

Evaluating alternative stem cell hypotheses for adult corneal epithelial maintenance

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Abstract

In this review we evaluate evidence for three different hypotheses that explain how the corneal epithelium is maintained. The limbal epithelial stem cell (LESC)

hypothesis is most widely accepted. This proposes that stem cells in the basal layer of the limbal epithelium, at the periphery of the cornea, maintain themselves and also produce transient (or transit) amplifying cells (TACs). TACs then move centripetally to the centre of the cornea in the basal layer of the corneal epithelium and also replenish cells in the overlying suprabasal layers. The LESCs maintain the corneal epithelium during normal homeostasis and become more active to repair significant wounds. Second, the corneal epithelial stem cell (CESC) hypothesis postulates that, during normal homeostasis, stem cells distributed throughout the basal corneal epithelium, maintain the tissue. According to this hypothesis, LESCs are present in the limbus but are only active during wound healing. We also consider a third possibility, that the corneal epithelium is maintained during normal homeostasis by proliferation of basal corneal epithelial cells without any input from stem cells. After reviewing the published evidence, we conclude that the LESCs and CESC hypotheses are consistent with more of the evidence than the third hypothesis, so we do not consider this further. The LESCs and CESC hypotheses each have difficulty accounting for one main type of evidence so we evaluate the two key lines of evidence that discriminate between them. Finally, we discuss how lineage-tracing experiments have begun to resolve the debate in favour of the LESCs hypothesis. Nevertheless, it also seems likely that some basal corneal epithelial cells can act as long-term progenitors if limbal stem cell function is compromised. Thus, this aspect of the CESC hypothesis may have a lasting impact on our understanding of corneal epithelial maintenance, even if it is eventually shown that stem cells are restricted to the limbus as proposed by the LESCs hypothesis.

Key words: Eye; Cornea; Corneal epithelium; Limbal epithelium; Stem cell; Lineage tracing

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Core tip: This review article evaluates the evidence for different hypotheses that have been proposed to explain how the corneal epithelium is maintained. It identifies core observations in favour of the conventional limbal epithelial stem cell (LESC) hypothesis and an alternative corneal epithelial stem cell hypothesis and describes how lineage-tracing experiments are helping to reconcile the two sets of conflicting evidence in favour of the LES C hypothesis.

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INTRODUCTION

It is widely accepted that adult corneal epithelium is maintained by stem cells located in a region called the limbus, at the corneal periphery. However, this limbal epithelial stem cell (LESC) hypothesis has been challenged by an alternative corneal epithelial stem cell (CESC) hypothesis, based on experimental studies with mice^[1]. This accepts that LESCs exist but proposes that they only contribute to corneal epithelial repair in response to wounding and that, during normal homeostasis, the corneal epithelium is maintained solely by stem cells scattered throughout the corneal epithelium itself. It has also been proposed that, in the absence of a wound, the corneal epithelium is maintained entirely by proliferation of its own basal cells without any involvement of stem cells^[2]. The main purpose of this review is to compare the evidence for the alternative LES C and CESC hypotheses in order to identify where there is common ground and where differences need further experimental investigation. However, we also consider whether the experimental evidence is consistent with the possibility that the corneal epithelium is maintained without stem cells.

THE CORNEAL AND LIMBAL EPITHELIA

The cornea is the specialised, avascular, transparent, dome-shaped region of the anterior ocular surface, which refracts light through the pupil to the lens and provides a protective, impermeable barrier. It consists of three cellular layers: (1) the inner corneal endothelium, which, despite its name, is a type of epithelium; (2) the middle corneal stroma, comprising specialised fibroblasts, called keratocytes, embedded in a collagen and proteoglycan matrix; and (3) the outer, non-keratinised, stratified squamous epithelium, comprising 5-6 layers of keratinocytes, which is kept moist by the tear film. The corneal epithelium is a very dynamic tissue. Differentiated cells are continuously shed from the outer layer and replaced by cells

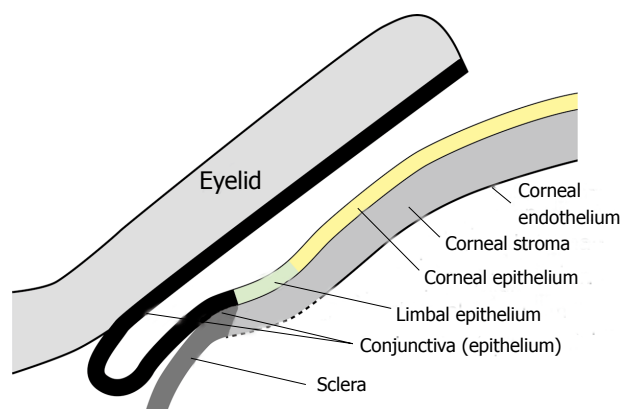


Figure 1 Diagrammatic representation of the tissues of the mouse ocular surface and eyelid.

produced in the proliferative basal layer. According to the conventional stem cell paradigm these proliferative basal cells are considered to be transient (or transit) amplifying cells (TACs) and are replenished by stem cells. The early generation TACs are sometimes referred to as progenitor cells.

The limbus is a narrow transition zone, which encircles the cornea (Figures 1 and 2). The stroma and epithelial layers of the cornea extend into the limbus where they become the limbal stroma and limbal epithelium. However, the corneal endothelium does not extend into the limbus and is replaced by the drainage channels of the trabecular meshwork. On the other side of the limbus, the stroma merges with the sclera, which forms most of the ocular surface (the white part of the human eye) and the limbal epithelium becomes the conjunctiva. The conjunctiva is an epithelium, which covers the anterior sclera, folds back to form the conjunctival sac and lines the inner surface of the eyelids (Figure 1). Thus, the conjunctiva attaches the eyeball to the eyelids and orbit and permits some rotation of the eyeball in the orbit. Unlike the transparent cornea, both the limbus and the conjunctiva are vascularised.

The human limbus contains radial fibrovascular ridges, called the palisades of Vogt, which project upwards from the stroma deep into the epithelium (Figure 2) but many species, including mice, do not have limbal palisade structures. Another anatomical difference between these species is that, in the mouse, the corneal epithelium is thickest in the centre of the cornea and has fewer cell layers in the peripheral cornea and limbus whereas, in humans, the limbal epithelium is thicker (about 8-10 cell layers) than the corneal epithelium (5-6 layers)^[3].

It is widely accepted that some basal limbal epithelial cells are stem cells^[4,5] and that the limbal stroma, vasculature and other associated cell types provides a suitable stem cell niche microenvironment, which is required to maintain the LESCs in a relatively undifferentiated state^[6,7]. The limbal palisades (Figure 2) increase the area of interface between the limbal

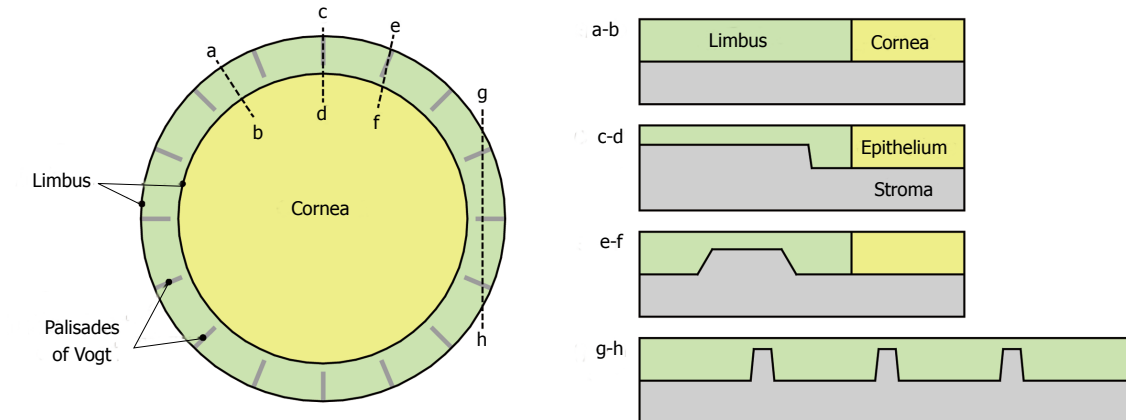


Figure 2 Palisades of Vogt. Diagram showing the arrangement of the palisades of Vogt (upward projections of limbal stroma into the limbal epithelium) in the human limbus in plan view (left) and how they might appear in differently orientated sections through the limbus (right). Only 16 palisades are shown but in reality there are many more. For anatomy see references^[114,115].

epithelium and stroma, so increasing the size of the region that is likely to harbour the LESC niches. Furthermore, it has been suggested that LESC niches may be particularly enriched in two types of epithelial crypts associated with the palisades. One type of crypt is formed by the regions of limbal epithelium between the upward-projecting stroma of the limbal palisades and these have been named "limbal crypts" (LCs)^[8,9]. The other type of crypt (named "limbal epithelial crypts"; LECs) are more sparsely distributed (only 6-7 per eye) and are formed from epithelial projections from the periphery of the limbal palisades, which extend either radially from the limbus into the conjunctival stroma or circumferentially within the limbus (perpendicular to the palisades)^[10,11]. However, many species do not have limbal palisades and associated crypts, which could, therefore, be considered to be species-specific adaptations, possibly related to eye size. Thus, if the limbus provides a niche microenvironment, presumably it is either not dependent on these structures or differs among species. Maintenance of the niche microenvironment is more likely to depend on the presence of the vasculature and other cell types that are present in the limbus in all species.

ALTERNATIVE HYPOTHESES OF CORNEAL EPITHELIAL MAINTENANCE

We consider two hypotheses, which propose alternative ways that stem cells may maintain the corneal epithelium, and a third hypothesis, which does not include stem cells. For the present purposes, we define a stem cell as an undifferentiated cell with high proliferative potential that is capable of renewing itself and also producing one or more differentiated cell types with lower proliferative potential. While most adult stem cells are multipotent, generating multiple cell types, stem cells that maintain the corneal epithelium are generally presumed to be unipotent,

only producing the corneal epithelial cells. Although one report shows that they may also produce the goblet cells that enter the corneal epithelium in response to large wounds^[12], we have not considered this possible additional role of the stem cells in this review.

Limbal epithelial stem cell hypothesis

According to the conventional LESC hypothesis (Figure 3A and D), LESC act as a source of new basal corneal epithelial cells (the TACs) during normal corneal epithelial homeostasis and become more active during episodes of significant wound healing^[13], although small wounds may be healed without upregulating stem cells. In this scheme LESC remain in the limbus where they maintain themselves and also generate the first generation of TACs. Some of these early TACs move to the overlying, non-mitotic suprabasal epithelial layers, and become terminally differentiated. Other early generation TACs continue to divide and move centripetally in the basal layer to maintain the corneal epithelium. Once cells leave the basal layer they differentiate and move rapidly through the suprabasal layers to the superficial layer from where they are shed. It seems that both daughter cells of a dividing basal cell usually share the same fate^[14] so that they either both remain in the proliferative basal layer or both move suprabasally. It is not known what determines whether cells leave the basal layer. For example, it could be a combination of declining suprabasal cell numbers, caused by cell loss, and overcrowding in the basal layer, caused by cell proliferation, as described for the *Drosophila notum*^[15].

Corneal epithelial stem cell hypothesis

The corneal epithelial stem cell (CESC) hypothesis accepts that there are stem cells in the limbus but proposes that these are only activated for repairing wounds and that during normal homeostasis the corneal epithelium is maintained by stem cells

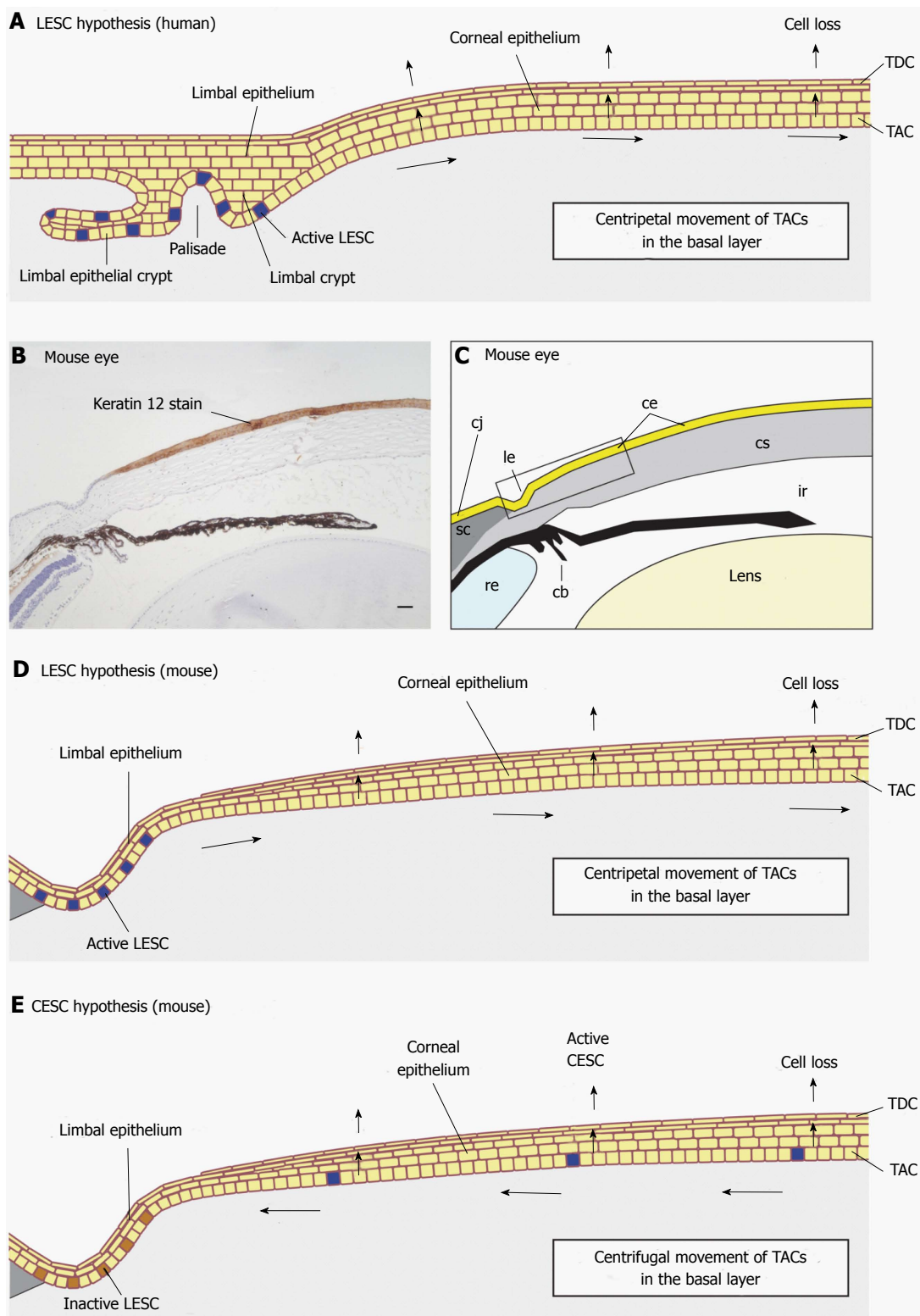


Figure 3 Limbal epithelial stem cell vs corneal epithelial stem cell hypotheses. A: Diagram of human corneal epithelial maintenance according to the limbal epithelial stem cell (LESC) hypothesis showing active LESC in the limbal epithelium in both a limbal crypt and a limbal epithelial crypt. The LESC divide slowly replacing themselves and producing daughter transient (or transit) amplifying cells (TACs), which divide more quickly and move centripetally from the basal layer of the limbal epithelium to the basal layer of the corneal epithelium. After a final cell division TACs leave the basal layer, move through the suprabasal layers and are shed from the surface as terminally differentiated cells (TDCs); B: Histological section showing mouse cornea, limbus and part of the conjunctiva immunohistochemically stained for keratin 12 (K12; dark brown staining) to show the border between the corneal epithelium (K12 positive) and limbal epithelium (K12 negative); C: Drawing of photograph shown in (B) with different tissues labelled. The boxed area shows part of the limbal and corneal epithelia, equivalent to that represented in (D) and (E); D: Diagram of mouse corneal epithelial maintenance according to the LESC hypothesis. The principles are the same as described for (A); E: Diagram of mouse corneal epithelial maintenance according to the corneal epithelial stem cell (CESC) hypothesis. The CESC divide slowly replacing themselves and producing daughter TACs, which divide more quickly and move centrifugally as originally proposed¹¹. After a final cell division TACs leave the basal layer, move through the suprabasal layers and are shed from the surface. cb: Ciliary body; ce: Corneal epithelium; cj: Conjunctiva; cs: Corneal stroma; ir: Iris; le: Limbal epithelium; re: Retina; sc: Sclera. Photograph (B) is reproduced from Mort *et al.*¹⁸ with kind permission of Springer Science + Business Media.

Table 1 Evidence discriminating between alternative hypotheses

Observations	Species	Ref.	Consistent with hypothesis?		
			LESC	CESC	GBL
Holoclonal-producing cells are present in limbus but not cornea	Human	[25]	++	±	±
Holoclonal-producing cells are present in limbus and cornea	Pig	[1]	±	++	±
Production of clonogenic spheres from limbus and central cornea	Human	[29]	±	++	±
Limbal epithelial cells are superior to corneal epithelial cells for corneal repair	Human	[32,33]	++	+	±
The central corneal epithelium can maintain itself when isolated from the limbus	Rabbit, mouse, human	[1,39-42]	+ ^a	++	++
Label-retaining cells are present in the limbus but not the corneal epithelium during normal homeostasis	Mouse, rabbit	[13,52-54,57]	++	+	0
After a 40-d chase ³ H-TdR-labelled cells are present in both the limbus and the corneal epithelium during normal homeostasis	Rabbit	[2]	+ ^b	+ ^b	+ ^b
Cells move centripetally from the periphery of the corneal epithelium during normal homeostasis	Human, mouse	[58,61-63]	++	± ^c	± ^c
Mosaic patterns change after birth and clones of labelled cells emerge from the limbus as radial stripes	Mouse	[27,72,77-79]	++	0	0
Distribution of rare stripes in corneas of <i>KRT5-LacZ</i> ^{+/+} transgenic mice	Mouse	[17]	++	±	±
Transplanted limbal tissue contributes to replacing experimentally debrided corneal epithelium	Rabbit, mouse	[1,76]	++	++	+
Transplanted limbal tissue does not contribute to the unwounded corneal epithelium	Mouse	[1]	0	++	++
More tumours arise from the limbal epithelium than corneal epithelium	Human	[80]	++ ^d	+	+
Diverse cell types and blood supply makes limbus a likely stem cell niche	All species	[6]	++ ^d	+	+
Distribution of markers associated with undifferentiated or stem cell phenotype	Human, mouse, rabbit	See Table 2	++	±	±
Lineage tracing studies show that limbal cells contribute to the unwounded corneal epithelium during normal homeostasis	Mouse	[64]	++	0	0

++: Expected for hypothesis; +: Consistent with hypothesis; ±: Not consistent with hypothesis unless specific assumptions are made or technical issues compromise the interpretation of the experiment; 0: Not consistent with hypothesis. ^aThis is compatible with the LESc hypothesis if self-maintenance of the central corneal epithelium is a back-up mechanism that is used when homeostasis is compromised and LEScs are unable to maintain the corneal epithelium. ^bThe chase period may not have been sufficient to identify label-retaining cells (see text). ^cThe CESC hypothesis, as originally stated^[1], proposed that corneal epithelial cell movement was centrifugal but this assumption is not necessary. However, it requires *ad hoc* assumptions to account for centripetal movement across the full radius. ^dEvidence is circumstantial. LESc: Limbal epithelial stem cell.

scattered throughout the corneal epithelium itself^[1]. This hypothesis is based largely on surgical transplantation experiments in mice. These experiments showed that labelled limbal epithelial tissue, transplanted to the limbus of immunologically compromised mice, only produced labelled clones in the cornea if the host corneal epithelium was subsequently removed. The authors reasoned that if LEScs were active during normal homeostasis, as the LESc proposes, the donor limbal tissue should colonise the cornea without being stimulated to do so by wounding. However, others have pointed out that the CESC hypothesis is not consistent with some of the earlier experimental evidence^[16-18].

Germinative basal layer hypothesis

A third possibility harks back to earlier explanations before the importance of tissue stem cells was recognised. Like the CESC hypothesis, this proposes that the corneal epithelium is normally maintained entirely from cells in the basal layer of the corneal epithelium but unlike the other two hypotheses it proposes there are no stem cells in either the limbal or corneal epithelia. Haddad *et al.*^[2] referred to the basal layer as the "germinative basal layer" and proposed this alternative mechanism for corneal epithelial maintenance to explain the results of their label-retaining cell experiment with rabbits. These

results are inconsistent with other label-retaining cell experiments, as discussed below. However, we have considered this hypothesis because there is evidence that some other adult tissues are maintained during normal homeostasis by proliferation of more differentiated cell types. In such cases, stem cells are either absent or only active during wound repair. For example, this type of tissue maintenance has been proposed for pancreas β -cells^[19], epidermis^[20], lung^[21] and liver hepatocytes^[22,23].

EXPERIMENTAL EVIDENCE AND EVALUATION OF ALTERNATIVE HYPOTHESES

The three alternative hypotheses are discussed below with respect to the available experimental evidence and evaluations are summarised in Table 1.

Cells with high proliferative potential

One of the hallmarks of stem cells is that they have a greater proliferative potential than TACs and this can be identified using *in vitro* colony-forming assays with cultured cells. The proliferation characteristics of cultures of explanted epithelial cells can be investigated in culture and clones derived from single cells can be classified as holoclones, meroclones and

paraclones. These are thought to represent *in vitro* descendants of stem cells, TACs and differentiated cells, respectively^[24]. (On indicator dishes in culture, holoclones form large, smooth-edged, fast-growing colonies with large numbers of small tightly packed cells. Meroclones form smaller colonies that are irregular in outline and include a mixture of small tightly packed cells and larger more loosely packed cells, which are predominantly at the edge. Paraclones form small, diffuse colonies and most cells are large, flattened and loosely packed). Clonal analysis of cells from the human ocular surface epithelia identified holoclone-producing cells in the limbal epithelium but not the corneal epithelium from a 54-year-old individual^[25], suggesting that stem cells are present in the limbal epithelium.

However, there is also evidence that some cells of the central cornea are self-sustaining and have high proliferative potential. Majo *et al.*^[1] showed that cultured corneal epithelial cells of many species produced colonies of cells *in vitro* although there were significant species differences. Pig corneal epithelial cells grew particularly well and clonal analysis of cultured pig cells identified holoclone-producing cells in the central corneal epithelium as well as the limbus. This suggests that stem cells are present in the central corneal epithelium of pigs as well as the limbus but this result cannot be evaluated fully as the age of pigs was not given and fetal cells with stem cell characteristics may persist in younger individuals^[26-28].

The production of clonogenic spheres of cells in culture has also been associated with the presence of stem cells and these have been isolated from the human limbus and central cornea, although isolation is most efficient from the limbus and from younger individuals^[29]. These culture experiments imply that both the limbal epithelium and the central corneal epithelium have cells that are able to behave like stem cells in clonogenic assays *in vitro* which argues against the LESC and germinative basal layer (GBL) hypotheses. However, if culture conditions unmasked proliferative potential of corneal epithelial cells, which is not expressed during normal homeostasis *in vivo*, this result would be compatible with all three hypotheses.

Despite their slow proliferation *in vivo* (see next section and reference^[30]), human limbal epithelial cells grow well in culture and have a higher mitotic rate than corneal epithelial cells^[31]. Furthermore, clinical observations indicate that human limbal tissue is superior to central corneal tissue for treating patients with severely wounded corneal epithelia, which is likely to reflect a greater proliferative potential. The corneal epithelium can be restored using grafts of human limbal epithelial tissue^[32,33] or cells cultured from explanted limbal tissue^[34-38]. Although the limbus is the preferred source of cells for clinical therapeutic use, this does not help determine whether LSCs are active during normal tissue homeostasis (LESC hypothesis)

or only during wound healing (CESC hypothesis).

There is also evidence that the central cornea of several species contains highly proliferative cells. For example, rabbit central cornea is able to survive for months after the limbus is removed or separated from the cornea^[39,40], although corneal integrity slowly degenerates and it does not heal properly after corneal wounding. Similarly, the mouse corneal epithelium was able to sustain itself for four months after the limbus was cauterised to destroy the limbal epithelium^[1]. It has also been reported that some patients with symptoms of total LESC deficiency retain central islands of normal corneal epithelium for several years^[41] and in one case this appeared to be sufficient to restore the corneal epithelium^[42]. These studies show that the central cornea can maintain itself to some extent when the limbus is eliminated or disconnected. This implies that the central corneal epithelium has cells that are able to act as progenitors, if LSCs are unable to maintain the corneal epithelium. However, again this does not show whether these cells act as progenitors during normal homeostasis so it does not provide conclusive evidence against the LESC hypothesis.

Cell division characteristics and identification of slow-cycling label-retaining cells

Stem cell populations maintain themselves and produce more differentiated cells throughout the lifetime of the organism. This is sometimes interpreted as requiring stem cells to divide asymmetrically (producing one stem cell and one TAC) but this does not mean that each division of every stem cell has to be asymmetric as long as the population average achieves this. There have been few attempts to identify asymmetrically dividing cells in the ocular surface^[43] and results are insufficient to discriminate among the three hypotheses.

A more widely studied characteristic of many stem cells is that they are relatively quiescent so divide infrequently. A slow cell division rate is not an obligatory phenotype of stem cells but it has been used to try to locate the stem cells that maintain the corneal epithelium. Slow-cycling cells (including putative stem cells) are usually identified as "label-retaining cells". These are cells that retain a DNA-label such as BrdU or ³H-TdR, or a chromatin label such as GFP-tagged histone-2B, after prolonged labelling and a chase period to dilute the label from more rapidly dividing cells. The prolonged period of labelling is to label as many cells as possible including relatively quiescent stem cells that divide infrequently. The chase period is calibrated to dilute label from most cells in the tissue but not any slow-cycling cells (which include putative quiescent stem cells). This method is useful for identifying the location of putative stem cells but is not specific and will also identify other slow-cycling cell types and cells that divide during the labelling

period and then stop dividing when they terminally differentiate.

The ocular surface is a suitable tissue for label-retaining cell experiments because the corneal epithelial TACs divide quite frequently so will readily dilute the label. (The average mitotic rate has been estimated for rats as 14.5% per day for the whole corneal epithelium^[44] and this equates to approximately 37% for just the mitotic basal layer, based on the relative basal and suprabasal cell numbers in mouse corneas^[45]). Similarly, BrdU experiments indicate that almost 50% of basal corneal epithelial cells are in S-phase of the cell cycle, during a 24-h labelling period^[46]. The effectiveness of the chase period is also helped by the constant loss of cells from the superficial layer, as stem cells will not be lost in this way. It has been estimated that once cells leave the basal corneal epithelial layer the time to cell loss (turnover time) is only about 7 d (range $3^{1/2}$ to 14 d) for mice, rats and humans^[45,47-50] but a longer turnover time of between 14 and 21 d has been estimated for rabbits^[51].

Most investigations have identified label-retaining cells in the basal limbal epithelium but not in the corneal epithelium either after wounding^[5,13] or during normal homeostasis in mice^[13,45,52-54], rats^[55,56] and rabbits^[57]. Two caveats about the exclusive location of label-retaining cells to the limbus in these experiments should be mentioned: (1) species differences in cell cycle kinetics and technical differences between studies may affect the number of cells that remain labelled so the chase period needs to be optimised for each species (for example, in these studies, chase periods for treatments without wounding varied from 4 to 11 wk); (2) using a relatively short chase period of 4 wk, Chen *et al.*^[56] showed that approximately 20% of the label-retaining cells were slow-cycling Langerhans cells rather than putative slow-cycling stem cells. (These Langerhans cells also shared two other characteristics of putative stem cells, discussed below, as they were positive for the marker ABCG2 and had a high nucleus to cytoplasm ratio). Nevertheless, the results of all these studies consistently identified label-retaining cells in the basal limbal epithelium but not in the basal corneal epithelium and it is likely that most of these will be stem cells. Thus, these studies favour the LESC hypothesis, unless there is an additional stem cell population in the corneal epithelium, which is not slow cycling. They also argue against the GBL hypothesis unless none of the limbal label-retaining cells are stem cells.

A completely different result was found for one study with rabbits^[2], which prompted the authors to conclude that the corneal epithelium is not maintained by LESC but by virtually all the cells of the basal corneal epithelium (referred to here as the GBL hypothesis). Rabbits were given 3 intravitreal injections of ³H-TdR at intervals of 4 d. After a 41-d chase (49 d after the first injection) the labelling index was higher in the corneal epithelium (17.8%) than the limbal

epithelium (3.8%)^[2]. However, the high labelling index suggests that many of the labelled cells were TACs, rather than slow-cycling stem cells, and the chase period was insufficient to detect label-retaining cells. Paradoxically, the chase period was comparable to that used in an earlier experiment, which identified label-retaining cells in the limbus but not corneal epithelium of rabbits^[57]. In this experiment, BrdU was infused from an osmotic mini-pump for 14 d, the pump was removed at 17 d, and the rabbits were left for a further 38-d chase period (*i.e.*, until 55 d after the beginning of labelling). In the ³H-TdR study^[2], autoradiography was used to detect label in high quality semi-thin sections and it is possible that this is more sensitive than the BrdU immunofluorescence used earlier^[57]. If so, more cell divisions and a longer chase period would be required to dilute the ³H-TdR below detectable levels in the majority of cells in order to identify any label-retaining cells. It would, therefore, be worth repeating the ³H-TdR experiment with a longer chase period before drawing conclusions that contradict the other label-retaining cell studies.

Movement of corneal epithelial cells

Early experimental evidence showed that cells moved from the limbus to the cornea to repair a corneal wound in guinea pigs^[58]. The observation that donor corneal epithelial cells, transplanted to the centre of rabbit corneas, were replaced by host cells more quickly at the periphery of the transplant also suggested that new host cells were moving centripetally from the periphery of the cornea to replace the older donor cells^[59]. Other indirect evidence that cells move centripetally from the limbus during homeostasis of unwounded corneas is reviewed elsewhere^[60]. More importantly, direct observations of radial epithelial movement during normal corneal homeostasis have consistently demonstrated that cells move centripetally from the periphery to the centre of the cornea. This supports the LESC hypothesis, which proposes that LESC remain in their limbal niche but TACs move centripetally to maintain the corneal epithelium.

These experiments also provided estimates of the rate of centripetal movement of corneal epithelial cells for the unwounded cornea. This was estimated to be 28 $\mu\text{m}/\text{d}$ from observations of one human subject over 24 h using *in vivo* confocal microscopy^[61]. For mouse corneas, the rate of centripetal movement has been estimated as 11-26 $\mu\text{m}/\text{d}$ using three different approaches involving direct observations of labelled cells. Corneal epithelial cells labelled with India ink moved 17 $\mu\text{m}/\text{d}$ over 7 d^[62], patches of brightly fluorescent cells moved 26 $\mu\text{m}/\text{d}$ over 7 wk in mosaic GFP transgenic mice^[63] and fluorescent clones of cells extended 11 $\mu\text{m}/\text{d}$ over 12 wk in K14-CreERT2;R26R-confetti transgenic mice (from 9 to 21 wk after tamoxifen-activation of the reporter transgene)^[64]. Furthermore, the evidence from the mosaic GFP

transgenic mice^[63] and tamoxifen-activated reporter transgenic mice^[64] demonstrated that the same clonal lineage of cells moved across the full radius from the periphery to the centre. This is in contrast to cells in the conjunctiva, which do not move significantly at all^[62,65].

When Majo *et al.*^[1] proposed the CESC cell hypothesis they also proposed that the corneal and conjunctival epithelia continuously expand towards the limbus, which they described as a zone of equilibrium, so any movement in the corneal epithelium was predicted to be centrifugal. This is inconsistent both with the absence of movement in the conjunctiva^[62,65] and the convincing, direct evidence that movement of corneal epithelial cells is centripetal not centrifugal^[62-64]. The evidence for centripetal cell movement in the corneal epithelium is inconsistent with the CESC hypothesis as originally proposed^[1] but there is no need to link the stem cell location and movement aspects of the original CESC hypothesis. In principle, it would be possible for the corneal epithelium to be maintained by stem cells, scattered throughout the tissue, without invoking centrifugal movement. It is likely that TACs produced by CESC would only move radially because evidence from various mosaics and chimaeras implies that lateral movement is constrained (discussed in the next section). In theory, radial movement of TACs could be either centripetal or centrifugal but, as noted above, evidence for centripetal movement is compelling.

The causes of centripetal movement are not known and suggestions include: (1) population pressure from the periphery due to production of new TACs by LESC^[66-68]; (2) preferential loss of epithelial cells from the central cornea^[30,60]; (3) differential stiffness of cornea and limbus^[69]; (4) chemotaxis^[62]; (5) stimulation by corneal nerves^[70]; and (6) response to endogenous electric currents^[71].

If the LESC hypothesis was incorrect, centripetal movement could still be explained by a mechanism other than population pressure from the limbus. However, if LESC were absent or only active during wound healing, a peripheral source of cells would be required to replace peripheral cells that move centripetally, during normal homeostasis. This might be provided by limbal TACs for the GBL hypothesis or CESC in or near the limbus for the CESC hypothesis. Thus, both the CESC and GBL hypotheses could account for centripetal movement of cells in separately maintained regions on the same radius. However, evidence from transgenic mice shows that a single clone of cells moves across the full radius^[63,64], implying that there is a single source of cells in the limbus or peripheral cornea, rather than multiple sources throughout the cornea. This is more difficult for the CESC and GBL hypotheses to explain unless it is argued that not all radial regions are maintained by a single CESC or progenitor TAC. Another problem for the CESC hypothesis is that the CESC would tend to

move centripetally with the TACs, and so accumulate in the centre, unless the CESC were somehow stabilised in unidentified niches and the TACs could move past them. Overall, centripetal movement strongly favours the LESC hypothesis and it is difficult to reconcile this with the other two hypotheses without *ad hoc* assumptions.

Change in mosaic patterns after birth

In addition to direct studies of cell movement in real time, changes in patterns in several types of mosaic mice have provided additional evidence that cells emerge from the limbus at the periphery of the corneal epithelium and continue to move centripetally across the cornea. Mosaic patterns in adult mouse and rat chimaeras and mouse X-inactivation mosaics (*XLacZ* mosaics) are arranged as radial stripes in the corneal epithelium^[27,72,73], which is consistent with either centripetal or centrifugal movement, without significant lateral dispersion. Similar radial stripes, have been observed with various endogenous markers in human corneas, including traces of pigment^[58] and various opacities, cell inclusions or drug-induced lipidosis associated with vortex keratopathy (cornea verticillata) or hurricane keratopathy^[60,66,74,75]. In many cases the stripes form a spiral-pattern in the centre, which fits well with the more direct evidence for centripetal movement, discussed above, because centripetal movement of labelled cells transplanted to the rabbit limbus sometimes formed a similar spiral^[76].

Before about 5 wk of age, the pattern in X-inactivation mosaics is completely different from the adult radial stripes and the β -gal-positive and β -gal-negative cell populations initially form randomly orientated patches^[27,72]. Groups of β -gal-positive and β -gal-negative cells emerge from the periphery by about 5 wk and extend as radial stripes across the cornea. The simplest interpretation is that the formation of stripes coincides with the onset of activation of stem cells in the limbus that generate new cells, which replace those produced during development^[27]. This is supported by similar observations with mosaic transgenic mice^[77,78] and lineage tracing with a GFP-tagged lentiviral marker^[79], as illustrated in Figure 4, and is consistent with the LESC hypothesis but not with the CESC or GBL hypotheses.

One problem with these mosaic systems is that similar proportions of labelled and unlabelled cells were present so many of the radial stripes may comprise more than one adjacent clone that are similarly marked. Observations on *KRT5-LacZ*^{+/-} transgenic mice showed that they had rare β -gal-positive stripes in a predominantly β -gal-negative corneal epithelium, so largely avoiding the problem of multiple adjacent clones^[17]. The distribution of β -gal-positive stripes was not consistent with predictions of centrifugal extension of clones of labelled cells from β -gal-positive CESC distributed randomly in the corneal epithelium and the simplest interpretation is that the stripes represent clonal lineages derived from LESC located

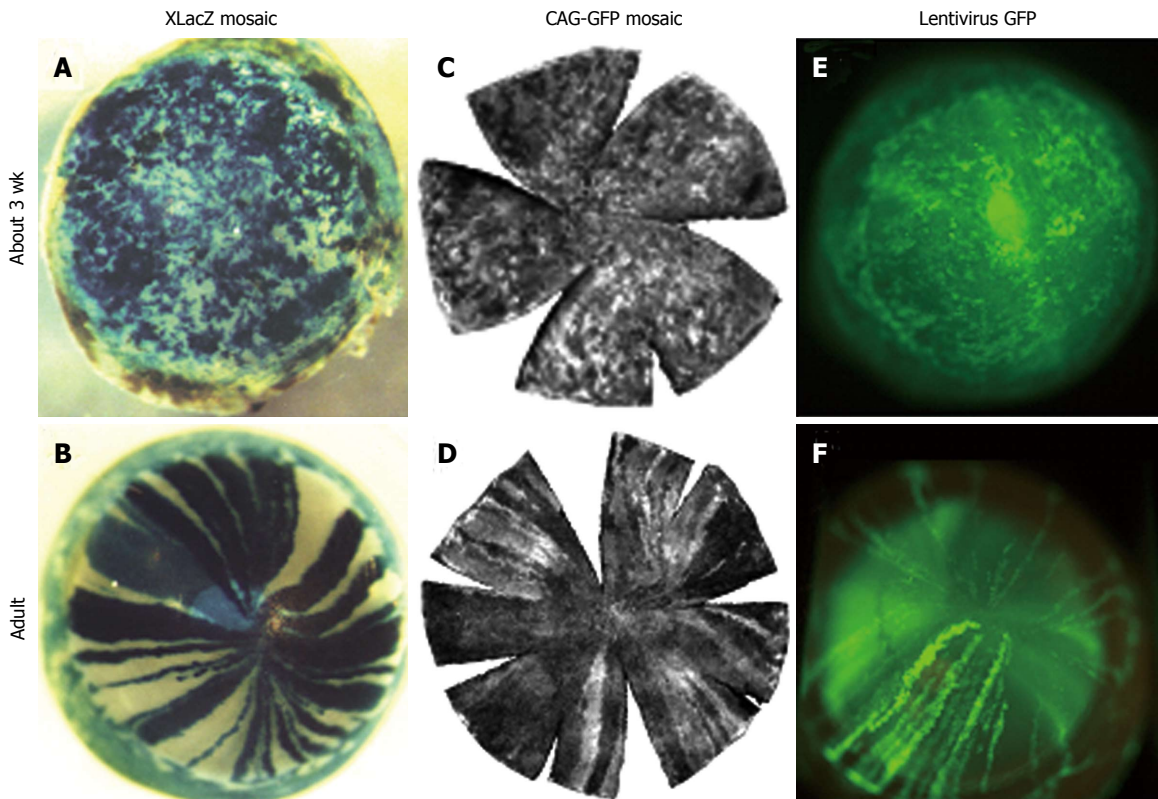


Figure 4 Transition from randomly orientated patches to radial stripes in corneal epithelia of different types of mosaic mice between 3 wk and adulthood. A and B: β -gal staining in XLacZ X-inactivation mosaics^[27]; C and D: Green fluorescent protein (GFP) fluorescence in CAG-GFP transgenic mosaics^[77]; E and F: GFP fluorescence in corneal epithelium after transfecting conceptuses with lentiviral vectors encoding GFP at embryonic day 9 or 10^[79]. Photographs (A and D) are reproduced from *Developmental Dynamics*^[27] with kind permission of John Wiley and Sons, (C and D) are reproduced from *Molecular Vision*^[77] with kind permission of the authors and editors, and photographs (E and F) are reproduced from *Molecular Therapy*^[79] with kind permission of the authors and the Nature Publishing Group. This combination of photographs was first published by Mort *et al.*^[6].

in the limbus. However, analysis of striped patterns in *KRT5-LacZ*^{+/-} corneas is not unequivocal and similar analyses with inducible lineage markers are required, as discussed below.

Transplantation experiments

Bradshaw *et al.*^[76] labelled rabbit limbal tissue *ex vivo* and transplanted it back to the limbus of the donor rabbits after first debriding the corneal epithelium across the full diameter. The labelled cells quickly colonised the corneal epithelium but, as the corneal epithelium was completely removed, this is equivalent to wound healing rather than normal corneal homeostasis. Majo *et al.*^[1] transplanted either β -gal-positive limbal or central corneal tissue from transgenic mice into the limbus of β -gal-negative, immunocompromised mice and both sources of tissue produced similar results. Consistent with the earlier experiment with rabbits^[76], labelled clones of donor cells moved centripetally into the corneal epithelium if the host corneal epithelium was removed but it failed to contribute to the corneal epithelium if the host cornea was left intact. Thus, although the transplanted limbal tissue contributed to corneal repair, it did not contribute to steady state corneal maintenance during normal tissue homeostasis, as predicted by the LESC

hypothesis. This was a key result, which prompted Majo *et al.*^[1] to propose the CESC hypothesis.

Circumstantial evidence

In addition to the specific investigations discussed so far, there are two circumstantial observations that favour the limbus as a site for stem cells. First, tumours of the ocular surface commonly involve the limbus^[80] and for other systems it has been suggested that tumour cells may preferentially arise from stem cells^[81,82]. This provides only weak, circumstantial evidence in favour of the LESC hypothesis.

Second, it is generally agreed that stem cells need a specialised niche environment to maintain the stem cell phenotype and this is likely to involve interactions with several cell types^[6,83]. For example, signalling from the microvasculature plays an important role in the mouse neural stem cell niche^[84]. Undeniably, the limbus provides a more diverse population of cell types than cornea and this is enriched further by its blood supply and for this reason it seems arguably a more likely location for a stem cell niche than the cornea. As already mentioned, an additional issue is that a stem cell niche in the basal corneal epithelium might be unstable because of the continuous centripetal movement of TACs. These considerations also make

Table 2 Examples of marker gene expression differences between the basal limbal and corneal epithelia during normal homeostasis identified by immunostaining

Positive markers			Negative markers		
Marker	Species	Ref.	Marker	Species	Ref.
Integrin $\alpha 9$	Mouse, human	[3,105,106]	Keratin 3	Rabbit	[4]
$\Delta Np63^1$	Human	[3,107,108]	Keratin 3/Keratin 12	Human	[3,108]
$\Delta Np63\alpha$	Human	[96]	NGF receptor (p75NTR)	Human	[3]
ABCG2	Human, rat, rabbit	[3,57,91-93,108]	Involucrin	Human	[3]
Vimentin	Human	[108]	Connexin 43	Human	[3,108]
Keratin 19	Human, mouse	[108,109]	E-cadherin	Human	[3]
Keratin 15	Human, mouse	[109]	Nestin	Human	[108]
N-cadherin	Human	[110]			
Bmi1	Human	[111]			
C/EBP δ	Human	[111]			
ABC5	Human, mouse	[54]			

¹ $\Delta Np63$ is not expressed in the human basal corneal epithelium^[107] but it is expressed in mouse and rat corneal epithelia^[27,112,113]. Positive markers are expressed in basal limbal but not basal corneal epithelium. Negative markers are expressed in basal corneal but not basal limbal epithelium.

it more likely that stem cell niches would be located preferentially in the limbus rather than the cornea.

Although some tissues are maintained during normal homeostasis by stem cells in the main body of the tissue, the limbus is not the only putative stem cell niche with a more peripheral location. For example, there are two types of stem cells that maintain the epithelium that lines the intestinal crypts and villi: crypt base columnar cells and position +4 reserve stem cells. These are both located near the base of the intestinal crypts, from where they produce TACs, which move up the crypt and generate the different functional cell types of the villus epithelium^[85]. Maintenance of the corneal epithelium by stem cells located in the limbal epithelium, as proposed by the LESC hypothesis, is essentially analogous to the way the intestinal epithelium is maintained. The circumstantial evidence that the limbus is a likely location for a stem cell niche supports the LESC hypothesis. However, it does not provide strong evidence against the CESC hypothesis, which accepts that LSCs exist, or the GBL hypothesis, which predicts there are no stem cells and so no niches.

Stem cell markers and phenotype

The Holy Grail of stem cell research is to find a phenotype or cell marker that allows the stem cells to be unequivocally distinguished from all neighbouring cells, including early generation TACs, and isolated for further study. This has not yet proved possible for the putative stem cell population(s) that maintain the corneal epithelium. Early evidence that the basal limbal epithelium contained stem cells was produced by an immunohistochemical study of keratin 3 (K3), which is considered to be a corneal differentiation marker^[4]. K3 is expressed in the basal and suprabasal layers of the rabbit corneal epithelium but only the suprabasal layers of the limbal epithelium, leading to the conclusion the basal limbal epithelium was less differentiated than the other epithelial layers. The

mouse has no K3^[86] but K12, which normally pairs with K3, is present and expression is restricted to the cornea^[87], as shown in Figure 3B. Several authors have also noted that cell morphology of cells in the basal limbal epithelium was more characteristic of stem cells (smaller, euchromatin-rich, high nucleus to cytoplasm ratio) than the corneal epithelium^[3,88] but, as already noted, Langerhans cells in the limbus also share this phenotype^[56]. These observations are consistent with the hypothesis that the limbus contains stem cells but no more than that.

The discovery of the K3 difference between basal limbal and corneal epithelia, as a whole, was followed by a quest for a specific cell marker to identify the LSCs within the limbal epithelium. Many candidate markers have been proposed based on differential expression studies (reviewed in reference^[18]) or conventional immunostaining (Table 2) but no definitive marker has been found, that is known to be expressed in putative stem cells in the limbus but not in neighbouring early generation TACs.

Some of the markers expressed in the limbal but not the corneal epithelium have been identified as putative stem cell markers in other tissues. ATP-binding cassette transporters (ABC transporters) are a family of transmembrane proteins whose functions include the transport of (potentially harmful) metabolic products out of the cells^[89]. Conceptually, they may form a component of the molecular mechanisms by which long-lived stem cells reduce the potential for genomic damage over their extended lives, and their expression has been correlated with stem cell activity^[90]. ABCG2 expression in the limbus is one such example and cells expressing this marker can be isolated as a "side population" by fluorescence-activated cell sorting (FACS)^[3,57,91-95]. However, as noted above, some of the ABCG2-positive, label-retaining cells with a high nucleus to cytoplasm ratio cells in the rat limbus have been identified as Langerhans cells rather than epithelial stem cells^[56].

It has recently been shown that ABCB5 appears to be a promising new marker for LESC and early TACs in both mice and humans, which should also allow enrichment by FACS sorting^[54].

Despite the absence of a marker that is only expressed in the stem cells, Δ Np63 α has proved useful for identifying cultures of human limbal cells with sufficient LESC and early TACs for clinical transplantation^[37,96].

RESOLVING THE LESC VS CESC DEBATE

Some of the evidence discussed so far (summarised in rows 1-15 of Table 1) is inconclusive. Evidence from holoclone experiments with pig and human tissues is inconsistent and, in any case, the critical thing is to understand how the corneal epithelium is maintained *in vivo* during normal homeostasis. Various studies have shown that some cells in the central corneal epithelium are capable of acting as long-term progenitor cells. On the face of it, this favours the CESC and GBL hypotheses. However, these observations are also consistent with the LESC hypothesis if some basal corneal epithelial TACs have a latent proliferative potential that is only used if LESC function is compromised so homeostasis is disrupted. Drawing attention to this latent proliferative potential is an important outcome of Majo *et al*'s^[1] investigations even if the CESC hypothesis ultimately proves to be incorrect.

Other evidence provides better discrimination. There are several strong arguments against the GBL hypothesis. It is inconsistent with the consensus of results from label-retaining cell experiments and the evidence that mosaic patterns in the mouse cornea change after birth, when clones of cells emerge from the peripheral cornea and form radial stripes. The GBL hypothesis also has difficulty accounting for the convincing evidence that corneal epithelial cells move centripetally across the full radius. In our view, this evidence (summarised in Table 1) is sufficient to exclude the GBL hypothesis and it is, therefore, not considered further.

These same observations also argue against the CESC hypothesis. However, results of the label-retaining cell experiments could be accommodated by the CESC hypothesis if CESC were not slow cycling. Nevertheless, the CESC hypothesis is inconsistent with the developmental switch from randomly orientated patches to stripes that emerge from the periphery in various types of genetic mosaics in mice and then extend across the full radius to the centre. By the same token, the CESC hypothesis requires *ad hoc* assumptions to account for centripetal movement, if the same clone of cells moves across the full radius.

Conversely, the LESC hypothesis is inconsistent with the observation that when genetically marked limbal tissue was surgically transplanted to the limbus of immunocompromised mice, donor cells failed to

move into the cornea unless the corneal epithelium was removed^[1]. Thus, the LESC and CESC hypotheses each have difficulty accounting for one type of evidence. In each case, critical evidence is based on experiments with mice so there are no grounds for suggesting that maintenance of the corneal epithelium differs between mice and humans or other species.

Clinically, it may not matter whether the LESC or CESC hypothesis is correct as both agree that the limbus is a suitable source of stem cells for therapeutic use. However, we need to know where the stem cells that maintain the corneal epithelium during normal homeostasis are located in order to understand the biology of this process. The key issue that needs to be resolved is why evidence from mouse mosaics and transfection with lentiviral markers conflict with results of surgical transplantation experiments. Analysis of mosaics show that, during normal homeostasis, clones of cells appear at the corneal periphery at around 5 wk after birth and extend centripetally across the corneal radius, consistent with activation of LESC. In contrast, surgical studies in mice show that transplantation of labelled cells to the limbus fail to colonise the cornea in a similar way.

One possible explanation is that the transplanted limbal tissue failed to colonise the corneal epithelium because the surgical manipulation or other aspects of the experimental procedure somehow perturbed normal homeostasis and affected the outcome. In principle, one way of testing this would be to label individual cells in the limbus of adult mice, using a genetic switch rather than surgical transplantation and test whether any of the labelled cells produce long-lived clones of cells that colonise the corneal epithelium. Similar genetic labelling of cells in the adult basal corneal epithelium would also allow investigation of whether these produce long-lived clones of cells in the corneal epithelium. This requires lineage tracing experiments and some possibilities are discussed below.

It should be borne in mind that some of the apparent contradictions between the different hypotheses may be more imagined than real, and arise as a result of different research groups attempting to subdivide and label what is, perhaps, a continuum of biological situations. It seems certain that limbal stem cells exist, that they can contribute to regeneration of the cornea, and yet that the basal corneal epithelial cells themselves also have massive regenerative potential. There is no reason why the balance between limbal-mediated and corneal-mediated corneal regeneration should not shift over the lifetime of the animal, with age, disease and injury, and no reason why the balance should necessarily be the same in different species.

Although we talk about wounded and unwounded corneas as if they are separate entities, in fact the corneal epithelium is, of necessity constantly regenerating, because of normal desquamation of cells. Desquamation rate is modulated by rate of blinking,

tear film composition, irritants and abrasive dust in the environment, chronic abrasion caused by *e.g.*, contact lens wear and diseases such as trachoma, acute minor scratches, ranging through to significant physical or chemical injuries and acute infections such as Herpes simplex keratitis. This continuum of insults to the corneal surface may require different levels of limbal response to support the regenerative potential of the corneal epithelial cells. It may be that the genuinely uninjured cornea does not require limbal input, but the genuinely uninjured corneal epithelium does not exist. Experimentally, factors such as the abrasive nature of bedding and dust from food that laboratory mice are exposed to, may modulate corneal regeneration and be a source of variation between research institutes. Furthermore, the large circular central corneal wounds that are so widely used as models of induced regeneration do not really recapitulate any of the most common injuries that happen in life.

There may also be problems with the practical definition of stem cells, particularly with regard to their property of "immortality". We accept that TACs do not renew indefinitely and therefore have a finite lifespan, whereas stem cells do not have this constraint. However, outside the laboratory, a stem cell cannot outlive the individual. We do not know the maximum lifespan of a TAC but this is likely to vary stochastically. If the longer-lived TACs survive for 6 mo or more, this will encompass the effective lifespan of most laboratory mice used experimentally. At that stage, the difference between a long-lived TAC and a stem cell becomes blurred. On the other hand, in larger animals such as humans, a lifespan of 6 mo or a year for a TAC is utterly insignificant in terms of the lifespan of the individual. Differences such as this may start to explain why stem-like regenerative ability may be assigned to the corneal epithelium on experimental small animals, while at the same time the data do not reflect clinical experience in humans.

Lineage tracing experiments

To test whether any cells in the limbal and/or corneal epithelia can generate long-lived clones of cells that colonise the corneal epithelium, an inducible lineage tracing method is required that can label some of the putative stem cells in the adult at a chosen time without surgical intervention or disturbing homeostasis. Sophisticated, inducible lineage tracing methods using *Cre/loxP* transgenic mice are now available that can be used to throw a genetic switch to label a chosen cell population with a fluorescent or histochemical marker that will identify them and all their mitotic progeny. Such methods have been used to trace stem cell lineages in other tissues, including the hair follicle^[97], intestinal epithelium^[98-100] and ovarian surface epithelium^[101], and this approach could be used to help resolve the LESC vs CESC debate. A similar approach has already been used, in conjunction with a multi-coloured reporter construct, to trace clonal lineages in

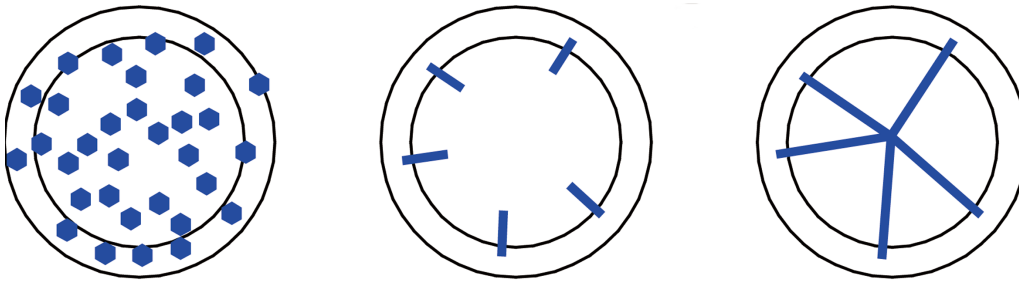
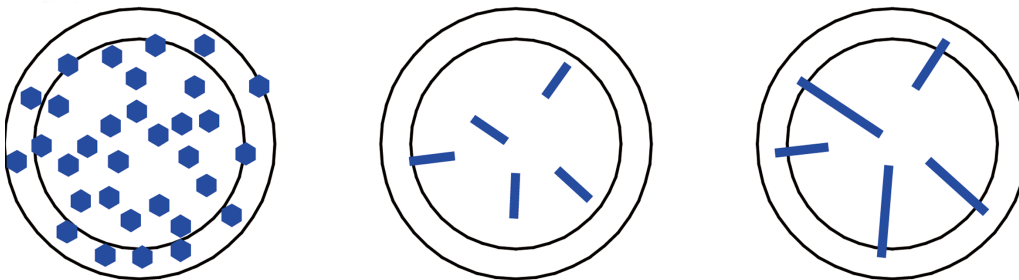
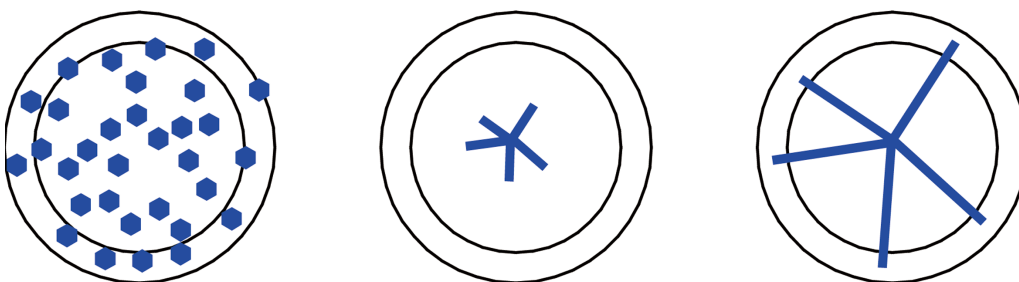
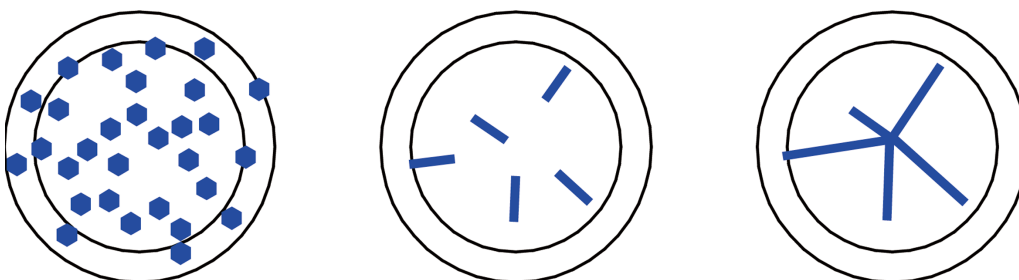
the ocular surface of zebrafish and demonstrate that, as in mice, the initial patchwork of cells established in the embryo is replaced by a radial pattern of clones that extend from the limbus^[102].

We have begun to explore this *Cre/loxP* lineage tracing approach using transgenic mice in which a reporter transgene is ubiquitously expressed once the flanking *loxP* sites are recombined by active *Cre* recombinase to remove an upstream stop sequence. *Cre* recombinase is provided in the form of a *CreER* fusion protein, which is produced by another transgene under the control of a ubiquitous promoter. The *CreER* fusion protein is normally sequestered in the cytoplasm unless the mouse is treated with tamoxifen. This binds to the modified oestrogen receptor (ER) and translocates *CreER* to the nucleus, where it can recombine the *loxP* sites, so removing the stop sequence and activating the reporter transgene. Use of a ubiquitous promoter to drive expression of *CreER* provides an unbiased approach, which allows putative stem cells in the limbus or cornea (and any other tissue) to be labelled. However it is not specific for stem cells so initially most of the labelled cells will not be stem cells but, by including a chase period, short-lived clones founded by labelled TACs will be shed, leaving long-lived clones founded by labelled stem cells.

The genetic switch is activated when the mouse is injected with tamoxifen. Delaying tamoxifen treatment, until well after the adult stem cells are activated, should ensure that the genetic switch is thrown to label individual adult stem cells, during normal homeostasis. This avoids labelling ancestral cells, which could each generate multiple labelled stem cells. Furthermore, by titrating the dose of tamoxifen it should be possible to label a relatively small proportion of cells so only a few stem cells will be labelled per eye. Together, this will ensure that most of the clones of labelled cells that remain as stripes or patches after the chase period are individual clones produced by single stem cells. The predicted results for such an experiment are shown, for the LESC hypothesis and three versions of the CESC hypothesis, in Figure 5.

Tamoxifen-inducible labelling of a low proportion of stem cells in adults is a significant advantage over analysis of other types of mosaics, where the cells are labelled early in development and many patches and stripes of labelled TACs are likely to be derived from multiple adjacent labelled stem cells. This is because labelling a cell in the embryo will produce a large clone of labelled cells, some of which will remain close together throughout development. Later, when adult stem cells are specified, some adjacent stem cells will probably be clonally related making it difficult to identify TACs descended from a single stem cell in these mosaic systems.

Examples of results of a preliminary experiment of this type are shown in Figure 6 and indicate that the strategy suggested in Figure 5 is feasible. The cornea

A LESC hypothesis (centripetal movement)**B** Original CESC hypothesis (centrifugal movement)**C** Central CESC and centrifugal movement**D** CESC throughout cornea and centripetal movement

Time 1

Time 2

Time 3

Figure 5 Hypothetical results from a lineage tracing experiment to distinguish between the limbal epithelial stem cell and corneal epithelial stem cell hypotheses. In each figure the inner disc represents the corneal epithelium and the outer ring represents the limbal epithelium. If a reporter transgene is driven by a tamoxifen-inducible, ubiquitous promoter, a proportion of all the cell types in the ocular surface (and other tissues) will be labelled shortly after tamoxifen treatment. The frequency of labelled cells will depend partly on the dose of tamoxifen, which could be titrated to ensure only a few stem cells are labelled per eye. Time 1 is shortly after tamoxifen-treatment and the labelled cells may have divided to produce a small clone of labelled cells. By Time 2, the short-lived labelled clones produced by labelling transient (or transit) amplifying cells should have been shed from the corneal epithelium but long-lived labelled clones produced by long-lived labelled stem cells will remain. Expectations for distributions of labelled cells at Times 2 and 3 vary for the different hypotheses. A: The limbal epithelial stem cell hypothesis predicts clones of labelled cells will extend radially from the limbus and, by Time 3, clones of labelled cells will span the full radius; B: The original corneal epithelial stem cell (CESC) hypothesis predicts clones of labelled cells produced by labelled stem cells may also extend radially but will extend centrifugally from stem cells located throughout the corneal epithelium itself. Clones of labelled cells that do not arise from the centre of the cornea will not span the full radius; C: If the CESC hypothesis is modified so that all the CESC are at the very centre of the cornea, centrifugal movement will produce clones of labelled cells that span the full radius by Time 3 but at Time 2 there should be no labelled peripheral cells produced by stem cells; D: If the CESC hypothesis is modified so that the CESC are located throughout the corneal epithelium but movement is centripetal, clones of labelled cells that do not arise from the periphery of the cornea will not span the full radius. To distinguish between the various hypotheses it will be necessary to compare patterns of labelled clones at different times after tamoxifen treatment.

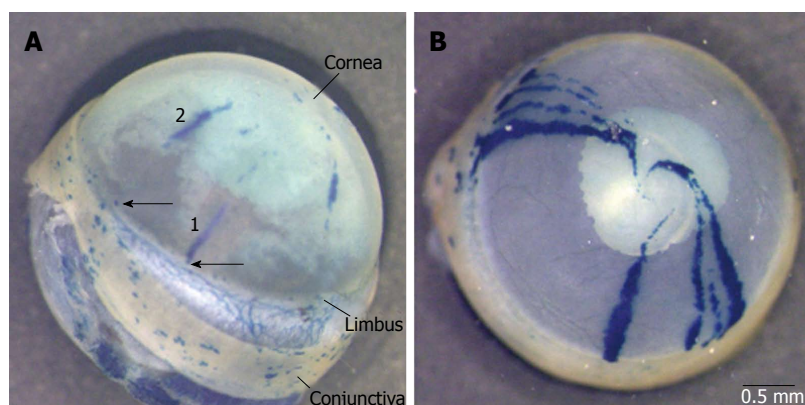


Figure 6 Preliminary results from a lineage tracing experiment to distinguish between the limbal epithelial stem cell and corneal epithelial stem cell hypotheses. Eyes from CAGG-CreER; R26R-LacZ mice that were injected with tamoxifen to induce LacZ reporter gene expression and stained for β -galactosidase (β -gal) activity after different chase periods. The pigmented iris is visible through the cornea and appears grey, whereas the β -gal staining is blue. A: Side view of a β -gal-stained eye, after a chase period of 9 wk and 4 d, with several radial β -gal-positive stripes and small patches in the cornea and numerous β -gal-positive patches in the conjunctiva. (The conjunctiva is torn near the limbus and hangs down at the bottom right of the photograph, so the sclera is visible between the limbus and conjunctiva.) Stripe 1 is a limbus-cornea (LC) stripe, with its more peripheral end in the limbus (arrow), consistent with expectations of the limbal epithelial stem cell (LESC) hypothesis (see Figure 5). It also appears to be aligned with other β -gal-positive patches towards the centre of the cornea so it may be part of a longer discontinuous stripe. Stripe 2 is a cornea-cornea stripe with both ends in the cornea, consistent with the corneal epithelial stem cell hypothesis. However, it is radially aligned with a small β -gal-positive patch in the limbus (arrow), which could be the location of a β -gal-positive LESC. If so, stripe 2 might be a discontinuous stripe, which extended from a LESC that was not continuously active (consistent with the LESC hypothesis); B: Anterior (frontal) view of a β -gal-stained eye, after a 14-wk chase period, with eight radial β -gal-positive stripes. All eight stripes are LC stripes with one end at the limbus and many extend the full radius and have a curved end, consistent with a central spiral pattern, as reported for other chimaeric and mosaic eyes^[27,72,73]. CAGG-CreER; R26R-LacZ mice were produced by crossing CAGG-CreER and R26R-LacZ mice [full names Tg(CAG-cre/Esr1*)5Amc and B6.129S4-Gt(ROSA)26Sortm1Sor/J; references^[116,117]]. LacZ reporter gene expression was induced at 12 wk by 3 injections of tamoxifen (100 μ g/g body weight per injection).

illustrated in Figure 6A was stained for β -gal activity after chase period of 9^{1/2} weeks. Stripe 1 has its peripheral end in the limbus and its more central end in the cornea so, using the stripe classification system described for mosaic *KRT5^{LacZ/-}* transgenic mice^[17], it is classified as a limbus-cornea (LC) stripe. This is consistent with expectations of the LESC hypothesis (Time 2 in Figure 5A), particularly as the stripe does seem to extend into the limbus itself (arrow in Figure 6A). A peripheral stripe could also be consistent with the original CESC hypothesis (Figure 5B) or the centripetal version shown in Figure 5D, although, in the latter case, peripheral stripes would be expected to be entirely within the cornea (CC stripes). This LC stripe fits less well with the hypothesis that all the CESC are located in the very centre of the cornea (Figure 5C) unless additional assumptions are made (e.g., stripes have already extended to the periphery by 9^{1/2} weeks).

Stripe 1 also appears to be radially aligned with other β -gal-stained tissue located more centrally so it may be a longer discontinuous stripe. Discontinuous stripes have been discussed elsewhere for *KRT5^{LacZ/-}* transgenic mosaics^[17] and also occur in mouse chimaeras and X-inactivation mosaics^[27,72,103]. The discontinuities in β -gal staining shown in Figure 6A could reflect: (1) separate clones of cells derived from β -gal-positive and β -gal-negative CESC or TACs that are radially aligned; (2) dispersal of a β -gal-positive clones by incursions from laterally adjacent β -gal-negative clones; or (3) alternating contributions of more than one stem cell to a single radial stripe if individual stem cells cycle through phases of activity

and quiescence.

Stripe 2 in Figure 6A is a cornea-cornea (CC) stripe with both ends in the cornea, consistent with the predictions of the original CESC hypothesis at Time 2 (Figure 5B) or the centripetal version shown in Figure 5D. However, it might also be consistent with the LESC hypothesis (Figure 5A) because this stripe is radially aligned with a small β -gal-stained patch in the limbus, which could mark the location of a β -gal-positive LESC. If so, stripe 2 might be a discontinuous stripe formed in two phases. During the first phase a β -gal-positive region might have extended from a β -gal-positive LESC, which was active during the early part of the chase period. This could have been followed by extension of a β -gal-negative region generated by an adjacent active β -gal-negative LESC, if the β -gal-positive LESC became inactive during the later part of the chase period.

The eye shown in Figure 6B was stained after a 14-wk chase period and has eight β -gal-positive stripes, all of which are LC stripes, consistent with the LESC hypothesis (Time 3 in Figure 5A) and both the original CESC hypothesis (Figure 5B) and the variant shown in Figure 5C. However, the variant CESC hypothesis shown in Figure 5D predicts that only a small proportion of stripes would extend right to the limbus. Some of these stripes span the full radius, consistent with the LESC hypothesis (Figure 5A) and the variant CESC hypothesis shown in Figure 5C. It could also be consistent with the original CESC hypothesis (Figure 5B) and the centripetal version, shown in Figure 5D, if full-radius stripes comprised several shorter stripes produced by β -gal-positive

CESCs aligned on the same radius. However, most of the cornea is β -gal-negative so it seems unlikely that the *LacZ* reporter would be activated in radially aligned CESC clones but not in many of the other CESC clones, located elsewhere in the corneal epithelium.

The preliminary results shown in Figure 6 and the alternative explanations of discontinuous stripes indicate that the interpretation of the stripe patterns may be more complicated than predicted in Figure 5, so detailed analysis of many more eyes and different chase periods will be required to resolve the LESC vs CESC debate. Nevertheless, this lineage-tracing approach appears to be a promising way of resolving the conflicting evidence from transplantation experiments and mosaics. Although important, the evidence from conventional mosaics is limited because the time of stem cell labelling cannot be controlled and occurs early in development. Even lineage tracing with a GFP-tagged lentiviral marker (Figure 4E and F) relied on marking one cell population before birth^[79]. In contrast, a tamoxifen-inducible transgenic reporter system enables cells to be labelled at a specific time in the adult without the risk of disturbing homeostasis with surgical intervention.

While this review was in preparation, a similar lineage tracing study was published online, in a preliminary form, and this has already been mentioned in the section on cell movement^[64]. This used K14-CreER^{T2}; R26R-confetti mice, where the keratin 14 (K14 or *Krt14*) promoter, rather than a ubiquitous promoter, was used to drive tamoxifen-inducible CreER^{T2}. The multi-colour "R26R-confetti" fluorescent reporter^[100] was used to identify labelled cells. This reporter is based on an earlier "rainbow" construct^[104] and is also similar to that used for the zebrafish cornea^[102]. According to the authors, immunofluorescence showed that K14 protein was present in basal epithelial cells in the mouse limbus but the central corneal epithelium had much lower levels. Thus, limbal epithelial cells will be preferentially targeted for labelling, making it difficult to test whether CESC clones exist. On the other hand, a chase period to remove short-lived clones formed by TACs rather than stem cells is less important than for the experimental design shown in Figure 5, where CreER is expressed ubiquitously.

Mice were treated with tamoxifen at 6 wk and corneas of the same mice were imaged repeatedly to allow changes in size and position of different clones to be tracked in real time. This elegant time-lapse study showed that labelled cells began to emerge from the limbus 5 wk after tamoxifen treatment and the same stripes were tracked at 9, 13, 17 and 21 wk, as they extended into the centre of the unlabelled cornea without overlapping. The frequency of labelled clones was higher than for the preliminary results illustrated for tamoxifen-treated CAGG-CreER; R26R-*LacZ* mice, shown in Figure 6. However, because the multi-coloured R26R-confetti reporter randomly labelled cells with one of up to ten different colours, individual

adjacent clones could be distinguished easily. In addition to the radial stripes, the authors reported the presence of some rare, small patches of labelled cells in the cornea, which they suggested might have arisen in the cornea itself.

This is an important study as it is the first lineage tracing investigation of the mouse cornea with an inducible marker that was activated in adults. In particular, the informative, real-time study with live mice proved that individual cells labelled in the adult limbal epithelium could form long-lived clones that extend centripetally across the complete radius of the cornea during normal homeostasis. This effectively provides a non-surgical equivalent of the limbal transplantation experiment described by Majo *et al.*^[11] and supports the interpretation that the outcome of the transplantation was adversely affected by the surgical procedures and homeostasis was perturbed. If so, this would undermine the evidence for the CESC hypothesis and reconcile the conflicting evidence from surgical transplantation experiments and conventional mosaics. In effect, all the available evidence would then be consistent with the LESC hypothesis. To further clarify the situation, additional investigations, driving CreER from a promoter expressed in the corneal epithelium, are now required to investigate whether long-lived clones can arise in the adult cornea as well as the limbus.

CONCLUSION

There is strong evidence that the corneal epithelium is maintained by stem cells rather than solely by proliferation of more differentiated cells in the basal corneal epithelium. Most evidence also favours the conventional LESC hypothesis, which proposes that limbal epithelial stem cells maintain the corneal epithelium during normal homeostasis. Although limbal transplantation experiments favour the alternative CESC hypothesis, this result could be reconciled with the LESC hypothesis if surgical transplantation perturbed normal homeostasis and affected the outcome. This possibility is supported by a recent non-surgical, lineage-tracing experiment, which demonstrated that clonal derivatives of cells in the limbal epithelium move into the corneal epithelium during normal homeostasis. Thus, the available evidence supports the conclusion that, during normal homeostasis, the corneal epithelium is maintained by stem cells in the limbus, which produce daughter TACs that migrate centripetally, rather than any stem cells in the corneal epithelium itself. However, if homeostasis is compromised, so the limbal epithelial stem cells are unable to maintain the corneal epithelium, it seems likely that TACs in the basal corneal epithelium can act as long-term progenitors and maintain the tissue for a considerable time in the absence of functional LESC clones.

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