LIM Homeobox Domain 2 Is Required for Corneal Epithelial Homeostasis

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ABSTRACT

The cornea requires constant epithelial renewal to maintain clarity for appropriate vision. A subset of stem cells residing at the limbus is primarily responsible for maintaining corneal epithelial homeostasis. Trauma and disease may lead to stem cell deficiency and therapeutic targeting to replenish the stemness capacity has been stalled by the lack of reliable corneal epithelial stem cell markers. Here we identified the location of Lhx2 in mice (mLhx2) cornea and conjunctival tissue using an Lhx2eGFP reporter model in human tissues (hLHX2). Lhx2 localized to the basal cells of central cornea, the conjunctiva and the entire limbal epithelium in humans and mice. To ascribe a functional role we generated Lhx2 conditional knockout (cKO) mice and the phenotypic effects in corneas were analyzed by slit lamp microscopy, in cell-based assays and in a model of corneal epithelium debridement. Immunodetection on corneal sections were used to visualize conjunctivalization, a sign of limbal barrier failure. Lhx2cKO mice produced reduced body hair and spontaneous epithelial defects in the cornea that included neovascularization, perforation with formation of scar tissue and opacification. Cell based assays showed that Lhx2cKO derived corneal epithelial cells have a significantly lower capacity to form colonies over time and delayed wound-healing recovery when compared to wildtype cells. Repeated corneal epithelial wounding resulted in decreased re-epithelialization and multiple cornea lesions in Lhx2cKO mice compared to normal recovery seen in wildtype mice. We conclude that Lhx2 is required for maintenance of the corneal epithelial cell compartment and the limbal barrier. Stem Cells 2016;34:493–503

SIGNIFICANCE STATEMENT

Over 1.2 million Americans suffer from cornea damage and over 40,000 people per year undergo corneal transplantation therapy to repair irreversible corneal injury. Corneal epithelial stem cell transplantation to stem cell deficient corneas has the potential to restore vision. In order to improve patient rehabilitation with limbal stem cell deficiency, the ability to better harness and induce the regenerative capacity of adult corneal epithelial stem cells (CESCs) using reliable genetic markers is required. The proposed research will aid in identifying, purifying and expanding stem cell populations and induce corneal epithelial cells to behave stem cell-like necessary for stem cell maintenance.

INTRODUCTION

Transparency of the cornea is essential for vision. The integrity of the corneal epithelium, the outermost layer of the cornea, is maintained by corneal limbal epithelial stem cells localized between the edge of the cornea and the conjunctiva, in a zone known as the limbus. Trauma, disease or genetic alteration in the limbus, can result in corneal limbal stem cell deficiency that manifests clinically as recurrent epithelial defects leading to significant corneal decompensation with signs of neovascularization, conjunctivalization, opacification, and in extreme cases ulceration, cornea perforation, or blindness [1–3]. Treatment of limbal stem cell deficiency requires the direct involvement and application of corneal epithelial stem cells, such as donor limbal stem cell transplantation. Presently there are no established limbal stem cell markers that enable sensitive and specific direct identification of corneal stem cells for selection and expansion for transplantation. Allogeneic limbal transplantation is at risk of rejection and requires systemic immunosuppression, as the corneal limbus is not an immune privileged tissue, unlike the rest of the cornea. Thus, further investigation of corneal epithelial stem cell biology remains a relevant and important area for
advancing the treatment options for limbal stem cell deficiency.

Lim homeobox domain 2 (Lhx2) plays an important role in a variety of epithelial–mesenchymal interactions and in the regulation of various stem/progenitor cell populations identified in skin and hair, liver, blood, and the brain [4–8]. Lhx2 has been shown to be crucial in the maintenance of stemness in murine hair follicle stem cells (HFSCs) [5, 9]. The cornea is an epithelial tissue derived from neuroepithelial ectodermal origin, similar to skin. As both tissues share a common developmental origin, our hypothesis is that previously identified stem cell markers in skin may also exist in the cornea. In support of this idea, there is evidence that cofactors of LIM domains (CLIMs), which interact with LIM domains such as Lhx2, regulate maintenance of HFSCs as well as corneal homeostasis [10]. Furthermore, Lhx2cko mice driven by the K14 promoter, results in reduced hair formation from the failure to maintain HFSC quiescence and hair anchoring [11]. Although the skin functions differently from the cornea, it has shown the potential to transdifferentiate into cells of a corneal phenotype [12]. This apparent connection between epidermal and corneal epithelial cells suggests that Lhx2 may not only be important in maintaining stem cells of the skin, but may also play a role in corneal epithelial stem cell maintenance. We used a mouse genetics approach to identify Lhx2 by using a green fluorescent protein (GFP) reporter gene tagged to the Lhx2 promoter, known as the Lhx2eGFP model and a conditional knockout mating K14-Cre with Lhx2flox/flox animals, conditionally ablating Lhx2 in keratin 14 driven cells. Our findings demonstrate that Lhx2 is required for the maintenance of corneal limbal stem cells and the preservation of the ocular surface structure.

**MATERIALS AND METHODS**

**Animals**

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) from Weill Cornell Medical College, in accordance with the US NIH Guide for the Care and Use of Laboratory Animals and guidelines of the Association for Research in Vision and Ophthalmology Statement for the use of Animals in Ophthalmic and Vision Research. Wild-type (WT) CD1 mice were obtained from Jackson Laboratories (Bar Harbor, ME). The transgenic mice Lhx2eGFP [13] and the floxed LIM homeobox 2 (Lhx2flox) mice [14] crossed with K14-Cre mice [15] to obtain Lhx2cko lines were obtained as a collaborative study with Dr. Elaine Fuchs (Rockefeller University, NY).

**Immunofluorescence and Preparation of Corneal–Conjunctival Wholemounts and Sections**

The Lhx2eGFP reporter allowed us to detect the expression of Lhx2 in corneal tissue. First, the expression of Lhx2 and GFP was detected in Lhx2eGFP corneal–conjunctival wholemount tissue. For Lhx2 detection, 20-week-old nonfixed mouse corneas were incubated with rabbit polyclonal LHX2Ab at 1:5,000 dilution (Gift from Dr. E. Fuchs, Rockefeller University) overnight at 4°C followed by secondary anti rabbit Cy3 (Jackson Immuno Research: 711-165-152, Westgrove, PA, https://www.jacksonimmuno.com/catalog/products/711-165-152). Samples were mounted in vectashield containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, http://vectorlabs.com/vectashield-mounting-medium-with-dapi.html).

Next, to detect corneal, limbal, and conjunctival expression of Lhx2eGFP reporter, 9-week-old corneas were fixed in 4% paraformaldehyde (PFA) for 40 minutes and embedded in Tissue Tek Optical Cutting Temperature compound (Sakura Finetek Japan Co., Tokyo, Japan) and snap frozen in liquid nitrogen. Cornea sections of 8 µm were mounted onto Superfrost Plus Gold slides (FishterScientific, Waltham, MA, http://www.thermoscientific.com/content/ts/en/product/superfrost-plus-gold-slides.html) and incubated with chicken polyclonal to GFP antibody, at 1:1,000 (Abcam: 13970, Cambridge, MA, http://www.abcam.com/gfp-antibody-chip-grade-ab290.html) followed by secondary antibody (as above) anti chicken Alexa 488 (Life Technologies: A11039, South San Francisco, CA, https://webshop.fishersci.com/insight2_uk/getProduct.do?productCode=10286672&resultSetPosition=0).

For LHX2 in human tissues, 10 µm corneal and conjunctival sections, were obtained from human corneas donated by the Eversight Eye Bank and processed for immunofluorescence as above using Cy3 as secondary antibody.

**Expression of Stem Cell Markers on Corneal Tissue**

The GFP and Lhx2 expression in mice harboring the Lhx2eGFP reporter was determined in the central and limbal corneal epithelium of 6-week-old mice. Corneal tissues from the center and limbus were isolated using a 2-mm biopsy punch (Miltex, Inc., York, PA) (n = 4 mice). Corneal epithelium were separated from stroma by incubating the tissues at 4°C overnight in CnT50 media (CELLnTEC, Bern, Switzerland) containing 100 mg/ml of dispase (Roche Diagnostics, Basel, Switzerland) and 100 mg/ml D-sorbitol (Sigma Aldrich, St. Louis, MO). Total RNA was extracted using the RNA Easy Mini Kit (Qiagen, Gaithersburg, MD, https://www.qiagen.com/us/shop/sample-technologies/rna/rna-preparation/rneasy-mini-kit), quantified and used in equal concentration of limbal and central corneal epithelium to generate cDNA using a reverse transcriptase polymerase chain reaction (RT-PCR) kit according to the manufacturer’s protocol (Invitrogen, Grand Island, NY, http://www.thermoscientific.com/us/en/home/life-science/pcr/reverse-transcription/rt-PCR.html). cDNA was used as a template for real-time PCR, using the Step One Plus Applied Biosystems (Thermo Fisher Scientific, Waltham, MA). For GFP detection, forward and reverse primers were diluted to 2 µM from 20 µM stock concentration in RNAsé free water. The GFP probe was diluted to 1 µM from 100 µM stock concentration. GFP primer sequence Egfp-fp: AGC AAA GAC CCC AGC TAC GGG ATG GGG GAT G-3’ (Applied Biosystems, Grand Island, NY, http://www.thermoscientific.com/us/en/home/life-science/pcr/reverse-transcription/rt-PCR.html).

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by 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds. All samples were tested in triplicate. For the detection of Lhx2, Trp63 and ABCG2 expression in WT and Lhx2cKO mice, RNA was isolated from the entire corneal epithelium (n = 10 mice), cDNA and RT-PCR was performed as described above. The primer pairs and TaqMan MGB probes (Applied Biosystems, Grand Island, NY) used included mouse GAPDH (Mm00465599m1), Trp63 (Mm00495788m1), ABCG2 (Mm00496364m1), and Lhx2 (Mm00839783_m1).

To study the Lhx2 expression on human samples, approval from Weill Cornell Medical College Institutional Review Board was obtained for use of human corneal specimens for research. Healthy corneas from male donors aged 57, 68, and 63 were separated into central and limbal segments using a trephine ring and the epithelium was scraped from the stroma by using a #64 Beaver blade (Becton Dickinson, Franklin Lakes, NJ). The RNA was extracted as described and cDNA was used as a template to test for the expression of LHX2 in the limbal versus central corneal epithelium. The GAPDH (Hs02758991g1) and LHX2 (Hs00180351m1) primers were obtained from Applied Biosystems, and the RT-PCR procedure was performed as described above.

Evaluation of Cornea Phenotypes

Mice were continuously evaluated to follow up the distinct phenotype that some Lhx2cKO mice presented along this study. Mice were when significant symptoms of distress were found. Cornea images from 2- to 26-week-old mice were obtained using a slit lamp (WT and Lhx2cKO, n = 12 mice each).

Isolation of Corneal Epithelial Cells

To obtain isolated corneal epithelial cells for cell culture or RNA analysis, the eyes from WT and Lhx2cKO mice were enucleated, washed in phosphate-buffered saline and incubated at 4°C overnight in CnT-50 media containing dispase and D-sorbitol as described above. The detached epithelial sheets were collected and incubated with 0.05% trypsin/0.03% EDTA (Life Technologies, Grand Island, NY, http://www.thermofisher.com/order/catalog/product/25300054) at 37°C for 10 minutes to isolate single cells using a spatula to peel off the corneal epithelium attached to the eyeball. The enzymatic reactions were stopped by adding CnT-50 media supplemented with fetal calf serum (FCS) (Sigma, St Louis, MO, http://www.sigmaaldrich.com/catalog/product/sigma/121133c?lang=en&region=US).

Colony Forming Assay

Isolated epithelial cells from adult WT and Lhx2cKO (6–10 weeks old) were seeded at 2,000 cells per well on Mitomycin C-treated (4 μg/ml) mouse embryonic fibroblast 3T3 feeder layer (Cell line: CCL-92) cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Grand Island, NY, http://www.thermofisher.com/us/en/home/life-sciences/cell-culture/mammalian-cell-culture/classical-media/dmem.html) containing 10% FCS in triplicate on six-well culture plates. The number of colonies generated expressed as a percentage of the total number of epithelial cells plated in a well was defined as the colony forming efficiency (CFE), determined at days 4–12. The clone growth capacity was evaluated on days 12–14 and the cultures stained with 1% crystal violet. SPSS statistics version 19 was used for statistical analysis; a Student’s t test was used for comparing the CFE (%) with a p < 0.05 as statistically significant.

In Vitro Scratch Assay

To determine the direct role of Lhx2 on epithelium renewal, we evaluated the wound healing capacity of WT corneal cells and compared with cells derived from Lhx2cKO mice. Corneal cells from adult WT and Lhx2cKO (adult 6–9 weeks old) were isolated as described above and seeded at a density of 2.7 × 10^4 per square centimeter on tissue culture dishes coated with rat-tail I collagen (Life Technologies, Grand Island, NY, http://www.thermofisher.com/order/catalog/product/A1048301) at a concentration of 5 μg/cm². Cells were grown to confluence over 7 days and scratched using a P200 pipette tip creating a cell-free area as described by Liang et al. [16]. The scratched areas were photographed in the same location at 0, 8, 16, 24, and 48 hours. The cell-free areas were measured using Image J Software (Image J 1.47v, NIH, Thornwood, MD, Bethesda) and only closely matching areas were selected for analysis. The experiments were repeated three times.

In Vivo Corneal Epithelial Wounding and Histological Processing

To evaluate the role of Lhx2 on corneal epithelium renewal in vivo, the right eyes of WT (n = 8) and Lhx2cKO (n = 9) mice were subjected to epithelial debridement without causing injury to the underlying stroma. The left eyes were untouched and serve as controls. For this procedure, mice were anesthetized with intraperitoneal injections of a combination of ketamine (100 mg/kg) and xylazine (5 mg/kg; Phoenix Scientific, St. Joseph, MO) and 10 μl of proparacaine hydrochloride ophthalmic solution (0.5%) as a local anesthesia. A 2-mm trephine was used to mark the area for the epithelial debridement and blunt scalpel used to gentle detach the epithelium. Animal received analgesic, and topical fluorescein solution was administered to the corneal surfaces and the epithelial defects imaged at 0, 8, 16, 32, 48, 72, 96, and 144 hours. To avoid infection of the eye, 10 μl of vigamox (moxifloxacin, Alcon, Inc., Fort Worth, TX) was applied after epithelial debridement. The epithelial debridement experiments were repeated three times, once per week during 3 weeks, allowing the corneal epithelium to heal between procedures. This challenge to the epithelial renewal was performed on the same experimental group of mice. After 144 hours from the final epithelial debridement surgery, WT and Lhx2cKO mice were euthanized, the eyes enucleated and an orbital exenteration was performed including the fornical, palpebral conjunctiva, and cornea. The tissues were then fixed in 4% PFA for 40 minutes at room temperature and embedded in OCT as described above, cut at 8 μm sections and mounted adjacent to each other for immunofluorescence and periodic acid staining (PAS). For the detection of conjunctival tissue, sections were processed as above and rabbit polyclonal Keratin 15 antibody (Protein Tech Group, IL:10137-1-AP, Rosemont, IL, http://www.ptglab.com/Products/KRT15-Antibody-10137-1-AP.htm) at 1:200 dilution was used.

Periodic Acid Staining

For the detection of goblet cells on the cornea, frozen sections from WT and Lhx2cKO corneas after the third debridement...
surgery were rinsed in water, incubated in 0.5% periodic acid solution, and washed in water. Slides were stained in Schiff (Electron Microscopy Sciences, PA, Hatfield, PA) reagent, washed in tap water, and counterstained in Harris hematoxylin. Slides were rinsed in water, dipped in 0.5% acid.

Figure 1. Lhx2 is expressed in the central cornea and limbus.
Lhx2 wholemount cornea and conjunctival nuclear staining (red) were detected on Lhx2eGFP corneal tissue in central, limbal, and conjunctival tissue (A). GFP expression of Lhx2eGFP in corneal wholemounts was detected in the central cornea and spanned the entire limbal region, while conjunctival tissue highly expressed Lhx2eGFP (B). In tissue cross sections, GFP-positive cells were detected in the basal layer of the central corneal epithelium. In the peripheral cornea where the limbus is located, GFP expression was more abundant including suprabasal and basal layers. Basal cells of the conjunctiva stained positive for GFP as well (C). In human tissue, LHX2 nuclear staining in the central cornea localized to few cells in the basal layer, and included basal and suprabasal cells in the limbus. The conjunctiva distinctly expressed LHX2 in basal cells (D). Using quantitative reverse transcriptase polymerase chain reaction analysis, expression of GFP and Lhx2 was found significantly higher in murine epithelial cells originating from the limbus when compared with those from the central cornea (GFP = 4.5-fold higher, Lhx2 = 1.6-fold higher vs. central corneal epithelium) (E). Similarly, human LHX2 was expressed at significantly higher levels in limbal versus central corneal epithelium (32-fold higher) (F). White arrows indicate Lhx2eGFP and hLHX2 cells, respectively; yellow dashed line separates the central cornea, from the limbus, conjunctiva, and sclera. Scale bar = 50 μm for images in (A–D). Data are means ± SE (human n = 3, mouse n = 4). *, p ≤ 0.05. Abbreviations: cc, central cornea; cj, conjunctiva; DAPI, * * *; GFP, green fluorescent protein; l, limbus; sc, sclera.
Phenotypes of Lhx2cKO Mice Exhibit Abnormal Skin and Corneal Abnormalities

Table 1. Corneal abnormalities in Lhx2cKO mice increase with age.

| Age (weeks) | 1. Normal | 2. NV | Opacification | Perforation |
|-------------|-----------|------|---------------|------------|
| 2           | 3. 100    | 4. 0 | 5. 0          | 6. 0       |
| 3           | 7. 100    | 8. 0 | 9. 0          | 10. 0      |
| 4           | 11. 100   | 12. 0| 13. 0         | 14. 0      |
| 5           | 15. 100   | 16. 0| 17. 0         | 18. 0      |
| 6           | 19. 83    | 20. 8| 21. 0         | 22. 17     |
| 7           | 23. 75    | 24. 17| 25. 0         | 26. 25     |
| 8           | 27. 75    | 28. 17| 29. 8         | 30. 25     |
| 9           | 31. 75    | 32. 17| 33. 17        | 34. 8      |
| 13          | 35. 60    | 36. 25| 37. 17        | 38. 33     |
| 22          | 39. 30    | 40. 50| 41. 25        | 42. 42     |
| 26          | 43. 16    | 44. 83| 45. 75        | 46. 42     |

This table represents the result of slit-lamp imaging obtained from animals followed from 2 to 26 weeks old. The percentage of normal corneal architecture decreased over time, with increased percentages of mice exhibiting neovascularization, opacification and perforation as age increased.

alcohol and further rinsed in water to mount in DePeX mounting medium to cover slip.

Imaging

Bright field and immunofluorescence images were captured using an observer Z1 fluorescent microscope with the AxioCam HRm digital camera and AxioVision software (Carl Zeiss, Thornwood, NY). Wholemount images were captured using a Zeiss LSM-710 confocal microscope (Carl Zeiss, Thornwood, NY). Images were compiled in Photoshop CS5.1 and Illustrator CS5.1 (Adobe systems Inc., San Jose, CA.).

Statistics for Wound Healing Assays

All wound healing experiments were statistically analyzed only in those mice that healed by calculating the mean ± SD for each group and a Student’s unpaired t test was applied by Prism (Graph Pad Software, La Jolla, CA) and graphed. Test results were reported as two-tailed p values, where p < 0.05 were considered statistically significant.

Figure 2. Lhx2cKO mice exhibiting spontaneous corneal phenotypes P10 littermates from K14Cre+/− and Lhx2f/f gave rise to K14Cre−/− Lhx2f/f or WT (with hair) and K14Cre+/− Lhx2f/f or Lhx2cKO (reduced hair) (A). Slit lamp images of adult Lhx2cKO corneas from 6 to 8 weeks old mice (B) exhibiting the phenotype of Lhx2cKO cornea as clear and devoid of vessels (a), vascularized (b), perforated (c), and opaque infiltrated with vessels (d). Black arrows point to vessels in the cornea, white arrow to the perforation and red arrow to neovessels in the opaque cornea. Quantitative reverse transcriptase polymerase chain reaction from freshly isolated WT and ABCG2 were considered statistically significant.

Table 1. Corneal abnormalities in Lhx2cKO mice increase with age.

RESULTS

Lhx2 Is Expressed in Murine Corneal Epithelium and Preferentially Located Within the Limbus

To detect Lhx2 expression, we used a mouse genetics approach where the Lhx2 gene promoter was fused to eGFP and could be detected by direct fluorescence or immunostaining for GFP. Our studies on wholemount cornea, limbus, and conjunctival tissue demonstrated consistent localization that was diffusely distributed in central cornea, with more focal expression in the limbus, and high levels of expression in the conjunctival tissue at 20 weeks old. There was a distinct area of localization at the limbus which was not seen in adjacent sclera (Fig. 1A, 1B indicated by dashed yellow lines). GFP expression in the cornea is found in few central basal cells and in all epithelial layers of the limbus, which showed high expression (Fig. 1C, yellow line separate central cornea from limbus). The localization of human followed the same expression pattern as observed in the mouse. The central cornea expressed Lhx2 in few basal epithelial cells, whereas limbal corneal expression included basal and a few suprabasal layers. The conjunctival expression, as seen in mouse was highly pronounced in the basal layer and included few cells above (Fig. 1D). To corroborate the immunostaining, we isolated cells from both areas of the cornea and performed a RT-PCR analysis. We found that both the expression of GFP and Lhx2 were significantly higher in the limbus as compared to central cornea (4.5 and 0.6 fold respectively) (Fig. 1E). We also investigated the relevance of the findings in mice to humans and found that this preferential expression of Lhx2 in limbal versus central cornea was even more pronounced in human cornea. Real-time PCR demonstrated a 32-fold higher levels of Lhx2 in human limbus versus central corneal epithelium (Fig. 1F).

Lhx2cKO Mice Exhibit Abnormal Skin and Corneal Phenotypes

We next generated Lhx2cKO mice to study how the deletion of Lhx2 influences corneal epithelium homeostasis. These...
mice were obtained by breeding K14Cre<sup>1/2</sup> and Lhx2fl/fl and gave rise to K14Cre<sup>2/2</sup>Lhx2fl/fl or WT and K14Cre<sup>1/2</sup>Lhx2fl/fl or Lhx2cKO. When comparing postnatal day 10 (P10) littermates, it was clearly visible that the Lhx2cKO mice have reduced body hair when compared with WT (Fig. 2A). When we analyzed the cornea, all WT mice were normal (data not shown) and Lhx2cKO corneas were normal up to 6 weeks and devoid of vessels or ocular surface disorders (Fig. 2Ba). Some adult Lhx2cKO corneas exhibited a spontaneous phenotype including neovascularization (Fig. 2Bb), perforation (Fig. 2Bc), and opacification (Fig. 2Bd). These phenotypic changes became more common and severe as the mice aged (Table 1). For instance, at 6 weeks of age, 8% of mice showed neovascularization, that increased to 17% at 9 weeks, and become more prominent as with age, resulting in 83% of animal presenting this phenotype at 26 weeks. Perforated corneas sometimes occurred independently of vascularization and sometimes together (recorded at 8 weeks). Opacified corneas were noticed around 8 weeks old and increased rapidly over the weeks likely due to the healing from those mice exhibiting perforations. At 13 weeks, 25% of mice showed signs of neovascularization, while greater numbers (33%) were perforated. By 26 weeks only 16% of mice were normal in appearance while the majority were neovascularized, perforated, or opacified.

The knockdown in Lhx2 expression and previously identified corneal stem cell markers was corroborated by quantitative RT-PCR on freshly isolated cornea epithelial cells derived from the entire cornea. The analysis revealed that P63 and ABCG2 expression were significantly reduced in Lhx2cKO as compared to WT mice. Lhx2 expression was decreased in the Lhx2cKO as expected (Fig. 2C).
Lhx2cKO-Derived Epithelial Cells Showed Reduced Colony Forming Efficiency

We assessed the proliferative capacity, population doublings, and colony forming efficiency (CFE) of WT versus Lhx2cKO corneal epithelial cells (CECs) in culture. Cells were isolated from the entire cornea and evaluated in a time dependent manner for CFE, and at day 12, the cultures were stained with crystal violet to visualize the colonies. In general, Lhx2cKO-derived cells showed reduced cell proliferation and CFE efficiency when compared with WT cells (Fig. 3A). Initially at day 5, Lhx2cKO CECs showed increased CFE (calculated by the number of colonies divided by the total number of plated cells on a 3T3 feeder layer) by 37% (CFE WT = 1 ± 0.0% vs. Lhx2cKO = 1.37 ± 0.06%, p = 0.0027) compared with the WT cells. The trend changed after day 7 and WT cells were 30.5% higher than Lhx2cKO cells (CFE WT = 2.3 ± 0.2% vs. Lhx2cKO = 1.6 ± 0.1%, p = 0.006) and at day 9, CFE were 50% higher (CFE WT = 4.1 ± 0.30% vs. Lhx2cKO = 2.1 ± 0.2%, p = 0.001) (Fig. 3B). Lhx2cKO corneal epithelial cells were able to form colonies in culture, though greater numbers of colonies were predominantly seen in WT cultures and rarely identified in the Lhx2cKO deficient cells.

Lhx2cKO Corneal Epithelial Cells Showed Delayed Wound Healing In Vitro

Tissue injury is known to activate stem cells to proliferate and form new tissue in various adult organs [17–20]. In culture, we mimicked the corneal wound healing procedure by scratching confluent cultures of the WT and Lhx2cKO murine CECs and monitored the closure of the wound. Images were taken at 0, 8, 16, 24, and 48 hours at the same locations in the cultures using phase-contrast microscopy. The cell-free area of the scratch in WT and Lhx2cKO cells was measured at time 0 and selected for direct comparisons only if they had approximately the same wound area between replicas to monitor healing over time. We found a significant decrease in Lhx2cKO wound closure when compared with WT (Fig. 4A). Quantification of the cell-free areas showed that Lhx2cKO cells delay wound healing as early as 8 hours (2.2 ± 1.16) when compared with the WT (7.5 ± 1.46, p = 0.0092). These differences remained significant overtime and at 24 hours WT cells completely close the scratch while Lhx2cKO-derived cells presented only 39.7 % closure. Lhx2cKO corneal cells fully recovered at 48 hours after wounding (Fig. 4B).

Lhx2cKO Mice Showed Impaired Corneal Epithelial Wound Healing

Corneal injury is repaired from proliferating corneal epithelial cells, originating from the limbus. Re-epithelialization can be visualized during this process by fluorescein that stains the cornea devoid of epithelium. To determine the role of Lhx2 in maintaining the stemness capacity of the limbus, we challenged the corneal epithelium with multiple epithelial debridements to establish if the re-epithelialization of the injured cornea proceeds in a similar fashion in Lhx2cKO and WT mice. The mice (WT n = 8, Lhx2cKO n = 9) were subjected to epithelial debridement repeatedly once a week for 3 weeks. We used the right eyes for debridement and the contralateral eye as a control. We found that Lhx2cKO mice have impaired capacity to heal the injured corneal epithelium. Slit lamp examination demonstrated that WT corneas routinely healed by 32 hours after a third debridement, whereas Lhx2cKO corneas had a marked delay in re-epithelialization with only 30% of these corneas healed at 32 hours post-injury, whilst a significant subset of Lhx2cKO mice demonstrated markedly delayed wound closure. After a third epithelial debridement, WT mice healed their epithelium at 32hrs while most Lhx2cKO had persistent epithelial defects at 144 hrs post wounding (typical example of this marked delay in healing shown) (A). A detailed graphical representation of all epithelial debridements showed that Lhx2cKO mice (far right panel) have a reduced healing capacity after repeated debridement and presented corneal complications, such as corneal neovascularization and perforation that were not observed in the WT (far left panel) (B). Abbreviation: WT, wild type.
144 hours (Fig. 5A). Experimental data from epithelial debridement 1, 2, and 3 from WT (left) and Lhx2 cKO (right) are collectively represented in Figure 5B. We registered the epithelial wound healing response found in the cornea of both WT and Lhx2 cKO after these series of debridements. We observed no phenotypic alteration in the cornea of WT mice in any of the experiments. WT mice showed faster epithelial wound healing in the second and third debridement when compared with the initial first debridement (Fig. 5B). However, in the corneas of Lhx2cKO mice, we found that some mice healed within the normal time frame after the first debridement and some took longer to heal. After the second and third debridement, we observed an increase in the number of mice that showed epithelial defects or did not heal (Fig. 5B). The epithelial defects usually started with cornea neovascularization (white squares) that lead to perforation (red squares), keratinization, opacification, and loss of vision.

Using data from only those mice that did heal in <96 hours, a mild but not significant delay in wound healing was shown in the transgenic Lhx2cKO mice after the first debridement (Supporting Information Fig. S1). After the second epithelial debridement, there was a statistically significant delay in wound healing seen in Lhx2cKO (mean = 3.64 ± 0.08%, p = 0.0067) at the early time points of 8 hours compared with WT mice (mean = 18.48 ± 10.29%). These differences extended to 16 hours between Lhx2cKO (mean = 42.7 ± 21.08%, p = 0.0399) versus WT control (mean = 61.9 ± 12.16%). After 16 hours, however, no significant difference was seen in the rate of wound healing of both mice. After the final debridement, the wound healing rate for Lhx2cKO (mean = 39.7 ± 16.6%, p = 0.0176) was also significantly lower at early time points up to 16 hours when compared with WT (mean = 61.97 ± 10.10%). However, no significant difference was seen at later time points (Supporting Information Fig. S1).

Lhx2 Contributes to Maintenance of the Limbal Barrier

In the normal eye, the limbus separates the avascular cornea from the conjunctiva. The limbus is rich in stem cells and highly vascularized and by mechanisms that are not totally understood, it prevents blood vessels penetrating into the cornea. We observed that after epithelial debridement, some corneas in our Lhx2cKO population showed signs of neovascularization. Therefore, we investigated whether WT and Lhx2cKO corneas showed histological signs of neovascularization and conjunctivization by looking for specific markers, such as presence of goblet cells (normally only present in the conjunctiva) using PAS and keratin 15 (K15), a marker of conjunctival and limbal epithelial tissue in mice [21]. Nondebrided and after the third debridement corneas from WT and Lhx2cKO mice were sectioned and stained, including the cornea, bulbar, fornical, and palpebral conjunctiva. Higher power images are shown (large white boxes) for immunofluorescence and PAS (Fig. 6). In the absence of epithelium injury, we found normal distribution of K15- and PAS-positive cells only in the conjunctiva of both WT (Fig. 6Aa) and Lhx2cKO (Fig. 6Bb) mice. After consecutive corneal epithelial injury induced by
debridement, WT mice showed normal K15 and PAS restricted to the conjunctiva (Fig. 6Cc). However, Lhx2cKO mice revealed PAS-positive cells and K15 expression in the corneal epithelium consistent with conjunctivalization of the cornea in all the mice that underwent the procedure, regardless of external clinical appearance (Fig. 6D–6F). This includes eyes that clinically did not exhibit any signs of corneal neovascularization or persistent epithelial defects (no NV, no PED) (Fig. 6Dd), eyes with signs of corneal neovascularization but no persistent epithelial defects (NV, no PED) (Fig. 6Ee), as well as eyes with both corneal neovascularization and persistent epithelial (NV and PED) defects (Fig. 6Ff). Thus conjunctivalization of Lhx2cKO corneas indicates loss of a limbal barrier, therefore, ascribing a new role for Lhx2 in maintaining the cornea free from epithelium derived from the conjunctiva.

**Discussion**

The corneal epithelium is constantly renewed via corneal stem cells that are classically believed to reside at the limbus [3, 22, 23]. The location of Lhx2 and the fate of corneal epithelial cells in K14 driven Lhx2KO mice in the cornea have not been described previously. In our study, Lhx2 is highly expressed at the limbus, although it was also identified in the basal layer of the conjunctiva and central cornea. However, the strong localization at the limbus may suggest that Lhx2 is mobilized to the center cornea during normal epithelial renewal, growth or upon injury induced re-epithelialization.

Reports from Majo et al. have shown that stem cells may not be exclusive to the limbal region of mice and that populations of central corneal cells also possess regenerative capacity in the context of wound healing [24]. Previous studies have shown that limbal ablation did not induce corneal opacity and that limbal grafts did not migrate centripetally to the central cornea for up to 4 months. These data suggest that the limbus may not exclusively participate in normal corneal homeostasis and that there may be stem cells within the central cornea [24, 25]. Amitai-Lange et al. used multicolor lineage tracing of limbal and corneal K14-positive cells in R26R-Confetti/K14Cre Cre mice and showed that limbal epithelial cells underwent slow centripetal migration of 4–5 months until reaching the corneal center [26]. Although the limbus greatly contributed to corneal renewal, the progenitor cells had longer turnover, while limbal clones contributed to renewing itself to remain in a cluster at the limbus, creating radial streaks migrating to the center for normal homeostasis to occur. Upon mild injury, the central corneal cells greatly contributed to the repair [26].

As Lhx2 is highly expressed at the limbus, we generated K14 driven Lhx2 conditional knockout mice to determine its role in corneal epithelial homeostasis and renewal upon injury. K14 has previously been localized to the full thickness of the limbal epithelium, occasionally restricted to the basal limbal epithelial layer, the central cornea and the conjunctiva in humans and mice [21, 25, 27], therefore, we expected a conditional deletion of Lhx2 to appropriately affect these tissues. The Lhx2cKO mice displayed various phenotypes in the unperturbed state. A subset of these animals were phenotypically normal; however, other groups demonstrated variable evidence of stem cell failure including neovascularization, persistent epithelial defect, or corneal melt with perforation. The dominant negative (DN)-Clims transgenic, a cofactor of the LIM domain, also driven by the K14 promoter portrayed a striking phenotype resulting in hair loss, similar to our Lhx2cKO model. Clim5 have shown to be prominently expressed in the corneal epithelium, including the stem cell rich limbal epithelium, as seen in our Lhx2eGFP murine model. Abnormal corneal homeostasis of the model K14-DN-Clims and Lhx2cKO exhibit many of the same phenotypic characteristics including neovascularization, opacification in which 50% of the K14-DN-CLIMS were affected by 1 month old (significantly earlier than we observed in Lhx2cKO mice), and by 6 months nearly all mice were affected as seen in our Lhx2cKO model. Histologically, the DN-Clims cornea in older mice was hyperplastic, cornified, and the stroma was vascularized as we observed in the Lhx2cKO (data not shown).

The abnormal phenotypes seen in our transgenic mice model were similar to those described by either adding high concentrations of topical benzalkonium chloride frequently to the corneal surface [28] or alkali-burn models [29, 30], both resulting in limbal stem cell deficiency and eventually corneal neovascularization within a similar time frame of 4–12 weeks. Corneal neovascularization has been associated with a number of human corneal ailments such as chemical injury, Steven Johnson’s syndrome and aniridia, and patients with these ailments exhibit limbal stem cell deficiency and goblet cells on the corneal surface. The presence of goblet cells in the corneal surface epithelium is an indication that the conjunctival tissue has migrated across the limbus that normally separates the avascular cornea from the highly vascularized conjunctiva.

In our observations of Lhx2cKO mice, we also noticed that there was a time-dependent phenotype change in those mice having corneal alterations, whereby a subset of mice appearing normal at 5 weeks of age developed corneal neovascularization at 6 weeks, perforation at 7 weeks and opacity at 8 weeks. In mice, above 13 weeks old, corneal anomalies were more frequent, suggesting that as the cornea is exposed to environmental stress seen during the lifetime of these mice, the lack of Lhx2 affected the natural epithelial cell turnover. Other genetic models such as the Pax6 KO, representing a model of aniridia, exhibit similar phenotypes as seen in Lhx2cKO mice, including the in-growth of blood vessels from the limbal region onto the corneal surface, the appearance of goblet cells and corneal opacity that contributed to visual loss later in life.

To further understand the role of Lhx2 in the corneal epithelium, we studied the capacity of Lhx2cKO corneal epithelial cells to behave as stem cells. Lhx2cKO-derived cells have reduced colony-forming efficiency as well as reduced wound healing in culture suggesting that Lhx2 can directly affect the renewal of the dividing population that includes stem cells and transient amplifying cells, both required for normal corneal epithelium homeostasis and fundamental for repair after injury. We further extended our studies by analyzing the role of Lhx2 in vivo in corneas subjected to epithelial injury. As seen in isolated cells in vitro, mice lacking Lhx2 showed an impaired capacity to renew the injured epithelium when the tissue was challenged by multiple debridements, an effect that was not observed in WT mice that renewed their corneal epithelium normally. In WT mice, the epithelium healed within 32–48 hours. In subsequent second and third debridement’s performed on the same mice, this decreased to 24–32 hours, most likely due to a higher proportion of transient amplifying...
CONCLUSIONS

Lhx2 was abundantly expressed in the limbus, the stem cell rich region of the cornea. Conditional deletion of Lhx2 in the corneal epithelium produced spontaneous corneal anomalies that resulted in impaired vision. In vitro, corneal epithelial cells from Lhx2 cKO mice have reduced capacity of forming colonies and wound closure. In vivo, Lhx2 deficiency resulted in loss of corneal epithelial renewal and the limbal barrier. Lhx2 could be responsible in part for maintaining or activating quiescent cells in the corneal epithelium and its loss in the limbus resembles features of limbal stem cell deficiency. Our data further enhance the need to identify novel genes that regulate stem and progenitor cell activation and proliferation for corneal stem cell treatments.

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AUTHOR CONTRIBUTIONS

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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