Circulation Research

ORIGINAL RESEARCH

Heterogeneous Cellular Contributions to Elastic Laminae Formation in Arterial Wall Development

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RATIONALE: Elastin is an important ECM (extracellular matrix) protein in large and small arteries. Vascular smooth muscle cells (SMCs) produce the layered elastic laminae found in elastic arteries but synthesize little elastin in muscular arteries. However, muscular arteries have a well-defined internal elastic lamina (IEL) that separates endothelial cells (ECs) from SMCs. The extent to which ECs contribute elastin to the IEL is unknown.

OBJECTIVE: To use targeted elastin (Eln) deletion in mice to explore the relative contributions of SMCs and ECs to elastic laminae formation in different arteries.

METHODS AND RESULTS: We used SMC- and EC-specific *Cre* recombinase transgenes with a novel floxed *Eln* allele to focus gene inactivation in mice. Inactivation of *Eln* in SMCs using *Sm22aCre* resulted in depletion of elastic laminae in the arterial wall with the exception of the IEL and SMC clusters in the outer media near the adventitia. Inactivation of elastin in ECs using *Tie2Cre* or *Cdh5Cre* resulted in normal medial elastin and a typical IEL in elastic arteries. In contrast, the IEL was absent or severely disrupted in muscular arteries. Interruptions in the IEL resulted in neointimal formation in the ascending aorta but not in muscular arteries.

CONCLUSIONS: Combined with lineage-specific fate mapping systems, our knockout results document an unexpected heterogeneity in vascular cells that produce the elastic laminae. SMCs and ECs can independently form an IEL in most elastic arteries, whereas ECs are the major source of elastin for the IEL in muscular and resistance arteries. Neointimal formation at IEL disruptions in the ascending aorta confirms that the IEL is a critical physical barrier between SMCs and ECs in the large elastic arteries. Our studies provide new information about how SMCs and ECs contribute elastin to the arterial wall and how local elastic laminae defects may contribute to cardiovascular disease.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: aorta ■ constriction, pathologic ■ elastic tissue ■ elastin ■ neointima

Meet the First Author, see p 934

A vital adaptation that made the vertebrate closed circulatory system possible was the appearance of the ECM (extracellular matrix) protein elastin,[1] which allows vessels to expand and store energy during the cardiac cycle. Elastin is found throughout the arterial system and is organized, predominantly, as protein sheets, or laminae, that separate smooth muscle cell (SMC) layers in large arteries and endothelial cells (ECs) from SMCs in all arteries. Elastin is required for postnatal cardiovascular function, since elastin knockout mice ($Eln^{-/-}$) die shortly after birth.[2,3]

In developing arteries, mural cells produce elastin soon after their recruitment to the nascent tubular vessel and after their commitment to the SMC lineage.[4] In large conducting arteries, each SMC layer produces fenestrated elastin sheets and connecting fibers that are designed to distend outward with the surge of blood pressure during cardiac contraction. In muscular and resistance arteries where pulse pressure is low, outward stretch is less important and medial elastin layers are absent. The exceptions are an occasional, and frequently discontinuous, elastin layer between the media and

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Novelty and Significance

What Is Known?

- Elastin is arranged in layers within the arterial wall that are critical for cardiovascular function.
- The internal elastic lamina (IEL) separates endothelial cells (ECs) from smooth muscle cells (SMCs).
- The relative contributions of ECs and SMCs to elastic fiber formation in vivo are unknown.

What New Information Does This Article Contribute?

- SMCs and ECs can independently form the IEL in most elastic arteries.
- ECs are the major source of elastin in the IEL for muscular and resistance arteries.
- Lack of SMC elastin in the ascending aortic IEL leads to disruptions that correlate with neointimal formation.

Elastin is arranged in concentric layers in the arterial wall, and the number of layers decreases with arterial size. Almost all arteries have at least 1 layer, the IEL, which separates ECs from SMCs. In Eln$^{-/-}$ (elastin knockout) mice, the lack of an IEL may contribute to aortic stenosis and eventual occlusion. We used EC- and SMC-Eln$^{-/-}$ mice to determine how each cell type contributes to elastin deposition and cardiovascular function. In EC-Eln$^{-/-}$ mice, the IEL was severely disrupted in small arteries that have 1 to 2 elastin layers but was normal in large arteries with multiple elastin layers. In SMC-Eln$^{-/-}$ mice, the IEL was normal in all arteries except the ascending aorta where there were IEL disruptions that correlated with neointimal formation. The midwall elastin layers in large arteries were absent in SMC-Eln$^{-/-}$ mice and the mice died around 2 weeks of age from cardiomyopathy secondary-to-severe aortic stenosis. Our results show heterogeneity in elastin deposition depending on cell type and arterial location that was previously unappreciated. Future studies to unravel mechanisms driving this heterogeneity may assist in repairing elastin defects that lead to cardiovascular diseases such as aortic stenosis.

Nonstandard Abbreviations and Acronyms

α-SMA	α-smooth muscle actin
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat–associated 9
EC	endothelial cell
ECM	extracellular matrix
EEL	external elastic lamina
***Eln*$^{-/-}$**	elastin knockout
IEL	internal elastic lamina
LV	left ventricular
P	postnatal day
SMC	smooth muscle cell

adventitia (the external elastic lamina [EEL]) and a layer of elastin that separates intimal ECs from SMCs (the internal elastic lamina [IEL]). The IEL is present in almost all arteries including muscular and resistance arteries and was assumed to be formed by SMCs, which produce the other elastic structures in the vessel wall. The absence of the IEL was suggested to contribute to aortic occlusion in *Eln*$^{-/-}$ mice.[2]

In this report, we generated mice with SMC- and EC-specific deletion of *Eln* to determine how the absence of elastin deposition by vascular wall cells affects structure and function in different arteries. We found an unexpected heterogeneity in elastin production by SMCs and ECs in different arterial beds and documented a contribution of elastin to the IEL by ECs. We also found that areas of malformed IEL in the ascending aorta were associated with neointimal formation by SMCs. These results suggest that elastin production by vascular wall cells is more complicated than heretofore appreciated and that disruption of the IEL correlates with neointimal formation in large arteries.

METHODS

The authors declare that all supporting data are available within the article and its Online Data Supplement.

Mice and Genotyping

LoxP sites were inserted surrounding the 4th and 29th exons of the *Eln* gene using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat–associated 9) technology (Sage Labs; Figure 1A). Validated Cas9 pairs and donor vectors were microinjected into 1-cell stage embryos from female C57BL6 embryos. Positive founders were identified by polymerase chain reaction genotyping using the following primer pairs: LoxP1F, CAGGCTTGGTTAAGCCACCA; LoxP1R, CCTACCTTTCTGGGGCCACT (LoxP1 knock in, 455 bp; wild type, 415 bp); LoxP2F, CAACTGGTCCCCTTGTCACT; LoxP2R, AGACAGTGTGGGTCTGGCTA (LoxP1 knock in, 583 bp; wild type, 543 bp). The directionality of the LoxP sites was confirmed by subjecting the amplicons to partial digestion with SalI (streptomyces albus) restriction enzyme, with expected band sizes indicated in Figure 1B. Sanger sequencing confirmed correct Cas9/sgRNA-mediated genome editing. A single *Eln*$^{f/+}$ founder was bred to produce an F1 breeding pair and subsequent *Eln*$^{f/f}$ mice.

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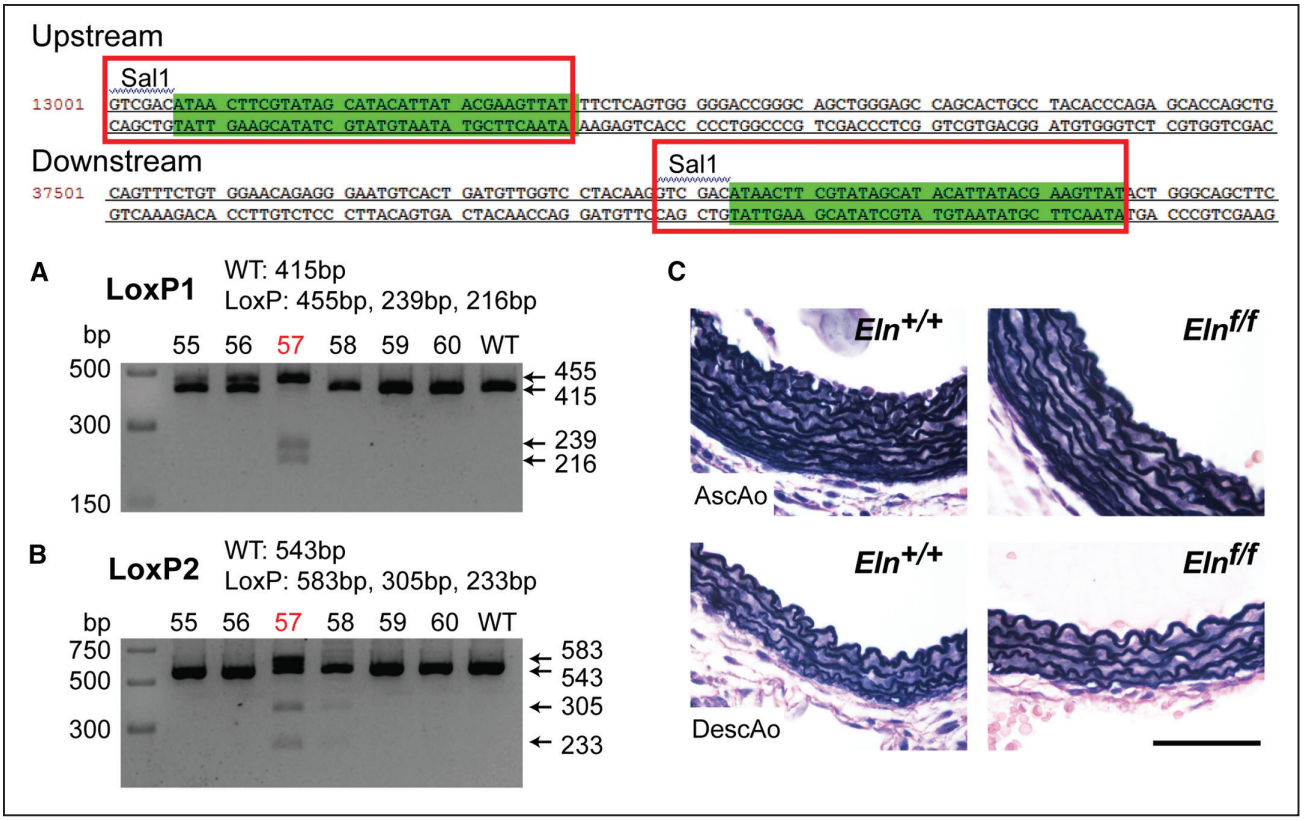

Figure 1. Generation and validation of Eln (elastin) floxed mice.
A, Genomic sequence of *Eln* floxed allele. Red boxes indicate sequences introduced by CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat–associated 9) near exons 4 and 29. Green highlighting indicates LoxP (locus of X-over P1) sequences. **B**, Genomic amplicon partially digested by SalI (streptomyces albus) restriction enzyme indicating presence and correct orientation of upstream and downstream LoxP sites (LoxP1 and LoxP2, respectively). Mouse No. 57 was confirmed and used as the founder for subsequent studies. **C**, Verhoeff-Van Gieson staining of postnatal day (P) 4 $Eln^{+/+}$ and $Eln^{f/f}$ ascending aorta (AscAo) and descending aorta (DescAo). Scale bar=50 μm. WT indicates wild type.

$Eln^{f/f}$ mice were bred to various *Cre* lines and maintained in mixed backgrounds. *Sm22aCre* (Jackson Labs No. 004746),[5,6] *Tie2Cre* (Jackson Labs No. 008863),[7] *Cdh5Cre* (Jackson Labs No. 006137),[8] and $ROSA26^{nT/mG}$ mice (Jackson Labs No. 007576)[9] were described previously. $Eln^{+/-}$ mice were also used.[2,10] Experimental mice were compared with littermate controls. $Eln^{f/f}$ and $Eln^{f/+}$mice were combined as controls in most instances. Males were used for the mechanical testing, as carotid artery diameter varies with sex.[11] Arterial blood pressure, which also varies with sex,[12] was measured in male mice before sacrifice for mechanical testing. Both sexes were used for all other studies. All animal studies were approved by the Institutional Animal Care and Use Committee at Washington University. Genotyping was performed by TransnetYX, Inc. The *Eln* alleles were amplified with the following primers: ElnF, CCATGTGGGTGCTGTAAGCT; ElnR, GCAGTGCTGGCTCCCA; probe 1, wild type, CCTGCCTGAGTTCTCA; probe 2, LoxP floxed, AGGTCGACATAACTTCG. Genotyping for *Cre*, *Sm22aCre*, and $ROSA26^{nT/mG}$ alleles was performed with TransnetYX proprietary primers.

Vascular Casting

Mice were euthanized by CO_2 and their chest wall excised. Blood was drained from the vasculature by injecting PBS through the left ventricular (LV) apex after transecting the left common iliac artery. Yellow latex (Ward's Science) was then injected through the LV.[13] Mice were fixed in 10% neutral buffered formalin (Thermo Fisher Scientific) at 4°C overnight, followed by fine dissection to reveal the aorta and aortic arch arteries.

Echocardiography

Mouse echocardiography was performed at the Washington University Mouse Cardiovascular Phenotyping Core facility using a Vevo 2100 Imaging System (FUJIFILM VisualSonics, Inc) and MS700 or MS550 probes. Postnatal day (P) 5 or 10 mice were weighed, anesthetized with 1% isoflurane, and then placed on a heated stage under a heat lamp. Two-dimensional and M-mode short-axis images of the LV were obtained, as well as 2-dimensional and continuous-wave Doppler flow images of the aortic arch. LV dimensions and aortic arch velocities were obtained using edge detection software and standard techniques.[14]

Blood Pressure and Mechanical Testing

P30 male mice were anesthetized with 1.5% isoflurane, and arterial blood pressure was measured with a solid-state catheter (1.2F; Transonic). Mice were then sacrificed by thoracotomy under anesthesia. Images were taken of the left common carotid artery before and after dissection to determine the in vivo axial stretch ratio. The artery was mounted at its in vivo

axial stretch ratio in a pressure myograph (110P; Danish Myo Technology) in physiological saline solution at 37°C. Arteries were inflated from 0 to 175 mm Hg in steps of 25 mm Hg (12 s/step) while pressure and outer diameter were recorded. The compliance was calculated as the average change in outer diameter for each pressure step.[15]

Protein Quantification

Ascending and descending aortic segments from P8 mice were hydrolyzed in 6 N HCl, dried, and resuspended in ultrapure water. Desmosine—an elastin-specific cross-link—was measured through a competitive ELISA and normalized to total protein as described in the study by Stoilov et al.[16]

Histology and Immunostaining

Harvested tissues were fixed overnight at 4°C in 4% paraformaldehyde in PBS solution (Electron Microscopy Sciences) or 10% neutral buffered formalin (Thermo Fisher Scientific). For frozen sections, fixed samples were embedded in Optimum Cutting Temperature Compound (Sakura Finetek), flash frozen, and sectioned at 5-µm thickness with a cryostat. For paraffin sections, fixed tissue was dehydrated, paraffinized with a tissue processor, embedded, and sectioned at 5 µm thickness using a microtome.

Hematoxylin and eosin staining was performed following standard protocols. Verhoeff staining was performed by first overstaining sections with alcoholic hematoxylin-ferric chloride-iodine, followed by differentiation with ferric chloride and then sodium thiosulfate. Verhoeff-stained slides were counterstained with van Gieson solution for Verhoeff-Van Gieson staining. All histological staining solutions were from American MasterTech.

For immunohistochemistry, paraffin tissue sections were rehydrated, immersed in antigen retrieval solution (10 mmol/L Tris, 1 mmol/L EDTA, pH 9), and then heated for 10 minutes in a pressure cooker. After cooling down to room temperature, the slides were blocked with 0.3% hydrogen peroxide and the mouse on mouse (MOM) blocking reagent (BMK-2202; Vector Labs), followed by incubation with the primary antibody for 1 hour at room temperature in the M.O.M. diluent. The slides were washed with PBS and incubated with biotin-conjugated secondary antibodies in the M.O.M. diluent. Visualization was accomplished using streptavidin-based ABC Kits (Vector Labs) following the manufacturer's instructions. Nuclei were counterstained with hematoxylin. Stained slides were dehydrated, cleared, proliferating cell nuclear antigen, and mounted with Cytoseal 60 (Thermo Fisher Scientific) or VectaMount Permanent (Vector Labs). Primary antibodies used include mouse anti–α-SMA (α-smooth muscle actin; 1A4, No. A2547; Sigma-Aldrich), mouse anti-PCNA (proliferating cell nuclear antigen; PC10, No. 2586; Cell Signaling Technology), rabbit anti-Myh11 (myosin heavy chain 11; JA03-35, No. NBP2-66967; Novus Biologicals), rabbit anti-calponin (SI67-01, No. NBP2-67401; Novus Biologicals), and rabbit anti-Sm22a (smooth muscle 22-alpha; ab155272; Abcam). Secondary antibodies used include biotinylated anti-mouse (M.O.M. kit) and biotinylated goat anti-rabbit antibody (No. 14708; Cell Signaling). Concentration of 1:200 was used for primary antibodies and 1:400 for secondary antibodies. Secondary antibody-only staining controls of ascending aorta slides were used for each antibody to ensure the specificity of staining. For fluorescence imaging, nuclei were counterstained with Hoechst 34580 (5 µmol/L; Life Technologies) or DAPI (4′,6-diamidino-2-phenylindole; Sigma-Aldrich). Elastin autofluorescence was obtained by illuminating the slides with a light-emitting diode bulb and capturing the image under a dark field.

Transmission Electron Microscopy

Arteries used for transmission electron microscopy were fixed in 2.5% glutaraldehyde and 0.1 M sodium cacodylate (both from Electron Microscopy Sciences) at 4°C overnight. They were sent to Washington University Center for Cellular Imaging for processing and thin sectioning following standard protocols. Images were taken using a JEOL JEM-1400Plus transmission electron microscope with an Advanced Microscopy Techniques XR111 high-speed, 4000×2000-pixel, phosphor-scintillated, 12-bit charge-coupled device camera.

Statistics and Animal Numbers

For survival analysis, the day of death was defined as the day the mouse was found dead in the cage. In some instances when no carcass was found, the day of death was defined as the midpoint since the day the mouse was last seen. Those mice euthanized for experiments were censored. Log-rank survival analysis was performed using RStudio, version 1.1.463 (RStudio, Inc, Boston, MA) and its survdiff function in the survival package, with default settings and significance level set at $P<0.05$. Kaplan-Meier curves were produced using Prism 8 (GraphPad Software, San Diego, CA). One hundred sixty-one total mice were used for the survival analysis, of which 22 were controls, 39 *SM22aCre;Eln*$^{f/+}$, and 84 *SM22aCre;Eln*$^{f/f}$. For gross phenotyping, ≈10 mice per group were examined. For echocardiography, 1 *SM22aCre;Eln*$^{f/f}$ and 1 control were imaged at P5 and 1 *SM22aCre;Eln*$^{f/f}$ and 3 controls were imaged at P10. For histological analyses, images shown are representative of 4 to 6 mice examined in each group. For transmission electron microscopy analysis, images shown are representative of 2 to 3 mice examined in each group. A priori power calculations were utilized to estimate group sizes for quantitative data using estimates of mean differences and variability from our previously published work on elastin insufficient mice[3,15,17] and assuming a normal distribution. The D'Agostino-Pearson omnibus normality test was used to confirm normality of the mechanical testing data. All other quantitative datasets were too small for formal normality tests. Four to 5 arteries per group were analyzed for quantification of elastic layers and protein amounts. Two-way ANOVA with Sidak multiple comparisons test was used to determine significant differences between genotypes for each artery type. For the blood pressure and mechanical testing, 6 to 8 male mice were used for each group. One-way ANOVA with Sidak multiple comparisons test was used to determine significant differences for experimental genotypes compared with *Eln*$^{+/+}$. For proliferation analysis, 3 to 4 mice were used for each group. An image of the PCNA staining was captured from a representative section from each ascending aorta. Quantification was done by counting the total number of PCNA-positive cells in tunica media and neointima (in the case of *SM22aCre;Eln*$^{f/f}$) divided by the number of total cells in the area. One-way ANOVA with Sidak multiple comparisons test was used to determine significant differences for experimental genotypes compared with control. Prism 8 (Graphpad Software) was used for statistical analysis, and significance was set to $P<0.05$. Researchers were blinded to animal genotypes during experimentation and analyses.

RESULTS

Floxed Eln Allele Has No Adverse Effect on Aortic Wall Structure

CRISPR/Cas9 technology was used to generate an *Eln*^{f} allele. LoxP sites were inserted near the 4th and 29th exons of *Eln*, with SalI sites inserted adjacent to both LoxP sites to facilitate genotyping (Figure 1A). Sanger sequencing confirmed correct Cas9/sgRNA-mediated genome editing (data not shown). Hemizygous offspring of a founder with LoxP sites in the correct location on the same chromosome (Figure 1B) was bred to produce mice homozygous for the *Eln*^{f} allele (*Eln*$^{f/f}$). *Eln*$^{f/f}$ mice have no discernable gross phenotype and live a normal life span. Histological analysis of the ascending and descending aorta showed normal morphology with the appropriate number of elastic layers (Figure 1C). These results confirm insertion of the LoxP sites in the *Eln* gene and show that mice homozygous for the floxed elastin allele have typical vascular wall structure.

Mice Heterozygous for SMC-Specific Deletion of Eln Show Cardiovascular Remodeling Similar to Eln$^{+/-}$ Mice but Have Normal Blood Pressure

To inactivate elastin expression in SMCs, we crossed *Eln*^{f} mice with mice bearing a transgenic *Sm22aCre* allele.[5,6] *Sm22aCre;Eln*$^{f/+}$ mice were grossly indistinguishable from *Eln*$^{f/f}$ littermates, were fertile, and showed no premature death. Similar to mice bearing a global knockout allele (*Eln*$^{+/-}$),[10] the ascending and descending aorta in *Sm22aCre;Eln*$^{f/+}$ mice had a slightly thicker tunica media and more circumferential elastic laminae than control littermates (Figure 2A and 2B). *Sm22aCre;Eln*$^{f/+}$ mice also had reduced diameter and altered compliance in the carotid artery, which is in agreement with arterial changes in *Eln*$^{+/-}$ mice[18] (Figure 2C). Unlike *Eln*$^{+/-}$ mice,[19] P30 *Sm22aCre;Eln*$^{f/+}$ mice did not show systolic hypertension (Figure 2D).

Homozygous Deletion of Eln in SMCs Results in Extensive Heart and Vascular Changes

Mice with SMC-specific *Eln* deletion (*Sm22aCre;Eln*$^{f/f}$) were born at the expected Mendelian ratio and were initially similar in appearance to their littermates. By P7 to P8, however, *Sm22aCre;Eln*$^{f/f}$ mice were appreciably smaller than control mice (Figure 3A) and most died between P9 to P18 (Figure 3B). Unlike the relatively mild cardiovascular phenotype seen in *Sm22aCre;Eln*$^{f/+}$mice, *Sm22aCre;Eln*$^{f/f}$ mice exhibited a grossly lengthened and thickened ascending aorta, coarctation of the aortic arch, and severe tortuosity of the carotid arteries with 100% penetrance. Their descending aorta, in contrast, was neither tortuous nor aneurysmal (Figure 3C). Echocardiography of P5 and P10 mice confirmed coarctation of the aortic arch with greatly increased peak flow velocity (peak pressure gradient, ≈53 mm Hg, consistent with moderate-to-severe stenosis; Figure 3D). By P5 to P10, *Sm22aCre;Eln*$^{f/f}$ mice exhibited cardiomegaly (Figure 3E) owing to a dilated, eccentrically hypertrophic LV with compromised fractional shortening (Figure 3F). Histologically, the *Sm22aCre;Eln*$^{f/f}$ heart exhibited chronic thrombosis, necrosis, and calcification in the left atrium and ventricle (Online Figure IA and IB) at P9 and P18. By contrast, these histopathologic changes were absent in P2 *Sm22aCre;Eln*$^{f/f}$ hearts (Online Figure IC), suggesting the cardiomyopathy was likely secondary to pressure overload by aortic coarctation.

Both the ascending and descending aorta in *Sm22aCre;Eln*$^{f/f}$ mice (Figure 4A) showed a markedly thickened tunica media with SMCs in the descending aorta maintaining their circumferential orientation in the absence of elastin, as did SMCs in the outer part of the ascending aorta. SMCs near the lumen of the ascending aorta, however, showed an axial or random orientation. There were no intact sheets of elastic fibers in the media of either artery, which was instead collagen laden, as demonstrated by red Verhoeff-Van Gieson staining. There were, however, random patches of elastin usually located in the outer half of the media near the adventitia. Amounts of desmosine—an elastin-specific cross-link—in *Sm22aCre;Eln*$^{f/f}$ ascending and descending aorta were 20% and 30% of controls, respectively (Figure 4B). Elastic fibers within the patches near the outer media were thin and ultrastructurally fragmented (Figure 4C). Lineage tracing with the *ROSA26*$^{nT/mG}$ reporter mouse confirmed *Sm22aCre* recombination in areas of the media where elastin was absent and no recombination where elastin was present (Figure 4D). However, cells throughout the *Sm22aCre;Eln*$^{f/f}$ ascending aorta media stained positively for *SM22a* regardless of the presence or absence of elastin (Figure 4E).

IEL Is Retained in Both SMC- and EC-Specific Eln Knockouts

An unexpected finding in *Sm22aCre;Eln*$^{f/f}$ mice was the persistence of the IEL, which suggests that ECs contribute to, or solely synthesize, this layer of elastin. The relative contribution of ECs to IEL formation was determined by targeting the floxed elastin gene in ECs using *Tie2Cre*. *Tie2Cre;Eln*$^{f/f}$ mice were fertile, grossly similar to control mice, and exhibited no early mortality (tracked ≤10 months of age). Histological analysis showed that the IEL and medial lamellae formed normally in *Tie2Cre;Eln*$^{f/f}$ ascending and descending aorta (Figure 5A and 5B). Lineage tracing with the *ROSA26*$^{nT/mG}$ reporter mouse confirmed recombination in ECs in *Tie2Cre;Eln*$^{f/f}$ mice (Figure 5C). Using the EC-specific cadherin gene (*Cdh-5Cre*) to inactivate *Eln* gave similar results (data not shown).

We used transmission electron microscopy to compare IEL ultrastructure in the ascending and descending aorta. The IEL appeared normal in the ascending and descending

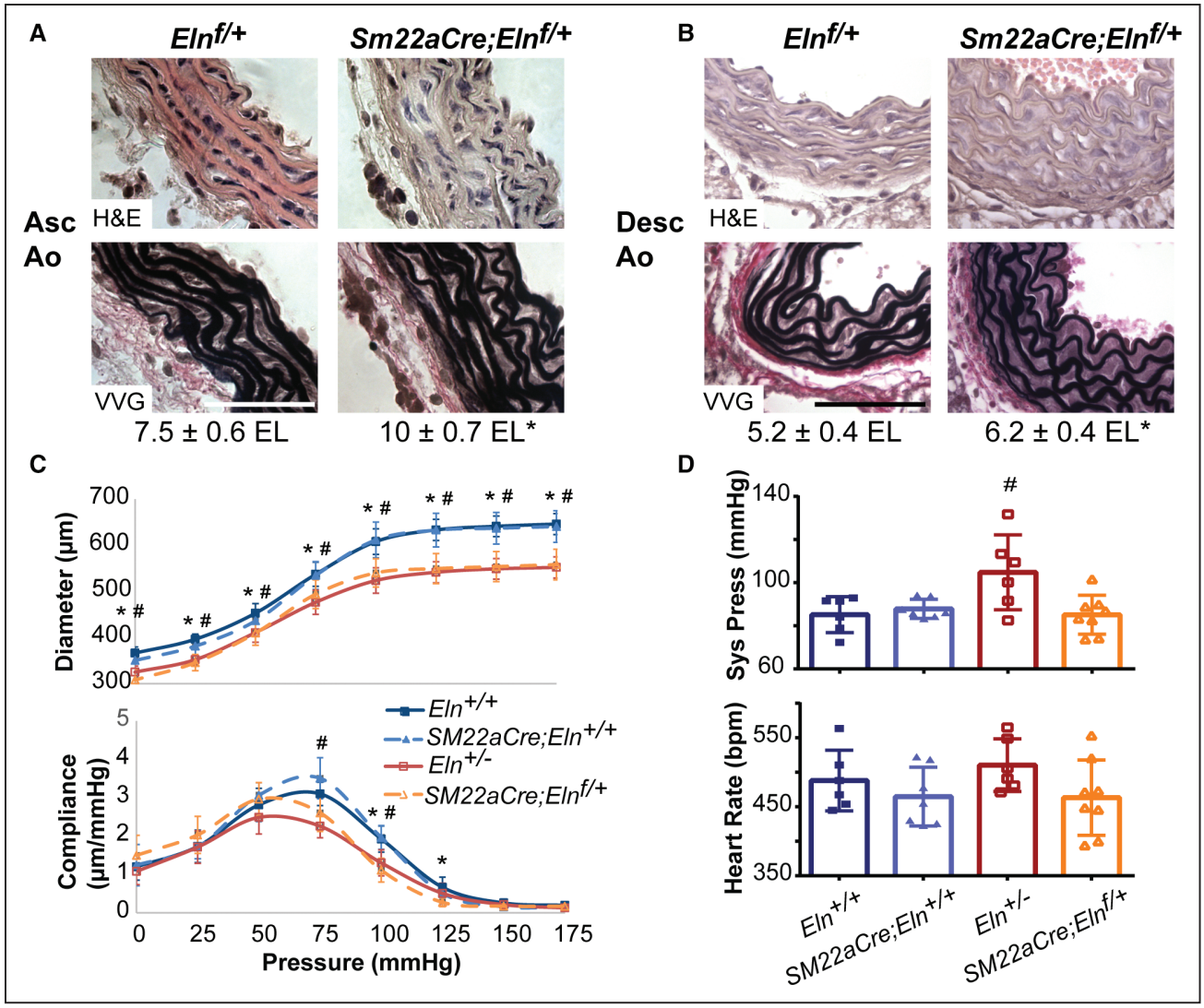

Figure 2. Mice heterozygous for smooth muscle cell (SMC)–specific deletion of Eln (elastin) show cardiovascular remodeling similar to Eln+/– mice but have normal blood pressure.

A, Hematoxylin and eosin (H&E) and Verhoeff-Van Gieson (VVG) staining of postnatal day (P) 18 ascending and (**B**) P12 descending aorta shows the elastic laminae structure. Scale bar=50 μm. Average number of elastic laminae (EL) are shown below the images. *P<0.05 between genotypes for each artery by 2-way ANOVA with Sidak multiple comparisons test. n=4 to 5/group. **C**, Reduced arterial diameter (**top**) and compliance (**bottom**) in P30 male *Sm22aCre;Eln$^{f/+}$* common carotid artery is similar to *Eln$^{+/-}$*. n=8/group. **D**, Unlike *Eln$^{+/-}$* mice, *Sm22aCre;Eln$^{f/+}$* mice do not have increased systolic (Sys) blood pressure. Heart rate is similar across groups. n=6 to 8/group. For **C** and **D**, * and # indicate P<0.05 compared with *Eln$^{+/+}$* for *Sm22aCre;Eln$^{f/+}$*and *Eln$^{+/-}$*, respectively, by 1-way ANOVA with Sidak multiple comparisons test. *Sm22aCre;Eln$^{+/+}$* was not significantly different from *Eln$^{+/+}$*. Mean±SD. AscAo indicates ascending aorta; DescAo, descending aorta; and Sys, systolic.

aorta of *Eln$^{f/f}$* mice (Figure 6A and 6C), confirming observations from light microscopy (Figure 1C) that insertion of the LoxP sites does not affect elastin integrity. Consistent with the light microscopy images in Figure 4A, the IEL in the ascending aorta of *Sm22aCre;Eln$^{f/f}$* mice was disrupted (Figure 6A and 6B), whereas the IEL in the descending aorta was normal (Figure 6C). The IEL in the ascending aorta of *Sm22aCre;Eln$^{f/f}$* mice frequently consisted of small aggregates of elastin that never fused to form a continuous elastin layer (Figure 6A). Where the IEL was more intact, the elastin had a moth-eaten appearance when viewed at higher magnification (Figure 6B). In contrast, the IEL in the ascending aorta appeared ultrastructurally normal when elastin was inactivated in ECs (*Tie2Cre;Eln$^{f/f}$*; Figure 6D). Together, the results from the SMC- and EC-specific *Eln* knockout studies suggest that ECs are capable of contributing elastin to the IEL but require a contribution from SMCs in the ascending aorta to produce an intact structure. SMCs in the ascending and descending aorta, in contrast, can produce an intact IEL with no elastin contribution from ECs.

ECs Produce the IEL in Resistance Arteries

A characteristic of muscular and resistance arteries is the absence of elastic laminae between the medial SMC

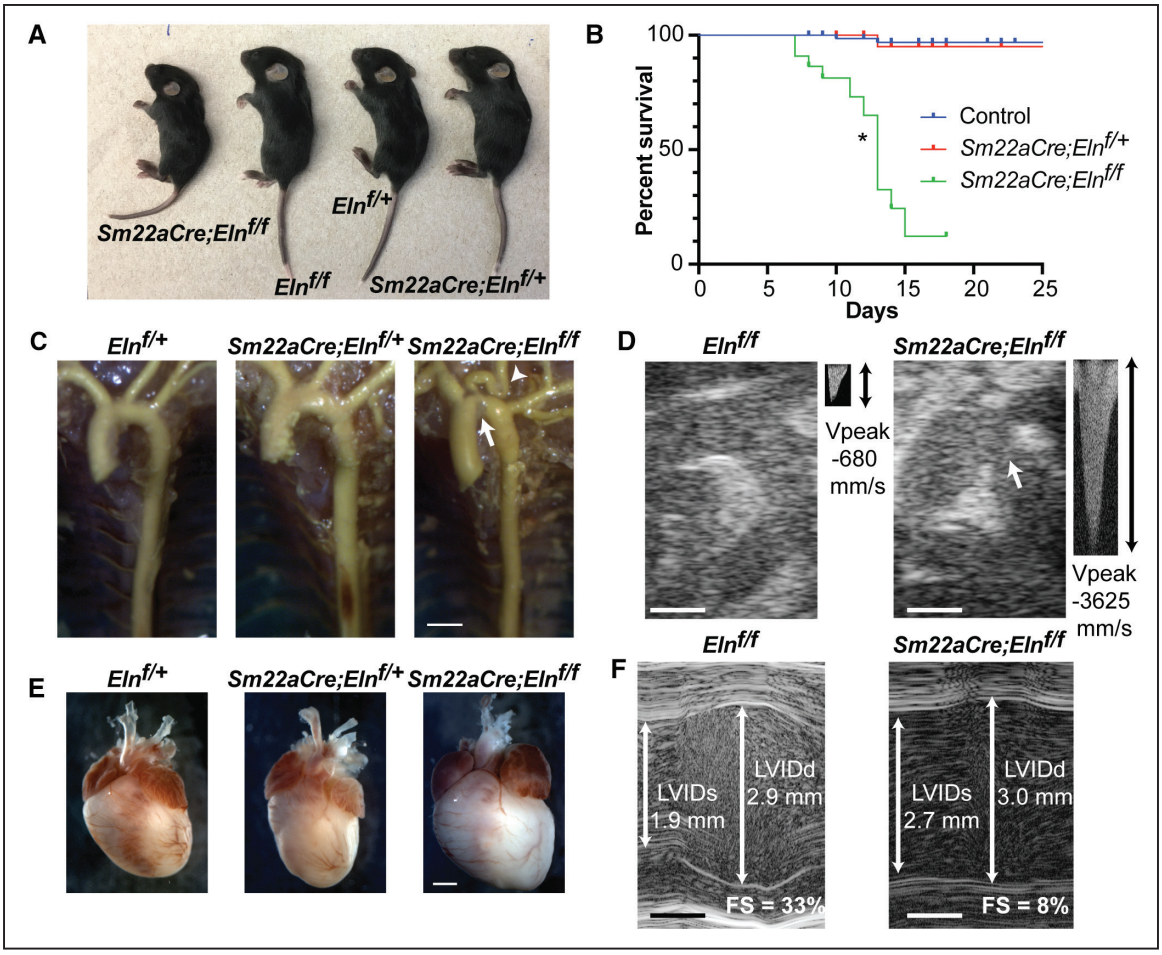

Figure 3. Homozygous deletion of Eln (elastin) in smooth muscle cells results in extensive cardiac and vascular changes. **A**, Pictures of members from a postnatal day (P) 14 litter with genotypes indicated. **B**, Kaplan-Meier curves showing premature death of *Sm22aCre;Eln$^{f/f}$* mice. Control indicates *Eln$^{f/+}$* and *Eln$^{f/f}$* combined. n=161 mice in total. $P=6\times10^{-16}$ (log-rank test) for *Sm22aCre;Eln$^{f/f}$* compared with control (*). **C**, Latex angiogram demonstrating elongated ascending aorta, coarctation of the aortic arch (arrow), tortuous carotid arteries (arrowhead), and straight descending aorta in P6 *Sm22aCre;Eln$^{f/f}$* mice. Scale bar=1 mm. **D**, Two-dimensional echocardiograms of ascending aorta and arch. Continuous-wave Doppler blood flow velocity was measured in the arch (small panels). Coarctation is visible in the *Sm22aCre;Eln$^{f/f}$* P10 aortic arch (arrow) leading to an increase in peak blood flow velocity (Vpeak). Scale bars=0.5 mm. **E**, Whole mount picture demonstrating cardiomegaly in P9 *Sm22aCre;Eln$^{f/f}$* mice. Scale bar=2 mm. **F**, M-mode echocardiograms showing representative measures of the left ventricular inner diameter at systole (LVIDs) and left ventricular inner diameter at diastole (LVIDd) and the associated fractional shortening (FS) in P10 mice. Scale bars=0.5 mm.

layers. These arteries do, however, have a well-defined IEL and, in many cases, a complete or partial EEL that separates the media from the adventitia. To see how the loss of cell type–specific elastin expression influences resistance artery structure, we studied second-order mesenterics. *Eln$^{f/f}$* mesenterics have an intact IEL and a thin discontinuous EEL in light and electron microscopy (Figure 7A). *Sm22aCre;Eln$^{f/f}$* mesenterics have an intact IEL, but the EEL was not detectable (Figure 7B), consistent with SMCs making elastin in the outer regions of the wall. When *Eln* was inactivated in ECs (*Tie2Cre;Eln$^{f/f}$* and *Cdh5Cre;Eln$^{f/f}$*), however, the IEL was severely disrupted and the EEL appeared normal or sometimes thicker (Figure 7C and 7D). These results suggest that SMCs in the second-order mesenteric arteries make small amounts of elastin that contribute partially to the IEL and solely to the EEL but cannot increase elastin levels enough to compensate for the absence of elastin production from ECs in the IEL.

Based on our findings of heterogeneous cellular contributions to elastin deposition in the ascending aorta, descending aorta, and second-order mesenteric arteries, we performed a histological survey of different arterial beds in mice with SMC- and EC-specific Eln deletion. The aortic root in *Sm22aCre;Eln$^{f/f}$* mice had a similar wall structure to the ascending aorta, with a fragmented IEL, neointimal formation, and patches of elastin in the external medial layer (Online Figure II). Medium-sized elastic and muscular/resistance arteries in *Sm22aCre;Eln$^{f/f}$* mice showed wall structures that were similar to the descending aorta; the arterial wall was generally thicker than controls, and all arteries had an intact IEL even

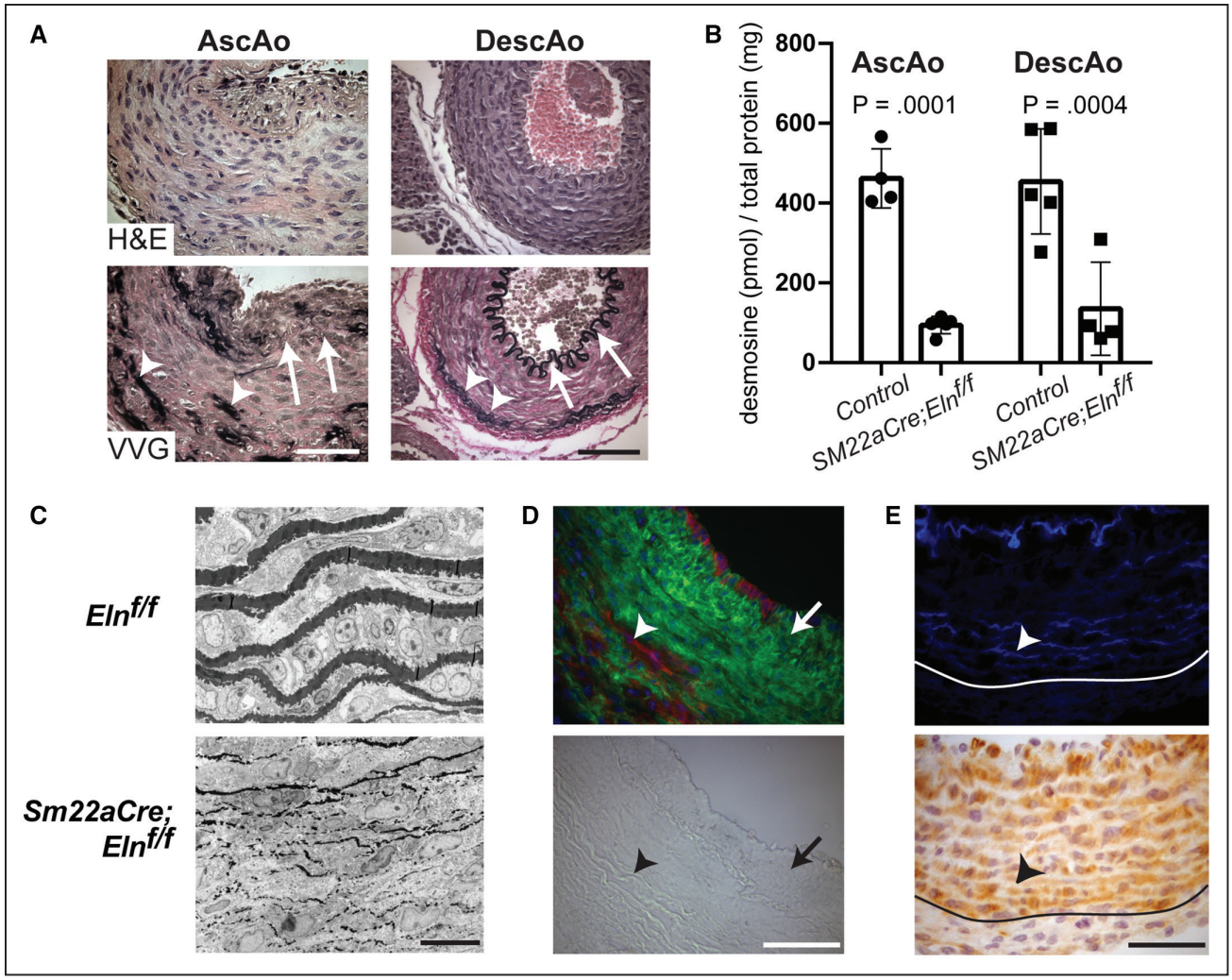

Figure 4. Analysis of the aorta in mice with homozygous deletion of Eln (elastin) in smooth muscle cells.
A, Hematoxylin and eosin (H&E) and Verhoeff-Van Gieson (VVG) staining of postnatal day (P) 18 ascending aorta (AscAo; **left**) and P12 descending aorta (DescAo; **right**) shows the elastic laminae structure. *Sm22aCre;Eln*$^{f/f}$ AscAo has a fragmented internal elastic lamina (IEL), whereas the DescAo has an intact IEL (arrows). Both arteries have patches of Eln (arrowheads) in the outer half of the media near the adventitia. Littermate control images are shown in Figure 2A and 2B. Scale bars=50 μm. **B**, Desmosine levels were expressed as a ratio of total protein in the AscAo and DescAo. Control is *Eln*$^{f/f}$ and *Eln*$^{f/+}$ combined. *P* values were determined between genotypes for each artery by 2-way ANOVA with Sidak multiple comparisons test. **C**, Electron microscopy images of the outer media show that the patches of Eln present in *Sm22aCre;Eln*$^{f/f}$ AscAo are thin and fragmented. Scale bar=10 μm. **D**, Lineage tracing with the *ROSA26*$^{nT/mG}$ reporter mouse demonstrates *Sm22aCre*-mediated recombination (green) in the media corresponding with the absence of Eln, as well as in the neointima (arrow). There are also cells in outer regions of the wall near the patches of Eln that do not show *Sm22aCre*-mediated recombination (red, arrowhead). Bright-field image of the same field is shown below for comparison. Scale bar=50 μm. **E**, Patches of Eln are shown with autofluorescence in P2 *Sm22aCre;Eln*$^{f/f}$ AscAo (arrowhead, **top**). The medial cells stain positive for SM22a protein even if they are near the patches of Eln associated with nonrecombination (arrowhead, **bottom**). The border between the media and adventitia is indicated. Scale bar=50 μm.

though medial elastic lamellae were missing (Online Figure III). When *Eln* was inactivated in ECs (*Tie2Cre;Eln*$^{f/f}$), the IEL, medial elastic fibers, and overall wall structure were indistinguishable at the histological level from *Eln*$^{f/f}$ controls in the medium-sized elastic arteries, but the IEL was less prominent and appeared fragmented in the muscular/resistance arteries (Online Figure IV). The abdominal aorta, carotid artery, subclavian artery, iliac artery, and internal thoracic artery in *Tie2Cre;Eln*$^{f/f}$ mice had a normal IEL (Online Figure IVA through IVE), whereas the femoral artery, renal artery, and inferior epigastric artery had a thin or fragmented IEL (Online Figure IVF through IVH).

Neointima Formation in *Sm22aCre;Eln*$^{f/f}$ Ascending Aorta Correlates With IEL Disruptions

Histological sections of *Sm22aCre;Eln*$^{f/f}$ ascending aorta frequently showed cells luminal to the IEL in areas where the IEL was absent or severely disrupted. Cells in this neointima express SMC markers including α-SMA, calponin,

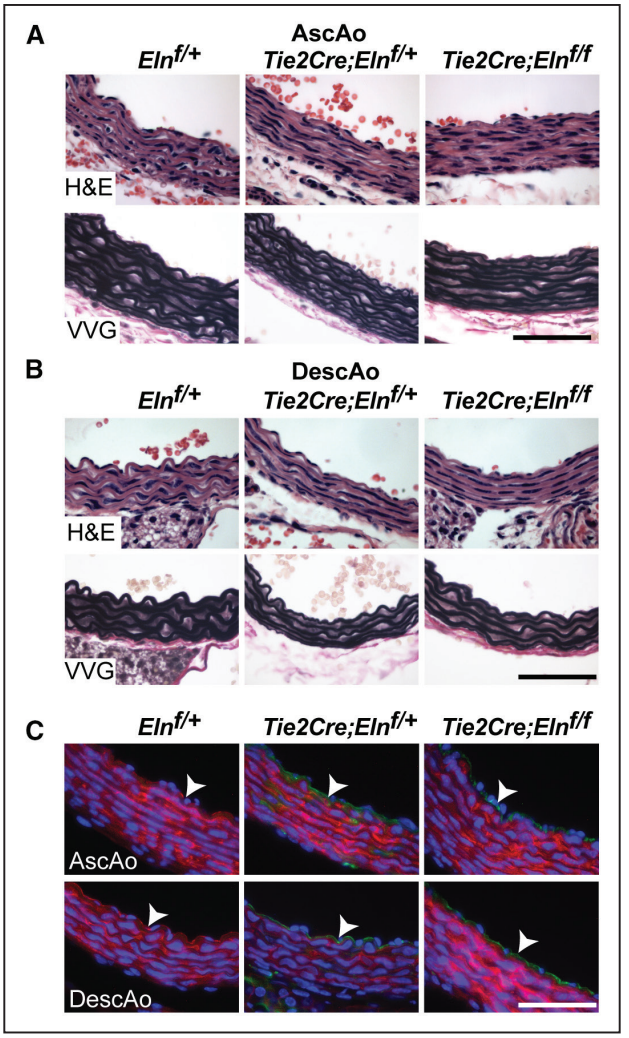

Figure 5. The internal elastic lamina (IEL) appears normal in the aorta despite endothelial cell (EC)–specific Eln (elastin) deletion.
Hematoxylin and eosin (H&E) and Verhoeff-Van Gieson (VVG) staining of postnatal day 10 ascending aorta (AscAo; **A**) and descending aorta (DescAo; **B**) shows normal IEL and medial lamellae formation despite EC-specific *Eln* deletion. **C**, Lineage tracing with the *ROSA26*$^{mT/mG}$ reporter mouse confirms green (recombined) ECs in *Eln*$^{f/+}$ and *Eln*$^{f/f}$ mice expressing *Tie2Cre* and red (not recombined) ECs in *Eln*$^{f/+}$ controls (arrowheads). All scale bars=50 μm.

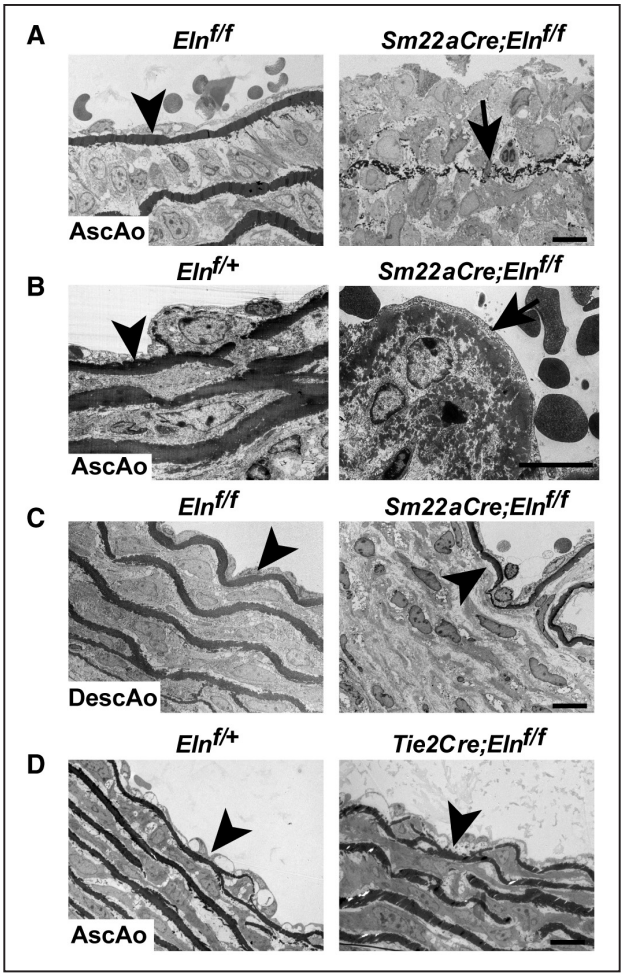

Figure 6. Electron microscopy of the internal elastic lamina (IEL) in smooth muscle cell–specific and endothelial cell–specific Eln (elastin) knockouts.
A, The IEL in the ascending aorta (AscAo) of postnatal day (P) 8 *Eln*$^{f/f}$ mice is intact (arrowhead), whereas in some regions, it is composed of small aggregates of Eln that never fused to form a continuous barrier in *Sm22aCre;Eln*$^{f/f}$ mice (arrow). **B**, In regions where the IEL is thicker and more intact in *Sm22aCre;Eln*$^{f/f}$ AscAo, it has a moth-eaten appearance (arrow). **C**, In contrast, the descending aorta (DescAo) in P8 *Eln*$^{f/f}$ and *Sm22aCre;Eln*$^{f/f}$ mice has an intact IEL (arrowheads). **D**, The IEL is also intact in the AscAo of P10 *Eln*$^{f/+}$ and *Tie2Cre;Eln*$^{f/f}$ mice (arrowheads). All scale bars=10 μm.

and smooth muscle myosin heavy chain 11, suggesting a smooth muscle identity (Figure 8A). Lineage tracing using the *ROSA26*$^{mT/mG}$ reporter allele confirmed that the neointimal cells were derived from the *Sm22aCre* lineage (Figure 4D). PCNA staining showed that the percentage of proliferating cells in the neointima was not different from the portion of proliferating cells in the media of control ascending aorta (Figure 8B), suggesting that neointimal cells arise by a mechanism other than uncontrolled proliferation. Areas of neointimal hyperplasia correlated with local IEL disruption (Figure 8C). These findings show that the IEL functions as a physical barrier to prevent medial SMCs from migrating into the arterial lumen and forming a neointima in the ascending aorta.

DISCUSSION

In our previous studies, we identified how global inactivation of one or both elastin alleles influences arterial development and cardiovascular function in mice.[3,15,20] In this report, we use SMC- and EC-targeted *Cre* drivers to inactive elastin expression in those 2 vascular cell populations. While there are several characteristics shared between the global and targeted knockouts, being able to specifically limit elastin production to a particular cell type has provided new information about how SMCs and ECs contribute elastin to the arterial wall and how defects in local elastin deposition may lead to cardiovascular disease.

The amount of elastin has a significant influence on arterial wall structure and function. With normal elastin

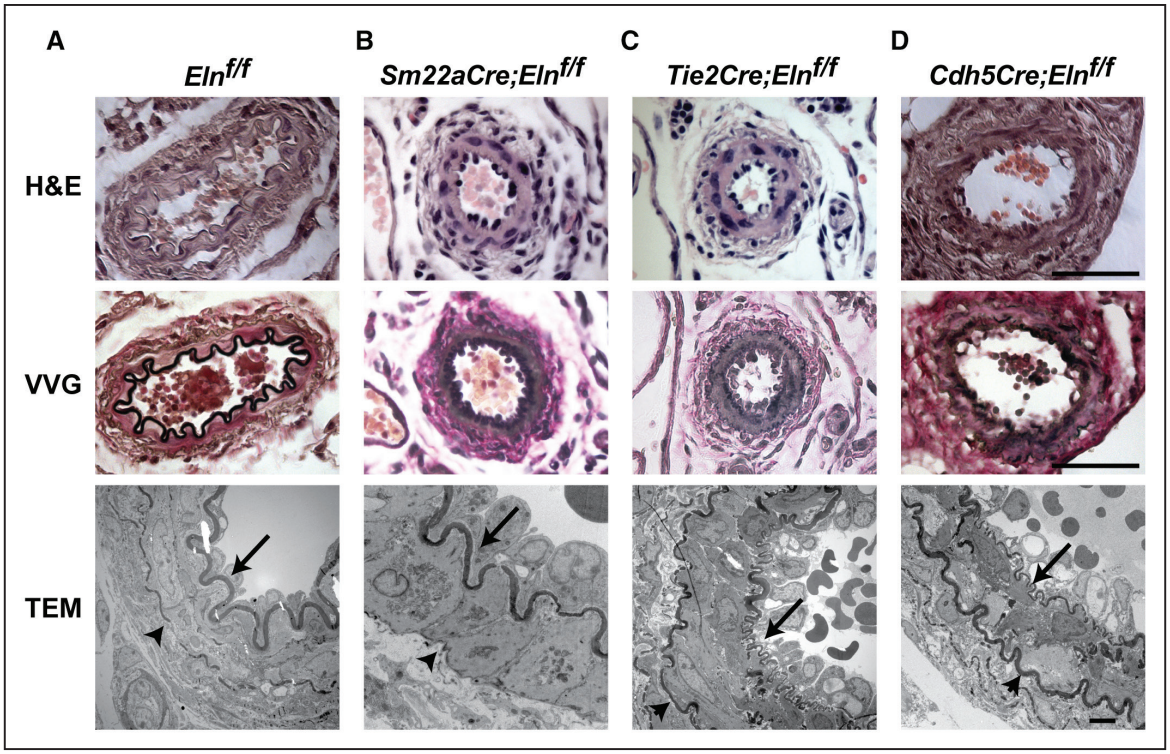

Figure 7. Endothelial cells produce the internal elastic lamina (IEL) in resistance arteries.
Hematoxylin and eosin (H&E; **top**), Verhoeff-Van Gieson (VVG; **middle**), and transmission electron microscopy (TEM; **bottom**) images of second-order mesenteric arteries from (**A**) postnatal day (P) 13 *Eln*$^{f/f}$, (**B**) P8 *Sm22aCre;Eln*$^{f/f}$, (**C**) P10 *Tie2Cre;Eln*$^{f/f}$, and (**D**) P13 *Cdh5Cre;Eln*$^{f/f}$ mice. *Eln*$^{f/f}$ and *Sm22aCre;Eln*$^{f/f}$ arteries have a prominent, intact IEL (**A** and **B**, arrows). *Eln*$^{f/f}$ arteries have a thin, discontinuous external elastic lamina (EEL; **A**, arrowhead), whereas the EEL in *Sm22aCre;Eln*$^{f/f}$ arteries is undetectable (**B**, arrowhead at expected location). In contrast, *Tie2Cre;Eln*$^{f/f}$ and *Cdh5Cre;Eln*$^{f/f}$ arteries have a thin, fragmented IEL (**C** and **D**, arrows) and a normal or slightly thicker EEL (**C** and **D**, arrowheads). Scale bars=50 μm for H&E and VVG and 2 μm for TEM.

levels, the number of SMC layers that are present before the onset of elastin production remains the same throughout the lifetime of the organism, including humans.[21] However, in response to loss-of-function mutations that reduce elastin levels (eg, to 50% in *Eln*$^{+/-}$ mice), additional SMCs are recruited from the adventitia to the media right before birth to form more elastic laminae and normalize wall stress.[10,20] Humans with supravalvular aortic stenosis caused by genetic mutations that lead to functional elastin haploinsufficiency show a similar increase in arterial SMC layers and elastic laminae.[10] Mice heterozygous for the SMC-specific *Eln* deletion show arterial wall changes remarkably similar to what occurs in *Eln*$^{+/-}$ mice, including recruitment of additional SMC layers and changes in arterial compliance, implying that the phenotype of *Eln*$^{+/-}$ mice is largely driven by reduced elastin amounts in the medial layer. Despite the structural and mechanical similarities in the arterial wall, one major difference is the lack of increased systolic blood pressure in P30 *Sm22aCre;Eln*$^{f/+}$ mice compared with *Eln*$^{+/-}$. Hypertension becomes more significant as *Eln*$^{+/-}$ mice mature,[19] and scales with elastin amounts in other elastin-deficient mouse models,[17] so it is possible that *Sm22aCre;Eln*$^{f/+}$ may have a delayed hypertensive phenotype because of changes in elastin amounts in the SMC versus global heterozygote. It is also possible that phenotype discrepancies are due to differences in the resistance arteries[22] or renin-angiotensin system signaling[18] between *Sm22aCre;Eln*$^{f/+}$ and *Eln*$^{+/-}$ mice, which were not investigated in the current study.

The arterial remodeling similarities mostly disappear when elastin inactivation in SMCs (*Sm22aCre;Eln*$^{f/f}$) is compared with the global knockout (*Eln*$^{-/-}$). A major difference is that *Sm22aCre;Eln*$^{f/f}$ mice live longer than global knockouts (10–15 days compared with 48 hours, respectively), which allows for a lengthier evaluation of how arterial wall development proceeds without elastin. *Eln*$^{-/-}$ mice die when SMCs in the aortic media reorient, proliferate, and move into the lumen where they obstruct blood flow.[2] This is not the case when elastin is inactivated only in SMCs. SMCs in the descending aorta of *Sm22aCre;Eln*$^{f/f}$ mice remain in the media, most likely because of an intact IEL made by ECs. Also, the characteristic tortuosity in the *Eln*$^{-/-}$ descending aorta[15] is absent in *Sm22aCre;Eln*$^{f/f}$ mice. The lack of tortuosity in the descending aorta suggests a tempered mechanical remodeling response,[23] likely a result of the small amount of elastin present in *Sm22aCre;Eln*$^{f/f}$ arteries compared with *Eln*$^{-/-}$. *Sm22aCre;Eln*$^{f/f}$ mice have approximately the same amount of arterial elastin as *mEln*$^{-/-}$*hELN*$^{+/+}$ mice that express human elastin in the mouse knockout background.[17] *mEln*$^{-/-}$*hELN*$^{+/+}$ mice have

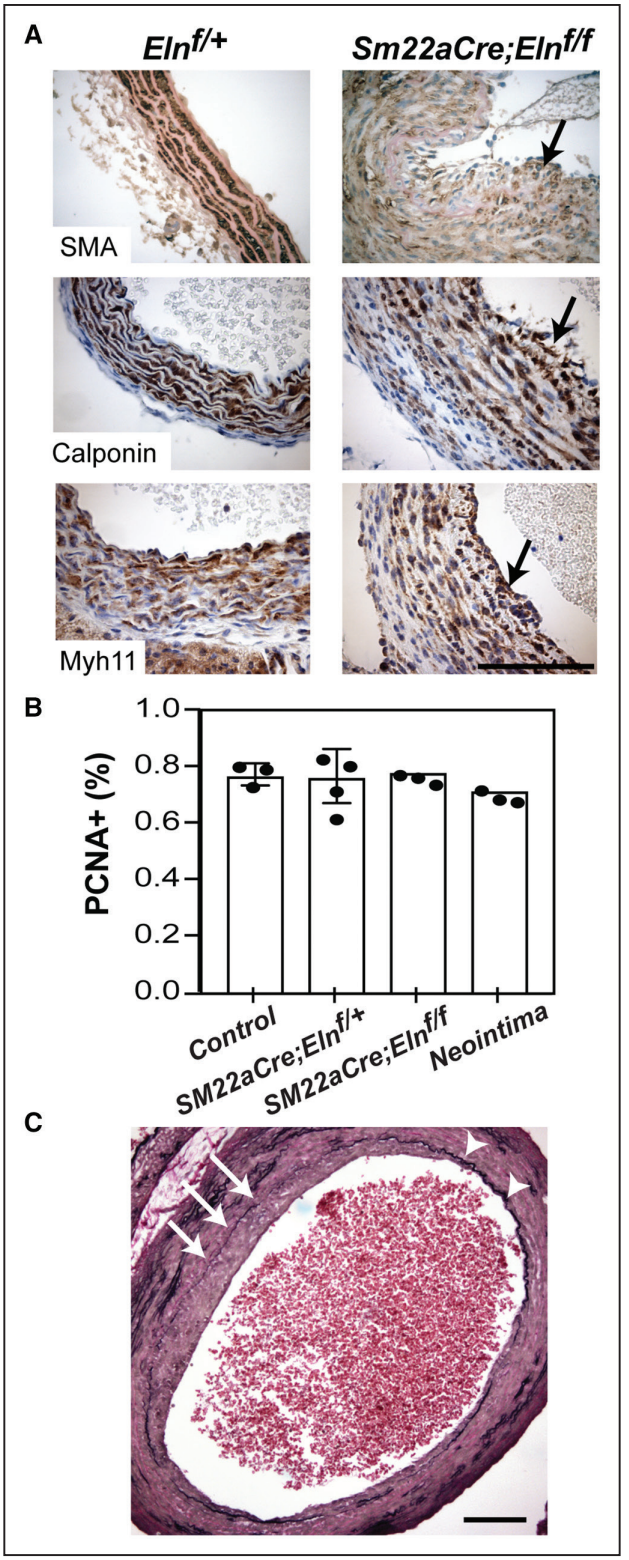

Figure 8. Neointima formation in Sm22aCre;Elnf/f ascending aorta.

A, Immunostaining revealed smooth muscle cell marker–positive neointima in the ascending aorta of *Sm22aCre;Eln$^{f/f}$* mice. Neointimal cells are positive for α-SMA (α-smooth muscle actin; postnatal day [P] 18; **top**), calponin (P9, **middle**), and myosin heavy chain 11 (P9, Myh11; **bottom**; arrows). Scale bar=50 μm. (*Continued*)

hypertension and narrow arteries but do not have focal aortic stenoses or a reduced life span,[17,24] indicating that elastin organization throughout the aortic wall must be considered in addition to elastin amounts. The cause of death for *Sm22aCre;Eln$^{f/f}$* mice is not clear, but the cardiomegaly, thrombosis, calcification, and necrosis found in the heart suggest that cardiomyopathy may be a factor. As the cardiac histopathology did not manifest by P2, it is likely that the cardiomyopathy was secondary to pressure overload caused by aortic coarctation. The fact that *Eln* SMC knockout mice live for ≈2 weeks indicates that medial elastin is not required for early postnatal survival.

The IEL is unique among elastic laminae in that it lies adjacent to the basal lamina of the endothelium, forming a common boundary between ECs in the intima and SMCs in the media. Because SMCs are the major elastin-producing cells in the arterial wall,[25–27] the assumption has been that they are responsible for producing the IEL. However, the presence of an IEL when elastin production is inactivated in SMCs indicates that ECs are capable of contributing elastin to this first elastic layer. The question of whether ECs contribute some, or all, of the elastin to the IEL was investigated by inactivating *Eln* in ECs using either *Tie2*– or *Cdh5Cre*. In both mouse lines, an IEL was present in the elastic arteries but was barely detectable or highly fragmented in the muscular/resistance arteries. Together, data from the SMC and EC-*Eln$^{-/-}$* suggest that both cell types contribute elastin to the IEL in elastic arteries, but ECs are mainly responsible for producing the IEL in resistance arteries where SMCs normally make little elastin.

In most elastic arteries, the *Sm22aCre;Eln$^{f/f}$* IEL was indistinguishable from controls. The exception was the ascending aorta and aortic root where regions of the IEL consisted of a linear arrangement of disconnected small elastin globules, as well as regions of more intact elastin with a moth-eaten appearance. These structural irregularities suggest that ECs in the ascending aorta and aortic root can make the necessary elastic fiber building blocks but are not able to structure a functional elastic lamina without SMC contributions. It is possible that the IEL produced solely by ECs is unable to withstand the unique high systolic and pulse pressure in the ascending aorta or alternatively that ascending aortic ECs require cross talk with SMCs to make an intact IEL. Elastin production by cultured ECs has been documented in several studies,[28–30] but EC contribution to elastic structures

Figure 8 Continued. B, Quantification of PCNA (proliferating cell nuclear antigen)-positive cells in ascending aortic media from mice of indicated genotypes. Quantification of PCNA-positive cells in neointima (only in *Sm22aCre;Eln$^{f/f}$*) is also shown. n=4 for *Sm22aCre;Eln$^{f/+}$* and n=3 for others. No significant differences for each group compared with control by 1-way ANOVA with Sidak multiple comparison test. **C**, Neointimal formation correlates with internal elastic lamina (IEL) disruption. Arrows indicate IEL disruption and neointima formation. Arrowheads indicate intact IEL without neointimal formation. Scale bar=50 μm.

in vivo has been difficult to establish. Our results show that ECs play a major role in the production of the IEL in vivo. Elastin production in the arterial wall begins only after mural cells associate with the vascular endothelium suggesting that SMCs provide a signal to ECs to initiate elastin production. Indeed, our previous studies showed that cultured ECs synthesize and secrete soluble elastin only when incubated in medium conditioned by SMCs.[30]

As discussed above, *Eln*$^{-/-}$ mice die within 48 hours of birth with an obstructive arterial occlusion resulting from SMC migration into the vascular lumen. The absence of an IEL was offered as an explanation for the unrestricted SMC ingrowth observed in *Eln*$^{-/-}$ mice.[2] With only an IEL and no other elastin in the arterial wall, luminal obstruction in *Sm22aCre;Eln*$^{f/f}$ mice is less pronounced. Neointimal cells were evident, however, around discontinuities in the IEL of the ascending aorta and aortic root in *Sm22aCre;Eln*$^{f/f}$ mice, and the extent of neointima correlated with local IEL integrity. These findings show that the IEL is an effective barrier against SMC migration into the lumen of the ascending aorta and aortic root. Neointima was not detected in the resistances arteries of *TieCre;Eln*$^{f/f}$ mice, despite there also being a defective IEL. These results suggest that additional factors beyond IEL integrity, such as hemodynamic forces or SMC developmental origin, which vary with artery location, play a role in neointimal formation.

The origin of cells that populate the neointima associated with disease in mature vessels has been a source of debate. Endothelial, mesenchymal, and hematopoietic lineages have been identified in neointima, as have arterial wall–resident stem/progenitor cells expressing Sca1 (stem cell antigen-1) or c-kit (tyrosine kinase receptor for stem cell factor).[31,32] In our study, the neointimal cells are of *Sm22aCre* lineage and are most likely derived from medial SMCs. Because of the constitutively expressed nature of the *Cre* line, we are unable to distinguish when the cells poised to become neointimal cells turned on smooth muscle genes. An inducible lineage-tracing technique would be better suited to answer such questions.

In addition to an intact IEL in most *Sm22aCre;Eln*$^{f/f}$ arteries, another unexpected finding was clusters of elastin-producing cells in the outer part of the media. Lineage tracing using the *ROSA26*$^{mT/mG}$ reporter allele and the *Sm22aCre* transgene confirmed that these elastin-producing cells did not undergo recombination with *Sm22aCre*. However, immunostaining with an antibody to SM22a protein established that cells within the elastin-positive clusters expressed SM22a, suggesting that *Cre* expression driven by the *SM22a* promoter in the transgene does not faithfully recapitulate expression of the native *SM22a* gene in these cells or that there are subtle differences in cell populations that produce differences in recombination efficiency. It has been shown that cells expressing the promoter gene in a transient or submaximal fashion can have lower *Cre* recombination efficiency.[33] Evidence that the cells are a unique cell population in terms of ECM production includes the following: (1) the unrecombined cells are not randomly distributed across the arterial wall but, for the most part, localize to the outer aspect of the media in both ascending and descending aorta in all mice examined, (2) few unrecombined cells are detected in *SMC22αCre;Eln*$^{f/+}$ aorta, and (3) elastic structures made by the cells are abnormal. *SM22a* is expressed around embryonic day 9.5 in mouse vascular SMCs shortly after expression of α-SMA, and expression continues into adulthood.[34] An intriguing possibility is that the clusters of elastin-producing cells are derived from adventitial SMC progenitors. Passman et al[35] have previously shown a population of adventitial Sca1+ cells capable of becoming SMCs. Our lineage-tracing analysis using the *ROSA26*$^{mT/mG}$ reporter showed that most adventitial cells in the aorta of *SM22aCre;Eln*$^{f/f}$ mice did not recombine. While the majority of these cells may be myofibroblasts, those at the medial-adventitial boarder are in the correct environmental niche to be SMC progenitor cells.[35,36] These cells may migrate into the media of *SM22aCre;Eln*$^{f/f}$ arteries because of the absence of defined elastic laminae that normally hamper their transmural movement.

Heterogeneity within the vascular population of elastin-producing SMCs has been observed in previous studies. In the developing avian vascular system, elastin production is first detected in the aorta near the aortic root and then expands outward along the SMCs of the ascending aorta. Interestingly, the onset of elastogenesis coincides with the loss of α-SMA staining, which reappears in later development. Arteries that develop a muscular phenotype, however, never lose α-SMA expression even though they express elastin.[37,38] Other complex patterns of elastin production have been observed, including radial gradients of elastin expression that also differed between elastic and muscular arteries.[39,40] In these studies, the highest levels of elastin mRNA in the developing ascending aorta were detected in the outer layers of the media, which is the location of the cell clusters observed in our study. Clearly, different populations of SMCs in the vasculature exhibit distinct ECM phenotypes. Future studies to unravel the mechanisms driving heterogeneity in cellular ECM expression may assist in repairing ECM defects in cardiovascular disease.

ARTICLE INFORMATION

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Disclosures

None.

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