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Fibulin-2 Is Dispensable for Mouse Development and Elastic Fiber Formation

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Fibulin-2 is an extracellular matrix protein belonging to the five-member fibulin family, of which two
members have been shown to play essential roles in elastic fiber formation during development. Fibulin-2
interacts with two major constituents of elastic fibers, tropoelastin and fibrillin-1, in vitro and localizes to
elastic fibers in many tissues in vivo. The protein is prominently expressed during morphogenesis of the heart
and aortic arch vessels and at early stages of cartilage development. To examine its role in vivo, we generated
mice that do not express the fibulin-2 gene (Fbhn2) through homologous recombination of embryonic stem cells.
Unexpectedly, the fibulin-2-null mice were viable and fertile and did not display gross and anatomical
abnormalities. Histological and ultrastructural analyses revealed that elastic fibers assembled normally in
the absence of fibulin-2. No compensatory up-regulation of mRNAs for other fibulin members was detected in
the aorta and skin tissue. However, in the fibulin-2 null aortae, fibulin-1 immunostaining was increased in the
inner elastic lamina, where fibulin-2 preferentially localizes. The results demonstrate that fibulin-2 is not
required for mouse development and elastic fiber formation and suggest possible functional redundancy
between fibulin-1 and fibulin-2.

Fibulins constitute a small family of five extracellular matrix proteins that share a distinctive carboxyl-terminal globular do-
main and a tandem array of calcium-binding epidermal growth factor-like modules (1, 25). Fibulin-1 and fibulin-2 contain an
additional domain of three anaphylatoxin modules preceding the calcium-binding epidermal growth factor-like repeats
and therefore are larger than fibulin-3, -4, and -5, which have identical modular structures. Fibulin-2 possesses a large aminoterminal globular domain of approximately 400 amino acids
that is not present in the other fibulins and hence is the largest member of the family (17). The protein is approximately 180
kDa in size and forms covalently linked dimers (22). The other four fibulins range between 50 and 100 kDa in size and are
present mainly as monomers (8), though dimers of fibulin-5 have recently been described (31).

The fibulins not only share structural similarities but also have overlapping expression patterns (4). A notable common
feature is that all fibulins are abundantly distributed in elastic tissues, and all except fibulin-3 have been localized to elastic
fibers by immunoelectron microscopy (8, 18, 19, 27). However, the fine localization of the fibulins within the elastic fibers is
not identical. While fibulin-2 and -4 are present at the interface between the central elastin core and its surrounding fibrillin
microfibrils (8, 18), fibulin-1 is located within the elastin core and fibulin-5 is associated with fibrillin microfibrils (8, 19).
Consistent with these observations, in vitro protein binding studies have shown that all fibulins are capable of binding to
tropoelastin, albeit with different affinities (8, 20, 27), and that
fibulin-2, -4, and -5 interact with the N-terminal region of
fibrillin-1 (5, 6, 18).

The biological roles of most fibulins have been elucidated through studies of gene-targeted mouse models. Fibulin-1-null mice
die perinatally, as a result of massive bleeding associated with abnormal endothelial lining of small blood vessels and
severe defects in the basement membranes of many organs, including the kidneys and lungs (10). There is no apparent
abnormality in elastic fiber formation. Mice deficient in fibu-
lin-3 show early aging and develop multiple large hernias in a
genetic background-dependent manner (12). A reduction in
elastic fibers specifically in fascia connective tissues may ex-
plain the herniation phenotype. Mice lacking either fibulin-4 or
fibulin-5 have highly disrupted and disorganized elastic fibers,
leading to developmental defects in skin, arterial blood vessels,
and lungs (13, 14, 27). Although the elastic fiber abnormalities
are similar in these two mouse mutants, the fibulin-4-null mice are
perinatally lethal, whereas the fibulin-5-deficient mice can
survive until adulthood. The animal models demonstrate that
fibulin-4 and fibulin-5 play essential yet nonredundant roles in
elastic fiber formation during development.

The in vivo function of fibulin-2 remains poorly understood.
Here we report the generation and characterization of mice deficient in fibulin-2. We show that the fibulin-2 null mice develop normally and are phenotypically indistinguishable from their wild-type littermates. Our study indicates that fibulin-2 is dispensable for mouse development and elastic fiber formation, possibly due to functional redundancy with fibulin-1.

**MATERIALS AND METHODS**

**Construction of the targeting vector.** A cosmide clone, T3b, containing the 5’ portion of the mouse fibulin-2 gene was isolated from a 129/Sv genomic library by screening with a mouse fibulin-2 cDNA clone as described previously (7). A gene targeting vector was prepared using a 9.0-kb BamHI fragment isolated from a 129/Sv genomic library by blunt-end ligation, resulting in the SpeI site of exon 2 (black box) by blunt-end ligation, resulting in the deletion of the SpeI site and addition of an EcoRI site located in the SpeI site of exon 2 in the PGKNeo fragment. Restriction sites shown are BamHI (B), EcoRI (E), XhoI (X), HindIII (H), and SpeI (Spe). The translation start site (ATG) and the probe used for Southern blotting are indicated.

**Generation of fibulin-2-deficient mice.** The targeting vector containing a 5.0-kb BamHI fragment isolated from the cosmide, which contains the first coding exon (exon 2 of 1,288 bp) and its flanking introns. A neomycin resistance gene driven by the phosphoglycerate kinase promoter (PGK-Neo) was inserted by blunt-end ligation into the SpeI site of exon 2. The correct targeting event was confirmed by Southern blot analysis using an ES genomic clone as a probe.

**Materials and methods**

**Histological evaluation.** Tissues were embedded in a mixture of EMbed 812, nadic methyl anhydride, dodecenyl ethanol series, followed by propylene oxide, the samples were infiltrated and embedded in paraffin. Sections 5 μm thick were stained with hematoxylin-eosin, Masson’s trichrome collagen stain, or Verhoeff’s elastin stain.

**Northern blot analysis.** Total RNA from mouse embryonic fibroblasts and adult mouse tissues were isolated using the Total RNA kit (Ambion, Austin, TX). Ten micrograms of the RNA samples were electrophoresed on 1% agarose gel containing 6% acrylamide, transferred to a nylon membrane, hybridized with a probe obtained by screening with a mouse fibulin-2 cDNA clone as described previously (7). A probe was prepared by blunt-end ligation of the whole-length mouse fibulin-2 cDNA as a probe (top panel). Ethidium bromide staining of 28S RNA is shown in the bottom panel. (D) Western blot analysis of culture medium from mouse fibroblasts of the three genotypes, using antibodies specific for fibulin-2 and the α1(VI) collagen (Colα1a).

**Elastin content measurement.** Elastin content in fibulin-2 null mice and their wild-type littermates was determined using a Beckman 6300 amino acid analyzer as previously described (3).

**Electron microscopy.** Tissues from 8-μm-thick sections of mouse tissues or fibroblasts grown on chamber slides by the method described elsewhere (26). Primary antibodies included those specific for the five fibulins (1:1,000 dilution) (8, 17), fibrinogen-1 (a generous gift of Lynn Sakai), elastin (PR387; Elastin Product Companies, Owensville, MO), and fibronectin (Sigma, St. Louis, MO). Cy3-conjugated anti-rabbit immunoglobulin G (1:800 dilution; Jackson Immunoresearch Laboratories, West Grove, PA) was used as the secondary antibody. Nuclei were counterstained with 4,6-diamidino-2-phenylindole hydrochloride. Images were captured using a Zeiss Axioskop epifluorescence microscope with a Toshiba 3CCD camera and ImagePro software (Media Cybernetics, Silver Spring, MD).

**Elastin content measurement.** Descending aorta from adult mice were dissected, and the desmosine/isodesmosine contents were determined using a Beckman 6300 amino acid analyzer as previously described (3).

**Electron microscopy.** Descending aorta and back skin were dissected from adult wild-type and fibulin-2 null mice, and samples for transmission electron microscopy were prepared as described previously (2). Briefly, tissues were fixed in 4% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium cacodylate (pH 7.4) with 8.0 mM CaCl2 and then post-fixed with 1% osmium tetroxide (containing 2% tannic acid for aorta samples only). After dehydration in an ethanol series, tissues were post-fixed with 1% osmium tetroxide, then embedded in a mixture of an EMBed 812, nadt methyl acrylate, dodecylbenzene sulfonic acid, and DMP-30 ( Electron Microscopy Sciences, Hatfield, PA). Thin sections were cut using a Reichert UCT ultramicrotome and post-stained with aqueous uranyl acetate followed by phosphotungstic acid or lead citrate.
RESULTS

Generation of fibulin-2-deficient mice. The gene targeting vector was designed to disrupt the fibulin-2 gene by inserting the PGK-Neo cassette after codon 282. Approximately 400 G418-resistant ES clones were screened by Southern blotting with the 3′ external probe, which detected a 17-kb EcoRI band from the wild-type allele and a 10-kb EcoRI band from the targeted allele (Fig. 1A and B). The screening revealed that approximately 10% of the clones were correctly targeted. Four of the targeted ES clones were microinjected into the C57BL/6 blastocysts, and Fbn2+/−/− mice were obtained from chimeric founders from each ES cell clone. Intercrossing of the heterozygous (Fbn2+/−) F1 mice from each ES cell clone yielded offspring of the three genotypes (Fbn2+/+, Fbn2+−/−, and Fbn2−/−) at the expected Mendelian frequencies. Embryonic fibroblasts originating from the Fbn2−/−, Fbn2+/+, and Fbn2+/− littermates were analyzed by Northern and Western blotting. The results confirmed the absence of fibulin-2 mRNA and protein in the Fbn2 null animals and indicated that the heterozygous animals expressed approximately half the amounts of mRNA and protein expressed in the wild-type mice (Fig. 1C and D).

Fibulin-2-deficient mice are fertile and have no gross abnormalities. The Fbn2−/− mice on either a 129S1/SvImJ or a C57BL/6j background did not display any apparent, abnormal phenotype. No statistically significant differences in body weights were observed between the Fbn2+/+, Fbn2+/−, and Fbn2−/− mice, indicating normal growth. Since fibulin-2 expression is specifically associated with cardiovascular morphogenesis (26, 28), anatomical analyses were performed on mice at days 11 and 13 of embryonic development and adult stages (6 to 8 weeks). There were no noticeable differences in the morphology of cardiac valves and major blood vessels between the Fbn2−/− and Fbn2+/+ animals. Inbreeding of Fbn2−/− mice resulted in litters with numbers of litter per litter and pups of normal weight and appearance equal to those of Fbn2+/+ litters. The number of litters produced by the male and female Fbn2−/− animals was indistinguishable from that of their wild-type counterparts. The Fbn2−/− mice had a normal life span, surviving beyond 2 years of age.

Loss of fibulin-2 does not affect elastogenesis. Histological and ultrastructural analyses were carried out with two elastic fiber-enriched organs, aorta and skin, from the adult mice. As shown in Fig. 2, the elastic laminae of the ascending aorta formed normally in the Fbn2−/− mice. Analysis of C57BL/6 Fbn2−/− congenic animals revealed that the number of aortic laminar units in the Fbn2−/− mice was not significantly different from that in the Fbn2+/+ mice. The amounts of cross-linked elastin (pmole desmosine/mg protein), determined by the desmosine contents of the aortae, were comparable between the Fbn2−/− and Fbn2+/+ mice (for Fbn2−/− mice, 47.6 ± 14.4 [n = 5]; for Fbn2+/+ mice, 41.7 ± 10.9 [n = 6]). Ultrastructural examination of the Fbn2−/− aortae by transmission electron microscopy confirmed that the elastic laminae were not disrupted (Fig. 3). The connective tissue layer, consisting mainly of collagen fibrils, between smooth muscle cells and the elastic laminae appeared to be decreased in the Fbn2−/− mice compared to that in the Fbn2+/+ animals. Skin from the Fbn2−/− mice appeared normal by histological staining with hematoxylin/eosin, Masson’s trichrome collagen stain, and Verhoeff’s van Gieson elastin (G and J) stains. Bars = 100 μm.

Altered distribution of fibulin-1 in the fibulin-2-deficient aorta. Cryosections of the skin and descending aorta were immunostained with antibodies specific for elastin, fibrillin-1, and each of the five fibulins. There was no significant difference in the expression of these proteins in the skin of the Fbn2−/− mice and Fbn2+/+ mice. However, immunostaining of aorta sections showed that fibulin-1 expression was significantly increased in the internal elastic lamina of the Fbn2−/− null mice compared to levels for wild-type mice (Fig. 5). In the wild-type aorta, fibulin-2 expression was most prominent in the inner elastic lamina along the endothelial basement membrane and fibulin-1 was evenly distributed in all elastic laminae. The distribution of fibulin-3, -4, and -5 protein in the Fbn2−/− aortae
was unchanged (data not shown). To determine whether fibulin-2-deficient cells deposited more fibulin-1 protein, Fbln2+/−/− and Fbln2+/−/+ embryonic fibroblasts were cultured for 7 days postconfluency and immunostained with antibodies against fibulin-1 and fibulin-2. The fibulin-1 fibrils deposited by fibroblasts from these two genotypes were comparable (Fig. 6).

No compensatory up-regulation of mRNA expression for other fibulin members. To determine whether loss of fibulin-2 results in a change in gene expression of the other fibulins, total RNA extracted from the aorta and skin of the newborn mice was evaluated by Northern blot analyses. The amounts of fibulin-1, -3, -4, and -5 mRNA were not significantly different in the Fbln2+/−/− and Fbln2+/−/+ animals (Fig. 7).

Matrix assembly by fibulin-2 null fibroblasts is not compromised. Previous studies have shown that fibulin-2 can be incorporated into both elastic fibers and the fibronectin matrix (18, 23). To determine whether the absence of fibulin-2 alters the assembly of fibronectin or elastic fibers, embryonic fibroblasts from the Fbln2+/−/− and Fbln2+/−/+ animals grown for 7 days after confluence were immunostained with antibodies against elastin, fibrillin-1, and fibronectin. There were no significant differences in the intensity or pattern of immunostaining of the extracellular matrix produced by fibroblasts from the two genotypes (Fig. 8).

DISCUSSION

We have generated a mouse deficient in fibulin-2 in order to understand its biological role in vivo. Our previous studies demonstrate that fibulin-2 expression is prominent and highly specific at embryonic sites of epithelial-mesenchymal transformation (26, 28, 29). Expansion of the fibulin-2 extracellular network is associated with mesenchymal cells that have migrated into the endocardial cushion tissue, aortic arch vessels,
and coronary vessels during cardiovascular development (26). High levels of fibulin-2 protein expression are also found in developing hair follicles and precartilage condensation sites (29). These observations suggest that fibulin-2 may promote proliferation, differentiation, and migration of mesenchymal cells during organogenesis. It is therefore surprising that a total absence of fibulin-2 does not have discernible effects on embryonic and postnatal development of the mice. In particular, the Fibln2−/− mice do not show developmental delay or abnormalities in the cardiovascular system.

Several lines of evidence suggest that fibulin-2 may have a role in male and female reproduction. A correlation has been found between postnatal development of testis and fibulin-2 expression in the basement membrane of seminiferous tubule in rats (11). Human ovary tissue has been shown to express high levels of fibulin-2 mRNA (30). Moreover, a recent study suggests that fibulin-2 and fibulin-1 may be involved in sequestering sex hormone-binding globulin within the uterine stroma and epididymis, thereby controlling sex-steroid access to target cells (15). However, the results presented here show that both male and female Fibln2−/− mice are fertile, indicating that fibulin-2 is not required for normal reproductive function.

Fibulin-2, like fibulin-4 and -5, binds to tropoelastin and fibillin-1 in vitro and localizes to elastic fibers in vivo (18, 20). However, unlike the case with fibulin-4 or fibulin-5-deficient mice, histological and ultrastructural analyses of the fibulin-2-deficient mice demonstrate that a lack of fibulin-2 does not affect elastic fiber formation in vivo. Consistent with this finding, embryonic fibroblasts deficient in fibulin-2 are capable of depositing the fibrillar matrix of elastin, fibrillin-1, and fibronectin. Since members of the fibulin family display overlapping developmental expression, tissue distribution, and molecular interactions (4, 25), a loss of fibulin-2 likely can be compensated by other family members. In particular, the tissue distribution of fibulin-2 is substantially more restricted than that of the other four fibulins. For instance, in the lung, fibulin-2 is present only in the blood vessels, whereas the other four fibulins are also found, to various extents, in the airways and parenchyma (8). Moreover, the content of fibulin-2 in protein extracts from most organs, as determined by radioimmunoinhibition assays, is considerably less than that of fibulin-1 and -5 and is similar to that of fibulin-3 and -4 (8).

Northern blot analyses show that the mRNA levels of the other four fibulins are not changed for the fibulin-2 null mice from that for controls. On the other hand, immunostaining studies of the fibulin-2 null mice show increased fibulin-1 protein expression in the inner elastic lamina of the aorta, where fibulin-2 is normally localized. This indicates that a loss of fibulin-2 leads to an alteration in the localization of the fibulin-1 protein within the aorta rather than a change in its gene expression. It is possible that in the inner elastic lamina of the fibulin-2 null mice, the fibulin-1 protein is bound to molecules that normally interact with fibulin-2 and is thereby less prone to removal or degradation. Fibulin-2, like fibulin-1 but unlike fibulin-3, -4, and -5, binds fibronectin and several basement membrane and cartilage proteins, including laminin α2 and γ2 chains, nidogen, collagen XVIII, vesican, and aggrecan (8, 16, 21). It is thus conceivable that a loss of fibulin-2 could readily be compensated for by fibulin-1 but not by fibulin-3, -4, and -5. On the other hand, compensation by other fibulin family members, though not detected in this study, cannot be excluded.

Our previous finding that the fibulin-1 null mice display a perinatally lethal phenotype (10) suggests that fibulin-2 is unable to functionally compensate for the loss of fibulin-1. The lack of compensation for fibulin-1 could be explained by the following points. Fibulin-1 is an integral component of all basement membranes, and consequently its expression initiates very early during embryonic development (26). The lethal phenotype of the fibulin-1 null mice results largely from bleeding due to a defective endothelial basement membrane of small but not large blood vessels (10). On the other hand, fibulin-2 has a more restricted expression pattern and is not present in the small blood vessels (26). Its expression during embryogen-
esis initiates substantially later than that of fibulin-1 (26). Moreover, fibulin-2 is significantly less abundant than fibulin-1, present at a level of only 10 to 30% of that of fibulin-1 in various organs (8). Though fibulin-1 and fibulin-2 share similar modular structures and binding interactions, the temporal, spatial, and quantitative expression differences prevent fibulin-2 from serving the full functions of fibulin-1.

In conclusion, our studies demonstrate that fibulin-2 is not essential for development, fertility, and elastogenesis. This could be attributed to functional compensation by other members of the fibulin protein family. Testing this hypothesis will depend on the characterization of mice deficient in two or more fibulins.

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