Dysfunction in elastic fiber formation in fibulin-5 null mice abrogates the evolution in mechanical response of carotid arteries during maturation

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Wan W, Gleason RL, Jr. Dysfunction in elastic fiber formation in fibulin-5 null mice abrogates the evolution in mechanical response of carotid arteries during maturation. Am J Physiol Heart Circ Physiol 304: H674–H686, 2013. First published December 15, 2012; doi:10.1152/ajpheart.00459.2012.—Elastin fragmentation is a common characteristic of vascular diseases, such as abdominal aortic aneurysms, peripheral arterial disease, and aortic dissection. Examining growth and remodeling in the presence of dysfunctional elastic fibers provides insight into the adaptive or maladaptive changes that tissues undergo in compensating for structural deficiencies. This study used the maturation of fibulin-5 knockout (KO) and wild-type mice to study the effects of fragmented elastic fibers on the growth and remodeling of carotid arteries. The microstructural content and organization and the biaxial mechanical behavior of common carotid arteries were measured, and parameter estimation performed from KO and WT mice aged 3, 4, 8, and 13 wk. Gross measurements and biaxial tests revealed significant differences in pressure-diameter behavior, in vivo axial stretch, opening angle, compliance, and wall stresses during maturation of wild-type arteries, but little change in these values in KO mice. Multiphoton microscopy used to image collagen fibers across the vessel wall in pressurized and stretched arteries suggests that there is little variation in fiber angles between different ages. Parameter estimation revealed significant differences in material parameters between genotypes and age groups. This study suggests that neonatal formation and cross-linking of functional elastic fibers, followed by increases in artery size due to growth with little remodeling of the elastic fibers, endow arteries with large distensibility and contribute to the evolution of mechanical behavior of arteries during maturation. Dysfunction in neonatal formation of elastic fibers abrogates many of the changes in mechanical response that take place during the maturation.

artery mechanics; extracellular matrix; elastin; arterial stiffening; fbln5

LOSS OF FUNCTIONAL ELASTIC fibers is associated with various vascular diseases, including abdominal aortic aneurysms (5, 46), peripheral arterial disease (42, 43), aortic dissection (30, 49), as well as pathologies in other organ systems (13, 20). Fibulin-5 (fbln5) is an extracellular matrix protein that binds to integrins and localizes tropoelastin to microfibrils (39, 62). In humans, altered expression of fbln5 is correlated with a variety of diseases, such as thoracic aortic dissection (59), age-related macular degeneration (52), as well as various cancers (26, 36, 63). Fbln5 knockout (KO) mice exhibit loose skin, pelvic organ prolapse, and tortuous arteries with disrupted elastic lamellae (39, 62). Previous studies have shown that arteries from adult fbln5 KO mice have altered biomechanical and microstructural properties, including disrupted and dysfunctional elastic lamellae (58); however, evolution of biomechanical and microstructural properties with postnatal maturation in this mouse model has not been studied.

During postnatal maturation, the cardiovascular system undergoes large hemodynamic changes that coincide with changes in biomechanical properties of arteries. Huang et al. found that both the axial stretch ratio and the mean physiological circumferential stress of wild-type (WT) mouse aortas increase with age (27). Wiesmann et al. (61) found that mean body weight of C57BL/6 mice increased from 2.2 g at postnatal day 2 to 26.6 g at week 16 and that the left ventricular cardiac output increased from 1.1 ml/min at postnatal day 2 to 14.3 ml/min at week 16. Observations of 129/SvEv and C57BL/6 strains of mice reveal that, within the first 30 days after birth, the mean arterial pressure more than doubles (27, 31), reaching an asymptotic value at ~50 days (53).

Despite the vast body of knowledge detailing cardiovascular changes during maturation, there remains a need to quantify the evolution of arterial mechanical behavior and the role of load-bearing constituents, such as collagen and elastin, in large arteries. It has been hypothesized that elastin-containing fibers are laid down and cross-linked neonatally and remain mechanically stable throughout maturation and early adulthood (18, 19, 34, 50). During maturation, the in vivo axial stretch ratio of arteries increases, the circumferential residual stress decreases, and the shape of the mechanical properties and response curves evolves with age; it is argued that these phenomena are due to an increase in strain in elastin-containing fibers, which maintain the stress-free configuration determined at the time of cross-linking (neonatally), but experience an increase in strain due to increases in arterial length and diameter that occur during maturation (7, 19, 21, 57). In contrast, it is argued that other structural constituents (e.g., collagen and smooth muscle) experience significant turnover and remodeling during maturation at rates that generally exceed the rates of increases in arterial diameter and length (34, 50); thus the organization and in vivo strain of these structural constituents are continually restored to homeostatic values (57).

This paper provides compelling evidence to support this hypothesis by comparing the microstructural properties and the mechanical response of common carotid arteries from fbln5 null mice, which lack functional, cross-linked, elastin-containing fibers and WT mice during maturation between 3 and 13 wk of age. Multiphoton microscopy was used to image collagen fibers in excised arteries under controlled pressure and axial stretch. We also performed constitutive modeling to quantify the biomechanical behavior during maturation. The results of this study suggest that neonatal formation of functional elastin-containing fibers, followed by increases in artery size due to organism growth with little remodeling of the elastin-containing fibers, endow arteries with large distensibility and contributes to the evolution of mechanical behavior of arteries during maturation.
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METHODS

Surgical preparation and vessel isolation. Adult male WT and fbn5 KO mice (3, 4, and 8 wk old) on the C57-Bl6 × 129/SvEv background were euthanized with an overdose of CO2. All mice were generated from a breeding pair originally obtained from Dr. Hiromi Yanagisawa (UT Southwestern, from Ref. 62). Both common carotid arteries were excised under sterile conditions, placed in Dulbecco’s phosphate-buffered saline, dissected free of perivascular tissue, and mounted on the glass cannulas of our biomechanical testing device using sterile suture (17). All animal procedures were approved by the Institute Animal Care and Use Committee at the Georgia Institute of Technology. Biaxial mechanical test results for 13-wk-old WT and KO arteries were previously reported (58).

Biaxial mechanical testing. Cylindrical biaxial biomechanical testing and multiphoton microscopy was performed as described previously (58). Briefly, pressure-diameter (P-D) data were collected from 0 to 160 mmHg at constant axial extensions, and axial force-length (F-L) data were collected over cyclic axial extensions at constant pressures of P = 60, 100, and 140 mmHg. The in vivo axial stretch ratio (L/H) was defined as the intersection of force-length tests performed at different transmural pressures. This property of arteries has been previously observed experimentally, and it is thought to help prevent buckling during movement over the cardiac cycle (54, 60). For each experimental group, the in vivo axial stretch ratio was used to determine axial stretch ratios for pressure-diameter tests. Axial stretch ratios for pressure-diameter tests were selected to include the in vivo axial stretch ratio, as well as those above and below the in vivo value.

Opening angle. When an arterial ring is cut radially, it springs open, indicating the presence of residual strains in the unloaded state. Changes in opening angle suggest nonuniform remodeling of load-bearing constituents within the arterial wall. Increases in the opening angle have been observed in organ culture and in vivo animal models of hypertension (23, 38). After biaxial testing, arteries were carefully cut into open sectors to measure opening angles to quantify the residual strains present in the unloaded artery. Opening angle, Φo, was calculated as

\[ \Phi_o = \pi - \left( \frac{L_o - L_i}{2H} \right) \text{ and } A = \frac{H(L_o + L_i)}{2} \]

where \( L_o \) and \( L_i \) are the outer and inner arc lengths of the stress-free sector, respectively; \( H \) is the wall thickness of the stress-free sector; and \( A \) is the cross-sectional area of the wall in the open sector (58). A mean value for \( H \) was calculated by measuring the area of the sector using a MATLAB script and solving for \( H \) using Eq. 1.

Compliance. The compliance provides a measure of stiffness based on the change in radius of the artery over a change in pressure. In diseased arteries, decreases in compliance can be caused by increased wall thickening or increased collagen production, along with fragmentation of elastic fibers (67). Compliance was calculated according to the relation

\[ C = \frac{\Delta P}{\Delta r_m} \]

where \( C \) is compliance, \( \Delta r_m \) is the difference in the midwall radii measurements at two different pressures, \( r_m \) is the midwall radius at the mean pressure, and \( \Delta P \) is difference in the two pressures.

Stress and strain. Pressure diameter tests and compliance measurements depend on both the material properties and geometry of the artery. Changes in opening angle suggest nonuniform remodeling of load-bearing constituents, stresses and strains were calculated. The mean circumferential (\( \sigma_o \)) and axial (\( \sigma_z \)) stresses were calculated according to the following relations

\[ \sigma_o = \frac{P a}{h} \text{ and } \sigma_z = \frac{f}{\pi(b^2 - a^2)} \]

where \( P \) is transmural pressure, \( b \) is the loaded outer radius, \( h \) is the loaded thickness, and \( f = r_m + \frac{P(a^2)}{E} \) is the force applied to the vessel wall, where \( r_m \) is the force measured by the force transducer. Mean circumferential (\( \lambda_o \)) and axial (\( \lambda_z \)) stretches were defined as

\[ \lambda_o = \frac{r_m}{R_m} \text{ and } \lambda_z = \frac{l}{L} \]

where \( r_m \) is the current midwall radius, \( R_m \) is the unloaded midwall radius, \( l \) is the current axial length, and \( L \) is the unloaded axial length. The mean circumferential (\( E_{o_+} \)) and axial Green strain (\( E_{z_+} \)) were calculated as

\[ E_{o_+} = \frac{\lambda_o - 1}{2} \text{ and } E_{z_+} = \frac{\lambda_z - 1}{2} \]

Multiphoton microscopy. Mouse carotid arteries were mounted on the biaxial testing device and imaged on an LSM 510 META inverted confocal microscope (Zeiss) fitted with a tunable multiphoton laser (Coherent). The laser was tuned to 800 nm and reached the sample through a ×40/1.3 numerical aperture oil immersion objective (Zeiss). The META module of the microscope was configured as a 350- to 450-nm bandpass filter to detect backwards scattering second harmonic generation signal from collagen (68, 69). WT vessels were imaged at \( \lambda_z = 1.54 \) and \( P = 110 \) mmHg for 8-wk-old vessels and at \( \lambda_z = 1.39 \) and \( P = 110 \) mmHg for 3-wk-old vessels. Knockout vessels were imaged at \( \lambda_z = 1.39 \) and \( P = 110 \) mmHg for 8-wk-old vessels and \( \lambda_z = 1.34 \) and \( P = 110 \) mmHg for 3-wk-old vessels.

Measurement of collagen fiber angle distribution. The angular distribution of collagen fibers for each optical slice in a z-stack was measured using a MATLAB (Mathworks) script modified from a previously reported fast Fourier series algorithm (40, 56). Only collagen fibers in the adventitia of the vessel were visible with the current imaging system (58). The first and last optical slices of collagen fibers within the image stack were determined by reconstructing the vessel and examining the orthogonal views. Each optical slice was low-pass filtered, converted to a binary image using Otsu’s method to threshold, and windowed with a two-dimensional Tukey window. A fast Fourier transform was performed on each optical slice, and a power spectrum was generated. This power spectrum was filtered and used to generate a histogram of frequency intensities between −90° and 90° binned into 4° increments. The relative location of each optical slice was normalized through the thickness of the adventitia, and corresponding wall locations of optical slices were averaged across samples within each experimental group to generate a surface of fiber angle distributions through the thickness of the adventitial layer. Collagen fiber angle quantification data for 8-wk WT arteries were previously reported (56).

Elastin content assay. Elastin mass fraction was measured using a quantitative dye binding assay, Fastin kit (Biocolor). Samples were
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Parameter estimation. A four-fiber constitutive model was used to estimate material and structural parameters (1). Cauchy stress (T) is defined according to

\[ T = -p I + \hat{T} \text{ where } \hat{T} = 2F(\partial W/\partial C)F^T \]  

where \( \hat{T} \) is the so-called “extra” stress due to the deformation, \( p \) is a Lagrangian multiplier that enforces the incompressibility constraint, \( I \) is the identity tensor, \( W \) is the strain energy density function, \( F = \text{diag}(\lambda_1, \lambda_2, \lambda_3) \) is the deformation gradient, and \( C = \text{diag}(\lambda_1^2, \lambda_2^2, \lambda_3^2) \) is the right Cauchy-Green strain tensor (29). Local stretch ratios are designated by \( \lambda_i \), \( \lambda_0 \), and \( \lambda_i \), defined according to

\[ \lambda_i = \frac{\partial r}{\partial r_i}, \lambda_0 = \frac{\pi r}{0 R}, \text{and } \lambda_i = \lambda A \]  

where \( r \) is the radius in the current configuration, \( R \) is the radius in the stress-free configuration, \( \theta = (L_o - L) / 2H \) is the half-angle of the open sector as defined by Chuong and Fung (3), \( \lambda \) is the stretch from the stress-free to the unloaded configuration, and \( A \) is the axial stretch from the unloaded to the current configuration. The four-fiber constitutive model is defined as

\[ W = \frac{b_0}{2}(I_1 - 3) + \frac{1}{2} \sum_{k=1}^{4} \frac{b_k}{2k^2} \exp \left[ b_k \left( \lambda^k - 1 \right)^2 \right] - 1 \]  

where \( W \) is a stress energy function; \( b_0, b_1, \text{ and } b_k \) are material parameters with \( k \) denoting a fiber family; \( I_1 = tr(C) = C_{11} + C_{22} + C_{33} \) is the first invariant of \( C \), \( \lambda^k \) is the stretch of the \( k \)-th fiber family, \( M^k = \sin(\alpha^k)\sigma + \cos(\alpha^k)\tau \) is the unit vector along the \( k \)-th fiber direction in the reference configuration, and \( \alpha^k \) is the associated angle between the axial and diagonal directions. This constitutive relation models the tissue as an isotropic amorphous solid embedded with four structural fibers oriented in axial (\( \alpha^1 = 90^\circ \)), circumferential (\( \alpha^2 = 0^\circ \)), and symmetric diagonal directions (\( \alpha^3 = -\alpha^4 = \alpha \)). For material symmetry, diagonal fibers are constrained to the same material properties, \( b_1 = b_2 \) and \( b_3 = b_4 \), and the fiber angle \( \alpha \) is determined along with the seven material parameters. This model has been shown in the past to capture the salient features of biaxial tests of arteries (16, 57, 58).

Material parameters were estimated using the Matlab optimization function *lsqnonlin* and minimizing the error function,

\[ \text{error} = \sum_{i=1}^{n} \left[ \frac{P_{\text{mean}}(i) - P_{\text{model}}(i)}{P_{\text{mean}}} \right]^2 + \sum_{i=1}^{n} \left[ \frac{f_{\text{mean}}(i) - f_{\text{model}}(i)}{f_{\text{mean}}} \right]^2 \]  

where \( P_{\text{mean}}(i) \) is the measured pressure for data point \( i \), \( P_{\text{model}}(i) \) is the pressure predicted by the model at data point \( i \), \( P_{\text{mean}} \) is the mean of all measured pressures, \( f_{\text{mean}}(i) \) is the measured axial force for data point \( i \), \( f_{\text{model}}(i) \) is the axial force predicted by the model at data point \( i \), and \( f_{\text{mean}} \) is the mean of all axial force measurements (24). Fitting error for each sample was calculated by dividing the error function, Eq. 9, by the total number of data points, 2n. Parameter estimation data for 8-wk WT arteries were previously reported.

Statistical analysis. The data were analyzed by fitting general estimating equations using an exchangeable correlation matrix (65, 66). This method can account for numerical factors (age), unbalanced group sizes, and correlations between experimental groups. The data were imported into the statistical software package R (44) and analyzed using the “geepack” R package (22). Wald \( \chi^2 \) tests were tested to calculate the effect of age, genotype, and interactions with effects considered significant for \( P < 0.05 \). Individual group means were then compared using Bonferroni’s test to correct for multiple pairwise comparisons. For fiber angle orientations, a circular mean angle and standard deviations were calculated according to Zar (64). Significance was taken at \( P < 0.05 \).

RESULTS

Body and artery growth. The mean body mass increased by 36, 101, and 126% for WT and 91, 161, and 129% for KO mice between 3, 4, 8, and 13 wk, respectively (Fig. 1). Differences in body mass between genotypes at each time point were not significant; however, age was a significant effect on body mass. The unloaded outer diameter increased by 4, 16, and 10% in WT and 7, 20, and 22% in KO mice between week 3 and weeks 4, 8, and 13; differences were significant between 3 and 8 wk, but differences between 8 and 13 wk were not significant. Both age and genotype were significant effects on unloaded outer diameter. In WT arteries, changes in the unloaded thickness were not significant; however, in KO arteries, the unloaded thickness was significantly lower at 3 wk than at other ages. At all ages, differences in unloaded outer diameter and thickness were not significant between genotypes.

Biaxial mechanical testing. Significant differences were found in the mechanical response between genotypes at various ages and between ages within genotypes. The pressure-diameter test results performed at the in vivo axial stretch ratio were used to calculate the midwall radii, defined as the mean of the outer and inner radii measured at each pressure (Fig. 2). The mean pressure-radius response at the in vivo axial stretch ratio shows that KO vessels at all ages have an overall stiffer circumferential response than WT vessels. Mean midwall radii were also compared at pressures of 40, 80, 120, and 150 mmHg. At all ages and all pressures compared, KO vessels had a statistically lower mean midwall radii, except for the 13-wk age groups at \( P = 40 \) mmHg. Age and genotype were significant effects on midwall radii at all pressures tested, and their interaction was a significant effect at pressures of \( P = 40 \) and 80 mmHg.

Mean local compliance of WT vessels varied with age, while the mean compliance of KO vessels remained at similar levels (Fig. 2). At all pressures analyzed, local compliance did not vary with age for KO vessels; however, in WT vessels, compliance at \( P = 80, 120, \) and 150 mmHg significantly increased with age (Fig. 2). At \( P = 40 \) mmHg, the compliance of WT vessels increased between weeks 3 and 4 and then decreased at later ages. Differences in local compliance between genotypes were only observed at \( P = 40 \) and 80 mmHg. Age, genotype, and their interaction were significant effects on compliance at all pressures except \( P = 40 \) mmHg, where only genotype and age-genotype interaction were significant effects.

The in vivo axial force (the axial force measured during pressure-diameter cycles performed at the in vivo axial stretch ratio) increased during maturation in WT vessels, whereas in KO vessels the in vivo axial force remained nearly constant (Fig. 3). At 13 wk, the axial force of WT vessels was significantly greater than that of KO vessels. The in vivo axial stretch ratio significantly increased with age between 3 and 8 wk in WT vessels, whereas, in KO vessels, the in vivo axial stretch ratio did not significantly change with age (Fig. 4). The mean in vivo axial stretch ratio was lower in KO vessels for 8- and 13-wk samples. Age, genotype, and their interaction were significant effects on the in vivo axial force at all pressures.

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whereas age and the age-genotype interaction were significant effects on the in vivo axial stretch ratio.

The mean opening angle decreased with age for WT vessels, whereas no significant differences were seen within KO vessels between any ages (Fig. 5). At the 4-, 8-, and 13-wk age groups, the opening angle was lower in WT vessels, whereas the difference in opening angle between genotypes was not statistically significant at 3 wk. The interaction between age and genotype was a significant effect on the opening angle.

The mean in vivo circumferential and axial stresses were calculated based on the in vivo axial stretch ratio and the mean adult blood pressure from Yanagisawa et al. (62). In WT arteries, both the circumferential and axial stress increased with age (Fig. 6). There was a slight decrease is mean circumferential stress between 3 and 4 wk, but the difference was not significant. The mean circumferential stress in WT arteries at 13 wk was higher than at 4 and 8 wk, and the mean axial stress in WT arteries at 13 wk was higher than at any other age. In KO arteries, means stresses decreased from 3 wk and remained stable with age after 4 wk. The mean circumferential stress, in KO arteries, at 3 wk was higher than for any other age group, and the mean axial stress was higher at 3 wk than at 4 and 8 wk. The interaction between age and genotype was a significant effect on the in vivo axial stress. Note that, although blood pressure reached an asymptotic value at ∼4 wk (53), the blood pressure at 3 wk is likely somewhat lower than the adult value used in these calculations at 3 wk; thus the 3-wk circumferential stress may be slightly overestimated. The mean circumferential stretch ratio was computed at the in vivo axial stretch ratio and mean adult blood pressure. The circumferential stretch ratio was not significantly different between genotypes and did not change significantly during maturation (Fig. 1).

Measurement of collagen fiber angle distribution. Fiber angle distributions were quantified for optical slices encompassing the thickness of the adventitia. The thickness of each image stack was normalized, and fiber angle distributions at corresponding image slices were averaged across all samples to generate a mean fiber angle surface (Fig. 7). Fiber angle distributions varied throughout the thickness of the adventitia, and fibers appeared more highly aligned in the optical slices taken farther away from the lumen. For both WT and KO arteries, the mean fiber angle distributions appeared to remain constant between 3 and 8 wk (Fig. 7). The mean fiber angle was also not significantly different between genotypes (Fig. 8).

Protein content assays. The elastin wet mass fraction decreased with age for both WT and KO vessels (Fig. 9A); however, the dry elastin mass fraction was not significantly different between ages and genotypes (Fig. 9B).

Parameter estimation. Age and genotype were significant effects for parameters $b_{2,1}$ and $b_{2,2}$. In addition, interactions between age and genotype were a significant effect for $b_{2,1}$, $b_{2,2}$, and $b_{3,2}$. Parameter estimation reveals that the fiber angle

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**Fig. 1.** The mean body mass (A) and mean unloaded outer diameter (B) significantly increased with age in both knockout (KO) and wild-type (WT) arteries. C: in WT arteries, the mean unloaded thickness did not significantly change with age, whereas in KO arteries the unloaded thickness increased with age. D: after 3 wk, the loaded thickness is significantly greater in KO arteries than in WT arteries. There is also a significant increase in thickness in KO arteries between 3 wk and all older time points. E: the mean circumferential stretch ratio ($\lambda_3$) also did not change with age for both WT and KO arteries, $*P < 0.05$ between genotypes at the same age. Differences between genotypes were not significant for mass, unloaded outer diameter, unloaded thickness, or mean circumferential stretch ratio. Age was a significant effect on body mass, and both age and genotype were significant effects on unloaded outer diameter. Values are means ± SE.
parameter, \( \alpha \), does not change with age or genotype (Fig. 10). In addition, the material parameters \( b_{1,2} \) and \( b_{3,2} \) did not change with age or with genotype. Material parameters were significantly different between genotypes at 3 wk for \( b_{1,1} \) and \( b_{3,1} \) and at 13 wk for \( b_{2,1} \) and \( b_{2,2} \). The material parameters \( b_{2,1}, b_{2,2}, \) and \( b_{3,1} \) varied with age in KO vessels. The material parameters for individual vessels are listed in Table 1.

**DISCUSSION**

Arterial growth and remodeling occur via delicate balances (or imbalances) in the production, removal, and remodeling of individual constituents of the wall (e.g., elastin, collagen, smooth muscle) at different rates, to different extents, and within different biomechanical states (28). Elastin appears to be laid down and cross-linked into functional elastic fibers neonatally, and it is argued that these fibers may remain mechanically stable, with production, removal, and remodeling rates remaining very low throughout growth and development.
Damage or loss of functional elastic fibers occurs during aging and disease and correlates with arterial stiffening, a key predictor of cardiovascular events. In contrast, collagen and other structural constituents exhibit much higher rates of turnover and remodeling. During aging and disease, decreases in the load-bearing capacity of elastin fibers by degradation and fragmentation of elastin are compensated by the increased load bearing of collagen (and smooth muscle), leading to a net increase in material stiffness.

As mice develop from 3 wk of age to 13 wk of age, they experience significant increase in size, and the large arteries experience increases in blood flow, blood pressure, and arterial length. It is well thought that arterial growth and remodeling in response to changes in blood flow, blood pressure, and axial stretch leads to geometric and material adaptations that restore the wall shear stress (33), circumferential (hoop) stress (4), cyclic circumferential strain (45), and axial stress (32) to target values.

Studies in KO mice have shown that disruption of load-bearing proteins can significantly alter the mechanical properties of arteries (9, 11, 12, 55, 58). In a mouse model for Marfan Syndrome, mutations in fibrillin-1 resulted in carotid arteries having a lower axial prestretch and were axially stiffer (11). In an elastin haplo-insufficient mouse, carotid arteries were stiffer circumferentially, had a lower axial prestretch, but exhibited no significant change in opening angle (55). Elastase treatment has also been shown to decrease collagen fiber undulation, leading to stiffer mechanical behavior (12). Finally, in adult fbln5 null mice, carotid arteries have a lower axial prestretch, are axially stiffer, and have larger opening angles (58). These studies suggest that the amount, configuration, and the level of cross-linking of elastin play important roles in determining an artery’s mechanical properties. With the exception of Le et al. (35), many of these studies of genetic KO mice examine the arteries at one point in time and did not track the time-

![Fig. 4](image-url) The mean in vivo axial stretch ratio ($\lambda_x$) was lower in KO vessels for 8- and 13-wk samples, and the in vivo axial stretch ratio for WT vessels increased with age. Differences in the in vivo axial stretch ratio were not significant during maturation for KO arteries. Horizontal lines indicate $P < 0.05$ between means at different ages for WT vessels. *$P < 0.05$ between genotypes at the same age. Age and the age and genotype interaction were significant effects on the in vivo axial stretch ratio. Values are means ± SE.

![Fig. 5](image-url) The opening angle was not significantly different between genotypes at 3 wk; however, the opening angle decreased in WT vessels after 3 wk. *$P < 0.05$ between genotypes at the same age, and horizontal lines indicate $P < 0.05$ between ages in WT vessels. The interaction between age and genotype was a significant effect on the opening angle. Values are means ± SE.

![Fig. 6](image-url) Differences in mean in vivo circumferential (A) and axial stresses (B) between KO and WT arteries became significant with age. Mean stresses tended to increase with maturation in WT arteries, while, in KO arteries, mean stresses remain stable at 4 wk. Beginning at 4 wk, arteries in KO mice are under lower mean stress than WT arteries at the same age. The interaction between age and genotype was a significant effect on the in vivo axial stress. Values are means ± SE. *$P < 0.05$ between genotypes at the same age. Horizontal bars indicate $P < 0.05$ between ages in WT (solid line) and KO (dotted line) arteries.
dependent changes in constituent properties that caused the corresponding changes in mechanics. Studying the time-dependent changes in the content and organization of matrix proteins in arteries may lead to insight into mechanisms of growth and remodeling of arteries under normal and pathological conditions.

During the normal maturation of WT vessels, significant biomechanical changes include a decreasing opening angle, an increase in in vivo axial force, an increase in in vivo axial stretch ratio, an increase in unloaded outer diameter, an increase in compliance over physiological pressures, and an increase in mean in vivo axial and circumferential stresses. In maturing KO arteries, these age-related changes are not observed. These data suggest that functional elastic fibers play a key role in the development of normal arterial biomechanics. However, differences between genotypes are affected by age; thus there are interactions between age and genotype. For example, the opening angle decreases over time in WT vessels and is significantly lower than KO vessels at 4 wk (Fig. 5). However, differences in in vivo axial force do not become significant until 13 wk (Fig. 3). In both WT and KO arteries, midwall radii in the pressurized state did not change with increasing age; however, KO arteries had a lower midwall radius than WT arteries at all ages. In addition, KO vessels at all age groups exhibited a stiffer mechanical response and lower in vivo midwall radii (with the exception at P = 40
mmHg in the 13-wk age group). The pressure-radius response in WT mice at 13 wk does not follow the same trend as younger mice. We speculate that this may be due to the beginning of elastic fiber fragmentation due to aging. This speculation will require additional study of much older mice. The pressure-radius responses measured at the in vivo axial stretch ratio suggest that normal circumferential growth is impaired when elastic fibers are disrupted; however, this does exclude effects from altered smooth muscle cell (SMC) migration or proliferation due to the lack of fbn5. The local compliance varied with age in WT vessels, while the local compliance in KO vessels did not undergo significant changes with age. At P = 120 and 150 mmHg, WT and KO vessels did not have significantly different compliance, suggesting that KO vessels may remodel to restore compliance over the physiological pressure range.

The in vivo axial stretch ratio increased over time in WT vessels, and differences between genotypes become significant at 8 and 13 wk. Our findings are similar to previous studies in dogs; namely, Dobrin et al. found that the axial stretch ratio, or prestretch, increases nearly linearly with age (7), and that this increase was due to elastin (8). In WT vessels, the in vivo axial force also underwent significant increases between 3 and 13 wk; however, differences between genotypes did not become significant until 13 wk. The half-life of elastin is on the order of the life of the organism, which is a slower turnover rate than other arterial constituents (34, 50); thus the stretching of elastic fibers may be the cause of the increase in in vivo axial force. This increase in in vivo axial force between WT and KO mice was also observed in previous experiments in a mouse model with reduced expression of fibrillin-1 (11).

The opening angle decreased with age in WT arteries, but did not change significantly in KO arteries (Fig. 5). In addition, there were no significant differences between genotype in opening angle in samples tested at 3 wk. A decrease in opening angle during maturation was previously observed in the mouse thoracic aorta between 6 and 30 days of age (27). In an elastin haplo-insufficient mouse model, there were no significant differences in the opening angle of the ascending aorta between 3 and 60 days. Because the half-life of elastin is much longer than that of collagen (34, 50), it is hypothesized that functional elastic fibers undergo increasing levels of stretch during maturation while collagen fibers undergo turnover at their homeostatic stretch ratio, thus inducing increased tension on the luminal surface of the unloaded vessel. This increased stretching of elastin may explain the decreasing opening angle in WT arteries. The decrease in elastin mass fraction over time suggests that the remodeling of other matrix proteins and cells may also contribute to the decrease in opening angles; however, the lack of functional elastic fibers in KO arteries suggests collagen and SMC in these arteries grow and remodel under conditions that cause little changes in mechanical properties. The lack of significant differences in unloaded thickness between WT and KO arteries (Fig. 1), suggests that residual strains between load-bearing constituents have a greater effect on the opening angle than any differences in thickness.

We observed changes in mean stress in the deformed configuration, as calculated by Laplace’s law (Eq. 3). Mean wall stresses are significantly higher in WT arteries at age 4 wk and above (Fig. 6). Previous reports have shown significantly higher systolic, but no significant differences in mean, blood pressure of KO mice (62). Thus it is likely that the decreased mean wall stresses of KO arteries are caused by both a reduction in midwall radius (Fig. 2A), and an increase in thickness in the loaded configuration (Fig. 1). There was also an increase in wall stresses in WT arteries between 8 and 13 wk, which may be explained in part by a decreasing trend in loaded wall thickness (Fig. 1). In hypertensive animal models, an increase in wall thickness leads to decreased compliance (38); however, in this study, the increase in wall thickness was not associated with significant changes in circumferential stretch ratio. The question of whether tissue remodeling is driven by stress or strain is an enduring question in the field of biomechanics. These quantities are inherently linked; however, advances in computational models provide the means to predict local stresses and strain at the scale of individual constituents within tissues. In the case of arteries, elastic fibers, collagen, and SMCs can remodel at different rates to different extents and to different mechanical states. Our group and others have shown that, when such detail is taken into consideration, predictions of tissue-level stress and strain become more complicated (15, 28, 57). In addition, when the quantity and mechanical states of constituents change, tissue properties evolve even when the material properties of the constituents remain constant. In a previous study, simulating hypertension...
using a microstructural model predicted seemingly contradictory results regarding stress and strain restoration when allowing different constituents to turnover at different rates (15). In the present study, we observed significant differences in mean stress at later time points while the mean circumferential stretch ratio did not change significantly. We argue that these findings may be partially explained by the WT arteries containing functional elastic fibers that are mechanically stable and stretch as the vessel grows. This results in the normal evolution of stress and strain. In contrast, KO arteries lack cross-linked elastic fibers; thus the stresses and strains do not evolve as dramatically with age. The results in this study support the hypothesis that fragmented elastic fibers, an increase in wall thickness, and maintenance of collagen fiber strain, as seen in hypertension, would result in the restoration of circumferential stress and a decrease in circumferential strain. Thus the maintenance of circumferential stretch is likely due to differences in the material properties, namely fragmented elastic fibers, and remodeling of the constituents in the \textit{fbln5} artery.

Mean collagen fiber distributions measured at different ages varied little within each genotype (Fig. 7), while distinct variations in fiber distribution shapes were seen between genotypes when comparing at the same age. No significant differences in material parameters were calculated for WT vessels at any age. *$P < 0.05$ between genotypes at the same age, and dashed horizontal lines indicate $P < 0.05$ between ages in KO vessels. Age and genotype were significant effects for parameters $b_{2,1}$ and $b_{2,2}$. In addition, interactions between age and genotype were a significant effect for $b_{2,1}, b_{2,2},$ and $b_{3,2}$. Values are means ± SE.
### Table 1. Best fit material parameters for the model represented by Eq. 8

<table>
<thead>
<tr>
<th>Week</th>
<th>Sample</th>
<th>$b_{0.1}$</th>
<th>$b_{1.1}$</th>
<th>$b_{2.1}$</th>
<th>$b_{0.2}$</th>
<th>$b_{1.2}$</th>
<th>$b_{2.2}$</th>
<th>$b_{3.1}$</th>
<th>$b_{3.2}$</th>
<th>$\alpha$</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>I</td>
<td>23,212</td>
<td>4,579</td>
<td>0.8628</td>
<td>4,306.30</td>
<td>0.5234</td>
<td>8,561</td>
<td>2,625.00</td>
<td>36,530</td>
<td>0.013</td>
<td>6,618.6</td>
</tr>
<tr>
<td>2</td>
<td>11,780</td>
<td>10,740</td>
<td>0.2840</td>
<td>20,504.00</td>
<td>0.0001</td>
<td>19,500.00</td>
<td>0.5372</td>
<td>35,300</td>
<td>0.036</td>
<td>6,912.8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>25,516</td>
<td>1.295</td>
<td>1.1856</td>
<td>804.45</td>
<td>4.0478</td>
<td>26,501</td>
<td>3.7000</td>
<td>33,443</td>
<td>0.033</td>
<td>18,578</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2,6634</td>
<td>0.006</td>
<td>1.9248</td>
<td>14,643.00</td>
<td>0.3594</td>
<td>295,720</td>
<td>1.6247</td>
<td>41,415</td>
<td>0.029</td>
<td>7,483</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15,429</td>
<td>1,047</td>
<td>0.4383</td>
<td>23,368.00</td>
<td>0.032728</td>
<td>749,620</td>
<td>1.3513</td>
<td>39,255</td>
<td>0.025</td>
<td>20,793</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>27,191</td>
<td>4.472</td>
<td>0.8483</td>
<td>23,368.00</td>
<td>0.032728</td>
<td>749,620</td>
<td>1.3513</td>
<td>39,255</td>
<td>0.025</td>
<td>20,793</td>
<td></td>
</tr>
</tbody>
</table>

**SD, standard deviation. Wild-type (WT) and knockout (KO) samples at 3-, 4-, 8-, and 13-wk time points are shown. See METHODS for definition of terms.**
Material parameters that changed during maturation were only observed in KO vessels. The high degree of axial fiber alignment in KO vessels may explain the increased axial stiffness of KO vessels. The results here suggest that, in an effort to compensate for fragmented elastic fibers, collagen fibers in KO vessels undergo turnover at an altered fiber angle orientation. Changes in collagen fiber organization due to damage have been previously documented in bioprosthetic aortic heart valves, and changes in fiber alignment with age and tissue depth have also been observed in human aortas.

Parameter estimation revealed significant differences in material parameters without significant changes in structural parameters in the stress-free state. These findings suggest that, under normal maturation, material properties of WT arteries undergo nonsignificant changes with age, and the lack of fibln5 causes material properties of KO arteries to evolve as vessels adapt to the lack of functional elastin. Specifically, there were significant changes in the circumferential material parameters, b2,1 and b2,2, during the maturation of KO vessels. These results suggest circumferential remodeling of material properties; however, previous studies of KO mice with elastic fiber deficiencies did not necessarily find this trend in the same parameters. There was also a decrease in b1,1, a parameter associated with diagonal fibers, during the maturation of KO arteries. However, b3,2, the other diagonal fiber parameter, did not change during maturation. The precise physical interpretation of these parameters as well as those associated with axial fibers (b1,1 and b1,2) is difficult due to their nonlinear nature, high variability between samples, and inconsistent trends across different KO mouse models. However, previous parameter estimation studies of KO mice have found trends in the neo-Hookean parameter, b. For example, there is a trend toward a lower value for b in mice with elastic fiber deficiencies. The structural parameter, α, represents a diagonal family of fibers in the stress-free configuration. Transforming to physiological conditions will likely change this parameter because WT and KO arteries are under different states of physiological stretch. The lack of statistical differences in mean fiber angles between genotypes and during maturation suggests that the connection between α and experimentally measured fiber angles requires further investigation. The lack of changes in the fiber angle parameter between genotypes is in contrast to previous studies of KO mice. The overall shape of the fiber angle distribution appears to be different between KO and WT arteries. In WT vessels, the lack of significant changes in material parameters coupled with the significant changes in mechanical response highlights the need for an additional material property to better validate constitutive models. Differences in material parameters and in vivo fiber distribution suggest that KO arteries remodel to the lack of functional fibers through changes in material properties, as well as through changes in fiber orientation. This observation warrants further investigation, because the present study did not examine SMCs, and fibln5 binds integrins and has been shown to affect SMC migration and proliferation.

There are a few limitations to this study that may be addressed in the future. We did not directly measure changes in blood pressure, cardiac output, blood flow in the common carotid artery, or changes in arterial length, but rather used data from previous work in adults and from the literature; these data could shed additional light on the observed growth and remodeling response. We measured elastin mass fractions, but did not measure collagen and SMC content. Active SMCs play a role in the mechanical response of an artery, and, during growth and remodeling, they undergo changes such as hypertrophy, proliferation, and migration. Measurement of SMC content and imaging of SMC orientation would provide additional insight into the role of fibln5 in the microstructural and mechanical properties of arteries. The protein content assay quantified total elastin content; however, quantification of the degree of cross-linking may provide further insight into changes in material properties of microconstituents. This study also performed multiphoton imaging and biaxial testing on separate groups of arteries. Performing both tests on a single artery would provide structural information unique to each sample for parameter estimation studies. Finally, because fibln5 binds to other proteins, such as integrins and extracellular superoxide dismutase, the effects observed in this study may not be isolated to fragmented elastin alone.

In conclusion, this study highlights the effects of dysfunctional elastin-containing fibers associated with deficiencies in fibln5 on the microstructural and mechanical properties of mouse carotid arteries during maturation. The lack of dysfunctional elastic fibers mitigates key changes in mechanical properties of arteries that occur during maturation. This study illustrates two systems in which the load-bearing constituents of arteries are turned over at different rates. In the WT artery, collagen and SMC turnover is normal, while elastin is likely negligible. In contrast, the KO artery consists of dysfunctional elastic fibers and is not a major load-bearing constituent, but collagen and SMC turnover is likely normal. This difference in constituent turnover between KO and WT vessels results in mechanical properties that remain nearly constant in KO arteries, while the mechanical response of WT arteries evolves throughout maturation. In addition, the reduced stretch of elastic fibers in 3-wk-old WT arteries results in mechanical properties similar to those of KO arteries. This study illustrates the advantages of a combined theoretical and experimental approach in analyzing differences in arteries from genetic KO mice. This study utilizes the collection of biaxial test data at the macroscopic tissue level, while quantifying fiber angle distribution at the microstructural level, and we use the experimental data in structurally motivated constitutive relations to elucidate material property relationships that are not yet experimentally tractable.

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GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: WW and RLG conception and design of research; WW performed experiments; WW analyzed data; WW and RLG interpreted results of experiments; WW prepared figures; WW drafted manuscript; WW...
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