Symposium - TRIP

Transforming ocular surface stem cell research into successful clinical practice

Virender S Sangwan, Rajat Jain[1], Sayan Basu[2], Anupam B Bagadi[3], Shraddha Sureka[4], Indumathi Mariappan[5], Sheila MacNeil[6]

It has only been a quarter of a century since the discovery of adult stem cells at the human corneo-scleral limbus. These limbal stem cells are responsible for generating a constant and unending supply of corneal epithelial cells throughout life, thus maintaining a stable and uniformly refractive corneal surface. Establishing this hitherto unknown association between ocular surface disease and limbal dysfunction helped usher in therapeutic approaches that successfully addressed blinding conditions such as ocular burns, which were previously considered incurable. Subsequent advances in ocular surface biology through basic science research have translated into innovations that have made the surgical technique of limbal stem cell transplantation simpler and more predictable. This review recapitulates the basic biology of the limbus and the rationale and principles of limbal stem cell transplantation in ocular surface disease. An evidence-based algorithm is presented, which is tailored to clinical considerations such as laterality of affliction, severity of limbal damage and concurrent need for other procedures. Additionally, novel findings in the form of factors influencing the survival and function of limbal stem cells after transplantation and the possibility of substituting limbal cells with epithelial stem cells of other lineages is also discussed. Finally this review focuses on the future directions in which both basic science and clinical research in this field is headed.

Access this article online

Website: www.ijo.in

DOI: 10.4103/0301-4738.126173

Quick Response Code:

Key words: Allograft, autograft, cell-based therapy, limbus, limbal stem cell deficiency, limbal transplantation, niche, stem cells

The ocular surface functional unit is the interface between the eye and the outer world. Structurally it consists of the tear film, non-keratinized stratified squamous corneal and limbal epithelium, stratified columnar conjunctival epithelium (interspersed with mucin producing goblet cells and accessory lacrimal glands) and the muco-cutaneous junction of the lids, posterior to which lie the outlets of lipid secreting meibomian glands. Structural integrity along with optimal functioning of this unit including proper blinking and closure of the lids protects the eyes from external insult and provides a uniform anterior refractive surface for sharp vision.

Corneal epithelial turnover and XYZ hypothesis

The corneal surface is constantly exposed to the environment and a uniformly thick and regularly arranged corneal epithelial cover can only be maintained by rapid turnover of these cells. The corneal epithelium is thought to be maintained by the balance of proliferation of basal epithelial cells (X) and proliferation and centripetal migration of limbal epithelial cells (Y) with the loss of epithelial cells from the surface (Z). This was proposed by Thoft and Friend in 1983 as the 'XYZ hypothesis' (X + Y = Z).[1] Thus, migration occurs centripetally and circumferentially from the limbus and vertically from the basal layer forwards. To ensure the normal health of the tissue, cellular proliferation and differentiation in a coordinated manner at different levels of this hierarchy is indispensable.

Identification and location of limbal stem cell niche

Stem cells are defined as undifferentiated, self-renewing cells capable of indefinite proliferation to a large number of differentiated progeny, responsible for cellular replacement and regeneration. Stem cells are present in all self-renewing tissues, have a slow cell cycle, long life span, high capacity for error-free self-renewal and capability for asymmetric division.[2-5] They constitute only a small subpopulation of the tissue, 0.5 to 10%, of the total cell population.[6-8] Schofield, in 1983, proposed the 'niche hypothesis' suggesting that stem cells exist in an optimal micro-environment, which promotes their maintenance in an undifferentiated fashion.[9] The corneal stem cell niche is believed to be located at the limbus, in the *palisades of Vogt*.[10] Davanger and Evensen, in 1971, first proposed the concept that epithelial cells in the limbal region are involved in the renewal of corneal epithelium.[11] Subsequently, their existence at the limbus has been suggested by indirect clinical[12] and experimental evidence.[13-17]

Limbal stem cells are adult stem cells and require adequate growth factors. This requirement is fulfilled by the distinct anatomical proximity of the basal cells of the limbus to the stromal vasculature. Besides, to increase the surface area of limbal stem cells with their microenvironment, the limbal basement membrane is undulated, seen clinically on the surface of the limbus as '*palisades of Vogt*'. These are more prominent at the more pigmented superior and inferior limbus where the melanocytes and eyelids probably provide protection from

Clinical Trial Center, Dr. Paul Dubord Chair in Cornea, L V Prasad Eye Institute, Hyderabad, [1]Cornea Services, L V Prasad Eye Institute, Bhubaneswar, [2]Cornea and Anterior Segment Services, L V Prasad Eye Institute, Hyderabad, [3]L.V. Prasad Eye Institute, Hyderabad, [4]Cornea Services, L.V. Prasad Eye Institute, Hyderabad, [5]Sudhakar and Sreekanth Ravi Stem Cell Biology Laboratory, L.V. Prasad Eye Institute, Hyderabad, Andhra Pradesh, India, [6]Department of Materials, Science and Engineering, University of Sheffield, Sheffield, UK

Correspondence to: Dr. Virender S Sangwan, Clinical Trial Centre, Dr. Paul Dubord Chair in Cornea, L V Prasad Eye Institute, Hyderabad, India. E-mail: vsangwan@lvpei.org

Manuscript received: 13.11.13; Revision accepted: 25.11.13

ultraviolet (UV) radiation induced DNA damage.[11,18-21] Dua *et al*. proposed the concept of limbal epithelial crypts, which are deeper epithelial ingrowths into the limbal stroma where the true limbal stem cells are believed to reside.[22]

The asymmetric cell division of the limbal stem cells (SC) allows one of the daughter cells to remain a stem cell whereas the other cell differentiates to become a transient-amplifying cell (TAC) located in the corneal epithelial basal layer. Both SCs and TACs are regarded as progenitor cells and give rise to post-mitotic cells (PMC) of the suprabasal layers and finally to terminally differentiated cells (TDC) of the superficial layers. The latter two cell types are incapable of further cell division.[4] We can thus appreciate the fact that the loss of TDC is compensated by the gradual terminal differentiation of the preceding higher hierarchy, PMC and, eventually by the source of cellular proliferation, SC, at the highest rank.

Limbal stem cell deficiency

Acquired or inherited conditions that result in acute or chronic inflammatory damage to limbal stem cells can lead to permanent limbal stem cell deficiency (LSCD). This can be unilateral or bilateral, partial/focal or total/complete depending on the extent of limbal involement.[2,23,24] Autoimmune disorders such as Stevens Johnson syndrome (SJS), ocular cicatricial pemphigoid (OCP) and ocular allergy or inherited conditions such as anridia usually cause bilateral involvement whereas acquired conditions such as ocular burns and iatrogenic limbal trauma from multiple ocular surgeries usually result in unilateral disease.[23,24] LSCD manifests clinically as poor corneal epithelial healing, persistent epithelial defects or progressive superficial corneal vascularization and replacement of the transparent corneal epithelial phenotype with that of the transluscent conjunctival phenotype. On fluorescein staining, the conjunctivalized cornea shows a stippled appearance,[25,26] and there may be loss of palisades of Vogt in an area known to have palisades prior to the insult.[27,28] Besides, it is useful to compare the limbus in the affected quadrants with the corresponding areas of the unaffected fellow eye in unilateral cases. Patients usually complain of redness, irritation, foreign body sensation, photophobia, decreased vision and blepharospasm. The histological proof of LSCD is the presence of conjunctival goblet cells on the corneal surface as seen on impression cytology.[29-31] However, LSCD is usually a clinical diagnosis and histological studies are seldom required.

LSCD- management principles

Principles of Management of LSCD

The limbal stem cells are limited in number and do not regenerate. This makes the deficiency of limbal stem cells impossible to treat by pharmacological means. The definitive management of LSCD is surgical transplantation of healthy limbal tissue to restore the damaged corneal surface followed subsequently by visual rehabilitation.[24] Corneal transplantation alone is not successful in LSCD because the central corneal tissue that is actually transplanted does not contain any epithelial stem cells and consequently the grafted cornea also develops epithelial healing problems in due time leading to recurrence of LSCD. Previous studies have found that only 33% to 46% of corneal grafts survive for one year and fewer survive longer in eyes with ocular surface damage.[32]

After more than two decades of experience with limbal transplantation ocular surface surgeons the world over now recognize that all cases of LSCD are not amenable to this procedure. Survival of the transplanted stem cells is largely dependent on wetness of the ocular surface and therefore this procedure is currently contraindicated in dry eyes. Correction of eyelid abnormalities prior to limbal transplantation is recommended and has been shown to correlate well with better success rates.[33] It is also worthwhile to note that limbal transplantation is not considered for acute SJS or acute ocular burns, because the ocular surface is too inflamed in the acute stage for the survival of the transplanted cells,[34] and perhaps if managed properly many of such acute cases may never develop LSCD in the long-run.

The donor limbal graft can either be in the form of a large annular conjunctival-limbal lenticule several clock-hours in arc-length or a small one-clock hour sized limbal biopsy. The source can either be the healthy fellow eye of the same individual (autologous) or eyes of another individual (allogeneic). Allogeneic (related or unrelated; HLA matched or unmatched) grafts can again be from living or cadaveric donors. The limbal graft should ideally be harvested in a manner such that the donor eye is not left susceptible to developing LSCD. It is important therefore to screen the donor eye for subtle signs of ocular surface disease before proceeding to obtain the limbal graft, unless the source is cadaveric. The recommended technique of obtaining limbal grafts involves: initiating dissection from the conjunctival side and not from the corneal side, to avoid an unnecessarily deeper plane of dissection; continuing to dissect superficially taking only a sliver of superficial limbal stromal tissue; and not proceeding more than 0.5 mm into the clear cornea. The recipient eye should be prepared by removing the pannus covering the corneal surface, which frequently leaves behind clear underlying stroma. If the stroma is found to be severely scarred, thinned or perforated anterior lamellar or penetrating keratoplasty along with the limbal transplantation should be considered.

Management of unilateral LSCD

Limbal autografting

Once the limbal location of the putative corneal epithelial stem cells was proven, the research on limbal stem cells gained substantial momentum. Tsai RJ, *et al*. conducted the first pre-clinical animal trial in which they compared limbal and conjunctival autograft transplantation for corneal surface reconstruction in rabbits.[34] They found that corneas transplanted with limbal transplantation retained the corneal phenotype and showed a progressive decrease of vascularity.[35] Soon thereafter Kenyon and Tseng in their landmark proof-of-principle study showed that a normal corneal surface could be regenerated by performing limbal transplantation in human eyes with LSCD.[36] This technique, known as limbal auto-transplantation (LAT) or later as conjunctival-limbal autografting (CLAu), involved obtaining two three-clock-hour limbal grafts from the healthy fellow eye and transplanting it onto the affected eye after pannus excision. Multiple reports of ocular surface reconstruction using minor modifications of this procedure were subsequently published.[37-48] However, some groups reported iatrogenic donor-site LSCD following this technique, which was possibly related to the large size of the donor

graft.[41,47,49]

Autologous *ex-vivo* cultivated limbal epithelial transplantation

To avoid the risk of iatrogenic donor-site LSCD[39,45,47] researchers explored the possibility of obtaining a tiny one-clock-hour limbal biopsies and expanding the cells *ex-vivo* on a suitable substrate before transplanting them onto the affected eye.[48] Subsequently, several groups around the world have described various techniques of culturing limbal stem cells using either a suspension[48-55] or an explant[56-69] culture with use of either animal-derived growth factors[48-63,70-73] or a completely xenofree cultivation technique.[64-69] Animal derived products in a cell culture system always have a theoretical risk of infection, rejection or acquisition of prion diseases. Elimination of feeder cells and use of autologous human serum as an alternative to fetal bovine serum (FBS) is therefore desirable.[68]

The authors have developed a cost-effective and safer xeno-free and feeder-free explant culture system that uses autologous serum, recombinant enzymes and human growth factors and is devoid of animal-derived products. It is a submerged culture technique which also promotes stem cell maintenance[74] [Figs. 1 and 2] Although the cell-culture protocols are standardized and extremely reliable, predicting the outcome of limbal transplantation for a patient is presently difficult because the risk factors predisposing to failure of surgery are not clearly known. To address this issue the authors decided to study a large number of cases followed over a long period of time to identify the clinical risk factors associated with failure of autologous CLET in the treatment of LSCD.

As an extension to the already published results,[68] this study included 526 eyes of 526 patients. Male: female ratio was 3:1. Children and adults constituted 47% (n = 248) and 53% (n = 278) patients, respectively. Fifty-eight percent (n = 304) patients had injury within the one year of surgical intervention and 42% (n = 222) had injury more than a year ago. Sixty-four percent of the ocular surface burns were due to alkali. The mean number of interventions in the affected eye after the injury was 0.82 ± 0.8 (range: 0-3). The mean follow-up period was 1.4 ± 1.6 years.

Three hundred and thirty (63%) patients underwent only CLET, 170 (32%) patients underwent CLET with symblepharon release and 26 (5%) patients underwent CLET with penetrating keratoplasty. The median extent of symblepharon was 2 (IQR 3; range: 0-12) clock hours. The median clock hours of limbal conjunctivalization was 12 (IQR 0; range: 3-12). Mean pre-op BCVA was 0.047 + 0.1 decimal units (range: 0.001-1.0). Outcome was a success in 55.5% (n = 292) patients and failure in others (44.4%, n = 234). Fig. 3 shows a composite of slit lamp images showing a case each with conjunctivalization

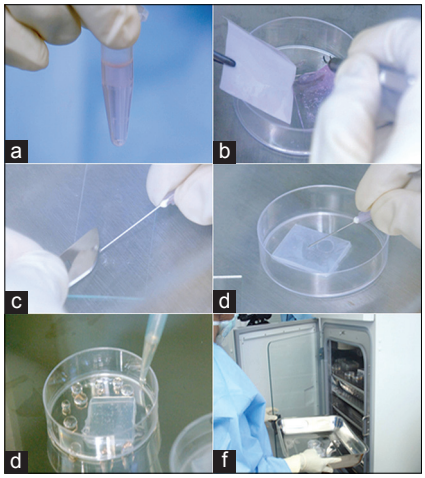

Figure 1: Stepwise display of the laboratory procedure: (a) Collection of tissue biopsy specimen in a sterile microcentrifuge tube containing HCE medium; (b) Denuded hAM spread and tucked around a glass slide; (c) Mincing of limbal tissue on a sterile glass slide; (d) Tissue bits being picked with a 24-gauge sterile needle and explanted onto the denuded hAM surface; (e) The medium in the culture dishes being replaced by 2 ml of fresh HCE medium; (f) Culture dished being incubated in a CO2 incubator

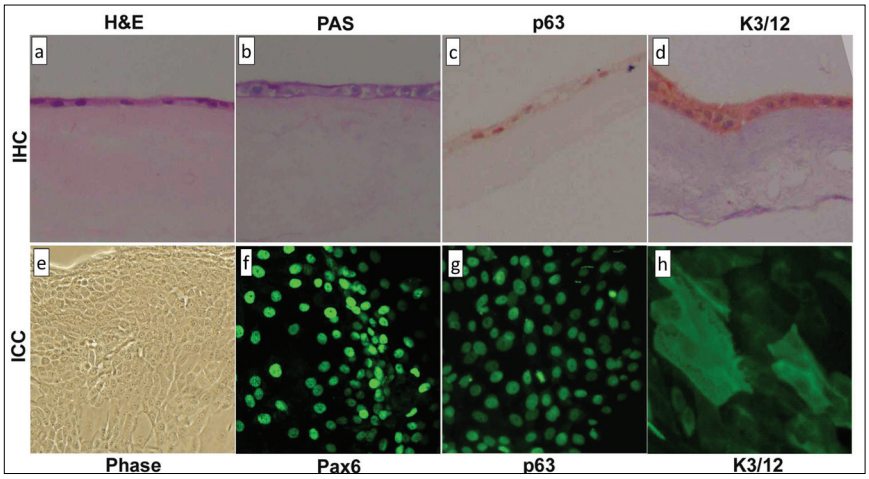

Figure 2: Limbal epithelial cell cultures on denuded human amniotic membrane (hAM): (a) Flat whole mounts were processed for H and E stain; (b) showing single monolayer of cells and PAS staining; (c) which confirms absence of goblet cells. Immunohistochemistry (IHC) confirms expression of epithelium and stem cell-specific marker, p63α; (d) differentiated corneal epithelium-specific marker, K3/12; (e) Bright field, phase image whole mount; (f) shows morphology of cell sheet. Direct IHC and fluorescence imaging also confirms expression of eye-specific transcription factor; (g) Pax6; h) p63α; (i) K3/12

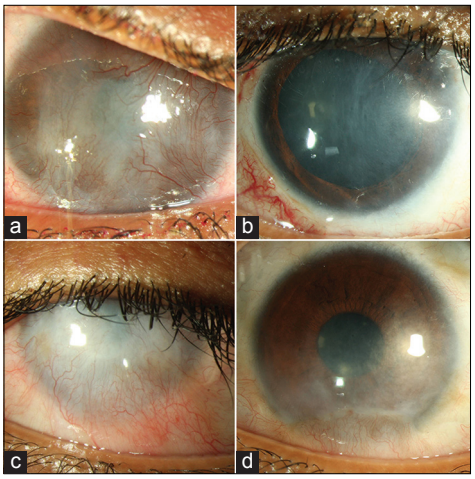

Figure 3: Composite of slit lamp images showing a) case of conjunctivalization due to LSCD preoperatively; b) same eye showing a stable ocular surface 1 year postoperative after CLET; c) another case of conjunctivalization due to LSCD preoperatively; d) showing a recurrence in the form of inferior conjunctivalization 8 months postoperative after CLET

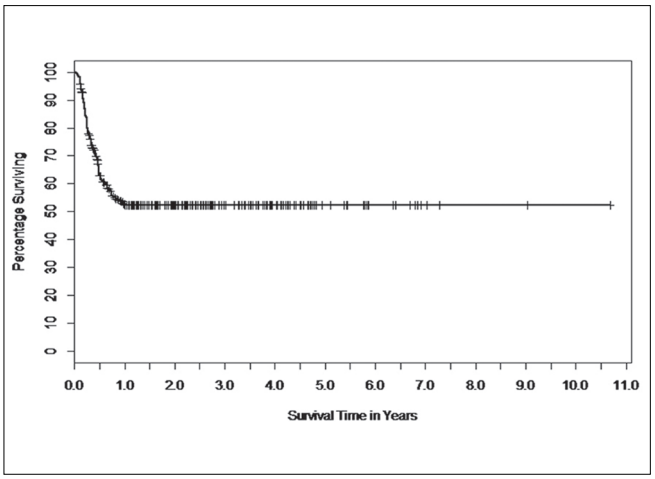

Figure 4: Kaplan-Meier survival analysis of 526 eyes that underwent autologous cultivated limbal epithelial transplantation for unilateral limbal stem cell deficiency following ocular surface burns

due to LSCD preoperatively and a stable ocular surface 1 year postoperative (a, b) or a recurrence in the form of inferior conjunctivalization 8 months postoperative after CLET (c, d). Fig. 4 shows the Kaplan-Meier survival analysis of these patients. The different risk factors found to be statistically significant were patients younger than 18 years (OR: 1.47, 95% CI: 1.01-2.15, P = 0.044), history of prior surgical intervention (OR: 1.31, 95% CI: 1.05-1.62, P = 0.015) and the presence of symblepharon (OR: 1.24, 95% CI: 1.09-1.4, P < 0.001). Patients who underwent penetrating keratoplasty with CLET (group 2) were more likely to have failure as an outcome than those who underwent CLET only (group 1). (OR: 3.24, 95% CI: 1.24-8.45, P = 0.016). However, symblepharon release done with CLET (group 3) was associated with a better outcome compared to CLET alone (group 1). (OR: 0.32, 95% CI: 0.2-0.52, P < 0.001**).** These results indicate that the cases likely to benefit maximally from autologous CLET were adults without prior ocular surface procedures and either without symblepharon or with symblepharon but having them released during the CLET procedure. Pellegrini *et al.*, found that the total number of clonogenic cells, colony size, growth rate and presence of conjunctival cells could not predict clinical results. Instead, the clinical data provided conclusive evidence that graft quality and likelihood of a successful outcome rely on an accurate evaluation of the number of stem cells detected before transplantation as holoclones expressing high levels of the p63 transcription factor.[75] Although an attractive theory, other groups have neither replicated such claims nor was the original analysis controlled for the confounding effect of clinical severity, which was also found to affect the clinical outcome.

Combining penetrating keratoplasty with autologous CLET Basu *et al.*,[76] compared the outcomes of a combining CLET with keratoplasty in a single stage (n = 12) or performing keratoplasty at least 6 weeks after CLET (n = 35). Most patients (76.6%) in that series were young (mean age: 18 ± 11.4 years) males with LSCD due to alkali burns (78.7%) and vision less than 20/200 (91.5%). The mean follow-up was 4.2 ± 1.9 years. Kaplan-Meier corneal allograft survival rate at 1 year was significantly greater in eyes undergoing two-stage (80 ± 6%, median survival: 4 years) as compared to single-stage (25 ± 13%, median survival: 6 months) limbal and corneal transplantation (P = 0.0003). Visual acuity of 20/40 or better was attained by 71.4% of eyes with clear corneal grafts. Allograft failure occurred in 26 (60.5%) eyes due to graft rejection (57.7%), graft infiltrate (26.9%) or persistent epithelial defects (15.4%). Recurrence of LSCD was more common after single-stage (58.3%) than two-stage (14.3%) surgery (P = 0.008). Two-stage approach of autologous cultivated limbal epithelial transplantation followed by penetrating keratoplasty (PK) successfully restores ocular surface stability and vision in eyes with unilateral LSCD due to ocular burns. Single-stage approach is associated with poorer clinical outcomes and should be avoided.

Repeat autologous CLET is an option in patients who develop recurrent LSCD after an autologous CLET for unilateral total LSCD. Basu *et al.*, in a series of 50 patients undergoing repeat autologous CLET, reported a 2-line improvement in visual acuity in 76% cases at a mean follow-up of 2.3 years. No donor eye complications were noted even after taking the second limbal biopsy. Kaplan-Meier curve showed that all failures occurred within 15 months of repeat surgery and graft survival at 1 year was 70 + 6% and at 2 years and thereafter was 63.7 + 7%.[77] To put these results into clinical perspective would be to say that over two-thirds of cases which fail after a primary autologous CLET can be treated successfully with a repeat procedure without any adverse impact on the donor eye. This is a major advantage of CLET over conventional CLAu/LAT which cannot be repeated from the same donor eye owing to the large amount of tissue that is required for the procedure.[76]

CLET in the pediatric population has certain specific risk factors as their treatment poses unique challenges to the surgeon due to issues related to delayed presentation, stronger inflammatory response to inciting injury as well as to transplanted graft, stimulation-deprivation amblyopia and strabismus, need for frequent examinations under anesthesia and lack of adherence to spectacle wear and patching therapy. In a series of 107 eyes of children less than 15 years, Sejpal

et al., showed that 50 eyes (46.7%) achieved a completely epithelialized, avascular and stable ocular surface at a mean follow-up of 3.4 years.[69]

Autologous CLET for unilateral and partial LSCD presents the ocular surface surgeon with the unique dilemma of whether to harvest the limbal biospy from the healthy part of the affected eye (ipsilateral) or the fellow eye (contralateral). Vazirani *et al.*, in 70 eyes with a mean follow up of 17.5 ± 7 months; found a 70.6% clinical success in the ipsilateral group (n = 34) and 75% success in the contralateral group (n = 36, P = 0.79). The authors concluded that the outcomes were similar irrespective of whether the limbal biopsy was taken from the healthy part of the ipsilateral eye or the contralateral eye.[78]

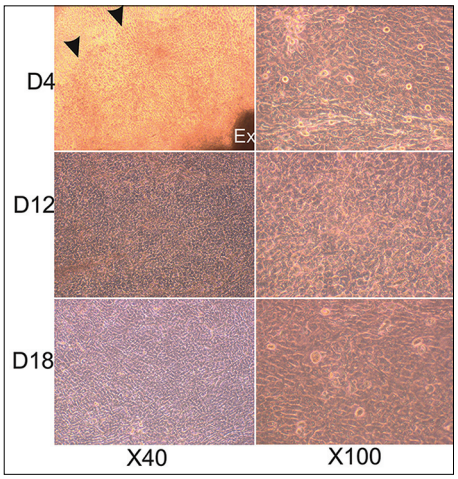

Figure 5: Growth pattern of oral mucosal epithelial cells on denuded hAM. Phase contrast images taken at day 4 in culture showing the initiation of cellular outgrowth from the explant (Ex) tissue and monolayer formation (arrows head-growing edge). Confluent monolayer formed after 12, 18 days in culture

In-vivo cultivation of autologous limbal epithelial cells using simple limbal epithelial transplantation

Sangwan *et al.*,[79] have also proposed a novel simplified technique of limbal transplantation which combines the advantages of CLAu and CLET by being a single-stage, easily affordable procedure which utilizes a minimal donor tissue and does not need a stem cell laboratory for cultivation of limbal epithelial cells.

The surgical steps are essentially similar to those of CLET for harvesting the limbal biopsy. The recipient eye surgery is performed in the same sitting. Human amniotic membrane (hAM) graft is placed over the bared ocular surface and is secured with fibrin glue (TISSEEL Kit from Baxter AG, Vienna, Austria). The donor tissue is subsequently cut into eight to ten small pieces and these limbal transplants are placed, epithelial side up, on the hAM, sparing the visual axis. These transplants are also fixed in place with fibrin glue. Authors have reported successful outcomes with SLET in eyes with and without high-risk clinical features of limbal transplantation.[80-82] The authors now have considerable experience with this technique. One-year success rate of primary SLET is better than autologous CLET in both adults (76% vs 71%) and children (74% vs 37%) possibly due to use of fresh tissue without lab processing and transplantation of the whole niche in SLET as compared to isolated epithelial cell transplantation in CLET.

Management of bilateral LSCD

Limbal allografting

Patients of bilateral LSCD do not have any autologous source of limbal cells which can be used for therapy. Limbal stem cells can hence be harvested from an allogenic source, either from living (related or unrelated) or cadaveric donors.[31,47,83,84] Though reports of good results without recipient immunosuppression have been also published,[47] systemic immunosuppression is usually necessary.[31,84] Although an-HLA matched transplantation is ideal, the disadvantages of

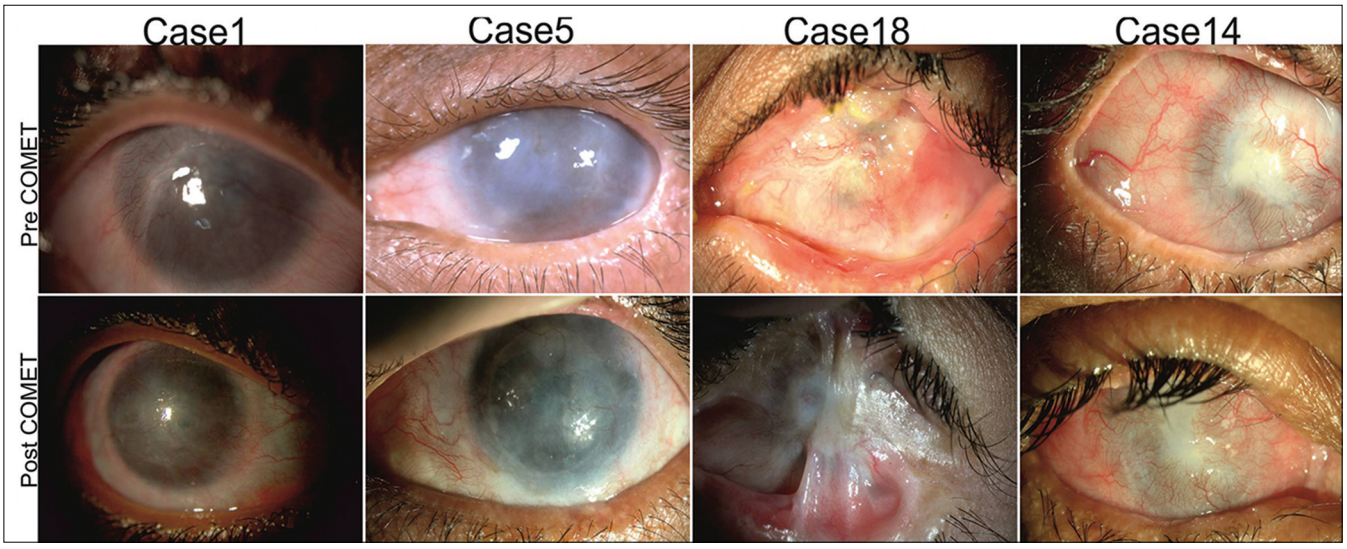

Figure 6: Clinical photographs before (top)/after 1 year (below) autologous COMET (1) stable ocular surface with superficial corneal vascularization, central corneal scarring (5) Living related conjunctival limbal allograft and penetrating keratoplasty failed, COMET was performed and ocular surface was stable with non-progressive peripheral corneal vascularization, corneal scarring and contact lens corrected vision of 20/125 (18) severe symblepharon formation with corneal conjunctivalization (14) conjunctivalized corneal surface

HLA matching are the possibility of stem cell dropout due to lengthy preservation time needed for HLA antigen matching[85] and the additional procedural costs. Besides, a complete immune-histocompatibility match between cadaveric donors and recipients is rarely obtained.[85] In living-related limbal allografts, there is an obvious risk of donor-site LSCD and therefore more than one donor is usually preferred.[86] Various surgical strategies have been reported to harvest the donor limbal grafts.[31,87-93] However, transplanted limbal stem cells are usually unable to sustain sufficient long-term epithelial cell production for the entire limbus.[86]

Allogeneic CLET

As stated, living-related limbal allograft transplantation is limited by the amount of limbal stem cells that can be harvested.[94] Herein lies the advantage of cultivation where minimal tissue biopsy is harvested and cultured *in-vivo* into an transplantable epithelial sheet on an amniotic membrane.

For allogenic transplantation, living donors are preferable as limbal cells obtained from cadavers have a lower proliferative rate *in-vitro*[93] and a poorer corneal epithelization rate *in-vivo*.[65] Multiple studies have earlier reported the results of allogenic cultivated limbal epithelial transplantation.[95-103] Authors earlier reported their results in xeno-free allogenic CLET.[103] The Kaplan-Meier allo-CLET survival rate at 12 months was 76.4 ± 8.7% with mean follow up of 58 ± 33 months. At last follow-up, 20 eyes (71.4%) had either maintained a healthy corneal surface or underwent PK. The corneal allograft survival rate following allo-CLET ($n = 13$) was 76.9 ± 11.7% at 12 months with median survival of 40 months. While the transplanted cells are of the limbal lineage, the main limitation is the life-long need of systemic immunosuppression.

Cultivated oral mucosal epithelial transplantation

Autologous transplantation of epithelial cells of a different lineage, like oral or nasal mucosa has the advantage of not requiring long term systemic immunosuppression, and therefore avoiding its associated complications. Nakamura *et al.*,[104] first described COMET as an alternative to allogeneic limbal transplantation for management of patients with bilateral LSCD. Subsequently, other groups have also reported the successful use of this technique.[105-117]

The authors performed COMET in 19 eyes of 18 patients with bilateral LSCD. Outcome measure was based on the clinical appearance of the corneal surface. Success was defined as a totally epithelized, stable and avascular corneal surface. Failure was defined as appearance of any superficial corneal vascularization (even if the corneal surface was epithelized and stable), epithelial defects lasting more than two weeks and conjunctival overgrowth on the cornea (conjunctivalization).

The mean age at the time of surgery was 23.7 ± 12.5 years with male to female ratio of 2.8:1. The median time period between the initial injury and autologous COMET was 34 months (range: 6 to 240) months. Other pre-operative clinical characteristics of the transplanted eyes are summarized in Table 1. Three patients underwent biopsy and transplantation under general anesthesia, whereas others were operated under local anesthesias. No anesthetic or intra-operative complications occurred during either biopsy or transplantation. Following the biopsy no donor site complications were noted. The mucosal defect created on the lower lip following the oral biopsy completely healed by one week. In the laboratory, a confluent monolayer of cells formed on the denuded-hAM [Fig. 5] in a mean duration of 19.3 days (range 15 to 27 days). No cultures showed microbial contamination or inadequate growth.

The mean follow-up was 22.3 (range: 7 to 48) months. Postoperatively on day one and at one week, fluorescein staining was negative over the grafted area and no folding or loosening of the hAM was noted. At six weeks all the grafted eyes had a completely epithelized and stable corneal surface but absence of peripheral superficial corneal vascularization was noted in 16 (84%) of 19 eyes. However, peripheral superficial corneal vascularization was seen in all eyes by three months. Therefore, none of eyes met the clinical criteria of success at 3 months and thereafter. In 7 (36.8%)

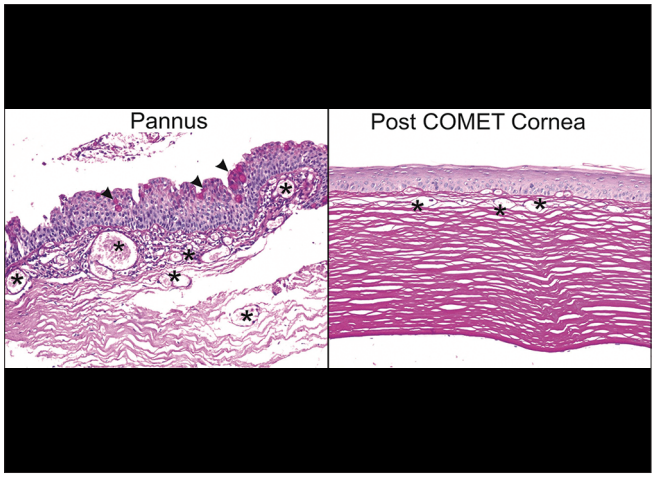

Figure 7: Periodic acid schiff (PAS) staining on pannus excised during COMET showing conjunctival epithelial cells with goblet cells (arrow head) and stromal tissue was vascularized (asterix), PAS stained post COMET corneal tissue showing hyperplasia of epithelial cells. Sub epithelial vasculatures (asterix) can also be seen. No goblet cells were observed in the stratified epithelium

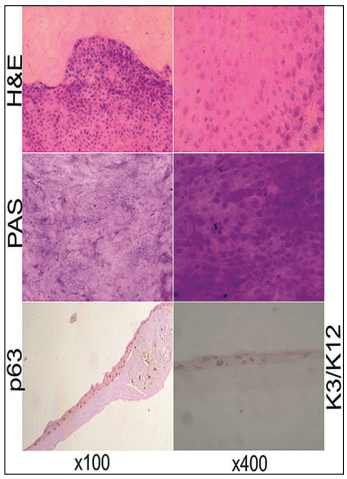

Figure 8: Hematoxylin-eosin and PAS stained whole mounts of cultured oral mucosal epithelial cells showing confluent monolayer on human amniotic membrane after three weeks of culture. The paraffin embedded cultured oral mucosal epithelial cells showing p63 and K3/K12 staining

eyes the peripheral vascularization did not progress and the corneal surface was completely epithelized and was stable at 12 months after COMET. In the remaining 12 (63.2%) eyes the central cornea became progressively vascularized or developed persistent epithelial defects with recurrence or worsening of symblepharon between 3 and 9 months of COMET [Fig. 6].

Figs. 7 and 8 describe the histopathological features of the excised pannus and the cultured oral mucosal epithelial cells, respectively. Fig. 9 describes the various immunohistochemistry markers expressed on the normal cornea, oral mucosa, conjunctiva and a post-COMET keratoplasty cornea. Fig. 10 shows the markers which confirm the presence of sub epithelial blood vessels.

The findings of our study suggested that transplantation of autologous oral mucosal epithelium cultivated using a xeno-free explant culture system was unsuccessful in restoring a stable ocular surface and improving vision in eyes with bilateral LSCD following ocular burns. Table 2 summarizes the comparison of the authors' experience with outcomes of COMET published by other groups. A cursory glance at Table 2 reveals the fact that visual results following COMET in all studies have been modest at best.

Table 1: Baseline demographic characteristics of patients with bilateral limbal stem cell deficiency who underwent autologous cultivated oral mucosal epithelial transplantation

Characteristic	*N* (%)
Age	
8 years or younger	2
9 to 16 years	1
Older than 16 years	16
Visual Acuity	
Light Perception	11 (58)
Hand Movements	8 (42)
Etiology of Ocular Surface Burns	
Lime	8 (42)
Sulphuric Acid	4 (21)
Fire-cracker	2 (11)
Sodium Sulphate	1 (6)
Ammonium Nitrate	1 (6)
Liquid Ammonia	1 (6)
Titanium Oxide	1 (6)
Formic Acid	1 (6)
Previous Ocular Surface Surgery	
None	6 (32)
Amniotic Membrane Grafting	8 (42)
Penetrating Keratoplasty	5 (26)
Allogeneic Limbal Transplantation	5 (26)
Symblepharon Release	3 (16)
Ocular Surface Status	
Conjunctivalization	15 (79)
Persistent Epithelial Defect	4 (21)
Symblepharon	8 (42)

Allogeneic SLET

Authors have earlier also reported allogeneic cadaveric simple limbal epithelial transplantation for bilateral LSCD due to alkali injury. The visual acuity improved to 20/100 unaided from hand-motions preoperatively with a stable, avascular and epithelialized corneal surface. However, 3 months later, she presented with allograft rejection. This also re-emphasized the importance of continued immunosuppression in allogeneic limbal transplantation.[83]

Summary and Conclusion

Limbal stem cell transplantation is currently the only approved human stem cell therapy in India other than mesenchymal stem cell therapy for hematological malignancies. Both the basic understanding of limbal cell biology and the techniques of limbal transplantation have evolved immensely over the last two decades. This can be both overwhelming and confusing to ophthalmologists simply because the science is progressing faster than the rate at which standard textbook editions are being revised. However, with the novel technique of limbal transplantation that the authors have recently described SLET, it may finally be possible for this effective procedure to be practiced around the world by corneal surgeons with modest resources; rather than only by an exclusive club of advanced institutes with sophisticated cell biology laboratories and grant funding support. The authors' group has had the unique opportunity of exploring all the techniques of stem cell-based therapy for ocular surface reconstruction that have been described in this review. This unique experience has given the authors an enviable perspective on the subject that no other group in the world can currently claim. Through this article the authors have tried to share their vast experience and clinical perspective on this subject and make recommendations based on rigorous scientific evidence.

The authors strongly believe that autologous limbal transplantation is the treatment of choice in unilateral total or partial LSCD and there is no role of allogeneic procedures in this condition. The authors recommend SLET over CLET as the preferred surgical technique not only because it is effective but also because it offers many other advantages such as: being single-staged, more affordable and technically feasible in a resource-limited setting. There is no unanimity yet among ocular surface surgeons regarding the preferred therapy for bilateral LSCD. In certain situations, autologous limbal transplantation may still be possible if at least one clock-hour of healthy limbus is present in either eye. However, if both eyes have total LSCD then the choice for cell-based therapy is between allogeneic-limbal transplantation and autologous COMET. This is a toss-up between long-term immunosuppression (allogeneic limbal transplantation) and modest visual outcomes (COMET) and needs to be decided on a case-to-case basis. It would be fair to state therefore that the optimal therapy for bilateral LSCD is still elusive.

Future challenges

With cultured cell therapy being used for two decades now, continued efforts are needed by major cell therapy centers to publish all clinical outcome data so that this informs surgeon's ability to decide on which treatment to offer which patients. Whereas *in-vivo* expansion of cells obviates the need for specialist tissue culture laboratories, developing off-the-shelf alternatives to the amniotic membrane should help in the uptake of this therapy by surgeons who do not have access

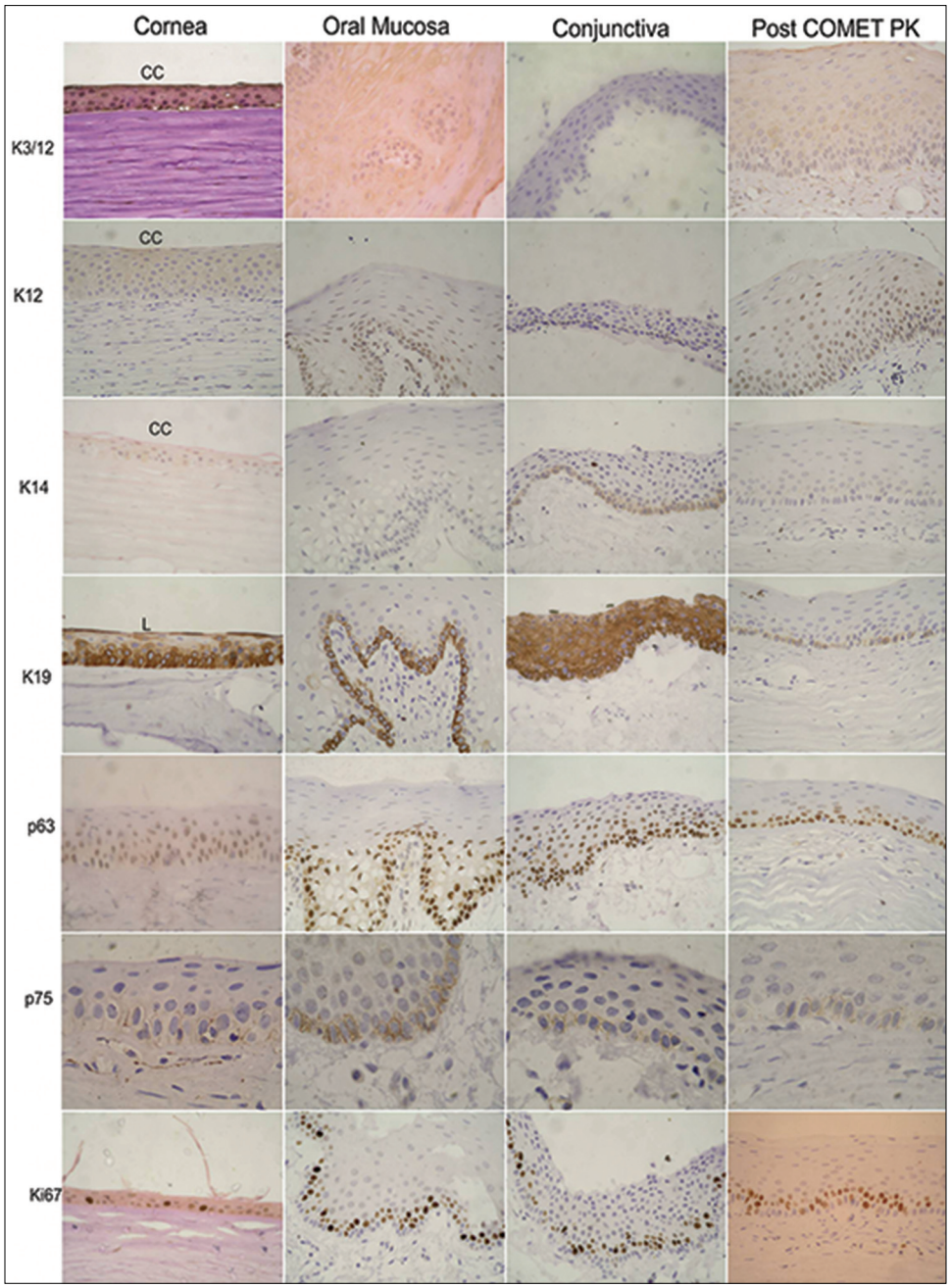

Figure 9: Immunohistochemistry for cytokeratin, proliferative and stem cell marker profile. Cytoplasmic K 3/12 staining is present in central corneal epithelium, oral mucosal epithelium and post COMET corneal epithelium but not present in conjunctiva. Cytoplasmic K12 staining was seen throughout the central corneal epithelium and was absent in oral mucosa, conjunctiva and post-COMET corneal epithelium. K14 was not expressed by the epithelial cells of the central cornea, oral mucosal epithelium and post COMET corneal tissue, but was expressed by the basal conjunctival epithelial cells. K19 is expressed in all the layers of the limbal and conjunctival epithelium but not expressed in the central cornea (data not shown). The basal cells of oral mucosal epithelium and the post COMET PK tissue showed K19 expression. p63 immunostaining showed nuclear staining in the basal and supra-basal cells of all the tissues tested. p75 immunostaining showed membrane staining only in the basal epithelial cells of the cornea, oral mucosa, conjunctiva and post COMET PK tissue. Ki 67 staining in corneal, oral mucosal, conjunctival epithelium and post COMET corneal tissue showed clear nuclear expression by the proliferating supra-basal cells. (All pictures magnification- ×400, p75 stained tissues- ×1000)

to Tissue Banked human amnion. The authors are currently seeking to develop such amniotic membrane alternatives.[117]

Even when the right therapy is selected for the right patient, there are patients where the regenerated epithelium does well long term and others where it only survives for a few years. Hence why do transplanted LSC cells fail to maintain a clear epithelium over long term is a question which still remains unanswered. Whether it is because enough cells with "stemness" to survive long term have not been transplanted or a protective environment for the limbal stem cell niches to repopulate is not there is still unexplored. Li *et al.,* have

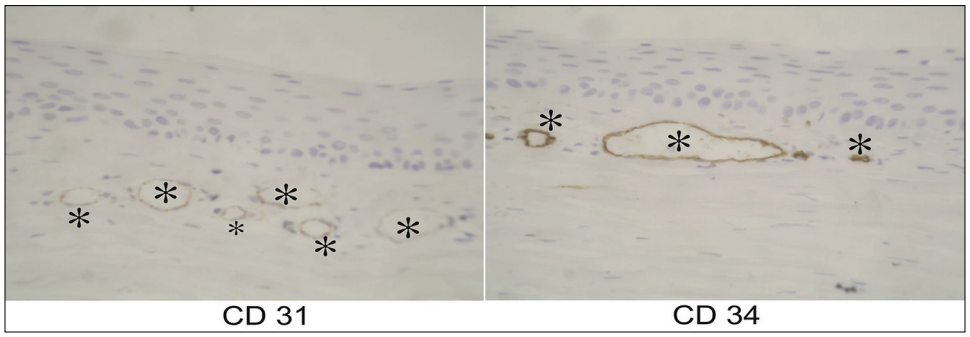

Figure 10: Immunohistochemical staining for vascular endothelial cells markers CD31 and CD34: Bright field microscopy pictures of post COMET corneal tissue stained for vascular endothelial markers CD31 and CD34 showing positive conform the presence of sub epithelial blood vessels

Table 2: Comparison of the indications, techniques and outcomes reported in previous studies on autologous cultivated oral mucosal epithelial transplantation that included nine or more eyes

Author	Year	Indications (Eyes)				Culture method (Substrate)	Xeno-biotics	Clinical outcomes			Other surgery (%)	Mean Follow- up (Months)
		SJS	OCP	Ocular Burns	Others			Avascular Cornea (%)	Stable surface (%)	BCVA >20/200 (%)		
Nakamura *et al*[105]	2004	6	-	3	-	Suspension (hAM)	FBS+3T3	0	9/9 (100)	1/9 (11)	None	13.8
Inatomi *et al*[106]	2006	7	-	6	2	Suspension (hAM)	FBS+3T3	0	SJS: 2/7 (28.5), Others: 10/10 (100)	1/15 (7)	PK: 2 (13), AM: 1 (7)	20
Ang *et al*[108]	2006	7	1	2	0	Suspension (hAM)	3T3	0	10/10 (100)	3/10 (30)	AM: 6 (60)	12.6
Nakamura *et al*[112]	2011	11	4	1	3	Suspension (hAM)	3T3	0	SJS: 6/11 (55), Others: 6/8 (75)	3/19 (16)	AM: 14 (74)	55
Satake *et al*[113]	2011	12	9	11	8	Suspension (hAM)	FBS+3T3	NA	SJS: 8/12 (75), Burns: 4/11 (36), OCP: 4/9 (44), Others: 7/8 (88)	8/40 (20)	PK: 12 (30), ALK: 3 (8)	25.5
Burillon *et al*[116]	2011	0	0	9	16	Suspension (Inserts)	3T3	0	Burns: 4/9 (44), Others: 12/16 (75)	5/25 (25)	PK: 2 (8), AM: 1 (4)	12
Current Study	2012	0	0	19	0	Explant (hAM)	None	0	7/19 (37)	1/19 (5)	PK: 1 (5), Kpro: 4 (20)	22.3

SJS=Stevens Johnson Syndrome; OCP=Ocular Cicatricial Pemphigoid; BCVA=Best Corrected Visual Acuity; hAM=Human Amniotic Membrane; 3T3=Murine 3T3 Fibroblasts; AM=Amniotic Membrane Transplantation; PK=Penetrating Keratoplasty; Kpro=Boston Type 1 Keratoprosthe

developed an *in-vitro* model of stem cell renewal of skin epithelia.[118,119] Certainly a similar approach could be undertaken with corneal stem cells. Such i*n-vitro* modelling can allow testing different scenarios and see to what extent they best mimic the available clinical or *in-vitro* data. Further, improved non-invasive imaging techniques are needed to look at the corneal surface and the palisades of Vogt and examine whether cultured cells have repopulated.

Finally, probably the most important hurdle which still remains a significant challenge is to devise a method to convert dry eyes to wet eyes. This would require in depth research into the lacrymal glands and their possible regeneration.

Having made significant strides in the last twenty-odd years, limbal stem cell biology and its clinical applications appear to have an exciting future ahead. In this review, the authors have tried to put in perspective the past, the present and the foreseeable future in the field of ocular surface regeneration. Better understanding of the molecular mechanisms of limbal cell biology and need-based innovations in surgical techniques promise to further simplify the management of limbal dysfunction and ocular surface disease through a symbiotic exchange between basic science research and clinical therapy.

References

1. Thoft RA, Friend J. The X, Y, Z hypothesis of corneal epithelial maintenance. Invest Ophthalmol Vis Sci 1983;24:1442-3.
2. Dua HS, Azuara-Blanco A. limbal stem cells of corneal epithelium. Surv Ophthalmol 2000;44:415-25.
3. Kinoshita S, Friend J, Thoft RA. Biphasic cell proliferation in transdifferentiation of conjunctival to corneal epithelium in rabbits. Invest Ophthalmol Vis Sci 1983;24:1008-14.
4. Leblond CP. The life history of cells in renewing systems. Am J Anat 1981;160:114-58.

5. Potten CS. Epithelial proliferative subpopulations. In: Stem Cells And Tissue Hemostasis. Cambridge: Cambridge university press; 1978. p. 317.
6. Potten CS, Leoffler M. Epithelial Cell Prolifration. 1. Changes with time in the proportaion of isolated, paired and clustered labelled cells in sheets of murine epidermis. Virchows Arch B Cell Pathol Incl Mol Pathol 1987;53:286:300.
7. Potten CS, Morris RJ. Epithelial stem cells *in vivo*. J Cell Sci Suppl 1988;10:45-62.
8. Pfister RR. Corneal stem cell disease: Concepts, categorization and treatment by auto- and homo- transplantation of limbal stem cells. CLAO J 1994;20:64-72.
9. Schofield R. The stem cell system. Biomed Pharamcother 1983;37:375-80.
10. Schlotzer-Schrehardt U, Kruse FE. Identification and characterisation of limbal stem cells. Exp Eye Res 2005;81:247-64.
11. Davanger M, Evensen A. Role of pericorneal papillary structure in renewal of corneal epithelium. Nature 1971;229:560-1.
12. Lee GA, Hirst LW. Ocular surface squamous neoplasia. Surv Ophthalmol 1995;39:429-50.
13. Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin *in vivo* and in culture suggests limbal location of corneal epithelial cells. J Cell Biol 1986;103:49-62.
14. Kurpakus MA, Stock EL, Jones JC. Expression of the 55-kD/64-kD corneal keratins in ocular surface epithelium. Invest Ophthalmol Vis Sci 1990;31:448-56.
15. Ebato B, Friend J, Thoft RA. Comparison of central and peripheral human corneal epithelium in tissue culture. Invest Ophthalmol Vis Sci 1987;28:1450-6.
16. Ebato B, Friend J, Thoft RA. Comparison of limbal and peripheral human corneal epithelium in tissue culture. Invest Ophthalmol Vis Sci 1988;29:1533-7.
17. Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: Implications of epithelial stem cells. Cell 1989;57:201-9.
18. Shortt AJ, Secker GA, Munro PM, Khaw PT, Tuft SJ, Daniels JT. Characterization of the limbal epithelial stem cell niche: Novel imaging techniques permit *in vivo* observation and targeted biopsy of limbal epithelial stem cells. Stem Cells 2007;25:1402-9.
19. Lauweryns B, van den Oord JJ, De Vos R, Missotten L. A new epithelial cell type in the human cornea. Invest Ophthalmol Vis Sci 1993;34:1983-90.
20. Wiley L, SundarRaj N, Sun TT, Thoft RA. Regional heterogeneity in human corneal and limbal epithelia: An immunohistochemical evaluation. Invest Ophthalmol Vis Sci 1991;32:594-602.
21. Higa K, Shimmura S, Miyashita H, Shimazaki J, Tsubota K. Melanocytes in the corneal limbus interact with K19-positive basal epithelial cells. Exp Eye Res 2005;81:218-23.
22. Dua HS, Shanmuganathan VA, Powell Richards AO, Tighe PJ, Joseph A. Limbal epithelial crypts: A novel anatomical structure and a putative limbal stem cell niche. Br J Ophthalmol 2005;89:529-32.
23. Fernandes M, Sangwan VS, Rao SK, Basti S, Sridhar MS, Bansal AK, *et al*. Limbal stem cell transplantation. Indian J Ophthalmol 2004;52:5-22.
24. Kruse EF. Stem cells and corneal regeneration. Eye (Lond) 1994;8 (Pt 2):170-83.
25. Dua HS, Gomes JA, Singh A. Corneal epithelial wound healing. Br J Ophthalmol 1994;78:401-8.
26. Dua HS, Forrester HV. The corneo-scleral limbus in human corneal epithelial wound healing. Am J Ophthalmol 1990;110:646-56.
27. Tseng SC, Chen JJ, Huang AJ. Classification of conjunctival surgeries for corneal disease based on stem cell concept. Ophthalmol Clin North Am 1990:595-610.
28. Kinoshita S, Kiritoshi A, Ohji M. Disappearance of palisades of Vogt in ocular surface disease. Jpn J Clin Ophthalmol 1986;40:363-66.
29. Puangsricharern V, Tseng SC. Cytologic evidence of corneal disease with limbal stem cell deficiency. Ophthalmology 1995;103:1476-85.
30. Egbert PR, Lauber S, Maurice DM. A simple conjunctival biopsy. Am J Ophthalmol 1977;84:798-801.
31. Tsubota K, Toda I, Saito H, Shinozaki N, Shimazaki J. Reconstruction of the corneal epithelium by limbal allograft transplantation for severe ocular surface disorders. Ophthalmology 1995;102:486-96.
32. Brown SI, Bloomfield SE, Pearce DB. Follow-up report on transplantation of the alkali burned cornea. Am J Ophthalmol 1974;77:538-42.
33. DiPascuale MA, Espana EM, Tzong-Shyue Liu D, Kawakita T, Li W, Gao YY, *et al*. Complications with eyelid cicatricial pathologies in patients with Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis Syndrome. Ophthalmology 2005;112 (5):904-12.
34. Subramaniam SV, Sejpal K, Fatima A, Gaddipati S, Vemuganti GK, Sangwan VS. Coculture of autologous limbal and conjunctival epithelial cells to treat severe ocular surface disorders: Long-term survival analysis. Indian J Ophthalmol 2013;61:202-7.
35. Tsai RJ, Sun TT, Tseng SC. Comparison of limbal and conjunctival autograft transplantation in corneal surface reconstruction in rabbits. Ophthalmology 1990;97:446-55.
36. Kenyon KR, Tseng SC. Limbal autograft transplantation for ocular surface disorders. Ophthalmology 1989;96:709-22.
37. Morgan S, Murray A. Limbal autotransplantation in the acute and chronic phases of severe chemical injuries. Eye (Lond) 1996;10 (Pt 3):349-54.
38. Frucht-Pery J, Siganos CS, Solomon A, Scheman L, Brautbar C, Zauberman H. Limbal cell autograft transplantation for severe ocular surface disorders. Graefes Arch Clin Exp Ophthalmol 1998;236:582-7.
39. Basti S, Mathur U. Unusual intermediate-term outcome in three cases of limbal autograft transplantation. Ophthalmology 1999;106:958-63.
40. Rao SK, Rajagopal R, Sitalakshmi G, Padmanabhan P. Limbal autografting: Comparison of results in the acute and chronic phases of ocular surface burns. Cornea 1999;18:164-71.
41. Dua HS, Azuara-Blanco A. Autologous limbal transplantation in patients with unilateral corneal stem cell deficiency. Br J Ophthalmol 2000;84:273-8.
42. Ozdemir O, Tekeli O, Ornek K, Arslanpençe A, Yalçindağ NF. Limbal autograft and allograft transplantations in patients with corneal burns. Eye (Lond) 2004;18:241-8.
43. Santos MS, Gomes JA, Hofling-Lima AL, Rizzo LV, Romano AC, Belfort R Jr. Survival analysis of conjunctival limbal grafts and amniotic membrane transplantation in eyes with total limbalstem cell deficiency. Am J Ophthalmol 2005;140:223-30.
44. Miri A, Al-Deiri B, Dua HS. Long-term outcomes of autolimbal and allolimbal transplants. Ophthalmology 2010;117:1207-13.
45. Miri A, Said DG, Dua HS. Donor site complications in autolimbal and living-related allolimbal transplantation. Ophthalmology 2011;118:1265-71.
46. Welder JD, Pandya HK, Nassiri N, Djalilian AR. Conjunctival limbal autograft and allograft transplantation using fibrin glue. Ophthalmic Surg Lasers Imaging 2012;43:323-7.
47. Tan DT, Ficker LA, Buckley RJ. Limbal transplantation. Ophthalmology 1996;103:29-36.
48. Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet 1997;349:990-3.
49. Schwab IR. Cultured corneal epithelia for ocular surface disease. Trans Am Ophthalmol Soc 1999;97:891-986.

50. Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. Cornea 2000;19:421-6.
51. Rama P, Bonini S, Lambiase A, Golisano O, Paterna P, De Luca M, *et al.* Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. Transplantation 2001;72:1478-85.
52. Shortt AJ, Secker GA, Rajan MS, Meligonis G, Dart JK, Tuft SJ, *et al. Ex vivo* expansion and transplantation of limbal epithelial stem cells. Ophthalmology 2008;115:1989-97.
53. Gisoldi Ram C, Pocobelli A, Villani CM, Amato D, Pellegrini G. Evaluation of molecular markers in corneal regeneration by means of autologous cultures of limbal cells and keratoplasty. Cornea 2010;29:715-22.
54. Rama P, Matuska S, Paganoni G, Spinelli A, De Luca M, Pellegrini G. Limbal stem-cell therapy and long-term corneal regeneration. N Engl J Med 2010;363:147-55.
55. Di Iorio E, Ferrari S, Fasolo A, Böhm E, Ponzin D, Barbaro V. Techniques for culture and assessment of limbal stem cell grafts. Ocul Surf 2010;8:146-53.
56. Tsai RJ, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. N Engl J Med 2000;343:86-93.
57. Grueterich M, Espana EM, Touhami A, Ti SE, Tseng SC. Phenotypic study of a case with successful transplantation of *ex vivo* expanded human limbal epithelium for unilateral total limbal stem cell deficiency. Ophthalmology 2002;109:1547-52.
58. Sangwan VS, Vemuganti GK, Iftekhar G, Bansal AK, Rao GN. Use of autologous cultured limbal and conjunctival epithelium in a patient with severe bilateral ocular surface disease induced by acid injury: A case report of unique application. Cornea 2003;22:478-81.
59. Nakamura T, Inatomi T, Sotozono C, Koizumi N, Kinoshita S. Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease. Acta Ophthalmol Scand 2004;82:468-71.
60. Kawashima M, Kawakita T, Satake Y, Higa K, Shimazaki J. Phenotypic study after cultivated limbal epithelial transplantation for limbal stem cell deficiency. Arch Ophthalmol 2007;125:1337-44.
61. Pauklin M, Fuchsluger TA, Westekemper H, Steuhl KP, Meller D. Midterm results of cultivated autologous and allogeneic limbal epithelial transplantation in limbal stem cell deficiency. Dev Ophthalmol 2010;45:57-70.
62. Baradaran-Rafii A, Ebrahimi M, Kanavi MR, Taghi-Abadi E, Aghdami N, Eslani M, *et al.* Midterm outcomes of autologous cultivated limbal stem cell transplantation with or without penetrating keratoplasty. Cornea 2010;29:502-9.
63. Prabhasawat P, Ekpo P, Uiprasertkul M, Chotikavanich S, Tesavibul N. Efficacy of cultivated corneal epithelial stem cells for ocular surface reconstruction. Clin Ophthalmol 2012;6:1483-92.
64. Nakamura T, Inatomi T, Sotozono C, Koizumi N, Yokoi N, Kinoshita S. Transplantation of autologous serum-derived cultivated corneal epithelial equivalents for the treatment of severe ocular surface disease. Ophthalmology 2006;113:1765-72.
65. Shimazaki J, Higa K, Morito F, Kawakita T, Satake Y, Shimmura S, *et al.* Factors influencing outcomes in cultivated limbal epithelial transplantation for chronic cicatricial ocular surface disorders. Am J Ophthalmol 2007;143:945-53.
66. Di Girolamo N, Bosch M, Zamora K, Coroneo MT, Wakefield D, Watson SL. A contact lens-based technique for expansion and transplantation of autologous epithelial progenitors for ocular surface reconstruction. Transplantation 2009;87:1571-8.
67. Kolli S, Ahmad S, Lako M, Figueiredo F. Successful clinical implementation of corneal epithelial stem cell therapy for treatment of unilateral limbal stem cell deficiency. Stem Cells 2010;28:597-610.
68. Sangwan VS, Basu S, Vemuganti GK, Sejpal K, Subramaniam SV, Bandyopadhyay S, *et al.* Clinical outcomes of xeno-free autologous cultivated limbal epithelial transplantation: A 10-year study. Br J Ophthalmol 2011;95:1525-9.
69. Sejpal K, Ali MH, Maddileti S, Basu S, Ramappa M, Kekunnaya R, *et al.* Cultivated limbal epithelial transplantation in children with ocular surface burns. JAMA Ophthalmol 2013;131:731-6.
70. Meller D, Pauklin M, Westekemper H, Steuhl KP. Autologous Transplantation of cultivated limbal epithelium. Ophthalmologe 2010;107:1133-8.
71. Thanos M, Pauklin M, Steuhl KP, Meller D. Ocular surface reconstruction with cultivated limbal epithelium in a patient with unilateral stem cell deficiency caused by Epidermolysis Bullosa Dystrophica Hallopeau-Siemens. Cornea 2010;29:462-4.
72. Marchini G, Pedrotti E, Pedrotti M, Barbaro V, Di Iorio E, Ferrari S, *et al.* Long-term effectiveness of autologous cultured limbal stem cell grafts in patients with limbal stem cell deficiency due to chemical burns. Clin Experiment Ophthalmol 2012;40:255-67.
73. Sharma S, Tandon R, Mohanty S, Kashyap S, Vanathi M. Phenotypic evaluation of severely damaged ocular surface after reconstruction by cultured limbal epithelial cell transplantation. Ophthalmic Res 2013;50:59-64.
74. Mariappan I, Maddileti S, Savy S, Tiwari S, Gaddipati S, Fatima A, *et al. In vitro* culture and expansion of human limbal epithelial cells. Nat Prot 2010;5:1470-9.
75. Pellegrini G, Rama P, Matuska S, Lambiase A, Bonini S, Pocobelli A, *et al.* Biological parameters determining the clinical outcome of autologous cultures of limbal stem cells. Regen Med 2013;8:553-67.
76. Basu S, Mohamed A, Chaurasia S, Sejpal K, Vemuganti GK, Sangwan VS. Clinical outcomes of penetrating keratoplasty after autologous cultivated limbal epithelial transplantation for ocular surface burns. Am J Ophthalmol 2011;152:917-24.
77. Basu S, Ali MH, Sangwan VS. Clinical outcomes of repeat autologous cultivated limbal epithelial transplantation for ocular surface burns. Am J Ophthalmol 2012;153:643-50.
78. Vazirani J, Basu S, Kenia H, Ali MH, Kacham S, Mariappan I, *et al.* Unilateral partial limbal stem cell deficiency: contralateral versus ipsilateral autologous cultivated limbal epithelial transplantation. Am J Ophthalmol 2013.
79. Sangwan VS, Basu S, MacNeil S, Balasubramanian D. Simple limbal epithelial transplantation (SLET): A novel surgical technique for the treatment of unilateral limbal stem cell deficiency. Br J Ophthalmol 2012;96:931-4.
80. Lal I, Panchal BU, Basu S, Sangwan VS. *In-vivo* expansion of autologous limbal stem cell using simple limbal epithelial transplantation for treatment of limbal stem cell deficiency. BMJ Case Rep 2013;2013.
81. Bhalekar S, Basu S, Lal I, Sangwan VS. Successful autologous simple limbal epithelial transplantation (SLET) in previously failed paediatric limbal transplantation for ocular surface burns. BMJ Case Rep 2013;2013.
82. Bhalekar S, Basu S, Sangwan VS. Successful management of immunological rejection following allogenic simple limbal epithelial transplantation (SLET) for bilateral ocular burns. BMJ Case Rep 2013;2013.
83. Tsai RJ, Tseng SC. Human allograft limbal transplantation for corneal surface reconstruction. Cornea 1994;13:389-400.
84. Tsubota K, Satake Y, Ohyama M, Toda I, Takano Y, Ono M, *et al.* Surgical reconstruction of the ocular surface in advanced ocular cicatricial pemphigoid and Stevens-Johnson syndrome. Am J Ophthalmol 1996;122:38-52.
85. Javadi MA, Baradaran-Rafii A. Living-related conjunctival-limbal allograft for chronic or delayed-onset mustard gas keratopathy.

Cornea 2009;28:51-7.

86. Rao SK, Rajagopal R, Sitalakshmi G, Padmanabhan P. Limbal allografting from related live donors for corneal surface reconstruction. Ophthalmology 1999;106:822-8.
87. Chuck RS, Behrens A, McDonnell PJ. Microkeratome-based limbal harvester for limbal stem cell transplantation: Preliminary studies. Am J Ophthalmol 2001;131:377-8.
88. Mannis MJ, McCarthy M, Izquierdo L Jr. Technique for harvesting keratolimbal allografts from corneoscleral buttons. Am J Ophthalmol 1999;128:237-8.
89. Aldave AJ, Wong IG. A novel technique for harvesting keratolimbal allografts from corneoscleral buttons. Am J Ophthalmol 2002;134:929-31.
90. Meisler DM, Perez VL, Proudfit J. A device to facilitate limbal stemcell procurement from eye bank donor tissue for keratolimbal allograft procedures. Am J Ophthalmol 2005;139:212-4.
91. Lim LT, Bhatt PR, Ramaesh K. Harvesting keratolimbal allografts from corneoscleral buttons: A novel application of cyanoacrylate adhesive. Br J Ophthalmol 2008;92:1550-1.
92. Nassiri N, Pandya HK, Djalilian AR. Limbal allograft transplantation using fibrin glue. Arch Ophthalmol 2011;129:218-22.
93. Vemuganti GK, Kashyap S, Sangwan VS, Singh S. *Ex-vivo* potential of cadaveric and fresh limbal tissues to regenerate cultured epithelium. Ind J Ophthalmol 2004;52:113-20.
94. Tsubota K, Shimmura S, Shinozaki N, Holland EJ, Shimazaki J. Clinical application of living-related conjunctival-limbal allograft. Am J Ophthalmol 2002;133:134-5.
95. Baylis O, Figueiredo F, Henein C, Lako M, Ahmad S. 13 Years of cultured limbal epithelial cell therapy: A review of the outcomes. J Cell Biochem 2011;112:993-1002.
96. Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. Ophthalmology 2001;108:1569-74.
97. Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S. Cultivated corneal epithelial transplantation for ocular surface reconstruction in acute phase of Stevens-Johnson syndrome. Arch Ophthalmol 2001;119:298-300.
98. Shimazaki J, Aiba M, Goto E, Kato N, Shimmura S, Tsubota K. Transplantation of human limbal epithelium cultivated on amniotic membrane for the treatment of severe ocular surface disorders. Ophthalmology 2002;109:1285-90.
99. Sangwan VS, Fernandes M, Bansal AK, Vemuganti GK, Rao GN. Early results of penetrating keratoplasty following limbal stem cell transplantation. Indian J Ophthalmol 2005;53:31-5.
100. Daya SM, Watson A, Sharpe JR, Giledi O, Rowe A, Martin R, *et al.*: Outcomes and DNA analysis of *ex vivo* expanded stem cell allograft for ocular surface reconstruction. Ophthalmology 2005;112:470-7.
101. Ang LP, Sotozono C, Koizumi N, Suzuki T, Inatomi T, Kinoshita S. A comparison between cultivated and conventional limbal stem cell transplantation for stevens-johnson syndrome. Am J Ophthalmol 2007;143:178-80.
102. Meller D, Fuchsluger T, Pauklin M, Steuhl KP. Ocular surface reconstruction in graft-versus-host disease with HLA-identical living-related allogeneic cultivated limbal epithelium after hematopoietic stem cell transplantation from the same donor. Cornea 2009;28:233-6.
103. Basu S, Fernandez MM, Das S, Gaddipati S, Vemuganti GK, Sangwan VS. Clinical outcomes of xeno-free allogeneic cultivated limbal epithelial transplantation for bilateral limbal stem cell deficiency. Br J Ophthalmol 2012;96:1504-9.
104. Nishida K, Yamato M, Hayashida Y, Watanabe K, Yamamoto K, Adachi E, *et al.* Corneal reconstruction with tissue engineered cell sheets composed of autologous oral mucosal epithelium. N Engl J Med 2004;16:1187-96.
105. Nakamura T, Inatomi T, Sotozono C, Amemiya T, Kanamura N, Kinoshita S. Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. Br J Ophthalmol 2004;88:1280-4
106. Inatomi T, Nakamura T, Koizumi N, Sotozono C, Yokoi N, Kinoshita S. Midterm results on ocular surface reconstruction using cultivated autologous oral mucosal epithelial transplantation. Am J Ophthalmol 2006;141:267-75.
107. Inatomi T, Nakamura T, Kojyo M, Koizumi N, Sotozono C, Kinoshita S. Ocular surface reconstruction with combination of cultivated autologous oral mucosal epithelial transplantation and penetrating keratoplasty. Am J Ophthalmol 2006;142:757-64.
108. Ang LP, Nakamura T, Inatomi T, Sotozono C, Koizumi N, Yokoi N, *et al.* Autologous serum-derived cultivated oral epithelial transplants for severe ocular surface disease. Arch Ophthalmol 2006;124:1543-51.
109. Ma DH, Kuo MT, Tsai YJ, Chen HC, Chen XL, Wang SF, *et al.* Transplantation of cultivated oral mucosal epithelial cells for severe corneal burn. Eye (Lond) 2009;23:1442-50.
110. Uchino Y, Uchino M, Shimazaki J. Combination treatment of intravenous immunoglobulin and cultivated oral mucosal epithelial transplantation for ocular cicatricial pemphigoid. BMJ Case Rep 2009;2009.
111. Chen HC, Chen HL, Lai JY, Chen CC, Tsai YJ, Kuo MT, *et al.* Persistence of transplanted oral mucosal epithelial cells in human cornea. Invest Ophthalmol Vis Sci 2009;50:4660-8.
112. Nakamura, T, Takeda K, Inatomi T, Sotozono C, Kinoshita S. Long-term results of autologous cultivated oral mucosal epithelial transplantation in the scar phase of severe ocular surface disorders. Br J Ophthalmol 2011;95:942-6.
113. Satake Y, Higa K, Tsubota K, Shimazaki J. Long-term outcome of cultivated oral mucosal epithelial sheet transplantation in treatment of total limbal stem cell deficiency. Ophthalmology 2011;118:1524-30.
114. Takeda K, Nakamura T, Inatomi T, Sotozono C, Watanabe A, Kinoshita S. Ocular surface reconstruction using the combination of autologous cultivated oral mucosal epithelial transplantation and eyelid surgery for severe ocular surface disease. Am J Ophthalmol 2011;152:195-201.
115. Hirayama M, Satake Y, Higa K, Yamaguchi T, Shimazaki J. Transplantation of cultivated oral mucosal epithelium prepared in fibrin-coated culture dishes. Invest Ophthalmol Vis Sci 2012;53:1602-9.
116. Burillon C, Huot L, Justin V, Nataf S, Chapuis F, Decullier E, *et al.* Cultured autologous oral mucosal epithelial cell sheet (CAOMECS) transplantation for the treatment of corneal limbal epithelial stem cell deficiency. Invest Ophthalmol Vis Sci 2012;53:1325-31.
117. Sotozono C, Inatomi T, Nakamura T, Koizumi N, Yokoi N, Ueta M, *et al.* Visual improvement after cultivated oral mucosal epithelial transplantation. Ophthalmology 2013;120:193-200.
118. Deshpande P, Ramachandran C, Sefat F, Mariappan I, Johnson C, McKean R, *et al.* Simplifying corneal surface regeneration using a biodegradable synthetic membrane and limbal tissue explants. Biomaterials 2013;34:5088-106.
119. Li X, Upadhyay AK, Bullock AJ, Dicolandrea T, Xu J, Binder RL, *et al.* Skin stem cell hypotheses and long term clone survival – explored using agent based modelling. Sci Rep 2013;3.1904.

Cite this article as: Sangwan VS, Jain R, Basu S, Bagadi AB, Sureka S, Mariappan I, *et al.* Transforming ocular surface stem cell research into successful clinical practice. Indian J Ophthalmol 2014;62:29-40.

Source of Support: Nil. **Conflict of Interest:** None declared.