Biol. Cell (2005) **97**, 265–276 (Printed in Great Britain)

Review

In search of markers for the stem cells of the corneal epithelium

Ahdeah Pajoohesh-Ganji*† and Mary Ann Stepp*‡[1]

*Department of Anatomy and Cell Biology, The George Washington University Medical Center, Washington, DC, U.S.A., †Department of Biological Sciences, The George Washington University, Washington, DC, U.S.A., and ‡Department of Ophthalmology, The George Washington University Medical Center, Washington, DC, U.S.A.

The anterior one-fifth of the human eye is called the cornea. It consists of several specialized cell types that work together to give the cornea its unique optical properties. As a result of its smooth surface and clarity, light entering the cornea focuses on the neural retina allowing images to come into focus in the optical centres of the brain. When the cornea is not smooth or clear, vision is impaired. The surface of the cornea consists of a stratified squamous epithelium that must be continuously renewed. The cells that make up this outer covering come from an adult stem cell population located at the corneal periphery at a site called the corneal limbus. While engaging in the search for surface markers for corneal epithelial stem cells, vision scientists have obtained a better understanding of the healthy ocular surface. In this review, we summarize the current state of knowledge of the ocular surface and its adult stem cells, and analyse data as they now exist regarding putative corneal epithelial stem cell markers.

The ocular surface

The surface of the eye consists of the cornea, the conjunctiva, and the border between the two, which is known as the corneoscleral junction, or limbus (Figure 1). A healthy cornea is required as a refractive surface to focus light on the retina, and as a protective barrier for the inner neuronal tissues. The cornea consists of epithelial, stromal and endothelial layers, with each layer separated by a specialized basement membrane (Figure 2). The corneal epithelium includes, from its superficial aspect, approximately one to three layers of flattened cells called squames, two to three layers of suprabasal or wing cells, and a single layer of columnar basal cells. The superficial squames of the corneal epithelium have a unique surface with extensive microvilli, called microplicae, that serve to increase the cells surface area and to facilitate the close association of the squames with the tear film. The presence of lateral tight junctions between squames prevents the entry of harmful substances into the intraocular tissues. The wing cells are located beneath the apical squames and have lateral, wing-like extensions. These cells are not directly involved in the spreading of the tears and do not undergo frequent cell division; however, recent studies have shown that wing cells do participate in re-epithelialization during wound healing and sometimes can be found at the leading edge in contact with the basement membrane (Danjo and Gipson, 2002).

The innermost cells in the corneal epithelium are called basal cells. These cells are a single layer of columnar cells with several important functions in the cornea (Farjo and Soong, 2004). They proliferate to generate new wing and squames, secrete numerous matrix molecules that are incorporated into the underlying basement membrane and stroma, organize the hemidesmosomes that maintain stable attachment to the underlying basement membrane and, finally, organize the more transient cell–matrix attachments called focal complexes that are important in mediating cell migration in response to an injury.

[1]To whom correspondence should be addressed (email mastepp@gwu.edu).
Key words: corneal epithelial stem cell (CESC), integrins, limbal stem cell deficiency, limbus, surface markers.
Abbreviations used: ABC, ATP-binding cassette; CESC, corneal epithelial stem cell; Cx, connexin; ECM, extracellular matrix; EGF, epidermal growth factor; eTAC, early transient amplifying cell; GFP, green fluorescent protein; HSC, haematopoietic stem cell; K, keratin; LSCD, limbal stem cell deficiency; TAC, transient amplifying cell; TGF-β, transforming growth factor β.

Microplicae: Characteristic ridge-like folds of plasma membrane present at the free surfaces of stratified squamous epithelial cells lining the alimentary tract, cornea, and conjunctiva, exhibited by scanning electron microscopy.

Figure 1 | The mouse ocular surface

The black arrowheads indicate the location of the limbus. The transition zone between the thin epidermis of the outer surface of the eyelid and the conjunctival epithelium on the eyelid inner surface, shown by the arrows, is called the mucocutaneous junction. Not shown is the fornix, which is the site where the conjunctival epithelium leaves the eyelid to begin covering the globe of the eye. The fornix has been proposed as the site where the conjunctival stem cells are concentrated (Wei et al., 1995).

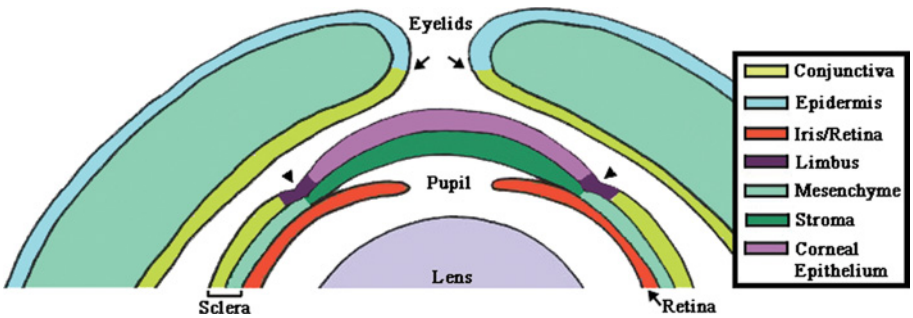
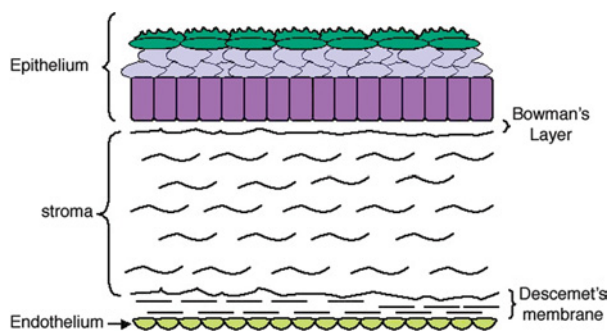


Figure 2 | Different layers of the cornea

The corneal epithelium consists of approximately five to seven cell layers. The superficial layer of this epithelium includes a layer or two of squames cells with extensive apical microvilli (green). Underneath the superficial layer are the suprabasal or wing cells (blue) and the final layer is the basal cells (purple). Beneath the corneal epithelium lies the basement membrane and Bowman's layer followed by the stroma. Descemet's membrane is directly below the stroma, which in turn overlies the corneal endothelium.



While the basal cells generate all of the cells that make up the other layers of the corneal epithelium, they themselves are derived from cells that are located at the limbus, a region between the cornea and conjunctiva (Figure 1).

In primates and birds, the epithelial layer and the stroma are separated by Bowman's layer. Bowman's layer, which has unknown functions, is present from limbus to limbus, and consists of both the underlying basement membrane zone and the acellular zone immediately beneath it. Beneath the epithelial basement membrane is the stroma. The cells that reside in the stroma are called corneal fibroblasts and are specialized in producing and organizing the stromal extracellular matrix (ECM). In humans, the stroma makes up over 90% of the bulk of the entire cornea. This dense connective tissue serves primarily as a structural support, but, unlike cartilage and tendon, it is transparent. By mechanisms still not fully understood, stromal fibroblasts regulate the synthesis, organization and spacing of the various types of collagen fibres and proteoglycans that make up the stromal matrix, and function to maintain stromal transparency. In addition, data also show that the stromal fibroblasts produce corneal crystallins that, similarly to the crystallins in the lens, participate in maintaining the clarity of the fibroblast cytoplasm (Jester et al., 1999; Piatigorsky, 2000). Without a transparent cornea, light would not come into focus on the retina and vision would be impaired.

Beneath the stroma is another basement membrane known as Descemet's membrane, which overlies the corneal endothelium, a single layer of flattened cells connected by tight junctions. The endothelial cells pump nutrients from the aqueous humour into the stroma and pump excess water out of the cornea. The aqueous humour is made primarily by the cells that make up the ciliary processes and is secreted into the anterior and posterior chambers of the eye.

Nutrients pumped into the stroma by endothelial cells passively diffuse throughout the cornea nourishing the stromal fibroblasts and the corneal epithelium. By functioning to regulate the hydration state of the corneal stroma, the endothelial cells play an important role in maintaining corneal transparency. Lack of this regulation may cause swelling, which in turn alters collagen fibril spacing and makes the stroma opaque (Gipson, 1994).

A healthy cornea requires nourishment as well as protection. The eyelids provide one form of protection, but another important protective element is the tear film, which contains water, mucins, lipids, and antimicrobial peptides and enzymes, including lysozyme. Mucins are highly glycosylated proteins secreted primarily by goblet cells present in the adjacent conjunctiva. The conjunctiva is a two to three layer epithelium that extends from the mucocutaneous junction, beginning at the eyelashes on to the inner surface of the eyelids, over the ocular surface on the eye proper and ending at the limbus (see Figure 1). The presence of goblet and epithelial cells in the conjunctiva makes this epithelial tissue distinct from the other nonkeratinizing squamous tissues. The lipid portion of the tears are made by the Meibomian glands located at the base of the hair follicles of the eyelashes with contributions from the glands of Zeis, also found in the eyelid. The aqueous component of the tears is made by the lacrimal glands located bilaterally in the superior temporal region of the orbit of the face. Deficiencies in the production of any one of the components of the tear film results in a condition known as 'dry eye'. Aging is the most common contributing factor for dry eye, and the condition is thought to be due to the slowing down of synthetic activity of the glands and cells that produce the tears. Some diseases, such as Sjogren's syndrome, which causes dryness in the mucous membranes of the body, also result in dry-eye-like symptoms. Since the air–water interface serves as the principle refractive surface of the eye, maintaining a stable uniform tear film is critical to good vision.

The tears are produced through the concerted action of several different cell types; similarly, the corneal epithelial stem cells (CESCs) are maintained by a variety of cells including cells from the conjunctiva, peripheral cornea and sclera. The CESCs are mixed in among the basal cells at the limbus, and they appear smaller and less columnar than those of the corneal epithelium and have a number of unique properties.

CESC properties

Cells derived from the limbus are accepted as the source for the cells that make up the corneal epithelium. Evidence for the limbal localization of the stem cells for the corneal epithelium is obtained from different studies. It has been shown that in injured human eyes during re-epithelialization, there is a circumferential migration and a centripetal movement of cells from the limbus towards the central cornea (Lemp and Mathers, 1989). It has also been shown that limbal basal cells lack differentiation markers, such as keratin-3 (K3), which can be recognized by the AE5 antibody; K3 is expressed on the more differentiated corneal epithelial cells and is uniformly present throughout the central epithelium, whereas it is absent from the limbal basal cells (Schermer et al., 1986; Espana et al., 2003). These data suggest that the limbal basal cells are less differentiated than the cells in the central cornea. The presence of K3-positive cells immediately above and adjacent to the limbal basal cells suggests that these cells give rise to the K3-positive cells in both the central epithelium and the limbus (Lavker et al., 2004).

Moreover, both tritiated thymidine and bromodeoxyuridine have been used in studies to identify stem-like cells that are slow cycling (Cotsarelis et al., 1989; Lavker and Sun, 2003; Lavker et al., 2004). In these experiments, the DNA of cells in S-phase is labelled over several days by repeated treatment of neonatal mice with the label. The treatment is then stopped and mice are allowed to grow and mature. The choice of neonates for this type of experiment is made based on the fact that the cornea is only a 2-cell layer thick at birth but within 7 days has stratified to 3–4 cell layers. This stratification, coupled with the rapid growth of the overall diameter of the eye over the same time period, indicates that the progenitor cells for the corneal epithelium are proliferating rapidly and are likely to acquire radiolabel. After treatment with label is stopped, the label begins to dissipate within cells going through multiple rounds of cell division. Cells that do not divide as frequently retain the label for long periods of time and are termed label-retaining or slow-cycling, one of the intrinsic properties of stem cells. Data from these kinds of experiments clarified the identity of the CESCs and

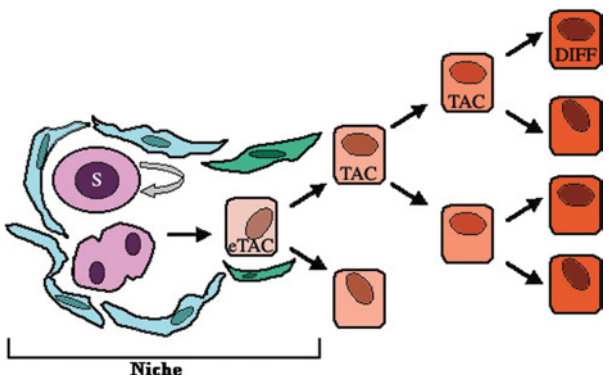
demonstrated that label-retaining cells were present at the limbal region in the basal cell population (Cotsarelis et al., 1989).

In humans, cells from the limbal region have recently been shown to have a greater proliferative potential in culture (Pellegrini et al., 1999), which is yet another characteristic of stem cells. Barrandon and Green (1987) classified epidermal keratinocytes in terms of colonies having three different sizes that were coupled with distinct differentiation characteristics. Holoclone colonies had the greatest proliferative potential, with less than 5% of the cells in each colony differentiating; paraclone colonies were the smallest and had the poorest proliferative potential and the highest percentage of differentiating cells; and meroclone colonies were intermediate in their properties. Using methods originally established for skin cells, Pellegrini et al. (1999) have shown that only the limbal basal cells can give rise to holoclone colonies whereas cells from the central cornea give rise exclusively to paraclone and meroclone colonies. These studies demonstrate that cells in the limbal region are more clonogenic and have a higher proliferative capacity. It has also been shown in rabbits that surgical removal of the limbus results in healing with non-corneal epithelium from the conjunctiva, and that in the absence of the limbus, the proliferative capacity of the corneal epithelium is limited (Huang and Tseng, 1991). The most compelling data on the limbal location for the CESCs come from the clinical studies. Patients with injuries to their limbus have been treated successfully for over 15 years with limbal autografts, which are transplants of limbal tissue taken from the patient's healthy fellow eye (Kenyon and Tseng, 1989). In addition, it has been shown that for some patients, treatment using cultured limbal cells can result in restoration of a clear cornea for up to 4 years (Pires et al., 2000; Stenevi et al., 2002).

The CESCs have a self-renewing capability: they are believed to undergo asymmetric cell division in the cornea to generate daughter cells with different proliferative potentials (Figure 3). In asymmetric cell division, one of the daughter cells stays in the stem cell niche to maintain the stem cell population, whereas the other daughter cell, called an early transient amplifying cell (eTAC), moves out of the stem cell niche. The eTAC is found mixed among the CESCs and is capable of dividing further to give rise to transient amplifying cells (TACs) and finally to the more differentiated K3-positive corneal epithelial basal cells. TACs migrate along the basement membrane of the cornea from the limbus making their way towards its centre. Along the way, some TACs leave the basement membrane, become wing cells, terminally differentiate into squames and are shed into the

Figure 3 | Asymmetric cell division of CESCs in the niche

CESCs are believed to undergo asymmetric cell division in the cornea, in which the stem cell (S) divides to generate two daughter cells with different properties. One of the cells, the cell destined to remain a stem cell, stays in the niche to maintain the stem cell population, whereas the other daughter cell, the eTAC, is 'pushed out' of the niche and has the capability of dividing further to give rise to TAC and eventually give rise to differentiated cells (DIFF). The bent arrow represents the self-renewing capability of the stem cells. The blue and green cells surrounding the stem cells represent supporting cells probably, but not necessarily, of mesenchymal origin, that together with the CESCs create the niche within which the CESCs maintain their stem-like phenotype.



Holoclone: A colony with large and smooth perimeter which is 10–30 mm^2 in area and contains (2–5) $\times$ 10^4 small cells with only 0–5% of colonies being terminal.

Paraclone: A colony with irregular perimeter which is 5 mm^2 or less in area. The cells of this colony are large and flattened and do not proliferate. All cells in this colony express involucrin, which is a marker for terminally differentiated cells.

Meroclone: A colony with wrinkled perimeter which grows progressively to macroscopic size but does not reach the same size as the typical colonies produced by holoclones (between holoclones and paraclones).

Clonogenic: The ability of a single cell to form a colony.

tear film (Figure 3). The length of time taken by a cell to go from the limbus to the central cornea and be shed from the surface can vary dramatically. The half-life of a TAC in the human cornea is generally thought to be one to several weeks, but numerous examples exist showing that an individual cell or cluster of cells can persist for months (Kinoshita et al., 2001).

The migration and proliferation of TACs from the limbus to the central cornea has been documented in the mouse recently using two different strategies, one involving creation of clonal patches of cells expressing Lac-Z in chimeric and X-inactivation mosaic mice (Collinson et al., 2002, 2004), and the other exploiting inherent variations in green fluorescent protein (GFP) expression levels in individual cells on the corneal surface of GFP transgenic mice (Nagasaki and Zhao, 2003). The former study showed elegantly the formation of stripes of enzyme-labelled cells migrating in swirling patterns from the limbus to the central cornea. The latter study documented a rate of migration of the TACs of 26 μm per day which compares favourably with the results of a study performed nearly 20 years ago using India ink to mark the limbal cells of the mouse; those data showed that cells moved centripetally from the limbus to the central cornea at a rate of 17 μm per day (Buck, 1985). Since the mouse cornea has a radius of approximately 1.5 mm, it would take around 8–9 weeks for a cell to traverse from the limbus to the centre of the cornea.

The TACs in the cornea are also capable of varying the number of times they undergo cell division prior to differentiating. Using a clever double-labelling technique, Lehrer et al. (1998) showed that in response to wounding and/or the administration of a protein kinase C activator, the TACs could increase the number of times they divide prior to undergoing terminal differentiation. In homoeostasis, the central corneal cells divide once before terminally differentiating, whereas the peripheral cells divide twice before differentiating. After wounding or administration of a protein kinase C activator, the number of cell divisions that the TACs underwent before differentiating was increased from 1–2 divisions to 3–4 divisions. Thus while the stem cell progeny, the TACs, are committed to being corneal epithelial cells, they also retain their ability to modulate their replication rate and the timing of their own terminal differentiation.

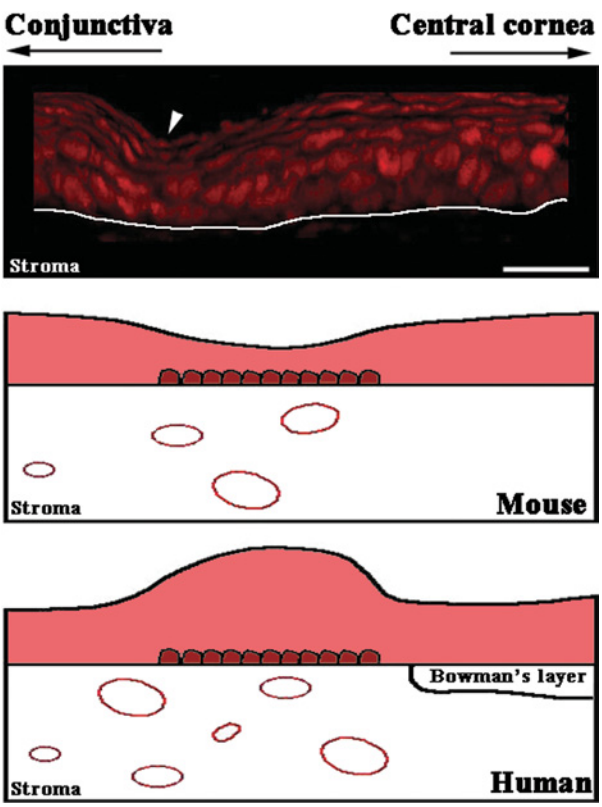
The regulation of proliferation and terminal differentiation of TACs in the cornea is similar to that of the skin. In skin, however, interfollicular stem cells are present at intervals in the stratum germinativum or basal cell layer, and form proliferative units giving rise to clonal clusters of cells that leave the basement membrane and move from their place of origin in the basal layer up through the various cell layers to eventually be shed from the stratum corneum. A primary difference between the interfollicular skin and cornea is that, prior to leaving the basement membrane and initiating terminal differentiation, the majority of the TACs in the cornea migrate for significant distances along their basement membrane away from their site of origin in the limbus. In skin, the placement of stem cells at regular intervals makes it unnecessary for TACs to migrate as far laterally before terminally differentiating. Of course in both tissues, after wounding, the epithelial cells can migrate as well as proliferate.

In cornea, as in skin, the stem cells are believed to be preferentially localized within a niche. The word niche is derived from the German word meaning nest and is believed to be the site where the CESCs are protected from the environment and surrounded by other non-stem cells and/or ECM, which provides the cues or signals required for cells to maintain their stem-like nature. In the mouse cornea, the niche is formed at the limbus by the slight infolding of the corneal epithelium (Figure 4), whereas in humans there is actually a thickening of the stromal tissues at the limbus (Kinoshita et al., 2001). Stem cells in the niche are protected in different ways. In the mouse, the anatomical limbus folds in and the stem cells are no longer subjected to direct abrasive forces. While the basement membrane is almost perfectly flat in the central cornea, at the limbus it becomes undulated, increasing the surface area for contact with the underlying basement membrane. In non-albino mice and humans, there is a rich supply of pigment underlying the limbal basal cells that serves to absorb energy from light that might otherwise cause DNA damage. Finally, at the limbus there are nearby blood vessels that can provide nutrients and growth factors that can function as survival factors to suppress cell differentiation and maintain the cells in their slow cycling state (Lavker et al., 1998; Lehrer et al., 1998).

Interestingly, the basement membrane beneath the CESCs has a distinct ECM composition compared with that of the cornea (Ljubimov et al., 1995; Tuori et al., 1996; Wessel et al., 1997). It is not clear

Figure 4 | The limbal niche

CESCs are preferentially located within a protected niche (white arrowhead), which is formed at the limbus by slight infolding of the corneal epithelium in the mouse, and is shown here using propidium iodide (a nuclear marker). The white line indicates where the corneal epithelium meets the underlying stroma. However, the human cornea differs from the mouse in two aspects: the thickness of the limbal region and the presence of the Bowman's layer exactly where the limbus starts, as depicted in the cartoons. The red ovals indicate the blood vessels present in the stroma. Image taken by a 60× oil objective of a confocal microscope. Bar = 10 μm.



whether these matrix proteins are made by the CESCs themselves or by mesenchymal cells within the stroma that might well form a part of a functional stem cell unit. For example, the stem cells present at the bulge region of the hair follicle lie in close apposition to mesenchymal cells called the dermal papilla that are thought to possess potential hairshaft-inducing ability (Alonso and Fuchs, 2003). In *Drosophila*, cell–cell contact between the stem cells that make up the ovaries and testes and mesenchymal cells, called cap or hub cells in female and male flies respectively, is necessary to maintain stem cells within their respective niches (Fuchs et al., 2004). In mammals, it is not yet clear whether the CESCs remain within their niche throughout life because of the distinct environment formed there, or whether they themselves induce the necessary microenvironment at the niche, due to the properties resulting from their stem cell nature.

A primary reason for understanding the cell biology of CESCs is to find treatments for limbal stem cell deficiency (LSCD). LSCD is a condition that leads to patient suffering and loss of quality of life. Symptoms include ocular pain and discomfort secondary to corneal neovascularization, poor re-epithelization after injury, conjunctival cell ingrowth, chronic inflammation, and overall, a loss of visual acuity. Transplantation of cultures of putative human CESCs onto the surface of eyes can restore clear corneas in some patients. Human amniotic membranes (Pires et al., 2000) and fibrin matrices (Rama et al., 2001) are now being used in trials as substrates to grow corneal stem cells in culture for transplantation purposes with clinically mixed results (Grueterich et al., 2003; Meallet et al., 2003; Ramaesh and Dhillon, 2003). If we could identify CESCs through a definitive set of surface markers, it would facilitate diagnosis of LSCD and enhance quality control on cells grown in culture and intended for transplantation. Clinicians often wait 2–3 weeks to determine if a given culture of limbal cells is highly clonogenic and suitable for transplantation; there are patients for whom waiting reduces their chances for full recovery. These clinical data prove that some rapidly proliferating TACs can reoccupy niches left empty by irradiation or disease. While the CESCs can persist for the life of the organism, the depletion of CESCs observed in LSCD indicates that they do not always do so. The reason why in LSCD the CESCs are lost or, if present, no longer functional, remains unclear. Reduction or loss of survival factors from their immediate environment is a probable cause, but the real causes are still unknown. We have no way at present to determine whether the cells are there and not working correctly, whether they have undergone apoptosis, or whether the CESCs have migrated out of the niche and then terminally differentiated.

We can conclude from our current knowledge that CESCs are located in a niche at the limbus, and are relatively undifferentiated cells that are slow or

rarely cycling. While generally quiescent themselves, CESCs can be induced, in response to injury or stimulatory factors, to proliferate and give rise to large numbers of their progeny, the TACs. One goal for the future is to improve the odds that transplantation procedures work, and another is to reduce LSCD by maintaining the overall health of the limbus and cornea in the aging eye. Both goals will be furthered by the availability of markers that will permit the identification of the CESCs *in vivo* in patients and allow better diagnosis and classification of disease states.

Markers

It is important to identify markers for the CESC and to validate candidate markers quickly and reliably. Progress in the analysis of adult stem cells from the bone marrow has outpaced that in other fields for several reasons, including the identification of markers for haematopoietic stem cells (HSCs). Markers for HSCs (Lin^-, $Sca1^+$, Kit^+) allowed the development of standardized methods for the purification and analysis of these cells (Calvi et al., 2003). Markers also allow for the refinement of diagnostic criteria for disease states. It is most likely that not all patients who present with symptoms of LSCD suffer from the same condition; however, separating patients into subsets is difficult based on symptoms alone. Treatments that work on one subset of patients may appear to be ineffective when patients are grouped together.

No standard criteria exist for the establishment of a protein or group of proteins as a marker(s) for a specific cell type or disease state. For the CESCs, we propose the following as criteria for markers. (1) While the markers do not have to be present exclusively on cells of the ocular surface, their presence or absence alone or in combination must provide a reliable means for the enrichment and/or isolation of the CESCs. (2) The markers must also allow for the identification of the CESCs cells within their native tissue in healthy individuals. (3) The number of CESCs bearing markers must remain relatively constant throughout the life of the organism in healthy individuals. Treatments or conditions that increase cell proliferation in the corneal epithelium should not increase the numbers of CESCs expressing markers in the long term. (4) The markers should identify fewer CESCs in individuals presenting with LSCD. Once markers are identified, the isolated cells need to show proof of their inherent 'stemness'. This could be done for CESCs as rigorously as it is for other adult stem cell populations, and should include demonstrating long-term repopulation, restoration of a clear cornea, expression of cornea-specific markers such as K3 and K12, and showing evidence for clonogenicity, *in vitro*.

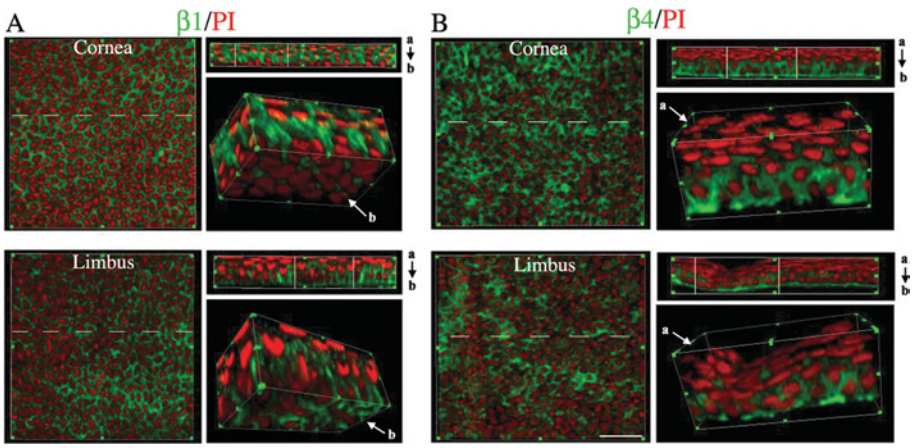
Table 1 | Putative markers for limbal stem cells
The presence (+) or absence (–) of a given protein is indicated, as determined using immunohistochemical and/or immunofluorescence methods on either whole mounts or tissue sections.

Marker	Limbal region
Keratins	
K3/K12	–
K5/K14	+
Gap junction proteins	
Cx43	–
Cx50	–
Integrins	
β1 integrin	+
β4 integrin	+
α6 integrin	+
α9 integrin	+
Metabolic enzymes	
α-enolase	+
Cytochrome oxidase	+
Carbonic anhydrase	+
Transporters	
Glucose transporter I	+
Na^+/K^+-ATPase	+
ABCG2	+
Growth factor receptors	
EGF receptor	+
TGF-β receptor I/II	+
Cell cycle mediators	
Cyclin D	+
Cyclin E	+
Others	
p63	+

Although markers are being sought for CESCs, so far none are available that meet the criteria stated above; however, several molecules have been suggested as possible markers. These can be divided into two major categories: negative and positive markers (Table 1). Negative markers are those that are absent or poorly expressed in the limbal basal cells. For

Figure 5 | Localization of integrins on the mouse ocular surface

Eyes of 8-week-old Balb/C mice were enucleated, fixed, and stained with sera against either β1 or β4 integrins, and propidium iodide (PI) to visualize nuclei. Using a 60× oil objective of a confocal microscope, 30 optical sections were acquired at 0.5 μm intervals and they were made into a 3-dimensional stack using image pro-plus software. On the left-hand side of each panel is a lower magnification view of the basal side of the optical stack. On the top right-hand side of each panel, the stack of 30 optical sections is shown rotated by 90 degrees to reveal a cross sectional view (apical to basal; a → b) of the tissue indicated by the dotted line. On the bottom right-hand side of each panel, the rectangular box from the cross section is digitally magnified to illustrate that both β1 (**A**) and β4 (**B**) integrins are more abundant in the central cornea than in the limbus. β1 integrin is present in the basal cells and gradually decreases towards the apical cells. β4, which forms heterodimer with α6 integrin to form the hemidesmosomes, is primarily expressed by the basal cells. The same pattern of expression was observed for α6 integrin (data not shown). Bar = 20 μm.



example, the keratin pair K3/K12 is not present in the limbal region but is found in the central cornea (Schermer et al., 1986; Kurpakus et al., 1990; Pearton et al., 2004). Thus the absence of K3/K12 might serve as a marker for the CESCs. However, keratins are intracellular proteins and would not be ideal as markers, since sorting cells based on the expression of intracellular proteins requires permeabilization of the cells. In this context, the gap-junction proteins Connexin 43 (Cx43) and Cx50 are most suitable. They are reduced or absent from the limbal epithelial cells but are abundant in the basal cells of the surrounding cornea and conjunctiva (Matic et al., 1997). Connexins are integral membrane proteins on the cell surface, and thus might be useful as negative markers, as long as conditions can be identified so that the proteins are not internalized or degraded when cells are prepared from tissues for cell sorting. In addition, we have observed that some of the epithelial integrins, which have been implicated as potential stem cell markers in the epidermis, including β1 and β4 integrins, appear less abundant overall in the limbal region of the eye compared with the central cornea (Figure 5). Integrins, which are cell adhesion molecules, would make ideal markers since they are surface proteins and are trypsin insensitive and thereby would remain intact after tissues are gently trypsinized. While β1, β4, and α6 integrins are less abundant on the basal cells of the limbal region compared with basal cells in the central cornea, it is not clear whether a reliable isolation of CESCs could be achieved via sorting for reduced surface expression of these integrins in limbal derived cells. As seen in Figure 5, some cells scattered among cells that are relatively poor at expressing β4, are in fact β4 bright. Our current studies include using BrdU and antibodies against various integrins to determine whether the integrin bright or dull cells are the slow cycling CESCs.

Positive markers would include molecules that are highly expressed in the limbal region versus the other regions of the ocular surface. For instance, in the human cornea, the keratin pair K5/K14 is expressed in the limbal basal cells (Pearton et al., 2004) and

not in the central cornea. However, keratins are not ideal markers since they are not present on the cell surface. High expression of some enzymes such as α-enolase (Zieske et al., 1992), cytochrome oxidase (Hayashi and Kenyon, 1983), and carbonic anhydrase (Steuhl and Thiel, 1987) were observed in the basal cells of the limbus but not in the basal cells of the central cornea. Growth factor receptors such as the epidermal growth factor (EGF) receptor and TGF-β (transforming growth factor β) receptor I and II have been shown to be highly expressed in the limbus (Zieske et al., 2000, 2001), as has p63 (Pellegrini et al., 2001), a transcription factor that belongs to the family that also includes p53 and p73 (Lohrum and Vousden, 2000). Cell cycle mediators, such as cyclins D and E (Joyce et al., 1996), and ATP-binding cassette (ABC) transporters, such as ABCG2 transporter protein (Chen et al., 2004), have also been shown to be preferentially localized in the limbal region. However, the expression of several of these proteins increases at least transiently in the limbal basal cells after injury or trauma (Chung et al., 1995, 1999; Joyce et al., 1996; Zieske et al., 2000, 2001). Further, the functions of many of the above proteins involve cell proliferation and activation whereas stem cells are known to be quiescent and metabolically inactive *in situ*. For these reasons, it is likely that most of these proteins are present on the eTACs intermixed with the stem cells at the limbus and not exclusively on the stem cells themselves. No data on the relationship between expression of any of these proteins in the limbus and slow cycling have been reported.

Finally, we have shown that α9 integrin, which forms heterodimers with β1 integrin, is expressed in a subpopulation of cells present in the limbus (Stepp et al., 1995; Stepp, 1999; Stepp and Zhu, 1997). α9 integrin is localized to the apical and basolateral aspect of the limbal basal cells, whereas the cells in the central cornea are negative for α9 integrin. Although α9 is abundant in the apical cytoplasm of the cells, it is also observed in the cell membrane where it colocalizes with E-cadherin (Pajoohesh-Ganji et al., 2004). Furthermore, a rodent model of LSCD shows that regions of the limbus adjacent to sites where goblet cells have invaded are depleted of α9 integrin (Pal-Ghosh et al., 2004). While α9 integrin expression appears to meet several criteria for consideration as a marker for the CESCs, in that it is a surface protein and its expression is reduced or absent in at least one model for LSCD, it does not meet all the criteria since the number of cells expressing it increases in the limbus after certain types of wounds to the mouse eye. Thus far, data support the idea that α9 integrin, like the other positive markers mentioned above, is present on subpopulations of eTACs intermixed with the stem cells at the limbus.

Although none of the above molecules appear to be definitive markers for CESCs, it remains possible that using a combination of the positive and negative markers, as discussed above and listed in Table 1, the CESCs could be isolated and purified. Finding CESC markers will expedite the isolation and culture of the CESCs, and facilitate transplantation, as well as treatment of diseases caused by LSCD. Progress towards identifying surface markers is being made but much more remains to be done.

Transdifferentiation of the CESCs

Transdifferentiation or plasticity, the differentiation of an adult tissue-specific stem cell into another type of cell or tissue, is a controversial topic. In studies initially reported by Ferraris et al. (2000) and recently expanded on by Pearton et al. (2004), it was shown that basal epithelial cells from the adult cornea can give rise to hair follicles. Their studies demonstrated that when corneal explants obtained from the centre of a rabbit cornea, and devoid of the limbal region, were implanted into a nude mouse along with embryonic mouse dermis, the K3/K12-positive cells in the basal epithelium of the rabbit cornea stopped making corneal keratins and begin to express K5/K14, keratins expressed on basal cells of the limbus and epidermis. These cells went on to give rise to hair follicles. They concluded from these experiments that adult TACs of the central cornea maintain the ability to produce hair follicles if presented with the appropriate environment.

Although these data support the concept of transdifferentiation, not everyone is convinced that transdifferentiation occurs *in vivo* in adult stem cell populations. Experiments have been hard to reproduce and it is unclear exactly what the sources of variability are in these studies. Wagers and Weissman (2004) argue that the purity of the stem cell population studied plays a major role in whether or not plasticity is observed in adult stem cells, and that cells with adult stem cell characteristics can be isolated from different tissue sources, a fact that is rarely

considered in many studies. Another source of variability arises from the fact that a subpopulation of adult stem cells may dedifferentiate into a more primitive cell type, given the right environment, giving rise to a new set of stem-like cells with different properties. Cell–cell fusion has also been cited as a possible explanation for some reports of transdifferentiation. According to Wagers and Weissman (2004), there are no published studies that demonstrate true transdifferentiation events.

Without a definitive set of markers for the CESCs, it remains difficult to disprove the transdifferentiation shown by Pearton et al. (2004). Even if we accept that transdifferentiation has been proven, it must have been regulated by factors provided by neonatal dermal fibroblasts, since only when the rabbit central cornea is transplanted along with neonatal dermis, does transdifferentiation occur. LSCD patients exist and, in these patients, the K3/K12-positive basal cells that remain in their central corneal epithelium do not give rise to CESCs; however, we do not know whether or not they are capable of doing so given the right environmental cues. The factors present in the neonatal dermis that convert the TACs on the central cornea into epidermal stem cells will in time be identified, and the knowledge of how these factors convert adult corneal TACs back into stem-like cells could help to reduce the incidence of LSCD.

Conclusions

Stem cells for the corneal epithelium, like other adult stem cells, reside in a niche, which is located between the corneal epithelium and the conjunctiva at a site called the corneal limbus. At the limbus, the CESCs, TACs, and corneal epithelial basal cells intermix making it difficult to isolate the CESCs without markers for selection. The fairly undifferentiated CESCs are slow cycling and, while generally quiescent themselves, can be induced to proliferate and give rise to large numbers of TACs. To date, there is no single protein or group of proteins that have been characterized to serve as a marker for the CESCs. However, the search for CESCs markers has lead us to a better understanding of the cells that make up the limbus. We now know that the limbal basal cells communicate with one another differently than do the surrounding corneal epithelial cells, since they lack Cx43-containing gap junctions. They express elevated levels of the transcription factor p63 and several growth factor receptors including the EGF receptor and the TGF-β receptors. Finally, we know that the cells in the limbal region interact differently with their underlying substrate, given their differential expression of several integrins coupled with the fact that their underlying basement membrane is distinct from that of the central cornea. While studying integrin expression during cell migration in the wounded cornea, a mouse model for the study of LSCD was described and characterized. Thus, despite a lack of progress in finding CESC markers, identifying and validating potential markers for the CESCs is leading to a better understanding of the limbus. Yet, finding markers remains essential to further our goal of finding effective treatments for diseases caused by LSCD.

Acknowledgements

We thank Ms Sonali Pal-Ghosh for her technical assistance and for careful reading of the manuscript. We also thank Dr Robyn Rufner, the director of CMIA at The George Washington University, and Dr Anastas Popratiloff for help with the confocal microscopy and image analysis. The funding was provided by NIH/NEI, Grant number: R01-EY13559 to M.A.S.

References

*Articles of special interest

***Alonso, L. and Fuchs, E.** (2003) Stem cells of the skin epithelium. Proc. Natl. Acad. Sci. U.S.A. **100**, 11830–11835

Barrandon, Y. and Green, H. (1987) Three clonal types of keratinocyte with different capacities for multiplication. Proc. Natl. Acad. Sci. U.S.A. **84**, 2302–2306

Buck, R.C. (1985) Measurement of centripetal migration of normal corneal epithelial cells in the mouse. Invest. Ophthalmol. Vis. Sci. **26**, 1296–1299

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R. et al. (2003) Osteoblastic cells regulate the haematopoietic stem cell niche. Nature (London) **425**, 841–846

Chen, Z., De Paiva, C.S., Luo, L., Kretzer, F.L., Pflugfelder, S.C. and Li, D. (2004) Characterization of Putative Stem Cell Phenotype in Human Limbal Epithelia. Stem Cells **22**, 355–366

Chung, E.H., DeGregorio, P.G., Wasson, M. and Zieske, J.D. (1995) Epithelial regeneration after limbus-to-limbus debridement. Expression of α-enolase in stem and transient amplifying cells. Invest. Ophthalmol. Vis. Sci. **36**, 1336–1343

Chung, E.H., Hutcheon, A.E., Joyce, N.C. and Zieske, J.D. (1999) Synchronization of the G1/S transition in response to corneal debridement. Invest. Ophthalmol. Vis. Sci. **40**, 1952–1958

***Collinson, J.M., Morris, L., Reid, A.I., Ramaesh, T., Keighren, M.A., Flockhart, J.H., Hill, R.E., Tan, S.S., Ramaesh, K., Dhillon, B. and West, J.D.** (2002) Clonal analysis of patterns of growth, stem cell activity, and cell movement during the development and maintenance of the murine corneal epithelium. Dev. Dyn. **224**, 432–440

Collinson, J.M., Chanas, S.A., Hill, R.E. and West, J.D. (2004) Corneal development, limbal stem cell function, and corneal epithelial cell migration in the Pax6+/− mouse. Invest. Ophthalmol. Vis. Sci. **45**, 1101–1108

Cotsarelis, G., Cheng, S.Z., Dong, G., Sun, T.T. and Lavker, R.M. (1989) Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell (Cambridge, Mass.) **57**, 201–209

Danjo, Y. and Gipson, I.K. (2002) Specific transduction of the leading edge cells of migrating epithelia demonstrates that they are replaced during healing. Exp. Eye Res. **74**, 199–204

Espana, E.M., Kawakita, T., Romano, A., Di Pascuale, M., Smiddy, R., Liu, C.Y. and Tseng, S.C. (2003) Stromal niche controls the plasticity of limbal and corneal epithelial differentiation in a rabbit model of recombined tissue. Invest. Ophthalmol. Vis. Sci. **44**, 5130–5135

Farjo, A.A. and Soong, K.H. (2004) Corneal epithelium. In Ophthalmology (Yanoof, M. and Duker, J.S., eds.), pp. 413–421, Mosby, MO, U.S.A.

***Ferraris, C., Chevalier, G., Favier, B., Jahoda, C.A. and Dhouailly, D.** (2000) Adult corneal epithelium basal cells possess the capacity to activate epidermal, pilosebaceous and sweat gland genetic programs in response to embryonic dermal stimuli. Development **127**, 5487–5495

***Fuchs, E., Tumbar, T. and Guasch, G.** (2004) Socializing with the neighbors: stem cells and their niche. Cell (Cambridge, Mass.) **116**, 769–778

Gipson, I.K. (1994) Anatomy of the conjuntiva, cornea, and limbus. In The Cornea (Smolin, G. and Thoft, R., eds.), pp. 3–24, Little Brown, MA, U.S.A.

Grueterich, M., Espana, E.M. and Tseng, S.C. (2003) *Ex vivo* expansion of limbal epithelial stem cells: amniotic membrane serving as a stem cell niche. Surv. Ophthalmol. **48**, 631–646

Hayashi, K. and Kenyon, K.R. (1983) Increases cytochrome oxidase activity in alkali burned corneas. Exp. Eye Res. **7**, 131–138

Huang, A.J. and Tseng, S.C. (1991) Corneal epithelial wound healing in the absence of limbal epithelium. Invest. Ophthalmol. Vis. Sci. **32**, 96–105

Jester, J.V., Moller-Pedersen, T., Huang, J., Sax, C.M., Kays, W.T., Cavangh, H.D., Petroll, W.M. and Piatigorsky, J. (1999) The cellular basis of corneal transparency: evidence for 'corneal crystallins'. J. Cell Sci. **112**, 613–622

Joyce, N.C., Meklir, B., Joyce, S.J. and Zieske, J.D. (1996) Cell cycle protein expression and proliferative status in human corneal cells. Invest. Ophthalmol. Vis. Sci. **37**, 645–655

Kenyon, K.R. and Tseng, S.C. (1989) Limbal autograft transplantation for ocular surface disorders. Ophthalmol. **96**, 709–722

Kinoshita, S., Adachi, W., Sotozono, C., Nishida, K., Yokoi, N., Quantock, A.J. and Okubo, K. (2001) Characteristics of the human ocular surface epithelium. Prog. Retinal Eye Res. **20**, 639–673

Kurpakus, M.A., Stock, E.L. and Jones, J.C. (1990) Expression of the 55-kD-64-kD corneal keratins in ocular surface epithelium. Invest. Ophthalmol. Vis. Sci. **31**, 448–456

Lavker, R.M. and Sun, T.T. (2003) Epithelial stem cells: the eye provides a vision. Eye **17**, 937–942

Lavker, R.M., Wei, Z.G. and Sun, T.T. (1998) Phorbol ester preferentially stimulates mouse fornical conjunctival and limbal epithelial cells to proliferate *in vivo*. Invest. Ophthalmol. Vis. Sci. **39**, 301–307

***Lavker, R.M., Tseng, S.C. and Sun, T.T.** (2004) Corneal epithelial stem cells at the limbus: looking at some old problems from a new angle. Exp. Eye Res. **78**, 433–436

Lehrer, M.S., Sun, T.T. and Lavker, R.M. (1998) Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. J. Cell Sci. **111**, 2867–2875

Lemp, M.A. and Mathers, W.D. (1989) Corneal epithelial cell movement in humans. Eye **3**, 438–445

***Ljubimov, A.V., Burgeson, R.E., Butkowski, R.J., Michael, A.F., Sun, T.T. and Kenney, M.C.** (1995) Human corneal basement membrane heterogeneity: topographical differences in the expression of type IV collagen and laminin isoforms. Lab. Invest. **72**, 461–473

Lohrum, M.A. and Vousden, K.H. (2000) Regulation and function of the p53-related proteins: same family, different rules. Trends Cell Biol. **10**, 197–202

***Matic, M., Petrov, I.N., Chen, S., Wang, C., Dimitrijevich, S.D. and Wolosin, J.M.** (1997) Stem cells of the corneal epithelium lack connexins and metabolite transfer capacity. Differentiation **61**, 251–260

Meallet, M.A., Espana, E.M., Grueterich, M., Ti, S.E., Goto, E. and Tseng, S.C. (2003) Amniotic membrane transplantation with conjunctival limbal autograft for total limbal stem cell deficiency. Ophthalmol. **110**, 1585–1592

***Nagasaki, T. and Zhao, J.** (2003) Centripetal movement of corneal epithelial cells in the normal adult mouse. Invest. Ophthalmol. Vis. Sci. **44**, 558–566

Pajoohesh-Ganji, A., Pal Ghosh, S. and Stepp, M.A. (2004) Regional distribution of $\alpha 9\beta 1$ integrin within the limbus of the mouse ocular surface. Dev. Dyn. **230**, 518–528

Pal-Ghosh, S., Pajoohesh-Ganji, A., Brown, M. and Stepp, M.A. (2004) A mouse model for the study of recurrent corneal epithelial erosions: $\alpha 9\beta 1$ integrin implicated in progression of the disease. Invest. Ophthalmol. Vis. Sci. **45**, 1775–1788

Pearton, D.J., Ferraris, C. and Dhouailly, D. (2004) Transdifferentitaion of corneal epithelium: evidence for a linkage between the segregation of epidermal stem cells and the induction of hair follicles during embryogenesis. Int. J. Dev. Biol. **48**, 197–202

***Pellegrini, G., Golisano, O., Paterna, P., Lambiase, A., Bonini, S., Rama, P. and De Luca, M.** (1999) Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. J. Cell Biol. **145**, 769–782

Pellegrini, G., Dellambra, E., Golisano, O., Martinelli, E., Fantozzi, I., Bondanza, S., McKeon, F. and DeLuca, M. (2001) p63 identifies keratinocyte stem cells. Proc. Natl. Acad. Sci. U.S.A. **98**, 3156–3161

Piatigorsky, J. (2000) A case for corneal crystallins. J. Ocul. Pharmacol. Ther. **16**, 173–180

Pires, R.T., Chokshi, A. and Tseng, S.C. (2000) Amniotic membrane transplantation or conjunctival limbal autograft for limbal stem cell deficiency induced by 5-fluorouracil in glaucoma surgeries. Cornea **19**, 284–287

Rama, P., Bonini, S., Lambiase, A., Golisano, O., Paterna, P., De Luca, M. and Pellegrini, G. (2001) Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. Transplantation **72**, 1478–1485

***Ramaesh, K. and Dhillon, B.** (2003) *Ex vivo* expansion of corneal limbal epithelial/stem cells for corneal surface reconstruction. Eur. J. Ophthalmol. **13**, 515–524

Schermer, A., Galvin, S. and Sun, T.T. (1986) Differentiationrelated expression of a major 64K corneal keratin *in vivo* and in culture suggests limbal location of corneal epithelial stem cells. J. Cell Biol. **103**, 49–62

Stepp, M.A. (1999) $\alpha 9$ and $\beta 8$ integrin expression correlates with the merger of the developing mouse eyelids. Dev. Dyn. **214**, 216–228

Stepp, M.A. and Zhu, L. (1997) Upregulation of α_9 integrin and tenascin during epithelial regeneration after debridement in the cornea. J. Histochem. Cytochem. **45**, 189–201

Stepp, M.A., Zhu, L., Sheppard, D. and Cranfill, R.L. (1995) Localized distribution of $\alpha 9$ integrin in the cornea and changes in expression during corneal epithelial cell differentiation. J. Histochem. Cytochem. **43**, 353–362

Steuhl, K.P. and Thiel, H.J. (1987) Histochemical, morphological study of the regenerating corneal epithelium after limbus-to-limbus denudation. Graefes Arch. Clin. Exp. Ophthalmol. **225**, 53–58

Stenevi, U., Hanson, C., Claesson, M., Corneliusson, E. and Ek, S. (2002) Survival of transplanted human corneal stem cells. Acta Ophthalmol. Scand. **80**, 105–109

Tuori, A., Uusitalo, H., Burgeson, R.E., Terttunen, J. and Virtanen, I. (1996) The immunohistochemical composition of the human corneal basement membrane. Cornea **15**, 286–294

***Wagers, A.J. and Weissman, I.L.** (2004) Plasticity of adult stem cells. Cell (Cambridge, Mass.) **116**, 639–648

Wei, Z.G., Cotsarelis, G., Sun, T.T. and Lavker, R.M. (1995) Label-retaining cells are preferentially located in fornical epithelium: implications on conjunctival epithelial homeostasis. Invest. Ophthalmol. Vis. Sci. **36**, 236–246

Wessel, H., Anderson, S., Fite, D., Halvas, E., Hempel, J. and SundarRaj, N. (1997) Type XII collagen contributes to diversities in human corneal and limbal extracellular matrices. Invest. Ophthalmol. Vis. Sci. **38**, 2408–2422

Zieske, J.D., Bukusoglu, G., Yankauckas, M.A., Wasson, M.E. and Keutmann, H.T. (1992) Alpha enolase is restricted to basal cells of stratified squamous epithelium. Dev. Biol. **151**, 18–26

Zieske, J.D., Takahashi, H., Hutcheon, A.E. and Dalbone, A.C. (2000) Activation of epidermal growth factor receptor during corneal epithelial migration. Invest. Ophthalmol. Vis. Sci. **41**, 1346–1355

Zieske, J.D., Hutcheon, A.E., Guo, X., Chung, E.H. and Joyce, N.C. (2001) TGF-β receptor typesI and II are differentially expressed during corneal epithelial wound repair. Invest. Ophthalmol. Vis. Sci. **42**, 1465–1471

Received 31 August 2004; accepted 10 November 2004

Published on the Internet 22 March 2005, DOI 10.1042/BC20040114

