of the ascending and proximal descending thoracic aorta in a mouse model of altered transforming growth factor-beta signaling, with and without treatment with rapamycin.

Approach and Results—Postnatal disruption of the gene (Tgfbr2) that codes the type II transforming growth factor-beta receptor compromises vessel-level contractility and elasticity. Daily treatment with rapamycin, a mechanistic target of rapamycin inhibitor that protects against aortic dissection in these mice, largely preserves or restores the contractile function while the passive properties remain compromised. Importantly, this increased smooth muscle contractility protects an otherwise vulnerable aortic wall from pressure-induced intramural delaminations in vitro.

Conclusions—Notwithstanding the protection afforded by rapamycin in vivo and in vitro, the residual mechanical dysfunction suggests a need for caution if rapamycin is to be considered as a potential therapeutic. There is a need for in vivo evaluations in cases of increased hemodynamic loading, including hypertension or extreme exercise, which could unduly stress a structurally vulnerable aortic wall. Given these promising early results, however, such studies are clearly warranted. (Arterioscler Thromb Vasc Biol. 2016;36:919-927. DOI: 10.1161/ATVBAHA.116.307436.)

Key Words: aneurysm ▪ aorta ▪ rapamycin ▪ stiffness ▪ strength ▪ transforming growth factor-beta

Vascular cells must establish and then maintain an extracellular matrix (ECM) that endows the aortic wall with appropriate compliance and resilience, but also sufficient strength. Decreased compliance can lead to hemodynamic consequences that are implicated in diverse hypertension- and aging-related conditions, including heart attack, stroke, and kidney failure. In contrast, decreased strength can lead to catastrophic aortic rupture or dissection.2 Frank rupture is most common in aneurysms of the infrarenal aorta, whereas dissection tends to occur in the thoracic aorta either in the presence or absence of an aneurysm. It is not clear why the thoracic aorta is particularly vulnerable to dissection,2 but different embryonic lineages of intramural cells, their differential responses to cytokines, such as transforming growth factor-beta (TGF-β), or hormones, such as angiotensin II, and the distinct pooling of mucoid material have been suggested.4-6

Altered activation of or signaling through TGF-β has also been linked to thoracic aortic aneurysms and dissections (TAADs). Such alterations have been reported in Marfan syndrome, Loeys-Dietz syndrome, and familial TAADs, including those because of ACTA2 and MYH11 mutations.7 Mouse models have been developed to study effects of altered TGF-β, its receptors, and downstream signaling in arterial morphogenesis, homeostasis, and TAAD progression.8-11 Such studies provide insight into molecular- and cellular-level mechanisms, but there has yet to be any rigorous study of vessel-level changes in the wall mechanics. It is the biomechanical mechanisms that largely dictate the ultimate fate—disSECTION and rupture—of the aortic wall. We study a conditional knockout mouse model wherein the gene (Tgfbr2) coding the TGF-β type II receptor, an essential ligand-binding component of the receptor complex, can...
be inactivated postnatally in vascular smooth muscle cells (SMCs) under tamoxifen control. Of particular note, this model suggests that basal TGF-β signaling in aortic SMCs promotes postnatal wall homeostasis and impedes early progression to TAADs. An additional finding is that rapamycin, an inhibitor of the mechanistic target of rapamycin serine/threonine protein kinase that regulates cell proliferation and protein synthesis, protects against the development of intramural hematoma in the thoracic aorta of mice in which Tgfbr2 is disrupted postnatally. Given that the material properties and structural integrity of the aortic wall are fundamental to its long-term biomechanical functionality, including resistance to TAADs, we assessed, for the first time, active and passive biaxial mechanical behaviors of the ascending thoracic aorta (ATA) and proximal descending thoracic aorta (DTA) in these mice, without and with rapamycin treatment.

Materials and Methods

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

Results

Fifty-two (52) male mice were studied at ≈8 weeks of age across 3 groups (Table): 17 controls (Ctrl), 19 Tgfbr2 disrupted via tamoxifen induction at 4 weeks of age (Tmx), and 16 treated daily with rapamycin for 4 weeks after Tgfbr2 disruption at 4 weeks of age (Tmx+Rapa). Of these, 30 were used for passive biaxial evaluation and histological examination, 17 for active biaxial evaluation, and 5 for final hypothesis testing.

Consistent with prior findings, loss of SMC-specific TGF-β signaling at 4 weeks of age resulted in an ≈42% incidence of thoracic aortic dissections at 8 weeks of age—with lesions arising in both the ATA and DTA—while daily treatment with rapamycin prevented dissection but resulted in a lower body mass on average (Table). ATAs from the Tmx group exhibited diverse gross phenotypes; some vessels appeared normal, some exhibited mild dilatation and fibrosis, and some had varying degrees of intramural hematoma in the absence of a clear intimal flap (Figure II in the online-only Data Supplement). In contrast, many DTAs from the Tmx group appeared normal, save some proximal intramural hematomas (Figure II in the online-only Data Supplement). Figure 1 shows histological images for some of these phenotypes at both aortic locations, with Tmx+Dis indicating vessels in which Tgfbr2 disruption led to an accumulation of blood within the medial layer in vivo. Consistent with prior findings in aortas that had not undergone in vitro biomechanical testing (Figure 2F–2G in Li et al), medial and adventitial cross-sectional areas were larger in dissected vessels (Figure III in the online-only Data Supplement). Calculating the percentage of total area that was occupied by media versus adventitia revealed slight adventitial thickening in the Tmx+Rapa group (Figure 1; Figure III in the online-only Data Supplement). The amount of elastin appeared to be preserved, which means that its area fraction tended to be lower with dissection or treatment with rapamycin because of increases in adventitial collagen (Figure III in the online-only Data Supplement). Measurements of residual stress relieving opening angles revealed marked differences because of Tgfbr2 disruption that were not prevented with rapamycin treatment (Figure IV and Table I in the online-only Data Supplement). Opening angles reflect transmural distributions in elastic fibers and fibrillar collagens; the differences were consistent with an increase in adventitial collagen in Tgfbr2 disruption. Finally, focal accumulations of mucoid material were observed in the media of some dissected vessels, not necessarily at the site of dissection (Figure V in the online-only Data Supplement). Henceforth, the term dissection will indicate the presence of an intramural accumulation of blood independent of its source; the term delamination will signify intramural separations (usually within the media) without evidence of associated blood.

Geometric and biomechanical metrics are listed in Table I in the online-only Data Supplement for all specimens that survived passive biaxial testing. That is, to focus on the potentially vulnerable, but not dissected, aortic wall, we excluded Tmx+Dis samples from quantitative comparisons of passive mechanical properties (Figure VI in the online-only Data Supplement). Figure 2 shows pressure–diameter (structural) and associated circumferential stress–stretch (material) behaviors as well as axial force–stretch responses for the 3 groups and both regions. Note that ATAs and DTAs from control mice (white symbols) exhibit the usual highly nonlinear and compliant behavior; the initial nonzero slope in the stress–stretch response indicates a normal elastin-dominated behavior at low pressure. This low-pressure behavior was similar in vessels from Tmx and Tmx+Rapa groups, consistent with histological evidence of nearly preserved elastin. Yet, the subsequent characteristic stiffening usually ascribed to the recruitment of previously undulated collagen fibers differed with Tgfbr2 disruption. Tmx and Tmx+Rapa ATAs exhibited qualitatively similar stress–stretch behaviors with a loss of distensibility.

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATA</td>
<td>ascending thoracic aorta</td>
</tr>
<tr>
<td>DTA</td>
<td>descending thoracic aorta</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
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<tr>
<td>TAAD</td>
<td>thoracic aortic aneurysm and dissection</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
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<tr>
<td>Tgfbr2</td>
<td>murine gene coding the TGF-β1 type II receptor</td>
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### Table. Group Composition, Age, Body Mass, and Occurrence of Aortic Dissections in Tgfbr2f/f Mice That Were Untreated (Ctrl), Treated With Tamoxifen to Disrupt TGF-β Type II Receptor Signaling (Tmx), and Additionally Treated With Rapamycin (Tmx+Rapa)

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>Tmx</th>
<th>Tmx+Rapa</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>17</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>Age, wk</td>
<td>8.2±0.1</td>
<td>8.5±0.2</td>
<td>8.4±0.1</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>22.8±0.5</td>
<td>22.0±0.5</td>
<td>16.4±0.4*</td>
</tr>
<tr>
<td>% Dissected</td>
<td>0%</td>
<td>42%*</td>
<td>0%</td>
</tr>
</tbody>
</table>

Ctrl indicates control; Rapa, rapamycin; TGF-β, transforming growth factor β; and Tmx, tamoxifen.

*P<0.05 vs control.
despite axial properties similar to controls (Figure 2, top row). In contrast, Tmx and Tmx+Rapa DTAs were circumferentially indistinguishable from controls (despite the latter having smaller diameters) while displaying a progressive loss of axial extensibility (Figure 2, bottom row). Finally, dissected DTAs had nearly normal circumferential behaviors (away from the site of dissection), whereas dissected ATAs showed circumferential dilation, a further loss of distensibility, and loss of extensibility (Figure VI in the online-only Data Supplement). Further examination of passive data revealed that Tgfbr2 disruption did not affect the biaxial material stiffness (Table I in the online-only Data Supplement), though biaxial stresses were lower at in vivo conditions (Figure 2).

Changes in elastic energy storage because of disrupted TGF-β signaling in the SMCs are shown in Figure 3. Consistent with data in Figure 2 and with respect to controls,
iso-energetic contours were compressed along the ordinate (indicating lower distensibility) for ATAs and especially along the abscissa (indicating lower extensibility) for DTAs for both the Tmx and Tmx+Rapa groups. Albeit not shown, control ATAs and DTAs displayed similar energy storage and degrees of anisotropy as wild-type mice. Importantly, the reduced ATAs and DTAs displayed similar energy storage and degrees of anisotropy as wild-type mice.

ATAs normally store more energy than DTAs because of a higher elastin content and nearly equi-biaxial loading. Loss of TGF-β signaling decreased energy storage in both regions, and rapamycin neither prevented nor restored this function (in ATAs, Tmx+Rapa energy storage is lower than Ctrl at P<0.1 while being not significantly different from Tmx). * indicates P<0.05 with respect to Ctrl samples from similar aortic locations. Ctrl indicates control; Rapa, rapamycin; TGF-β, transforming growth factor; and Tmx, tamoxifen.

Figure 3. Passive elastic energy storage during biaxial mechanical testing of ascending (ATA; top row) and descending (DTA; bottom row) thoracic aortas for 3 groups: Ctrl (first column), Tmx (second column), and Tmx+Rapa (third column). Iso-energetic contour plots highlight biaxial strain dependencies, whereas individual values reveal in vivo states (bar plot, fourth column). Each iso-energy level is indicated as a curve (with values of 0.1, 1, 5, 10, 20, 40, 60, 100, 250, and 500 kPa), while in vivo values are shown as filled circles. ATAs normally store more energy than DTAs because of a higher elastin content and nearly equi-biaxial loading. Loss of TGF-β signaling decreased energy storage in both regions, and rapamycin neither prevented nor restored this function (in ATAs, Tmx-Rapa energy storage is lower than Ctrl at P<0.1 while being not significantly different from Tmx). * indicates P<0.05 with respect to Ctrl samples from similar aortic locations.

Figure 4 shows an unexpected, but striking, observation in passively loaded DTAs from Tgfbr2 disrupted mice, both Tmx (1 of 9) and Tmx+Rapa (6 of 11). In this subset of specimens (7 of 20 or 35%), grossly normally appearing specimens often delaminated during passive mechanical testing, beginning during the initial period of static loading (acclimation) and progressing during subsequent mechanical testing (Figure 4A). In all cases, the P-d and f-l protocols were completed because the apparently intact adventitia allowed pressurization (Figure 4B; Figure VI in the online-only Data Supplement); as noted above, however, we excluded data from these specimens in the quantitative assessments in Figures 2 and 3 and Table I in the online-only Data Supplement. Nevertheless, by comparing raw P-d data between intact and delaminated DTAs, we observed dilatation and increased hysteresis (ie, energy dissipation, as revealed by different loading and unloading curves) in delaminated samples (Figure 4B). The latter likely resulted from an accumulation of physiological solution within the delaminated wall, which would increase intramural viscous dissipation during cyclic loading. As seen in Figure 4C–4D, using both in vitro imaging via optical coherence tomography and normal histology, the intramural delaminations were extensive and involved multiple medial lamellar units. Some of the delaminations may have initiated near branch sites in the DTAs, which necessarily were not present within the tested regions of the ATAs.

Active biaxial tests on the DTA revealed that daily treatment with rapamycin largely preserved or improved the contractile function that was otherwise diminished because of Tgfbr2 disruption. In particular, we measured changes in outer diameter on stimulation with 80 mmol/L KCl while monitoring changes in axial force. For each vessel, we adjusted the fixed value of axial stretch so that changes in axial force (total minus passive) varied from positive (tensile) to negative (compressive) because of contraction (Figure VII in the online-only Data Supplement). We defined an active in vivo axial stretch as that value at which no change in transducer-measured axial force occurs on contraction, assuming that this would be a preferred (homeostatic) value under physiological conditions. When contracted at individual active in vivo stretches and 70 mm Hg transmural pressure, changes in outer diameter were −250±22 μm (Ctrl), −163±21 μm (Tmx), and −223±25 μm (Tmx+Rapa; Figure 5). Hence, rapamycin either nearly preserved or restored toward normal the active circumferential properties of DTAs in Tgfbr2 disrupted mice. Values of the active in vivo stretches were yet statistically lower for Tmx (1.38±0.03) and Tmx+Rapa (1.41±0.01), relative to Ctrl (1.52±0.02), similar to the lower passive in vivo stretches (Table I in the online-only Data Supplement) for Tmx (1.40)
and Tmx+Rapa (1.34) relative to Ctrl (1.50). These findings suggest that the axial direction is controlled largely by passive properties and rapamycin neither prevented nor reversed the decreased axial stretch because of disrupted TGF-β signaling (Figure 2, right). A decreased axial stretch can unload the wall biaxially, which could be protective in many cases of altered hemodynamic loading or genetic mutations.13

Anecdotally, in contrast to observations under passive conditions (Figure 4), none of the DTA specimens exhibited intramural delaminations while tested under active conditions. We thus tested the hypothesis that increased SMC contractility could protect an otherwise structurally vulnerable aortic wall from intramural delamination. Figure 6 shows illustrative results from a series of experiments on Tmx+Rapa DTAs. None of the specimens exhibited a visible or optical coherence tomographic-detected intramural delamination when pressurized while actively contracted with 80 mmol/L KCl at 70 mmHg (Figure 6, top). In stark contrast, a subset of vessels (2 of 5) began to delaminate at the same pressure soon after being rendered passive by washing out the high K+ Krebs with a normal Hanks’ solution (Figure 6, middle); indeed, these specimens delaminated further on cyclic pressurization over physiological ranges (Figure 6, bottom). Table II in the online-only Data Supplement shows calculated reductions in wall stress in circumferential and axial directions when the vessels were contracted. This reduction resulted, in large part, by decreasing the inner radius and increasing wall thickness (Figure 6) and may have protected the wall from damage.

Discussion

Data from patients demonstrate that mutations to the gene (TGFBR2) that codes the TGF-β type II receptor predispose to TAADs.14,15 Among other cellular-level effects, these mutations lead to a decreased expression of contractile proteins in medial SMCs; indeed, cultured mutant SMCs fail to elaborate or organize increased contractile proteins in response to exogenous TGF-β.16 Importantly, mutations to genes that encode SMC contractile proteins (eg, ACTA2 and MYH11) and associated molecules (eg, MLCK and PRKG1) similarly predispose to TAADs.17-20 There is, therefore, increasing evidence that dysfunctional SMC contractility plays important roles in humans presenting with TAADs.21,22

There are 3 main findings of this study. First, postnatal disruption of Tgfbr2 in SMCs compromises both active (contractile) and passive (structural) biaxial biomechanical properties in the murine thoracic aorta. Second, daily in vivo treatment with rapamycin largely preserves or restores biaxial contractile properties, but not passive structural properties. Third, stimulated in vitro contraction of SMCs in DTAs from rapamycin-treated mice with postnatal disruption of Tgfbr2 prevents pressure-induced intramural delamination (0 of 11), whereas in vitro inactivation of SMCs allows delaminations (6 of 11 total, or 55%). Indeed, delaminations initiated even at a modest transmural pressure (70 mmHg or lower) in a subset of rapamycin-treated vessels (2 of 5 or 40%) immediately after the contracted vessel was rendered passive.
Postnatal disruption of Tgfbr2 reduced the contractile range of the DTA by ≈37% (at transmural pressures of 70 and 80 mm Hg and in vivo axial stretches), and fundamentally changed its biaxial character (Figure VII in the online-only Data Supplement). Because of circumferential–axial coupling, contraction at a fixed pressure and axial stretch just above the in vivo value typically results in positive (ie, tensile) axial force generation in normal vessels.23 DTAs from Tgfbr2−disrupted mice exhibited weak or negative changes in axial force, which was either prevented or reversed by rapamycin treatment. As noted in the online-only Data Supplement, these differences may allow Tmx+Rapa vessels to increase wall thickness on contraction similar to controls. Related to this, unloaded wall thickness was not different between Ctrl and Tmx DTAs, but it was ≈11% greater in Tmx+Rapa DTAs (Table I in the online-only Data Supplement). Both contraction-induced and intrinsic increases in thickness can reduce wall stress (Table II in the online-only Data Supplement), which together may have kept active Tmx+Rapa vessels from exceeding threshold levels for delamination.

The compromised vessel-level contractility in Tgfbr2-disrupted aortas is consistent with our prior findings that there is decreased contractile protein gene expression (eg, Acta2 and Myh11), decreased phosphorylation of myosin light chain, and decreased cell-induced compaction in a collagen gel assay.11 The present data reveal further, however, that rapamycin largely protects against or reverses vessel-level contractile deficits. This finding is consistent with reports that rapamycin promotes SMC differentiation from a synthetic to contractile phenotype.24 Indeed, rapamycin did not protect against or reverse the marked changes in passive biaxial properties, which may require adequate synthesis of ECM to ensure structural integrity. Tgfbr2 disruption, with or without treatment with rapamycin, resulted in a significant decrease in elastic energy storage (Figure 3), which marks decreased mechanical functionality.12 Although circumferential material stiffness was not affected significantly by Tgfbr2 disruption, or its treatment with rapamycin, there was a trend in the ATA and significance in the DTA toward a decrease in the passive in vivo axial stretch (Figure 2), which likely contributed to the overall decreases in wall stress (Figure 2). Notwithstanding slightly lower blood pressures in the Tmx mice11 and near normalized pressures in the Tmx+Rapa mice, lower passive wall stresses could be either protective or detrimental. Lower stresses typically reduce the risk of a structural failure; yet, lower than normal stresses can also signal an atrophic mechanobiological response.25 The latter could contribute to diminished wall integrity, provided that the intramural cells can sense the wall stress. Mechanosensing and mechanoregulation of ECM requires functional actomyosin activity,22 however,
which may have been compromised similar to the diminished vessel-level contractility caused by the Tgfbr2 disruption. Given that remodeling responses to decreased wall stress tend to take much longer to manifest on a tissue level than responses to increased wall stress, rapamycin could possibly improve passive properties by improving mechanosensing and mechanoregulation of ECM if vessels were treated for a longer period (>4 weeks). Future studies will be needed in this regard.

Rapamycin inhibits the mechanistic target of rapamycin pathway that serves as an important node for cell signaling. Among its diverse actions, rapamycin is a strong attenuator of cell proliferation, particularly T cells, which contributes to its potent immunosuppression. Interestingly, T cells play important roles in structurally stiffening the murine aorta in an angiotensin II model of hypertension, primarily by increasing collagen deposition in the adventitia. Angiotensin II also plays important roles in structurally stiffening the murine aorta in an angiotensin II model of hypertension, primarily by increasing collagen deposition in the adventitia. Angiotensin II also plays important roles in TAADs, and there is considerable cross talk between angiotensin II and TGF-β signaling, both of which promote ECM production. Our results in normotensive mice showed, however, that rapamycin (which presumably blocked T cell activity) associated with a slight increase in adventitial area in the Tgfbr2-disrupted mice because of a modest increase in collagen and a slight decrease in medial area (Figure III in the online-only Data Supplement). The latter may have resulted from a reduction in SMC number because rapamycin attenuates proliferation. Recalling that rapamycin largely preserved or improved biaxial contractility in the DTAs of the Tgfbr2 mice (and that ATAs were not studied under active conditions because they never delaminated in vitro), note that increased contractility typically associates with decreased proliferation and decreased protein synthesis. Indeed, rapamycin decreases angiotensin II/mechanical stress-mediated protein synthesis. Our results are thus consistent with an increased contractility and decreased proliferation/synthesis by medial SMCs, with less apparent effects on adventitial fibroblasts. Indeed, a logical mechanobiological response by adventitial fibroblasts in cases of medial atrophy would be to increase ECM deposition, but there is a need to delineate medial and adventitial biomechanics to address this expectation. The situation could also be different in patients having TGBFR2 mutations. Tgfbr2 disruption in the current mouse model is both SMC-specific and postnatal, whereas adventitial (myo)fibroblasts in TGFBR2 patients may have reduced contractile protein expression and decreased mechanosensing and mechanoregulation capability.

That rapamycin increased vessel-level contractility and prevented in vivo dissection in all mice with tamoxifen-induced Tgfbr2 disruption is consistent with the emerging concept that decreased SMC contractility predisposes to TAADs. Three possibilities seem tenable. First, increased contractility could off-load some stress from an otherwise mechanically vulnerable ECM. Second, increased actomyosin activity could improve the SMC mechanosensing and mechanoregulation of the ECM that is fundamental to maintaining mechanical functionality and structural integrity. Third, increased microvascular tone could decrease both flow within and permeability of a vasa vasorum, if present (ie, although a stimulated vasa vasorum and associated angiogenic progression in murine arteries remains controversial, an invading pathologic vasa vasorum could enable entry of blood into the outer medial layers [Figure 1] and increased microvascular contractility could attenuate this possibility).

Although each of these effects could contribute to the in vivo protection afforded by rapamycin, it is particularly noteworthy that in vitro pressurization to physiological levels resulted in gross delaminations in vessels under passive, but never active, conditions (Figure 6). This observation supports the hypothesis that improved SMC tone may help to stress shield an otherwise mechanically vulnerable ECM (Table II in the online-only Data Supplement).
Supplement). If true, there is a pressing need to determine if such shielding would remain sufficient in cases of increased intramural stress, as in extreme exercise or hypertension, both of which are risk factors for aortic dissection.3 There is similarly motivation to determine whether adverse effects of calcium channel blocking in mice and patients predisposed to TAADs35 might be due, in part, to the loss of otherwise beneficial stress shielding because of SMC contraction. Indeed, increased vessel-level contractility could not only stress shield the ECM, it could attenuate a localized accumulation of interstitial fluid (cf. Figure 4) that could otherwise follow from an increased pooling of glycosaminoglycans or increased permeability of vasa vasorum. Additional, potential roles of rapamycin in these other cases are noted in the online-only Data Supplement.

In conclusion, this study confirms that rapamycin protects against intramural hematoma in vivo in mice with postnatal Tgfbr2 disruption.11 Although reasons for this therapeutic benefit remain unknown, rapamycin preserved or restored SMC contractility toward control values and reduced localized pooling of mucoid material, both of which may have been protective.6,22 Nevertheless, rapamycin did not restore the passive biaxial mechanical properties to normal; axial stretch and axial stiffness tended to remain reduced as did overall elastic energy storage. Because blood pressure is normal or slightly lower than normal in mice with Tgfbr2 disruption, the lower than normal stresses may not have been high enough to exploit the apparent underlying structural vulnerability that was revealed via in vitro mechanical testing under passive conditions. Given that uncontrolled hypertension and acute increases in blood pressure (eg, exercise related) pose significant risks for aortic dissection, we caution against the therapeutic use of rapamycin until further investigation can elucidate the protective mechanisms and potential trade-offs are considered carefully. Indeed, although there is no obvious vascular phenotype in Myh11R247C/R247C mice under normal hemodynamic loading, induced hypertension results in both rupture-related, premature deaths and evident aortic fragility during mechanical testing.32 There is, therefore, a need for further study of the potential role of ECM vulnerability under increased hemodynamic loads. Nevertheless, the potentially protective role of vessel-level contractility against dissection of a vulnerable thoracic aorta emphasizes the need for further biomechanical studies to complement molecular findings.

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Disclosures
None.

References
The present results represent the first observation of spontaneous in vitro delaminations under physiological loading of a structurally vulnerable thoracic aorta in a mouse model predisposed to aortic dissection. Importantly, induced SMC contractility afforded 100% protection against intramural delamination, which could be an initiator of dissection. Biomechanical calculations suggest that contraction markedly reduces intramural wall stresses and thereby may stress shield a vulnerable extracellular matrix. Pharmacological treatments should thus balance the need to reduce hemodynamic loads while preserving smooth muscle cell contractility in large arteries.
Pharmacologically Improved Contractility Protects Against Aortic Dissection in Mice With Disrupted Transforming Growth Factor-β Signaling Despite Compromised Extracellular Matrix Properties

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Figure I. Protocol for active biaxial testing. Video-images (left) of a representative cannulated descending thoracic aorta (DTA), within the biaxial testing device, both (a) before and (b) 15 minutes after SMC stimulation with high K\(^+\) while maintained at a 70 mmHg luminal pressure and near the in vivo axial stretch. Actual time course (right) of respective changes in outer diameter (along the ordinate), where contractions via 80 mM KCl are highlighted in pink and relaxations via wash-out with a standard Krebs solution are highlighted in green; the three sections show contraction-relaxation at three different axial stretches. This protocol was repeated for each specimen at 3 different pressures, hence resulting in 9 different contractile states. See Figure VII below. Note, too, the ligated intercostal arteries in the video images; based on visual observations in DTAs and the lack of delaminations in the ascending thoracic aortas (ATAs), which did not have branches within the region of testing, the intercostal branches may have served to help nucleate the in vitro delaminations discussed below.
Figure II. Gross phenotypes of three representative ascending thoracic aortas (ATAs) and one descending thoracic aorta (DTA) after 4 weeks of Tgfbr2 disruption (Tmx). ATAs (left panels) showed diverse phenotypes ranging from normal (top-left) to dilated and fibrotic (middle-left) or dramatic dissection, which is revealed by intramural blood (bottom-left). Note that phenotypic abnormalities involved the ascending and the arch regions while leaving the aortic root mostly unaffected. In contrast, the DTA (right panels) showed a milder phenotype with normal unloaded dimensions. DTA dissections manifested primarily as blood pools localized immediately after the left subclavian branch (top-right). Optical coherence tomography, or OCT, images of the intramural hematomas (middle- and bottom-right) never revealed an intimal flap, but rather multiple apparently separate intramural hematomas that often contributed to the formation of the main dissection. Finally, despite these different manifestations, the percentage of lesions was only slightly higher in the ATA than in the DTA, with an overall dissection rate in vivo about 42%. The white scale bars represent 1 mm.
Figure III. Quantification of the microstructural composition (left panels) and layer organization (middle and right panels) in thoracic aortas after Tgfbr2 disruption. ATA is ascending thoracic aorta whereas DTA is descending thoracic aorta. Elastin areas measured from VVG images did not show any significant differences among the four experimental groups (Ctrl, Tmx, Tmx + Dis, Tmx + Rapa), but did confirm that the elastin content is higher in the ATA than the DTA. Collagen areas were measured from MTC-stained cross sections, thus accounting for total collagen content, which was significantly higher in Tmx + Dis DTAs and Tmx + Rapa ATAs (with respect to Tmx). Medial and adventitial areas were measured from MOV images and showed a clear thickening of both layers in dissected DTAs, with similar – albeit not significant – trends in ATAs. Despite the smaller size of Tmx + Rapa DTAs (Table II), which correlated well with the smaller body mass of the animals (Table in main paper), such vessels displayed a moderate increase in adventitial area (p < 0.1) while maintaining medial area unchanged. Similar trends were seen in Tmx + Rapa ATAs without reaching statistical significance. Differences in total aortic areas can be due to treatment or aortic location, thus we calculated the percent aortic cross section that is occupied by media and adventitia. Daily treatment with rapamycin following Tgfbr2 disruption led to an increased percentage of adventitial area in both ATAs and DTAs due to adventitial thickening. The * and † indicate, respectively, p < 0.05 compared with Ctrl and Tmx samples from similar aortic locations.
Figure IV. Estimation of the stress-free configuration via the introduction of a radial cut in ascending thoracic aortas (ATA – top row) and descending thoracic aortas (DTA – bottom row) for all three primary groups: controls (Ctrl – first column), tamoxifen-induced disruption of Tgfbr2 without dissection (Tmx – second column), and tamoxifen-induced disruption with daily treatment with rapamycin (Tmx + Rapa – third column). Residual strains are reflected by the opening angle $\Phi_0$ (bar plot – fourth column). Tgfbr2 disruption associated with a marked decrease in opening angles in ATAs, with no significant protection or improvement with rapamycin treatment. Similar trends were observed in DTAs despite the lack of statistical significance. Larger opening angles reflect the presence of larger residual stresses (i.e., greater compressive stresses in the inner wall and greater tensile stresses in the outer wall, both in unloaded but intact specimens). The scale bar represents 1 mm. * indicates $p < 0.05$ with respect to Ctrl samples from similar aortic locations.
Figure V. Cross-sectional views of DTAs that dissected in vivo and were stained with Verhoeff Van Gieson (VVG - left), Masson's trichrome (MTC - center), or Movat's pentachrome (MOV - right). These images summarize the main pathological phenotypes occurring with dissection after tamoxifen-induced, postnatal disruption of Tgfbr2. The red arrows indicate dramatic intramural hematomas (red in MTC and MOV) contained within multiple elastic laminae (black in VVG and MOV sections). The aqua arrows instead highlight localized accumulations of mucoid material (aqua / green in the MOV sections), likely glycosaminoglycans. The mucoid pools often manifested distant from the dissected region, but were nevertheless often within the same intramural layers in which the dissections occurred. Albeit not shown, rapamycin treatment tended to prevent the pooling of mucoid material even though it did not reduce the overall amount. Insets show magnified views of the aortic wall. The scale bars represent 200 μm.
Figure VI. Passive biaxial mechanical behaviors from ascending thoracic aortas (ATA) comparing two subgroups of tamoxifen-induced disruption of Tgfrbr2: specimens without dissection (Tmx) and specimens that experienced aortic dissection in vivo with an accumulation of blood within the media (Tmx + Dis). Dissection associated with circumferential dilation of the ATA wall (P-d data – first column), loss of distensibility shown as a leftward shift in the associated circumferential Cauchy stress-stretch data for cyclic P-d tests (second column), and loss of extensibility (f-l data – third column). Such changes were not seen in dissected DTA samples (not shown) due to the localized proximal nature of their dissections and the measurement of mechanical quantities away from the lesions (Figure II).
**Figure VII.** Representative active biaxial data for all three groups: controls (Ctrl – top row), tamoxifen-induced disruption of Tgfbr2 without dissection (Tmx – middle row), and tamoxifen-induced disruption with daily treatment with rapamycin (Tmx + Rapa – bottom row). Changes in outer diameter over time (left column) indicate that smaller contractions are obtained for higher axial stretches (around the in vivo condition), whereas changes in axial force over time (right column) show that for each vessel there exists a value of axial stretch (called the active in vivo axial stretch) for which measured axial force does not change much upon SMC stimulation. Albeit not shown here, isochoric changes in wall thickness during contraction appeared to reflect differences in “active and passive” in vivo axial stretches. When the active in vivo axial stretch is less than the passive value, the vessel will expand axially during contraction (negative change in axial force), as observed in Tgfbr2 disrupted DTAs which exhibit a small active increase in wall thickness. Conversely, when the active in vivo axial stretch is greater than the passive value, the vessel will retract axially during contraction (positive change in axial force), as observed in rapamycin treated DTAs which exhibit larger active increases in wall thickness. Increased wall thickness reduces wall stress, which should be protective.
<table>
<thead>
<tr>
<th></th>
<th>ATA</th>
<th></th>
<th>DTA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl</td>
<td>Tmx</td>
<td>Tmx + Rapa</td>
<td>Ctrl</td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>7</td>
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<tr>
<td>Unloaded and Stress-Free</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer Diameter (μm)</td>
<td>1071 ± 27</td>
<td>1249 ± 52*</td>
<td>1076 ± 46</td>
<td>921 ± 18</td>
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<tr>
<td>Wall Thickness (μm)</td>
<td>106 ± 3</td>
<td>134 ± 9*</td>
<td>120 ± 6</td>
<td>105 ± 4</td>
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<tr>
<td><em>In-vitro</em> Length (mm)</td>
<td>2.8 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>5.0 ± 0.3</td>
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<td>Opening Angle (deg)</td>
<td>74 ± 8</td>
<td>8 ± 4*</td>
<td>17 ± 3*</td>
<td>43 ± 8</td>
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<tr>
<td>Loaded at 100 mmHg</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Outer Diameter (μm)</td>
<td>1778 ± 17</td>
<td>1802 ± 45</td>
<td>1590 ± 35*†</td>
<td>1532 ± 37</td>
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<tr>
<td>Wall Thickness (μm)</td>
<td>34 ± 2</td>
<td>53 ± 8*</td>
<td>46 ± 4</td>
<td>38 ± 1</td>
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<tr>
<td><em>In-vivo</em> Axial Stretch</td>
<td>1.74 ± 0.04</td>
<td>1.69 ± 0.08</td>
<td>1.63 ± 0.03</td>
<td>1.50 ± 0.01</td>
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<tr>
<td>Cauchy Stresses (kPa)</td>
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<tr>
<td>Circumferential</td>
<td>344.2 ± 23.4</td>
<td>240.8 ± 38.5</td>
<td>226.5 ± 20.1*</td>
<td>255.7 ± 8.4</td>
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<tr>
<td>Axial</td>
<td>355.0 ± 25.9</td>
<td>220.3 ± 45.1*</td>
<td>254.6 ± 28.7</td>
<td>211.0 ± 11.5</td>
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<tr>
<td>Material Stiffness (MPa)</td>
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<tr>
<td>Circumferential</td>
<td>1.68 ± 0.13</td>
<td>1.56 ± 0.15</td>
<td>1.31 ± 0.10</td>
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<td>Axial</td>
<td>2.15 ± 0.17</td>
<td>1.42 ± 0.34</td>
<td>1.53 ± 0.21</td>
<td>2.47 ± 0.12</td>
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<td>Stored Energy (kPa)</td>
<td>104.3 ± 10.2</td>
<td>59.7 ± 14.5*</td>
<td>66.2 ± 8.7</td>
<td>67.1 ± 2.3</td>
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<td>Energy Dissipation (%)</td>
<td>2.57 ± 0.50</td>
<td>5.70 ± 0.44*</td>
<td>3.71 ± 0.31†</td>
<td>3.26 ± 0.43</td>
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</tbody>
</table>

*p<0.05 with respect to Ctrl
†p<0.05 with respect to Tmx
Table I. Morphological and passive mechanical metrics for ascending thoracic aortas (ATA) and descending thoracic aortas (DTA) for all three primary groups: controls (Ctrl), tamoxifen-induced disruption of Tgfbr2 (Tmx), and tamoxifen-induced disruption with daily treatment with rapamycin (Tmx+Rapa). The mechanical metrics were evaluated at the group specific in vivo values of axial stretch but a common distending (transmural) pressure of 100 mmHg. Note, therefore, that mean (luminal) arterial pressures, measured using an invasive central catheter under anesthesia, were 84.3±4.3, 77.4±3.1, and 77.6±3.3 mmHg, respectively. Consistent with Figures 2 and 3 in the main paper, Tgfbr2 disruption resulted in lower energy storage and biaxial stresses but little difference in material stiffness. Rapamycin neither preserved nor restored passive wall properties. On the other hand, Tgfbr2 disruption caused an increase in energy dissipation in the ATA that was maintained at or brought towards normal values with rapamycin treatment. The same trends were observed in the DTA, despite lacking statistical significance. The * and † indicate, respectively, $p < 0.05$ compared to Ctrl and Tmx samples from similar aortic locations. Data are shown only for vessels that did not delaminate prior to data collection.
Table II

<table>
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<tr>
<th>P = 70 mmHg</th>
<th>Ctrl</th>
<th>Contracted</th>
<th>Change</th>
<th>Axial Stress (kPa)</th>
<th>Relaxed</th>
<th>Contracted</th>
<th>Change</th>
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<tr>
<td>P = 70 mmHg</td>
<td>Tmx</td>
<td>96 ± 8</td>
<td>70 ± 9</td>
<td>26 ± 4*</td>
<td>58 ± 5*</td>
<td>48 ± 5*</td>
<td>10 ± 1*</td>
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<tr>
<td>P = 70 mmHg</td>
<td>Tmx + Rapa</td>
<td>95 ± 2*</td>
<td>56 ± 5</td>
<td>39 ± 3</td>
<td>67 ± 10*</td>
<td>51 ± 10*</td>
<td>16 ± 1*</td>
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<tr>
<td>P = 80 mmHg</td>
<td>Ctrl</td>
<td>187 ± 14</td>
<td>149 ± 12</td>
<td>38 ± 2</td>
<td>128 ± 10</td>
<td>116 ± 10</td>
<td>12 ± 0.5</td>
</tr>
<tr>
<td>P = 80 mmHg</td>
<td>Tmx</td>
<td>121 ± 11*</td>
<td>103 ± 14*</td>
<td>19 ± 3*</td>
<td>59 ± 8*</td>
<td>47 ± 7*</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>P = 80 mmHg</td>
<td>Tmx + Rapa</td>
<td>120 ± 3*</td>
<td>85 ± 6*</td>
<td>35 ± 3</td>
<td>68 ± 7*</td>
<td>53 ± 7*</td>
<td>15 ± 2</td>
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<tr>
<td>P = 90 mmHg</td>
<td>Ctrl</td>
<td>216 ± 20</td>
<td>206 ± 17</td>
<td>10 ± 7</td>
<td>141 ± 11</td>
<td>130 ± 10</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>P = 90 mmHg</td>
<td>Tmx</td>
<td>144 ± 13*</td>
<td>140 ± 14*</td>
<td>4 ± 2</td>
<td>59 ± 9*</td>
<td>55 ± 10*</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>P = 90 mmHg</td>
<td>Tmx + Rapa</td>
<td>143 ± 5*</td>
<td>127 ± 9*</td>
<td>16 ± 6</td>
<td>71 ± 5*</td>
<td>59 ± 6*</td>
<td>12 ± 4</td>
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</table>

*p<0.05 with respect to Ctrl

Table II. Mean wall stresses during active and passive biaxial testing in descending thoracic aortas (DTA) for all three groups: controls (Ctrl), tamoxifen-induced disruption of Tgfbr2 (Tmx), and tamoxifen-induced disruption with daily treatment with rapamycin (Tmx+Rapa). Biaxial stresses were evaluated from raw data by enforcing equilibrium at the three levels of intraluminal pressure (70, 80, and 90 mmHg) and individual estimated value of in vivo axial stretch. SMC contraction reduces both circumferential (expected from Laplace’s equation due to the reduced radius and increased wall thickness) and axial stress. While both relaxed (passive) and contracted (active) stresses are influenced by long-term remodeling of the aortic wall, the displayed change in biaxial stress here (relaxed minus contracted) is due to acute contractility of intramural SMCs. Tgfbr2 disrupted DTAs display consistently lower changes in stress when stimulated with high KCl due to a reduced contractile capacity, whereas treatment with rapamycin either preserved or restored nearly to normal the values of wall stress. Together with the observation of in vitro delaminations under passive conditions (Figures 5 and 6 in the main text), these data suggest that normal SMC contractility can protect an otherwise vulnerable aortic wall. * indicates p < 0.05 with respect to Ctrl samples from similar aortic locations.
Pharmacologically Improved Contractility Protects Against Aortic Dissection in Mice with Disrupted Transforming Growth Factor-β Signaling Despite Compromised Extracellular Matrix Properties

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Materials and Methods

Mouse Model. All animal procedures were approved by the Institutional Animal Care and Use Committee of Yale University. Details can be found elsewhere1, but, briefly, the TGF-β type II receptor was disrupted in smooth muscle cells (SMCs) beginning at 4 weeks of age in male, tamoxifen-inducible, Myh11-CreER12, Tgfbr22/ mice (Tmx). The tamoxifen was administered for 5 consecutive days via daily 1 mg intraperitoneal injections while untreated animals served as controls (Ctrl). A third group of mice received daily intraperitoneal injections of rapamycin at 2 mg/kg for 4 weeks following the initial tamoxifen treatment (Tmx+Rapa). All mice were euthanized at about 8 weeks of age (corresponding to 4 weeks of treatment in the Rapa group) and the ascending thoracic aorta (ATA) was excised from the aortic root to the brachiocephalic artery whereas the proximal descending thoracic aorta (DTA) was excised from the left subclavian artery to the third pair of intercostal arteries.

Passive wall mechanics. Employing methods established in our laboratory2,3, excised segments of the ATA and DTA were cannulated on custom-drawn glass micropipets, secured with ligatures at each end, and mounted within a custom biaxial testing system. The vessels were acclimated at 80 mmHg for 30 minutes within a Hanks buffered testing solution at 37°C, then subjected to a short period of standard cyclic preconditioning (pressurization from 10 to 140 mmHg with the vessel held fixed near its in vivo length). Next, the vessels were subjected to cyclic pressure-diameter (P-d) testing at three fixed values of axial stretch (the passive in vivo value and ±5% of this value) followed by cyclic axial force-length (f-l) testing at four fixed values of intraluminal pressure (10, 60, 100, and 140 mmHg). Pressure, outer diameter, axial force, and axial length were measured on-line using standard transducers and a video-microscope, and used for feedback control of the 7 protocols as well as subsequent data analysis. In some cases, the transmural organization of the aortic wall was monitored during and after testing using an optical coherence tomography (OCT) system having an axial (depth) resolution <7 microns and lateral resolution of 8 microns (Callisto Model, Thorlabs, Newton, NJ).

Testing within a Hanks solution yields a passive biomechanical behavior2. As shown previously3, it is useful to quantify passive biaxial data in terms of circumferential and axial wall stress and material stiffness as well as elastic energy storage. Thus, nonlinear regression was used to fit an 8-parameter stored energy function W (see Appendix below) to unloading portions of P-d and f-l data from the final cycle of testing for each of the 7 protocols. Biaxial stress and stiffness were calculated from W by taking appropriate derivatives with respect to measured deformations and evaluated at vessel-specific values of the passive in vivo axial stretch and pressure (e.g., 100 mmHg). We emphasize, therefore, that all of these mechanical quantities depend nonlinearly on the biaxial deformation4; one cannot, for example, simply compute a modulus for stiffness from a 1-D Hooke’s law \( E = \sigma_0 / \epsilon_0 \), as is common in the literature. Following testing, short rings (~0.5 mm long) were excised from the specimens and used to measure unloaded wall thickness and residual-stress related opening angles4, the latter by suspending the unloaded rings in the Hanks solution and imaging the cross-section before and after introducing a radial cut to relieve any residual stored energy.

Active wall mechanics. In a separate set of tests, DTAs from each of the three groups were similarly mounted in the biaxial testing system to evaluate active properties. Briefly, these vessels were immersed in a bicarbonate-buffered Krebs-Ringer solution that was kept at 37°C and bubbled with 95% O2 / 5% CO2 to maintain pH at 7.4. Following estimation of the unloaded configuration, contractility was verified by increasing the KCl concentration to 80 mM in the adventitial bath at low intraluminal pressures and axial stretches: first contraction at \( P=40 \text{ mmHg and } \lambda_z=1.1 \), second at \( P=60 \text{ mmHg and } \lambda_z=1.2 \). After verifying that the vessels were responsive to KCl, similar contractions were elicited at nine combinations of transmural pressure (70, 80, and 90 mmHg – which pilot studies revealed did not cause smooth muscle damage due to overstressing the wall) and axial stretch (at and just above or below the active in vivo value, which is defined as the axial deformation at which axial force remains nearly constant upon contraction5). Vessels were relaxed for 10 minutes between each test by washing out the high K+ solution with a normal Krebs solution; the fixed values of pressure and axial stretch were then changed and the vessel was allowed to acclimate to the new conditions for 5 minutes, after
which it was contracted again for 15 minutes with 80 mM KCl. Figure I (below) shows a sequence of three tests (different axial stretches at a constant pressure) in terms of outer diameter changes during contraction (K+ loading) and relaxation (wash-out); pilot studies showed that responses were insensitive to the order of the pressure-stretch combinations. The 80 mM KCl produces a near maximal and sustained value of contraction suitable for biomechanical testing. Unfortunately, there are no data on the actual in vivo value of basal tone to which to compare and identification of basal tone is complicated by the need for measurements in mice under anesthesia, which changes this tone.

Histology. Following mechanical testing, the aortas were unloaded and fixed overnight in 4% formalin, then stored in 70% ethanol at 4°C. Vessels were embedded in paraffin, sectioned serially, and stained with Verhoeff Van Gieson (VVG), Masson’s trichrome (MTC), or Movat’s pentachrome (MOV). Cross-sections were imaged at 400× magnification and individual sectors were stitched together to obtain high resolution images. A custom computer code6 was used to extract area fractions of total elastin and collagen from VVG- and MTC-stained sections, while percent areas occupied by media or adventitia were obtained from MOV-stained sections. The presence of intramural blood (erythrocytes and/or fibrin) and glycosaminoglycans was assessed qualitatively from the MOV sections.

Statistics. Data are presented as mean ± SEM. Differences across experimental groups were assessed separately for each region (ATA and DTA) using a one-way ANOVA. Post-hoc pairwise comparisons were performed using the Bonferroni correction, and $p < 0.05$ was considered significant. All analyses were performed using the anova1 function in MATLAB.

Discussion

Selection of Animal Age. Our prior data1 show that Tgfrb2 disruption at 4 weeks of age yields in vivo dissections in 40 to 50% of the mice after a subsequent 4 weeks (i.e., at 8 weeks of age) whereas receptor disruption at 6 weeks yields dissections in less than 25% of the mice after 4 weeks (i.e., at 10 weeks of age) and disruption at 3 weeks of age yields dissections in over 75% of the mice after 4 weeks (i.e., at 7 weeks of age). We sought to balance the need to have non-dissected vessels suitable for biomechanical testing with the desire to yet have structurally vulnerable vessels to study. The 8-week age provided a good compromise. Moreover, studying mice at 8 weeks of age was consistent with two other studies using these mice7,8, which permits study-to-study comparisons, if desired. Finally, although the developing biomechanical properties have not reached steady state by 8 weeks of age, they are approaching those of the adult mouse9. This observation is consistent with two other findings. First, arterial mechanical homeostasis appears to be reached between 6 and 8 weeks of age (i.e., between P45 and P60) based on computed values of elastin maturity10 as well as wall shear stress and intramural tension (unpublished). Second, postnatal growth of the aorta is completed prior to that of the organism. In unpublished studies, we measured the growth of the murine thoracic aorta every 3 weeks after birth. The ascending and descending thoracic aorta reach >90% of their final diameter and length and ~85% of their final mass by 9 weeks of age. Hence, for many reasons, 8 weeks appeared to be a good age to study potential biomechanical vulnerability in these mice, noting that all mice were compared at the same age.

Study Group Sizes. As noted in the main paper, we used 52 male mice. There were no premature deaths, hence all animals / vessels were studied. There were multiple types of comparisons, however, which yielded different numbers in many groups. For example, all vessels were examined histologically and considered in the computation of numbers dissected or delaminated. In contrast, we excluded from detailed biomechanical quantifications (e.g., data in Table I) any vessel having an in vivo dissection (grossly visible, detected with in vitro imaging, or revealed histologically by intramural blood) within the region of interest or an in vitro delamination (similarly assessed) prior to data collection to ensure robust analyses and to focus our attention in vitro on potentially vulnerable, not already dissected, vessels. Similarly, we focused on DTAs in the active study since they alone delaminated in vitro under passive conditions.
Selection of Treatment Protocol. Related to the selection of age is the timing and dosing of the therapeutic agent. Our administration of rapamycin – daily beginning at 4 weeks of age when the receptor was disrupted and continuing to 8 weeks of age when the vessels were harvested – was based on the protocol developed in our prior study\textsuperscript{1}. Again, consistency across studies permits direct comparisons, if desired. In that study, the same dose of rapamycin in the same strain of mice resulted in whole-blood trough levels of 27.3 ± 5.0 ng/ml and achieved a therapeutic effect of preventing aortic dissection in 100% of the mice so treated. This dose is also similar to that used in other mouse studies of aortic disease\textsuperscript{11,12} and the blood levels are comparable to that recommended for clinical application (the therapeutic range of rapamycin is 4-12 ng/ml when used with cyclosporin and 12-20 ng/ml when used without cyclosporine)\textsuperscript{13}. Our previous work also showed that three-fold higher doses of rapamycin are required to prevent arterial remodeling than to achieve immunosuppression in mouse models\textsuperscript{14}.

Nevertheless, there is much to learn with regard to the timing and dosing of rapamycin, as for any drug. For example, a recent study showed that that appropriate timing of two drugs (early for an angiotensin type 2 receptor antagonist and later for a TGF-β neutralizing antibody) prevented aneurysmal rupture in a mouse model of Marfan syndrome whereas inappropriate timing could exacerbate the disease\textsuperscript{15}. There is a need for further study of the efficacy of the timing of rapamycin treatment, including a need to delineate potential differences in young versus older mice as well as to assess differences when rapamycin is given concurrent with the disease progression or after the disease has reached a clinically diagnostic threshold. As noted in the main paper, however, we suggest that the most pressing need at present is to evaluate the potential protection afforded by the current timing and dosing in the case of increased hemodynamic loading, as in hypertension.

Other Potential Roles of Rapamycin. Drug treatment may have had other beneficial effects in vivo. For example, rapamycin can block the accumulation of hyaluronan within the arterial wall\textsuperscript{16}; hyaluronan is the primary aggregating glycosaminoglycan (GAGs) in arteries\textsuperscript{17}. Recall, therefore, that untreated mice with Tgfbr2 disruption showed marked pooling of mucoid material, particularly in the outer portion of the media in those that dissected (Figure V). Whether functional or degraded, such mucoid material represents an increased accumulation of negatively charged matrix (presumably mainly GAGs), which could be an initiator of dissection in TAADs due to increases in intramural Donnan swelling pressures\textsuperscript{18}. Because of the potential importance of the localization of GAGs, its presence within MOV sections was quantified as a function of circumferential position. Sections in the Tmx group with evidence of intramural delamination or dissection consistently had one region with highly localized GAGs; those without delaminations and dissections had distributions of GAGs similar to Ctrl. Although rapamycin did not decrease overall mucoid area within whole cross-sections, it did reduce localized pooling of GAGs (not shown), similar to controls. Importantly, localized accumulations of GAGs constitute a common histopathological feature in human TAADs\textsuperscript{17}, and could represent a therapeutic target. Histological quantification of overall elastin and collagen suggested further that rapamycin increased adventitial area / collagen, with an associated decrease in medial area / elastin, but these were not dramatic. Increased adventitial collagen could protect against frank rupture.

Finally, related to the above comment regarding the importance of hemodynamic loading, rapamycin is thought either to not affect blood pressure much or to elevate it\textsuperscript{19}. Elevated blood pressures would be expected to increase aneurysmal or dissection risk, other effects notwithstanding. Note, therefore, that blood pressures were measured under isoflurane anesthesia using an invasive central pressure catheter (Millar, Inc., Houston, TX). Tgfbr2 disruption alone resulted in a slight (non-significant) decrease in blood pressures at 8 weeks of age, which was not changed significantly by rapamycin. Specifically, blood pressures were 99.8±6.0 / 76.5±4.0 mmHg in Ctrl, 92.5±4.1 / 69.8±4.1 mmHg in Tmx, and 98.0±3.2 / 67.4±4.6 mmHg in Tmx + Rapa, all at 8 weeks of age. Mean arterial pressures (84.3, 77.4, and 77.6 mmHg, respectively) were similarly not statistically different. Hence, neither the increased risk of dissection due to Tgfbr2 disruption nor the complete protection afforded in vivo by rapamycin were due to changes in hemodynamic loading alone.

Other Confounding Factors. Our in vitro observations are also consistent with the intramural delamination potentially arising at branch sites\textsuperscript{20,21}. That is, branches are abundant within the aortic root...
and thoracic aorta (e.g., the coronary ostia, three major branches off the arch, and intercostals) and spontaneous in vitro delaminations in the DTA may have nucleated at intercostal branches. That ATAs did not delaminate in vitro may have been due, in part, to the cannulation and mounting of the specimens excluding branches within the region that was pressurized. This possible contributor to TAADs requires further investigation, as do possible reasons why increased SMC contractility could be protective in this regard.

References


**Appendix**

Passive biaxial data were analyzed using a nonlinear stored energy function of the form

$$W(C,M^t) = \frac{C}{2} (I_C - 3) + \sum_{i=1}^{4} \frac{C_i}{4C_2} \left\{ \exp \left[ c_i \left( IV^i_C - 1 \right)^2 \right] - 1 \right\},$$

where $I_C = trC$, $IV^i_C = C : M^t \otimes M^t$ (with $C = \text{diag} \left[ \lambda^2_x, \lambda^2_y, \lambda^2_z \right]$), a measure of the finite deformations, and $\lambda_i = 1/\lambda_{x_i} \lambda_{y_i}$ by incompressibility. $M^t = (0, \sin \alpha^t_o, \cos \alpha^t_o)$ is a unit vector denoting the orientation of the $i^{th}$ family of locally parallel fibers, with angle $\alpha^t_o$ computed relative to the axial ($z$) direction in a reference configuration. One family of fibers is oriented axially ($\alpha^t_o = 0$), one circumferentially ($\alpha^t_o = \pi/2$), and two symmetric diagonally ($\alpha^t_o = -\alpha^t_o = \alpha^t_o$). Finally, $c$, $c_1^t$ and $c_2^t$ are material parameters.

Best-fit values of the 8 model parameters (i.e., 7 material parameters and 1 fiber angle) were determined from data sets combined from the seven biaxial testing protocols (with $N$ the total number of equilibrium configurations) using a nonlinear least squares minimization of the error $e$, where

$$e = \sum_{i=1}^{N} \left[ \left( \frac{P_{th} - P_{exp}}{P_{exp}} \right) + \left( \frac{f_{th} - f_{exp}}{f_{exp}} \right) \right]^2,$$

with $P$ and $f$ the distending pressure and total axial force, respectively, and $th$ and $exp$ denoting theoretically determined and experimentally inferred values, respectively. Regression analysis was performed in MATLAB R2013b using the built-in function *lsqnonlin* and assigning random initial guesses.
and appropriate physical constraints on the parameters. Six to ten minimization cycles per specimen ensured that best-fit parameters were independent of initial guesses. See\textsuperscript{3,4} for further details.