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Stem cell therapies for ocular surface disease

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Transparency of the cornea on the front surface of the eye is essential for vision. A variety of blinding ocular surface diseases involve the cornea. This review focusses on vision loss caused by disruption of the integrity and function of the outermost corneal layer (the epithelium) and the stem-cell-based therapeutic strategies in use and under development to restore sight in affected patients.

Structure and function of the cornea

The cornea is the clear window at the front of the eye. The cornea serves two main purposes: first, it allows accurate focussing of light to produce a sharp image on the retina for subsequent visual perception and second, it protects the eyeball and its contents. The cornea consists of five main layers, namely the corneal epithelium, Bowman's layer, the corneal stroma, Descemet's membrane and the corneal endothelium (Fig. 1a). The outermost layer of the cornea, which is exposed to the external environment, is the corneal epithelium, and even subtle changes to the structure and functioning of the corneal epithelium will have marked consequences on both visual acuity and ocular protection.

The corneal epithelium covers the entire surface of the avascular cornea up to the limbus, which is an area at the outer edge of the cornea that forms a barrier between the corneal epithelium and conjunctival epithelium (Fig. 1b).

The corneal epithelium consists of a non-keratinizing stratified squamous epithelium, which makes up approximately 10% of the total corneal thickness (Fig. 1c). It is made up of five to seven layers of cells consisting of a single layer of columnar cells resting on a basement membrane, which are capable of dividing. Above this are two to three layers of wing cells topped by two to three layers of squamous cells. The neighbouring conjunctival epithelium is also shown (Fig. 1c). The limbal epithelium that separates the corneal epithelium and conjunctival epithelium is made up of a non-keratinizing stratified squamous epithelium but is much thicker than the corneal epithelium (up to ten cell layers; Fig. 1c). It is thought to contain the source of the stem cells (SCs) that provide the source of corneal epithelial renewal and also provide a barrier preventing the conjunctival epithelium from encroaching onto the corneal surface. These cells are known as corneal epithelial SCs, or limbal stem cells (LSCs).

Maintenance of the corneal epithelium: LSC theory

During homeostasis, cells are constantly lost from the surface of the corneal epithelium. The ultimate source of cell renewal is the LSC population. LSCs are defined as unipotent, or 'progenitor', cells because they only give rise to corneal epithelial cells.

An SC exists in an optimal microenvironment or 'niche' that promotes its maintenance in an undifferentiated state [1]. When SCs undergo asymmetric division, only one of the daughter cells can re-enter the niche to replenish the SC population. The other cell loses the protection of the niche and is destined to differentiate and become a transient amplifying cell (TAC). The role of the TAC is to divide at an exponential rate to provide increased cell numbers. The ability of the TAC to multiply is limited and will eventually differentiate into a post-mitotic cell (PMC) that can no longer multiply. The PMCs are committed to cellular differentiation and mature to form terminally differentiated cells that represent the final phenotypic expression of the tissue type (Fig. 2a).

Precise LSC identification is difficult because of a lack of specific and reliable markers. However, there are several pieces of evidence that indicate the presence of an LSC population in the basal layer of the limbal epithelium: (i) migration of pigmented epithelium lines from the limbus centrally, after central corneal epithelial

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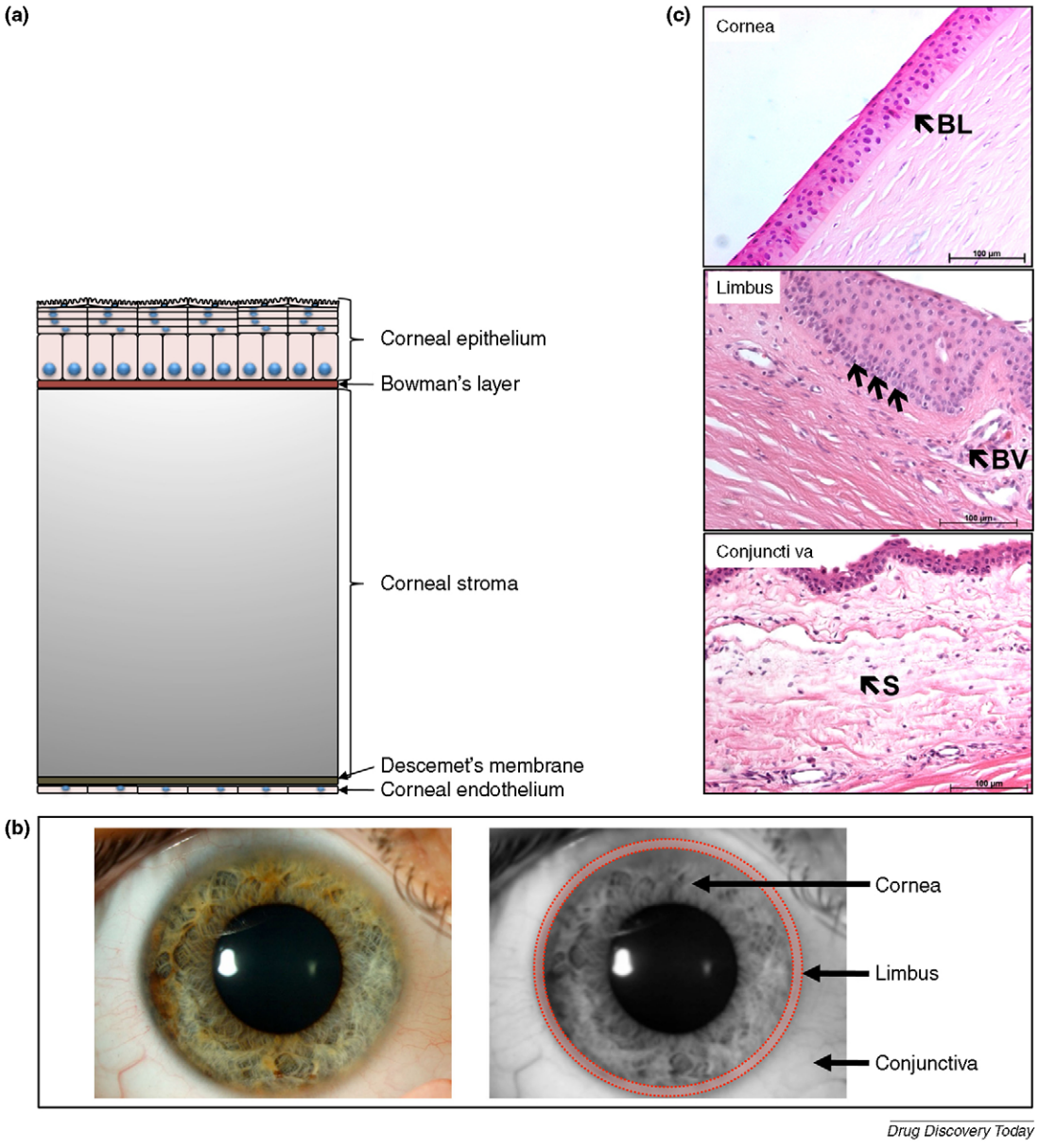

FIGURE 1

The structure of the human cornea. **(a)** Schematic diagram of the five layers of the human cornea. **(b)** Colour photograph of the anterior segment of a human eye (shown on the left) with corresponding greyscale image showing the locations of the cornea, limbus and conjunctiva (shown on the right). **(c)** Histology of the corneal, limbal and conjunctival epithelium, as seen with a haematoxylin and eosin stain. The corneal epithelium is seen to be very regular with a uniform stratified squamous epithelium of uniform thickness. Beneath the corneal epithelium is a modified acellular area of the corneal stroma named Bowman's layer (BL). The limbal epithelium is seen to be thicker with a larger number of cell layers. The basal layer is seen to consist of much smaller cells (labelled with a triple arrow) with a large nucleus:cytoplasm ratio, in keeping with the presence of a stem cell (SC) phenotype. In addition, the epithelium is seen to be folded and closely associated with blood vessels (BV). The conjunctival epithelium is seen to be thin and loosely arranged and sits on a thick vascular stroma (S).

wounding [2]; (ii) the presence of the corneal differentiation marker, cytokeratin K3, in all layers of the corneal and limbal epithelium except the basal limbal epithelium [3]; (iii) the presence of slow-cycling cells only in the basal limbal epithelium [4]; (iv) high proliferative capacity of the basal limbal epithelium [5]; (v) absence of normal corneal epithelial healing after the removal of limbal epithelium leading [6]; and (vi) the success of limbal epithelial grafts in reversing limbal SC deficiency [7].

Current understanding is that LSCs are located in low numbers exclusively in the basal layer of the limbal epithelium (Fig. 2b). On division, the LSCs give rise to TACs in the basal layer of the corneal epithelium. These TACs proliferate, migrate and differentiate to maintain the layers of the corneal epithelium. The role of the limbus in homeostatic maintenance of the corneal epithelium has been challenged recently in the mouse [8]; however, the relevance of this work to the human eye has yet to be fully verified.

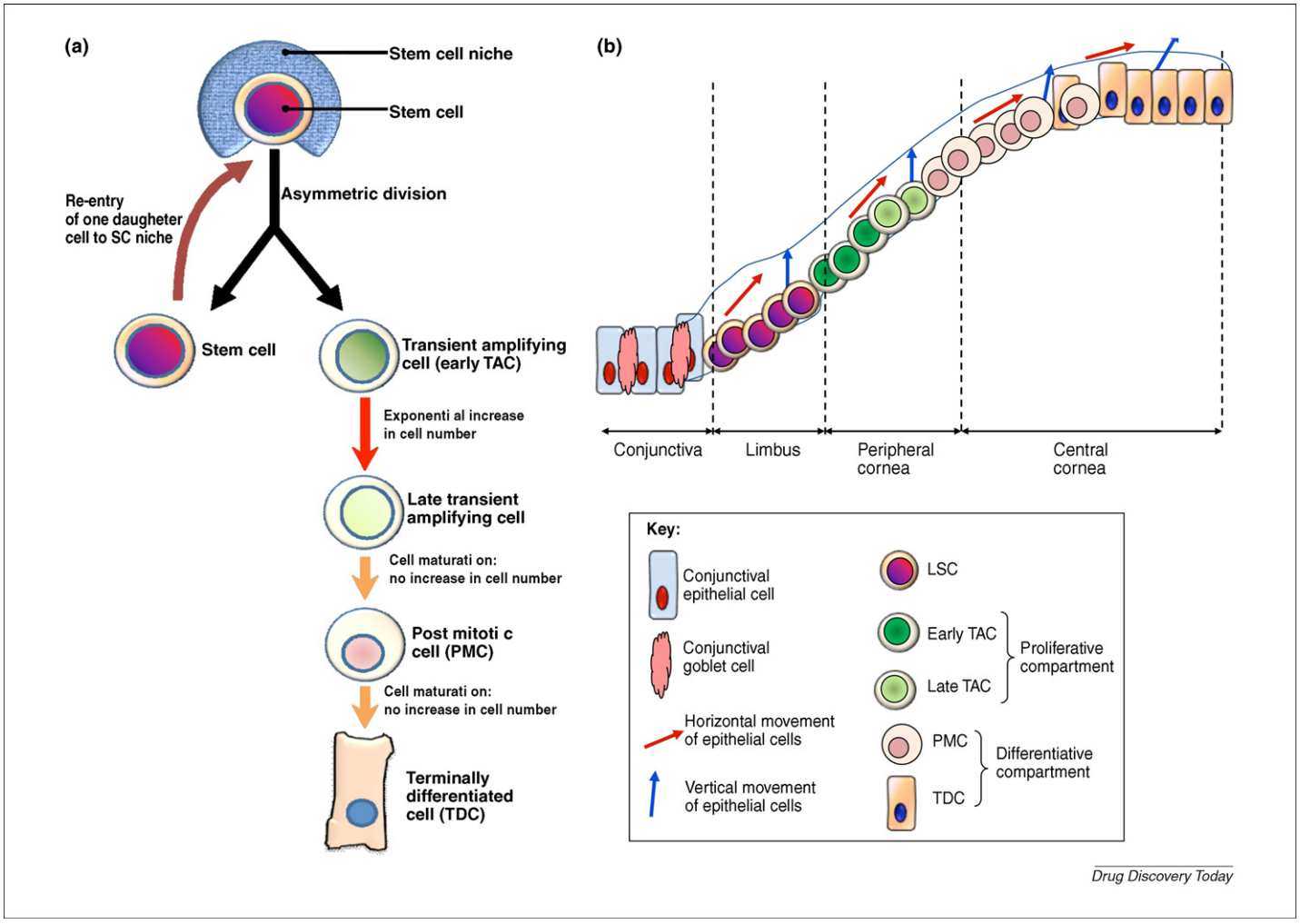

FIGURE 2

Diagrammatic representation of SC and daughter cells in the limbus. **(a)** Schematic diagram of the SC hierarchy. **(b)** Schematic representation of limbal SCs and their differentiated products in relation to the ocular surface.

LSCs can be identified indirectly by features including slow cell cycling [9], high proliferative potential, small size [10] and high nuclear to cytoplasmic ratio [11]. There is consensus that an LSC-enriched population can be defined by a signature of putative LSC marker expression [12], especially if combined with nuclear to cytoplasmic ratio [13]. Those that are the most reliable include the transcription factors ΔNp63α [14] and C/EBPδ [15] and the transporter molecule ABCG2 [16]. Reliable negative markers include cytokeratin K3 [3], the cell structural protein involucrin [17] and the cell–cell interaction molecules Connexin 43, 49 and 50 [18,19].

LSC niche

Normal SC functioning might depend more on their location than their inherent gene expression patterns. This location defines a group of surrounding cells that constitute a specific environment, or niche [20]. The cells making up this niche might control SC behaviour via intercellular contacts and signals [21].

A recent report shed light upon the development of the human LSC niche. Observations of human foetal corneas (8.5–22 weeks gestation) identified a ridge-like structure around the circumference of the cornea. SCs were found across the cornea at early time points. As gestational age increased, however, the SCs located to this ridge. Hence, the authors suggest the ridge to be the rudimentary niche for LSC [22].

Many findings suggest that the adult LSC niche exists in the basal epithelium of the palisades of Vogt found at the corneoscleral limbus (Fig. 3). The palisades of Vogt consist of a series of radially orientated fibrovascular ridges, which are concentrated along the superior and inferior limbus. Clinical confocal microscopy techniques have revealed in greater detail the 3D structure of the limbal niche and how it can be affected by disease or injury [23]. The stromal matrix and basement membrane of the palisades of Vogt differ considerably from those of the remainder of the cornea, in keeping with their role as LSC niche [24–26] by providing protection from physical insult. The LSC population is positioned to receive influences from a wide variety of cells that includes cell-to-cell contact, cell–extracellular matrix contact and paracrine signalling factors and their receptors. These influences arise from the adjacent conjunctival epithelial cells and fibroblasts, corneal epithelium and fibroblasts, limbal blood vessels, limbal melanocytes, corneal nerves and Langerhans cells.

Cytokine studies in the cornea and limbus imply specific differences in the microenvironment, suggesting fibroblast-mediated

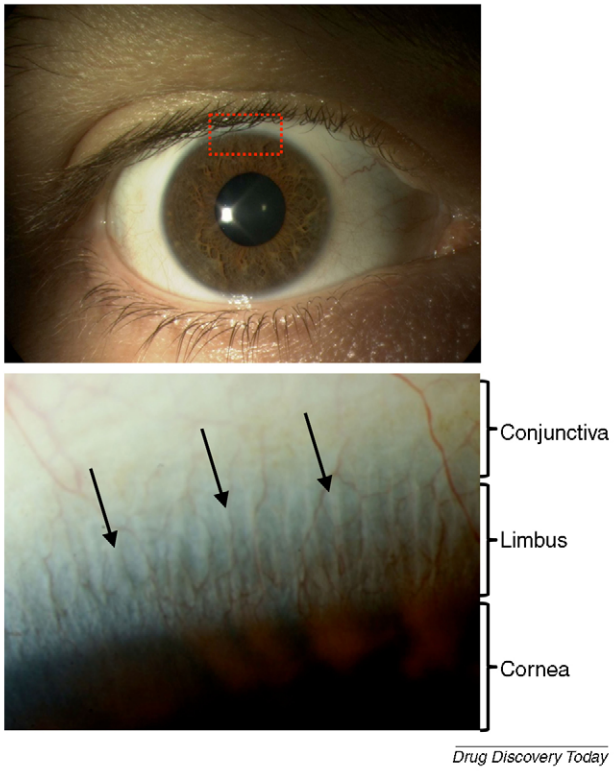

FIGURE 3

Colour photograph of the human eye (top panel). The area demarcated by the red dotted line corresponds to a small part of the superior limbus (of the same eye), which is shown magnified in the lower panel. Note the undulations of the limbal epithelium corresponding to the palisades of Vogt (indicated by the black arrows), which are richly supplied with blood vessels.

paracrine regulation of LSCs. Studies have also shown that replication of the limbal niche *in vitro* (collagen-IV-coated plates and conditioned medium from limbal fibroblasts) results in directed differentiation of human embryonic stem cells (hESCs) to corneal progenitors, thus pointing to a specific role of paracrine-mediated growth factors signalling from the feeders to the limbal cells themselves [27]. For example, limbal fibroblasts secrete keratinocyte growth factor (KGF), whereas corneal fibroblasts release hepatocyte growth factor (HGF) [28]. Most importantly, epithelial cells at the basal layer of the limbus express high levels of the KGF-R, in contrast to the central corneal epithelium, which expresses lower levels' of this receptor. KGF is a potent stimulator of proliferation in epithelial cells, and its presence in the limbus results in the proliferation of LSCs and TACs [28]. HGF is known to stimulate the migration of epithelial cells, and it is proposed that HGF aids the migration of new TACs produced at the limbus by the action of KGF [29].

Causes and consequences of LSC failure

Limbal stem cell deficiency (LSCD) results from the loss or dysfunction of LSC, most often because of injury or inflammation [30,31]. LSCD or dysfunction can result from severe chemical and thermal burns to the surface of the eye, inflammatory diseases (such as Stevens–Johnson Syndrome and ocular cicatricial pemphigoid), infection or long-term contact lens wear. There are also various iatrogenic causes of LSCD, which include extensive limbal surgery or cryotherapy and therapeutic radiation. Exposure of the limbus to cytotoxic agents such as mitomycin C has also been known to cause LSCD [32]. Hereditary causes of LSCD include aniridia and ectodermal dysplasia. It is probably in these cases that the niche for LSCs is altered and this results in subsequent LSC dysfunction and loss [33,34]. This is best characterized in aniridia, which results from *PAX6* mutations [35,36]. More recently, down-regulation of *PAX6* has been linked to abnormal epidermal differentiation of cornea epithelial cells [37]. Idiopathic cases of LSCD have also been described.

The limbus has two important functions with regard to the corneal surface. First, it is the niche for the corneal epithelial SCs, or LSCs, as they are commonly known. Second, it acts as a barrier preventing the phenotypically and functionally different conjunctival epithelium from encroaching onto the surface of the cornea and bringing with it its underlying blood vessels. In LSCD, precisely these two functions of the limbus fail. The corneal epithelium cannot be maintained or renewed and chronic epithelial defects result. The conjunctiva, its epithelium and blood vessels invade the surface of the cornea. The hallmarks of LSCD, therefore, are chronic epithelial defects on the corneal surface and conjunctivalization of the cornea. Both of these result in a chronically painful and visually impaired eye. The cause of the LSCD often dictates whether the disease is unilateral or bilateral (i.e. affecting one eye or both).

Historical review of the management of LSCD

In total LSCD, corneal grafting has been the historical treatment option. These grafts can fail over a short period of time, however, owing to the lack of healthy recipient LSCs. LSCD can also be treated by the transplantation of healthy limbal tissue at the diseased limbus [7]. The main disadvantage of taking limbal tissue from the patient's own contralateral healthy eye or from that of a living related donor is the risk of creating LSCD in the donor eye if a large amount of tissue is required [38]. If the limbal tissue is taken from a donor (e.g. a cadaveric donor), then the tissue is allogeneic [39,40] and the recipient requires potent immune suppression, with the added risks of systemic neoplasia and infection. For this reason, this treatment option is often not a viable one.

Cultured LSC therapy

In the late 1990s, cultured autologous limbal epithelial cells were used successfully to improve vision in two patients with chemical-injury-induced LSCD. A 1–2 mm^2 limbal biopsy from the contralateral healthy eye was harvested. From this tissue, limbal epithelial cells were isolated and expanded in the laboratory on tissue culture plastic, in the presence of growth-arrested 3T3 mouse fibroblast feeders, before transfer to the eye on a temporary fibrin carrier [41]. Since this landmark report, a variety of culture techniques have been developed to produce contiguous epithelial cell sheets for transplantation, which can broadly be defined as explant culture (in which cells migrate out from limbal tissue attached to a surface) and suspension culture (in which cells are released from enzymatically digested extracellular matrix before culture; for a review, see Ref. [42]).

Tissue-engineered constructs comprising limbal epithelial cells cultured on a substrate could also be used to transfer the cells to the eye and ideally promote their survival post-transplantation.

TABLE 1

A summary of cultured LSC transplantation procedures and clinical outcomes.

Study	Number of transplants	Autografts			Allografts		
		Number successful	Total number	Success (%)	Number successful	Total number	Success (%)
Pellegrini et al. [41]	2	2	2	100			
Schwab [47]	19	12	17	71	0	2	0
Tsai et al. [43]	6	6	6	100			
Schwab et al. [64]	14	6	10	60	4	4	100
Rama (2001)	18	14	18	78			
Koizumi (2001a)	13				12	13	92
Koizumi (2001b)	3				3	3	100
Shimazaki (2002)	13				6	13	46
Grueterich et al. [53]	1	1	1	100			
Nakamura et al. [57]	3				3	3	100
Sangwan et al. [48]	125	125	125	100			
Nakamura (2004)	1	1	1	100			
Daya et al. [49]	10	7	10	70			
Sangwan (2005)	2	1	1	100	1	1	100
Sangwan (2006)	78	57	78	73			
Nakamura et al. [50]	9	7	7	100	2	2	100
Shortt et al. [46,54]	10	1	3	33	5	7	71
Pauklin (2009)	5	4	5	80			
Kolli (2009)	8	8	8	100			
Total	340	252	292	84	36	48	75

Only cases with data available in the papers are included.

Currently, the most commonly used substrate in this context is human amniotic membrane, which forms the inner wall of the membranous sac surrounding the embryo during gestation. Because of the recognized anti-inflammatory and anti-angiogenic properties of amnion, its use in ocular surface reconstruction is well established; hence, it was a natural choice for attempting *ex vivo* expansion and transplantation of cultured limbal epithelial cells [43].

Clinical results

The clinical outcomes of cultured LSC therapy within each study and between studies remain difficult to interpret. First, the culture technique between studies varies considerably – for example, the use of 3T3 mouse fibroblasts or amniotic membrane or both, or the use of foetal calf serum or autologous serum in the culture medium, or the use of suspension or explant cultures [44]. Second, case selection between studies varies – with patients with different causes of LSCD treated in the same study, the use of clinical impression alone to gauge LSCD versus corneal impression cytology, and the inclusion of patients with severe partial and total LSCD in the same study. Third, even within studies, both autologous and allogeneic transplants are performed. Fourth, although stability of the corneal surface and visual improvement are two of the most important outcomes of limbal epithelial transplantation, in some studies these outcomes are not included. Fifth, the follow-up period within and between the studies varies. These variables make the task of assessing outcomes of a particular treatment difficult.

Despite the above limitations, an overall impression of the success of transplantation of cultured limbal epithelium can be formed (Table 1). Of the 15 studies describing autografts, the mean success rate is 84% (in a total of 292 transplants), with eight studies having a success rate of 100%, including our own recent experience [45]. Of the nine studies describing allografts, the mean success rate is 75% (in a total of 48 transplants), with five studies having a success rate of 100%. The slightly lower rate of success in allograft cases is understandable because of the added issues of immune rejection.

When looking at the success of the technique, the most important question to address is why some cases are successful and others are not. This is where the study variables make this question difficult to answer. It might help to look at those studies where the success rates were lower. In one study, two out of three autografts failed, and the authors suggested that this might have been because of the different culture conditions used for autografts and allografts [46]. In another study, of the two allografts performed, neither was successful [47]. One failed because of a corneal infection, and it is difficult to know whether immune suppression played a part in this. In the second case, immune suppression treatment was not taken as prescribed and the protective contact lens was lost. In both these studies, one with a lower than expected autograft success rate and the other with a lower allograft success rate, the number of cases was too small to make a reasonable objective judgement as to the reason for the failure. If we look at those studies that had a 100% success rate, the number of cases in all but one study is small (from one to eight for autografts and from

one to four for allografts). The largest study with a 100% success rate had 125 cases of autografts, however, and although the cases were deemed a success, there were insufficient details to determine the criteria for assessing success or failure [48] and little can be determined about the reason for the high success rate in this study. Improvement in visual acuity is clearly an important outcome criterion for the transplantation of cultured limbal epithelium. Although some of the studies do not give the details of visual acuity, out of a total of 85 cases (both autografts and allografts) where some form of visual acuity data is available, in 65 cases (or 76% of cases), there was an improvement in visual acuity of two Snellen lines or better. This includes data from cases after subsequent corneal transplantation following initial limbal epithelial transplantation. Although in many of these studies, the outcomes are difficult to interpret owing to the variables present, the above results certainly indicate that the outcomes from these studies are at least promising. Further studies in which the variables are standardized will make it easier to interpret the overall success of this technique and to assess the reasons for success or failure in particular cases. In addition, although the longest published follow-up lasted 50 months [49], the technique has now been used for 12 years, and long-term follow-up results in the early cases would be of particular interest in assessing the long-term success of this technique.

Current challenges

To make direct comparisons between different LSC therapy clinical outcome studies, adoption of universal culture (and clinical) protocols would be needed. Before this could be achieved, several challenges should be addressed. One of the key issues is removal of all animal-derived products from the culture process. Often, limbal epithelial cells are cultured in bovine serum. Although in the EU and other nations there are strict criteria regarding the source of bovine serum for use in clinical applications, the risk of adventitious agent transmission remains. Furthermore, serum batch variability can have a profound effect on the ability to culture epithelial cells, especially SCs. Bovine serum has been substituted for autologous human serum in clinical limbal epithelial cell cultures [50]; however, the suitability of human serum from patients who might be unwell or taking systemic medications has not been investigated thoroughly. A more complete understanding of signalling in the SC niche could be beneficial for the replacement of serum sources used for LSC expansion.

Growth-arrested murine 3T3 fibroblasts have been used for more than 30 years in the production of epithelial cell sheets for skin transplantation in humans, apparently without adverse effects. 3T3s provide a plentiful supply of cells with arguably the best-known ability to maintain epithelial SCs *in vitro*. Although it is possible to screen 3T3s for all known human and murine viruses, a risk of disease transmission or other unintended pathological consequence is possible. Human MRC-5 fibroblasts could be a suitable and safer feeder cell line for clinical limbal epithelial cell cultures [51].

Feeder cells are most often used to support limbal epithelial cells cultured using the cell-suspension method. The use of explant culture, especially on a substrate such as human amniotic membrane, might negate the requirement for feeders completely because the explant retains the natural LSC niche. It has been demonstrated, however, that very few LSCs migrate out of a limbal biopsy explant [52]. It is unknown how many LSCs are required for optimal long-term functionality of transplanted corneal epithelium. It is not uncommon for a limbal epithelial cell culture to be unsuccessful owing to the detachment of limbal explants during the long culture process.

The use of amniotic membrane in LSC therapy production protocols introduces notable biological variability in terms of cell-growth-promoting properties. In addition, amnion is semi-opaque, expensive and not readily available. The method of membrane preparation and storage has also been shown to influence LSC preservation [53,54]. Research efforts, therefore, are currently aiming to produce a tissue-engineered alternative.

Temperature-responsive plastics that release cultured cell sheets without the use of potentially destructive enzymes have also been used to produce epithelial sheets for corneal transplantation [55]. Recently, contact lenses were used successfully for the culture and transplantation of limbal epithelial cells to patients [56]. In this study, autologous serum was used to culture the cells. This study represents a important advance; however, it remains unclear whether the cultured cells will remain functional in the absence of an underlying substrate in all patient conditions to which this therapy might be applied.

Alternative cell sources for replacing corneal epithelium

All the current methods of *ex vivo* expansion of LSCs and subsequent transplantation for the treatment of LSCD have the advantage that they only require a very small amount of healthy limbal tissue (typically 1 mm^2), which means the risk of causing LSCD in the donor eye is negligible. There is, however, a proportion of patients with bilateral total LSCD who lack any healthy LSCs to expand in the first place. *Ex vivo* expansion of allogeneic limbal tissue from a living relative or cadaveric donor are possible options in these patients. Despite a lack of evidence for long-term donor cell survival [49], in cases of bilateral LSCD, cadaveric donor tissue has been used to produce cultured limbal epithelial sheets that have improved vision and comfort post-transplantation [46].

Because the tissue would be allogeneic, however, these methods would still have the problems associated with traditional whole-tissue allografts – namely, tissue rejection and the requirement for immunosuppression with its associated and potentially serious side effects.

Alternative sources of adult epithelial stem cells

In an attempt to overcome the problems inherent in the transplantation of allogeneic tissue, there has been recent interest in the possibility of using alternative autologous epithelial cells. There are several potential sources of non-keratinizing stratified squamous epithelium in the adult human including oral mucosal, conjunctival, nasal, oesophageal, vaginal and rectal epithelia. Of these, the use of oral mucosal epithelia on the surface of the eye has received the most interest because of their lack of advanced differentiation, high proliferative potential, cytokeratin K3 expression, easy access and rapid healing. Although intact oral epithelium has been used to resurface the ocular surface in the past, this is associated with a very thick epithelium that is opaque, leading to discomfort and poor vision. It seems, however, that oral epithelium can be

expanded *ex vivo* to produce an epithelium that is very similar in appearance and function to corneal epithelium. Nakamura *et al.* [57] have successfully expanded rabbit oral mucosa epithelial SCs on amniotic membrane and subsequently transplanted the cells onto the ocular surface of rabbits with total LSCD. This led to the successful re-epithelialization of the corneal surface in this animal model. This approach has been applied to humans with encouraging early and longer term results [50,58]. This new approach provides an exciting possibility of treating this difficult group of patients with blindness from bilateral total LSCD and warrants further study.

Potential use of embryonic stem cells

hESCs are derived from blastocysts generated through *in vitro* fertilization. They are pluripotent (i.e. they can give rise to any cell type in the body). In theory, differentiation of ESCs to a corneal epithelial lineage could be achieved by replication of the LSC niche environment. This approach was first applied to the generation of corneal epithelium from mouse ESCs in 2004 [59]. Recently, this approach has been achieved successfully using hESCs [27]. In brief, the *in vitro* replication of the LSC niche environment was achieved by culturing hESCs on an extracellular matrix of collagen-IV and fed with medium conditioned by limbal fibroblasts. As with other hESC work, however, the translation of this approach to human therapeutic use requires further work to overcome problems associated with functionality, complete hESC differentiation immune rejection and ethical concerns.

Potential use of induced pluripotent stem cells

The need for blastocyst-derived hESC as a source of pluripotent cells might be overcome with the development of protocols to transcriptionally induce pluripotency in adult cells by the expression of certain genes to produce ES-like cells, the so-called 'induced pluripotent stem cell' (iPS cell). Takahashi and Yamanaka [60] were the first to achieve the generation of mouse iPS cells. Recently the generation of human iPS cells has been achieved in several laboratories [61–63]. Current research aims to avoid potential iPS cell transformation [62].

In conclusion, the generation of iPS cells as a source of producing required cell types, as with all other methods available, still has challenging problems that require resolution before it can be considered for clinical application. These can be summarized as difficulties with purifying a specific population, safety concerns regarding the potential formation of tumours, potential immune rejection and difficulties in finding an appropriate model for preclinical studies. It is possible that many of these concerns will be addressed in the years to come.

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