Supplemental Material

Pharmacologically Improved Contractility Protects Against Aortic Dissection in Mice with Disrupted TGFβ Signaling Despite Compromised ECM 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 elsewhere[1], 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-CreER^{T2}.Tgfbr2$^{f/f}$* 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 laboratory[2,3], 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^{o}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 behavior[2]. As shown previously[3], 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 deformation[4]; one cannot, for example, simply compute a modulus for stiffness from a 1-D Hooke's law ($E = \sigma_\theta / \varepsilon_\theta$), 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 angles[4], 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^{o}C and bubbled with 95% O_2 / 5% CO_2 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 mmHg and λ_z=1.1, second at *P*=60 mmHg and λ_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 contraction[5]). 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 code[6] 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 data[1] show that *Tgfbr2* 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 mice[7,8], 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 mouse[9]. 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 maturity[10] 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 pre-mature 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[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[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)[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[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[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[16]; hyaluronan is the primary aggregating glycosaminoglycan (GAGs) in arteries[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[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[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[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[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.

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Appendix

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

$$W\left(\mathbf{C},\mathbf{M}^i\right)=\frac{c}{2}\left(I_C-3\right)+\sum_{i=1}^{4}\frac{c_1^i}{4c_2^i}\left\{\exp\left[c_2^i\left(IV_C^i-1\right)^2\right]-1\right\},$$

where $I_C=tr\mathbf{C}$, $IV_C^i=\mathbf{C}:\mathbf{M}^i\otimes\mathbf{M}^i$ (with $\mathbf{C}=diag\left[\lambda_r^2,\lambda_\vartheta^2,\lambda_z^2\right]$, a measure of the finite deformations, and $\lambda_r=1/\lambda_\vartheta\lambda_z$ by incompressibility). $\mathbf{M}^i=(0,\sin\alpha_o^i,\cos\alpha_o^i)$ is a unit vector denoting the orientation of the *i*th family of locally parallel fibers, with angle α_o^i computed relative to the axial (*z*) direction in a reference configuration. One family of fibers is oriented axially ($\alpha_o^1=0$), one circumferentially ($\alpha_o^2=\pi/2$), and two symmetric diagonally ($\alpha_o^3=-\alpha_o^4=\alpha_o$). Finally, *c*, c_1^i and c_2^i 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

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

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[3,4] for further details.