SEE COMMENTARY

Forkhead box transcription factor FoxC1 preserves corneal transparency by regulating vascular growth

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Normal vision requires the precise control of vascular growth to maintain corneal transparency. Here we provide evidence for a unique mechanism by which the Forkhead box transcription factor FoxC1 regulates corneal vascular development. Murine Foxc1 is essential for development of the ocular anterior segment, and in humans, mutations have been identified in Axenfeld–Rieger syndrome, a disorder characterized by anterior segment dysgenesis. We show that *FOXC1* mutations also lead to corneal angiogenesis, and that mice homozygous for either a global (*Foxc1*$^{-/-}$) or neural crest (NC)-specific (NC-*Foxc1*$^{-/-}$) null mutation display excessive growth of corneal blood and lymphatic vessels. This is associated with disorganization of the extracellular matrix and increased expression of multiple matrix metalloproteinases. Heterozygous mutants (*Foxc1*$^{+/-}$ and NC-*Foxc1*$^{+/-}$) exhibit milder phenotypes, such as disrupted limbal vasculature. Moreover, environmental exposure to corneal injury significantly increases growth of both blood and lymphatic vessels in both *Foxc1*$^{+/-}$ and NC-*Foxc1*$^{+/-}$ mice compared with controls. Notably, this amplification of the angiogenic response is abolished by inhibition of VEGF receptor 2. Collectively, these findings identify a role for FoxC1 in inhibiting corneal angiogenesis, thereby maintaining corneal transparency by regulating VEGF signaling.

avascularity | soluble form of VEGF receptor 1 | VEGF bioavailablility

Pathological angiogenesis occurs in a wide range of disorders (1), including highly prevalent causes of blindness (2–4). In the cornea, such neovascularization or abnormal growth of blood vessels from the limbus into the adjacent (normally avascular) corneal stroma is sight-threatening (5–7). Thus, an adequate understanding of the mechanisms by which the stroma impedes vascular growth and maintains corneal transparency is important for vision and the development of new therapies. Furthermore, the avascular nature of the cornea provides an excellent model for studying the regulation of angiogenesis, offering insights potentially applicable to multiple diseases and tissues. Here we present findings from human and murine studies of Forkhead box (Fox) transcription factor FoxC1* that reveal a novel mechanism of regulating corneal avascularity.

To better understand the molecular and cellular mechanisms that control abnormal vascular growth in disease, the roles of angiogenic factors such as VEGF have been studied intensively (8). This led to recognition that in the eye, expression of a soluble form of VEGF receptor 1 (sVEGFR-1) represents a potent natural inhibitor of corneal angiogenesis (9). More broadly, numerous clinical trials are underway to investigate the efficacy of antiangiogenic agents that block VEGF signaling, and several angiogenesis inhibitors have been approved for oncologic and ocular disease therapy (10). Although neovascularization is known to be tightly regulated by a balance between proangiogenic and antiangiogenic factors, the precise mechanism(s) by which pathological angiogenesis occurs in multiple diseases, including the angiogenically privileged cornea, remain incompletely understood.

Our study centers on FoxC1, a member of the large Forkhead box transcription factor family, which has essential roles in development and disease. *Fox* genes regulate an array of fundamental processes, including cell fate determination, proliferation, and differentiation (11–14). Of note, some family members, including Foxc1, have recently been implicated in vascular development (15, 16). These genes' closely related functions in multiple species (13, 14) correspond with evolutionary conservation of the forkhead (DNA binding) domain from yeast to humans and illustrate the value of model organism analyses for characterizing human disease mechanisms. Such approaches allowed identification of common causative pathways for the two main phenotypes thus far attributed to *FOXC1* mutation or gene dosage changes: Axenfeld–Rieger syndrome (ARS) and cerebellar/posterior fossa malformations. Perturbed neural crest (NC) function is central to both groups of disorders (17, 18), with the central nervous system malformations secondary to altered retinoic acid signaling from NC-derived meninges (19–21).

ARS comprises a range of malformations affecting primarily the iris and the iridocorneal angle, through which the aqueous humor drains (22–26), with comparable phenotypes observed in heterozygous *Foxc1* null mutant (*Foxc1*$^{+/-}$) mice (27). Homozygous null mutant (*Foxc1*$^{-/-}$) mice exhibit more severe anomalies, including disorganization of the corneal stromal extracellular matrix (ECM) and failure of corneal endothelial and anterior chamber formation (28, 29). These severe corneal phenotypes correspond to initial Foxc1 expression in presumptive corneal mesenchyme cells (located between the corneal epithelium and the lens) and, subsequently, in stromal cells. Of note, the great majority of these Foxc1-expressing cells are NC-derived (30).

Corneal transparency, which is an essential prerequisite for normal vision, requires precise control and inhibition of blood vessel growth. Here we present findings from human and murine studies that have identified FoxC1 as a key component of the mechanisms that maintain corneal avascularity. We report that patients with *FOXC1*-attributable ARS have pathological corneal angiogenesis, comparable to the anterior segment anomaly and angiogenesis phenotypes present in mice carrying either conventional null mutations (global *Foxc1*$^{-/-}$) or *Foxc1*-null mutations confined to the NC cells (NC-specific *Foxc1*$^{-/-}$), which give rise to

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*Gene names are in all uppercase letters for human genes (e.g., FOXC1). Only the first letter is capitalized for mouse genes (e.g., Foxc1), and the first and subclass letters are capitalized for all chordates (e.g., FoxC1) (55).

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the corneal stroma. Although homozygous *Foxc1* murine mutants die at birth, their corneas are extensively vascularized and have perturbed ECM and elevated levels of several proangiogenic matrix metalloproteinases (MMPs). Heterozygous mutant mice survived through adulthood, and although their corneas were avascular, their limbal vasculature was disrupted, and the growth of vessels after corneal alkali burn injury was enhanced. This amplification of the angiogenic response was abolished by inhibition of VEGF signaling. These results identify a previously unknown mechanism by which FoxC1 regulates vessel growth, and suggest that targeting FoxC1 may represent a useful strategy for developing novel therapies to combat blindness as well as other diseases involving pathological angiogenesis.

Results

***FOXC1*-Attributable ARS Is Associated with Corneal Angiogenesis.** Here we demonstrate that patients with ARS attributable to either *FOXC1* mutation or altered dosage exhibit corneal neovascularization. Affected individuals from an ARS pedigree secondary to a 29-kb *FOXC1*-encompassing deletion (19, 31) (Fig. S1) had profound perinatal corneal vascularization, which almost necessitated corneal transplantation before partial involution occurred (Fig. 1 *B*, *C*, *E*, and *F* and Fig. S1). Affected individuals from a pedigree with a *FOXC1* frameshift mutation (p.P274RfsX41) that results in early termination of the protein exhibited corneal vascularization (Fig. 1*G* and Fig. S1). Comparable, but milder, neovascularization was observed in patients with ARS and a 492-kb duplication that encompassed *FOXC1* and the adjacent Forkhead gene *FOXF2* (19, 31) (Fig. 1 *H* and *J* and Fig. S1), as well as in occasional patients with duplications of *FOXC1* alone. Thus, perturbed FOXC1 function attributable to either mutation or to increased or decreased gene copy number can lead to pathological corneal angiogenesis in humans, which is consistent with the essential role of *FOXC1* gene dosage in normal ocular and central nervous system development (19, 31).

Foxc1 Is Required for NC-Dependent Formation of the Ocular Anterior Segment. Recent murine cell fate mapping studies show that the majority of corneal stromal and endothelial cells are NC-derived (30, 32), as are the great majority of Foxc1-expressing cells in the eye (30). Thus, we began our investigation of the cell type-specific functions of *Foxc1* during embryonic development by generating mice with a conditional, NC-specific, *Foxc1*-null mutation (*Wnt1-Cre;Foxc1*$^{F/F}$). These mice exhibit a 97% reduction in Foxc1 expression compared with controls (*Foxc1*$^{F/F}$) (Fig. S2*A*). Mice heterozygous for the NC-specific *Foxc1*-null mutation (NC-*Foxc1*$^{+/-}$) survived to adulthood, but some (5 of 11) had abnormally shaped and/or positioned pupils (Fig. 2*A*), reminiscent of ARS patients with heterozygous *FOXC1* mutations. NC-*Foxc1*$^{-/-}$ mice died postnatally with hydrocephalus and craniofacial abnormalities comparable to those seen in conventional *Foxc1*$^{-/-}$ mice (29).

Although NC cells migrate normally to the eyes of NC-*Foxc1*$^{+/-}$ and NC-*Foxc1*$^{-/-}$ embryos (Fig. S3), collagen matrix accumulation and anterior segment formation is severely impaired (Fig. 2*B*). Ultrastructural analysis of the corneal stroma of NC-*Foxc1*$^{-/-}$ mice revealed abnormally shaped and less-ordered mesenchymal cells, with collagen deposited in swirls (Fig. 2*C*). Furthermore, whereas mutant corneal epithelium remains positive for keratin 14, alterations in NC-*Foxc1*$^{-/-}$ mice include failure of corneal endothelial formation (i.e., absence of *N*-cadherin^{+} cells), an absent anterior chamber, severely disorganized and thickened corneal stroma (Fig. S4*A*), hypoplasia of the prospective trabecular meshwork and iris (Fig. S4 *B* and *C*), and impaired iris smooth muscle differentiation (i.e., decreased staining of smooth muscle α-actin) (Fig. S4*D*), all of which are in agreement with findings in conventional *Foxc1*$^{-/-}$ mutants (28, 29). These data demonstrate that Foxc1 is essential for NC-dependent formation of the ocular anterior segment.

Foxc1 in the NC Is Required to Maintain Corneal Transparency by Preventing Blood and Lymphatic Vessel Growth. The developmental anomalies observed in NC-specific and conventional *Foxc1*-null mice were accompanied by corneal neovascularization. Ectopic outgrowth of CD31^{+} blood vessels from limbal vessels was observed in NC-*Foxc1*$^{-/-}$ (Fig. S5*A* and Fig. 3*A*) and *Foxc1*$^{-/-}$ (Fig. S5*B*) embryos starting at embryonic day 14.5 (E14.5). In NC-*Foxc1*$^{-/-}$ embryos, the blood vessels formed a fine vascular network across the entire cornea by postnatal day 0 (P0), and limbal vessels were severely disrupted (Fig. 3*A*). NC-*Foxc1*$^{+/-}$ mice

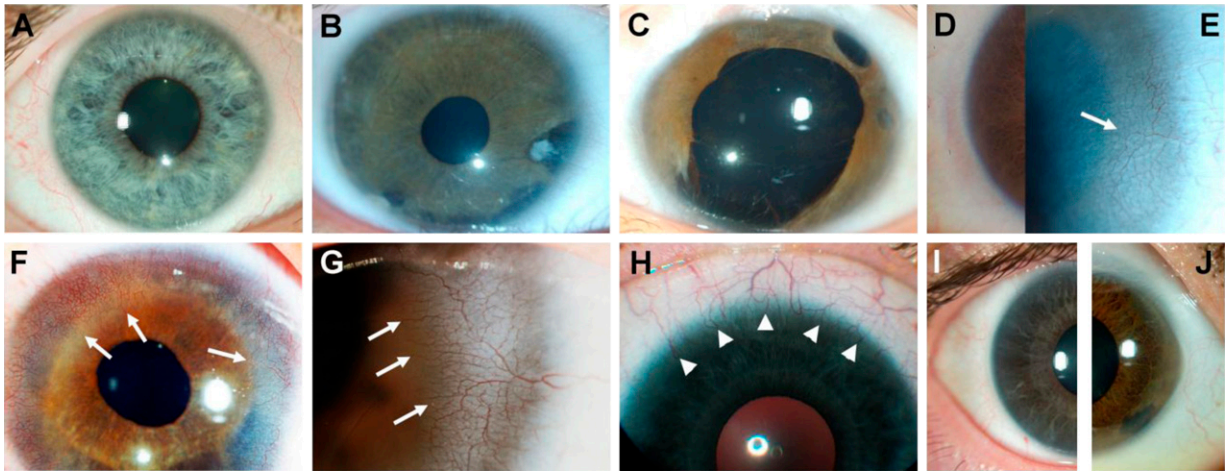

Fig. 1. FOXC1-attributable ARS is associated with a spectrum of corneal angiogenesis phenotypes. (*A*) In contrast to the normal anatomy (*A*), affected individuals from the deletion pedigree exhibit extensive iris (*C* and *D*) and corneal (*E* and *F*) anomalies. Of note, compared with the normal limbus (*E*, *Inset*), marked vascularization of the peripheral cornea (arrow in *E*) extends midway to the pupillary aperture (arrows in *F*). (*G*) The proband from the frameshift mutation pedigree (p.P274RfsX41) exhibits similar corneal vascularization (arrows). (*H*) Analogous but milder changes were observed in a pedigree with a 492-kb segmental duplication encompassing *FOXC1* and *FOXF2* with regional neovascularization extending across the superior cornea (arrowheads). (*I* and *J*) In a second member of this pedigree, in contrast to the normal circular limbus (*I*), there is squaring of the contour (*J*) implicating perturbed *FOXC1* gene dosage in more extensive limbal anatomical changes.

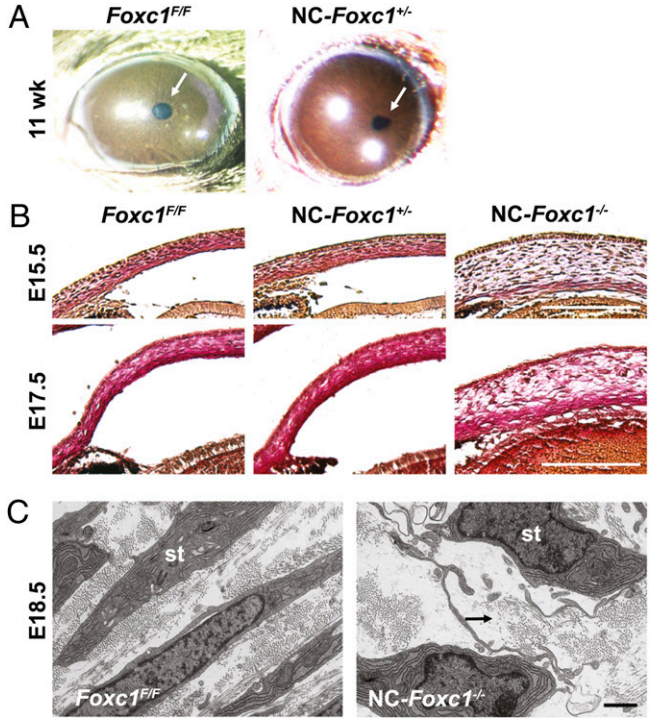

Fig. 2. NC-specific loss of *Foxc1* in mice induces pupillary abnormalities and impaired collagen formation in the corneal stroma. (*A*) Compared with the eye of a control ($Foxc1^{F/F}$) mouse, the eye of a mouse heterozygous for the NC-*Foxc1*-null mutation (NC-$Foxc1^{+/-}$) had a displaced and misshapen pupil at age 11 wk. (*B*) Impaired collagen formation in NC-$Foxc1^{-/-}$ embryos demonstrated by van Gieson staining of corneas at E15.5 and E17.5. NC-$Foxc1^{-/-}$ embryos show a remarkable reduction of collagen formation (pinkish red) in the corneal stroma. Nuclei stained with hematoxylin are shown in brown. (*C*) The ultrastructure of E18.5 corneas. Note that the collagen fibers (arrow) are disorganized and surround in swirls the disrupted corneal stromal cells of NC-$Foxc1^{-/-}$ embryos. st, corneal stromal cells. (Scale bars: 100 μm in *B* and 1 μm in *C*.)

exhibited partially disrupted limbal vessels, but their corneas, like those of control ($Foxc1^{F/F}$) mice, remained avascular through adulthood (Fig. 3*A*). Consistently, adult NC-$Foxc1^{+/-}$ mice exhibited increased sprouting of blood vessels at the limbus (Fig. 3 *A* and *C*). Corneal avascularity also was observed after the conditional deletion of *Foxc1* expression in murine vascular endothelial cells (29, 33–36) (Figs. S2*B* and S6). Collectively, these data demonstrate that deficiency of *Foxc1* expression in NC-derived corneal stroma cells, but not vascular endothelial cells, induces subsequent corneal angiogenesis. Furthermore, in view of the comparable human and murine phenotypes, this key role in preserving corneal avascularity during embryonic development is evolutionarily conserved.

Because lymphangiogenesis generally parallels angiogenesis (7, 37, 38), we next evaluated the growth of lymphatic vessels in NC-*Foxc1* mutant mice. At E17.5 and P0, $Prox1^+$ and $Lyve1^+$ lymphatic vessels extended from the limbus into the cornea of NC-$Foxc1^{-/-}$ mice (Fig. 3*B*). The corneas of NC-$Foxc1^{+/-}$ mice and control ($Foxc1^{F/F}$) mice remained avascular through adulthood, but the limbal lymphatic vessels of NC-$Foxc1^{+/-}$ embryos (E17.5) and adults were partially disrupted. Similar to the limbal blood vessels, lymphatic sprouting from the limbus of adult NC-$Foxc1^{+/-}$ mice was significantly increased (Fig. 3 *B* and *C*). Thus, NC-*Foxc1*–deficient mice exhibit a dose-dependent effect on vascular growth, and angiogenesis (E14.5) precedes lymphangiogenesis (E17.5), which mirrors the sequential induction of corneal angiogenesis and lymphangiogenesis by VEGF-A (39). Collectively, these findings indicate that Foxc1 appears to maintain corneal transparency by preventing both blood vessel and lymphatic vessel growth during embryonic development.

Foxc1 Regulates MMP Expression in the Corneal Stroma. NC-*Foxc1* deficiency did not alter corneal protein levels of VEGF-A or fibroblast growth factor (FGF)-2, which are frequently up-regulated during vascular growth, and was associated with increased sVEGFR-1 (or sFlt-1), an antiangiogenic factor (9, 40). However, several MMPs (e.g., MMP3, MMP9, and MMP19) were remarkably up-regulated in the corneas of NC-*Foxc1*–deficient mice (Fig. S7 and Fig. 4). MMPs degrade the ECM, possibly explaining the severely disorganized corneal ECM in NC-$Foxc1^{-/-}$ mice (Fig. 2 *B* and *C*) despite normal expression levels of collagens, such as *Col1a1* (Fig. S7). These observations also are consistent with the function of parologous Forkhead genes, which modulate MMP9 expression during cell migration and malignancy (41–43). These findings suggest that elevated MMP levels result in degraded ECM in the cornea of NC-$Foxc1^{-/-}$ mice.

Heterozygous *Foxc1* Mutations Enhance Growth of Blood and Lymphatic Vessels After Corneal Alkali Burn Injury. Alkali burns represent an extreme corneal injury, inducing pathological angiogenesis in both mice and humans. To determine whether Foxc1 has a role in the growth of corneal vessels in adult mice, we evaluated the angiogenic response to alkali burn injury (44, 45) in NC-$Foxc1^{+/-}$ and $Foxc1^{+/-}$ mice because of the perinatal lethality associated with the homozygous mutation. At 14 d after injury, corneal $CD31^+$ blood vessel networks were significantly more extensive in both NC-$Foxc1^{+/-}$ and $Foxc1^{+/-}$ mice compared with their respective controls ($Foxc1^{F/F}$ and WT) (Fig. 5). The heterozygous mutations also demonstrated increased growth of lymphatic ($Lyve\text{-}1^+$) vessels (Fig. 5) and up-regulated corneal MMP9 expression in NC-$Foxc1^{+/-}$ mice relative to controls (Fig. S8). Taken together with the observation of corneal angiogenesis in patients with *FOXC1* mutations (Fig. 1), these findings suggest a requirement for FoxC1 in keeping the postnatal cornea devoid of blood and lymphatic vessels.

Increased Angiogenic Response Attributable to Foxc1 Haploinsufficiency Is Abolished by Blockade of VEGF Signaling. Because several MMPs regulate the extracellular bioavailability of VEGF via proteolytic release or cleavage from the ECM (46–48), and because corneal MMP levels are up-regulated by the loss of *Foxc1* in NC-derived stromal cells (Fig. 4 and Figs. S7 and S8), we investigated whether VEGF signaling contributes to the enhanced vascular growth observed in NC-$Foxc1^{+/-}$ mice after corneal injury. Daily i.p. injections of SU5416, a VEGF receptor 2 (VEGFR-2) tyrosine kinase inhibitor (49, 50), after corneal alkali burn injury significantly reduced the growth of $CD31^+$ blood vessels at day 7 postinjury, abolishing the difference between control and NC-$Foxc1^{+/-}$ mice (Fig. 6 *A* and *B*). Because angiogenesis and lymphangiogenesis are induced by different VEGF concentrations (39), and corneal lymphangiogenesis is less robust than angiogenesis after corneal injury (Fig. 6 *A* and *B*), the lack of an effect of SU5416 on lymphangiogenesis might be attributable to SU5416 dosage. Taken together, these data indicate that stromal Foxc1 suppresses corneal neovascularization, at least in part, by blocking the angiogenic response to VEGF.

Discussion

Although much progress has been made toward defining the mechanisms underlying neovascularization, including identification of the antiangiogenic role of sVEGFR-1 in limiting corneal vessel growth (9), the pathogenesis of corneal angiogenesis remains incompletely understood. The findings reported here identify a second, complementary mechanism that

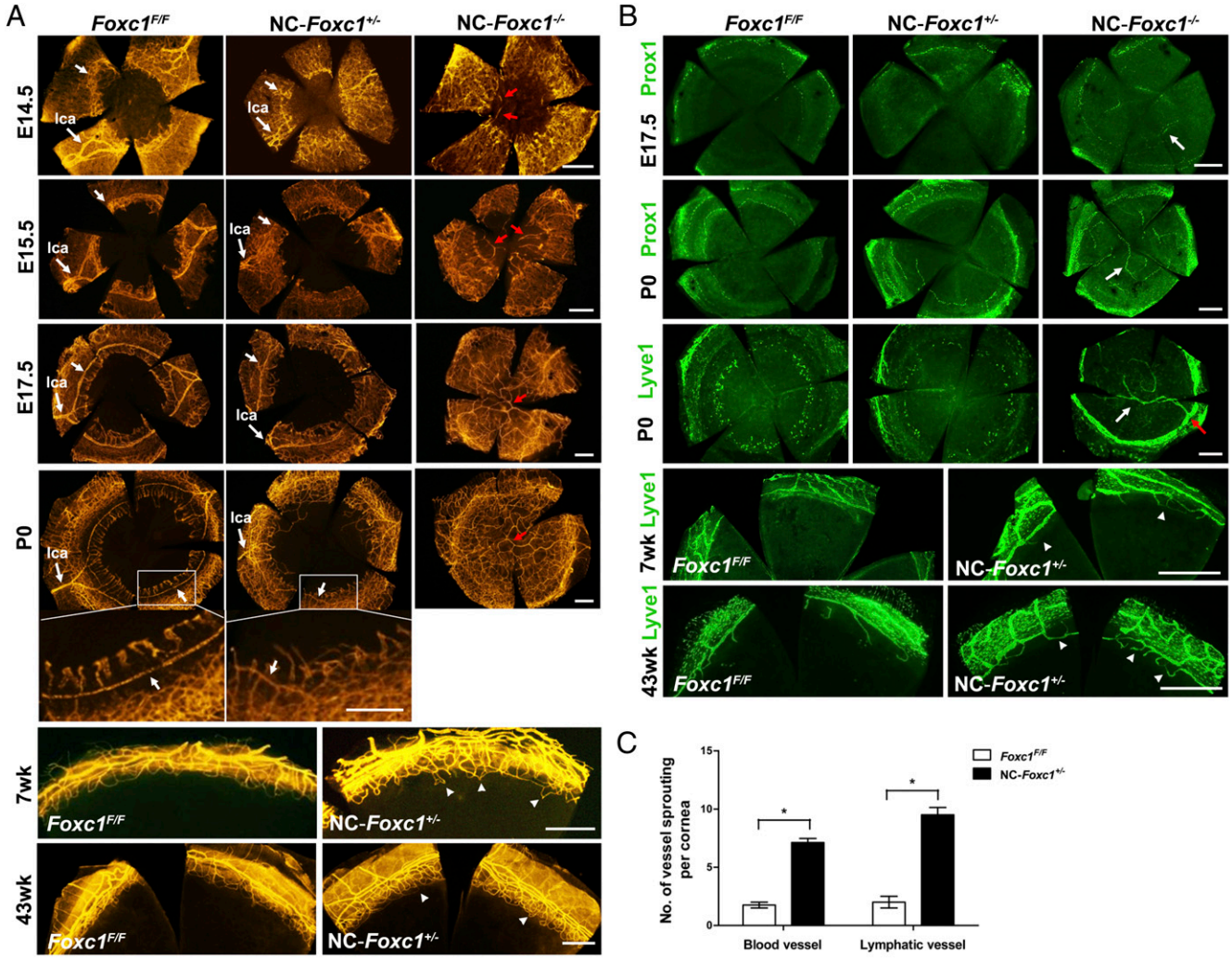

Fig. 3. NC-specific loss of *Foxc1* induces angiogenesis and lymphangiogenesis in the mouse cornea during development. (*A*) Neovascularization was evaluated in CD31-stained corneas from control (*Foxc1*$^{F/F}$), NC-*Foxc1*$^{+/-}$, and NC-*Foxc1*$^{-/-}$ mouse embryos (E14.5, E15.5, and E17.5) and newborn mice (P0), and from adult (7-wk-old and 43-wk-old) control (*Foxc1*$^{F/F}$) and NC-*Foxc1*$^{+/-}$ mice. The corneas of control (*Foxc1*$^{F/F}$) embryos and mice were avascular with developing long ciliary arteries (long arrows) and limbal blood vessels (short arrows). The corneas of NC-*Foxc1*$^{+/-}$ embryos and mice remained avascular through adulthood, but the long ciliary arteries (long arrows) and limbal blood vessels (*Inset*; short arrow) of embryos and newborn mice were disrupted. Increased sprouting of limbal blood vessels (arrowheads) was observed in adult NC-*Foxc1*$^{+/-}$ mice. In NC-*Foxc1*$^{-/-}$ embryos, corneal vascularization (red arrows) was observed by E14.5 and extended throughout the cornea at later time points; the disruption of limbal blood vessels was apparent on E17.5 and P0. (*B*) NC-specific loss of *Foxc1* increases the growth of corneal lymphatic vessels in mice. Lymphangiogenesis was evaluated in corneas from control (*Foxc1*$^{F/F}$), NC-*Foxc1*$^{+/-}$, and NC-*Foxc1*$^{-/-}$ embryos (E17.5) and newborn mice (P0), and also from adult (7-wk-old and 43-wk-old) *Foxc1*$^{F/F}$ and NC-*Foxc1*$^{+/-}$ mice. Embryonic corneas were stained with the lymphatic vessel marker Prox1, corneas from newborn mice were stained with Prox1 or the lymphatic vessel marker Lyve-1, and corneas from adult mice were stained with Lyve-1. Growth of lymphatic vessels from the limbus (red arrow) into the cornea was observed in NC-*Foxc1*$^{-/-}$ embryo at E17.5 and in newborn mice (arrows). The corneas of adult NC-*Foxc1*$^{+/-}$ mice remained free of lymphatic vessels, but showed increased sprouting of lymphatic vessels (arrowheads). (*C*) Quantification of blood and lymphatic vessel sprouting in 7-wk-old adult *Foxc1*$^{F/F}$ and NC-*Foxc1*$^{+/-}$ mice. The sprouting of both blood and lymphatic vessels was significantly greater in NC-*Foxc1*$^{+/-}$ mice compared with control *Foxc1*$^{F/F}$ mice. Values are mean ± SEM. Compared with control mice: blood vessel sprouting in NC-*Foxc1*$^{+/-}$ mice, $P = 0.0000006$; lymphatic vessel sprouting in NC-*Foxc1*$^{+/-}$ mice, $P = 0.00003$. *$P < 0.05$, Student *t* test. lca, long ciliary artery. (Scale bars: 100 μm.)

maintains corneal transparency through the FoxC1-mediated regulation of ECM alterations and subsequent VEGF-mediated angiogenesis (Fig. 6*C*). This interpretation is supported by phenotypic observations in patients with a spectrum of *FOXC1* variations and in mice with global and NC-specific *Foxc1* alterations.

The present study provides evidence that Foxc1 in the NC plays a specific role in ocular development. NC-specific *Foxc1*$^{+/-}$ mice have abnormally shaped pupils (Fig. 2*A*), whereas NC-specific *Foxc1*$^{-/-}$ mice have abnormal formation of the ocular anterior segment, including severely disorganized corneal stroma (Fig. 2 *B* and *C*) and corneal neovascularization (Fig. 3 and Fig. S5). These ocular abnormalities are very similar to those seen in global *Foxc1*$^{-/-}$ mutant mice and are consistent with the observation that the majority of Foxc1-expressing cells in the periocular mesenchyme of the mouse embryo are NC-derived (30). This finding also suggests that the corneal neovascularization seen in patients with *FOXC1* variations is likely caused by impairment of NC-derived corneal stroma.

One striking phenotype of corneal defects in NC-specific *Foxc1*$^{-/-}$ mice during embryonic development is the significant up-regulation of MMPs, including MMP3, MMP9, and MMP19 (Fig. 4 and Fig. S7). These mutants have abnormal ECM in the corneal stroma with normal expression of collagen genes,

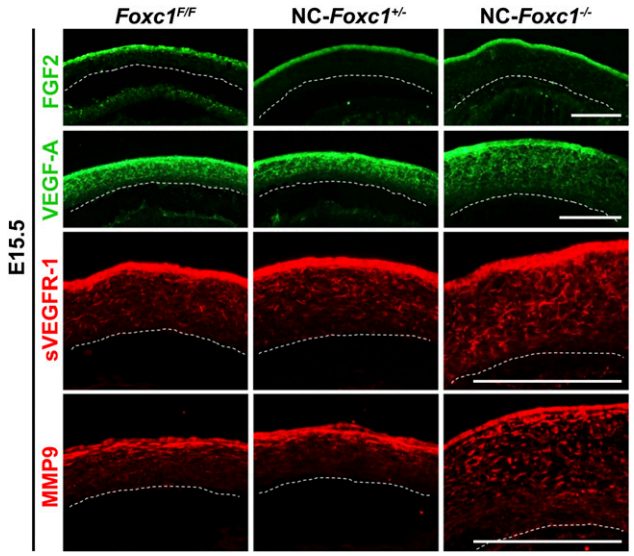

Fig. 4. Up-regulation of MMP expression in the cornea of NC-*Foxc1*$^{-/-}$ mice. (*A*) Gene expression was evaluated in the corneas of E15.5 control (*Foxc1*$^{F/F}$), NC-*Foxc1*$^{+/-}$, and NC-*Foxc1*$^{-/-}$ embryos via real-time RT-PCR. Values are mean ± SEM. *$P < 0.05$, Student *t* test. Expression of FGF2, VEGF-A, sVEGFR-1, and MMP9 protein was evaluated in E15.5 control (*Foxc1*$^{F/F}$), NC-*Foxc1*$^{+/-}$, and NC-*Foxc1*$^{-/-}$ embryos via immunofluorescent staining. MMP9 expression was observed in the corneal epithelium and superficial stroma of NC-*Foxc1*$^{+/-}$ embryo and throughout the corneal stroma of NC-*Foxc1*$^{-/-}$ embryo. (Scale bars: 100 μm.)

suggesting that excessive proteolytic ECM degradation is caused by the elevated MMP levels. Because MMP-mediated ECM degradation is involved in pathological neovascularization (51),

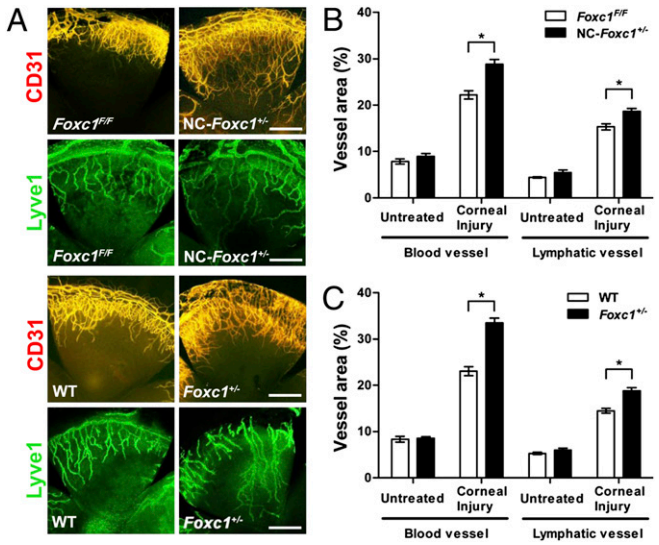

Fig. 5. Foxc1 regulates corneal angiogenesis and lymphangiogenesis after corneal injury in adult mice. Corneal alkali burn injury was induced in 6-wk-old control (*Foxc1*$^{F/F}$) mice, NC-*Foxc1*$^{+/-}$ mice, WT mice, and mice heterozygous for a global *Foxc1*-null mutation (*Foxc1*$^{+/-}$ mice). (*A*) At 14 d after injury, angiogenesis and lymphangiogenesis were evaluated in corneas stained for CD31 (red) and Lyve-1 (green) expression, respectively. (*B* and *C*) Both the NC-*Foxc1*$^{+/-}$ mutation and the *Foxc1*$^{+/-}$ mutation enhanced the sprouting of blood and lymphatic vessels, and the blood and lymphatic vessel areas were significantly larger in NC-*Foxc1*$^{+/-}$ mice than in control (*Foxc1*$^{F/F}$) mice (*B*) and in *Foxc1*$^{+/-}$ mice than in WT mice (*C*). Values are mean ± SEM. *$P < 0.05$, Student *t* test. (Scale bars: 100 μm.)

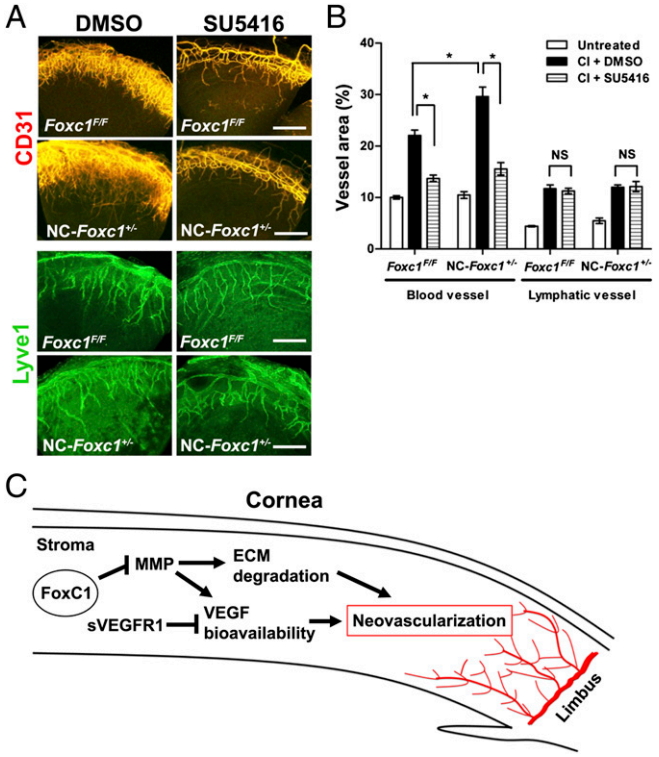

Fig. 6. Corneal angiogenesis after alkali burn injury in NC-*Foxc1*$^{-/-}$ mice is blocked by inhibition of VEGF signaling. (*A*) Mice were treated with DMSO (control) or the VEGFR-2 blocker SU5416 for 7 consecutive days after corneal alkali burn injury, after which angiogenesis and lymphangiogenesis were evaluated in corneas stained for CD31 (red) and Lyve-1 (green) expression, respectively, on day 7 after corneal injury. The blockade of VEGFR-2 dramatically reduced the growth of corneal blood vessels but not that of lymphatic vessels. (*B*) The blood vessel area, but not the lymphatic vessel area, was significantly smaller after treatment with SU5416 than after DMSO treatment. Values are mean ± SEM. CI, corneal injury; NS, not significant. *$P < 0.05$, Student *t* test. (Scale bars: 100 μm.) (*C*) Model for FoxC1 function in corneal transparency. FoxC1 is essential for the establishment and maintenance of corneal avascularity during development and in postnatal life by regulating MMP expression in corneal stroma. Corneal avascularity is tightly controlled by angiogenic and antiangiogenic responses. In *Foxc1*-mutant corneas, elevated MMP levels degrade ECM and enhance VEGF bioavailability, thereby inducing corneal neovascularization, whereas sVEGFR1 suppresses activation of VEGF signaling by neutralizing VEGF.

this process may lead to the outgrowth of blood and lymphatic vessels from the limbus (Fig. 6*C*). We found consistently increased MMP9 expression in the corneal stroma of NC-specific *Foxc1*$^{+/-}$ mice after alkali burn injury compared with controls (Fig. S8).

The inhibition of corneal angiogenesis in NC-specific *Foxc1*$^{+/-}$ mice observed with VEGF blockade (Fig. 6 *A* and *B*) indicates that *Foxc1* haploinsufficiency in the NC enhances VEGF-dependent corneal neovascularization. Although VEGF expression is not altered in the corneas of NC-specific *Foxc1* mutants, and the antiangiogenic molecule sVEGFR-1 is up-regulated (Fig. 4 and Fig. S7), MMPs have been shown to enhance VEGF bioavailability in the ECM (51, 52). Thus, it is highly likely that Foxc1 deficiency, by elevating MMP levels, shifts the delicate balance between proangiogenic and antiangiogenic factors, thereby inducing neovascularization from the limbus (Fig. 6*C*). The conserved function of FoxC1 in corneal angiogenesis between human and mouse has intriguing parallels to the contribution of altered FOXC1 levels to decreased breast cancer survival (53, 54), a phenomenon in which tumor neovascularization plays

a central role (1, 8). If dysregulated FoxC1 expression contributes to abnormal angiogenesis in multiple tissues, then our identification of a specific target for combating corneal angiogenesis may offer insight into a variety of conditions characterized by pathological neovascularization.

Materials and Methods

Studies included immunohistochemistry, real-time RT-PCR, corneal flat mounting, and corneal alkali burn injury. Detailed information is provided in *SI Materials and Methods*. PCR primer sequences are listed in Table S1.

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