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REVIEW

Limbal stem cells: Central concepts of corneal epithelial homeostasis

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Abstract

A strong cohort of evidence exists that supports the localisation of corneal stem cells at the limbus. The distinguishing characteristics of limbal cells as stem cells include slow cycling properties, high proliferative potential when required, clonogenicity, absence of differentiation marker expression coupled with positive expression of progenitor markers, multipotency, centripetal migration, requirement for a distinct niche environment and the ability of transplanted limbal cells to regenerate the entire corneal epithelium. The existence of limbal stem cells supports the prevailing theory of corneal homeostasis, known as the XYZ hypothesis where X represents proliferation and stratification of limbal basal cells, Y centripetal migration of basal cells and Z desquamation of superficial cells. To maintain the mass of cornea, the sum of X and Y must equal Z and very elegant cell tracking experiments provide strong evidence in support of this theory. However, several recent studies have suggested the existence of oligopotent stem cells capable of corneal maintenance outside of the limbus. This review presents a summary of data which led to the current concepts of corneal epithelial homeostasis and discusses areas of controversy surrounding the existence of a secondary stem cell reservoir on the corneal surface

Key words: Limbal stem cell; Corneal epithelium; XYZ hypothesis; Corneal homeostasis; Corneal wound repair

Core tip: It is a long held belief that stem cells reside only at the limbus. However, there are recent reports that present evidence of corneal repair and maintenance independent of limbal involvement. These findings call to light the possibility of previously undiscovered reservoirs of corneal stem/progenitor cells located at the central and peripheral cornea. A new secondary reservoir of stem cells has a significant clinical implication as new therapeutics for corneal degenerative disorders. This review outlines the historic evidence for limbal stem cells and discusses the role of these putative central and peripheral corneal stems cells in corneal homeostasis.

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INTRODUCTION

The transparent front surface of the eye, the cornea (Figure 1A) overlies the iris, pupil and anterior chamber. The structures that compose the anterior chamber are

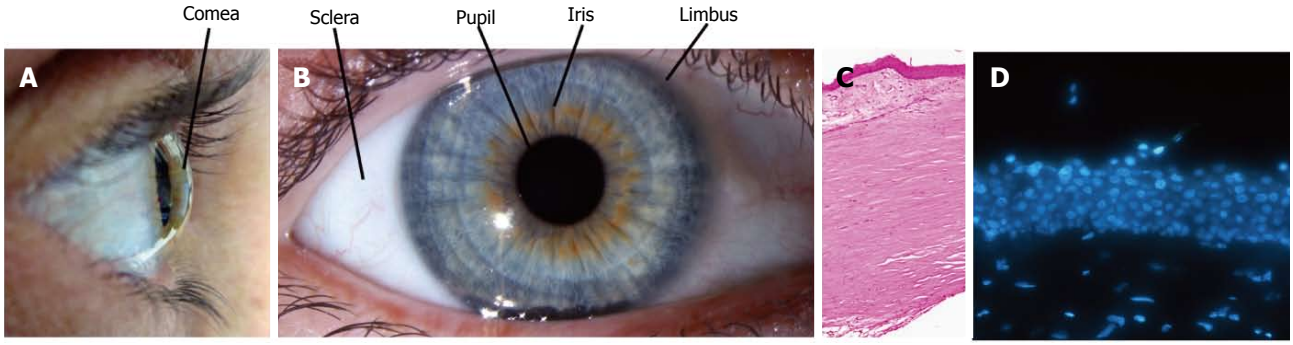

Figure 1 Anatomy of the eye. The cornea (A) comprises the colourless front portion of the eye immediately anterior to the iris and pupil (B). The limbus, located at the corneoscleral junction (B) is the transitional zone where the corneal and conjuctival epithelia merge, is shown in section using Haematoxalin and Eosin stain (C) and is considered a reservoir of stem cells which migrate centripetally to form the 5-7 cell layer corneal epithelium (DAPI fluorescence to highlight cell nuclei in corneal section, D).

surrounded by the white opaque sclera with the tissues meeting at the limbus. Maintenance of corneal integrity is imperative to light entry and refraction onto the correct position on the retina.

The anterior-most ocular surface is composed of corneal and conjunctival epithelia with the limbus at the transition zone between the two (Figure 1B and C). The corneal epithelium undergoes continuous renewal throughout life (Figure 1D). The central dogma of corneal homeostasis states that the mass of the epithelium remains constant so that the rate of cellular addition must equal that of cellular loss[1]. The predominant theory for corneal homeostasis is the XYZ hypothesis proposed by Thoft *et al*[2] in 1983. This theory proposes that the limbus serves as a reservoir of ocular stem cells. Asymmetric division of these stem cells produces a stem-like daughter cell which remains within the limbus and a transient-amplifying cell (TAC) (Figure 2A) which migrates centripetally and anteriorly (Figure 2B). TACs undergo multiple rounds of replication and progressively lose "stemness" (Figure 2C) as they migrate anteriorly and progress to post-mitotic suprabasal wing cells, and then terminally differentiated superficial squamous cells (Figure 2D). The superficial cells are lost from the surface by normal exfoliation (squamification) or traumatic injury (Figure 1E). Therefore anterior migration from cells of the basal epithelium "X" and centripetal migration from the limbus "Y" equals desquamation from the surface "Z". The entire human corneal epithelium is renewed in 9 to 12 mo[3].

Whilst the research underpinning the limbus as the main reservoir for corneal epithelial stem cells has been consolidated with sophisticated cell tracking assays, an additional emerging view of the existence of stem cells outside of the limbus is supported by findings from several independent groups. This review analyses the data in support of limbal stem cells (LSCs) and looks at the possibility of a secondary reservoir of stem cells for the corneal epithelium.

LIMBAL EPITHELIAL STEM CELLS: HISTORICAL REVIEW

Studies reporting differences between central corneal and limbal cells were published as early as the 1940s. These early studies showed increased frequency of mitoses in the basal layer of peripheral cornea using mitotic figure counts and radiated thymidine[4,5]. Centripetal migration of cells expressing melanin pigment was observed in rabbit as well as human corneas, suggesting the limbus as a source of new cells[6,7]. Since then, various studies have established the limbus as the location of corneal epithelial stem cells based on a set of unique properties observed within this cell population:

Slow cell turnover rate

DNA label-retention studies have shown the limbus contains cells in a growth-arrested or slow cycling state. Retention of radiated thymidine or 5-bromo-2'-deoxyuridine (BrdU) has been reported in limbal cells of mice cornea *in situ*[8-10], human limbal explant cultures[11] and whole cornea organ cultures[12]. The retention of DNA label was observed for up to nine weeks in these studies. The labelling index, or the percentage of BrdU-retaining cells, was 1%-4% in mice corneas[9,10,13], and approximately 4% in human limbal explant cultures[11]. The nuclear label was lost progressively as the labelled cells moved towards the central cornea, indicating increased cell division during centripetal migration[8].

Slow turnover rate in the limbus has also been demonstrated by resistance to 5-fluorouracil (an anti-metabolite which specifically targets proliferative cells)[14], cytoplasmic staining for cyclins D, E and A (indicator of a growth-arrested state)[15] and susceptibility to malignant transformation[16-18]. The susceptibility to tumour formation is thought to be a property of stem cells as oncogenic mutations are more likely to accumulate in cells with long life span[19].

Clonogenicity and proliferative potential

Life-long maintenance of any stratified epithelium necessitates a self-renewing pool of stem cells, asymmetric division of precursor cells and a rapid proliferative response upon injury[20]. Studies have suggested that these attributes are unique to the limbal cell population.

Self-renewal capacity or clonogenicity of limbal cell populations has been shown by their ability to form

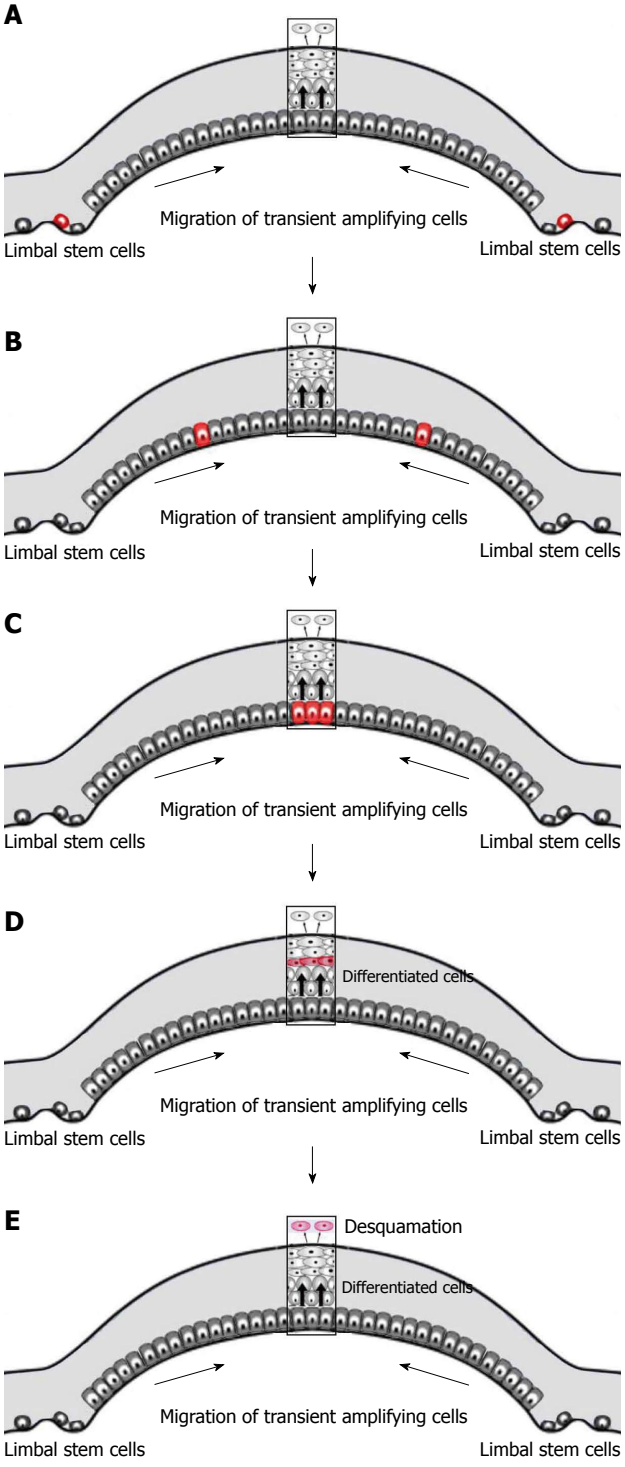

Figure 2 The X, Y, Z hypothesis of corneal maintenance. Limbal stem cells at the peripheral cornea divide and give rise to transient amplifying cells (TACs) (A). These TACs migrate centripetally through the basal epithelium (B) and undergo a limited number of divisions on the central cornea (C). The differentiated daughter cells move anteriorly to replenish the upper layers of the cornea (D) where they are eventually shed from the corneal surface (E). Hence the sum of X (proliferation and anterior migration) and Y (centripetal migration) must equal Z (desquamation of superficial cells) for corneal maintenance. Red cells: Continuum of transient amplifying migrating and/or differentiated cells.

sphere colonies on a 3T3 fibroblast feeder layer[21]. These authors showed that the holoclone, meroclone and paraclone colony formation system previously identified in human skin could be translated into spheres derived from human corneal biopsies. The single cell-derived sphere colonies from the limbus (equivalent to holoclones) were capable of undergoing 80 to 100 cell division cycles and could be propagated up to 14 passages before senescence. Single cell isolates from central cornea only formed paraclones (mostly consisting of terminally differentiated cells and capable of 15 cell divisions at maximum) and meroclones (intermediate form between holoclones and paraclones).

Asymmetric cell division has been suggested by uneven distribution of cell fate determinants across the corneal epithelium. Molecules implicated in asymmetric cell division and early cell fate decision, such as Musashi-1[22], Notch-1[23], p75[24], C/EBPδ[25] and ΔNp63α[26] have been almost exclusively localised in the mouse and human limbus.

Proliferative potential of limbal cells has been demonstrated by both *in vitro* and *in vivo* studies. Primary human limbal epithelial cell cultures showed high proliferative potential with a mean of 23 population doublings *in vitro,* while central corneal cells could not be propagated[27]. Explant cultures of human limbal epithelium showed larger outgrowth and higher mitotic rate compared to explants from central epithelia[28,29]. When transplanted into the flanks of athymic mice, single cell suspensions from limbal cell culture produced cysts which had more organised structure and longer life span than those derived from central corneal cell suspensions[30]. Furthermore, *in vivo* animal studies have shown that the slow cycling limbal basal cells can rapidly divert to proliferative status upon damage to cornea[8,13].

Cellular morphology

Morphological differences between limbal and corneal cells have been highlighted using a variety of imaging technologies including synchrotron infrared microspectroscopy[31], morphometric analysis of DAPI-stained nuclei[9], transmission electron microscopy[32,33], *in vivo* confocal microscopy and flow cytometry[34]. These studies commonly identified cuboidal cells 10 μm in diameter with a high nucleus-to-cytoplasm ratio in the limbal basal layer. The sparse cytoplasm in these cells appears smooth due to the paucity of organelles and intracellular junctions, another indicator of low metabolic activity and protein turnover. In contrast, basal cells of the central epithelium are more columnar and have a lower nucleus-to-cytoplasm ratio[31].

Biochemical characteristics

The identification of exclusive biochemical markers of corneal stem cells has been for many years a highly desirable endeavour. A number of putative stem cell markers have been suggested based on the biochemical transition that takes place in the basal cell layer of the corneolimbal junction[35-37]. Limbal basal cell layers preferentially express certain structural proteins (vimentin, cytokeratin 14, 15 and 19), cell adhesion molecules (integrin α6, β1,

β4, P-cadherin and N-cadherin), enzymes (α-enolase, aldehyde dehydrogenase, cytochrome oxidase, Na^+/K^+-ATPase and carbonic anhydrase), metallothionein, growth factor receptors (KGF-R and NGF-R), cell fate/cycle regulators (notch-1, Musashi-1, ΔNp63α, p75, Bmi-1 and C/EBPδ) and ABCG2, an ATP-binding cassette transporter protein. ABCG2 has been shown to be responsible for the efflux of the nuclear dye Hoechst 33342, enabling isolation of ABCG2-positive cells using flow cytometry[38]. This dye efflux property is an established marker of a stem cell in many cell lineages including haematopoietic[39], neuronal[40], muscle[41], and epithelium[42]. The ABCG2 proteins are thought to protect LSCs from oxidative stress by transporting small regulatory molecules required for their proliferation, differentiation and apoptosis[43]. ABCG2-positive cells are termed side population (SP) cells, and only a small proportion of limbal basal cells are SP cells. The SP cells have been shown to possess a number of stem cell properties including up-regulation in response to central corneal wounding[44], small cells with high nucleus-to-cytoplasm ratio, slow cycling, expression of ΔNp63α and ABCG2, absence of cytokeratin 3, 12 and involucrin, and increased colony-forming efficiency and growth capacity[45,46].

As limbal basal cells migrate out of the limbus, their protein expression profile gradually changes. Central corneal epithelium is characterised by the loss of α-enolase and melanin pigmentation and the expression of cytokeratin 3 and 12, connexin 43 and 50, involucrin and *CLED*, a Ca^{2+}-linked protein associated with early epithelial differentiation. The expression of a large amount of metabolic enzymes and proteins in the central corneal cells is thought to contribute to the increase in cell size[47]. Furthermore, increase in cell size has been correlated with loss of colony-forming efficiency[48].

Centripetal migration

Centripetal migration of corneal epithelial cells is a well-documented phenomenon[49,50]. Imaging studies have directly visualised centripetal migration of limbal cells towards the centre of the cornea. One of earliest studies used India ink to mark limbal cells which then migrated centripetally over the wounds of the mice cornea[51,52]. Centripetal migration was observed in rabbit lamellar keratoplasty model where the host corneal epithelial cells invaded the grafted donor tissue[53]. Similar results were obtained in the explants of human donor corneal buttons, where all donor corneal epithelial cells were replaced by recipient cells as early as three months post-penetrating keratoplasties[54]. Both Collinson *et al*[55], and Nagasaki *et al*[56] used transgenic mice with reporter genes to visualise centripetal migration in normal mice cornea. Interestingly, Matsuda *et al*[57] and Srinivasan *et al*[58] found that wounds close to the limbus or repeated insult to the central epithelium accelerated the healing rate, the latter implying that rapidly dividing TACs of the periphery have moved to more central areas after the first trauma and respond more quickly to the second.

The chemotactic signal for centripetal migration may be provided in the form of cytokines and/or the difference between the composition of extracellular matrix between the limbus and the cornea[59]. KGF, a paracrine hormone secreted by stromal cells, has been shown to enhance outgrowth in rabbit limbal explant culture on human amniotic membrane[60]. While the inflammatory cytokine interleukin-6[61], fibronectin[62], and hyaluronan[63], all of which are highly up-regulated upon injury, have been shown to play a role in drawing rabbit limbal cells towards the wound.

Recently, a very elegant study by Di Girolamo *et al*[64] has shown the centripetal movement of cells generated in the limbus using inducible multicolour tagging technology *in vivo*. Furthermore, this study linked the inducible multicolour tagging system with K14, one of the cytokeratin molecules that has been shown to mark an association with limbal stem cells. This study clearly showed that coloured K14 positive cells originated from the basal limbal epithelium and formed narrow corridors of epithelial cells that radiated centripetally onto the corneal surface. These authors do acknowledge that K14 is not an absolute limbal stem cell marker and that they could not exclude the existence of stem cells outside the limbal niche as K14 was targeted because of its limbal location.

Multipotency

Limbal basal cells characteristically lack differentiation markers indicating they are in an undifferentiated state. Several studies however, have implied a high multipotent differentiation potential when appropriate combinations of cellular signalling molecules are encountered: Rabbit limbal epithelial cell sheets transformed into fibroblasts when transplanted onto limbal stroma[65]; during the culture of human limbal explants, the limbal epithelial cells which invaded into the stroma underwent epithelial-mesenchymal transition[66]; mouse limbal epithelial cells expressed opsin when transplanted onto mice retina, indicating their potential to differentiate into rod photoreceptors[67]; and the potential to transdifferentiate to neuronal cells was demonstrated by Zhao *et al*[68]. In their study, rat limbal cell isolates maintained in growth factor-driven culture system expressed neuronal progenitors, β-tubulin, nestin and neurofilament. When subject to serum-containing differentiation medium, the limbal cell isolates expressed glial markers such as GFAP and O4. The limbus-derived neuron-like cells not only expressed neuronal markers and neurotransmitter receptors, but also exhibited electrical responses to GABA and kainic acid[69].

Stem cell niche

A stem cell niche is an anatomically defined area that is thought to provide a variety of intrinsic and extrinsic factors such as the physical protection, survival factors and cytokines and deemed essential to the maintenance of a stem cell population while preventing entry into differentiation[70,71]. Over the past decade, much progress

has been made in characterising the putative niche in the limbus. The limbal areas are rich in melanin pigments, highly innervated, well-vascularised and have a different array of extracellular matrix components than the central epithelium. Melanocytes or melanin granules within the cytoplasm of progenitor cells are thought to play a role in protection against ultraviolet radiation[8,72]. Blood-derived growth factors and nutrients provide for the active cell division[8,73].

The epithelial-stromal interface in the limbus differs from that in the central cornea. Bowman's layer, a densely interwoven collagen sheet lying between the basement membrane of the central corneal epithelium and the stroma, is absent in the limbus[74]. In the limbus, stroma directly underlies the epithelial basement membrane. The limbal epithelial basement membrane also differs from that of central cornea in its composition[75-80]. The limbal basement membrane labelled positive for type IV collagen α1 chain, laminin α2, β1, β2, γ1, γ3 chains, nidogen, agrin, BM40/SPARC, tenascin-C and thrombospondin-4, whereas central cornea showed positive immunoreactivity to type Ⅳ collagen α3 chain, type V collagen, thrombospondin-1 and endostatin. Limbal-specific basement membrane components were co-localised with putative stem cell markers such as ABCG2, p63 and cytokeratin 19, but not with differentiation markers including cytokeratin 3, connexin 43, desmoglein and integrin α2. In addition, the cornea-limbal transitional zone showed strong immunostaining to type XVI collagen, fibrillin-2, tenascin-C/R, vitronectin, bamacan, chondroitin sulfate and versican, and were co-localised with vimentin-positive cell clusters.

To date, four anatomic structures have been proposed as the corneal stem cell niche; Palisades of Vogt, limbal epithelial crypts, limbal crypts and focal stromal projections. The Palisades of Vogt are ridges of epithelium in the limbus that extend centripetally from the bulbar conjunctiva, and are easily visible by slit lamp microscopy, especially in young donors or those with dark skin[7,81,82]. More recently, Shanmuganathan*et al*[83] and Dua *et al*[84] identified limbal epithelial crypts located at the interpalisade epithelial rete ridges of the Palisades of Vogt. The limbal epithelial crypts radiate either peripherally into conjunctival stroma or circumferentially into limbal stroma. Shortt *et al*[85] proposed two additional niches using *in vivo* confocal microscopy; limbal crypts which are projections of limbal epithelium from the peripheral cornea into the limbal stroma, and focal stromal projections which are finger-like projections of limbal stroma with central blood vessels extending upward into the epithelium. These papillary structures offer physical protection for the deeply seated cells from injuries and shearing forces, and a large surface area that can accommodate increased cell numbers, blood vessels, and other supportive cells such as melanocytes, macrophages and stromal cells. Limbal crypts and focal stromal projections predominantly occur within regions of the cornea normally covered by the eyelids, which is a potential protective mechanism of these proposed niches[85]. Some of the putative stem cell features such as expression of ABCG2, p63 and p75, and high nucleus-to-cytoplasm ratio have been identified in the limbal basal cells lining these papillary structures[24,77]. In patients with limbal stem cell deficiency (LSCD), these four proposed niche structures are absent[84,85].

Recent studies have identified stromal stem cells which are directly subjacent to limbal basal cells[86,87]. An arising view of the limbal niche environment is that the limbal basal cells, stromal stem cells and the extracellular matrix molecules function as one unit to maintain the reservoir of ocular stem cells[88-90]. Human limbal epithelial cells co-cultured with stromal stem cells produced colonies with average diameter five times as large as those obtained with murine 3T3 feeder layer, indicating enhanced proliferation of limbal cells in the presence of stromal stem cells[91]. Recently, it was shown that limbal epithelial cells actively merge with stromal cells *via* chemokine receptor-mediated signalling in sphere-forming conditions, and this interaction seemed crucial for the maintenance of stem cell phenotype[92].

Limbal stem cell deficiency

The ability of limbal cells to regenerate corneal epithelium is robust evidence for the existence of stem cells in the limbus. Limbal stem cell deficiency (LSCD) is a complex corneal disorder resulting from functional and/or anatomical loss of limbus due to chemical or thermal burn, radiation, genetic/autoimmune disorders, multiple surgeries, contact lens use, infection or drug use[93,94]. Signs and symptoms of LSCD include conjunctivalisation, corneal vascularisation, pain, tear, redness, oedema, poor vision and blindness, which are thought to be associated with failure of epithelial regeneration[95,96]. Similar symptoms and a delayed wound healing response could be reproduced in rabbits by surgically removing the limbus[95,97]. The degree of loss of limbal tissue has been shown to correlate with the severity of pathology[98]. Clinical studies have shown that LSCD can be successfully treated with application of limbal cells[99-102]. Currently the sources of limbal cells are limbal autograft for unilateral LSCD, allogenic limbal graft from living related or cadaveric donors and *ex vivo* expanded limbal cells on transplantable substrate[93]. The overall success rate of limbal cell transplant is estimated at 76%, ranging from 50% to 100%[103]. The success rate varies between studies because outcome parameters, *ex vivo* expansion protocol, length of follow-up and aetiology of LSCD are different in each study[103]. Standard corneal transplants do not appear to provide a cure for patients with LSCD[104].

LIMBAL STEM CELL CONUNDRUMS

The body of evidence for the presence of stem cells at the limbus is impressive and convincing if largely circumstantial. The final piece of the jigsaw that remains to be revealed is the identification of an absolute stem cell

marker that is definitive of stem cell functionality. Likewise the body of evidence of the origin of epithelial cells at the limbus and their contribution to corneal epithelial homeostasis through the centripetal movement over the corneal surface has been elegantly shown by several research groups in several mammalian systems both *in vitro* and *in vivo*. However, despite this body of evidence, the proof that stem cells of the corneal epithelium reside only at the limbus and nowhere else is lacking and several pieces of knowledge remain unexplained by our current understanding of corneal maintenance by limbal stem cells:

Specificity of putative LSC indicators, criteria and markers

The traditional defining features of stem cells of the corneal epithelium include slow turnover rate, clonogenicity, proliferative potential, characteristic morphology, expression of certain proteins, centripetal migration *in vivo*, multipotency, specialised niche structures and ability to regenerate corneal epithelium. Despite the obvious biochemical changes at the cornea-limbal junction, selection of a consensus LSC marker has not been straightforward because each of these candidate markers has limitations resulting in inevitable ambiguities in separating stem cells from early progenitors[33,105]. In fact, there is mounting evidence showing that some of the putative markers of LSCs are not unique to the limbal basal cells.

Slow turnover rate has been demonstrated by label retaining studies in animal models. However, there are several pitfalls related to the use of label retention as a marker of stem cells[106]. The duration of the DNA labelling period was typically less than one week in most label retaining studies[8-11]. Cells quiescent during the labelling period will not take up DNA label and never be identified by this method. On the other hand, cells that have undergone a few rounds of cell division may still show DNA label albeit at a weaker level. Furthermore, label retention is not an essential property of stem cells as stem cells such as those underlying mammalian intestinal mucosa have short cycle time[107]. Not all label retaining cells are stem cells and vice versa.

The slow cycling property of the limbal cells has also been inferred from their resistance to 5-fluorouracil and predisposition to cancer. However, cells resistant to 5-fluorouracil are also found in the central epithelium although smaller in number than in the limbus[14]. Predisposition to cancer is also common in cells at the transitional zone where two types of epithelia unite in non-ocular tissue systems. The endo-ectocervical and oesophagus-stomach junctions are such examples.

Clonogenicity and asymmetric division are not unique properties of the limbal cells. Central corneal cells isolated from various mammalian species including humans have been shown to form clonogenic spheres *in vitro* although the number of spheres formed was smaller than when limbal cells isolates were used[108,109].

Asymmetric division as a means of self-renewal of stem cells is a widely accepted concept, but is difficult to show in experimental settings, and therefore it is as yet largely hypothetical due to a lack of compelling evidence. Recent evidence suggests mitotic spindle orientation and direction of asymmetric division are under the influence of specific environmental cues from the limbus rather than intrinsic polarity[110,111]. Possible environmental cues include growth factors, adhesion molecules and components of basement membrane that are specifically found in the limbus[112].

In terms of morphological criteria for LSCs, different groups have reported contradictory results. The amount of melanin granules[8,32,33], prominence of nucleoli and basal membrane invaginations[9,32,33,73] appear to vary from study to study. The reason for this contradiction is unknown but the lack of clear morphological distinction between stem cells and TACs could be responsible. As yet, TACs cannot be distinguished from true stem cells based on cellular morphology alone.

The expression of the protein markers of the LSCs either occurs in other cell types of the ocular surface, or is subject to change depending on environmental input. Cytokeratin 19, a well-established marker of limbal basal cells is also expressed in conjunctival epithelial cells[113]. ΔNp63α was identified in the corneal panni excised from patients with LSCD using western blot[114]. The free-floating spheres generated from human central corneal cells expressed ΔNp63α and ABCG2[109]. ABCG2 was found to be weakly expressed in the central cornea with what appeared to be an increasing gradient of expression towards peripheral cornea and finally the limbus[109,115].

Furthermore, the link between limbal location and stem cell indicators is further compounded as several studies have indicated that the components of the niche influence the expression of LSC markers. Espana *et al*[116] transplanted rabbit central corneal or limbal epithelial sheets onto either limbal or corneal stroma, and investigated the expression profile of two differentiation markers, cytokeratin 3 and connexin 43. Regardless of the type of epithelium transplanted, corneal stroma promoted expression of cytokeratin 3 while limbal stroma suppressed it. Expression of connexin 43 and apoptosis only occurred when corneal epithelium was cultured on corneal stroma. Li *et al*[87] showed that when human limbal epithelial cells were co-cultured with stromal stem cells, p63α was up-regulated and cytokeratin 12 down-regulated. The opposite expression pattern was observed when corneal fibroblasts were used instead of stromal stem cells. Kurpakus *et al*[117] showed that bovine conjunctival cells on corneal substrate expressed the differentiation marker cytokeratin 12 only when the basement membrane was left attached to the substrate, suggesting corneal basement membrane may encourage differentiation.

Since there is not one consensus marker for LSCs, a combination of functional, morphological and immunohistochemical markers is perhaps the most useful identifier for LSCs at present. To date, the "SP" property is the only marker that has been aligned with functionality.

ABCG2-positive cells in the limbus exhibited proliferative capacity, label retention and clonogenicity. However, heterogeneity exists even within the limbal SP cells as suggested by the lack of intracellular complexities in 60% to 80% of limbal SP cells[47].

At the time of writing this article, a newly published study in Nature has defined a new gene, ABCB5, as a novel limbal stem cell marker[118]. The authors have shown ABCB5 positive cells were predominantly BrdU label retaining cells from the limbus and co-localised with ΔNp63α in both mice and humans. Furthermore, the authors showed that ABCB5 positive cell numbers were reduced in LSC deficient patients and that ABCB5 positive cells isolated from mouse and human corneas had the ability to rescue the cornea in LSC deficient mice in both syngeneic and xenogeneic transplant models. Finally, the paper demonstrated that ABCB5 knockout mice showed disorganised corneal epithelial organisation and reduced wound healing capabilities, although bizarrely the knockout mouse was indistinguishable from wild type littermates by physical examination and contained all anterior and posterior segment components.

This appears to be the first description of a molecular limbal marker with stem cell functionality, and may be the missing jigsaw piece required to define limbal stem cells beyond doubt.

Limbus-independent corneal maintenance

A number of independent studies have challenged the long held belief that the limbus is the sole repository of stem cells in the corneal epithelium. These studies show that wound healing and normal corneal homeostasis can take place in the absence of limbus.

In 1994, Sandvig *et al*[119] showed that small lesions made in the rat central corneas did not evoke proliferative responses in the limbus, while medium-sized and large lesions did. This suggests wound healing of small lesions does not require limbal input. Our laboratory developed a "donut" excimer laser ablation model to demonstrate that human corneal epithelial regrowth occurs bi-directionally from both central and peripheral cornea[115]. In our model, the cell proliferation and migration response to wounding appeared to be as rapid from the central cornea as from the limbus, with central corneal epithelial cells fully capable of corneal epithelial regeneration. When the limbus was also ablated to remove any LSC's, re-growth occurred from the remaining central corneal epithelium and extended right out to the limbus.

Corneal maintenance without limbal input has also been observed by several other researchers. Huang *et al*[97] created a rabbit LSCD model by performing 360° cornea-limbal peritomy. After six months, two thirds of the corneas were completely normal while one third showed mild vascularisation. Kawakita *et al*[120] blocked communication and migration between the limbus and the cornea by transplanting a stainless steel ring on rabbit peripheral corneas. In their study, the isolated central corneas remained free of epithelial defects for at least six months. In a mouse LSCD model where the limbus was cauterised, the corneas remained transparent for four months[108]. In this study, portions of athymic mice limbus were excised and replaced with limbal grafts from β-gal-ROSA26 mice whose cells were β-galactosidase labelled. After four months they observed that β-galactosidase-labelled limbal cells never migrated out of the grafts and hence made no contribution to corneal homeostasis. However, when the eyes with limbal transplants were chemically or physically wounded, the labelled cells rapidly migrated out of the graft, along with unlabelled recipient limbal cells, to create a mosaic in the resulting healed corneal epithelium.

One criticism that these studies commonly face is that their observations may be due to the result of a TAC response as the periods of observation were rather short. If stem cells do exist in the central cornea, one would expect to see long-term corneal maintenance in the animal LSCD models.

Indeed, long-term corneal maintenance in the absence of limbal input has been described in a few case reports. Some patients who had 360° LSCD were found to have normal corneas for up to 12 years[121]. Also in LSCD patients who received *ex vivo* expanded limbal cell transplants, donor limbal cells that only lasted for 28 wk[122] or 9 mo[123] still resulted in the long-term restoration of the central corneal epithelium. What is maintaining the central cornea in these cases? Assuming desquamation of superficial cell layer occurs constantly, there are a few possible scenarios; (1) the amount of limbal stem cells remaining is undetectable but just enough to maintain homeostasis; (2) TACs in the basal cell layer of the central epithelium have an unexpected life span and a greater than previously thought proliferative potential; or (3) a self-renewing pool of precursor cells exist in the central cornea. Two independent groups have proposed the existence of a conceptual type of cell in the central corneal epithelium which is a TAC with more stem cell-like characteristics[121,124]. Further research efforts are required to explore and clarify these possibilities although a TAC cell with more stem cell-like characteristics sounds uncommonly similar to a stem cell. Thus the question arises - is there a different type of stem cell that exists on the corneal surface that may be activated by different mechanisms, may serve different purposes and may be defined by different markers than the limbal stem cells?

Ex vivo expansion of LSCs on amniotic membrane

A further strong argument against the existence of stem cells in the central cornea is the absence of anatomic niche structure in the central cornea to maintain stemness. However, there is evidence for survival and self-maintenance of LSCs outside of the described limbal niches.

The most frequently used substrate for limbal stem cell expansion is human amniotic membrane, the innermost wall of the placenta consisting of an epithelial monolayer, basement membrane and avascular stroma[125].

Isolated limbal cells, when cultivated on amniotic membrane, formed stratified epithelium much resembling cornea *in situ* and exhibited limbal stem cell phenotype such as increased expression of ΔNp63, p75, p63, ABCG2, integrin β1, Pax6, cytokeratin 3 and 19, decreased expression of connexin 43, increased resistance to phorbol ester-induced differentiation[126], label retention and clonogenicity[127]. Paulkin *et al*[128] analysed corneal buttons from LSCD patients who had previously received limbal cell transplants on amniotic membrane. The regenerated epithelial specimens had normal stratified structures and expressed central corneal markers cytokeratin 3 and 12 but not 19. These techniques provide evidence that limbal stem cells can survive, proliferate and expand outside of their niche which has been previously thought to be necessary for LSC maintenance.

It is not fully understood how an avascular structure like amniotic membrane can maintain the phenotype and metabolic needs of the LSCs[36,129]. The amniotic basement membrane is thought to promote adhesion, migration and differentiation of limbal epithelial cells, while amniotic stroma provide growth factors and anti-angiogenic and anti-inflammatory cytokines such as KGF, HGF, NGF, TGF-β and bFGF that prevent apoptosis and help maintain the stem cell phenotype.

Cytokine signalling is becoming increasingly recognised as a key component of a niche, regulating stem cell morphology and behaviour[130]. The Wnt/β-catenin signalling system has been shown to be responsible for preventing apoptosis of limbal cells *in vitro*[131]. The authors suggested that as long as survival factors are present, limbal stem cells are likely to survive outside their niche. Indeed, in a mouse model, LSCD was successfully treated with human limbal fibroblast-conditioned culture medium but not with skin fibroblast-conditioned medium, again emphasising the importance of chemical signals produced in the limbus[132].

There are studies which question the longevity of *ex vivo* expanded limbal epithelial cells. Li *et al*[66] showed progressive loss of clonogenicity and proliferative potential of limbal explant cultures on intact amniotic membrane in subsequent passages. The reason for this contradictory result is unknown but slight differences in expansion protocol and donor tissue variability might be responsible.

Furthermore, one study has proposed the existence of compound niches of cells that exist in the limbus of the mouse in unwounded corneas[133]. However, after wounding these compound niches were able to migrate onto the surface of the cornea and express corneal epithelial cytokeratins while also retaining both features of the compound niche and features of goblet cells. This study serves to illustrate that a niche may not be an immovable structure to which cells attach but may be inherent to the cellular components and therefore able to migrate with those components.

Developmental origin of limbus

Epithelia of skin, gut wall and cornea are outer most coverings of our body and share the same developmental origin. In all types of epithelia, with the exception of cornea, desquamated cells are replaced with newly generated cells from stem cells located in the basal layer[8]. Only corneal epithelium is thought to be renewed from a distant repository of stem cells. This is somewhat peculiar in evolutionary sense especially when the directly adjacent conjunctiva is maintained in the same way as any other epithelia[134].

In fetal eyes, adult LSC markers are found in the basal layer across the cornea[135,136] and it is unknown how the markers become segregated in the limbus during development. Investigation of limbal organogenesis has raised a possibility that the limbal papillary structures are mere developmental remnants. The limbus does not develop until eyelids open and the ocular surface is exposed to amniotic fluid[135,136]. The papillary structures of the limbus do not form until post-natal life[137]. The question remains as to why a microenvironment essential for the support of stem cell maintenance only appears after birth and why stem cells can be maintained on the central cornea prior to birth.

CONCLUSION

A strong body of evidence has accumulated over the past few decades, showing that markers of stemness are exclusively localised at the limbus. Furthermore the centripetal migration of corneal epithelial cells after generation at the limbus has been definitively shown. Therefore, the limbus has been designated as the single repository of stem cells of the corneal epithelium. However, there is mounting evidence showing that the expression of the stem cell markers are largely determined by extrinsic signals provided by the regional microenvironment[130,138], and the markers themselves do not indicate intrinsic stemness. As shown by the clinical success of LSC transplant on amniotic membrane in LSCD, a niche structure is not an absolute requirement for the survival of ocular stem cells, as long as the right survival signals are provided. The existence of the limbus as the sole repository of corneal epithelial stem cells also does not explain a number of clinical observations which have demonstrated corneal wound healing without limbal input and also does not explain the developmental origin of the limbus.

A vast majority of studies consider central cornea as a lineage-committed, post-mitotic tissue, but some groups have independently suggested a possibility that stem cells exist outside the limbus. Until more definitive data becomes available, the possibility of the existence of progenitor cells outside the limbus should not be excluded as central cornea may provide a new source of stem cells that can serve as a sustainable repository of high quality, evaluated, optimised tissue for the treatment of corneal degenerative disorders.

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