Discrete Contributions of Elastic Fiber Components to Arterial Development and Mechanical Compliance

Luca Carta, Jessica E. Wagenseil, Russell H. Knutsen, Boubacar Mariko, Gilles Faury, Elaine C. Davis, Barry Starcher, Robert P. Mecham, Francesco Ramirez

Objective—Even though elastin and fibrillin-1 are the major structural components of elastic fibers, mutations in elastin and fibrillin-1 lead to narrowing of large arteries in supravalvular aortic stenosis and dilation of the ascending aorta in Marfan syndrome, respectively. A genetic approach was therefore used here to distinguish the differential contributions of elastin and fibrillin-1 to arterial development and compliance.

Methods and Results—Key parameters of cardiovascular function were compared among adult mice haploinsufficient for elastin (Eln\(^{+/−}\)), fibrillin-1 (Fbn1\(^{+/−}\)), or both proteins (dHet). Physiological and morphological comparisons correlate elastin haploinsufficiency with increased blood pressure and vessel length and tortuosity in dHet mice, and fibrillin-1 haploinsufficiency with increased aortic diameter in the same mutant animals. Mechanical tests confirm that elastin and fibrillin-1 impart elastic recoil and tensile strength to the aortic wall, respectively. Additional ex vivo analyses demonstrate additive and overlapping contributions of elastin and fibrillin-1 to the material properties of vascular tissues. Lastly, light and electron microscopy evidence implicates fibrillin-1 in the hypertension-promoted remodeling of the elastin-deficient aorta.

Conclusions—These results demonstrate that elastin and fibrillin-1 have both differential and complementary roles in arterial wall formation and function, and advance our knowledge of the structural determinants of vascular physiology and disease. (Arterioscler Thromb Vasc Biol. 2009;29:2083-2089.)

Key Words: elastin ■ fibrillin-1 ■ hypertension ■ Marfan syndrome ■ supravalvular ■ aortic ■ stenosis

Elastin fibers together with smooth muscle cells (SMCs) form the lamellar units that distribute hemodynamic tension uniformly throughout the vessel wall and confer elastic recoil to large arteries. Elastic fibers consist of fibrillin microfibrils surrounding and embedded within an amorphous core of elastin. Fibrillins are large glycoproteins that polymerize into microfibrils that associate with elastin and other elastic fiber proteins. Elastin is secreted as soluble tropoelastin molecules that are subsequently cross-linked by lysyl oxidase enzymes to become insoluble elastin. Fibrillin-rich microfibrils are believed to provide a structural scaffold that guides elastin deposition and assembly in concert with other elastic fiber molecules.

In spite of being part of the same extracellular macroaggregate, elastin haploinsufficiency in supravalvular aortic stenosis (SVAS, OMIM #185500) leads to narrowing of large elastic arteries, whereas mutations of fibrillin-1 in Marfan syndrome (MFS, OMIM #154700) result in aortic dilation. Mouse models of SVAS and MFS have yielded mechanistic insights underlying the vascular phenotype of these 2 conditions. Elastin haploinsufficient mice are viable and display an increased number of lamellar units attributable to a developmental adaptation to abnormally high blood pressure. Mice underexpressing fibrillin-1 die from aortic rupture between 2 to 4 months of age and show no changes in blood pressure or in the number of lamellar units. No comparable information is currently available for fibrillin-1 haploinsufficient mice.

To characterize the contributions of elastin and fibrillin-1 to arterial function, we examined several different properties of vessels haploinsufficient for both proteins. The phenotypes of mice haploinsufficient for elastin or fibrillin-1 or both proteins suggest no major involvement of fibrillin-1 microfibrils in elastin-dependent maintenance of baseline blood pressure and vessel morphology, reiterate the discrete functions of fibrillin-1 and elastin in arterial compliance, and reveal cooperation between the 2 proteins in conferring material properties to arterial tissues. Additional evidence indicates that fibrillin-1 participates in the hypertension-driven process of adaptive remodeling of the aortic wall.

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2083
Methods

Animals

Seven-month-old C57Bl/6J wild-type (WT) mice and mice haploinsufficient for elastin (Eln<sup>−/−</sup>),<sup>6</sup> fibrillin-1 (Fbn1<sup>−/−</sup>),<sup>15</sup> or both proteins (dHet) were used in the studies following the institutional guidelines.

ECM Analyses

The amounts of elastin and collagen incorporated into the arterial walls of various mutant mice were quantified using standard measurements of tissue desmosin and hydroxyproline content, respectively.<sup>16,17</sup> Lacking a comparable biochemical assay, relative levels of fibrillin-1 in wild-type and mutant aortae were estimated by measuring the intensity of immunoreactive material in tissues treated with increasing dilutions (from 1:100 to 1:400) of antibody measuring the intensity of immunoreactive material in tissues treated of fibrillin-1 in wild-type and mutant aortae were estimated by

Surgical Procedures, and Physiological and Morphological Analyses

Mice were anesthetized with 1.5% isoflurane, and body temperature was maintained through a heating pad system with feedback control (Fine Science Tools). Arterial blood pressure was measured using a catheter (Millar Instruments) inserted into the right common carotid artery.10,11 Isoflurane was reduced to 0.5%, and blood pressure was measured using a pressure arteriograph (Danish Myotechnology), and the unloaded length was recorded. Each artery was preconditioned for 3 cycles during which the length was adjusted to the in vivo length at

Mechanical Tests

Arteries were placed on stainless steel cannulae mounted in a pressure and force arteriograph (Danish Myotechnology), and the unloaded length was recorded. Each artery was preconditioned for 3 cycles during which the length was adjusted to the in vivo length at

which the force decreased slightly with pressure. The in vivo length was further verified by comparing the stretch of the artery (with respect to the unloaded length) to previously determined in vivo stretch ratios.<sup>12</sup> Stretch of carotid arteries during mechanical testing was similar to the in vivo value. The artery was then pressurized for 3 cycles from 0 to 175 mm Hg in increments of 25 mm Hg (12 seconds/step) while pressure, outer diameter, and longitudinal force were recorded at 1 Hz. After mechanical testing, 2 to 3 rings (1 to 2 mm long) were cut from the center of the artery and imaged to obtain the unloaded diameter and thickness. Artery boundaries were determined manually and measured using Image J software. Pressure, diameter, longitudinal force, and longitudinal stretch data were converted to stress and stretch ratios.<sup>13</sup> Aortic rings (1 to 2 mm long) were cut radially to measure the opening angle. Opening angle was not measured in carotid arteries because this parameter is unaffected by elastin haploinsufficiency.<sup>11</sup> The opening angle is defined as the angle subtended by the lines connecting the midpoint of the inner circumference with the ends of the ring.<sup>19</sup>

Light and Electron Microscopy

For histology, ascending aortae were fixed overnight in 4% paraformaldehyde at 4°C and processed for paraffin embedding. Sections were excised where the pulmonary artery courses behind the ascending aorta and then stained with Weigert solution.<sup>15</sup> Two individuals, blinded to the genotype, counted the number of elastic lamellae at 4 equal intervals in sections of the entire ring of the ascending aorta.<sup>6</sup> This approach was applied to 4 equally distanced rings along the vessel length of each mouse. All measurements were averaged for each aorta. For electron microscopy (EM), aortae were fixed by cardiac perfusion of 3% glutaraldehyde in 0.1 mol/L sodium cacodylate (pH 7.4) after clearing blood with normal saline. Aortae were trimmed to a 1.5-mm ring and treated en bloc with osmium tetroxide, tannic acid, and uranyl acetate.<sup>20</sup> After dehydration through a graded series of methanol and infiltration with Epon, tissues were embedded in pure Epon and polymerized. Sixty-nm sections were counterstained with 7% methanolic uranyl acetate and lead citrate and viewed using a Tecnai 12 transmission electron microscope at 120 kV.

Statistical Analysis

Four to 8 mice of each genotype were examined in various experiments. Results were evaluated using Student t test and are presented as mean values±SD; probability values ≤0.05 were chosen as statistically significant.

Results

ECM Composition, Anatomy, and Physiology of dHet Arteries

The relative amounts of elastin, fibrillin-1, and collagen proteins incorporated in the WT, Fbn1<sup>−/−</sup>, Eln<sup>−/−</sup>, and dHet aortic walls are proportional to the number of the respective genes in each of the mutant mouse lines (Table 1). Concordance between gene expression and tissue protein levels thus excludes the formal possibility of a substantial adaptation in

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Table 1. Desmosine and Hydroxyproline Content (Normalized to Total Protein) and Relative Intensity of Immunoreactive Fibrillin-1 (Normalized to Tissue Area) in Ascending Aortae

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Fbn1&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Eln&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>dHet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmosine, pm/mg protein</td>
<td>2976±250</td>
<td>2769±598</td>
<td>1385±326†</td>
<td>1413±399†</td>
</tr>
<tr>
<td>Hydroxyproline, µg/mg protein</td>
<td>31±3</td>
<td>34±5</td>
<td>34±6</td>
<td>36±4</td>
</tr>
<tr>
<td>Fibrillin-1 immunoreactivity, signal intensity/µm² tissue</td>
<td>22.66±0.83‡</td>
<td>9.90±0.634</td>
<td>20.61±9.61</td>
<td>13.99±1.83</td>
</tr>
</tbody>
</table>

No. of mice | 5 | 4 | 6 | 6

*P<0.05 between Eln<sup>−/−</sup> and WT or Fbn1<sup>−/−</sup>.
†P<0.05 between dHet and WT or Fbn1<sup>−/−</sup>.
‡P<0.05 between WT and Fbn1<sup>−/−</sup> or dHet.
Like dHet with a significant increase of left ventricular wet weight in twisting in vivo, residual torsion ex vivo, and ECM assembly that is secondary to the mechanical changes of mutant vessels.

The aortae of adult Fbn1+/−, Eln+/−, and dHet mice are 10%, 44%, and 49% longer than WT, respectively (Table 2). Like Eln+/− mice, the carotid artery of dHet mice shows twisting in vivo, residual torsion ex vivo, and ∼10% to 15% lower stretch ratio than the Fbn1+/− and WT artery (Figure 1A and 1B, and Table 2). Additionally, dHet and Eln+/− mice are both hypertensive (Table 3). Hypertension is associated with a significant increase of left ventricular wet weight in dHet compared to WT mice, and with an appreciable trend toward the same increase in Eln+/− mice (Table 3). These genetic data are at least consistent with the notion that elastin has a greater role than fibrillin-1 in establishing baseline blood pressure, maintaining arterial stretch, and preventing residual torsion, and that fibrillin-1 plays a significantly lesser role than elastin in controlling arterial length.

Despite comparable ages and body weights, the average unloaded outer diameter of the ascending aorta is 12% to 19% smaller in Eln+/− and dHet than in WT and Fbn1+/− mice, respectively (Table 2). Moreover, the average thickness of the ascending aorta is 11% lower in Eln+/− than WT mice, and the outer diameter and thickness of the carotid artery is 11% to 19% smaller in dHet than in WT and Fbn1+/− mice, respectively (Table 2). These results suggest that fibrillin-1 haploinsufficiency has little impact on aortic adaptation to elastin haploinsufficiency, and may enhance the carotid artery phenotype in dHet mice.

**Mechanical Properties of dHet Arteries**

The mean outer diameter of the Eln+/− ascending aorta is smaller than WT at all pressures except between 50 and 100 mm Hg, and the Fbn1+/− aorta is larger than WT at pressures between 50 and 150 mm Hg (Figure 2A). Importantly, the dHet aorta has a significantly smaller diameter at extreme pressures (0, 150, and 175 mm Hg) and a significantly larger diameter at intermediate pressures (75 and 100 mm Hg) compared to WT (Figure 2A). Additionally, the average diameter of the dHet aorta at all pressures is more similar to WT than either the Eln+/− or Fbn1+/− aorta (Figure 2A). These observations support the notion that elastin and fibrillin-1 endow the aortic wall with distinct mechanical properties, elastic recoil and tensile strength, respectively.

Whereas the mean outer diameter of the Fbn1+/− and WT carotid artery is comparable at all pressures, the Eln+/− value is significantly smaller than WT or Fbn1+/− at pressures greater than 75 or 0 mm Hg, respectively (Figure 2B). Additionally, the dHet artery has a substantially smaller diameter than the Eln+/− artery at extreme pressures (0 and 150 mm Hg), the WT artery at all pressures except 50 mm Hg,
analyses demonstrate that reducing either elastin or fibrillin-1 of the dHet diameter change and the incremental elastic modulus (Einc) of elastic fiber protein was evaluated by calculating the percent impacts the ascending aorta, the mechanical role of each high-pressures. Because fibrillin-1 haploinsufficiency mostly suggests that fibrillin-1 imparts tensile strength at midto ical pressure (dotted black and gray arrows; Figure 2A) suggests that fibrillin-1 imparts tensile strength at midto high-pressures. Because fibrillin-1 haploinsufficiency mostly impacts the ascending aorta, the mechanical role of each elastic fiber protein was evaluated by calculating the percent diameter change and the incremental elastic modulus (Einc) of WT and mutant aortae. The percent diameter change is an inverse measure of vessel stiffness that does not account for differences in thickness. Fbn1 +/−, Eln +/− and dHet aortae display a biphasic profile with more change in diameter than WT at pressures below 100 mm Hg and less change in diameter at pressures above 100 mm Hg (Figure 2C). Moreover, the percent diameter change of the Eln +/− and Fbn1 +/− aorta is exacerbated when the amount of both proteins is reduced (Figure 2C). Einc is the local slope of the stress-stretch ratio curve and thus represents a measure of stiffness that accounts for differences in both unloaded diameter and thickness. When plotted against pressure, Einc shows an increased stiffness of Eln +/− and Fbn1 +/− aortae compared with WT above 125 mm Hg, and greater than normal stiffness of the dHet aorta above 100 mm Hg (Figure 2D). These analyses demonstrate that reducing either elastin or fibrillin-1 content increases arterial stiffness at midto high pressures (>100 mm Hg), and that reducing both proteins further increases stiffness in an additive fashion.

Material Properties of dHet Arteries
Eln +/−, Fbn1 +/−, and dHet ascending aortae exhibit higher than normal circumferential stresses at pressures between 75 and 100 mm Hg (Figure 3A). Compared to WT, the circumferential stretch ratio is significantly higher in the Fbn1 +/− aorta between 75 and 100 mm Hg, in the Eln +/− aorta between 50 and 125 mm Hg, and in the dHet aorta at all pressures except 0 mm Hg (Figure 3B). Whereas the stretch ratios of Eln +/− or Fbn1 +/− aortae are comparable at any pressure, there are significant differences between dHet and both Eln +/− and Fbn1 +/− aortae at pressures between 50 and 125 mm Hg (Figure 3B). At similar stretch ratios, the stress decreases in the Eln +/− and Fbn1 +/− aorta and is further decreased in the dHet aorta (Figure 3C). The findings that physiological stress and stretch ratio are higher than normal in Eln +/− and Fbn1 +/− aortae and further increase in dHet aortae strongly argue for independent contributions of elastin and fibrillin-1 to the material properties of the aortic wall. Circumferential residual strain, which is expected to increase during vascular remodeling in response to hypertension, normalizes the circumferential strain gradient through the arterial wall.23 Comparable results from opening angle measurements of WT and mutant aortae indicate that halving the amount of elastin or fibrillin-1 or both proteins has no impact on residual strain (Table 1).

Morphological Changes in dHet Aortae
The Eln +/− aorta exhibits more lamellar units than the WT or Fbn1 +/− aorta (10.16 versus 7.03), and a less pronounced increase (8.92) also characterizes the dHet aorta (Figure 4A). These findings correlate with appreciably thinner elastic lamellae in the ascending aorta of Eln +/− and dHet compared to WT and Fbn1 +/− mice (Figure 4B). Using physiological pressures, radii, and number of lamellar units, the tension per lamellar unit was calculated to be 1.0, 1.3, 1.0, and 1.1 Pa-m in the WT, Fbn1 +/−, Eln +/−, and dHet aorta, respectively. Although within the physiological range, the Fbn1 +/− and dHet values are higher than those of the WT and Eln +/− aorta. These results suggest that fibrillin-1 levels influence the developmental adaptation of the aortic wall to elastin deficiency.

Discussion
Characterization of the vascular phenotype in dHet mice has correlated hypertension, decreased vessel diameter, increased vessel length, and vessel tortuosity with elastin haploinsufficiency, and fibrillin-1 deficiency with aortic dilation and significantly less with increased vessel length. Together with

Table 3. Body Weight (BW), Normalized Left Ventricular Weight (LVW/BW), Normalized Total Heart Weight (THW/BW), Systolic, Diastolic, and Mean Blood Pressures, and Heart Rate of Various Mouse Lines

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Fbn1 +/−</th>
<th>Eln +/−</th>
<th>dHet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>28.96±3.79</td>
<td>31.00±2.17</td>
<td>34.26±4.76$</td>
<td>28.96±5.03</td>
</tr>
<tr>
<td>LWV/BW, mg/g</td>
<td>3.02±0.34</td>
<td>3.28±0.34</td>
<td>3.28±0.45</td>
<td>3.58±0.43†</td>
</tr>
<tr>
<td>THW/BW, mg/g</td>
<td>3.76±0.42</td>
<td>4.06±0.40</td>
<td>4.09±0.57</td>
<td>4.41±0.52</td>
</tr>
<tr>
<td>Systolic pressure, mm Hg</td>
<td>107±4</td>
<td>111±7</td>
<td>139±22*</td>
<td>132±16†</td>
</tr>
<tr>
<td>Diastolic pressure, mm Hg</td>
<td>77±6</td>
<td>78±8</td>
<td>91±14*</td>
<td>85±7</td>
</tr>
<tr>
<td>Mean pressure, mm Hg</td>
<td>91±5</td>
<td>94±7</td>
<td>112±17*</td>
<td>105±10†</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>569±59</td>
<td>587±28</td>
<td>566±44</td>
<td>562±22</td>
</tr>
<tr>
<td>No. of mice</td>
<td>7</td>
<td>6</td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>

*P<0.05 between Eln +/− and WT or Fbn1 +/−.
†P<0.05 between dHet and WT.
‡P<0.05 between dHet and WT or Fbn1 +/−.
§P<0.05 between Fbn1 +/− and WT.
estimates of mutant ECM composition, the analyses also indicate that perturbations of some material properties are mostly accounted for by reduction in the total amount of both proteins, which operate in either additive (aortic stiffness) or partially overlapping (carotid artery compliance) manners. Lastly, preliminary evidence suggests that fibrillin-1 participates in the developmental adaptation of Eln\(^{-/-}\) arteries (number of elastic lamellae) to hypertension.

Combined reduction of elastin and fibrillin-1 levels leads to changes in the pressure-diameter relationship of the arterial wall, which restricts vessel expansion at extreme pressures and promotes expansion at intermediate physiological values. Reduced expansion of Eln\(^{-/-}\) aortae at high pressures may reflect an increase in the relative ratio between collagen and elastin, which causes stiff collagen fibers to become the load-bearing elements at lower pressures. On the other hand, increased vessel expansion at intermediate physiological pressures in Fbn1\(^{-/-}\) aortae conceivably reflects the mechanical role of microfibrils in opposing pressure-induced dilation.\(^1\)\(^,\)\(^2\) Consistent with evolutionary considerations,\(^2\) the fact that dHet aortae display abnormalities in both pressure ranges supports the notion that the 2 proteins have distinct functions in vessel compliance, with elastin providing elastic recoil and fibrillin-1 providing tensile strength. In accordance with previous work,\(^10\)\(^,\)\(^11\) our data also indicate that normalization of aortic diameter and blood flow occurs at physiological pressures in WT or Eln\(^{-/-}\) but not in Fbn1\(^{-/-}\) or dHet aortae. This last finding strongly suggests that vascular compensation requires optimal fibrillin-1 levels.

Further analyses of our data indicate that elastin levels are critical for the mechanical behavior of both the ascending aorta and carotid artery, whereas fibrillin-1 levels mostly affect the ascending aorta. These findings are in agreement with the restricted vascular manifestations in MFS patients.
and mouse models of the disease, and imply spatially defined role(s) of fibrillin-1 microfibrils in the circulatory system. Circumferential stress and stretch ratio of Eln\+/H11001/H11002 and Fbn1\+/H11001/H11002 aortae show similar increases at intermediate pressure values, suggesting that elastin and fibrillin-1 cooperate in endowing the aortic wall with discrete material properties that influence the mechanical behavior. This conclusion is also supported by the observation that the stretch ratio of dHet aortae differs significantly from that of Eln\+/− or Fbn1\+/− in the same pressure range. A similar argument applies to the incremental stiffness of dHet aortae. Previous studies have highlighted the associations between increased arterial stiffness and decreased compliance with both systemic hypertension and left ventricular hypertrophy. They have also shown that aortic stiffness is abnormally high in either elastin or fibrillin-1 deficiency.

Figure 3. Circumferential stress and stretch ratio in wild-type and mutant aortae. Average circumferential stress (A) and stretch ratio (B) vs pressure, and circumferential stress vs circumferential stretch ratio (C) in the ascending aorta of the indicated genotypes. Compared with the WT aorta, line graphs in A show increased circumferential stress of the dHet or Eln\+/− aorta at 75 and 100 mm Hg, and of the Fbn1\+/− aorta at 75, 100, and 125 mm Hg (P<0.05). Compared with the WT aorta, line graphs in B show greater circumferential stretch ratio of the Eln\+/− aorta at 50 to 125 mm Hg, and of the Fbn1\+/− aorta at 75 to 100 mm Hg (P<0.05). Line graphs also show that the stretch ratio of the dHet aorta is greater than the WT, Eln\+/−, or Fbn1\+/− aorta between 25 and 175 mm Hg, 50 and 125 mm Hg, or 25 and 125 mm Hg, respectively (P<0.05). There are no statistical differences between the singly haploinsufficient vessels (P>0.05). Line graphs in C represent circumferential stress and stretch ratio plotted against each other for all genotypes; Fbn1\+/− and Eln\+/− profiles are very close to each other, Fbn1\+/− and WT profiles overlap at both ends, and the dHet profile is appreciably shifted to the right. Values are means±SD; n=4 to 6 per genotype.

Figure 4. Aortic wall morphology. A, Elastic fiber staining in the ascending aortae of the indicated genotypes. Bar graphs at the bottom summarize the average number of lamellar units in the WT (black), Fbn1\+/− (white), Eln\+/− (dark gray), or dHet (light gray) aorta. Asterisks indicate statistically significant differences in the number of elastic lamellae (P<0.05). Values are means±SD; n=4 for each genotype. Scale bar=100 μm. B, EM images of the central medial layer of the ascending aortae in mice of the indicated genotypes. Scale bar=5 μm.
Although increased stiffness characterizes both $Eln^{+/−}$ and $Fbn1^{+/−}$ aortae, only the former mice are hypertensive probably because reduced fibrillin-1 levels do not decrease the unloaded aortic diameter thus maintaining normal blood flow. This observation supports our contention that hypertension is primarily caused by elastin deficiency, which restricts vessel size and causes hypertension in both $Eln^{+/−}$ and $dHet$ mice. Association of elastin haploinsufficiency and hypertension with a greater number of lamellar units is also consistent with the notion that increased hemodynamic force induces wall remodeling to maintain a constant tension per lamellar unit.6,10–13 Lamellar unit number is established during arterial development in coordination with blood pressure increases, SMC differentiation, and elastin accumulation.1,27 Elastin deficiency causes perinatal hypertension leading to more lamellar units and lamellar tension normalization in $Eln^{+/−}$ mice.10,11,14 The fact that $dHet$ mice are hypertensive and have fewer lamellar units than $Eln^{+/−}$ mice indicates that a threshold level of fibrillin-1 is required to normalize the tension/lamellar unit ratio and thus aortic mechanics. This line of reasoning is consistent with our earlier report that the organization and maturation of lamellar units are grossly impaired in $Fbn1$-null mice.15 Collectively, these findings advance substantially our understanding of the structural determinants of vascular physiology and the pathological mechanisms that underlay disease progression in SVAS and MFS, and in age-related processes of vascular tissue degradation.

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Disclosures

None.

References


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