**FoxC1 Is Essential for Vascular Basement Membrane Integrity and Hyaloid Vessel Morphogenesis**

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**PURPOSE.** Alterations in FOXC1 dosage lead to a spectrum of highly penetrant, ocular anterior segment dysgenesis phenotypes. The most serious outcome is the development of glaucoma, which occurs in 50% to 75% of patients. Therefore, the need to identify specific pathways and genes that interact with FOXC1 to promote glaucoma is great. In this study, the authors investigated the loss of foxC1 in the zebrafish to characterize phenotypes and gene interactions that may impact glaucoma pathogenesis.

**METHODS.** Morpholino knockdown in zebrafish, RNA and protein marker analyses, transgenic reporter lines, and angiography, along with histology and transmission electron microscopy, were used to study foxC1 function and gene interactions.

**RESULTS.** Zebrafish foxC1 genes were expressed dynamically in the developing vasculature and pericellular mesenchyme during development. Multiple ocular and vascular defects were found after the knockdown of foxC1. Defects in the hyaloid vasculature, arteriovenous malformations, and coarctation of the aorta were observed with maximal depletion of foxC1. Partial loss of foxC1 resulted in CNS and ocular hemorrhages, defects in intersegmental vessel patterning, and increased vascular permeability. To investigate the basis for these disruptions, the ultrastructure of foxC1-depleted hyaloid vascular cells was studied. These experiments, along with laminin-111 immuno-reactivity, revealed disruptions in basement membrane integrity. Finally, codepletion of laminin α-1 and foxC1 uncovered a genetic interaction between these genes during development.

**CONCLUSIONS.** Genetic interactions between FOXC1 and basement membrane components influence vascular stability and may impact glaucoma development and increase stroke risk in FOXC1 patients. (Invest Ophthalmol Vis Sci. 2009;50: 5026–5034) DOI:10.1167/iovs.09-3447

Gene dosage changes in FOXC1 result in a spectrum of ocular anterior segment dysgenesis (ASD), including Axenfeld-Rieger anomaly and iridogoniodysgenesis.1-8 All disease forms are developmental, dominantly inherited, and highly penetrant. The most debilitating outcome for ASD patients is the development of glaucoma, which occurs in 50% to 75% of cases and is often refractive to treatment.9-11 Glaucoma is a progressive blinding disease that results from atrophy of the optic nerve and death of retinal ganglion cells. In patients with ASD, increased intraocular pressure (IOP) is the greatest risk factor for glaucoma.12 IOP is regulated by aqueous humor flow in the anterior segment of the eye. Typically, elevated IOP is caused by reduced drainage of aqueous humor. The precise mechanistic relationship between elevated IOP and glaucoma is still unknown. In ASD, development of the outflow structures is compromised, and it is hypothesized that defects in these structures lead to elevated IOP and glaucoma. However, in FOXC1 disease, it is not understood why glaucoma occurs only in some patients despite the high penetrance of ASD phenotypes.

The role of FOXC1 in development has been studied in a number of experimental systems, including cell culture, mice, zebrafish, and frogs. FoxC1 is widely expressed during embryogenesis, but it is not ubiquitous. Mice with null foxC1 mutations show defects in the differentiation of chondrocytes, the renal system, the eyes, and the vascular system.13,14,15 In addition to null mutations, a hypomorphic allele revealed later developmental requirements of FOXC1.14 Defects of later onset include cortical dyslamination and skull anomalies. Interestingly, the cortical defects were suggested to be secondary to altered meningeal differentiation. Haploinsufficiency of FoxC1 in mice has also been used to model human disease. Interestingly, ASD phenotypes were found only in specific mouse strains, whereas other genetic backgrounds did not show observable defects.15 These observations reinforce the role of genetic modifiers on FoxC1-dependent phenotypes.

A similar distribution of phenotypic defects was found with loss of the closely related gene Foxc2. FoxC1 and Foxc2 share nearly identical DNA binding domains and developmental expression patterns. Compound mutant analysis in mice revealed redundant functions between these factors.15-18 Vascular phenotypes of FoxC1/Foxc2 double knockout mice included arteriovenous fates defects and vessel morphogenesis and remodeling anomalies.17,19-21 In the zebrafish, foxC1 is duplicated, and there is no evidence for a foxC2 homolog.22 However, the combined expression of foxC1a and foxC1b during zebrafish embryogenesis is similar to that of FoxC1 and FoxC2 in higher vertebrates,22,23 suggesting that zebrafish foxC1b is the functional homolog of mammalian FoxC2.

In this study, we explored the mechanisms of the eye and vascular phenotypes associated with loss of foxC1 function in zebrafish. Overall, the depletion of foxC1 resulted in multiple, dose-sensitive vascular phenotypes. In particular, we found that loss of foxC1 results in defects to vascular basement membrane integrity. These studies provide insight into the mechanisms for how altered FoxC1 function can affect glaucoma and suggest that FOXC1 mutations in humans may also increase the risk for stroke.
**Materials and Methods**

**Specimens**
Wild-type zebrafish (*Danio rerio*) of the AB/AB and LF/LF backgrounds were reared under standard conditions with a light cycle of 14 hours light/10 hours dark. Before experimental manipulation or tissue fixation, fish were anesthetized in 0.2 mg/mL ethyl 3-aminobenzoate methanesulfonate (tricaine). All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**In Situ Hybridization**
Probes against zebrafish foxC1a (foxC1.1), foxC1b (foxC1.2), and flt4 were produced as previously described.22–24 Antisense RNA probes were produced and, when holomeshed in situ hybridization was conducted as previously described.25–26 Ten to 15 embryos, treated with 0.003% phenylthiouracil (PTU), were analyzed in each in situ experiment. PTU inhibits melanin synthesis. For posthybridization sectioning, embryos were fixed in 4% paraformaldehyde/PBS and infiltrated with 15% sucrose, 30% sucrose, and 100% embedding medium (Tissue-Tek OCT; Miles Inc., Elkhart, IN). Embryos were oriented in a freezing mold, and 10-μm sections were cut on a cryostat and mounted on gelatin-coated glass slides.

**Morpholino Knockdown**
Previously described antisense morpholinos (MOs) used for knockdown were ordered from Gene Tools (Philomath, OR), resuspended in sterile water, and injected into one to four cell-stage embryos. MOs used were (Start codon underlined): foxC1a MO2 (GeneID 79374), 5'- CCTGTACGCTGGCTCCTACAAAACGG-3'; foxC1b MO1 (GeneID 79375), 5'- GCATGCATCACCCCTTCCTCGGTACA-3'; lammintin a-1 MO1 (GeneID 77995535), 5'- ATTAAAGCTAAAGCTGTGCTGAAATC-3'; control MO, 5'- CCTCTTACCTCAGTTACAATTTATA-3'. Embryos injected with the individual morpholinos plus control MOs used to verify phenotypes were: foxC1a MO1, 5'- GTCAAGAAAGACTGAAGCAATCCACA-3'; foxC1b MO1, 5'- AAGTGAATGAACTACGTCAGGG-3'.

**Transmission Electron Microscopy and Histologic Analysis**
Histologic speciments for light microscopy were processed as previously described.30 In brief, embryos were fixed in primary fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 3% sucrose, 0.06% phosphate buffer [pH 7.4]) at 4°C for 24 hours and then were washed in 0.1 M PBS, dehydrated through an ethanol series and propylene oxide, and infiltrated with a resin mixture (EMbed-812/Araldite; Electron Microscopy Sciences, Hatfield, PA). Semithin plastic sections were cut with a glass knife on a JB4 microtome and stained with 1% toluidine blue in 1% sodium borate. For transmission electron microscopy (TEM), an additional fixation in 1.0% osmium was included, followed by dehydration in MeOH/araldite. Embryos were then embedded in fixative (EMBed-812/DER 736; Electron Microscope Sciences). Ultrathin sections (60–70 nm) were collected on coated grids and stained with uranyl acetate and lead citrate for contrast. Images were captured with a transmission electron microscope (H600 TEM; Hitachi, Tokyo, Japan).

**Frozen Section Immunohistochemistry**
Wild-type and morphant embryos were fixed in 4% paraformaldehyde/PBS and infiltrated with 2-hour steps of 15% sucrose, 30% sucrose, and 100% embedding medium (Tissue-Tek OCT; Miles Inc.). Embryos were oriented, sectioned, and mounted on gelatin-coated glass slides. Antibodies that recognize laminin-111 (Laminin-1 epitope, L9395; 1:200 dilution; Sigma, St. Louis, MO) were used in a 5% goat serum/PBT blocking solution. This antibody was raised against purified basement membranes and affinity purified against entire laminin-111 heterotrimer. Immunoreactivity was detected with a fluorescent secondary antibody and imaged with a confocal microscope (C1; Nikon, Tokyo, Japan).

**Microangiography**
Fluorescently labeled 2-MDa dextrans were injected into the tail vein or sinus venosus of 2 to 5 days postfertilization (dpf). PTU-treated, anesthetized Tg(fli1a:GFP) zebrafish embryos, as previously described.31 Embryos were maintained in 1% low-melt agarose, and injections were made (Nanoject; Drummond Scientific Co., Broomall, PA) with micropipettes cut to a bore size of 5 to 10 μM. Distribution of dextrans throughout the vasculature was monitored through a fluorescent dissecting microscope, and imaging was performed with a confocal microscope (C1; Nikon).

**Results**

**Expression of foxC1a and foxC1b in Periocular Mesenchyme and Vasculature**
Expression of foxC1a and foxC1b was assessed during development by in situ hybridization. As previously reported, we found both genes expressed in the paraxial mesoderm, head mesenchyme, and vascular system before 24 hours postfertilization (hpf; data not shown).22 By 24 hpf, strong expression of foxC1a was found in the periocular mesenchyme (Figs. 1A, 1C). Levels in the periocular mesenchyme peaked between 24 and 36 hpf and then gradually decreased over time (Figs. 1A, 1C). In the developing hyaloid vasculature, levels of foxC1a expression correlated with expression in the periocular mesenchyme (Fig. 1C). Expression of foxC1a was also maintained in endothelial cells of the developing trunk vasculature (Fig. 1D). From 72 to 96 hpf, the strongest expression of foxC1a was found in the branchial arches (Figs. 1A, 1C). foxC1b showed both overlapping and distinct patterns and timing of expression compared with foxC1a. In the periocular mesenchyme, expression lagged behind foxC1a, with levels not detectable until 36 hpf but then persisting later during development (Figs. 1B, 1C). Expression in trunk vascular endothelial cells was evident at all times assessed, though levels in the hyaloid were below detection by wholeomeshed in situ hybridization (Figs. 1B–D). Overall, the strongest expression of foxC1b was in the branchial arch region and the fin buds (Figs. 1B, 1C).

**Vascular Defects Due to Loss of foxC1**
To address loss-of-function phenotypes during zebrafish development, morpholinos were used to knock down both foxC1 genes. The morpholinos used were originally described to assess phenotypes during early somitogenesis (<18 hpf).27 For both foxC1a and foxC1b, two independent morpholinos were designed to specifically target each gene. Because each gene contains only one exon, morpholinos were designed to target the ATG start codon and the 5′-untranslated regions. For both genes, injection of either morpholino resulted in similar phenotypes. The morpholinos targeting the ATG start codons were effective at lower doses, and these were used for the remainder of our studies. Phenotypes were unchanged when a morpholino targeting p53 was coinjected, indicating the morpholinos do not promote phenotypes through off-target–mediated cell death.32 Knock-down of foxC1a resulted in a number of gross phenotypes, including hydrocephaly, heart edema, small eye, and variable hemorrhage within the CNS and eyes (Fig. 2A). When foxC1b was targeted, no obvious phenotypes were observed except a slight developmental delay common with morpholino injections (Fig. 2A). Based on gene similarity, we hypothesized that the lack of gross phenotypes might have been attributed to the compensation by foxC1a. This also raised the possibility that foxC1b may compensate for some degree for the loss of foxC1a. To address this, morpholinos targeting both genes were coinjected and compared with embryos injected with the individual morpholinos plus control morpholino to maintain equal total morpholino concentra-
Disruption of Vascular Endothelial Fate and Morphology in foxC1 Morphant Embryos

To further investigate the vascular phenotypes in foxC1 morphants, knockdown experiments were conducted in the vascular endothelial reporter transgenic line Tg(fli1a:EGFP). This transgenic line allows visualization of the entire vasculature throughout development. A number of interesting phenotypes were observed with injection of the high morpholino dose described. Confocal imaging of the dorsal aorta and cardinal vein revealed inappropriate luminal connections throughout the tail, suggestive of arteriovenous cell fate defects (Fig.

**Figure 1.** Expression of foxC1a and foxC1b. (A) Wholemount expression of foxC1a during development. Strong expression was found in the pericardial mesenchyme surrounding the eye at 24 to 36 hpf. Expression was observed in the branchial arch region (arrows) and in the pericardial mesenchyme at 36, 48, and 96 hpf. (B) Wholemount expression of foxC1b during development. High levels were found in the branchial arches (arrows) throughout development and in the fin buds (arrowheads) at 24 to 48 hpf. Pericardial mesenchyme expression was absent at 24 hpf but increased thereafter. (C) Sections through the eyes showed dynamic expression in the pericardial mesenchyme for both foxC1a and foxC1b. Expression of foxC1a was also found in the hyaloid vasculature (arrows). (D) Schematic of the zebrafish trunk vasculature (left) and expression of foxC1a and foxC1b in vascular endothelial cells of the dorsal aorta and the cardinal vein at 36 hpf. SC, spinal cord; NC, notochord; DA, dorsal aorta; CV, cardinal vein.
FIGURE 2. Gross phenotypes of foxC1 morphants. (A) Comparison of control (Cntl) MO, foxC1a + Cntl MO, foxC1b + Cntl MO, and foxCa+b double morphants (foxC1dMO) at 48 hpf. Hydrocephaly, heart edema, and small eyes were found in foxC1a morphants. Only a slight delay was found with injection of foxC1b morpholino. Severity of phenotypes found in foxC1a morphants increased in foxC1dMO. (B) Quantification of phenotypes after injection of high-dose foxC1 morpholinos. Heart rates represent an average of five embryos, rounded to the nearest integer. Blood flow indicates that blood cells were directly observed flowing throughout the vasculature of the embryo. (C) Dose-dependent phenotypes of foxC1dMO in 48 hpf PTU-treated embryos: one control morphant (upper left), one high-dose foxC1dMO embryo (3.25 ng each morpholino; upper right), and two lower-dose foxC1dMO embryos (1.63 ng each morpholino). At high doses, blood flow and hemorrhaging were only occasionally observed. At lower doses, both blood flow and CNS or ocular hemorrhaging was found in the majority of embryos (arrowsheads). (D) Quantification of observed phenotypes with changing dosages of foxC1 morpholino.

4A). Consistent with this interpretation, the venous marker flt4 showed ectopic expression in vessels of the dorsal aorta (Fig. 4B). Analysis of vessels in the anterior embryo also showed disruption. In this region, the paired lateral dorsal aortae normally join to form the single midline dorsal aorta (Fig. 4A). Embryos depleted of foxC1 showed thin and disorganized vessels in this region (Fig. 4A). The use of the Tg(fli1a:GFP) line also helped resolve defects in the hyaloid vasculature. By 48 hpf in control embryos, an organized vascular basket envelops the lens (Fig. 4A). In some foxC1dMO morphants, fli1a:GFP-positive cells were present but collected in an undifferentiated mass behind the lens (Fig. 4A). In other morphant embryos, hyaloid vessel differentiation did occur, but, as suggested by histologic examination, resulted in a less branched and dilated morphology (Fig. 4C). Expression of flt4 in the hyaloid was similar in foxC1dMO morphants and control embryos (Fig. 4B), indicating that the cellular defects did not result from altered endothelial cell fate determination but from disrupted differentiation.

Vascular Basement Membrane Defects Due to Loss of foxC1

To explore the observed vascular phenotypes in more depth, hyaloid vessels from high-dose foxC1dMO embryos were analyzed by TEM. In foxC1dMO embryos, endothelial cells were dilated but showed normal morphology and cell-cell junctions compared with controls (Fig. 6). As predicted by histology, undifferentiated mesenchymal cells were also often found behind the lenses of foxC1dMO embryos (Fig. 6E). In addition to mesenchymal cell differentiation and vessel morphology defects in foxC1dMO eyes, TEM revealed disruptions to basement membrane between hyaloid vessels and both the lens and the
retina (Fig. 6B). In controls, a smooth, continuous basement membrane was present along the entire basal surface of the lens and the retina (Figs. 6F, 6H, 6J, 6L). In foxC1 dMOs, the basement membrane was disorganized and showed focal disruptions (Figs. 6G, 6L, 6K, 6M).

To confirm the basement membrane disruptions in foxC1 dMOs, immunostaining for laminin-111 was performed. Laminins, along with type IV collagens, nidogen, and sulfated proteoglycans make up the normal components of all basement membranes. Laminin-111 is a heterotrimeric glycoprotein composed of one alpha, one beta, and one gamma chain. In control embryos, a smooth, uninterrupted staining pattern was observed along both the basal surface of the retina and the lens

(FIG. 6F–M). In contrast, laminin-111 staining was more diffuse with interruptions along the lens and retinal surfaces in foxC1dMO eyes (Fig. 7).

**Genetic Interaction Shown by foxC1 and laminin α-1**

Many of the vascular defects in foxC1dMO embryos are reminiscent of vascular anomalies caused by loss of laminin α-1 (lama1) function. The lama1 gene encodes for the alpha subunit of the laminin-111 complex. To investigate the relationship between foxC1 and the basement membrane component lama1, zebrafish embryos were coinjected with morpholinos targeting the foxC1 genes and lama1. lama1 has been previously investigated in the zebrafish, and homozygous mutations in this gene (bashful) or morpholino knockdown result in lens degeneration, hyaloid and trunk vascular defects, and body truncations. When previously described concentrations of this morpholino were coinjected with high or low doses of the foxC1 morpholinos described, severe body truncation phenotypes were observed (data not shown). To further investigate the combined effects of foxC1 and lama1 knockdown, submaximal doses of all morpholinos were injected. Very mild hydrocephalic and hemorrhaging phenotypes were observed when 1.3 ng each foxC1 morpholino was injected alone (Figs. 8A, 8B). When 3.9 ng lama1 morpholino was coinjected with these concentrations of foxC1dMO, the incidence and severity of both hemorrhaging and hydrocephaly were increased (8. 8A, B). This concentration of lama1 morpholino injected alone showed only occasional, mild lens and tail defects, and a small number of embryos had mild hydrocephaly and hemorrhage (Figs. 8A, 8B). Finally, we also studied the knockdown of foxC1 in homozygous bashful/ lama1 mutants (balwin). Injection of submaximal foxC1dMO into these embryos resulted in more severe phenotypes than mutants alone, including increased severity of the tail and lens phenotypes and a more pronounced hydrocephaly (data not shown). We also tested foxC1 knockdown in embryos heterozygous for the balwin mutation but did not observe any differences compared with the wild-type siblings.

**Discussion**

In this study we describe the effects of foxC1 knockdown on zebrafish larvae. We found that the overall spectrum of phenotypes attributed to loss of foxC1 in the zebrafish is similar to that found with loss of FOXC1 in mammals, indicating a conservation of function across species. We focused our investigation on how the loss of foxC1 impacts ocular and CNS vascular development. Knockdown of foxC1 resulted in defects in vascular endothelial cell fate and differentiation and disrupted vessel morphology and integrity. Interestingly, we found that loss of foxC1 leads to the disruption of vascular basement membrane structure and that foxC1 genetically interacts with lama1, which encodes a key component of the basement membrane.

In mouse, combined deletion of Foxc1 and Foxc2 results in severe vascular phenotypes, including defects in arteriovenous fate decisions, concomitant arteriovenous malformations, and aorta coarctation. Arterial versus venous specification occurs by differential VEGF activity and subsequent reciprocal Eph/Ephrin signaling (reviewed in Ref. 37). EphrinB2 expression in arterial cells occurs by activation of the Notch pathway and Hey2 activation. FOXC1 and FOXC2 have been implicated in multiple steps in this pathway, and both have been shown to directly activate Notch target genes in response to VEGF signaling. In our studies, we found similar arteriovenous defects in severe loss of foxC1dMO embryos. In addition to expansion of the venous marker flt4, arteriovenous malforma-

**Figure 3.** Assessment of ocular and CNS development in foxC1 dMO. (A) Histologic examination of 48 hpf WT and foxC1 dMO. Sagittal sections of the brain and eye show expanded territories of the brain (asterisk) and the loosely organized perioocular mesenchyme (arrows) in foxC1 dMOs. Areas of cell loss are also noted along the ventricles within the brain (arrowhead). High magnification of the cornea and anterior lens shows the corneal endothelium is absent and defects in corneal stroma (arrowbeads) are found in foxC1 dMOs. High magnification of the iridio-corneal angle shows undifferentiated and poorly organized perioocular cells (arrows) in foxC1 dMOs. High magnification of the posterior lens and hyaloid vasculature shows fewer dilated hyaloid vessels behind the lens (arrows) in foxC1 dMOs. (B) Analysis of foxD3-positive neural crest cells in 48 hpf foxC1 dMOs. Fewer foxD3-GFP-positive cells were found migrating to the anterior of the eye. Cells that have migrated show abnormal morphology compared with controls.
tions were revealed by fli1a:GFP. Vessel defects in the tails and the aorta coarctation of foxC1 dMO embryos are similar to those described for zebrafish gridlock mutants, in which Hey2 function is disrupted.31 Defects in the trunk vessels and aorta help explain the lack of proper blood flow found in severely affected foxC1 dMO embryos.

Although foxC1 is important for arterial specification in the trunk vessels, it is unclear whether this also accounts for the severe phenotypes observed in the hyaloid vasculature. In mammals, the hyaloid is an entirely arterial system and is devoid of veins.38 This led to an initial hypothesis that defects in arterial specification may cause the hyaloid defects in foxC1 dMO embryos. However, in zebrafish, the venous marker flt4 was expressed in both wild-type and foxC1 dMO hyaloid vessels, indicating differences exist in the zebrafish hyaloid compared with mammals. This may be indicative of the different fates these vascular beds have in zebrafish and mammals. The hyaloid system is a transient structure in mammals, and its regression is required for proper retinal vessel development (reviewed in Ref. 38). In contrast, the zebrafish hyaloid system does not regress but instead migrates and grows with the retina to form the mature vitreoretinal vascular system.35

Although endothelial cell fate defects cannot explain the hyaloid defects in foxC1 morphants, clues were provided by additional experiments. Fluorescent dextrans injected into the vasculature of foxC1 dMO embryos showed that vessel integrity was compromised and permeability was increased. We hypothesized this might have resulted from defects in endothelial

**Figure 4.** Vascular phenotypes in high-dose foxC1 dMO embryos. (A) Knockdown of foxC1 in 48 hpf Tg(fli1a:GFP) embryos. Defects in dorsal aorta development, axial vessel development, and hyaloid vasculature were found in foxC1 dMOS. Dorsal aortae vessels (arrows) are dysmorphic preceding their fusion (asterisk). In the trunk region, arteriovenous malformations (AVM) are found between the cardinal vein (CV) and the dorsal aorta (DA) in foxC1 dMOS. Vascular endothelial cells are present in the hyaloid of foxC1 dMOS, but are less organized than wild-type vessels. (B) Expression of the venous marker flt4 was found expanded into the region of the dorsal aorta in foxC1 dMO embryos (upper). See Figure 1D for schematic of anatomy. No differences were observed in flt4 expression between foxC1 dMOS and WT within the hyaloid vasculature (lower). (C) Microangiography in foxC1 dMO, Tg(fli1a:GFP) embryos revealed defects in vessel integrity, as shown by breakage of vessels and leakage of dye into the yolk (left, arrows). Increased permeability in CNS vessels as revealed by microangiography (center, asterisk). Dye also flowed behind the eye but was enclosed by fli1a:GFP-positive cells (right).

**Figure 5.** Vascular phenotypes in low-dose foxC1 dMO embryos. (A) Hyaloid vasculature as revealed by fli1a:GFP in wild-type (left) and in low-dose foxC1 dMO morphant embryos (right) at 48 hpf. No differences in overall morphology were noted in low-dose morphants. (B) Intersegmental vessels in wild-type (left) and low-dose foxC1 dMO morphant (right) embryos at 48 hpf. No differences in overall morphology were noted in low-dose morphants. (C) Intersegmental vessels in wild-type (left) and low-dose foxC1 dMO morphant embryos (right) at 4 dpf. Note the increased leakage of dye into the anterior chamber of foxC1 dMO eyes.
cell-cell junction formation. Indeed, junctional complexes are disrupted in the cornea of Foxc1 knockout mice. However, TEM analysis of vessels in foxC1 dMOs showed morphologically normal interendothelial cell junctions. Ultrastructural analyses did reveal disruptions in the basement membranes between hyaloid vessels and the lens and retina. Staining for laminin-111 in foxC1 dMO embryos confirmed basement membrane defects. Given that lama1 mutants show defects in the development of the hyaloid and trunk vascular system similar to those of foxC1 morphants, we also explored the genetic interaction between foxC1 and lama1 genes. Combined submaximal knockdown of foxC1 and lama1 genes resulted in vascular phenotypes similar to those observed in full-dose, severe foxC1 dMO embryos. These data suggest that FoxC1 regulates factor(s) essential for normal basement membrane formation or maintenance.

We posit that basement membrane defects result in increased permeability and hemorrhaging in CNS vessels that were observed on tracer dye injection. Basal membrane defects may also disrupt proper differentiation of vascular cells in the hyaloid, leading to the dilated vessel morphology and accumulation of undifferentiated pericellular mesenchymal cells behind the lens in foxC1 morphants. The delay in retinal lamination found in foxC1 dMO eyes may also be explained by the lack of a mature basement membrane on the basal surface of the retina. This is supported by the recent finding that focal disruptions in meningeal basement membrane result in lamination defects in the cortex of the mouse Foxc1 hypomorph. Additionally, zebrafish mutant for either pdk2a or β-pix results in increased permeability and hemorrhaging in hyaloid vasculature in 48 hpf foxC1 dMO embryos. Hyaloid vascular endothelial cells (black arrowheads) in wild-type (A, C) and foxC1 dMO eyes (B, D). In some foxC1 dMO embryos, undifferentiated mesenchymal cells (E; black asterisks) occupied the space where vessels normally reside. Vascular endothelial cells (black arrows) associated with the basal surfaces of the lens cells in wild-type (F) or foxC1 dMO embryos (G). Vascular endothelial cells (black arrows) associated with the basal surface of the retina in wild-type (H) or foxC1 dMO embryos (I). (I, asterisk) Undifferentiated mesenchymal cell. Boxed areas: magnified regions (J–M). Basement membranes (white arrowheads) or disorganized extracellular matrix (white asterisk) associated with the lens cells (J, K) or the ILM of the retina (L, M). Note the discontinuous and disorganized basement membranes associated with foxC1 dMO vascular cells. Scale bars: (A, B) 10 μm; (C–E) 4 μm; (F–I) 0.5 μm. RGC, retinal ganglion cell; ILM, inner limiting membrane of the retina; a, artifact (section fold).
in hydrocephalus and CNS hemorrhaging, similar to that found in foxC1dMO embryos.59–61 pak2a and β-pix function together downstream of integrin signaling and were shown to be required for proper maintenance of the basement membrane surrounding CNS vessels.62–64 Cumulatively, these findings suggest that defects in the production or maintenance of basement membrane components may underlie aspects of vascular defects found with the loss of foxC1.

The disruption of basement membrane integrity in foxC1 morphants provides insight into FOXC1 disease phenotypes. Vascular basement membrane defects have implications for the development of glaucoma in foxC1 patients. Integrity of retinal vessels is important to maintain the blood-retinal barrier, and defects affecting vascular permeability are known to impact glaucoma (reviewed in Ref. 40). Vascular tone and permeability are also under the influence of systemic factors, both environmental and genetic, and this may represent a focal point at which FOXC1 disease can be modified. Finally, mutations in the basement membrane constituent collagen IV alpha 1 (COL4A1) correlate with an increased incidence of stroke and are associated with Axenfeld-Rieger anomaly in humans.42–46 Indeed, one recognized cause of stroke is the localized breakdown of vessel integrity, and COL4A1 knockout mice show hemorrhagic stroke and ASD phenotypes.44–47 Neither increased mortality rates in foxC1 patients nor potential relationships between FOXC1 mutations and vascular compromise have been reported, but together these data suggest that FOXC1 patients may have an increased risk for stroke.

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References


