Differences in genetic signaling, and not mechanical properties of the wall, are linked to ascending aortic aneurysms in fibulin-4 knockout mice

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Kim J, Procknow JD, Yanagisawa H, Wagenseil JE. Differences in genetic signaling, and not mechanical properties of the wall, are linked to ascending aortic aneurysms in fibulin-4 knockout mice. Am J Physiol Heart Circ Physiol 309: H103–H113, 2015. First published May 1, 2015; doi:10.1152/ajpheart.00178.2015.—Fibulin-4 is an extracellular matrix protein that is essential for proper assembly of arterial elastic fibers. Mutations in fibulin-4 cause cutis laxa with thoracic aortic aneurysms (TAA). Sixty percent of TAA occur in the ascending aorta (AA). Newborn mice lacking fibulin-4 (Fbln4−/−) have aneurysms in the AA, but narrowing in the descending aorta (DA), and are a unique model to investigate locational differences in aneurysm susceptibility. We measured mechanical behavior and gene expression of AA and DA segments in newborn Fbln4−/− and Fbln4+/+ mice. Fbln4−/− AA has increased diameters compared with Fbln4+/+ AA and Fbln4−/− DA at most applied pressures, confirming genotypic and locational specificities of the aneurysm phenotype. When diameter compliance and tendon modulus were calculated from the mechanical data, we found few significant differences between genotypes, suggesting that the mechanical response to incremental diameter changes is similar, despite the fragmented elastic fibers in Fbln4−/− aortas. Fbln4−/− aortas showed a trend toward increased circumferential stretch, which may be transmitted to smooth muscle cells (SMCs) in the wall. Gene expression data suggest activation of pathways for SMC proliferation and inflammation in Fbln4−/− aortas compared with Fbln4+/+. Additional genes in both pathways, as well as matrix metalloprotease-8 (Mmp8), are upregulated specifically in Fbln4−/− AA compared with Fbln4+/+ AA and Fbln4−/− DA. Mmp8 is a neurelline collagen that targets type I collagen, and upregulation may be necessary to allow diameter expansion in Fbln4−/− AA. Our results provide molecular and mechanical targets for further investigation in aneurysm pathogenesis.

fibulin-4; elastin; mechanics; gene array; aneurysm

THORACIC AORTIC ANEURYSMS (TAA) are associated with genetic mutations that affect extracellular matrix (ECM) proteins, components of the transforming growth factor-β (TGF-β) signaling pathway, and smooth muscle cell (SMC) contractile apparatus (49). Sixty percent of TAA occur at the aortic root or ascending aorta (AA) (16, 39). Mutations affecting the ECM protein fibrillin-1 (FBN1) lead to Marfan syndrome, which is characterized by AA aneurysms (19). Mutations affecting another ECM protein, fibrillin-4 (FBLN4), cause cutis laxa with AA aneurysms (15, 36). Mouse models with reduced expression of Fbn1 (40, 62, 63), Fbln4 (31), or SMC-specific knock-out of Fbln4 (SMKO) (34) recapitulate the AA aneurysm phenotype observed in humans. Fibrillin-1 and fibrillin-4 are both critical in the assembly of elastic fibers. Elastic fibers provide energy storage in the large arteries during systole and help dampen flow to distal vessels (82). The combination of elastic fiber protein mutations and elastic fiber fragmentation in TAA led to early theories that mechanical tissue failure due to abnormal elastogenesis or elastic fiber homeostasis was a key component in aneurysm pathogenesis (49). Recent studies, however, have shown that ECM proteins regulate the availability of signaling molecules in addition to providing structural support. This is highlighted by studies demonstrating the importance of the TGF-β signaling pathway in Marfan syndrome (29) and cutis laxa (66).

SMC contractility has also been implicated in TAA development. Mutations in SMC contractile proteins cause TAA (26, 61, 89) and altered SMC phenotype and/or contractility is observed in TAA caused by other genetic defects (12, 34, 38, 57). SMCs, ECM, and signaling molecules in the arterial wall are a tightly integrated system, and the emerging genetic evidence suggests that disruptions at many points of the system (i.e., SMC contraction, specific ECM components, and TGF-β signaling) can lead to TAA. While the early views of TAA as solely a structural defect have been revised, there is still likely a mechanical component to TAA progression. SMCs sense and respond to mechanical stimuli by altering expression of signaling molecules. The stimuli experienced by the SMCs depend on the mechanical properties of the cells themselves (i.e., contractility), mechanical properties of the surrounding matrix (i.e., collection of ECM proteins), and the connection between the cells and their matrix (i.e., integrins) (37). Alterations at any of these points can lead to TAA.

Despite the advances in genetic and molecular understanding of TAA, there have been fewer advances in understanding the mechanical changes involved in TAA. It is also still not well understood why 60% of TAA occur in the AA. Susceptibility of different vascular regions to TAA has been attributed to locational differences in blood flow (10), endothelial permeability (42), ECM content and SMC mechanical properties (20), SMC gene expression (88), SMC embryonic origin (53), or SMC response to signaling molecules (60, 80). Fibrillin-4 knockout (Fbln4−/−) mice display opposite vascular phenotypes depending on the thoracic location, with aneurysms developing in the AA and narrowing occurring in the descending aorta (DA) (34, 55), and represent a unique model to study differences in TAA susceptibility of specific locations. We hypothesized that there would be changes in mechanical properties and gene expression of Fbln4−/− AA compared with Fbln4+/+ AA and Fbln4−/− DA that may provide clues to
TAA pathogenesis and reveal targets for future therapies. To
explore our hypothesis, we collected AA segments (aneu-
rysmal) and DA segments (narrowed) from newborn Fbln4+/−
and wild-type (Fbln4+/+) mice. Aortic ultrastructure and
morphology were quantified through imaging methods, mechanical
changes were quantified by inflation testing of the aortic
segment, molecular changes were quantified by gene array, and
expression of specific genes was confirmed by quantitative
(q)PCR.

MATERIALS AND METHODS

Animals. Mice with heterozygous germline expression of Fbln4
(34) were bred to produce Fbln4−/− and Fbln4+/+ pups. Pups were
used between 0 and 18 h after birth and killed by thoracotomy under
2.5% isoflurane or by CO2 inhalation. Fbln4−/− pups die within 24 h
of birth. Segments of the AA, from the aortic valve to the left common
carotid aorta, and the DA, from the ductus arteriosis to the diaphragm,
were harvested. Groups for comparison were Fbln4−/− AA, Fbln4−/−
DA, Fbln4+/+ AA, and Fbln4+/+ DA. Aortic segments were used for
electron microscopy (n = 2 for each group), mechanical testing (n = 8
for each group, excluding 3 outliers), gene array, gene array (n = 2 for
each pooled group of 8 samples), or qPCR (n = 2 for each pooled group
of 8 samples). All animal protocols were approved by the Institutional
Animal Care and Use Committee at Washington University.

Electron microscopy. Aortas were fixed with 2.5% glutaraldehyde
in 0.1 M sodium cacodylate buffer. They were sequentially stained
with 1.25% osmium tetroxide, 2% tannic acid, and 6% uranyl acetate.
Aortas were dehydrated in ethyl alcohol and propylene oxide and
embedded in Polybed 812 (Polysciences). Thin sections were cut, placed on
a copper grid, and examined on a transmission electron microscope
(JEM 1400; JEOL).

Mechanical testing and data analysis. Images of the aortas before
and after dissection were taken to measure the length change from in
vivo to ex vivo state and calculate the in vivo longitudinal stretch ratio
(IVSR). The aortas were stored in PSS (22) at 4°C for up to 3 days (4)
before testing. Inflation tests were performed using a Myograph 110P
(Danish Myotechnology) for the proximal sections of AAs or DAs, as
previously described (5). The aorta was mounted on cannulae in a
37°C PSS bath and secured with 11-0 suture. The unloaded length of
the mounted aorta was measured with calipers. The aorta was
stretched at a fixed longitudinal stretch ratio of 1.05 for AA and 1.10
for DA and pressurized from 0 to 60 mmHg in 5-mmHg increments.
Three preconditioning cycles were performed, and then three more
cycles were performed while the outer diameter, lumen pressure, and
longitudinal force were recorded at 1 Hz. After mechanical testing,
three 150- to 250-μm thick rings were cut, placed in PSS, and imaged
to determine the unloaded outer diameter and wall thickness of the
aorta. A radial cut was then made in each ring and imaged to
determine the residual strain, as measured by the opening angle (14).

Due to aortic collapse at 0 mmHg, data at 5 mmHg and above were
used for analysis. Aortas that exhibited consistent behavior for mul-
tiple inflation cycles were included in the analysis. Three outliers were
excluded due to high longitudinal forces, large outer diameters, or
almost no change in outer diameter with increased pressure. High
longitudinal force can be caused by overstretch of the aorta during
mounting on the cannulae; large outer diameter can be caused by
failure to remove loose connective tissue; and no change in diameter
with pressure can be due to leaks in the aorta that are difficult to
detect.

Compliance of the aorta represents a structural property of the wall
(depends on geometry and material). Diameter compliance (C) at each
pressure (P1) was calculated by,

\[ C_p = \frac{\frac{\partial R}{\partial p}}{P_1} \]  

where \( d_o = a_1 + a_2 \left( \frac{1 - e^{-P/a_2}}{P/a_2} \right) \) is the outer diameter of
the aorta (23), P is the lumen pressure, and \( a_i \) are constants
determined by regression in Matlab (Mathworks).

The circumferential stretch (\( \lambda_o \)) was calculated as the average
stretch of the inner (i) and outer (o) wall of the aorta,

\[ \lambda_o = \frac{1}{2} \left( \frac{R_i}{R_o} + \frac{R_o}{R_i} \right), \]

where \( r \) is the loaded radius and \( R \) is the unloaded radius. The mean
circumferential stress (\( \sigma_o \)) was calculated by,

\[ \sigma_o = \frac{P R}{R_o - R_i}. \]

The slope of the circumferential stretch-stress plot is the tangent
modulus and represents a material property of the wall (independent
of geometry). The tangent modulus (\( E \)) at each pressure was calculated by,

\[ E_p = \frac{\partial \sigma_o}{\partial P} \]  

where \( c = b_1 + b_2 \exp(\frac{P}{b_3}) \) and \( b_i \) are constants determined by
regression in Matlab.

Gene array and qPCR. Aortas were flash-frozen and stored at
−80°C. Like-genotypes were pooled in groups of eight and two
pooled samples were run for each group. RNA was isolated using the
RNeasy Plus Mini Kit (Qiagen). Only samples with 260/280 nm
absorbance ratios of 1.8–2 were used for further analysis. RNA was
processed for use on the Affymetrix mouse gene 2.0 array by the
Genome Technology Access Center (GTAC) at the Washington
University School of Medicine. Resulting gene array data was eval-
uated by GTAC with the Affymetrix GeneChip Command Console
and raw. CEL files were analyzed using Expression Console software
with Affymetrix default RMA Gene analysis settings. Probe summa-
tion (Robust Multichip Analysis), quality control analysis, and
probe annotation were performed according to recommended guide-
lines via Expression Console software by GTAC. Resulting CEL files
were uploaded into Partek Genomics Suite version 6.6 (Partek Incor-
porated) for further analyses. All gene array data have been de-
posited in National Center for Biotechnology Information’s Gene Expression
Omnibus and are accessible through GEO Series Accession No.
GSE67707.

Expression of genes of interest from the array was confirmed by
qPCR. Reverse transcription was done using the High Capacity cDNA
Reverse Transcription Kit (Applied Biosystems). qPCR was per-
formed with TaqMan Fast Advanced Master Mix on a QuantStudio
12k machine and analyzed with accompanying software (Applied
Biosystems). The reaction conditions consisted of initial template
denaturation at 95°C for 20 s, followed by 40 cycles of amplification
(95°C for 1 s, 60°C for 10 s). Primers were Taqman Gene Expression
Assays (Life Technologies). All experiments were run in triplicate
with 10.22 ± 0.33.3 on April 2, 2017 http://ajpheart.physiology.org/ Downloaded from
For the gene array data, Partek software was used to compare all two-way combinations of groups and highlight genes with significant up- or downregulated expression (fold change > 1.5 and \( P < 0.05 \)). For the qPCR data, we were specifically interested in expression differences of \( Fbln4^{-/-} \) AA compared with \( Fbln4^{+/+} \) AA and \( Fbln4^{-/-} \) AA compared with \( Fbln4^{-/-} \) DA, so two-tailed student's \( t \)-tests between these groups were used to evaluate the results. Data are shown as means ± SD. \( P < 0.05 \) was considered significant.

RESULTS

The organization of elastic fibers and SMCs in the aortic wall for each genotype and vascular location were evaluated by electron microscopy. \( Fbln4^{-/-} \) aortas have distinct aggregates of dark staining elastic fibers between layers of SMCs, compared with almost continuous elastic laminae in \( Fbln4^{+/+} \) aortas (Fig. 1). The space between laminae is larger in \( Fbln4^{-/-} \) aortas, with an increased amount of nonstaining (white) material. There are an increased number of SMCs in each layer of \( Fbln4^{-/-} \) aortas, and they are not aligned in the circumferential direction. At the intima, \( Fbln4^{-/-} \) aortas have disorganized endothelial cells (ECs) and a fragmented internal elastic lamina (IEL), while \( Fbln4^{+/+} \) aortas have radially aligned ECs and a nearly continuous IEL. The degree of fragmentation in the elastic fibers and disorganization of SMCs and ECs is similar in \( Fbln4^{-/-} \) AA and DA, compared with the respective wild-type controls.

Residual strain in the longitudinal and circumferential directions and unloaded dimensions were measured to evaluate residual stresses in the aortas and to calculate circumferential stresses from the mechanical test data. IVSR of \( Fbln4^{+/+} \) DA is 13% more than \( Fbln4^{+/+} \) AA (Fig. 2A). Opening angle of \( Fbln4^{+/+} \) DA is 34% less than \( Fbln4^{+/+} \) AA (Fig. 2B). In contrast, there are no significant differences between \( Fbln4^{-/-} \) AA and DA for IVSR or opening angle. In agreement with the ultrastructural images (Fig. 1), \( Fbln4^{-/-} \) aortas have 22–39% increased unloaded wall thicknesses compared with \( Fbln4^{+/+} \) (Fig. 2C). \( Fbln4^{-/-} \) AA has a 17–19% larger unloaded outer diameter than \( Fbln4^{+/+} \) AA and \( Fbln4^{-/-} \) DA (Fig. 2D).

Fig. 1. A–D: representative electron microscopy images of aortic cross sections from newborn \( Fbln4^{+/+} \) and \( Fbln4^{-/-} \) mice. The lumen is at the left of the images. \( Fbln4^{-/-} \) aortas have fragmented elastic fibers (arrows), increased numbers of disorganized smooth muscle cells (SMCs; arrowheads), larger spaces between elastic laminae, and thicker walls compared with \( Fbln4^{+/+} \) aortas. AA, ascending aorta; DA, descending aorta. Scale bars = 2 \( \mu \)m.
Structural properties for each group were determined by inflation tests with the aortas held at a fixed longitudinal stretch ratio. The aortic outer diameter increases nonlinearly with applied pressure in all groups (Fig. 3A). Fbn4<sup>+/−</sup> AA has 20–25% larger outer diameter than Fbn4<sup>+/+</sup> AA at all pressures and Fbn4<sup>+/−</sup> DA at high pressures (40–60 mmHg). The outer diameter of Fbn4<sup>+/−</sup> DA is similar to Fbn4<sup>+/+</sup> AA and DA. The DA outer diameters for both genotypes increase rapidly with pressure at low pressures (up to 25 mmHg) and then show little increase with pressure at high pressures. This behavior is quantified in Fig. 3B where the DAs have high compliance at low pressures and low compliance at high pressures. Compliance of Fbn4<sup>+/+</sup> DA is higher than Fbn4<sup>+/−</sup> AA at 5 mmHg and lower at 25–50 mmHg. Compliance of Fbn4<sup>−/−</sup> DA is lower than Fbn4<sup>−/+</sup> AA at 40–60 mmHg. Despite the larger diameter in Fbn4<sup>−/+</sup> AA, the compliance curve resembles the DA curves and Fbn4<sup>−/−</sup> AA compliance is higher than Fbn4<sup>+/+</sup> AA at 5 mmHg.

Mechanical test data and unloaded dimensions were used to determine circumferential material properties of the aortas for each genotype and vascular location. In general, Fbn4<sup>−/−</sup> aortas have a larger circumferential stretch than Fbn4<sup>+/+</sup> (Fig. 4A), although the differences are only significant for Fbn4<sup>−/−</sup> DA compared with Fbn4<sup>−/+</sup> DA at 25–60 mmHg. There are no significant differences in the circumferential stress at each pressure between groups (Fig. 4B). The stress-stretch curves for Fbn4<sup>−/−</sup> aortas are shifted to the right of the curves for Fbn4<sup>−/+</sup> aortas, and the curves for DAs are shifted to the right of the curves for AAs in each genotype (Fig. 4C). To compare the material properties in a quantitative manner, the tangent modulus was calculated from the local slope of the stress-stretch plots. While DAs have a tendency toward higher tangent modulus than AAs, a significant increase is found only in Fbn4<sup>−/−</sup> DA compared with Fbn4<sup>−/+</sup> AA at 20–25 mmHg (Fig. 4D).

A microarray was used to investigate changes in gene expression between groups. The numbers of up- or downregulated genes from the microarray data are shown in Table 1. The largest changes in gene expression are between Fbn4<sup>−/−</sup> AA and Fbn4<sup>−/+</sup> AA. Heat maps of comparisons by genotype (Fig. 5A) and vascular location (Fig. 5B) were generated to visualize the gene expression changes. We chose several genes from the microarray results for verification of expression by qPCR. Based on previous research involving fibulin-4 mutations, we focused on genes involved in SMC migration and proliferation, ERK1/2 activation (34), inflammation (57, 65), and ECM remodeling (31) (Table 2). Genes were chosen that were up- or downregulated in Fbn4<sup>−/−</sup> compared with Fbn4<sup>−/+</sup> at both AA and DA locations. These genes included collagen8a1 (Col8a1), ankyrin repeat domain-containing protein 1 (Ankrd1), serine peptidase inhibitor 3n (Serpina3n), and fibulin-4 (Efemp2). Genes were also chosen that were up- or downregulated in Fbn4<sup>−/−</sup> compared with Fbn4<sup>−/+</sup> at both AA and DA locations. These genes included collagen8a1 (Col8a1), ankyrin repeat domain-containing protein 1 (Ankrd1), serine peptidase inhibitor 3n (Serpina3n), and fibulin-4 (Efemp2). Genes were also chosen that were up- or downregulated in Fbn4<sup>−/−</sup> compared with Fbn4<sup>−/+</sup> AA at 20–25 mmHg (Fig. 4D).

Microarray results for the genes in Table 2 were verified by qPCR. Compared with Fbn4<sup>−/+</sup> AA, gene expression is upregulated in Fbn4<sup>−/−</sup> AA for Col8a1 (16.0-fold), Hbegf (2.4-fold), Ere (4.5-fold), Ankrd1 (2.7-fold), Emr1 (1.7-fold), Ptg2 (8.0-fold), Serpin3a (2.8-fold), and Mmp8 (4.0-fold) (Fig. 6A). Expression of Efemp2 is 1,900-fold higher in Fbn4<sup>−/−</sup> AA compared with Fbn4<sup>−/+</sup> AA (not shown), confirming genotype of the animals. Compared with Fbn4<sup>−/+</sup>
...overproliferation of SMCs throughout the wall thickness in aortas from elastin knockout (Eln−/−) mice with fragmented elastic fibers have decreased opening angles in AA and DA but only increased diameter in the AA. Thus the morphologic changes specific to Fbln4−/− AA cannot be explained alone by changes in residual stress distribution across the aortic wall.

IVSR is a measure of the longitudinal strain experienced by the aortic segments in vivo. IVSR varies with location in the vascular tree (28, 30) and age (84). Our data confirm that locational differences exist in newborn Fbln4−/− mice and are consistent with previous observations. Fbln4−/− mice, however, do not show differences in IVSR for AA and DA segments, suggesting that functional elastic fibers are necessary to generate locational differences in IVSR. Arteries from mice with reduced amounts of elastin (Eln−/−) (83), defects in another elastic fiber protein, fibulin-5 (Fbln5−/−) (84), and SMC-specific knockout of Fbln4 (SMKO) (46), also show reductions in IVSR. Additionally, arteries from Eln−/− (81), Fbln4−/− (34), and Fbh5−/− (59, 87) mice show increased length and tortuosity, suggesting that defects in elastic fibers promote SMC proliferation in the longitudinal, as well as the radial direction.

Elastic laminae provide a physical constraint between radial layers of SMCs. Loss of this barrier may provide a permissive environment for SMC proliferation in Fbln4−/− and Eln−/− aortas. Changes in genetic signaling may facilitate SMC proliferation in Fbln4−/− aortas. For example, we found that Col8a1 is highly upregulated in Fbln4−/− AA compared with Fbln4+/+ AA, and Col8a1 expression in Fbln4−/− DA is even higher than Fbln4−/− AA. Col8a1 is a member of the short-chain nonfibrillar collagen family and is present in small amounts in normal arteries as a result of production by ECs and mast cells (73). Col8a1 is proliferative (2) and promigratory (33). Upregulation of Col8a1 is found in atherosclerosis (52) and injury (74) models where inflammation is induced, vascular wall remodeling is increased, and SMC proliferation and migration are elevated. Col8a1 upregulation may be involved in the SMC overproliferation and arterial wall thickening in Fbln4−/− mice.

Although both Fbln4−/− AA and DA show increased arterial wall thickness, only Fbln4−/− AA shows the increased unloaded diameter associated with aneurysm formation. Gene expression changes specific to Fbln4−/− AA may be involved in the diameter increase. Hbegf and Ereg expression are upregulated in Fbln4−/− AA compared with Fbln4+/+ AA and compared with Fbln4−/− DA. Hbegf and Ereg are members of the epidermal growth factor (EGF) family and share similar conserved cysteine residues (72). Both can bind to and phosphorylate the EGF receptor (EGFR) (44, 67), resulting in potent SMC mitogenesis and proliferation (7, 45). Amounts of phosphorylated ERK1/2, a downstream target of EGFR, are increased in embryonic Fbln4−/− AA (34). The binding of Hbegf and Ereg to EGFR is hypothesized to stimulate SMC proliferation in aortic development, remodeling, and disease

**DISCUSSION**

Elastic fibers distribute stress throughout the aortic wall, with fragmented elastic fibers and disorganized SMCs in the media, consistent with previous studies (34, 55). Improper assembly of elastic fibers due to the lack of fibulin-4 is likely the main cause of elastic fiber fragmentation. Disorganized SMCs are also observed in aortas from elastin knockout (Eln−/−) mice (48, 81). Eln−/− AA shows inward remodeling and overproliferation of SMCs at the intima, compared with outward remodeling and overproliferation of SMCs throughout the wall thickness in Fbln4−/− AA. Proliferation of SMCs in Fbln4−/− and Eln−/− aortas may be stimulated by changes in the transmural stress distribution. Fbln4−/−, Eln−/−, and elastase-treated aortas (23, 25) have smaller opening angles than wild-type aortas, indicating that reduced amounts of functional elastic fibers decrease residual strain and the resulting residual stress. Elastase-treated aortas also have larger unloaded outer diameters compared with untreated aortas (23). This was interpreted as a release in compressive residual stress through elastic fiber fragmentation that leads to increased diameter and decreased opening angle. However, in our study, newborn Fbln4−/− mice...
Fig. 4. Material property data for inflation tests of AA and DA segments from newborn Fbln4+/− and Fbln4−/− mice. The mean circumferential stretch of Fbln4−/− DA is significantly larger than Fbln4+/− DA (A). There are no significant differences between groups for the circumferential stress at each applied pressure (B). The circumferential stress-stretch plots are shifted to the right in Fbln4−/− aorta compared with Fbln4+/− and in DA segments compared with AA (C). The tangent modulus is significantly higher in Fbln4−/− DA than Fbln4+/− AA at 20–25 mmHg (D). Symbols indicate significant differences between Fbln4−/− DA and Fbln4+/− DA (†) and Fbln4+/− AA and Fbln4−/− DA (&).

Table 1. Summary of gene expression changes

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<thead>
<tr>
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<th>Upregulated</th>
<th>Downregulated</th>
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<tbody>
<tr>
<td>Fbln4+/− AA vs. Fbln4+/− AA</td>
<td>742</td>
<td>649</td>
</tr>
<tr>
<td>Fbln4−/− AA vs. Fbln4−/− DA</td>
<td>235</td>
<td>358</td>
</tr>
<tr>
<td>Fbln4−/− DA vs. Fbln4+/− DA</td>
<td>592</td>
<td>291</td>
</tr>
<tr>
<td>Fbln4+/− AA vs. Fbln4+/− DA</td>
<td>356</td>
<td>223</td>
</tr>
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</table>

Microarrays were performed on 8 pooled ascending aortas (AAs) and descending aortas (DAs) from Fbln4+/− and Fbln4−/− mice using Affymetrix Mouse Gene 2.0 ST expression arrays (n = 2). The number of genes up- and downregulated in selected comparisons is shown, based on minimum fold changes of ±1.5 and significance of P < 0.05.

(56, 68, 79). Increased expression of Hbegf and Ereg may be linked to Fbln4−/− AA diameter expansion.

Inflation tests show that the outer diameter of Fbln4−/− AA is increased at all pressures compared with the other groups. However, when the compliance (change in diameter with each pressure step) is calculated, Fbln4−/− AA is only significantly different from Fbln4+/− AA at 5 mmHg. AAs from newborn Eln−/− mice have smaller diameters and compliance compared with wild-type AA (81), demonstrating that the absence of elastic fibers and fragmented elastic fibers do not have the same effects on the structural properties of the aorta. AAs from 6 wk-old SMKO mice have reduced compliance compared with wild-type, showing that differences in the structural properties may become more apparent with age, since the elastic laminae are not completely formed until ~1 wk old in the mouse (17). Like IVSR, compliance normally varies with vascular location (27). Fbln4+/− mice show differences in compliance with vascular location, but these differences are attenuated in Fbln4−/− mice. Our Fbln4−/− data suggest that functional elastic fibers are necessary for locational differences in compliance. It is possible that Fbln4−/− AAs are more susceptible to aneurysm formation because they do not exhibit the normal locational differences in compliance and IVSR.

When material behavior is determined through plots of the circumferential stress and stretch behavior, the data clusters by genotype and Fbln4−/− AA alone does not stand out. The tangent modulus (slope of the stress-stretch curve) is not sensitive to genotype, suggesting that the material behavior is similar in Fbln4−/− and Fbln4+/+ aortas for small changes in stretch, despite the fragmented elastic fibers in Fbln4−/− aorta. The tangent modulus in 6-wk-old SMKO AA is an order of magnitude higher than wild-type at high stretch (46), supporting our assertion that mechanical differences may become more significant with age as the elastic laminae mature. There is a tendency toward larger circumferential stretches in Fbln4−/− aortas compared with Fbln4+/+, while circumferential stress is similar between genotypes. For newborn Eln−/− AA, circumferential stretch is similar to wild-type, but circumferential stress is lower than wild-type (81), highlighting the differences between the mechanical effects of absent vs. fragmented elastic fibers. The increased circumferential stretches in Fbln4−/− AA may be transmitted to the SMCs in the aortic wall and cause changes in gene expression (6).

Cyclic stretch activates ERK1/2 signaling in cultured SMCs through the insulin-like growth factor receptor 1 (IGF-1) (8). Activation of ERK1/2 leads to SMC overproliferation in mice.
ERK1/2 signaling may be present specifically in and aneurysm development in fibulin-4 is unclear. Our study suggests that wall thickening although the mechanism of interaction between TGF-activity has been linked to fibulin-4 mutations (31, 57, 65), tion of ShcA also activates ERK1/2 signaling (47). TGF-H9252 due to upregulation of Fbln4 (34) and is consistent with the wall thickening observed in Fbln4−/− AA and DA. ERK1/2 signaling can also be activated by Hbegf and Ereg binding to EGFR (67, 77), so increased ERK1/2 signaling may be present specifically in Fbln4−/− AA due to upregulation of Hbegf and Ereg. TGF-β phosphorylation of ShcA also activates ERK1/2 signaling (47). TGF-β activity has been linked to fibulin-4 mutations (31, 57, 65), although the mechanism of interaction between TGF-β and fibulin-4 is unclear. Our study suggests that wall thickening and aneurysm development in Fbln4−/− AA may be due to proliferation and migration of SMCs through an activated ERK1/2 pathway that depends on cyclic stretch and upstream signaling molecules such as Hbegf, Ereg, IGF-1, and TGF-β. TGF-β-mediated ERK1/2 phosphorylation has been implicated in aneurysm formation in mice, and this is often suggested to be MMP-2 and/or -9 dependent (3, 13, 86). However, MMP8 is elevated in humans with abdominal aortic aneurysms compared with healthy aortas (85). MMP8 is typically stored in neutrophils and is a prolific type 1 collagenase in chronic inflammatory models (54). MMP8 expression is seen in ECs, SMCs, and macrophages in atherosclerotic models (24). Mechanically, degradation of type 1 collagen may be necessary to allow continued expansion of the arterial diameter in late aneurysm formation and rupture (21). While collagen degradation by Mmp8 may not be detectable as a change in arterial wall mechanical behavior by our methods, it may be enough to allow the outward diameter expansion necessary for aneurysm growth in Fbln4−/− AA. As aneurysms are located exclusively in Fbln4−/− AA and elevated Mmp8 gene expression is observed in Fbln4−/− AA compared with both Fbln4+/+ AA and Fbln4−/− DA, Mmp8 could be a potential therapeutic target for mitigating the formation and rupture of AA aneurysms in diseases like cutis laxa.

The AA is a region of disturbed blood flow due to the curvature and large branches of the aortic tree. This results in an upregulation of proinflammatory genes that are not upregulated in straight arteries with small or no branches where flow is laminar (10). The upregulation of proinflammatory genes leads to EC and SMC activation (18) and consequent recruitment of macrophages and monocytes to the region (70, 71). This eventually leads to activated macrophages and monocytes infiltrating the arterial wall, a positive feedback mechanism that can significantly contribute to the pathogenesis of TAAs (32). Upregulation of inflammatory genes from disturbed flow, as well as facilitated diffusion of signaling molecules and increased migration of leukocytes across the aortic wall due to fragmented elastic fibers, may be linked to aneurysm development in Fbln4−/− AA. SMKO mice, with normal IEL, but fragmented medial laminae develop TAAs by ~2 wk of age (35). The delay in aneurysm formation may be related to the physical barrier between the lumen and the wall provided by the intact IEL that is not present in Fbln4−/− AA.

Two inflammatory markers, Emr1 and PtgS2, are upregulated in Fbln4−/− AA compared with Fbln4+/+ AA and Fbln4−/− DA. Emr1 is a unique cell-surface receptor that contains six EGF repeats coupled to a G protein-coupled receptor (GPCR). Emr1 is restricted to leukocytes and is thought to play a significant role in the immune response by moderating cell adhesion via the EGF repeats with concomitant intracellular signaling via its GPCR (76). PtgS2 is inducible in the presence of cytokines, proinflammatory stimuli, and mitogens (75) and is upregulated in aneurysms in mice (50). Two other inflammatory markers, Ankrd1 and Serpina3n, are upregulated in Fbln4−/− AA compared with both Fbln4+/+ AA and Fbln4−/− DA. As aneurysms are located exclusively in Fbln4−/− AA and elevated Mmp8 gene expression is observed in Fbln4−/− AA compared with both Fbln4+/+ AA and Fbln4−/− DA, as well as previous data on signaling pathways associated with fibulin-4 mutations. ECM, extracellular matrix; SMC, smooth muscle cell.

Table 2. Selected genes for validation by quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein Encoded</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col5a1</td>
<td>Collagen 8 alpha 1</td>
<td>ECM component</td>
</tr>
<tr>
<td>Hbegf</td>
<td>Heparin-binding EGF-like growth factor</td>
<td>SMC proliferation/migration</td>
</tr>
<tr>
<td>Ereg</td>
<td>Epiregulin</td>
<td>SMC proliferation/migration</td>
</tr>
<tr>
<td>Ankrd1</td>
<td>Ankyrin repeat domain-containing protein 1</td>
<td>ERK1/2 activation</td>
</tr>
<tr>
<td>Emr1</td>
<td>EGF-like module containing hormone-receptor-like sequence 1</td>
<td>ERK1/2 activation</td>
</tr>
<tr>
<td>PtgS2</td>
<td>Cyclooxygenase-2</td>
<td>Inflammation</td>
</tr>
<tr>
<td>Serpina3n</td>
<td>Serine peptidase inhibitor 3n</td>
<td>Inflammation</td>
</tr>
<tr>
<td>Mmp8</td>
<td>Matrix metalloproteinase-8</td>
<td>ECM remodeling</td>
</tr>
</tbody>
</table>

Genes were chosen based on fold changes between Fbln4+/+ AA vs. Fbln4−/− AA and Fbln4+/+ AA vs. Fbln4−/− DA, as well as previous data on signaling pathways associated with fibulin-4 mutations. ECM, extracellular matrix; SMC, smooth muscle cell.
ameliorate the inflammatory response and limit the diameter only in

Hbegf
Fbln4
compared with

Fig. 6. Quantitative PCR validation of genes of interest identified from the microarray. Expression was compared between Fbln4−/− AA and Fbln4+/+ AA (A) and between Fbln4−/− AA and Fbln4−/− DA (B). Collagen 8a1 (Col8a1), heparin-binding EGF-like growth factor (Hbegf), epiregulin (Ereg), ankyrin repeat domain-containing protein 1 (Ankrd1), EGF-like module containing hormone receptor-like sequence 1 (Emr1), cyclooxygenase-2 (Ptgs2), and matrix-metalloprotease-8 (Mmp8) are increased in Fbln4−/− AA compared with Fbln4+/+ AA (*P < 0.05). Hbegf, Ereg, Emr1, Ptgs2, and Mmp8 are increased in Fbln4−/− AA compared with Fbln4−/− DA (*P < 0.05).

regulated in Fbln4−/− AA compared with Fbln4+/+ AA, but not compared with Fbln4−/− DA. Induction of Ankrd1 expression has been observed in inflammatory models (11, 41) and may be localized to activated ECs and SMCs (18). Serpina3n is increased in rats injected with LPS to induce inflammation (78). Our data indicate an active inflammatory response in Fbln4−/− mice, with additional inflammatory genes increased only in Fbln4−/− AA. Emr1 and Ptgs2 may represent targets to ameliorate the inflammatory response and limit the diameter expansion in Fbln4−/− AA.

It has been shown previously that diseased aortic tissue from TAA patients has different gene expression patterns than normal aortic tissue from control patients (1, 9, 43, 58). Patients with connective tissue disorders, such as Marfan syndrome, or familial forms of TAA were excluded from these studies, so they do not provide insight into molecular mechanisms of aneurysm progression in these cases. Additionally, no significant gene expression differences were found when tissue from normal and diseased aortic regions were compared from the same TAA patient (9, 58), so these studies do not give information about why aneurysms develop in specific vascular locations. Schwill et al. (69) investigated changes in aortic gene expression for a Marfan mouse model (mgR) and found increases in inflammatory pathway genes, consistent with our results. However, they pooled thoracic and abdominal aortic samples so locational differences in gene expression cannot be compared. Pfaltzgraff et al. (64) show that gene expression profiles in the mouse AA and DA are distinct in embryos, but not in adults, suggesting that SMCs from different embryonic origins converge into a common phenotype with maturation. Our data in newborn mice are consistent with Pfaltzgraff et al. (64) and support the hypothesis that locational differences in gene expression are important for disease progression. Our current findings extend previous gene array studies to identify expression patterns associated with aneurysm pathogenesis in the AA for mice with a connective tissue disorder.

Limitations and future directions. As Fbln4−/− mice die at birth or earlier (34), we may have selected for the healthiest Fbln4−/− pups that survived until birth in this study. The measured in vitro mechanical behavior is not identical to the in vivo behavior, but presumably comparisons between genotypes and vascular locations are relevant. Due to the small size of the aortic segments, end effects during mechanical testing and incomplete removal of loose tissue can affect the observed behavior. We quantified only circumferential mechanical behavior in this study, although longitudinal mechanical behavior and interactions between the two directions are certainly important. Microarray and qPCR data were from pooled groups of aortas with two samples for each group. Data from individual aortas and additional samples would be useful for confirming the results of this study. Protein expression data are necessary in future work to show that gene expression changes are translated into altered amounts of protein in the aortic wall. Additional factors such as blood pressure, blood flow, shape of the aorta, branching geometry, and peripheral tissue connections may also contribute to aneurysm formation in Fbln4−/− AA. Our study identifies mechanical changes and candidate genes that may be related to aneurysm pathogenesis but does not determine if these factors are necessary or sufficient for aneurysm formation. Future work will involve inhibition of the upregulated pathways identified in this study to determine if aneurysm formation can be delayed or prevented in Fbln4−/− AA.

Conclusions. Fbln4−/− aortas have thick walls, fragmented elastic fibers, and disorganized SMCs. Gene expression of Col8a1, Ankrd1, and Serpina3n suggest activation of pathways for SMC proliferation and inflammation in Fbln4−/− aortas. Despite the changes in geometry, ultrastructure, and gene expression in Fbln4−/− aortas, there are few significant differences for the diameter compliance and tangent modulus of Fbln4−/− aortas compared with Fbln4+/+. Fbln4−/− aortas demonstrate a trend toward increased circumferential stretch compared with Fbln4+/+, which may be transmitted to SMCs in the wall. Gene expression of Hbegf, Emr1, Ereg, Mmp8, and Ptgs2 are upregulated in Fbln4−/− AA compared with Fbln4+/+ AA and Fbln4−/− DA suggesting that ERK1/2 activation, degradation of collagen type 1, and increased inflammation are involved in aneurysm pathogenesis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.K. and J.D.P. performed experiments; J.K., J.D.P., and J.W. analyzed data; J.K., J.D.P., H.Y., and J.W. interpreted results of experiments; J.K., J.D.P., and J.W. prepared figures; J.K. and J.D.P. drafted manuscript; J.K., J.D.P., H.Y., and J.W. approved final version of manuscript; H.Y. and J.W. conceived and design of research; H.Y. and J.W. edited and revised manuscript.

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