Notch1 signaling in keratocytes maintains corneal transparency by suppressing VEGF expression

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https://doi.org/10.1016/j.stemcr.2022.04.017

SUMMARY

The cornea fends off chemicals, dirt, and infectious particles and provides most of the eye's focusing power. Corneal transparency is of paramount importance to normal vision, yet how it is established and maintained remains unclear. Here, we ablated *Notch1* in keratocytes using *Twist2-Cre* mice and found that *Twist2-Cre*; *Notch1^{f/f}* mice developed stroma expansion and neovascularization, followed by hyperproliferation and metaplasia of corneal epithelial progenitor cells and plaque formation at central cornea, leading to loss of transparency. Development of these phenotypes does not involve bacteria-caused inflammation; instead, *Notch1* deletion upregulates *Vegfa* and *Vegfc* via Hif1α in keratocytes. Vascular endothelial growth factor (VEGF) receptor inhibitor axitinib prevented development of these anomalies in *Twist2-Cre*; *Notch1^{f/f}* mice, suggesting that VEGFs secreted by keratocytes promote not only neovascularization but also proliferation and metaplasia of epithelial progenitor cells at central cornea. This study uncovers a Notch1-Hif1α-VEGF pathway in keratocytes that maintains corneal transparency and represents a potential target for treatment of related corneal disorders.

INTRODUCTION

The cornea provides about two-thirds of the eye's focusing power and acts as a barrier to protect the underlying tissues from damage (Shah et al., 2021; Sugioka et al., 2021). Mammalian cornea consists primarily of three layers: an outer non-keratinized squamous epithelium layer (Nowell and Radtke, 2017), a stroma layer at the middle with collagen-rich matrix and interspersed keratocytes (corneal stromal cells/fibroblasts), which provides strength to the cornea (Espana and Birk, 2020; Fukuda, 2020; Lagali, 2020; Medeiros et al., 2018; Yam et al., 2020), and an inner endothelial layer, which maintains hydration of the cornea (Faye et al., 2021). The corneal epithelia exhibit a dynamic turnover, driven by corneal epithelial stem cells (CESCs), while keratocytes and endothelial cells remain quiescent in adults (Funderburgh et al., 2016; Kamil and Mohan, 2021; Yazdanpanah et al., 2017). While limbal CESCs have been established as slow-cycling or quiescent stem cells (Altshuler et al., 2021; Yu et al., 2021), a number of studies have shown that transit-amplifying cells at the central cornea have stem cell activities (termed epithelial progenitor cells hereafter) (Chang et al., 2011; Majo et al., 2008; Nasser et al., 2018). Visual acuity requires corneal transparency, which is attributable to proper organization of the collagen fibrils, expression of crystalline, the lack of keratin, and blood vessels (Meek and Knupp, 2015; Mittal et al., 2016).

Trauma, infection, inflammation, and contact-lens wear can injure the cornea and may lead to scar formation and loss of corneal transparency (Kim et al., 2018; Mobaraki et al., 2019), which constitute a major risk of blindness and affect around 10 million people worldwide (Gain et al., 2016; Maddula et al., 2011). In particular, loss of corneal angiogenic privilege is a major contributing factor to ocular pathologies (Hadrian et al., 2021; Whitcher et al., 2001). Angiogenic privilege is preserved by balanced proangiogenic and antiangiogenic factors in the cornea (Abdelfattah et al., 2015; Di Iorio et al., 2019). Vascular endothelial growth factors (VEGFs) play critical roles in angiogenesis, with VEGF-A being upregulated under various vascular pathological conditions (Meurette, 2020). Expression of VEGFs is positively regulated by HIF-1α and HIF-1β, which are activated by hypoxia (Schito and Semenza, 2016). However, how the avascular status is established and maintained in the cornea remains less understood.

Notch signaling, an evolutionarily conserved pathway, regulates a wide range of developmental processes via controlling cell-cell communication and cell-fate determination (Akil et al., 2021; Henrique and Schweisguth, 2019; Lloyd-Lewis et al., 2019) and is also involved in cancer development (Meurette and Mehlen, 2018; Weng et al., 2004). The mammalian Notch pathway consists of five ligands (Jagged1, Jagged2, Dll1, Dll3, and Dll4) and four
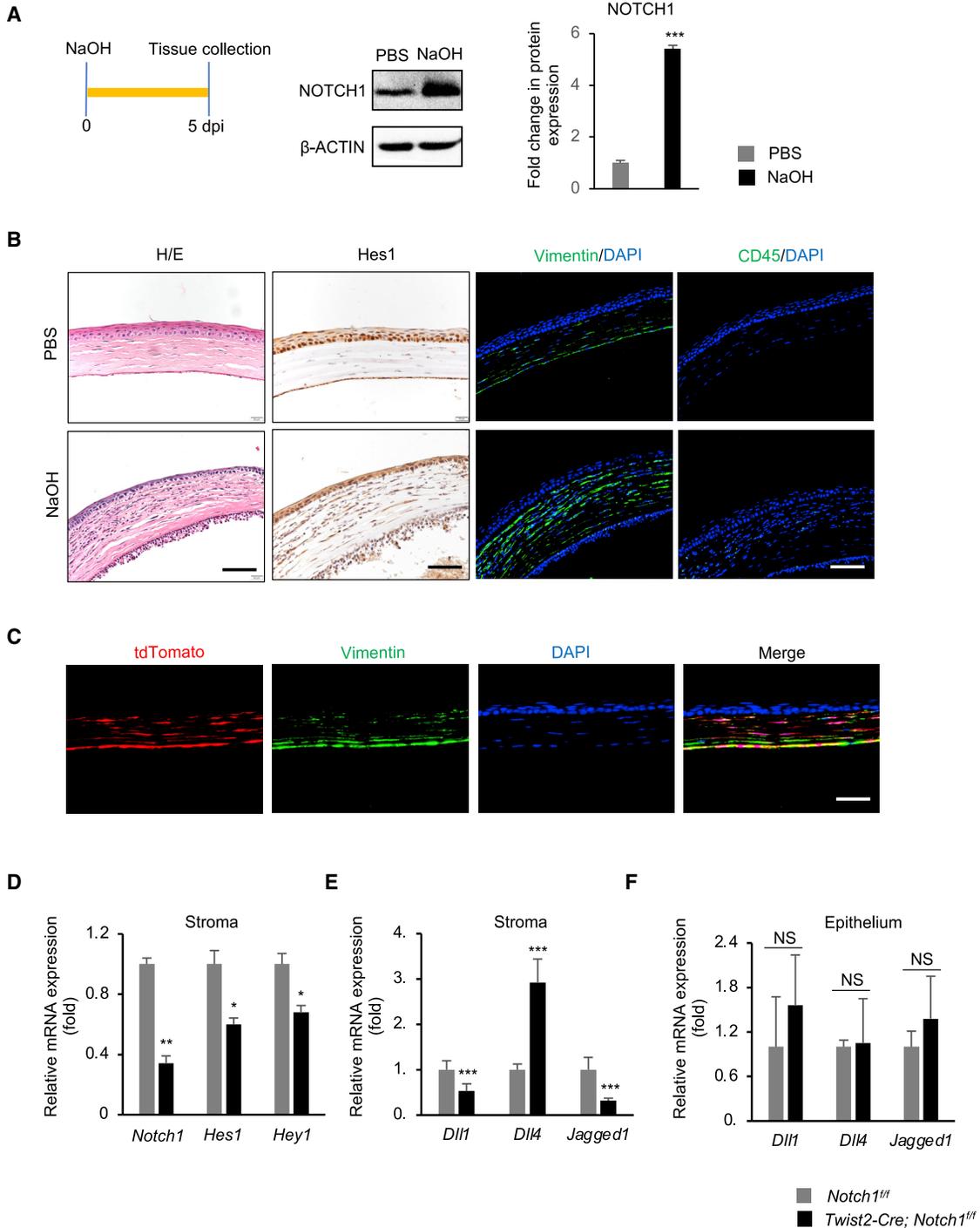
Figure 1. Tracing of Twist2 lineage keratocytes and generation of *Twist2-Cre; Notch1^{f/f}* mice

(A) Diagram showing the time of alkali-burn injury and mouse euthanization. Representative western-blot results show increased expression of NOTCH1 in the corneal tissues of standard housed mice during regeneration. Right panel: quantitation data. The ratio was normalized to loading control β-ACTIN. The value of control samples was set at 1.0. n = 3 mice. Data are shown as mean ± SD of three independent experiments. **p < 0.01.

(B) H&E and immunostaining of the corneal sections of NaOH- or PBS-treated C57BL/6 mice 5 days post alkali injury. Representative immunohistochemical staining results showed nuclear localization of Hes1 in keratocytes of regenerating cornea. Sections were developed

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receptors (Notch 1–4). Upon ligand binding to Notch receptors, γ-secretase-mediated proteolytic cleavage generates the Notch intracellular domain (NICD), which translocates to the nucleus, where it interacts with recombination signal-binding protein Jκ (RBP-J) and other transcription regulators to control the transcription of downstream genes such as *Hes1* and *Hey1* (Bray and Gomez-Lamarca, 2018; Kovall et al., 2017). In particular, Dll4 and Notch1 are expressed in endothelial cells and play critical roles in angiogenesis, vascular differentiation, and maintenance of vascular patterns (Blanco and Gerhardt, 2013; Krebs et al., 2000). During blood-vessel sprouting, Notch signaling maintains the specification of tip and stalk cells (Blanco and Gerhardt, 2013). VEGFs upregulate Dll4 expression in tip cells (high VEGF and low Notch), which binds to Notch1 on stalk cells (low VEGF and high Notch) and suppresses the tip cell fate via limiting expression of VEGFR2 (Benedito et al., 2012; Hellstrom et al., 2007; Roca and Adams, 2007). Inactivation of Notch signaling increases neovascularization in mice (Briot et al., 2016). Thus, the crosstalk between VEGFs and Notch signaling plays critical roles in endothelial cells and angiogenesis.

Previous studies suggest that Notch1 signaling plays critical roles in corneal development (Grisanti et al., 2016; Pang et al., 2021). Notch signaling also regulates CESC and corneal epithelial cell proliferation and differentiation (Dhamodaran et al., 2020; Gonzalez et al., 2020; Lu et al., 2012; Movahedan et al., 2013; Nakamura et al., 2008; Vauclair et al., 2007). Interestingly, *Notch1* deletion not only promotes epithelial cell proliferation and fate switch to keratinized skin-like cells but also induces stromal fibrosis and blood-vessel formation (Vauclair et al., 2007) and causes defects in barrier formation (Movahedan et al., 2013). Here, we show that Notch signaling is activated in corneal keratocytes and that its activation was enhanced in response to corneal injury. Deletion of *Notch1* in keratocytes using a *Twist2-Cre* mouse line led to keratocyte activation and expansion after 5 weeks of age with disarrayed extracellular matrix and neovascularization, followed by corneal CESC hyperproliferation and epidermal conversion at the central cornea but not at the limbus. Mechanistically, *Notch1* ablation upregu-

lates the expression of Hif1α and its downstream genes *Vegfa* and *Vegfc* in keratocytes, which are responsible for development of the cornea phenotypes. This study uncovers an important role for keratocyte-specific Notch1 signaling in constraining progenitor cell proliferation at the central cornea and maintaining corneal transparency and highlights the importance of *Notch1* in regulating neovascularization via stromal-epithelial cell interactions.

RESULTS

Activation of Notch1 in keratocytes during cornea homeostasis and regeneration

Previous studies have shown that Notch signaling plays important roles in corneal epithelial cell proliferation and differentiation (Movahedan et al., 2013; Vauclair et al., 2007). We reexamined NOTCH1 expression by western blot and found that NOTCH1 was expressed in mouse cornea samples, which was increased upon alkali-induced injury (Figure 1A). In this study, we briefly exposed the mouse cornea to NaOH so that the damage was limited to the epithelial layer with the underlying stroma unscathed (Ma et al., 2006; Yu et al., 2021). The injured epithelial layer is recovered within 14 days (Yu et al., 2021). We carried out immunohistochemical staining for Hes1, a downstream target of Notch signaling. Under basal conditions, Hes1 is observed in the nucleus of corneal epithelial cells and, to a lesser extent, of corneal keratocytes (Figure 1B). In alkali-injured cornea, the expression levels of Hes1 were elevated drastically in cells with keratocytes morphology, which were positive for vimentin, a marker for fibroblasts and keratocytes (Figure 1B). It is noted that CD45^{+} immune cells were rare (Figure 1B). These results suggest that Notch1 in keratocytes may play a role in corneal homeostasis and regeneration.

Ablation of *Notch1* in keratocytes using *Twist2-Cre* mice

To determine the physiological functions of Notch1 in corneal keratocytes, we wanted to ablate *Notch1* specifically

using DAB chromogen, and nuclei were counterstained with hematoxylene. Vimentin and CD45 were stained on consecutive slides with Hes1. Nuclei were counterstained with DAPI (blue). n = 3 mice. Scale bar: 20 μm.

(C) Lineage-tracing experiment showed that keratocytes were labeled by Twist2, which co-localized with vimentin. The eyeballs were dissected from *Twist2-Cre*; *Rosa-tdTomato* mice, frozen sectioned, immunostained for vimentin, and observed under a fluorescence microscope. Red and green colors represent tdTomato and vimentin, respectively. Nuclei were counterstained with DAPI (blue). n = 3 mice. Scale bar: 20 μm.

(D) Quantitative PCR analyses showed reduced mRNA levels of *Notch1*, *Hes1*, and *Hey1* in the stroma of *Twist2-Cre*; *Notch1^{f/f}* mice. The value of control samples was set at 1.0. n = 3 mice. Data are shown as mean ± SD of three independent experiments. **p < 0.01.

(E and F) Quantitative real-time PCR analysis showed altered expression of *Dll1*, *Dll4*, and *Jagged1* genes in corneal stroma (E) but not in epithelial (F) samples of *Twist2-Cre*; *Notch1^{f/f}* mice. The value of control samples was set at 1. n = 3 mice. Data are shown as mean ± SD of three independent experiments. ***p < 0.001. NS, not significant.

A

	Notch1^{f/f}	*Twist2-Cre; Notch1^{f/f}*			
	5	5	6	8	16 (weeks)

B

	5	6	8	12	16 (weeks)
Notch1^{f/f}					
Twist2-Cre; Notch1^{f/f}					

C

Vimentin/DAPI α-SMA/DAPI CD45/DAPI F4/80/DAPI

Col1α/DAPI CD31/DAPI NG2/DAPI Lyve-1/DAPI

Notch1^{f/f}

Twist2-Cre; Notch1^{f/f}
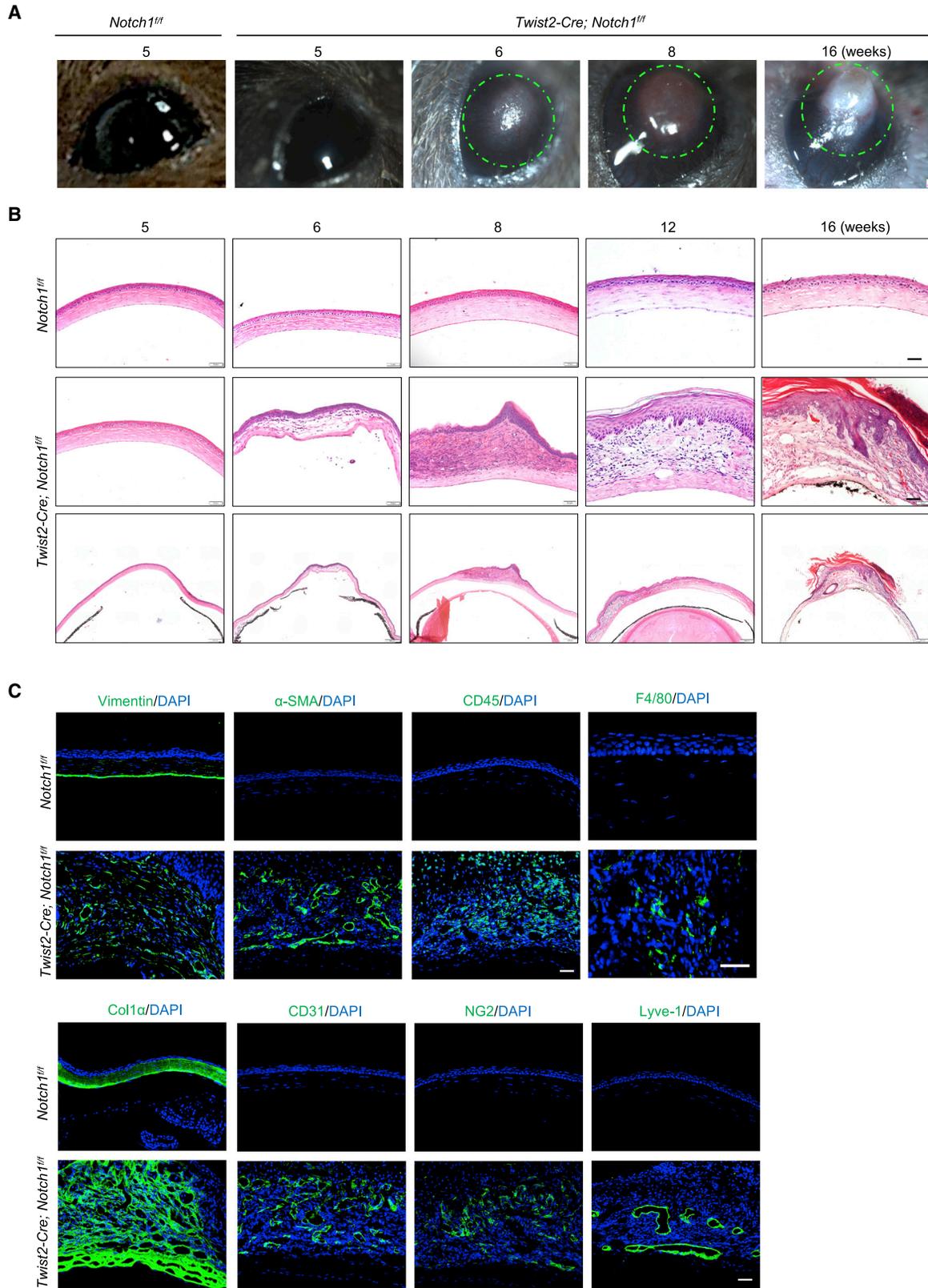
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in keratocytes using the Cre/Lox P system. Previous studies have shown that ablation of *Twist2*, a marker for bone marrow mesenchymal stromal cells, led to thinning of the corneal stroma without affecting the epithelial cells (Weaving et al., 2010), suggesting that Twist2 is specifically expressed in keratocytes. To verify that Twist2 marks corneal keratocytes, we crossed *Twist2-Cre* to *Rosa-tdTomato* reporter mice to trace Twist2 lineage cells in the cornea. Tomato fluorescent signal was found in keratocytes and endothelial cells, but not in epithelial cells, as well as in stromal cells in the limbus (Figures 1C and S1A). Immunostaining revealed that most of the tdTomato^{+} cells were positive for vimentin in the cornea (Figure 1C). These results suggest that the *Twist2-Cre* line can be used to mark corneal keratocytes. Moreover, we observed tdTomato^{+} stromal cells surrounding K14^{+} epithelial cells in the meibomian glands (Figure S1B).

We then generated *Twist2-Cre*; *Notch1^{f/f}* mice by crossing the *Twist2-Cre* and *Notch1^{f/f}* lines. Quantitative PCR analysis revealed decreased expression of Notch1 and its downstream signaling molecules Hey1 and Hes1 in the corneal stroma, confirming successful ablation of *Notch1* gene in keratocytes (Figure 1D). The stroma of the knockout mice also showed reduced expression of *Dll1* and *Jagged1* but increased expression of *Dll4* (Figure 1E). However, expression of the ligands was not significantly altered in corneal epithelia of *Twist2-Cre*; *Notch1^{f/f}* mice (Figure 1F). These results suggest that there may exist a feedback regulation between Notch1 and its ligands in corneal keratocytes, which may help to maintain low-level activation of Notch signaling in corneal stroma.

Notch1 ablation results in stromal remodeling and neovascularization

The cornea appeared normal in *Twist2-Cre*; *Notch1^{f/f}* mice up to 5 weeks of age (Figure 2A), suggesting that Notch1 signaling in keratocytes is not required for cornea development or early postnatal growth or establishment of corneal transparency. Starting at 6 weeks of age, *Twist2-Cre*; *Notch1^{f/f}* mice showed progressive changes in the cornea, and visible plaques were observed at 8 weeks of age at the central cornea (Figure 2A, encircled by dotted lines).

H&E staining revealed a progressive increase in the thickness of the stroma, as well as the epithelial layer (Figure 2B), suggesting that *Notch1* ablation in keratocytes leads to expansion of not only the stroma but also the epithelial layer.

Immunostaining revealed that *Twist2-Cre*; *Notch1^{f/f}* mice displayed keratocyte activation, manifested by increased expression of alpha-smooth muscle actin (α-SMA) and vimentin, and leukocyte infiltration, manifested by the emergence of CD45^{+} leukocytes and F4/80^{+} macrophages in the stroma (Figure 2C). Col1α staining revealed an increase in extracellular matrix with disrupted alignment of collagen fibrils in the knockout mice. Starting at 7–8 weeks of age, stromal neovascularization and lymphatic vessels were detected. Immunofluorescence staining showed strong signals for CD31, NG2, and Lyve-1, markers for endothelial cells, pericytes, and lymphatic vessels, respectively (Figure 2C). These results suggest that *Notch1* ablation in keratocytes induced stroma expansion as well as neovascularization.

Loss of *Notch1* in keratocytes leads to corneal epithelial overgrowth

Further analyses revealed thickening of the epithelial layer at the central cornea starting from 8 weeks of age (Figure 2A). H&E staining revealed invagination of the epithelial layer into the underlying stroma in 12-week-old knockout mice (Figure 2B). Immunofluorescence staining revealed increased numbers of Ki67^{+} proliferating epithelial cells at the basal and suprabasal layers of central cornea, as well as keratocytes in the stroma, in 6- or 12-week-old knockout mice (Figure 3A). Moreover, the number of epithelial progenitor cells at the central cornea, marked by p63 (Li et al., 2015), was also increased in 12-week-old knockout mice (Figure 3A). These results suggest that *Notch1* ablation in keratocytes led to the expansion and increased proliferation of epithelial progenitor cells at the central cornea.

However, the peripheral cornea and limbal regions remained normal in the knockout mice (Figures 2B and S2A). The numbers of Ki67^{+} cells and p63[+] CESCs in the limbus of the knockout mice were similar to those of control

Figure 2. Loss of *Notch1* in keratocytes led to progressive development of corneal defects including stromal remodeling and neovascularization

(A) Representative images showed the age-dependent changes in the corneas of *Twist2-Cre*; *Notch1^{f/f}* mice. Opacity and plaque formation started to appear from 6 to 8 weeks of age, respectively, and the plaque thickened considerably in 16-week-old mutant mice. The dotted line encircles the plaque.

(B) Illustrative histological analyses (H&E staining) results showed changes in corneal structures of *Twist2-Cre*; *Notch1^{f/f}* mice compared with control littermates. n = 3 mice. Scale bar: 50 μm.

(C) Representative immunostaining results showed the expression of vimentin, α-SMA, CD45, F4/80, and Col1α in corneas of the mutant mice and also showed the presence of CD31, NG2, and Lyve-1 signals in mutant corneas but not in control corneas. n = 3 mice. Scale bars: 50 and 20 μm (F4/80 only).
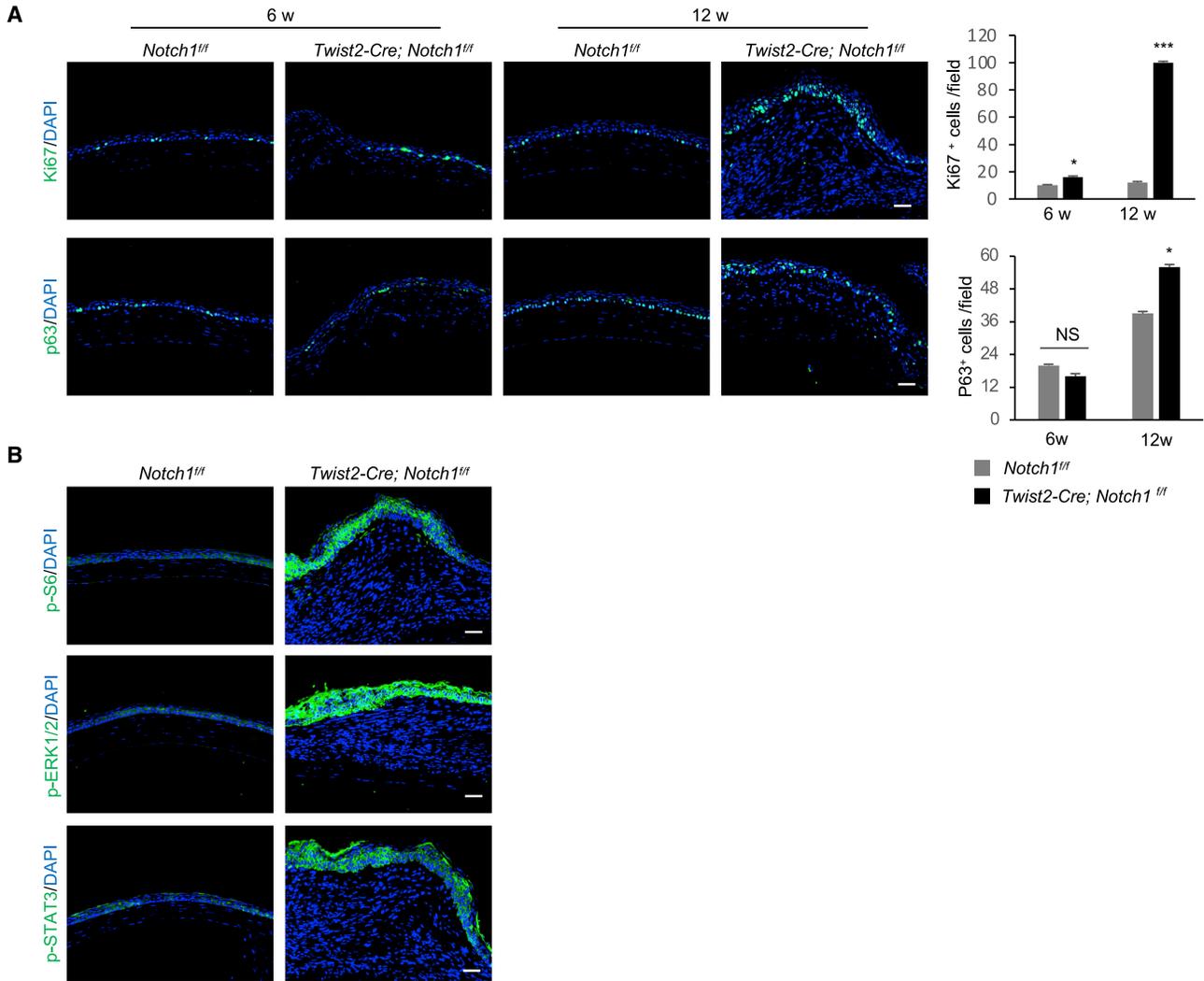
Figure 3. *Notch1* deletion leads epithelial overgrowth and increased number of p63⁺ progenitors at the central cornea

(A) Ki67 staining showed the hyperproliferative corneal epithelia and keratocytes and an increase in the number of p63⁺ progenitors in *Twist2-Cre*; *Notch1^{f/f}* mice compared with control littermates. Scale bar: 50 μm. Right panel: quantification of Ki67⁺ and p63⁺ cells per field. For each corneal section, six different fields were quantified and averaged. n = 6 mice. Data are shown as mean ± SD of three independent experiments. *p < 0.05, ***p < 0.001. NS, not significant.

(B) Representative immunostaining results confirmed the activation of mTOR, Erk1/2, and Stat3 in corneas of *Twist2-Cre*; *Notch1^{f/f}* compared with control mice. n = 3 mice. Scale bar: 50 μm.

mice (Figure S2A). It is possible that the limbal CESCs are not activated by *Notch1* ablation in keratocytes due to their slow-cycling nature and lack of stimulatory environment (Altshuler et al., 2021). Collectively, these results suggested that Notch1 signaling in keratocytes suppresses epithelial progenitor cell proliferation at the central cornea with minimal effect on quiescent CESCs in the limbus under homeostatic conditions. In addition, we observed that the knockout mice had normal meibomian glands at 6 weeks of age, revealed by H&E, oil red O staining, and immunostaining for K14, K1, and vimentin (Figure S2B).

Overproliferation of epithelial progenitor cells at the central cornea was accompanied by enhanced activation of mitogenic signaling molecules ERKs, mTOR, and STAT3, but not AKT1 and β-CATENIN, in cornea samples of *Twist2-Cre*; *Notch1f/f* mice, manifested by western-blot-analysis results (Figure S3). Immunostaining confirmed the activation of mTOR, Erk, and Stat3 in corneal epithelial cells and, to a much lesser extent, in the stroma of the knockout mice (Figure 3B). These results suggest that *Notch1* deletion in keratocytes activated multiple mitogenic pathways, consistent
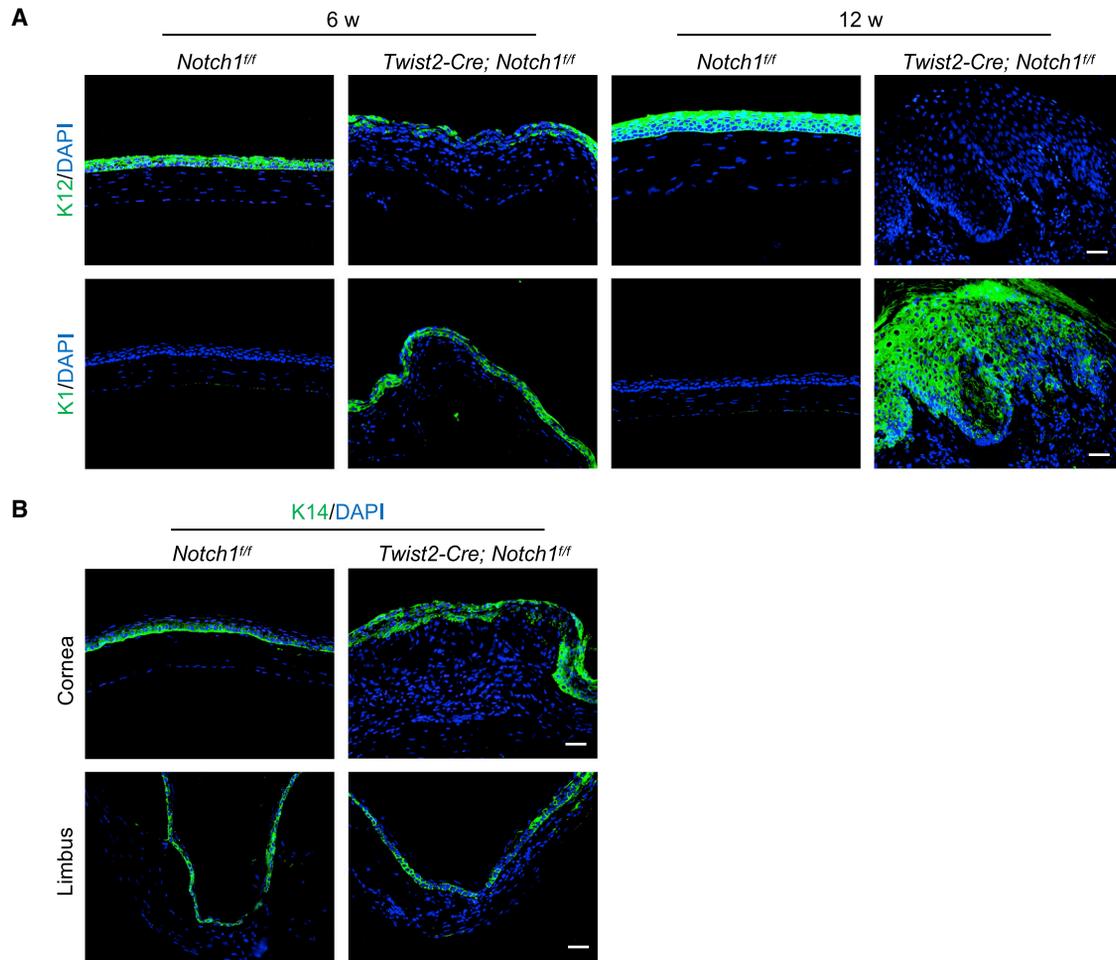
Figure 4. *Notch1* deficiency leads to conversion of corneal epithelial to epidermal cells
(A) Immunofluorescence-staining results showed a switch from expression of cytokeratin K12 to skin-specific marker K1 in corneal epithelia in *Twist2-Cre; Notch1^{f/f}* mice (left panel: 6-week-old mice; right panel: 12-week-old mice). n = 3 mice. Scale bar: 50 μm.
(B) Immunofluorescence-staining results showing the labeling of K14⁺ cells in the cornea and limbus of *Twist2-Cre; Notch1^{f/f}* and control mice. n = 3 mice. Scale bar: 50 μm.

with hyperproliferation of corneal epithelial and stromal cells.

Notch1 deletion in keratocytes induces epithelial progenitor fate switch to epidermal cells

It is known that corneal transparency requires expression of proper cytokeratins by the epithelia (Shah et al., 2021). We tested expression of several keratins in *Notch1*-deficient and control mice. Immunofluorescence staining revealed that the corneas of *Twist2-Cre; Notch1f/f* mice were devoid of K12, a keratin specific to corneal epithelium, in contrast to normal corneas (Figure 4A). This defective epithelial differentiation started as early as 6 weeks of age and progressed with age in the mutant mice (Figure 4A). On the other hand, the

cornea of *Notch1*-deficient mice was positive for epidermis-specific cytokeratin K1 starting at 6, but not at 5, weeks of age (Figures 4A and S4), indicating a switch from corneal epithelia to skin-like epidermal cells after 6 weeks of age. Several studies suggest that metaplasia occurred mainly to CESCs or corneal epiethlial progenitor cells rather than terminally differentiated cells (Nowell and Radtke, 2017; Vauclair et al., 2007). We found that the expanded corneal epithelial cells of *Twist2-Cre; Notch1f/f* mice were positive for K14 in contrast to the restricted expression of K14 in corneal epithelia of normal mice (Figure 4B). Since K14 is expressed in epidermal basal cells, a progenitor cell population, and CESCs, we conceive that these overproliferating cells maintain some stemness. However, K14 expression in

the limbus was similar between *Twist2-Cre; Notch1f/f* and control mice (Figure 4B). Overall, these data suggested that *Notch1* deficiency in keratocytes results in epithelial progenitor cell fate switch to epidermal-like cells at the central cornea but does not activate the quiescent limbal CESCs.

Anti-inflammatory drug showed minimal effect on the corneal phenotypes

Inflammation has been reported to induce epithelial metaplasia and neovascularization in the cornea (Nowell and Radtke, 2017). We detected immune cell infiltration in the stroma of *Twist2-Cre; Notch1f/f* mice (Figure 2C). To investigate whether inflammation is responsible for the abnormalities observed in the cornea of *Twist2-Cre; Notch1f/f* mice, we treated the knockout mice with Tobradex, which contains antibiotic tobramycin and anti-inflammatory dexamethasone, for 2 months starting at 4 weeks of age. Although immune cell infiltration was largely blocked, as evidenced by diminished immunofluorescent signals for CD45, keratocytes activation, neovascularization, and the development of squamous metaplasia were still observed in Tobradex-treated knockout mice (Figure S5). These results suggest that epithelial metaplasia and neovascularization were not caused by corneal inflammation in *Twist2-Cre; Notch1f/f* mice.

Notch1 suppresses VEGF expression via Hif1α in the keratocytes

Neovascularization often involves VEGF molecules (Lobov and Mikhailova, 2018; Meurette, 2020). We indeed found that stromal neovascularization in *Twist2-Cre; Notch1f/f* mice was accompanied by an increase in the mRNA levels of *Vegfa and Vegfc*, but not of *Vegfb* or *Vegfd*, in corneal stroma samples (Figure 5A). How does *Notch1* deletion upregulate the expression of VEGF molecules in keratocytes? A previous study showed that NICD and Hif1α form a complex to promote the expression of NICD target genes (Qiang et al., 2012). Our immunohistochemical staining revealed an increase in expression of Hif1α, which was mainly located in the nucleus, in the keratocytes of *Twist2-Cre; Notch1f/f* mice compared with control mice (Figure 5B). Quantitative PCR analysis revealed an increase in *Hif1α* mRNA levels as well in the stroma of the mutant mice (Figure 5C). Overall, these results suggest that *Notch1* ablation induces Hif1α expression especially in corneal keratocytes but not in corneal epithelial cells (Vauclair et al., 2007).

We also confirmed this finding in corneal keratocytes/fibroblasts *in vitro*. Primary corneal keratocytes were isolated from normal mice and plated to obtain 80% confluency. We used interference RNA (small interfering RNA [siRNA]) to knock down Notch1 and/or Hif1α in these cells. We found that transient Notch1 knockdown resulted in an increase in expression of *Vegfa* and *Vegfc* (Figure 5D), confirming our *in vivo* findings. The increase in *Vegfa* and *Vegfc* expression induced by Notch1 knockdown was diminished by knockdown of Hif1α (Figure 5D). Overall, these results suggest that Notch1 suppressed the expression of Hif1α and, subsequently, the transcription of *Vegfa* and *Vegfc*. In *Notch1*-deficient keratocytes, increased expression of VEGF molecules may underlie the increase in *Dll4* expression (Figure 1E), as VEGF is a well-known inducer of Dll4 expression.

Axitinib alleviates neovascularization and other cornea phenotypes

We then tested whether keratocyte-secreted VEGFs play a role in the cornea anomalies in *Twist2-Cre; Notch1f/f* mice. To this end, we used axitinib, an inhibitor of VEGFR, to treat the mutant and control mice. Four-week-old mice were given intraperitoneal injections of axitinib daily for 2 months. Macroscopically, no obvious anomaly was seen in axitinib-treated mutant mice. Histological and immunological analyses revealed normal corneal epithelial and stromal structures in 3-month-old axitinib-treated mutant mice (Figures 6A and 6B). Axitinib not only diminished neovascularization but also suppressed epithelial cell metaplasia and overproliferation in the knockout mice (Figure 6B). Moreover, immunostaining revealed that axitinib prevented the overactivation of mTOR and ERKs in central corneal epithelial cells (Figure S6), consistent with the decrease in proliferation of epithelial cells. These results suggest that stroma-generated VEGFs might have caused most of the corneal phenotypes in *Twist2-Cre; Notch1^{f/f}* mice. Noticeably, since epithelial metaplasia and overproliferation occurred earlier than neovascularization (Figure 2B), VEGFs generated by the stroma may directly act on corneal epithelial cells to promote their proliferation and metaplasia, independent of the formation of functional blood vessels (Akil et al., 2021).

Ablation of *Notch1* in the keratocytes leads to defective corneal regeneration

Lastly, we determined the role of Notch1 signaling in keratocytes in cornea regeneration, a function of epithelial progenitor cells, as Notch signaling was activated upon alkali burn in our epithelial injury model (Figure 1B). Four-week-old mice were wounded by alkali burn at the central cornea. We used young mice to exclude possible effects of cornea growth defects on regeneration as *Twist2-Cre; Notch1^{f/f}* mice started to show anomalies after 5 weeks of age (Figure 2B). We collected the corneas 10 days after injury. The epithelial layer of *Twist2-Cre; Notch1f/f* mice was much thinner than that of control mice and was accompanied by decreased numbers of Ki67+ proliferating epithelial cells (Figures 7A and 7B), suggesting that *Notch1* in keratocytes
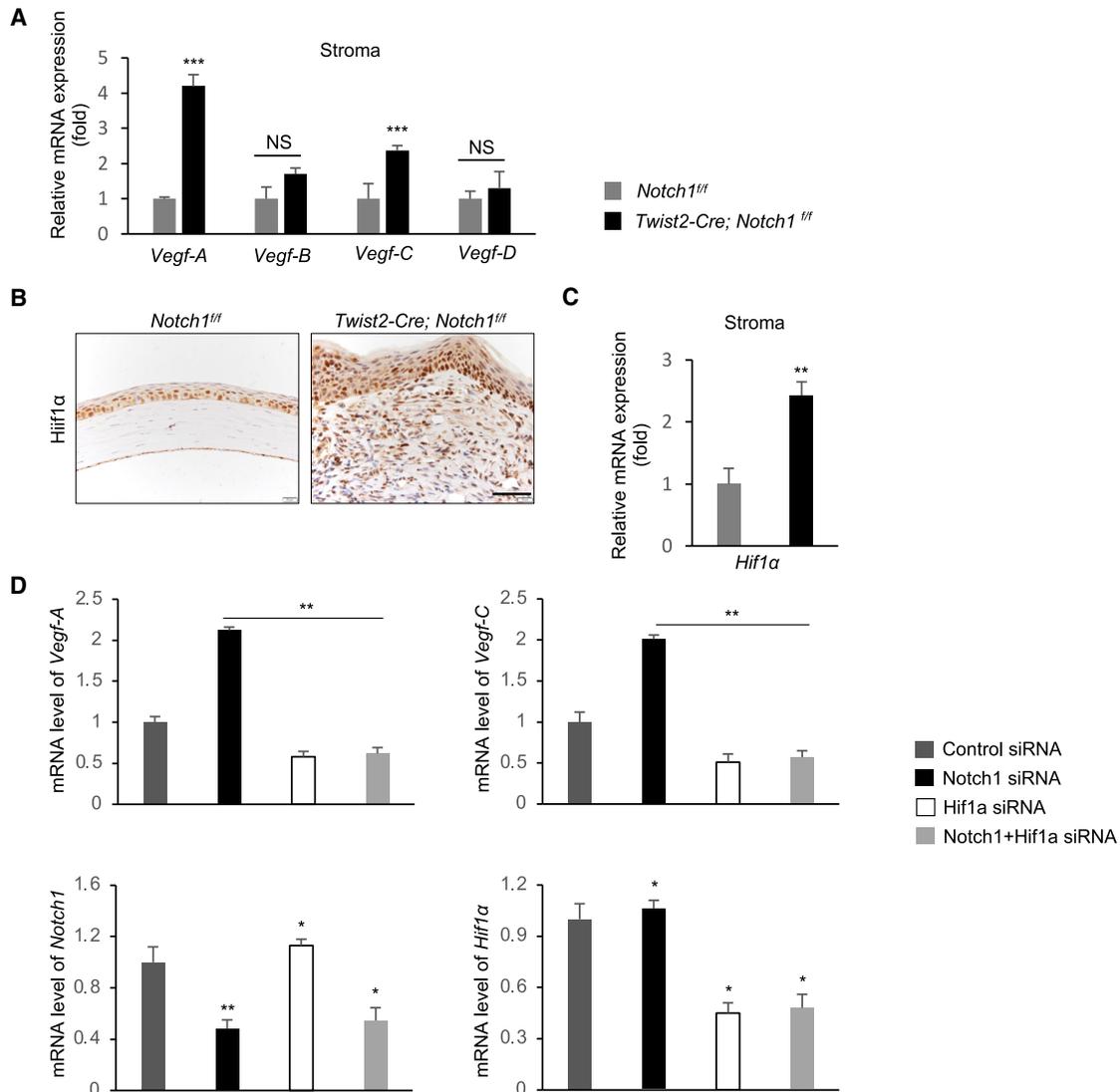
Figure 5. *Notch1* deletion leads to increased expression of *Hif1α* and *Vegfa* and *Vegfc*

(A) Quantitative real-time PCR analysis showed the mRNA levels of VEGF family members in the corneal stroma of the mutant and control mice. The values of control samples were set at 1. n = 3 mice. Data are shown as mean ± SD of three independent experiments. ***p < 0.001. NS, not significant.

(B) Representative immunohistochemical staining results showed increased expression of Hif1α in the stroma of *Twist2-Cre; Notch1^{f/f}* mice. Sections were developed using DAB chromogen, and nuclei were counterstained with hematoxylene. n = 3 mice. Scale bar: 20 μm.

(C) Quantitative real-time PCR results showed that Hif1α expression was upregulated at the mRNA levels in the corneal stroma of *Twist2-Cre; Notch1^{f/f}* mice. The value of control samples was set at 1. n = 3 mice. Data are shown as mean ± SD of three independent experiments. **p < 0.01.

(D) Quantitative real-time PCR results showed the effects of knockdown of Notch1, Hif1α, or both on the expression of *Vegfa* and *Vegfc*. The values of control samples were set at 1. n = 3 mice. Data are shown as mean ± SD of three independent experiments. *p < 0.05. **p < 0.01.

affects epithelial cell proliferation. Immunostaining revealed a lack of K12, a marker for matured corneal epithelial cells, in regenerated epithelia of the mutant mice, indicating defective epithelial cell differentiation. However, expression of Col1α was increased in the injured corneas of *Twist2-Cre; Notch1f/f* mice (Figure 7B), consistent with our observation

that *Notch1* suppresses stroma expansion under homeostatic conditions (Figure 2C). Taken together, these data indicate that Notch1 signaling in the stroma is required for corneal epithelial progenitor cell proliferation and differentiation during regeneration, highlighting the importance of stroma-epithelia interactions during cornea regeneration
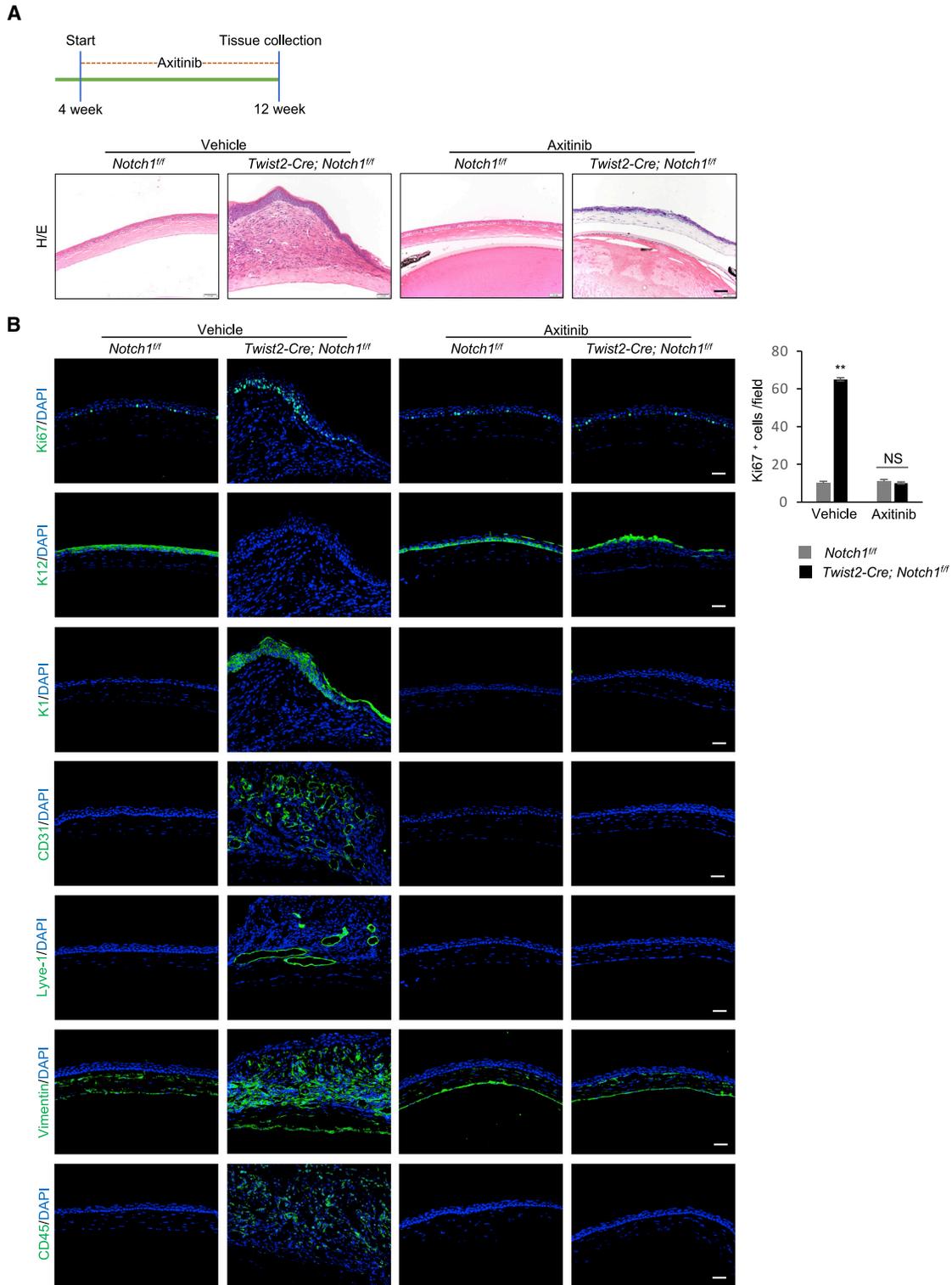
Figure 6. VEGFR inhibitor axitinib prevents development of corneal anomalies in *Twist2-Cre; Notch1*^{f/f} mice

(A) Representative histological analyses (H&E staining) showed that axitinib could rescue the corneal anomalies of *Twist2-Cre; Notch1*^{f/f} mice. n = 3 mice. Scale bar: 50 μm. Top panel: diagram showing the time of axitinib administration and mouse euthanization.

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(Dziasko and Daniels, 2016; Stassen et al., 2020; Vauclair et al., 2007).

DISCUSSION

Corneal transparency is of crucial importance for normal vision. Here, we provide genetic evidence that Notch1 signaling in corneal stroma is required for maintaining transparency and integrity of the cornea but is not required in cornea development. We show that the deletion of *Notch1* in Twist2⁺ corneal keratocytes leads to keratocyte activation and increased *Hif1α* expression and VEGF production, which result in corneal stroma expansion, neovascularization, and lymphatic vessel invasion, followed by increased proliferation and metaplasia of epithelial progenitor cells at the central cornea. Although Twist2 marks some endothelial cells in *Twist2-Cre*; *Notch1f/f* mice, Notch1 signaling in corneal endothelial cells has been reported to mainly promote endothelial-to-mesenchymal transition (Li et al., 2013). This, together with our findings that the original endothelial layer remains intact in *Twist2-Cre*; *Notch1f/f* mice (Figure 2B), suggests that the phenotypes observed in the mutant mice are largely if not exclusively due to the loss of *Notch1* in keratocytes.

Notch signaling regulates multiple organ development by affecting cell proliferation and differentiation during embryogenesis, as well as adult tissue renewal and maintenance, often in a context-dependent manner (Siebel and Lendahl, 2017). Previous studies described the corneal phenotype of *Notch1* ablation in corneal epithelial cells (K14-Cre mediated) (Movahedan et al., 2013; Vauclair et al., 2007), which bear some resemblance to the *Twist2-Cre*; *Notch1f/f* mice, i.e., stroma expansion, epithelial metaplasia, and defective wound-healing responses. However, the newly generated stroma was much thinner and less vascularized whereas the epidermis-like epithelial layer was much thicker than those in *Twist2-Cre*; *Notch1f/f* mice. These results suggest that Notch1 has a cell-autonomous effect in corneal epithelia. Thus, Notch1 signaling in epithelia influences keratocytes and vice versa, confirming a critical role for Notch1 signaling in communication between the epithelial and stroma layers in the cornea. More importantly, Notch1 signaling in either cell type is required to maintain corneal transparency.

Moreover, we show that VEGFs are key factors in mediating Notch1 ablation-induced cornea anomalies in our *Twist2-Cre*; *Notch1f/f* mice; this is in contrast to *K14-Cre*;

Notch1f/f mice, which did not show alteration in VEGF expression. Instead, epithelial-cell-specific *Notch1* ablation induced development of cornea anomalies via FGF2 and likely the retinoic-acid pathway, which may explain why neovascularization is not as obvious in these mice (Vauclair et al., 2007). Taken together, these results suggest that Notch1 signaling in keratocytes and epithelial cells uses different mechanisms to maintain corneal transparency. Our mouse model is also different from inflammation-induced corneal plaque formation, which elicits aberrant mechanotransduction and triggers the YAP-TAZ/β-catenin pathway in the epithelia (Nowell et al., 2016). Moreover, we show that inhibition of bacteria-induced inflammation does not affect development of cornea anomalies in *Twist2-Cre*; *Notch1f/f* mice.

A functional interaction between VEGF and Notch pathways has been established in tip and stalk cells during blood-vessel sprouting (Benedito et al., 2012; Blanco and Gerhardt, 2013; Gridley, 2007; Selvam et al., 2018). VEGF upregulates Dll4 expression in tip cells, which then binds to Notch receptors on stalk cells to suppress neovascularization. In general, the functions of Notch signaling in blood-vessel sprouting are endothelial cell autonomous. Here, we show that Notch1 signaling acts in keratocytes and upstream of VEGF expression to maintain the avascular status of the cornea. Whether this pathway plays a similar role under other conditions, for example, in solid tumors (Aster et al., 2017), needs further investigation. The ligands for Notch1 may be provided by neighboring keratocytes. Moreover, Notch1 regulates the expression of Notch ligands in keratocytes but not in corneal epithelial cells (Figure 1E). Based on these findings, we propose the existence of feedback regulatory circuits in keratocytes that maintain avascular status of the corneal stroma. Disruption of the balanced network produces an environment that supports neovascularization. For example, contact-lens wear or trauma may change the oxygen levels in the cornea, which may overcome the suppression of Hif1α by Notch signaling and lead to neovascularization in the stroma. In addition, Notch1 activation in keratocytes is enhanced upon injury, which may suppress VEGF expression and help to prevent stroma overexpansion and neovascularization during corneal regeneration.

Interestingly, in both *K14-Cre*; *Notch1f/f* and *Twist2-Cre*; *Notch1f/f* mouse models, the limbus region remains unaffected. We found no differences in the number and localization of p63⁺ CESCs in the limbus of *Twist2-Cre*; *Notch1f/f* mice. Since the limbal stem cells reside at the crypt of the palisade structure of limbus, it is possible that they are

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(B) Representative immunostaining results showed that axitinib could alleviate stromal remodeling and expansion, neovascularization, epithelial metaplasia, and immune cell infiltration in *Twist2-Cre*; *Notch1f/f* mice. Nuclei were counterstained with DAPI (blue). n = 3 mice. Scale bar: 50 μm. Right panel: quantification of Ki67⁺ cells per field. For each corneal section, six different fields were quantified and averaged. n = 6 mice. Data are shown as mean ± SD of three independent experiments. **p < 0.01. NS, not significant.
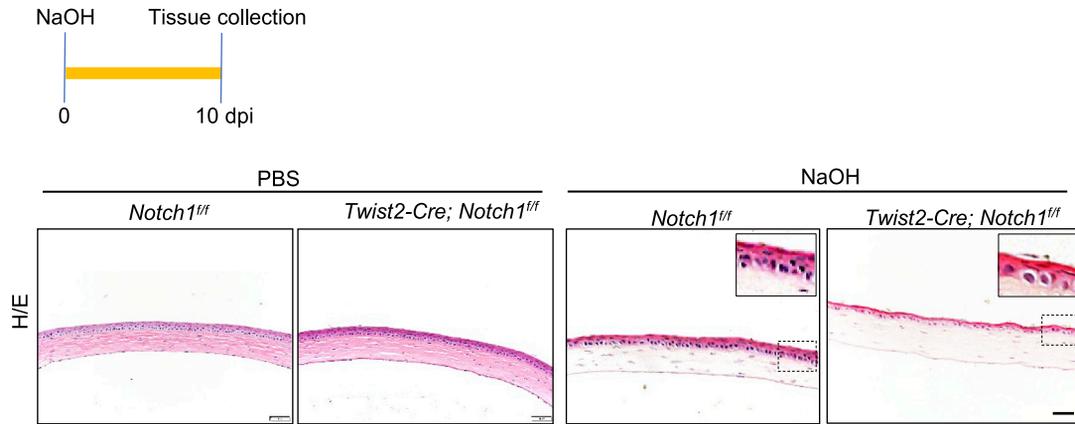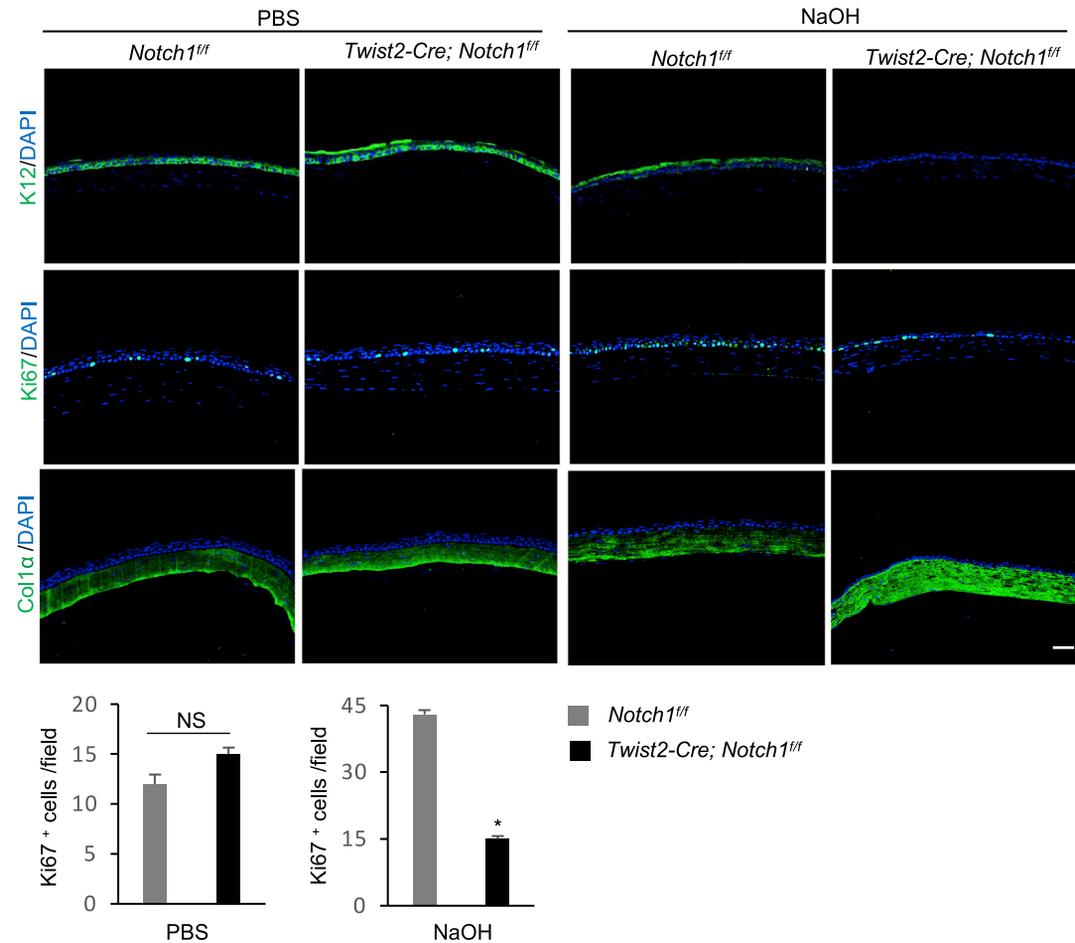
Figure 7. *Notch1* ablation in keratocyte impairs corneal epithelial regeneration

(A) Histological analyses revealed that cornea repair was slower in *Twist2-Cre; Notch1$^{f/f}$* compared with age- and sex-matched control mice after alkali burn. Inset: enlarged view of the indicated region. n = 3 mice. Scale bar: 50 μm. Top panel: Diagram showing the time of NaOH injury to mice and tissue collection.

protected from disruptive signals from the stroma. Another possibility is that limbal stem cells are not sensitive to signals from the newly formed vascularized stroma due to their quiescent nature, since the stroma underneath the limbus region are normally vascularized. While limbal CESCs have been shown to be mostly quiescent (Altshuler et al., 2021; Yu et al., 2021), studies have suggested that the transit-amplifying cells at the central cornea have stem cell activities (Majo et al., 2008; Nasser et al., 2018). Here, we show that the cells at the central cornea undergo proliferation, express p63, expand further in injury models, and can convert into epidermal-like cells, properties possessed by stem cells but not terminally differentiated epithelial cells.

In summary, we provide genetic evidence that Notch1 signaling in keratocytes plays an indispensable role in maintaining corneal transparency. *Notch1* executes its function by suppressing the expression of VEGFs via Hif1α to constrain stromal cell proliferation, proliferation and metaplasia of epithelial progenitor cells at the central cornea, and neovascularization. These findings suggest that the Notch1-Hif1α-VEGF axis may represent a major pathway underlying the pathogenesis of various corneal diseases and may thus serve as a potential therapeutic target for patients with corneal abnormalities. In addition, this study identifies stroma cells as an important source of VEGFs, which are under the control of Notch1 signaling.

EXPERIMENTAL PROCEDURES

Mouse lines and maintenance

The knockin *Twist2-Cre*, *Notch1^{f/f}*, and *Rosa-tdTomato* mouse lines were purchased from the Jackson Laboratory. *Rosa-tdTomato* mice were crossed with *Twist2-Cre* mice to generate *Twist2-Cre; Rosa-tdTomato* mice. *Notch1^{f/f}* mice were crossed with *Twist2-Cre* mice to generate *Twist2-Cre; Notch1^{f/f}* mice. All animals used in the study were bred and housed in the specific pathogen-free (SPF) facility of Shanghai Jiao Tong University, and food and water were provided *ad libitum*. All mouse work was carried out following the recommendations by the National Research Council Guide for the Care and Use of Laboratory Animals, with the protocols approved by the Institutional Animal Care and Use Committee of Shanghai, China (SYXK(SH)2011-0112).

Lineage tracing

The lineage-tracing experiments were performed in both male and female adult *Twist2-Cre; Rosa-tdTomato* mice, with similar results obtained. To trace tdTomato+ cells, cryostat sections of tissues were used. Mouse eyeballs were fixed and embedded in optimal

cutting temperature (OCT) compound (Leica) and were snap frozen in liquid nitrogen. Sections were cut 5-μm-thick at -20°C and then were counterstained with DAPI solution (Invitrogen, D1306), and the images were taken under a microscope (Nikon ECLIPSE 80i, Nikon Instruments).

Alkali injury on the cornea

Mice were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital. A proparacaine hydrochloride eye drop (0.5%, Bausch + Lomb) was applied to the cornea 3 min before the application of a 2-mm-diameter sodium hydroxide (NaOH)-soaked filter paper on the central cornea. The filter paper was prepared by soaking in 1 M NaOH solution for 10 s and dried of excess NaOH by a light touch on a paper towel. The filter paper stayed on cornea for 20 s. Complete adherence of the filter paper on the corneal surface was confirmed by a gentle push of the perimeter using forceps. The cornea was irrigated with sterile saline using a 50-mL syringe with a 23G needle for 10 s immediately after the removal of the filter paper. The mouse was then placed on a heating pad, and the eye irrigated gently for another 15 min with sterile saline. Erythromycin ophthalmic antibiotic ointment (Alcon Laboratories) was then applied to the eye, and the mice were kept on the heating pad until fully awake.

Separation of corneal epithelium and stroma

Cornea was carefully excised from the enucleated eye at the limbal-conjunctiva boundary using microsurgical techniques under a dissecting microscope. The protocol used to separate the epithelium from the stroma is described in detail in the supplemental experimental procedures.

Treatment with VEGFR inhibitor or antibiotics

Axitinib (Selleck Chemicals) dissolved in dimethyl sulfoxide (DMSO) was reconstituted in 44.44% PEG300, 5.56% Tween80, and 44.44% water. Mice were treated at a dosage of 10 mg/kg Axitinib or diluent through i.p. administration every other day for 60 days. For antibiotic treatment, Tobradex (Alcon Laboratories) containing tobramycin and dexamethasone was applied topically on the eyes.

Histological analyses

For histologic evaluation, paraffin-embedded eyeballs or excised corneas were sectioned 4-μm-thick and were used for H&E staining following standard protocols.

Assessment of lipid content of meibomian glands (MGs) of eyelid

To stain lipid of MGs, 5-μm-thick frozen eyelid sections were used for oil red O staining following standard protocols. In brief, after washing in PBS, sections were incubated in 60% isopropanol for 5 min and then stained with freshly prepared and filtered oil red O

(B) Immunofluorescence-staining results showed defects in epithelial proliferation (Ki67) and differentiation (K12) in *Twist2-Cre; Notch1^{f/f}* mice during corneal regeneration. The sections were stained with antibodies against K12, Ki67, and Col1α. Nuclei were counterstained with DAPI (blue). n = 3 mice. Scale bar: 50 μm. Bottom panel: Quantification of Ki67+ cells for both NaOH- and PBS-treated corneas. For each section, six different fields were quantified and averaged. n = 3 mice. Data are shown as mean ± SD of three independent experiments. *p < 0.05. NS, not significant.

solution (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Finally, the sections were washed in PBS and counterstained with hematoxylin.

Immunofluorescence staining

For paraffin-embedded tissues, 4-μm-thick sections were deparaffinized, rehydrated in graded ethanol, and subjected to antigen retrieval. Frozen sections were used without pre-treatment. Images were viewed and captured using Nikon ECLIPSE 80i (Nikon Instruments). See supplemental experimental procedures for the detailed protocol.

Immunohistochemistry

Paraffin sections prepared as mentioned in the previous section were treated with 3% H_2O_2 in methanol for 30 min in the dark at room temperature (RT) to inactivate endogenous peroxidase. Images were viewed and captured using the Olympus DP72 microscope (Olympus Microsystems). See supplemental experimental procedures for details.

Protein extraction and western-blot analysis

Proteins from pooled corneal tissues were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with protease and phosphatase inhibitors. Protein concentration was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific). The protein band was detected and visualized using Super Signal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and FluorChem E system (Protein Simple, Santa Clara, CA, USA, USA). Actin was probed for normalization of protein loading. Densitometric values of desired bands were used to compare the expression of specific proteins. For a detailed protocol, see the supplemental experimental procedures.

RNA extraction and quantitative real-time PCR

Total RNA was extracted with Trizol Reagent (Invitrogen, Thermo Fisher Scientific) following the manufacturer's instruction. cDNA was reversed transcribed using Transcriptor First Strand cDNA synthesis kit (Roche, Basel, Switzerland) with random anchored-oligo (dT) 18 primers and random hexamer. Quantitative PCR analyses were carried out using FS Universal SYBR Green Master Premix (Roche) in a final volume of 20 μL on Light Cycler 480 II (Roche). Each PCR reaction was run in triplicates. The delta-delta Ct (ΔΔCt) method was used to quantify the relative expression levels of mRNA species. The expression of *Gapdh* was used as the endogenous control. Primer sequences are listed in Table S1.

Primary culture of mouse corneal keratocytes

Corneal stroma keratocytes were isolated from 8-week-old mice. The cornea tissue was excised, and the epithelial sheet was peeled off as described above. The stroma tissue was transferred into digestion buffer containing collagenase type I in DMEM (3 mg/mL) and incubated at 37°C for 3–4 h with gentle shaking. Following the digestion, the cells were collected by low-speed centrifugation (300 x *g* for 5 min) and resuspended in trypsin-EDTA for an additional digestion of 15 min at 37°C. After the digestion, the cells were collected again, washed in PBS, resuspended in DMEM

containing 10% fetal bovine serum (FBS), and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO_2. The medium was changed 48 h later with DMEM containing 10% FBS without antibiotics. In culture, these cells acquired fibroblast-like morphology.

Gene silencing with siRNA-mediated transfection

For siRNA transfection, corneal keratocytes were plated into 12-well tissue culture plates and cultured until 80% confluency. Transfection was carried out following the manufacturer's protocol. For full details, see the supplemental experimental procedures.

Statistical analysis

Results were expressed as means ± SD. The number of mice used for each experiment is stated in the figure legends. For histology, at least 3 mutant and control littermates were used, and representative results are presented. Quantitative real-time PCR was performed using RNA isolated from 3 mice. Western blot was carried out using samples from 3 mice as well. Comparisons between two groups were analyzed using two-tailed unpaired Student's t test, and a p value of less than 0.05 was considered to indicate statistical significance (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ when mutant mice were compared with control mice). Each experiment was repeated three times independently.

Data and code availability

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplemental information.

AUTHOR CONTRIBUTIONS

B.L., P.Z., J.L., and S.B. designed the study. S.B., M.S., G.Y., P.L., and Q.Y. performed the research. S.B. and M.S. analyzed the data. S.B., M.S., J.L., and B.L. wrote the paper.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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