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Abstract

The narrow intersection between the cornea and conjunctiva, otherwise known as the limbus, is purported to harbor stem cells (SCs) that replenish the ocular surface epithelium throughout life. Damage to this site or depletion of its SCs can have dire consequences for eye health and vision. To date, various SC and keratin proteins have been used to identify the limbus, however, none could definitively mark its boundaries. Herein, we use the mouse as a model system to investigate whether structural and phenotypic features can be used to define the limbus and its boundaries with adjacent tissues. We demonstrate that differentially aligned blood and lymphatic vessels, intraepithelial nerves, and basal epithelial cellular and nuclei dimensions can be used as structural landmarks of the limbus. Identification of these features enabled approximation of the limbal expanse, which varied across distinct ocular surface quadrants, with the superior nasal and inferior temporal limbus being the widest and narrowest, respectively. Moreover, label-retaining SCs were unevenly distributed across the ocular circumference, with increased numbers in the superior temporal and inferior temporal moieties. These findings will heighten our current understanding of the SC niche, be beneficial for accurately predicting SC distribution to improve their isolation and devising efficacious cell therapies, and importantly, aid the ongoing search for novel SC markers.

Key words: boundary; conjunctiva; cornea; epithelium; limbus; stem cells.

Graphical Abstract

In mice, the stem cell-containing limbus can be distinguished by the presence of thin looped blood vessels, circumferential intraepithelial nerves and perpendicularly aligned lymph vessels. These structural features, including limbal span and quantity of stem cells are unevenly distributed across distinct ocular surface quadrants.
Significance statement

Mouse models are frequently exploited to recapitulate human ocular surface diseases. However, the area encompassing the SC-harboring limbus in the mouse eye is poorly detailed as it bears no distinguishing anatomical features. This report provides a comprehensive structural survey of this region, focusing on the organization of epithelia, blood vessels, lymphatics, and nerves, and the arrangement of these structures across distinct coordinates, in conjunction with specific features that demarcate its boundaries with adjacent tissues. These findings will be imperative for accurately pinpointing SC location to improve their isolation for therapeutic use, evaluating the efficacy of a treatment modality to restore tissue architecture, and assisting the ongoing search for reliable SC biomarkers.

Introduction

The anterior surface of the eye consists of 3 anatomically and functionally distinct regions, including cornea, limbus, and conjunctiva. The cornea comprises the outermost part, protecting internal structures, and acting as an avascular transparent window that enables light refraction and transmission onto the retina to facilitate optimal visual perception. The vascular conjunctiva surrounds the cornea and extends into the inner eyelids to form a mucosal layer that lubricates and protects the eye from desiccation and noxious external insults.1 The limbus is a highly vascular and innervated narrow annular zone that partitions these 2 tissues.2

The limbus is of particular interest because its epithelia prevent cells on either side from mixing. It is posited that the physical presence of the limbus, generation of soluble chemicals, and constituents of its local microenvironment, including the underlying basement membrane, stroma, nerves, vasculature, and various supporting cells contribute to the maintenance of this barrier function.3,4 Limbal epithelia are also purported to harbor a rare population of stem cells (SCs), otherwise referred to as limbal epithelial stem cells (LESCs),5,6,7 which possess a lifelong capacity to replenish aged, damaged, or diseased corneal epithelia. Depleted LESCs or damage to the limbal niche, results in a blinding disease known as limbal stem cell deficiency (LSCD), which is characterized by loss of barrier function concomitant with the incursion of an inflamed, fibrovascular conjunctival pannus into corneal territory.8

Currently, SC transplantation is the main treatment modality for restoring tissue function and sight in LSCD. However, patients continue to face disappointing mid-to-long-term outcomes, despite significant advances in SC-based therapies.8,11 Importantly, the progress in establishing novel strategies for ocular surface reconstruction is hampered by the challenge of identifying unique markers for isolating LESCs to improve graft quality and longevity. This is difficult to achieve because precision boundary markers that detail the limbal frontier with the cornea and conjunctiva are lacking.

Despite the wide use of mice in modeling ocular surface diseases that arise in patients, the limbus in this species is poorly defined and bears no distinguishable landmarks such as the palisades of Vogt (POVs) found in humans and other animals.12-15 Established characteristic features of the mouse limbus include thick stromal nerve trunks,16 and particular blood vessel (BV) and cell nuclei traits.17 However, none are definitive boundary markers due to their variable distribution and inability to discriminate the conjunctival neighborhood. Moreover, while mouse corneal and conjunctival epithelia can be phenotypically identified by specific keratin (K) proteins,18 including K12,19,20 and K8/K13,21,22 respectively, limbal keratins such as K14, K15, or K19 are less specific because they are also expressed in the conjunctiva.21,22 Putative LESCs can be exploited to identify the corneo-limbal border in mice,24,25 however, definitive identifiers of the limbal-conjunctival divide remain elusive.

Herein, we took advantage of the simple organizational layout of the mouse limbus, including differential keratin expression and physical parameters of epithelial and neurovascular patterns to define its coordinates in specific locations across the ocular surface. Insights gained from these results may bridge the current knowledge gap concerning LESCs location by enabling reliable prediction of their distribution, including the search for definitive biomarkers, and improving isolation strategies to develop longer-lasting therapeutic interventions for patients with LSCD.

Materials and methods

Animals

Male and female wildtype C57BL/6 mice (n = 25) were obtained from Australian BioResources (Moss Vale), housed in temperature-controlled rooms under pathogen-free conditions and given standard chow and water ad libitum. All experimental procedures were approved by the University of New South Wales Animal Care and Ethics Committee, under protocols, 20/59A and 23/27B. Unless specified, mice were euthanized by cervical dislocation at 8-10 weeks of age and whole eyes (left and right) consisting of the globe and conjunctiva up to the eyelids were procured. For the aging study, mice (n = 3) were euthanized at 56 weeks of age.

Histology, electron microscopy, and immunofluorescence

Eyes were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 5 hours at 4 °C. For histological assessment, eyes were embedded in paraffin blocks, sectioned, and stained with hematoxylin and eosin. Transmission electron microscopy (TEM) was performed as previously described.26 For phenotypic assessments, eyes were dissected into 4 quadrants with orientation recorded prior to fixation. Afterward, immunofluorescence staining for BVs, lymphatic vessels (LVs), nerves, nuclei, and cell markers was performed on sectioned or whole ocular surface tissues as previously described.27 Confocal images obtained from whole flat-mounted tissues were used for analysis of morphometric parameters and calculation of landmark dimensions. Full details of experimental procedures are described in Supplementary Information: Materials and Methods.

Determination of label-retaining cells across the ocular surface

To map the location of slow-cycling, label-retaining cells, bromodeoxyuridine (BrdU; Sigma-Åldrich) was injected intraperitoneally into 6-week-old C57BL/6 mice at 100 μg/g
of body weight, twice a day (6-hour intervals each day) for 4-days. Mice were euthanized 6-weeks after the last injection and whole eyes including the conjunctiva extracted. Tissues were processed and stained with a rat anti-BrdU antibody (Supplementary Table S1) as previously described. Three regions from each quadrant were randomly selected and imaged, and the number of BrdU+ cells in each limbal sector was counted.

Vessel width analyses

To analyze vessel width, a randomly selected 2048 pixels × 2048 pixels (425.10 μm × 425.10 μm) area consisting of conjunctival and limbal BVs or LVs was imaged. Vessels were detected by applying an algorithm where difference of a Gaussian method is used to identify bright objects, followed by manual cleanup to eliminate undesired structures. Instances of incorrect breaks in vessel outline were initially addressed through binary dilation and erosion procedures, with manual corrections applied in scenarios where this process was ineffective. Width measurements were obtained by skeletonizing the mask to derive the vessel’s centreline. Additionally, a distance map of the mask was generated to determine the number of pixels away from the center of the vessel for each pixel along its length.

Statistical analyses

All data are presented as ±SEM and analyzed using GraphPad Prism (v9.0.2) software (GraphPad). One-way ANOVA was performed to assess parameters among cornea, limbus, and conjunctiva as well as among SN, ST, IN, and IT quadrants. Tukey’s test was used to correct for multiple comparisons. Unpaired Student’s t test was used to compare differences among superior vs inferior, nasal vs temporal, and 8-10 week vs 56-week groups. Statistical significance was reached when \( P < .05 \).

Results

Determining limbal characteristics using brightfield and electron microscopy

In live mice, the limbus cannot be visualized with certainty because eyelids obscure this region (Figure 1A). However, when eyes are dissected, a circumferential demarcation zone is revealed at the corneoscleral junction, which we believe represents part of the limbal anatomy (Figure 1B). To determine whether this region can be accurately defined microscopically, histological sections spanning the ocular surface were examined (Figure 1Ca). The cornea is distinguishable by a stratified squamous epithelium (~6 to 8 cell layers) comprising tightly packed cells that possess large spherical nuclei in its basal layer and densely packed connective tissue matrices in the underlying stroma (Figure 1Ci). The transition into the limbus is characterized by a thinner epithelium (~2 cell layers), which forms a depression or trench, and a uniform stroma harboring numerous cells (Figure 1Cii), some of which are keratocytes, others likely leukocytes surveying the local microenvironment.29 The adjacent conjunctiva exhibits a less stratified epithelium (~4-5 layers) with cuboidal-shaped basal cells possessing large spherical nuclei (Figure 1Ciii). Cellular ruffling was occasionally observed in the superficial layer (Figure 1Ciii, thin arrows), which coincided with an undulating epithelial basement membrane (Figure 1Ciii, arrowheads). The conjunctival stroma was distinguishable by loose extracellular matrices (Figure 1Ci) and visible blood vessels (Figure 1Ciii, thick arrows).

TEM confirmed histological features at an ultrastructural level (Figure 1D). Additional features included elongated, eccentric nuclei, and more prominent nucleoli in the limbus, and high mitochondrial density in basal conjunctival epithelia (Figure 1Db and c). However, the corneal and conjunctival margins of the limbus were difficult to delineate using subjective morphological observations alone.

Defining the limbal boundaries using markers of stem cells and ocular surface epithelia

Because the limbus is purported to harbor SCs,30 markers that discriminate these cells31 and its epithelia were exploited to better resolve its extremities. Surprisingly, all putative SC and otherwise regarded limbal-specific keratin markers we tested were also expressed in the limbal and conjunctival compartments, with some detected in the peripheral cornea (Supplementary Figures S1 and S2), suggesting that none are specific for this transition zone. Therefore, we interrogated whether corneal (K12) and conjunctival (K8) keratins could be used to approximate the limbus, firstly to establish the dimensions of this zone, and secondly to identify any landmark features. This strategy revealed a zone between the K12+ cornea and K8+ conjunctival epithelia, devoid of immunoreactivity (Figure 2A), which we posit to represent the limbal sanctum.

Since the human limbal dimensions are reported to differ across ocular surface quadrants,32 we further ascertained regional differences by recording specimen orientation prior to procurement. Immunofluorescence on tissue sections revealed that the stretch of K12−/K8− epithelia was widest in the superior nasal (SN) sector and narrowest in the inferior temporal (IT) quadrant (Figure 2A). The limits of the K12/K8 region visually appeared similar in the superior temporal (ST) and inferior nasal (IN) sectors (Figure 2A).

To overcome the limitations of subjective determinations from tissue sections, K12/K8 expression was ascertained on whole flat-mounted ocular surface. Concordantly, a stretch of K12/K8− epithelia was detected between the cornea and conjunctiva (Figure 2Ba-d). Regional quantification showed the width in the SN quadrant (190.39 ± 15.46 μm) was significantly larger than ST (110.10 ± 7.21 μm), IN (97.98 ± 7.18 μm), and IT (18.64 ± 8.38 μm) quadrants (Figure 2C). No significant difference was observed between the ST and IN quadrants. Accordingly, average width measurement in relation to the vertical (superior/inferior) and horizontal (nasal/temporal) axes showed the limbus to be significantly wider in the superior vs inferior and nasal vs temporal sectors (Figure 2D, E). To avoid potential subjectivity in manual width calculation, this difference was further confirmed by computing K12−/K8− area, where values for the SN quadrant as well as superior and nasal halves were significantly larger than other sectors (Supplementary Figure S3A-C). However, a clear gap was not detected in the IT quadrant (Figure 2A-C). We hypothesize that this may be attributed to K12+ expression in suprabasal limbal epithelia (Figure 2Ba-d, asterisks),35,34 which may conceal the gap occurrence due to a potentially narrower limbal perimeter in the IT quadrant. Taken together, these results suggest that more definitive structural markers are required to precisely demarcate the limbal divide, especially on the corneal aspect.
Figure 1. Histology and electron microscopy of the mouse ocular surface. (A) Representative photograph of a live mouse eye observed using anterior segment optical coherence tomography. (B) Representative photograph of a dissected mouse eye imaged using a stereo microscope with a 4× objective lens. White arrows point to the corneoscleral junction. Limbus is denoted by an opaque ring between the transparent cornea and corneoscleral junction. (C) a. Representative tissue section through the mouse ocular surface (n = 6; 6 eyes) imaged using a brightfield microscope with 20× objective lens. Scale bar = 100 μm. Panels i-iii are higher magnification (100× objective lens) images of the corneal, limbal and conjunctival regions. Scale bars, 50 μm. Thick arrows point to BVs, thin arrows indicate epithelial in-folds, and arrowheads point to undulations in the basement membrane. D) Representative TEM images (800 000× magnification) of the corneal, limbal, and conjunctival epithelia (n = 3; 3 eyes). Panels a-c represent higher resolution images (1200 000×) of regions. Hatched lines represent the basement membrane which separates epithelium (Epi) from the stroma. Asterisks (*) are marked on dark areas which represent nucleoli. White arrows point to mitochondria. Scale bars, 1 μm. Abbreviations: Co, cornea; Conj, conjunctiva; Epi, epithelium; L, limbus; Nu, nucleus.
Figure 2. Corneal and conjunctival keratins demarcate the limbal expanse. (A) Representative images of K8 and K12 expression on tissue sections from different quadrants of the ocular surface (n = 6; 6 eyes). Cell nuclei were visualized with Hoechst. Scale bars, 50 μm. (B) Representative maximum intensity projection image of K8 and K12 expression on whole flat-mounted ocular surface tissue (n = 6; 12 eyes). Limbus (L) is represented by the gapped area. Scale bar = 500 μm. Panels a-d represent higher resolution images of the region encompassed by hatched squares in B. Asterisks (*) represent suprabasal K12+ expression. Scale bars, 50 μm. (C) Mean span of the unstained K12-/K8- gap in each quadrant from flat-mounted tissues (n = 6; 12 eyes). Data were analyzed by one-way ANOVA. Mean values were obtained by averaging data points from all 12 eyes. (D and E) Mean gap width between superior vs inferior, and nasal vs temporal quadrants on whole flat-mount ocular surface (n = 6; 12 eyes). Data were analyzed by unpaired Student's t test. Mean values were calculated by averaging data points from all 12 eyes. All Error bars represent ±SEM. All images were acquired using a scanning confocal microscope at 20× objective lens. ****P < .0001; ns = not significant. Abbreviations: Co; cornea, Conj; Conjunctiva; L; limbus.
Identifying the limbal divide using epithelial structural and functional traits

Because basal limbal epithelia harbor label-retaining SCs, often marked by incorporation of tritiated thymidine or BrdU, their nuclei bear features that discriminate them from neighboring epithelia. Therefore, various cellular and nuclei parameters were quantified on flat-mounted ocular surface tissue to corroborate our assertion that the K12−/K8+ region defines the limbal perimeter. As expected, basal nuclei in the K12−/K8+ limbal zone were morphologically distinct from corneal counterparts (Figure 3Aa-c), exhibiting significantly larger average nucleus area, perimeter, and eccentricity, while displaying significantly lower circularity and density (Figure 3B-F). However, statistical significance was not reached when these parameters were compared to conjunctiva, except for decreased circularity and increased eccentricity (Figure 3B-F), rendering elongated nuclei as a distinguishing limbal feature, consistent with our TEM results (Figure 1D). Moreover, BrdU+ nuclei were detected in basal cells harboring morphologically distinct elongated nuclei within the K12−/K8+ zone (Figure 3Ae). Few if any BrdU+ nuclei were detected in the peripheral cornea and bulbar conjunctiva (Figure 3Ae), thus, functionally validating the K8−/K12− region as the SC-harboring limbal boundary with the cornea but could not clearly delineate the limbal zone.

Phalloidin staining revealed that basal corneal and conjunctival epithelial cells appeared uniformly arranged and tightly packed, whilst those in the limbus were larger and irregularly oriented (Figure 3Af-h). Quantification of morphometric parameters revealed that cells within the limbus displayed significantly larger limbal cell area, perimeter, eccentricity, and significantly reduced circularity and density, compared to corneal counterparts (Figure 3G-K). However, basal conjunctival cell parameters were similar to those displayed by limbal counterparts (Figure 3G-K). Collectively, these findings imply that cell and nuclear morphology can be used to distinguish the limbal boundary with the cornea but could not clearly demarcate its margin with the conjunctiva.

To overcome this limitation, K8 staining was performed to discriminate the limbal frontier with the conjunctiva and basal cell morphology was used to ascertain the corneal-limbal margin. This strategy was instrumental in computing limbal width, revealing a significantly wider mean limbal span in the SN (312.41 ± 19.02 μm) and narrower in the IT (181.87 ± 19.02 μm) sectors (Figure 3L, M). Accordingly, the superior vs inferior and nasal vs temporal hemispheres displayed wider limbal span (Figure 3L, M). Similar results were obtained upon calculating the area between the 2 designated limbal margins (Supplementary Figure S3D-F).

Due to this variation in width, the number of BrdU+ nuclei in the limbal region were quantified to gain functional insights. Interestingly, BrdU+ nuclei quantity was significantly higher in the ST compared to SN and IN sectors (Figure 3P), although no statistical difference was noted when compared with the IT region. Comparisons between horizontal and vertical meridians revealed significantly higher BrdU+ nuclei number in the temporal vs nasal sector, but no statistical difference arose between superior vs inferior hemispheres (Figure 3Q, R). These results suggest that limbal width does not correlate with the proportion of label-retaining progenitor cells, and that cells in each region may possess distinct functional attributes.

Defining the limbal divides through assessment of vascular patterns

Although the cornea is an avascular tissue, vascular networks are known to differ between the limbus and conjunctiva, and within different hemispheres of the ocular surface. However, their spatial distribution across specific quadrants and the extent to which such characteristics distinguish this tissue intersection remains to be elucidated. Therefore, BV and LV architecture was explored against a K8 backdrop to ascertain whether these features can demarcate the limbal perimeter.

Morphologically, BVs in the conjunctiva (present within the K8+ zone) appeared thicker and were aligned in a parallel and circumferential manner compared to those present in the limbus which were thin, loopy, and oriented perpendicularly to those in the conjunctiva (Figure 4A). Basal nuclear morphology was used as a guide to locate the cornea’s frontier, where no BVs were detected. Notably, consistent with limbal width calculation (Figure 3L), the mean limbal BV span was significantly larger in the SN and lower in the IT zones compared to the ST and IN sectors (Figure 4B). Average limbal BV span in the superior and nasal hemispheres was also significantly larger compared to inferior and temporal regions (Figure 4C, D). The average conjunctival BV diameter was significantly larger than those within the K8+ limbal edge (Figure 4E). These results corroborated data for mean limbal BV area across all quadrants (Supplementary Figure S3G-I).

Similar features were uncovered for LVs. Conjunctival LVs were aligned circumferentially whilst those within the limbal stretch sprouted perpendicular to conjunctival equivalents (Supplementary Figure S4A). Moreover, conjunctival LVs were also significantly thicker than limbal counterparts (Supplementary Figure S4B, C). Interestingly, unlike the loopy BV arcades within the limbal precint, LV branches often projected beyond the limbal radius into the peripheral cornea where they terminated (Supplementary Figure S4A, thick arrows). Because LV traits fluctuated between quadrants and samples, they were not considered key boundary discriminators on the corneal aspect. Therefore, the span containing perpendicular LVs was not measured. Taken together, these findings suggest that BV (and to some extent LV) morphology can be exploited as structural landmarks to distinguish the limbal-conjunctival border, given their size and differential alignment.

Defining the limbal boundary through intraepithelial nerve distribution

Given the recorded differences in vascular features, we further hypothesized that other structures such as nerves may differ in their distribution and trajectory within the ocular surface. K8/βIII-tubulin immunostaining showed nerve fibers within the limbal epithelium aligning circumferentially, whilst those in the K8+ skirt branched from limbal axons in a centrifugal direction toward the conjunctiva (Figure 4F). In contrast, nerves in the peripheral cornea sprouted axons that navigated centripetally toward the central cornea (Figure 4F). The limbus consisted of parallel-aligned fibers, the span of which was significantly wider in the SN vs ST, IN, and IT quadrants (Figure 4F, G) as well as superior and nasal halves compared to the inferior and temporal moieties (Figure 4H, I). Similarly, the area occupied by parallel-aligned epithelial nerves in the SN, as well as superior and nasal regions was significantly wider in the SN vs ST, IN, and IT quadrants (Figure 4F, G) as well as superior and nasal halves compared to the inferior and temporal moieties (Figure 4H, I).
Figure 3. Epithelial and nuclear characteristics delineate the limbal fringe. (A) Morphological features of basal epithelia and their nuclei within the corneal K12+, limbal K12-/K8− (unstained), and conjunctival K8 + regions. a. Representative maximum intensity projection image of K8 and K12 expression on a flat-mounted ocular surface (n = 6; 12 eyes) captured using 20× objective lens. Scale bar = 50 μm. Panels b-d represent high magnification (63× objective lens) images of basal cell nuclei present within regions denoted by hatched squares in a (n = 17; 34 eyes). Scale bars, 20 μm. e. Representative maximum intensity projection image of BrdU+ nuclei located in ocular surface epithelia, 6-weeks following BrDU incorporation at 6 weeks of age (n = 3; 6 eyes). Scale bar = 20 μm. Panels f-h depict high magnification (63× objective lens) images of phalloidin-stained basal...
larger than other sectors (Supplementary Figure S3J-L). To further distinguish the cornea, limbus, and conjunctiva, other nerve parameters were computed, however, the focus was on the SN quadrant due to its larger expanse, enabling more accurate analyses (Supplementary Materials and Methods). In this instance, intraepithelial conjunctival nerves were significantly shorter and less dense compared to those within the cornea and limbus (Supplementary Figures S5A). However, no statistical differences were recorded between the cornea and limbus for any parameter (Supplementary Figure S5B-E).

**Limbal width is influenced by age**

Finally, to determine whether limbal width actively changes or remains static throughout life, old (6-week) mice were screened to quantify its breadth using K8 and basal nuclei characteristics. As expected, the limbal span in the SN and IT quadrants of these mice was wider and narrower, respectively (Figure 5A, B). This coincided with a significantly broader range in the superior and nasal hemispheres (Figure 5C, D). However, upon comparing limbal amplitude in relation to age, old mice had significantly reduced limbal dimensions in the SN quadrant and superior hemisphere compared to younger counterparts, despite no statistical differences observed in other regions (Figure 5E-L). These results suggest that the limbus in mice is dynamic and changes according to age.

**Discussion**

This study comprehensively characterized the morphological features encompassing the murine SC-harboring limbus and features that distinguish it from the adjacent cornea and conjunctiva. While numerous keratin21,23 and SC markers31 have been exploited to survey this region, its boundaries with adjacent epithelia are difficult to ascertain in the mouse, given the absence of anatomical landmarks such as the POVs, crypts, and stromal projections.14,15 Nonetheless, studies conducted in rodents are an important foundational step toward delineating the limbal coordinates in humans with greater precision, as fresh disease-free tissue can be procured and oriented. Exploring physical parameters that distinguish the mouse limbus and its boundaries, is important to accurately pinpoint LESC distribution, understand their regulatory microenvironment, and improve their isolation to develop new therapeutic interventions that effectively restore tissue architecture and function.

Our initial observations suggested that the mouse limbus could be approximated based on a trench-like formation and an epithelium that tapers into a few cell layers as it recedes from the cornea and merges with conjunctiva (Figure 1); however, this could not be considered an accurate and definitive boundary indicator for each junction. Therefore, keratin expression was used to approximate the limbal frontier, which we defined as a K8/K12-free zone that also contains label-retaining BrdU+ stem/progenitor cells (Figure 3). Although the conjunctival fringe was identified by its sharp K8 staining, K12 expression was less truncated and detected in suprabasal limbal epithelia near the peripheral cornea.35 Additionally, we visualized the distribution of several well-accepted limbal-specific keratins and putative LESC markers, which were ineffective at framing the limbal frontier due to positive staining in adjacent tissue compartments (Supplementary Figures S1, S2). This expression pattern does not suggest specificity for the limbus or LESC, and is consistent with reports in porcine34 and equine38 ocular surface tissue. The notion that these are high-fidelity LESC markers could be attributed to the procurement of corneas without conjunctiva, which prevents detailing neighboring epithelia. Our investigations included corneas with conjunctiva attached to identify the limbal extremities more reliably, and an antigen retrieval protocol that enhanced epithelial immunofluorescence signal37 in opaque conjunctival tissue. This approach was instrumental in unmasking unique structural features we deem limbal landmarks.

Our findings are supported by observations of limbal morphology in other mouse strains. Distinct limbal basal epithelial nuclei dimensions compared to adjacent locations have been demonstrated in CAG-EGFP mice.17 The existence of thick parallel BVs in the conjunctiva as opposed to thinner arching vessels in the limbus, has also been observed in Krt15-GFP mice,36 suggesting such morpho-physical facets are similar across different mouse strains. However, the inability to pinpoint the limbal-conjunctiva boundary, which renders measurements of the limbal expanse challenging, is a major limitation of prior investigations. Therefore, we used the expression of a previously validated conjunctival marker, K8,21,35 as the primary reference point from which to peg the limbal intersection with conjunctiva and features of nuclei belonging to basal epithelia were used as a secondary guide to identifying the limbal-corneal fringe. This strategy identified thick circumferentially coursing BVs and LVs as the start of the conjunctival precint, while thin looping BVs defined the limbal terrain at the corneal margin (Figure 4B). Likewise, thick centrifugal intraepithelial nerve axons discriminated the conjunctiva, while in the limbus, intraepithelial nerves aligned in a parallel manner and perpendicular to those infiltrating the cornea and conjunctiva (Figure 4F). To our knowledge, none of these features have been used to accurately define the limbal confines. The reason for thicker neurovascular structures at the limbal-conjunctival boundary remains unexplored, however, we speculate that vascular arcades need to diminish in size upon reaching the peri-corneal space and disappear altogether in the cornea so that vision is not obstructed. Additionally, thin LVs that pass through the limbus and terminate in the peripheral cornea suggest that lymphatics play...
Figure 4. Vascular features and intraepithelial nerve patterns as discriminatory limbal landmarks. (A) Representative maximum intensity projection images of conjunctival K8 and vascular CD31 stained whole flat-mount ocular surface tissue (n = 6; 12 eyes) obtained from SN, ST, IN, and IT quadrants. Hatched line represents border between limbus and conjunctiva. Scale bars, 50 μm. (B) Comparing looped BV span within the limbus of each quadrant in whole flat-mounts (n = 6; 12 eyes). (C and D) Comparing looped BV span in superior vs inferior and nasal vs temporal moieties on whole flat-mount ocular surface tissues (n = 6; 12 eyes). (E) Comparing mean BV diameter in the limbus and conjunctiva (n = 10; 17 eyes). (F) Representative maximum intensity projection images of conjunctival K8 and intraepithelial nerves stained with βIII-tubulin on ocular surface tissue (n = 5; 10 eyes), depicting distinct axonal arrangement within the cornea, limbus, and conjunctiva across SN, ST, IN, and IT quadrants. Hatched line represents the corneal and conjunctival borders of the limbus. Scale bars, 50 μm. (G) Comparing mean span of parallel-aligned axons in the limbus of each quadrant in whole flat-mount tissues (n = 5; 10 eyes). (H and I) Comparing parallel-aligned nerve span in superior vs inferior and nasal vs temporal regions on whole flat-mount ocular surface tissues (n = 5; 10 eyes). All images were acquired using a scanning confocal microscope at 20× objective lens. All error bars represent ±SEM. All mean values were calculated by averaging data points from the designated number of eyes in each panel. Quadrants were compared using one-way ANOVA. BV diameter, superior vs inferior, and nasal vs temporal data were analyzed using unpaired Student’s t test. **P < .05; ***P < .01; ****P < .001; ***P < .0001. Abbreviations: Co: cornea; Conj: conjunctiva; L: limbus; ns, not significant.
Figure 5. Comparison of limbal width in young and old mice. (A) Representative images of the limbal span based on nuclei morphology and K8 expression across each quadrant at 56 weeks of age (n = 3; 5 eyes). Scale bars, 50 μm. (B) Average limbal width in each quadrant (n = 3; 5 eyes), measured using basal nuclei characteristics at 56 weeks of age. (C and D) Comparing limbal width between superior vs inferior and nasal vs temporal sectors of the ocular surface at 56 weeks (n = 3; 5 eyes). (E-H) Comparing limbal width between 8-10-week-old and 56-week-old mice across individual quadrants of the ocular surface (n = 3; 5 eyes). (I-L) Comparing limbal width between 8-10-week-old and 56-week-old mice across superior, inferior,
a key role in cell signal transduction across the ocular surface that is independent of BV function. Collectively, our findings imply that alterations in morpho-physical features are linked to the functional properties of each tissue. It is unclear whether such differences arise from changes in tissue architecture, cell lineage, direction of epithelial cell movement, or a combination of these factors.

Having identified several structural landmarks that frame the limbus, its span was measured across specific locations. We revealed that the limbus is widest in the SN orientation and narrowest in the IT quadrant, with similar measurements for the ST and IN sectors (Figures 2 and 4). While a limited number of studies have explored structural features or detailed the limbal circumference in mouse, our data supports the nasal-dominant vascular distribution reported in the limbus of normal BALB/c and C57BL/6 mice. However, in such investigations, vascular density within the superior and inferior meridians was not computed, thereby making it difficult to ascertain whether this inconsistency is imposed by a functional difference in both these regions, as demonstrated in the present study. Our results corroborate those collated from humans where regional heterogeneity of the limbal span exists, including the greatest limbal width being in the superior pole. Although the functional significance remains unexplored, possible explanations for this nasal dominance include greater receptivity to inflammatory stimuli due to increased immune cell density in this region. Alternatively, it is to ensure barrier function is maintained because the conjunctiva in this sector is thicker compared to the temporal hemisphere. Moreover, the heightened vascular span within this region may stem from developmental cues, since neovessels are purported to begin sprouting in the nasal sector soon after birth. Optical coherence tomography indicates that the superior quadrant of the human corneoscleral junction exhibits the most angled and roughest position, while the temporal quadrant displays the smoothest and flattest surface. This implies that limbal width differential is attributed to alterations in the angle at which the eyeball is attached to the orbit or the percentage coverage of each region by the eyelids. It would be of interest to determine whether physical parameters such as eye positioning and associated mechanical forces influence limbal functionality in a region-specific manner. Interestingly, estimates of limbal width in older compared to younger mice revealed that this is mostly static, except for the SN quadrant, which appears to regress during aging (Figure 5). Certainly, this finding warrants further investigation but is supported by an aging effect in humans, including significant adverse impacts on limbal niche topography and LESC function.

While keratin expression, as well as BV and nerve patterns assessed in uncovering the greatest limbal expanse in the SN region and superior hemisphere, the fact that more label-retaining BrdU+ cells were detected in the ST and IT quadrants (temporal divide), implies that stem/progenitor cell functionality does not correlate with limbal span. Nonetheless, our results are consistent with observations in CAG-EGFP mice, where ST and IT quadrants exhibited the highest number of BrdU+ cells, and support studies in adult BALB/c mice, that display higher label-retaining cell density in the superior and inferior compared to nasal and temporal regions. Notably, the human superior and temporal quadrants harbor the greatest number of label-retaining cells. Disparities between studies may result from differences in species, method of tissue procurement, and age. In the present study, fresh ocular surface tissue was divided into 4 equal sectors within the cornea’s circumference with horizontal and vertical medians joining at a 90° angle. We deem this strategy accurate given that each quadrant’s limits can be standardized in the analysis. Moreover, our functional data suggests that SCs dominate the temporal residence (Figure 3P-R). It is possible that this precint offers greater protection for SCs, whilst the opposite (nasal) aspect, which contains denser vascular and neuronal networks, renders them vulnerable to inflammatory, angiogenic, and neuronal signals that activate, damage, or cause them to become dysfunctional.

Recent findings have shown that LESC are compartmentalized according to their function. Those located in the inner limbus (closest to the cornea) actively partake in corneal epithelial renewal, whilst those housed in the outer limbus (adjacent to the conjunctiva) are quiescent and called upon when the corneal epithelium is placed under duress. Curiously, we did not register any morphological attributes to suggest the limbus is physically subdivided into 2 distinct zones. Perhaps it is not necessary for the niche to bear topographical features that distinguish these 2 populations. However, it is important to note that tissue orientation was not disclosed, and whole eyes were not surveyed in those investigations. If multiple SC populations exist, the question of whether they are evenly or preferentially distributed across the limbal annulus, begs an answer.

The limbal frontier varies in span, as to why we cannot definitively say, nonetheless it houses label-retaining stem/progenitor cells and is clearly defined using a series of physical attributes as boundary markers. Knowing the limbal limits under steady state will provide detailed information about what transpires during aging, wound healing, chronic inflammation, LSCD and inform whether SC treatments can restore tissue architecture and function in this disease. Using our surveying strategy, the ongoing search for authentic LESC markers will be simplified and their validation against a structural marker achievable. Moreover, the relationship between LESC distribution and niche width has significant clinical ramifications. First, to ensure this site is spared during ocular surgery, and second, for SC isolation where the ST and IT sectors may be a more reliable location for their extraction to create better-quality, longer-lasting grafts for patients.

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Author contributions


Conflicts of interest

The authors declared no potential conflicts of interest.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Supplementary material

Supplementary material is available at Stem Cells Translational Medicine online.

References