Materials and Methods

Deficient Circumferential Growth is the Primary Determinant of Aortic Obstruction Attributable to Partial Elastin Deficiency

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Animals

The original elastin null (*Eln*$^{-/-}$) mice [1] had been back-bred to the C57BL/6 strain for an estimated 5 generations, then further back-bred for 3 generations during hBAC introduction [2]. The founder animals of our colony were back-bred to wild-type C57BL/6J mice (Stock #000664, Jackson Laboratory) one additional generation because of initial difficulties in breeding. There were no unexpected deaths of mice over the duration of the studies. Male littermates of all three genotypes from hBAC-mHET matings were studied to maintain uniform body stature; similar arterial phenotypes were observed in female mice.

Human Studies

The Human Investigation Committee of Yale University and the New England Organ Bank approved the human studies. Thoracic aortas were obtained from 3 male organ donors (43 ± 1.7 years old, 2 Caucasian and 1 African American) without vascular disease as well as at autopsy from a male WS subject (46 year old, Caucasian), without clinical symptoms or previous treatment for aortic obstruction, who died suddenly of unknown cause. Of possible relevance for sudden death, postmortem examination of the WS subject revealed rudimentary supracoronary membranes at the sinotubular junction that did not appear capable of obstructing the aortic lumen, but may have obstructed flow to the coronary ostia (cf. Fig. 5*A* of the main paper). The diagnosis of WS was confirmed by chromosome microarray (Agilent 180K) at the Yale Cytogenetic Laboratory, which demonstrated a 1.407 Mb deletion at 7q11.23 (chr7:72,726,572-74,133,332.GRCh37/hg19) from *TRIM50* to *GTF2I*, including *ELN*.

In Vivo Functional Analysis

Transthoracic B-mode images of the murine aorta were obtained in lightly isoflurane-anesthetized animals using a Vevo 770 high frequency ultrasound system (VisualSonics). Transverse diameters of the proximal-ascending aorta were measured at end-diastole and end-systole. Measurements were performed in triplicate. Aortic distension (change in diameter) was calculated as the diameter change during the cardiac cycle and was also indexed to end-diastolic diameter to render it closer to the common clinical definition of distensibility (see below). Blood pressure was measured noninvasively in conscious animals using a CODA volume-pressure recording sensor and occlusion tail-cuff (Kent Scientific Corporation). Mice were placed in warmed restraining chambers and acclimatized to the experimental procedure for a week before data acquisition. Systolic and diastolic pressures were recorded for 10 cycles after discarding the first 5 data points. A measure of aortic “compliance” was calculated as the change in diameter per change in blood pressure over a cardiac cycle.

Aortic Segments

Mice were euthanized at prescribed ages (3, 6, or 9 weeks) and their ascending and descending thoracic aortas were examined. We chose not to analyze the abdominal aorta as this segment is an uncommon site of obstructive disease in WS and it has a normal histological appearance in hBAC-mNULL mice [2]. Additionally, only a limited portion of the thoracic aorta of the WS subject was donated to our laboratory and other tissues were not available for analysis. Similarly, the height and weight of this individual were not available to index the diameters of the ascending and descending aortic segments.

In Situ and Ex Vivo Gross Examination

After euthanasia, the body cavities of the mice were widely opened and in situ images of the thoracic aorta were obtained using a SZX16 dissecting microscope with a camera attachment (Olympus). The unpressurized width (external diameter) of the proximal-, mid-, and distal-ascending aorta, mid-aortic arch, and proximal-descending aorta was measured from the in situ images. Measurements were performed in triplicate and ascending aorta width was averaged from proximal, mid, and distal sites of this segment. The unpressurized length of the

thoracic aorta was measured along its outer curvature from the ventricular-aortic junction to the proximal origin of the brachiocephalic artery for the ascending segment, to the distal origin of the left subclavian artery for the arch segment, and to the diaphragmatic hiatus for the descending segment. The aorta was then excised from heart to diaphragm, imaged ex vivo, and weighed. The heart and body were also weighed and body length was measured from snout to anus. Aorta dimensions were measured from digital images using a calibrated software tool, whereas body lengths were directly measured with a ruler.

Ex Vivo Biomechanical Phenotyping

Biaxial mechanical tests were performed on excised segments of the ascending and proximal descending thoracic aorta from hBAC-mWT and hBAC-mNULL mice at 9 weeks of age using methods described previously [3]. Briefly, following euthanasia and blunt excision, specimens were gently cleaned of excess perivascular tissue, cannulated with custom glass pipets, mounted within a custom computer-controlled biaxial test device, allowed to acclimate to a Hanks balanced salt solution (HBSS; GIBCO) at 37 ^{o}C, and subjected to a series of cyclic pressure-diameter (at multiple fixed axial lengths) and cyclic axial force-length (at multiple fixed luminal pressures) tests. The biaxial data (external diameter, pressure, axial length, and axial force) were collected on-line and used both for feedback control of the seven different cyclic testing protocols and to determine best-fit values of material parameters in a validated nonlinear constitutive relation. Additionally, unloaded dimensions were measured using a dissection microscope or optical coherence microscopy system (ThorLabs, Newton, NJ).

Written in terms of the elastic energy stored in the vessel upon biaxial deformation, first and second derivatives of our constitutive descriptor provided information on biaxial wall stresses and material (or intrinsic) stiffness. Since a key function of the aorta is to use elastic energy stored during systole to work on the blood during diastole to augment flow and since elastic fibers are the primary energy storage constituents in the wall, constant energy contour plots were used to compare across the experimental groups the overall mechanical functionality of the wall [4]. For comparison with in vivo measurements of pressure and diameter, the distensibility D was also calculated based on the cyclic ex vivo data, where $D = (d_{sys}-d_{dias})/d_{dias}PP$ where d is diameter, PP is pulse pressure, and sys and dias denote systolic and diastolic values, respectively. Biaxial wall stress, intrinsic material stiffness, energy storage and distensibility were computed for in vivo conditions, namely a systolic pressure of 129 mmHg and a group specific value of in vivo axial stretch. Finally, for purposes of further comparison, results were contrasted with those obtained previously for 9 week old male C57BL/6 WT mice [5] to assess possible changes in properties with increased elastin (hBAC-mWT).

Microscopic Examination

The thoracic aorta was fixed in 4% paraformaldehyde overnight. Segments of the ascending aorta, aortic arch, and proximal descending aorta were embedded in paraffin wax. Serial 5 µm-thick transverse sections were stained by Yale's Research Histology Laboratory with hematoxylin and eosin (H&E), elastin-Van Gieson (EVG: Verhoeff's hematoxylin with acid fuchsin-picric acid counterstain), sirius red, and elastin (Verhoeff's hematoxylin alone) stains using standard techniques. Images were obtained using either an Axioskop2 microscope (Carl Zeiss) or, for the sirius red-labeled sections, a polarized light BX/51 microscope (Olympus). Measurements were performed in triplicate.

Histomorphometric Analysis

Morphometry of EVG-stained transverse sections was performed using ImageJ software (http://rsbweb.nih.gov/ij/) after outlining the internal and external elastic laminae. Lumen area was calculated within the internal elastic lamina (since it closely approximates the adjacent endothelial perimeter). Medial area and mean thickness was calculated between the internal and external elastic laminae. The number of medial cells in murine aortas was calculated by

counting hematoxylin-stained nuclei between the inner and outer elastic laminae and expressed as a total number of cells (per cross-section) or as cell density (per medial area). The elastic laminae were also counted. Elastin and collagen levels were derived from elastin- and sirius red-stained transverse sections, respectively. Histological stains of aortic sections to be compared were performed in the same batch using an automated system. Within a particular vascular compartment, the absolute (area of positive staining) and relative (% area of positive staining) levels or intensity (optical density as pixels x 10^{-6}) of expression were calculated using ImageJ. Measurements were performed in triplicate. Analyses of human aortas were similar to those for murine aortas, except that sections of the entire aorta circumference could not be obtained because of vessel size. Medial area was calculated using the external diameter measured on gross examination and the average medial thickness from 4 different points on microscopic examination. The density of medial cells and the relative levels of elastin and collagen were averaged from 9 randomly selected high power fields per section. The total number of SMCs was extrapolated from their density and the cross-sectional medial area.

Immunohistochemistry and Immunofluorescence Analysis

A subgroup of animals was treated with bromodeoxyuridine (BrdU; Sigma-Aldrich, Cat. # B9285) at 1 mg s.c. q.o.d. x 5 doses from 3 to 4.5 weeks of age. Ascending and proximal descending aortas were procured, fixed in 4% paraformaldehyde overnight, and embedded in paraffin. Transverse sections were deparaffinized, rehydrated to distilled water, placed in Tris-buffered saline with 0.1% Tween 20, and labeled with an antibody to SMA (ThermoFisher Scientific, Cat. # MS-113) or isotype-matched, nonbinding immunoglobulin. Binding of secondary antibodies was detected with the HRP-based EnVision+ System and Liquid DAB+ Substrate-Chromogen kit (DAKO), counter-stained with hematoxylin (Sigma-Aldrich), and imaged using an Axioskop2 microscope (Carl Zeiss MicroImaging). Alternatively, tissue sections were incubated with an antibody to BrdU (Abcam, Cat. # ab6326) or isotype-matched, irrelevant IgG overnight at 4 ^{o}C followed by Alexa Fluor 594-conjugated secondary antibody (Invitrogen, Cat. # 21209) for 1 hour at room temperature and mounted on slides with Pro-Long Gold Mounting Reagent with DAPI (Life Technologies). Immunofluorescence images were acquired using an Axiovert 200M microscopy system (Carl Zeiss MicroImaging).

Quantitative (q)RT-PCR.

Aortic tissue (including the intimal and adventitial layers) was flash-frozen and crushed or cultured cells were collected and immersed in RLT lysis buffer (QIAGEN), vigorously vortexed, and total RNA was isolated using RNeasy mini kits and DNA digestion kits (QIAGEN) according to the manufacturer's protocol. Real-time RT-PCR reactions were prepared with TaqMan PCR Master Mix and pre-developed assay reagents from Applied Biosystems for *Acta2*, *Myh11*, *Tagln*, *Myl6*, *Col1a1*, *Col3a1*, *Col8a1*, *Col11a1*, *Itga11*, *Itgb1*, (murine) *Eln*, and (human) *ELN*. Samples were analyzed on an iCycler (Bio-Rad Laboratories). RNA-free ddH_2O was used as negative controls for qPCR instead of cDNA samples. The level of each transcript was normalized to that of a reference transcript as $\log_2$ (reference gene - gene of interest quantification cycle) and expressed as relative to controls (i.e., hBAC-mWT). We confirmed stable expression of several housekeeping genes, including *Hprt1*, *Actb*, and *Gapdh*, under our experimental conditions.

In Vitro Cell Culture

Individual thoracic aortas were lightly enzyme digested in HBSS containing 1 mg/mL collagenase A (Roche) for 15 minutes at 37 ^{o}C, the adventitia was stripped away under a microscope, and the aortic media was cultured overnight in Claycomb medium containing a proprietary mixture of vasoactive agents and growth factors (Sigma-Aldrich) and supplemented with 10% fetal bovine serum (FBS; Gibco); we refer to this cocktail as a growth medium. The tissue was further digested in HBSS solution containing 2 mg/mL collagenase A and 0.5 mg/mL

elastase (Worthington) for 30 minutes at 37 ^{o}C, growth medium was added, washed, and the pellet was resuspended in growth medium. The cells that populated the culture flasks were considered passage 0 and were serially expanded. For cell counting experiments, passage 1 SMCs were plated at 20 x10^3 cells per well of uncoated 24-well plates (Nunc), growth medium was changed every other day, and the cells were manually counted using a hemocytometer. For BrdU uptake experiments, passage 2 SMCs were plated at 100 x10^3 cells per well in 35 mm glass bottom dishes (MatTek) coated with 0.1% gelatin (Sigma-Aldrich) in growth medium overnight. The cells were growth arrested in serum-free Dulbecco's Modified Eagle Medium (DMEM; Gibco) for 24 hours, cultured in growth media containing 10 μM BrdU (Sigma-Aldrich, Cat. # B9285) for 2 hours, washed in phosphate buffered saline (PBS), fixed with 4% formaldehyde in PBS for 15 minutes, permeabilized with 0.1% Triton X-100 (AmericanBio) for 20 minutes, denatured in 1 M HCL for 20 minutes, labeled with rat anti-BrdU antibody (Abcam, Cat. # ab6326) overnight, and then exposed to Alexa Fluor 594-conjugated, donkey anti-rat antibody (Invitrogen, Cat. # 21209) for 1.5 hours. Nuclei were labeled with DAPI (Life Technologies) and random high power field (HPF) fluorescence images were acquired using an Axiovert 200M microscopy system with AxioVision 4.6 software. Alternatively, the cells were labeled with antibody to SMA (ThermoFisher Scientific, Cat. # MS-113) as for tissue sections and imaged using an Axioskop2 plus microscope.

Statistical Analyses

Data represent mean ± SEM and values conformed to Gaussian distributions by Shapiro-Wilk normality test. Comparisons among multiple groups were by one-way ANOVA, or among groups with two independent variables by two-way ANOVA, followed by Tukey's multiple comparisons test. *P* values were two-tailed and values < 0.05 were considered to indicate statistical significance. Data were analyzed using Prism 6.0 software (GraphPad).

References

1. Li DY, Brooke B, Davis EC, Mecham RP, Sorensen LK, Boak BB, Eichwald E, Keating MT. Elastin is an essential determinant of arterial morphogenesis. *Nature*. 1998;393:276-80.
2. Hirano E, Knutsen RH, Sugitani H, Ciliberto CH, Mecham RP. Functional rescue of elastin insufficiency in mice by the human elastin gene: implications for mouse models of human disease. *Circ Res*. 2007;101:523-31.
3. Ferruzzi J, Bersi MR, Humphrey JD. Biomechanical phenotyping of central arteries in health and disease: Advantages of and methods for murine models. *Annl Biomed Engr.* 2013;41:1311-1330.
4. Ferruzzi J, Bersi MR, Mecham RP, Ramirez F, Yanagisawa H, Tellides G, Humphrey JD. Loss of elastic fiber integrity compromises common carotid artery function: Implications for vascular aging. *Artery Res.* 2016;14:41-52.
5. Bellini C, Korneva A, Zilberberg L, Ramirez F, Rifkin DB, Humphrey JD. Differential ascending and descending aortic mechanics parallel aneurysmal propensity in a mouse model of Marfan syndrome. *J Biomech*. 2016;49:2383-9.