Immunofluorescence Tomography of Mouse Ocular Surface Epithelial Stem Cells and Their Niche Microenvironment

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PurposE. Currently, there are no definitive immunomarkers for epithelial stem cells (corneal and conjunctival) or their poorly understood niche microenvironment. The H2B-GFP/K5tTA mouse enables visualization of label-retaining cells (LRCs), which exhibit the functional marker of stem cell quiescence. We used immunofluorescence tomography to evaluate putative stem cell markers and LRCs of the mouse ocular surface.

Methods. H2B-GFP/K5tTA mice were pulsed for 56 days and then chased with doxycycline to label LRCs. Limbus and eyelid tissue was 3-dimensionally (3-D) reconstructed using immunofluorescence tomography to identify and characterize LRCs using the putative stem cell markers sox9, keratin 19, ilrig1, blimp1, and abcb5.

Results. After 28 days of chase, LRCs were localized to the entire limbus epithelium and, infrequently, the anterior limbal stroma. Label-retaining cells comprised 3% of limbal epithelial cells after 56 days of chase. Conjunctival LRCs were localized to the fornix and comprised 4% of the total fornix epithelial cells. No stem cell immunomarker was specific for ocular surface LRCs; however, blimp1 enriched for limbal basal epithelial cells and 100% of green fluorescent protein-positive (GFP+) cells at the limbus and fornix were found to be ilrig1-positive.

Conclusions. Label-retaining cells represent a larger population of the mouse limbus than previously thought. They decrease in number with increased doxycycline chase, suggesting that LRC populations with different cell cycle lengths exist at the limbus. We conclude that current immunomarkers are unable to colocalize with the functional marker of epithelial stem cell quiescence; however, blimp1 may enrich for limbal epithelial basal cells.

Keywords: 3-D reconstruction, limbal epithelial stem cells, cornea, conjunctiva, H2B-GFP
Mouse Ocular Surface Epithelial Stem Cells

Highly clonogenic cells\(^9\) that may contribute to epithelial and keratin 5 (K5) promoter driving the to generate bigenic H2B-GFP/K5tTA mice. K5tTA mice with a sclera. Evidence indicates that the fornix contains LRCs\(^24\) and the most superficial layer of epithelium across the front of the eye is attached to the eyelid and the bulbar conjunctiva, which forms the tear film stability and constitutes the palpebral conjunctiva. The conjunctiva is likely to be renewed by quiescent cells at regular intervals (3-D) reconstruct the limbus of the H2B-GFP/K5tTA mouse to colocalize putative stem cell immunomarkers, such as sox9, keratin 19, abcb5, and irig1, with LRCs. Furthermore, LRCs with different rates of cell division may exist at the limbus as they decrease in number with increased doxycycline chase. The conjunctiva is likely to be renewed by quiescent cells at the fornix. Gene analysis of LRCs isolated from the ocular surface epithelial tissues, including cornea and conjunctiva, will shed light on the similarities and differences of gene expression in LRCs from separate tissues and is required to fully elucidate a unique protein marker of limbal stem cells.

**Methods**

H2B-GFP/K5tTA Transgenic Mouse Generation and Pulse-Chase Labeling

At all times mice were treated according to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research, and experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Irvine (PI: Jester, protocol # 2011-3002, approved September 8, 2011). To breed H2B-GFP/K5tTA mice, we crossed K5tTA transgenic mice generously provided by Stuart Yuspa, MD, at the National Cancer Institute (Bethesda, MD, USA)\(^28\) with TRE/H2B-GFP transgenic mice obtained from Jackson Laboratory (005104; Bar Harbor, ME, USA),\(^6\) and donated by the Fuchs lab, to generate bigenic H2B-GFP/K5tTA mice. K5tTA mice with a keratin 5 (K5) promoter driving the tTA transgene are crossed with transgenic mice expressing a tightly regulated tetracycline-responsive element (TRE) driving H2B-GFP expression. Intra-nuclear GFP expression within keratin 5\(^+\) epithelial cells was achieved using the “tet-off” strategy where histone H2B-GFP expression is dependent on the doxycycline-controlled trans-activator protein (tTA). In the progeny of these mice, GFP fluorescence is expressed in epithelial cells of the ocular surface. When these mice are fed doxycycline in their diet and the chase phase is initiated, GFP fluorescence is diluted 2-fold with each division and GFP is retained in slow-cycling, putative stem cells only over long-term chase.

To ensure all epithelial cells are labeled, H2B-GFP/K5tTA mice were pulsed for 56 days at P0 before introducing a doxycycline diet (2 g/kg; Bio-Serv, Flemington, NJ, USA). After 0 to 56 days doxycycline chase mice were killed by carbon dioxide asphyxiation and cervical neck dislocation to evaluate label dilution and epithelial cell quiescence through GFP label retention. Low magnification fluorescent imaging was done using a Leica MZ 16 A dissecting microscope (Leica Biosystems, Nussloch, Germany) and x/50/0.5 LWD objective.

**Tissue Embedding, Sectioning, and Immunostaining**

Mouse corneas were excised, fixed in 2% paraformaldehyde in PBS for at least 24 hours and embedded in low melting point agarose necessary to orient the tissue appropriately. Tissues were increasingly dehydrated with ethanol (EtOH; 50–75–90–100% at 30-minute intervals) before resin infiltration with butyl methacrylate (BMMA; Sigma-Aldrich Corp., St. Louis, MO, USA; 2:1; 1:1; 1:2; EtOH:BMMA). The BMMA-embedded blocks then were polymerized for a minimum of 8 hours using UV light at 4°C in a temperature-regulated ice cooler box (Ted Pella, Redding, CA, USA). Additionally, selected tissues were embedded in OCT and cryo-sections cut at 10 µm using a Leica cryostat. After drying, sections were labeled with 4',6-diamidino-2-phenylendole (DAPI; BMMA; Sigma-Aldrich Corp.) which was added to the mounting agent (1:1 Glycerol/PBS) at a concentration of 1:15,000.

The BMMA plastic blocks of corneas were serially sectioned at 2 µm using a Leica EM UC7 Ultramicrotome equipped with a diamond knife (DIATOME, Nidau, Switzerland). The protocol for sequential immunostaining and image acquisition has been described previously.\(^29\) All immunostaining steps were done using a TedPella BioWave microwave (Ted Pella) for antigen retrieval as well as rapid and consistent staining under vacuum and at regulated temperatures.

Before immunofluorescence staining, GFP fluorescence was imaged to preserve endogenous signal. Sections then were treated with acetone for 10 minutes to remove BMMA and immunostained with fluorescent antibodies before being mounted with 1:1 Glycerol/PBS with 1:15,000 DAPI. Serial sections were sequentially immunolabeled with either sox9 (1:500; Millipore, Billerica, MA, USA), collagen IV (1:500; Abcam, Cambridge, UK), abcb5 (1:500; Abcam), α-smooth muscle actin (1:250; Sigma-Aldrich Corp.), blimp1 (1:500; Abcam), irig1 (1:500; Abcam), and keratin 5 (1:1000; Abcam). The total epithelial cell, LRC, and immunostained LRC count from all epithelial layers of the 3-D reconstructed limbus, cornea and fornix conjunctiva was quantified through physical and computational counting with ImageJ (available in the public domain at http://imagej.nih.gov/ij/). Epithelial cells were segmented using Amira software (Visage Imaging, San Diego, CA, USA) and cells underneath the basement membrane were removed before quantification.

**Immunofluorescence Tomography 3-D Reconstruction**

Fluorescence imaging of GFP and AlexaFluor 546 tagged antibody markers was done using a Leica DMi6000B inverted
epi-fluorescence microscope (Leica Biosystems) with an ASI automated stage driven by Metamorph software (Molecular Devices, Sunnyvale, CA, USA). Sections were mosaic imaged using a Leica 320 0.75NA objective and a CCD camera capturing images with a pixel area of 0.46 μm². Thus, the volume of each pixel (voxel) in the 3-D reconstructions is 0.46 × 0.46 × 2 μm. The 3-D reconstructions of the H2B-GFP/K5tTA mouse limbus comprised of 186 (sox9, 372 μm), 122 (lrig1, 244 μm), and 87 (blimp1, 174 μm) BMMA plastic sections.

Stitching of tiled mosaics was completed with a 10% overlap using Metamorph. The stitched mosaics that make up the image of each section were converted to 8-bit and contrast enhanced in ImageJ before semiautomated alignment and 3-D reconstruction using Amira software. The segmentation of basement membrane and vasculature from the collagen IV immunostaining of limbus also was done using Amira.

RESULTS

At P0, H2B-GFP/K5tTA mice were fed normal chow, without doxycycline, for 56 days, which constitutes the pulse phase. At 56 days pulse, the entire epithelial layer of the H2B-GFP/K5tTA mouse ocular surface, including cornea, conjunctiva, and meibomian gland, was labeled green due to the nuclear expression of histone H2B tagged with GFP (Fig. 1A). This

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/934655/)

**FIGURE 1.** Slow-cycling limbal stem cells responsible for corneal epithelial cell renewal were localized to the limbus in the H2B-GFP/K5tTA mouse after >28 days doxycycline chase. (A) At 56 days pulse (no doxycycline), keratin 5+ cells express nuclear GFP, which includes all the basal keratinocytes of the ocular surface: the cornea, conjunctiva, and meibomian gland. (B) Low-magnification image of H2B-GFP/K5tTA mouse whole eye after 28 days doxycycline chase. At 56 days pulse - 28 days chase, LRCs were seen throughout the circumference of the corneal limbus, although regions devoid of LRCs were also observed (*). (C) After 56 days doxycycline chase, LRCs at the limbus are GFP+ because they divide infrequently compared to other epithelial cells which lose half of their GFP signal with every round of cell division.

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/934655/)

**FIGURE 2.** LRCs in the H2B-GFP/K5tTA mouse were localized to the limbus epithelium and infrequently, the anterior limbal stroma. (A) At 56 days pulse, all keratin 5+ corneal epithelial cells express nuclear GFP tagged to histone H2B. (B, C) A 2 μm BMMA cross-section of the H2B-GFP/K5tTA mouse limbus after 56 days doxycycline chase. GFP+ cells were primarily localized to the limbus epithelium (red arrow); however, on rare occasion, they were observed in the limbus stroma with varying fluorescence intensities (blue arrow). (D) Keratin 5 staining (B, C) shows that the GFP+ stromal cells are Keratin 5+. This suggests that migration of limbal epithelial cells may occur or there is ectopic expression of keratin 5.
LRCs. The vasculature network beneath the limbus epithelium, which harbors the LRC population with longer chase, and the GFP intensity in a cross-section of frozen tissue (Fig. 1C). The decrease in barrier between conjunctiva and corneal epithelium, as shown markedly fewer and localized to the limbus region at the cent imaging (Fig. 1B). By 56 days chase, GFP were seen to occupy the entire peripheral corneal limbus of division. After 28 day doxycycline chase, an abundance of LRCs epithelial cells lose fluorescence signal by 50% with every cell pulse-chase, unlike BrdU.15,30 cells express GFP and is not toxic to the mouse over long-term extended pulse phase of 56 days ensures all keratin 5 epithelial proteins also were found in basal cells that had lost GFP label (Fig. 4B), lrig1 (Fig. 4H), and blimp1 (Fig. 4I), but these retaining cells were observed to express sox9 (Fig. 4A), abcb5 (Figs. 4B, 5E), collagen IV and surrounding niche, including sox9 (Figs. 4A, 5D), abcb5 (Figs. 4B, 5E), collagen IV and a-SMA (Figs. 4C, 4F), keratin 19 (Figs. 4G, 4J), lrig1 (Figs. 4H, 4K), and blimp1 (Figs. 4I, 4L). Label-retaining cells were observed to express sox9 (Fig. 4A), abcb5 (Fig. 4B), lrig1 (Fig. 4H), and blimp1 (Fig. 4I), but these proteins also were found in basal cells that had lost GFP label through multiple divisions and were not specific for long-term chased LRCs. Collagen IV highlights the well-vascularized basement membrane and extracellular matrix that lies underneath the limbal epithelial stem cells (Fig. 4C). No immuno-markers were found here to exclusively colocalize with LRCs and the slow-cycling functional marker of stem cell quiescence (Figs. 4A–L).

No LRCs were observed in the central corneal epithelium or the bulbar/palpebral conjunctival epithelium, lending support to the theory of specialized “niches” of epithelial stem cells. In the H2B-GFP/K5tTA mouse ocular surface after 56 days chase, LRCs were found only in the limbus epithelium and fornix conjunctiva, as shown in Figure 5A (blue arrow) favoring the hypothesis that renewal of the bulbar and palpebral conjunctiva is directed from slow-cycling stem cells at the fornix. Furthermore, no LRCs were observed at the eyelid margin. Label-retaining cells of the fornix conjunctiva were either sox9+ or sox9+ (Fig. 5B).

To quantify LRCs and their expression of the above markers, the 3-D objects counter in ImageJ was used on the 3-D reconstructions and the percentage of sox9+, lrig1+, and blimp1+ cells of the total epithelial cell nuclei and LRC count were evaluated for the limbus, cornea, and fornix conjunctiva epithelium (Fig. 6). We found that these markers are not specific differences among LRCs, suggests that distinct populations of LRCs exist at the limbus with varied cell cycle lengths.

Immunofluorescence tomography 3-D reconstructions captured infrequent events at high-resolution in a fixed tissue through serial sectioning of volumes >100 μm at a section thickness of 2 μm. Basal corneal epithelial cells fluorescent green at 56 days because of the expression of the H2B-GFP fusion protein in the nucleus, which is driven by the keratin 5 promoter (Fig. 2A). At 56 days doxycycline chase, rare GFP+ LRCs were identified in the limbal epithelium (Figs. 2B–D, red arrow) and extremely rarely in the limbal stroma (Figs. 2B–D, blue arrow). Keratin 5 immunostaining on the same sections revealed that while the limbal epithelial LRC were K5+, stromal LRCs did not stain for K5, implying that they may have migrated from the limbal epithelium within the 56-day chase period without enough proliferation to completely dilute the GFP label. In Figure 2C, three GFP+ cells (blue arrow) in the stroma display different green fluorescence intensities, suggesting that these cells have, in fact, divided, albeit infrequently. Another possibility here is ectopic expression of K5 in these cells before the doxycycline chase phase; however, only epithelial cells were seen to be GFP+ at the end of the pulse phase, as shown in Figure 2A.

In the 3-D reconstructions generated from immunostained and aligned BMMA serial sections of the H2B-GFP/K5tTA mouse limbus (Fig. 3), LRCs were few in number compared to sox9+ cells, which were present in a large fraction of cornea and conjunctiva basal cells (Fig. 3A). Blimp1+ cells were found more frequently in the limbus and conjunctiva rather than the corneal epithelium (Fig. 3B). Segmenting the vasculature and basement membrane from 3-D reconstructions generated through collagen IV immunostaining (Fig. 3C), we observed a flat continuous basement membrane in the mouse, which was well-vascularized but devoid of any crypt-like structures like the palisades of Vogt seen in human limbus tissue. The BMMA serial sections of the H2B-GFP/K5tTA mouse limbus and central cornea were cut at 2 μm and immunostained with putative markers of stem cells and their surrounding niche, including sox9 (Figs. 4A, 5D), abcb5 (Figs. 4B, 5E), collagen IV and a-SMA (Figs. 4C, 4F), keratin 19 (Figs. 4G, 4J), lrig1 (Figs. 4H, 4K), and blimp1 (Figs. 4I, 4L). Label-retaining cells were observed to express sox9 (Fig. 4A), abcb5 (Fig. 4B), lrig1 (Fig. 4I), and blimp1 (Fig. 4J), but these proteins also were found in basal cells that had lost GFP label through multiple divisions and were not specific for long-term chased LRCs. Collagen IV highlights the well-vascularized basement membrane and extracellular matrix that lies underneath the limbal epithelial stem cells (Fig. 4C). No immuno-markers were found here to exclusively colocalize with LRCs and the slow-cycling functional marker of stem cell quiescence (Figs. 4A–L).

FIGURE 3. Immunofluorescence tomography 3-D reconstruction of the H2B-GFP/K5tTA mouse limbus. (A) Reconstruction of sox9 immunostaining and LRCs after 56 days doxycycline chase using 180, 2-μm BMMA serial sections (572 μm). (B) 3-D Reconstruction of blimp1 immunostaining and LRCs in the mouse limbus using 87 serial sections (174 μm). (C) 3-D reconstruction of the basement membrane and vasculature network beneath the limbus epithelium which harbors LRCs.
for LRCs; however, blimp1 is enriched in limbal basal epithelial cells and lrig1 is expressed in 100% of LRCs found at the limbus and fornix. Lrig1 is expressed in the majority of ocular surface epithelial cells according to our immunostaining.

**DISCUSSION**

Slow-cycling limbal stem cells necessary for the maintenance of corneal epithelial cell turnover surround the entire periphery of the H2B-GFP/K5tTA mouse cornea. In 3-D reconstructions generated at 56 days chase, foci of LRCs are observed in much lower populations than those seen at 28 days chase, where low magnification imaging reveals a large band of GFP \(^+\) LRCs at the peripheral limbus. Through 3-D quantification, we determined that approximately 3% of limbal epithelial cells were LRCs at 56 days doxycycline chase. This raises the possibility that stem and/or progenitor epithelial cells exist with different rates of cell division at the limbus and questions the current theory of transient amplification and asymmetric division of epithelial stem cells. Lineage tracing experiments in the cornea have shown cell renewal from the limbus involves proliferation and homogeneous migration of clusters of cells from the limbus to the central cornea.\(^31\)

The limbal stromal translocation of LRCs is a new finding that raises important questions about epithelial-mesenchymal transition (EMT) and stromal migration of limbal epithelial cells. It has been shown previously that limbal epithelial cells invade the stroma by EMT after air exposure through in vitro culture.\(^32\) Our data support these findings and suggest that limbal epithelial cells may undergo EMT in vivo. Isolation of LRCs and further characterization by immunofluorescence tomography and in vitro analysis in 3-D cultures will help to determine their differentiation potential and capacity to undergo EMT.

From the LRC data obtained from the H2B-GFP/K5tTA mouse limbus, it is reasonable to suggest that a higher proportion of mouse limbal basal cells than those isolated from human tissue by the ATP-binding cassette ABCG2\(^33\) or by the high nuclear:cytoplasm side-population \(^34\) are, in fact, corneal epithelium progenitors. Purification of human corneal epithelial cells with cell surface markers, such as ABCG2, ABCB5, and \(\beta4\)-integrin, does provide cells with high clonogenic potential and, therefore, is a good method to enrich basal limbal epithelial cells and progenitors. However, in this study we found no immunomarker that colocalizes with the functional marker of epithelial stem cell quiescence. Sox9 expression in >20% of epithelial cells of ocular surface tissues, including the cornea and conjunctiva, suggests that sox9 is not

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**Figure 4.** Immunohistochemistry staining of the H2B-GFP/K5tTA mouse limbus and central cornea with putative stem cell markers at 56 days doxycycline chase. The 2 \(\mu\)m BMMA sections of the H2B-GFP/K5tTA mouse limbus (A–C, G–I) and central cornea (D–F, J–L) after 56 days doxycycline chase were immunostained for (A, D) sox9, (B, E) abcb5, (C, F) \(\alpha\)-smooth muscle actin, and collagen IV, (G, I) keratin 19, (H, K) lrig1, (I, L) blimp1. Sox9 is localized to LRCs and dividing basal cells of the ocular surface while collagen IV is expressed in the basement membrane and the vasculature network, which terminates at the limbus. Keratin 19 is found in the basal and suprabasal layers of the ocular surface, while the putative stem cell markers lrig1 and blimp1 are found expressed in LRCs and other basal cells of the limbus and the cornea.
a candidate marker of epithelial progenitor cells. While the SOX9 gene has been suggested to be an essential adult stem cell gene, its role in regulating limbal epithelial stem cell function remains unclear. Lrig1 was found in 100% of LRCs quantified, although it did not exclusively colocalize with LRCs as non-LRCs also exhibited lrig1 expression. Interestingly, blimp1 was found in most LRCs (66%) and enriched in the nucleus of basal limbal epithelial cells, whereas the low expression of blimp1 in the corneal epithelium was mostly cytoplasmic.

The LRCs that most likely represent a stem cell population for the bulbar and palpebral conjunctiva are localized to the fornix conjunctiva. In the H2B-GFP/K5tTA mouse conjunctiva after long-term chase of 56 days, the bulbar and palpebral conjunctiva regions do not exhibit label-retaining epithelial cells. The lid margin has been postulated previously to be a niche zone for quiescent epithelial cells, but no label-retention was seen at the lid margin in 3-D reconstructions of the conjunctiva. The localization of the quiescent epithelial cells to the fornix further supports the hypothesis that the fornix is the region of progenitor or adult stem cells in the conjunctiva.

In conclusion, the quiescent epithelial cell population of the cornea occupies almost the entire peripheral limbus of the mouse cornea at 28 days chase; however, only 3% of limbal epithelial cells are LRCs at 56 days chase. This suggests that two LRC populations with different cell cycle lengths exist, with the more quiescent cells likely to represent the true limbal epithelial stem cell population. Immunostaining with putative limbal stem cell markers confirms the absence of a definitive protein marker that colocalizes with the functional marker of slow-cycling LRCs. In the future, isolation and functional analysis in vitro of these LRCs at different chase timepoints will enable us to characterize their gene expression, reveal new potential markers, and help determine their clonogenic capacity and multipotency.

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