Targeted disruption of fibulin-4 abolishes elastogenesis and causes perinatal lethality in mice

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Elastic fibers provide tissues with elasticity which is critical to the function of arteries, lungs, skin, and other dynamic organs. Loss of elasticity is a major contributing factor in aging and diseases. However, the mechanism of elastic fiber development and assembly is poorly understood. Here, we show that lack of fibulin-4, an extracellular matrix molecule, abolishes elastogenesis, fibulin-4−/− mice generated by gene targeting exhibited severe lung and vascular defects including emphysema, artery tortuosity, irregularity, aneurysm, rupture, and resulting hemorrhages. All the homozygous mice died perinatally. The earliest abnormality noted was a uniformly narrowing of the aorta in fibulin-4−/− embryos at embryonic day 12.5 (E12.5). Aorta tortuosity and irregularity became noticeable at E15.5. Histological analysis demonstrated that fibulin-4−/− mice do not develop intact elastic fibers but contain irregular elastin aggregates. Electron microscopy revealed that the elastin aggregates are highly unusual in that they contain evenly distributed rod-like filaments, in contrast to the amorphous appearance of normal elastic fibers. Desmosine analysis indicated that elastin cross-links in fibulin-4−/− tissues were largely diminished. However, expression of tropoelastin or lysyl oxidase mRNA was unaffected in fibulin-4−/− mice. In addition, fibulin-4 strongly interacts with tropoelastin and colocalizes with elastic fibers in culture. These results demonstrate that fibulin-4 plays an irreplaceable role in elastogenesis.

Elastic fibers with morphologically distinct architectures are present in the extracellular matrix (ECM) to accommodate elastic requirements and mechanical stresses imposed on different tissues. They are particularly abundant in elastic tissues such as large blood vessels, lung, and skin. Loss of elasticity is a major contributing factor in aging and a myriad of pathological conditions including emphysema, artery diseases, and cutis laxa (39, 41, 44). Elastic fibers undergo irreversible structural and compositional changes with age and in some pathological conditions (41). Regardless of morphology, all elastic fibers consist of cross-linked elastin, fibrillin-rich microfibrils, and several associated molecules (23, 37, 38, 46). Elastin endows the fiber with the characteristic property of elastic recoil. It is chemically inert, extremely hydrophobic, and insoluble under most conditions. Monomeric elastin, called tropoelastin, is secreted from the cell as a soluble protein. Isolated and purified tropoelastin has been shown to exhibit a great tendency to aggregate (coacervation) in physiological solution and at temperatures in the physiological range, giving rise to supramolecular structures very similar to those found in natural elastic fibers (4, 5, 11). This self-aggregation property of tropoelastin is thought to contribute to elastic fiber assembly in vivo. However, self-aggregation alone is insufficient to explain the efficiency of the assembly process and the variable form of elastic fibers in different tissues.

The formation of elastic fibers has been proposed to require the deposition of tropoelastin on a preexisting scaffold, cross-linking of tropoelastin monomers by lysyl oxidase (LOX) family enzymes, and organization of the resulting insoluble elastin matrix into mature fibers (37). Fibrillin-rich microfibrils are thought to provide the scaffold for the deposition of elastin. Unexpectedly, normal elastic fiber assembly was found to occur in fibrillin-1 or fibrillin-2 mutant mice (2, 9, 42, 43). Therefore, the molecular mechanism of elastic fiber assembly remains elusive.

A significant insight into elastogenesis comes from two recent studies of fibulin-5−/− mice. These mice exhibit disrupted and disorganized elastic fibers throughout the body, indicating that fibulin-5 (also known as DANCE or EVEC) plays an important role in elastic fiber formation (40, 56). fibulin-5−/− mice grow to adulthood without lethality but have loose skin, vascular abnormalities, and emphysematous lungs. Fibulin-5 has an RGD motif and interacts with cell surface integrins and elastin. Thus, it has been proposed to promote elastic fiber formation by linking elastic fibers to cells (40, 56).

Fibulin-5 belongs to the fibulin family of six known ECM proteins that share tandem arrays of calcium-binding epidermal growth factor domains and a characteristic carboxyl-terminal fibulin domain (1, 10, 15, 52). Although little is known about the functions of fibrulins, mutations of individual members have been associated with several diseases. A single mutation of an arginine to tryptophan in fibulin-3 (also known as...
EFEMP1, S1-5, or FBNL1) causes an inherited macular degenerative disease termed maillotteine or Doyne honeycomb retinal dystrophy (51). Missense variations in other fibulins have been detected in patients with age-related macular degeneration (47, 50), the most common cause of incurable blindness (6). Mutations in fibulin-5 have also been found in some cutis laxa patients (29, 32). So far, fibulin-5 is the only fibulin reported to be necessary for elastogenesis, whereas fibulin-1-null mice are reported to die perinatally as a result of hemorrhages, due to defects associated with capillary endothelial cells (25). Knockout mice for other fibulins have not been reported. Among the fibulins, fibulin-3, fibulin-4 (also known as EFEMP2, MBP1, H411, or UPH1), and fibulin-5 share highest homology with each other. These three fibulins are the smallest members of the family, share >50% amino acid identity, and are nearly identical in their structural organization (1, 10, 15, 52). Despite this homology, fibulin-5 deficiency is not compensated for by fibulin-3 or -4, suggesting that fibulin-3, -4, and -5 are not functionally redundant.

The function of fibulin-4 is poorly understood. Several studies have consistently found that fibulin-4 promotes cell growth, exhibits oncopgenic properties, and is upregulated in tumor tissues (14, 16, 19). fibulin-4 mRNA has been shown to be widely expressed in various tissues throughout the body (14, 16, 18). High protein levels are present in blood vessel walls (18). During development, fibulin-4 mRNA is expressed in mouse embryos as early as embryonic day 7 (E7) (14). In this study, we investigated the biological role of fibulin-4 through targeted gene inactivation in mice. Remarkably, mice lacking fibulin-4 do not form elastic fibers, with resulting severe vascular and lung defects; they die perinatally. These results demonstrate that fibulin-4 plays an irreplaceable role in elastic fiber formation.

MATERIALS AND METHODS

fibulin-4°/° mouse generation, Southern blot analysis, and RT-PCR. The targeting vector was constructed using 2.5-kb (5′) and 3-kb (3′) mouse fibulin-4 genomic DNA fragments as homology arms. The two arms flanked a promoter-H11032/hemoglobin beta-promoter-H11032 locus. Germ line-transmitting chimeric mice generated from one of these mice. Several different clones producing monoclonal antibodies (MAbs) against fibulin-4 (OriGene Technologies) without signal peptide was cloned into vector pGEX-4T-2 (Pharmacia) to generate fibulin-4 fused with glutathione S-transferase (GST). GST-fibulin-4 or GST was purified as described previously (36). Four mice were immunized with GST-fibulin-4. Sera (polyclonal antibodies) from all the mice were characterized with both GST fusion proteins and lysates of transfected 293T cells expressing recombinant fibulin-4. Hydroxynitriles were derived from one of these mice. Several different clones producing monoclonal antibodies (MAbs) against fibulin-4 were identified by enzyme-linked immunosorbent assay, clones 7B9 (immunoglobulin G2a [IgG2a]), and 11E2 (IgG2b) are of high specificity and titer.

Transfection, immunoprecipitation, and immunoblotting. 293T cells were transfected with a control plasmid, pCMV-XL4-BS (human fibulin-4, CDNA), pCMV-XL6-heln (human tropoelastin CDNA obtained from OriGene), or both pCMV-XL4-BS and pCMV-XL6-heln with Lipofectamine (Invitrogen). At 48 h after transfection, cells were lysed. Immunoprecipitation and immunoblotting using antibodies against fibulin-4 and human elastin (Elastin Products Company) were performed as previously described (34) with modifications. To avoid the interference of the IgG heavy chain in interpreting results of immunoblotting following immunoprecipitation with the same or similar antibodies, immunoprecipitation was performed with antibodies covalently coupled to protein A-Sepharose. Anti-fibulin-4 MAb 7B9 or a rabbit anti-human tropoelastin polyclonal antibody (Elastin Products Company) was cross-linked to protein A-Sepharose CL-4B (Pharmacia) using dimethylimideimide (Sigma) as previously described (35). For each immunoprecipitation, cell lysate containing 200 μg of total protein was diluted to 1 ml with lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 0.5% NP-40, 0.5% Triton X-100, 1 ml phenylmethylsulfonyl fluoride, and a 1:100 dilution of protease inhibitor mixture III (Calbiochem). A total of 25 μl of antibody-protein A beads was incubated with the cell lysate for 4 h at 4°C. The immunoprecipitates were washed and resuspended in 30 μl of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer; 10 μl of the suspension was loaded to each well, resolved by SDS-PAGE on a 10% gel, and transferred onto a polyvinylidene difluoride membrane (Millipore). Anti-fibulin-4 MAb 11E2 or the anti-human tropoelastin antibody was used in immu-
RESULTS

Targeted inactivation of the fibulin-4 gene results in perinatal lethality. The mouse fibulin-4 gene was inactivated by insertion of a promoterless lacZ and a neomycin-resistant gene cassette into exon 4 (Fig. 1A). Homologous recombinants were identified by Southern blot analysis (Fig. 1B). The absence of fibulin-4 mRNA was confirmed by RT-PCR (Fig. 1C) and Northern blot analysis (see Fig. 7). We found no adult homozygous offspring of fibulin-4+/- crosses, raising the possibility that ablation of fibulin-4 causes early lethality. Genotype analysis of offspring at several developmental stages indicated that the number of wild-type, heterozygous, and homozygous animals were distributed in a normal Mendelian pattern at embryonic stages E11.5 and E18.5 (Table 1). However, most fibulin-4-/- mice died during birth, only 10% survived to P1, and all the homozygous mice died by P2 (Table 1). The frequency of heterozygous animals was about twice that of the wild type and thus was not affected by the disruption of one fibulin-4 allele. These results demonstrate that lack of fibulin-4 causes perinatal lethality in mice.

Severe vascular and lung defects in fibulin-4-/- mice. The gross appearances of wild-type, fibulin-4+/-, and fibulin-4-/- mice at P1 were similar. However, on dissection, fibulin-4-/- mice exhibited severe vascular and lung defects. The arteries were tortuous, with irregularities including narrowing, dilatation, aneurysms, rupture, and resulting hemorrhages. The abnormalities were most severe in the aorta (Fig. 2B) and large arteries but occurred in other arteries as well. In addition, all live-born fibulin-4-/- mice were found to have expanded lungs. Histological examinations showed markedly enlarged distal airspaces, similar to those of emphysematous lungs (Fig. 2D). No obvious difference was observed in other organs. Despite the severe phenotype exhibited by homozygous animals, heterozygous (fibulin-4+/+) mice are fertile, have a normal life span, and appear to be indistinguishable from the wild-type littermates.

The earliest abnormality appears in fibulin-4-/- embryos at E12.5. To determine the onset time of defects in fibulin-4-/- mice, we studied different developmental stages of the mice. The earliest abnormality noted was a uniformly narrowing of the descending aorta in fibulin-4-/- embryos at E12.5 (Fig. 3B). The outer diameter of the aorta in fibulin-4-/- mice was only

<table>
<thead>
<tr>
<th>Stage</th>
<th>No. of animals (%) of genotype:</th>
<th>Total no.</th>
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<tbody>
<tr>
<td></td>
<td>+/+</td>
<td>+/-</td>
</tr>
<tr>
<td>E11.5</td>
<td>16 (31)</td>
<td>23 (45)</td>
</tr>
<tr>
<td>E18.5</td>
<td>18 (21)</td>
<td>45 (52)</td>
</tr>
<tr>
<td>P1</td>
<td>33 (32)</td>
<td>61 (59)</td>
</tr>
<tr>
<td>P2</td>
<td>46 (32)</td>
<td>97 (67)</td>
</tr>
<tr>
<td>P3</td>
<td>53 (31)</td>
<td>118 (69)</td>
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one-half to two-thirds that of wild-type littermates. Histological analyses of cross sections showed that the aortic walls of fibulin-4−/− mice were nearly twice as thick as those of wild-type mice (Fig. 3D). However, the thickening of the fibulin-4−/− aortic wall did not appear to be the result of subendothelial overproliferation of cells, as the number of cells was similar in both fibulin-4−/− and wild-type aortic wall cross sections. There were 193 ± 3 cells for the wild type (data represent means and standard deviations for results with four mice) and 188 ± 7 cells for the homozygote (four mice) in a cross section of the aortic wall at a similar level of the thoracic aorta as shown in Fig. 3C and D. In contrast to the elongated, spindle-shaped cells (E, arrow) in wild-type mice, aortic wall cells were round and appeared to be less stretched (Fig. 3F). The narrowing and the cell shape difference of the fibulin-4−/− aorta became less obvious at E13.5 and at older embryonic ages, likely due to passive expansion of the aorta caused by the dramatic increase in systemic blood pressure during these stages (22). Aortic tortuosity and irregularity were noticeable at E15.5 and became more pronounced with age in homozygous animals. These aorta abnormalities were not observed in fibulin-4+/+ embryos.

fibulin-4−/− mice do not form elastic fibers. The vascular and lung abnormalities of fibulin-4−/− mice were suggestive of elastic fiber defects. The appearance of abnormalities, coincident with the onset of elastogenesis in mice, suggests that genesis of elastic fibers may be impaired in fibulin-4−/− mice. Thus, we stained tissue sections from E12.5 to P1 with elastin van Gieson staining, which specifically stains elastic fibers. In the wild type, the innermost elastic lamina of the aorta was identifiable at E13.5 under a light microscope (Fig. 4A). By E14.5, four elastic laminae could be distinguished (Fig. 4C). All five elastic laminae were present at E16.5 or older ages (Fig. 4E and G). In contrast, no continuous elastic lamina was observed in the fibulin-4−/− aorta at any stage (Fig. 4B, D, F, and H). Instead, irregular elastin aggregates were visible at E14.5 (Fig. 4D), and more and larger aggregates accumulated with age (Fig. 4F and H). In the lung and skin, elastic fibers were not distinguishable by light microscopy at any embryonic stages. At P1, fine elastic fibers could be observed in lung and the hypodermal connective tissue of the skin of wild-type mice (Fig. 5A and C) but not in fibulin-4−/− mice (Fig. 5B and D). Despite this, the skin of fibulin-4−/− mice did not show obvious
To determine whether mature elastin content is affected in fibulin-4 mice, we assessed the level of desmosine, an elastin cross-link, in the aorta and lungs of wild-type, heterozygous, and homozygous mutant mice. As shown in Table 2, elastin cross-linking was nearly absent in fibulin-4−/− mice. There was a 94% decrease in the amount of desmosine in the aorta and 88% decrease in lungs of fibulin-4−/− mice compared with wild-type mice. Interestingly, there was a near 20% increase in the amount of desmosine in heterozygous mutants compared with wild-type mice (Table 2), although this difference was not statistically significant (P > 0.05 in a t test). We did not find any difference in the level of hydroxyproline, an indicator of collagen content, between wild-type and fibulin-4−/− mice (data not shown), indicating that the amount of collagen did not differ in fibulin-4−/− mice.

**Tropoelastin and LOX expression is not affected in fibulin-4−/− mice.** Elastin cross-linking is catalyzed by LOX family enzymes. Among the five known members, LOX has been shown to be necessary for elastic fiber development (20, 31). To determine whether lack of fibulin-4 causes elastinopathy by affecting tropoelastin or LOX expression, we examined the mRNA levels of fibulin-4, tropoelastin, and LOX in wild-type, heterozygous, and homozygous littermates at P1. As shown in Fig. 7, while heterozygotes showed reduced fibulin-4 mRNA levels compared to those in wild-type mice and homozygotes had no detectable fibulin-4 mRNA, all of them had similar levels of tropoelastin and LOX expression. Elastin has been shown to have an antiproliferative effect on vascular smooth muscle cells, and mice lacking elastin (ELN−/−) die of vascular occlusion resulting from subendothelial cell proliferation (27). The unaffected tropoelastin expression is consistent with the lack of cell overproliferation and the accumulation of irregular elastin aggregates in fibulin-4−/− mice.

**Fibulin-4 interacts with tropoelastin and assembles into elastic fibers.** To investigate possible mechanisms by which fibulin-4 affects elastogenesis, we assessed the potential for interaction between fibulin-4 and elastin. Recombinant mouse fibulin-4 was expressed and purified from stably transfected 293T cell medium (Fig. 8A). A FLAG tag and a His6 tag were added at the N terminus of fibulin-4 without its signal peptide to facilitate the purification and characterization of fibulin-4.
The tagged protein was secreted from a preprotrypsin signal sequence. Although we found that untagged fibulin-4 was secreted efficiently from its native signal peptide, the C-terminal-tagged fibulin-4 was poorly secreted. It is possible that the C-terminal domain affects protein folding and is sensitive to modification. In a solid-phase binding assay, purified tropoelastin was used as an immobilized protein substrate, and fibulin-4 was used as a soluble ligand. As shown in Fig. 8B, fibulin-4 bound strongly to tropoelastin in the presence of Ca$^{2+}$ and the binding was inhibited in the presence of EDTA, suggesting that calcium is required for the binding and the calcium-binding epidermal growth factor domains of fibulin-4 may be necessary for this binding. However, it has not been demonstrated experimentally that fibulin-4 binds calcium. No binding was observed between fibulin-4 and a control substrate (bovine serum albumin). These results indicate that fibulin-4 and tropoelastin can interact directly.

To assess whether fibulin-4 and tropoelastin interact in solution, we performed a coimmunoprecipitation assay. Human fibulin-4 cDNA, human tropoelastin cDNA, or both were trans-

**FIG. 6.** Electron microscopy of elastic laminae in fibulin-4$^{-/-}$ mice. (A to D) Descending aorta cross sections from E14.5 (A and B) and P1 (C and D) mice. The wild-type aorta contained continuous elastic lamina (A and C). In contrast, irregular elastin aggregates (arrows) were randomly distributed in the fibulin-4$^{-/-}$ aorta (B and D). Under higher magnification (E and F), the wild-type elastic lamina appeared to be amorphous (E). But distinct, dark rod-like filaments (F, arrowhead) were evenly distributed in the fibulin-4$^{-/-}$ elastin aggregates. SMC, smooth muscle cell. Scale bars, 400 nm (A to D) and 100 nm (E and F).

**TABLE 2.** Desmosine in picomoles per milligram of protein in aorta and lung at P1

<table>
<thead>
<tr>
<th>Genotype (n)</th>
<th>Desmosine ± SD (% of wild type) in:</th>
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<tr>
<td></td>
<td>Aorta</td>
</tr>
<tr>
<td>++ (12)</td>
<td>419.05 ± 125.94 (100)</td>
</tr>
<tr>
<td>+/- (21)</td>
<td>501.86 ± 133.85 (120)$^a$</td>
</tr>
<tr>
<td>-- (6)</td>
<td>25.35 ± 8.69 (6)$^c$</td>
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$^a$ P = 0.09, compared to the wild type by t test.

$^b$ P = 0.23, compared to the wild type by t test.

$^c$ P < 0.0001, compared to the wild type by t test.
fected into 293T cells. Tropoelastin was expressed at a very low level. We could detect tropoelastin in the cell lysate, but it was difficult to detect it in the culture medium, presumably due to its self-coacervation and formation of insoluble elastin in the medium. Fibulin-4 was expressed at a relatively high level and could be readily detected in both lysate and medium. Thus, we chose to use transfected cell lysates for the coimmunoprecipitation assay. As shown in Fig. 8C, tropoelastin was immunoprecipitated from lysates transfected with either tropoelastin alone (lane 2) or both tropoelastin and fibulin-4 (lane 1) by the antitropoelastin antibody. It was also coimmunoprecipitated by an anti-fibulin-4 monoclonal antibody from the lysate cotransfected with tropoelastin and fibulin-4 (lane 3). The coimmunoprecipitation did not appear to be due to a nonspecific association of tropoelastin with the fibulin-4 antibody beads, as no tropoelastin signal was detected from the immunoprecipitate by the same antibody from the lysate transfected only with tropoelastin (lane 4). Reciprocally, fibulin-4 was coimmunoprecipitated by the anti-tropoelastin antibody from the lysate transfected with both tropoelastin and fibulin-4 (lane 7) but not from the lysate transfected with fibulin-4 alone (lane 8), while it was immunoprecipitated by the fibulin-4 antibody from both lysates (lanes 5 and 6). These results demonstrate that fibulin-4 binds specifically with tropoelastin.

To further determine whether exogenous fibulin-4 is colocalized to elastic fibers, we added FLAG-tagged recombinant fibulin-4 to cultured human fibroblasts. These cells are capable of developing a network of elastic fibers in vitro. Double labeling of antitropoelastin and anti-FLAG antibodies showed colocalization of fibulin-4 and elastin (Fig. 9). These data indicate that fibulin-4 assembles into elastic fibers.

DISCUSSION

Elastic fiber formation is thought to involve extrinsic proteins and an intrinsic capacity for elastin coacervation. How extrinsic proteins cooperate with each other and with elastin coacervation to form functional elastic fibers is unknown. Over 30 molecules have been reported to associate with elastic fibers in morphological studies and in vitro assays (23). But many

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**FIG. 7.** Unaffected tropoelastin and lysyl oxidase expression in fibulin-4<sup>−/−</sup> mice. Northern blot analysis of equal amount of mouse lung total RNA shows a fibulin-4 mRNA transcript of 1.8 kb detectable in wild-type (+/+ ) and heterozygous (+/−) but not homozygous (−/−) mice (top). The intensity of the signal is reduced in heterozygous mice. The same blot stripped and rebotted with a tropoelastin probe shows a 3.8-kb tropoelastin transcript with similar intensity in all the mice (second panel from the top). Probing with a Lox probe indicates a 6-kb Lox transcript with similar levels in all mice (third panel). A GAPDH probe was used as a control (bottom).
fibroblasts were cultured with FLAG-tagged recombinant fibulin-4 protein. (A) Cells stained with antitropoelastin antibody, showing a network structure. (B) Cells stained with anti-FLAG antibody, also showing a network structure. (C) Superimposed image of panels A and B, with DAPI nuclear staining showing fibulin-4 localization on elastic fibers.

have been found to have no effect or marginal effects on elastic fiber formation in vivo. In this study, we demonstrated that mice lacking fibulin-4 do not form elastic fibers. This is the first report that lack of a protein other than elastin itself completely abolishes the formation of elastic fibers in vivo, indicating that fibulin-4 plays an indispensable role in elastogenesis.

The initial abnormality present in fibulin-4−/− mice, arterial narrowing, is similar to that observed with ELN−/− mice (27). In ELN−/− mice, however, the change is caused by subendothelial cell overproliferation due to the lack of elastin, a process that eventually obliterates the vascular lumen (27). In fibulin-4−/− mice, tropoelastin mRNA levels are similar to those in wild-type mice, there is no sign of cell overproliferation and vascular occlusion, and irregular elastin aggregates accumulate with age, suggesting that fibulin-4 does not affect the synthesis of tropoelastin. The formation of functional elastic fibers requires the deposition of tropoelastin at the fiber assembly site, cross-linking of tropoelastin monomers by lysyl oxidase family enzymes, and the organization of resulting insoluble elastin matrix into mature fibers. Fibulin-4 likely affects one or more of these processes.

The irregular elastin aggregates observed in fibulin-4−/− mice are highly unusual in that they contain electron-dense rod-like filaments. These filaments are evenly distributed in the aggregates in fibulin-4−/− mice, as if, without fibulin-4, a molecule(s) associated with tropoelastin is incorporated together with each tropoelastin monomer into elastin aggregates. Alternatively, the rod-like filament may be a regular elastic fiber component whose presence is revealed by the absence of fibulin-4. The morphology of fibulin-4−/− elastin aggregates is similar to that of abnormal elastin aggregates permeated by proteoglycans in the presence of lysyl oxidase inhibitors (13). Also, ECM proteoglycans containing sulfated glycosaminoglycans visualized by a cationic copper phthalocyanin dye, cupromeronic blue, exhibit morphology very similar to the rod-like filaments observed in irregular elastin aggregates in fibulin-4−/− mice (48). These observations suggest that the filaments in fibulin-4−/− elastin aggregates may be proteoglycans. Glycosaminoglycans containing sulfate groups (chondroitin, dermatan, and heparan sulfate) and their associated proteoglycans have been shown to directly interact with tropoelastin and are normal components of elastic fibers (3, 7, 17, 55). Both chondroitin sulfate and heparan sulfate can mediate tropoelastin’s coacervation (17, 24, 45, 53, 55). Decreased elastin deposition was observed when the matrix was depleted of sulfated molecules by chlorate treatment of the cells (8, 53). We also found that fibulin-4 interacts with tropoelastin directly and assembles into elastic fibers in culture. Thus, fibulin-4 may play a role in the initial deposition of tropoelastin, such as scaffolding and facilitating the formation of homogeneous elastin polymers by preventing the association of other molecules with tropoelastin or coordinating tropoelastin and other elastic fiber components during elastic fiber assembly. The identity of the rod-like filaments, the precise roles of proteoglycans in elastogenesis, and their relationships with fibulin-4 remain to be determined.

Elastin cross-linking is severely affected in fibulin-4−/− mice. Desmosine was reduced by over 85% in fibulin-4−/− mice, compared to levels in wild-type mice. Interestingly, there was a near-20% increase in desmosine in fibulin-4−/− mice compared to wild-type mice, although this difference was not statistically significant with the number of animals we analyzed. It is possible that there is more cross-linking in heterozygous mutants than in wild-type mice to compensate for fibulin-4 haploinsufficiency. Elastin cross-linking is catalyzed by lysyl oxidases, a family of enzymes that catalyzes the oxidative deamination of lysine residues in elastin and collagen (21). Five members have been described so far (12, 30). LOX has been shown to be necessary for elastic fiber and collagen fiber development (20, 31), and LOX-like 1 (LOXL1) is required in elastic fiber homoeostasis (28). Similar to fibulin-4−/− mice, Lox−/− mice exhibit severe vascular defects and die perinatally (20, 31). The vascular defects include artery tortuosity, irregularity, and ruptured aneurysms with fragmented elastic lamina in the arterial walls. Desmosine content is decreased by 60%; hydroxyproline, which represents collagen content, is decreased by 30% in Lox−/− mice (20). The similarities in gross defects between fibulin-4−/− mice and Lox−/− mice and loss of desmosine content in fibulin-4−/− mice suggest that lack of fibulin-4 may affect the function of lysyl oxidase. However, Lox mRNA expression is similar in wild-type and fibulin-4−/− mice, hydroxyproline content is not altered in fibulin-4−/− mice, and no unusual elastic fiber content such as rod-like filaments found in fibulin-4−/− mice has been reported with Lox−/− mice. Lox1−/− mice survive to adulthood but do not deposit normal elastic fibers in the uterine tract postpartum; they develop
pelvic prolapse, enlarged airspaces of the lung, loose skin, and vascular abnormalities. Desmosine content in Loxl1−/− mice is reduced by 30 to 50%, depending on tissues (28). The difference in elastic fiber defects in fibulin-4−/−, Lox−/−, and Loxl1−/− mice suggests that fibulin-4 has different roles in elastic fiber assembly, even if it also affects the activities of lysyl oxidases.

Fibulin-4 shares high homology with fibulin-5, with a similar domain structure and >50% amino acid identity. Fibulin-5 null mice grow to adulthood but exhibit loose skin, lung airspace enlargement, and a stiff and tortuous aorta, due to disorganized and fragmented elastic fibers (40, 56). It has been proposed that fibulin-5 may be involved in elastogenesis by tethering elastic fibers onto cell surface integrins and by affecting cross-linking of elastin through direct binding with LOXL1 (28, 40, 56). Loxl1−/− mice exhibit similar but less-severe elastic fiber defects than fibulin-5−/− mice. Despite the high homology between fibulin-4 and -5, the fibulin-4−/− phenotype is not compensated for by fibulin-5, fibulin-4−/− mice exhibited almost complete loss of elastic fibers and perinatal lethality, suggesting a more essential role of fibulin-4 in elastogenesis than that of fibulin-5.

With age and some pathological conditions, elastic fibers exhibit interwoven filaments (41) that are similar to the morphology of elastin aggregates of fibulin-4−/− mice. Thus, alteration of the function or structure of fibulin-4 may be a major mechanism behind aging and elastic fiber-related diseases. fibulin-4−/− mice exhibit the most severe elastinopathy described to date. Understanding the role of fibulin-4 is a prerequisite for understanding the mechanism responsible for elastogenesis. In addition to the crucial role in elastogenesis, fibulin-4 may also have other important functions in cell proliferation and differentiation. Several studies have found that fibulin-4 stimulates cell growth and is upregulated in tumors (14, 16, 19). Fibulin-4 may also have other important functions in cell proliferation and differentiation. Several studies have found that fibulin-4 stimulates cell growth and is upregulated in tumors (14, 16, 19). Fibulin-4 is present in human dermis elastic fibers. Matrix Biol. 16:497–480.

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