Characterisation of Cultured Oral Mucosal Epithelial Cells and the Outcome after Autologous Transplantation to Diseased Ocular Surface

SYNOPSIS

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SYNOPSIS

Limbus is the niche for the stem cells of human corneal epithelium [1-7]. Epithelial cells are continuously renewed from the limbal region and they migrate centripetally on the cornea[8]. Corneal epithelium is renewed in 9 to 12 months [9] as opposed to every month in case of human epidermis[10]. Limbal stem cell deficiency (LSCD) is a rare cause of corneal blindness which results from physical, chemical or immunological damage to the corneal epithelial stem cells located at the limbus[1, 3]. In unilateral cases LSCD can be treated by either conventional or cultured autologous limbal transplantation from the unaffected fellow eye[11, 12]. However, in bilateral cases there is no autologous source for limbal stem cells so either a living or a cadaveric allogeneic donor is required[13]. An alternative to allogeneic limbal grafting, which necessitates long-term systemic immunosuppression, is transplantation of autologous epithelium from non-ocular sources[14].

The possibility of oral mucosa being used as a substitute for limbal epithelium was considered because of the phenotypic semblance between the two epithelial lineages[15, 16]. Animal trials and preliminary human trials also demonstrated that the *ex-vivo* cultured oral mucosa could be a suitable therapeutic alternative to limbal epithelium in eyes with LSCD [17-22]. However the cell culture protocols described for cultivating oral mucosal cells for human transplantation utilized various animal derived or xeno-biotic materials [18-28]. Use of xeno-biotic materials in cell culture for clinical use is undesirable as it carries the risk of transmitting known or unknown infections to the transplant recipient [29]. To avoid xeno-biotic usage, we developed a xeno-free technique of culturing oral mucosal cells[15], adopted from our standardized protocol for limbal epithelial

cultivation[30], which has been used successfully to treat over 500 eyes with unilateral LSCD[31-33]. In this study we reported an improved method for culture of oral epithelial cells on de-epithelialized amniotic membrane using explant culture technique, without the use of any feeder cells. To check the feasibility of using these cells as an alternative to limbal cells, we have compared the characteristics of cultured oral cells with those of cultured limbal and conjunctival cells. In addition we have also investigated the clinical outcomes and immunohistochemical findings in eyes with bilateral LSCD following ocular surface burns, treated by xeno-free autologous cultured oral mucosal epithelial transplantation (COMET).

Phase I

Studies were carried out after the protocol was approved by the Institutional Review Board. Experiments were carried out using oral tissue harvested from healthy adult volunteers and patients after obtaining informed consent and performing pre-surgical evaluation of ocular and oral health. A mucosal biopsy of 3 mm X 3 mm was harvested from the buccal surface of the lower lip. Human amniotic membrane was de-epithelialised using 0.25% Trypsin-EDTA at 37°C for 30 minutes followed by mechanical scraping and washing with phosphate-buffered saline (PBS). The tissue was cut into small pieces using a sterile surgical blade and placed on de-epithelialized HAM. Tissue explants were allowed to adhere to de-epithelialized HAM and explants were cultured in human corneal epithelial medium (HCE, which contains Minimal Essential Medium and Ham's F12 in 1:2 ratio), along with epidermal growth factor, insulin, penicillin, streptomycin, amphotericin-B, gentamycin and 10% autologus serum, for a period of three to four weeks in a humidified incubator at 37°C with 5% CO_2. The

medium was changed every other day. Limbal and conjunctival biopsies were cultured in a similar manner. Characterisation of the cultured cells was undertaken by hematoxylin-eosin (HE) staining, periodic acid schiff (PAS) staining, Transmission Electron Microscopy, Reverse Transcription-Polymerase Chain Reaction, Immunohistochemistry, cell cycle analysis, FACS.

RT-PCR analysis was carried out to check presence [34] of stem cell markers, namely, isoforms of p63, which is a stem cell-associated marker in all stratified epithelia and p75, a marker for stem cells in oral epithelia [35]. Previous studies have shown that the limbal cultures express p63, specifically ΔN isoforms of p63 [34,36]. All three cultured cells expressed ΔNp63α, ΔNp63β and ΔNp63γ. Recently p75 was shown to be a marker for oral stem cells [35] and expression of p75 was also observed in basal cells of the limbus [37,38]. All three cell types expressed p75.

IHC followed by RT-PCR showed both limbal and oral epithelial cells expressed cytokeratin K3. The oral epithelial cultures however did not express cytokeratin K12, conjunctival cultures also expressed cytokeratin 3 and K12. The cells also expressed cytokeratins K4 and K13, markers of non-keratinized stratified oral epithelia [39,40], as seen earlier in rabbit oral epithelial cultures [18]. These cytokeratins were also expressed by cultured limbal and conjunctival cultures. Cytokeratin 15, expressed in basal and suprabasal cells of limbus [41] , was also observed in all three cultured cells. Connexin 43, another marker for differentiated epithelial cells was also expressed by all these cells. Cultured oral cells also express Pax-6, a marker for ocular tissues.

We report here successful cultivation of oral mucosal epithelial cells using de-epithelialized amniotic membrane without the use of feeder cells, using the explant

culture technique. Phenotypic studies indicate that the cultures are a heterogeneous population expressing markers of differentiated epithelial cells as well as stem cells. These oral epithelial cultures were considered as suitable for ocular surface reconstruction in patients with bilateral LSCD. The advantage of the technique used here is it precludes the use of feeder cells, which are of animal origin. Although feeder cell-free cultures of oral epithelial cells have been established using temperature-responsive culture surface [42] , such a technique has not been reported so far using amniotic membrane. As these cells can also be cultured in the presence of autologous serum as has been reported earlier [23], This technique reduces the risk of introducing xenobiotic agents in the patient.

Our studies indicate that, the three cultures express markers of stratified epithelia such as cytokeratin K3, K4, K13, K15, Pax-6 and connexin 43. The cultured oral cells do not express cytokeratin K12. The cultured oral epithelial cells thus maintain their original phenotype as has been reported by other groups [18,19]. The absence of cytokeratin K12 may not interfere with ocular surface regeneration. Although it is required for the integrity of corneal epithelium as suggested by knock-out studies [43], it is not necessary for the integrity of oral epithelium. The oral cells expressed stem cell-associated marker such as all α, β and γ isoforms of ΔN p63 as seen by RTPCR analysis. This was also confirmed by immunohistochemistry. Where cultures underwent stratification; the expression of p63 was observed in the basal layer. Expression of p75 was also seen by RT-PCR. However in immunohistochemistry few cells expressing p75 were observed. It is possible that p75 is a more appropriate marker for the stem cells of oral epithelium and as the number of stem cells in the culture may not be very high, few cells expressing p75

were observed. p75 was also expressed in limbal and conjunctival cultures as has been reported earlier [38,44].

To summarize, we have established cultures of oral mucosal epithelial cells on human amniotic membrane without the use of feeder cells. The cultured cells are morphologically and phenotypically similar to the cultured limbal cells. Using this technique, we have now initiated clinical trial for ocular surface reconstruction in patients suffering from bilateral limbal stem cell deficiency.

Phase II

At the L V Prasad Eye Institute, Hyderabad, India autologous COMET was offered as an alternative to allogeneic cultivated limbal epithelial transplantation, between October 1, 2007 and November 1, 2010, to patients with bilateral and total LSCD (defined clinically as 360^{o} superficial corneal vascularization, diffuse fluorescein staining of the corneal surface with or without persistent epithelial defects, conjunctivalization of the corneal surface and absence of limbal palisades of Vogt) following ocular surface burns.

All patients were seen on post-COMET day one, at one week, at six weeks, and thereafter every six to eight weeks. Each examination included a complete history, including any new ocular or systemic symptoms, a complete ocular examination including fluorescein staining, and any signs of neovascularization or surface instability. The post-operative clinical assessment was performed by one ocular surface specialist [45].

Based on the clinical appearance of the corneal surface an impression of success or failure of therapy was made. 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 secondary clinical outcomes were improvement in BCVA from baseline and ocular and oral complications.

The data retrieved from the medical records included age and sex of the patient, type and date of injury, details of prior ocular procedures, Snellen's best spectacle corrected visual acuity (BCVA) and at each follow-up visit, presence or absence of lid abnormalities, dry eye disease, symblepharon, degree of limbal involvement, intra-operative surgical details, post-operative complications, duration of follow-up and status of ocular surface at each visit (slit-lamp findings including fluorescein staining).

During the entire study period 19 eyes of 18 patients with bilateral and total LSCD following ocular surface burns underwent autologous COMET. 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. The mean follow-up was 22.3 (range: 7 to 48) months. Post-operatively 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 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%) eyes the peripheral vascularization did not progress and the corneal surface was completely epithelized and 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. Prior to COMET the BCVA ranged from hand movements to perception of light in all eyes. On the last date of follow-up or before undergoing keratoplasty or keratoprosthesis surgery the BCVA had not improved in 12 (63%) eyes, had improved to counting fingers in 6 (32%) eyes and to 20/125 (5%) in one eye. Of the 7 eyes with a stable ocular surface, one eye underwent PK and four eyes underwent Boston type 1 keratoprosthesis surgery for visual improvement. Following PK the corneal graft developed repeated epithelial defects and a permanent tarsorrhaphy had to be performed three months later. Three years after PK the BCVA with an intact tarsorrhaphy was hand movements. The final BCVA in the four eyes that underwent Boston type 1 keratoprosthesis ranged from 20/20 to 20/30 with a maximum follow-up of 26 months. (a) hematoxylin and eosin and PAS staining of the pannus excised during COMET showed eight to ten layer thick stratified columnar epithelium with presence of goblet cells and underlying loose fibrovascular stromal tissue. These findings were consistent the clinical impression of LSCD. (b) hematoxylin and eosin and PAS staining of the unused back-up oral mucosal culture showed a monolayer of epithelium on a thick eosinophilic membrane. (c) hematoxylin and eosin staining of the corneal buttons excised during keratoplasty or keratoprosthesis surgery following COMET showed a six to eight cell stratified epithelium with basement membrane. No remnants of the hAM were seen. Goblet cells were not observed in PAS staining. A few sub-epithelial blood vessels were also seen in close proximity to the basement membrane both at the periphery and at the centre. Bowman's membrane was

absent and variable stromal scarring was noted. The Descemet's and endothelial complex was noted to be normal in all eyes.

Immunohistochemistry of excised corneal buttons showed (1) K19 being expressed in the basal layer of the epithelial cells of post-COMET corneas, in the basal layer of the limbal epithelium in control corneas, in all layers of the conjunctiva and in the basal cells of the oral mucosa; (2) expression of K14 was absent in post-COMET corneas, absent in control corneas, present in the basal cells of conjunctiva, absent in oral mucosa; [46] Cytoplasmic K3/K12 expression was seen in all epithelial layers of post-COMET corneas, control corneas and oral mucosa but absent in conjunctiva; (4) Cytoplasmic K12 staining was seen only in the control corneal epithelium and was absent in oral, conjunctival and control corneal epithelium; (5) Ki-67 expression was seen in the supra-basal layer of all specimens;(6) p63 expression was seen in basal and supra-basal layers of the post-COMET corneas, control corneas, conjunctiva and oral mucosa; (7) p75 expression was seen in basal epithelial cells of post-COMET corneas, basal epithelial cells of the limbus in control corneas, basal cells of conjunctiva as well as oral mucosa; (8) CD31 and CD34 expression was seen in sub-epithelial layers of the central and peripheral post-COMET corneas.

A comparison between this study and previous studies on COMET with those on allogeneic limbal transplantation is again difficult, because the indications and sample sizes vary among different studies. Indeed, there are no comparable published studies (with a sample size of five eyes or more) of allogeneic cultivated limbal transplantation in eyes with ocular burns [47,48]. With regards to keratolimbal allografts, in two series of 16 and 17 eyes with ocular burns among other indications, Solomon and associates [49] and

Maruyama-Hosoi and associates [50].reported long-term corneal epithelial stability in 71.3% and 58.8% eyes respectively. Similar to ocular surface stability, the proportion of patients who gained 20/200 or better vision, after keratolimbal allografting (43.5% to 44.6%) [49,50] was also greater as compared to that after COMET (7% to 30%) [21-23,26-28]. This limitation of COMET is particularly significant because unlike patients with unilateral LSCD, who usually have good vision in the unaffected eye and may be satisfied with a stable and symptom free ocular surface in the affected eye, the primary need of a patient with bilateral blindness is improvement in vision. Therefore the benefit of COMET of being an autologous therapy not requiring immunosuppression, does not outweigh its poor clinical outcomes. In view of these results, currently we do not offer COMET to patients with bilateral LSCD.

Other findings of our study were similar to Chen and associates and Nakamura and associates who performed histopathology and immunohistochemical analysis in four and six post-COMET eyes, respectively [24,51]. On histopathology, they found the transplanted epithelium to be five to twelve layers thick without goblet cells or apical microvilli. On immunohistochemistry, they also found that K3 was present in all epithelial layers, K12 was present occasionally at the peripheral portion of corneal tissue, p63 and p75 was present in the basal epithelial layers. These findings along with ours suggest that the transplanted oral mucosal epithelium maintains its original phenotype without any trans-differentiation to the corneal phenotype. Additionally we showed expression of vascular endothelial markers CD31 and CD34 in the sub-epithelial region of the post-COMET corneas to corroborate with the clinical findings of superficial vascularization.

This is the first report on transplantation of oral mucosal cells cultivated using a xeno-free technique of cell culture. Another strength of this study is the homogeneity of the patient cohort; being the largest such study in cases with bilateral ocular burns. Unlike others we used an explant culture technique and transplanted at a monolayer stage, like we do for cultivated limbal epithelial transplantation [30-33]. It may be argued that these unconventional techniques of cultivation and transplantation may have affected the results. But similar poor outcomes of COMET in ocular burns have been reported with conventional cell culture protocols as well [27,28]. We also found that the oral epithelium does not convert to a corneal phenotype when transplanted onto the ocular surface and because of the associated vascularization, which is probably essential to its survival, a conjunctivalized ocular surface and one reconstructed after COMET are virtually indistinguishable.

In summary, the findings of our study suggest that transplantation of autologous oral mucosal epithelium cultivated using a xeno-free explant culture system, is limited success in restoring a stable ocular surface and improving vision in eyes with bilateral LSCD following ocular burns so clinical application should be judiciously decided by the patient and the surgeon weighing the risk benefit ratio. However, our results do not apply to other causes of LSCD or other cell-culture protocols.

References

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

2 Lavker, R. M. *et al.* Relative proliferative rates of limbal and corneal epithelia. Implications of corneal epithelial migration, circadian rhythm, and suprabasally located DNA-synthesizing keratinocytes. *Invest Ophthalmol Vis Sci* **32**, 1864-1875 (1991).

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

4 Tseng, S. C. & Tsai, R. J. Limbal transplantation for ocular surface reconstruction--a review. *Fortschr Ophthalmol* **88**, 236-242 (1991).

5 Davanger, M. & Evensen, A. Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature* **229**, 560-561 (1971).

6 Thoft, R. A. The role of the limbus in ocular surface maintenance and repair. *Acta Ophthalmol Suppl* **192**, 91-94 (1989).

7 Thoft, R. A., Wiley, L. A. & Sundarraj, N. The multipotential cells of the limbus. *Eye (Lond)* **3 (Pt 2)**, 109-113 (1989).

8 Sharma, A. & Coles, W. H. Kinetics of corneal epithelial maintenance and graft loss. A population balance model. *Invest Ophthalmol Vis Sci* **30**, 1962-1971 (1989).

9 Wagoner, M. D. Chemical injuries of the eye: current concepts in pathophysiology and therapy. *Surv Ophthalmol* **41**, 275-313 (1997).

10 Green, H. The keratinocyte as differentiated cell type. *Harvey Lect* **74**, 101-139 (1980).

11 Kenyon, K. R. & Tseng, S. C. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology* **96**, 709-722; discussion 722-703 (1989).

12 Pellegrini, G. *et al.* Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet* **349**, 990-993 (1997).

13 Fernandes, M. *et al.* Limbal stem cell transplantation. *Indian J Ophthalmol* **52**, 5-22 (2004).

14 Abrez, H., Biswas, J. & Sudharshan, S. Clinical profile, treatment, and visual outcome of serpiginous choroiditis. *Ocular immunology and inflammation* **15**, 325-335, doi:10.1080/09273940701375162 (2007).

15 Madhira, S. L. *et al.* Culture and characterization of oral mucosal epithelial cells on human amniotic membrane for ocular surface reconstruction. *Mol Vis* **14**, 189-196 (2008).

16 Krishnan, S., Iyer, G. K. & Krishnakumar, S. Culture & characterisation of limbal epithelial cells & oral mucosal cells. *The Indian journal of medical research* **131**, 422-428 (2010).

17 Gipson, I. K., Geggel, H. S. & Spurr-Michaud, S. J. Transplant of oral mucosal epithelium to rabbit ocular surface wounds in vivo. *Arch Ophthalmol* **104**, 1529-1533 (1986).

18 Nakamura, T. *et al.* The successful culture and autologous transplantation of rabbit oral mucosal epithelial cells on amniotic membrane. *Invest Ophthalmol Vis Sci* **44**, 106-116 (2003).

19 Hayashida, Y. *et al.* Ocular surface reconstruction using autologous rabbit oral mucosal epithelial sheets fabricated ex vivo on a temperature-responsive culture surface. *Invest Ophthalmol Vis Sci* **46**, 1632-1639 (2005).

20 Nishida, K. *et al.* Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N Engl J Med* **351**, 1187-1196 (2004).

21 Nakamura, T. *et al.* Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. *Br J Ophthalmol* **88**, 1280-1284 (2004).

22 Inatomi, T. *et al.* Midterm results on ocular surface reconstruction using cultivated autologous oral mucosal epithelial transplantation. *Am J Ophthalmol* **141**, 267-275 (2006).

23 Ang, L. P. *et al.* Autologous serum-derived cultivated oral epithelial transplants for severe ocular surface disease. *Arch Ophthalmol* **124**, 1543-1551 (2006).

24 Chen, H. C. *et al.* Persistence of transplanted oral mucosal epithelial cells in human cornea. *Invest Ophthalmol Vis Sci* **50**, 4660-4668 (2009).

25 Ma, D. H. *et al.* Transplantation of cultivated oral mucosal epithelial cells for severe corneal burn. *Eye (Lond)* **23**, 1442-1450 (2009).

26 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* **95**, 942-946 (2011).

27 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* **118**, 1524-1530 (2011).

28 Burillon, C. *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* (2011).

29 Schwab, I. R., Johnson, N. T. & Harkin, D. G. Inherent risks associated with manufacture of bioengineered ocular surface tissue. *Arch Ophthalmol* **124**, 1734-1740 (2006).

30 Mariappan, I. *et al.* In vitro culture and expansion of human limbal epithelial cells. *Nat Protoc* **5**, 1470-1479 (2010).

31 Sangwan, V. S. *et al.* Clinical outcomes of xeno-free autologous cultivated limbal epithelial transplantation: a 10-year study. *Br J Ophthalmol* **95**, 1525-1529 (2011).

32 Basu, S. *et al.* Clinical outcomes of penetrating keratoplasty after autologous cultivated limbal epithelial transplantation for ocular surface burns. *Am J Ophthalmol* **152**, 917-924 e911 (2011).

33 Basu, S., Ali, H. & Sangwan, V. S. Clinical Outcomes of Repeat Autologous Cultivated Limbal Epithelial Transplantation for Ocular Surface Burns. *Am J Ophthalmol* (2011).

34 Salehi-Had, H., Alvarenga, L. S., Isseroff, R. & Schwab, I. R. Factors modulating p63 expression in cultured limbal epithelial cells. *Cornea* **24**, 845-852 (2005).

35 Nakamura, T., Endo, K. & Kinoshita, S. Identification of human oral keratinocyte stem/progenitor cells by neurotrophin receptor p75 and the role of neurotrophin/p75 signaling. *Stem Cells* **25**, 628-638 (2007).

36 Di Iorio, E. *et al.* Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci U S A* **102**, 9523-9528 (2005).

37 Qi, H. *et al.* Patterned expression of neurotrophic factors and receptors in human limbal and corneal regions. *Mol Vis* **13**, 1934-1941 (2007).

38 Qi, H. *et al.* Nerve growth factor and its receptor TrkA serve as potential markers for human corneal epithelial progenitor cells. *Exp Eye Res* (2007).

39 Clausen, H. *et al.* Differentiation-dependent expression of keratins in human oral epithelia. *J Invest Dermatol* **86**, 249-254 (1986).

40 Presland, R. B. & Jurevic, R. J. Making sense of the epithelial barrier: what molecular biology and genetics tell us about the functions of oral mucosal and epidermal tissues. *J Dent Educ* **66**, 564-574 (2002).

41 Yoshida, S. *et al.* Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. *Invest Ophthalmol Vis Sci* **47**, 4780-4786 (2006).

42 Murakami, D. *et al.* Fabrication of transplantable human oral mucosal epithelial cell sheets using temperature-responsive culture inserts without feeder layer cells. *J Artif Organs* **9**, 185-191 (2006).

43 Kao, W. W. *et al.* Keratin 12-deficient mice have fragile corneal epithelia. *Invest Ophthalmol Vis Sci* **37**, 2572-2584 (1996).

44 Touhami, A., Grueterich, M. & Tseng, S. C. The role of NGF signaling in human limbal epithelium expanded by amniotic membrane culture. *Invest Ophthalmol Vis Sci* **43**, 987-994 (2002).

45 Sorsby, A. & Symons, H. M. Amniotic membrane grafts in caustic burns of the eye (burns of the second degree). *Br J Ophthalmol* **30**, 337-345 (1946).

46 Gallico, G. G., 3rd, O'Connor, N. E., Compton, C. C., Kehinde, O. & Green, H. Permanent coverage of large burn wounds with autologous cultured human epithelium. *N Engl J Med* **311**, 448-451 (1984).

47 Shortt, A. J., Tuft, S. J. & Daniels, J. T. Ex vivo cultured limbal epithelial transplantation. A clinical perspective. *Ocul Surf* **8**, 80-90 (2010).

48 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* **112**, 993-1002 (2011).

49 Solomon, A. *et al.* Long-term outcome of keratolimbal allograft with or without penetrating keratoplasty for total limbal stem cell deficiency. *Ophthalmology* **109**, 1159-1166 (2002).

50 Maruyama-Hosoi, F., Shimazaki, J., Shimmura, S. & Tsubota, K. Changes observed in keratolimbal allograft. *Cornea* **25**, 377-382 (2006).

51 Nakamura, T. *et al.* Phenotypic investigation of human eyes with transplanted autologous cultivated oral mucosal epithelial sheets for severe ocular surface diseases. *Ophthalmology* **114**, 1080-1088 (2007).