Characterization and Safety Assessment of Bioengineered Limbal Epithelium

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SUMMARY

Transplantation of cultivated limbal epithelium on substrates such as amniotic membrane is an established treatment for severe ocular surface disease with limbal stem cell deficiency. In this study, we adapted an established method to generate sheets of limbal epithelium on amniotic membrane and characterized the cells contained in these sheets and tested them for safety with regard to microbial contamination. Human limbal biopsies were cultivated on denuded amniotic membranes. After three weeks of culture, the phenotypes of cultivated cells were analyzed by immunohistochemistry and real-time RT-PCR for the expression of a panel of specific markers. Cultivated limbal epithelial cell sheets were also analyzed by scanning (SEM) and transmission (TEM) electron microscopy. Sterility tests and mycoplasma assays were conducted for the safety of product. A confluent layer of polygonal cells was formed in 2 weeks and 1-3 stratified layer of cells were observed after three weeks of culture. Cultivated cells were positive for p63, K3, K19, and involucrin but negative for K14, integrin α9 and ABCG2 when analyzed by immunohistochemistry. Expression of molecular markers was detectable with real-time RT-PCR. SEM showed multilayer of flat squamous polygonal epithelial cells. Desmosomal and hemidesmosomal attachments were evident. Our study showed that cultivated limbal epithelium consists of limbal progenitors as well as differentiated corneal epithelial cells. SEM and TEM analysis showed cultivated cells demonstrated typical features of corneal epithelium. The risk of contamination is low and can be prevented by culturing the cells in a clean room facility complying to Good Manufacturing Practice standard.

KEY WORDS:
Amniotic membrane, Corneal epithelial stem cells, Limbus, Tissue engineering, Stem cell transplantation

INTRODUCTION

Clinical and laboratory evidence has indicated that the human corneo-scleral limbus is a repository for corneal epithelial stem cells, also referred to as limbal stem cells (LSC). The limbus is a very specialized zone around the circumference of the cornea. LSC are believed to be located in the palisades of Vogt and more specifically, concentrated in the limbal epithelial crypts[1-3]. Conventionally it is accepted that the maintenance of the corneal epithelial cell mass is achieved by the proliferation, migration and differentiation of LSC and their progeny, under both normal conditions as well as following injury. Recent evidence however suggests that physiological homeostasis of the corneal epithelium can be maintained by the central corneal epithelial cells[4,5].

In severe ocular surface diseases, such as Steven-Johnson syndrome and chemical burns, partial or total damage to the limbus can have severe consequences for corneal wound healing and ocular surface integrity secondary to limbal stem cell deficiency (LSCD)[6-8]. LSCD is characterized by conjunctivalisation of the cornea, vascularization, chronic inflammation, recurrent erosions and persistent ulcers, destruction of the basement membrane; and fibrous tissue ingrowth leading to severe functional impairment of the cornea[9,10].

An important treatment modality for LSCD involves the ex vivo expansion and transplantation of cultivated corneal epithelial cells. This approach for reconstructing damaged ocular surfaces was first reported in 1997 by Pellegrini *et al*[11]. Ex vivo expansion of human limbal epithelial cells on amniotic membrane or other substrates for the purpose of transplantation[12-19] has been reported. Although these techniques have been adopted into clinical practice, the question whether expanded limbal epithelial cells maintain their undifferentiated nature under culture condition still remains unanswered[20]. Furthermore, the safety and efficacy of the bioengineered ocular surface tissue still remains an issue. Risk factor which caused graft failure such as microbial contaminations from the use of material from animal origin is the major concern related to the use of this bioengineered tissue for ocular surface reconstruction[21].

In this country, the study of limbal stem cells and the use of bioengineered ocular surface tissue for transplantation is still in its infancy stage. In this study we bioengineered the ocular tissue in our centre with the aim of introducing it into clinical practice. We cultivated limbal epithelial cells on amniotic membrane (AM) and characterized the cell sheets with a panel of surface and molecular markers considered to be indicative of stemness of limbal epithelial cells [22]. We also conducted sterility tests and mycoplasma assay of the cultivated cells to evaluate the safety of the cell sheets for human transplantation.

MATERIALS AND METHODS
Limbal Epithelial Cells Culture
The method for limbal epithelial cells culture was adapted,

with minor modifications from the explants method reported by the LV Prasad Eye Institute, Hyderabad, India[23]. Human limbal biopsies and AM were handled according to the procedures approved by (excluded for identification). Informed consent was obtained prior to the collection of tissues from all donors. A total of 35 limbal biopsies, of 2x1 mm[2] in size, were obtained from patients who underwent the procedure of pterygium excision. Amniotic membranes were procured as per protocol from donated placenta after screening the donor for the human immunodeficiency virus (HIV), hepatitis B surface antigen (HBs Ag) and syphilis (venereal disease research laboratory [VDRL] test)[23]. Briefly, the limbal explants were placed on de-epithelialized amniotic membranes (3 x 3 cm[2]). For de-epithelialization, the AM was spread in a petri dish and 1 mL trypsin-EDTA was added on the surface and incubated for 25 min at 37 °C. The trypsin solution was then discarded and 2 mL of human corneal epithelium medium (HCEM) which contained Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Invitrogen Corporation, Carlsbad, CA, USA), epidermal growth factor, insulin, hydrocortisone (all from Sigma-Aldrich Chemic, Steinheim, Germany), antibiotics-antimycotics and 10% fetal bovine serum (both from Invitrogen Corporation, Carlsbad, CA, USA) was added to the AM. The AM was mechanically scraped with cell scraper for complete de-epithelialization and washed three times with phosphate buffered saline (PBS). After washing, the AM was spread taut on to a sterile grass slide (2.5 x 2.5 cm2) and carefully placed in a 35 mm culture dish. Fresh limbal biopsy tissue that was held in HCEM was minced into 4-6 tiny bits and explanted onto two denuded AM. The explants were allowed to adhere to the AM for 30 min before 4 mL of HCEM was added to the culture dishes. The medium was changed every alternate day and growth of the cells was monitored under phase contrast microscope. Cells were cultured for 2-3 weeks before being subjected to various tests. Cultivated limbal epithelial sheets were either whole mounted or paraffin sectioned before staining with hematoxylin and eosin (H&E) for morphological evaluation.

Scanning and Transmission Electron Microscopy
Cultivated cells on AM were fixed in 4% glutaraldehyde in PBS, post fixed in 2% osmium tetroxide for 2 h, dehydrated through ascending concentration of ethanol and sputter coated with gold before being examined by a scanning microscope (Philip SEM XL-30, FEI, Holland). For transmission electron microscopy, the limbal epithelial sheet that was fixed overnight in the 2% Glutaraldehyde in 0.1M Phosphate buffer was exposed to 2% Osmium Tetraoxide in 0.1M Phosphate buffer for 1 h. The specimen was dehydrated in ethanol, infiltrated with a mixture of ethanol and epoxy resin (1:1) for 1 h and polymerized with pure epoxy resin at 60°C for 18 h. Ninety nm ultra thin sections were mounted on 200 mesh thin bar copper grids (Agar) and stained with uranyl acetate and Reynold's stain. TEM was carried out with a Technai G2 Transmission Electron Microscopy (FEI, Holland) at an accelerating voltage of 100 kV.

Immunohistochemical Staining
The IHC Select® Immunoperoxidase Secondary Detection System (Millipore, Temecula, CA, USA) was used to stain paraffin sections of cultivated limbal epithelium. Briefly, 4 μm of paraffin sections were deparaffinized and antigens

retrieval were carried out with either proteinase K or Dako retrieval solution (all from DakoCytomation Inc., Carpinteria, California, USA) as per the manufacturer's instructions. The sections were washed with PBS and then covered with 3% hydrogen peroxide for 10 min. Sections were rinsed with rinsing buffer and incubated with blocking reagent for 5 min. Prediluted (1:100) primary monoclonal antibodies (mAb) of ABCG2, cytokeratins (K) 3 (Millipore, Temecula, CA, USA); K19, p63, involucrin, integrin α9 (DakoCytomation Inc., Carpinteria, California, USA); K14 (GeneTex Inc., Irvine, California, USA) were added separately to each section and incubated for 10 min. Primary mAb was excluded for negative control. After rinsing, secondary antibodies were added followed by streptavidin HRP. Chromogen reagent, 3, 3' diaminobenzidine (DAB substrate) was added for 10 min and counterstained with hematoxylin. Finally slides were mounted with aqueous-based mounting media with cover slips and examined with a Nikon microscope.

Immunofluorescent staining for K3
Immunofluorescent staining was performed according to the method reported by De Paiva *et al.*[24]. Cultivated sheet was fixed in fresh 2% paraformaldehye at 4C° for 10 min. After blocking with 5% normal goat serum in PBS for 30 min, mAb reactive with K3 was applied at a concentration of 1:100 for 1 h at room temperature. Fluorescein isothiocynate (FITC) conjugated secondary antibody (1:100) was applied for 1 h and the tissue was counterstained with DNA binding dye propidium iodide for 5 min. The specimen was spread flat on a glass slide and mounted with fluorescence mounting medium (DakoCytomation Inc., Carpinteria, California, USA) using a cover slip. The slides were examined with a Nikon Microscope.

Real time RT-PCR
Total RNA was extracted from 3-week cultivated limbal epithelial cells. First strand cDNA was synthesised with Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Nonnenwald, Penzberg, Germany) as per protocol[25]. Real time polymerase chain reaction (RT-PCR) was carried out using a LightCycler instrument (Roche Diagnostics, Nonnenwald, Penzberg, Germany). The oligodeoxynucleotide primers used for a panel of genes i.e. ABCG2, ΔNp63, K3, K12, Connexin 43, integrin α9 and GAPDH are listed in Table 120. Amplication products were detected via intercalation of the fluorescent dye SYBR green from LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics, Nonnenwald, Penzberg, Germany). Cycling conditions for amplification were as follows: initial enzyme activation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s, 58 °C for 5 s and 72 °C for 20 s. All cycling reactions were performed in the presence of 4 mM MgCl2. Gene specific products were confirmed by melting curve analysis. Expression of the genes were normalised by the expression of GAPDH. Expression ratio of genes from cultivated limbal epithelial cells as compared to limbal biopsy was calculated by REST software[26]. One μL of the gene products was loaded on 1.5% agarose gel for visualization.

Sterility Check for Amniotic membrane, HCEM and Cultures Amniotic membrane, HCEM and cultivated limbal epithelium were randomly subjected to laboratory culture for

Table I: Human primer sequences used for real time RT-PCR

Gene	Accession	Sense primer	Antisense primer	PCR Product
ΔNp63	XM_036421	CAGACTCAATTTAGTGAG	AGCTCATGGTTGGGGCAC	440 bp
ABCG2	AY017168	AGTTCCATGGCACTGGCCATA	TCAGGTAGGCAATTGTGAGG	379 bp
K3	NM_057808	GGCAGAGATCGAGGGTGTC	GTCATCCTTCGCCTGCTGTAG	145 bp
K12	D78367	ACATGAAGAAGAACCACGAGGATG	TCTGCTCAGCGATGGTTTCA	150 bp
Connexin 43	M65188	CCTTCTTGCTGATCCAGTCCAGTGGTAC	ACCAAGGACACCACCAGCAT	154 bp
Integrin α9	NM_002207	TGGATCATCGCCATCAGTTTG	CCGGTTCTTCTCAGCTTCGAT	123 bp
GAPDH	M33197	GCCAAGGTCATCATCCATGACAAC	GTCCACCACCCTGTTGCTGTA	498 bp

microbes (n=10 for each). Cultures which culture medium changed into yellowish and turbid were considered contaminated by bacteria and excluded from the study. A mycoplasma detection kit (Roche Applied Science, Nonnenwald, Penzberg, Germany) based on ELISA technique was used to detect the most common Mycoplasma/Acholeplasma species *(M. arginini, M. hyorhinis, A. laidlawii, M. orale)* contaminating mammalian cell cultures (n=5). Briefly, coating antibodies of four types of mycoplasma species were added to a 96-well microplate. The plate was incubated for 2 h at 37 °C. The antibody solution was removed and the coated wells were then incubated with blocking solution for 30 min at 37 °C. After washing, an aliquot (0.2 mL) of medium from limbal epithelium culture dish, negative control medium and positive control medium provided with the kit were added to the wells. The microplate was covered tightly and incubated overnight at 4 °C. The wells were then washed and incubated with detection antibodies for 2 h at 37 °C. Final incubation with streptavidin-AP-solution was carried out for 1 h at 37 °C and substrate solution was added for 30 min. Wells that developed a yellow colour were considered as positive for mycoplasma.

RESULTS

Morphological Evaluation
Sixty four successful cultures were established from 32 limbal biopsies. Clusters of round cells were seen on day 2-5 at the edge of the explants. A closely packed compact monolayer of polygonal cells with a growing age was seen on subsequent days (Figure 1A, B). These cells then expanded to confluency covering the entire AM within a period of 2-3 weeks. Polygonal cells were clearly visible with hematoxylin and eosin staining in all 10 whole mounts thus stained. The nucleus was vesicular with two-three nucleoli (Figure 1C). Frequent mitotic figures were noted. One-three layers of stratified limbal epithelium were observed (Figure 1 D).

Ultrastructure of cultivated limbal epithelium
Examination of the apical surface of the cultivated epithelium by scanning electron microscope showed a multilayer of flat polygonal epithelial cells. Desquamating cells were observed on the surface of the epithelium (Figure 2A). When analyzed by transmission electron microscope, basement membrane material from AM was evident, and the basal cells were attached to the denuded AM by hemidesmosomes. Desmosomal junctions were seen between neighboring basal cells. A prominent nucleolus was seen in the nucleus (Figure 2B).

Immunohistochemistry and immunofluorescence analysis
Immunohistochemistry using a panel of antibodies showed that the 3-week cultivated limbal epithelial cells were strongly positive for the corneal epithelial differentiation marker K3 (Figure 3A). The cultivated cells were also partially positive for involucrin, K19 and p63 in the basal layer (Figure 3B-D). Cells were totally negative for K14, Integrin α9 and ABCG2 (Figure 3E-G). Immunofluorescence staining with anti-K3 antibody was especially positive at the edge of the cell sheet (Figure 3F).

Relative gene expression of limbal epithelial cells from culture and limbal biopsy
The panel of genes comprised of ABCG2, ΔNp63, K3, K12, connexin 43, integrin α9 and GAPDH was successfully amplified from cultivated cells. However with fresh limbal biopsy cells, ABCG2 and integrin α9 were not amplified. Gene expression of p63, from cultivated limbal epithelial cells were upregulated (p=0.01); whereas K12, K3 and connexin 3 were down regulated (p=0.01) as compared to limbal biopsy cells (Figure 4).

Sterility of Medium, AM and Cultures
Freshly prepared AM and HCEM did not show any contamination. Similarly, samples of cultivated limbal epithelium were also clear of any contamination. Out of 70 cultures, six cultures (8.6%) were discarded when the culture medium turned turbid and yellowish in colour after two weeks of culture. Mycoplasma *(M. arginini, M. hyorhinis, A. laidlawii, M. orale)* contamination was not detected in any culture.

DISCUSSION
Clinical application of cultivated limbal epithelial cells on amniotic membrane has been reported from several centres in the world. This treatment has produced encouraging results for patients with severe ocular surface diseases[11-19]. However, this treatment is currently not available in this country yet. In this study, we adapted methods established by the LV Prasad Eye Institute, Hyderabad, India in the preparation of cultivated limbal epithelial sheets. Our results showed that multilayered cultivated limbal epithelial sheets were successfully obtained. The polygonal morphology of the cells was similar to that reported by others[23,27]. This submerged culture system as introduced by Sangwan *et al.* is only able to produce 1-3 layers of epithelial cells as compared to air lifted methods used by Koizumi *et al.*[14,28] which produced 5-6 layers of stratified cells. In this study, when we air lifted the cultivated cells after 2-weeks of submerged culture, the number of layers could be increased up to five (data not shown). The advantage of this culture system is that an
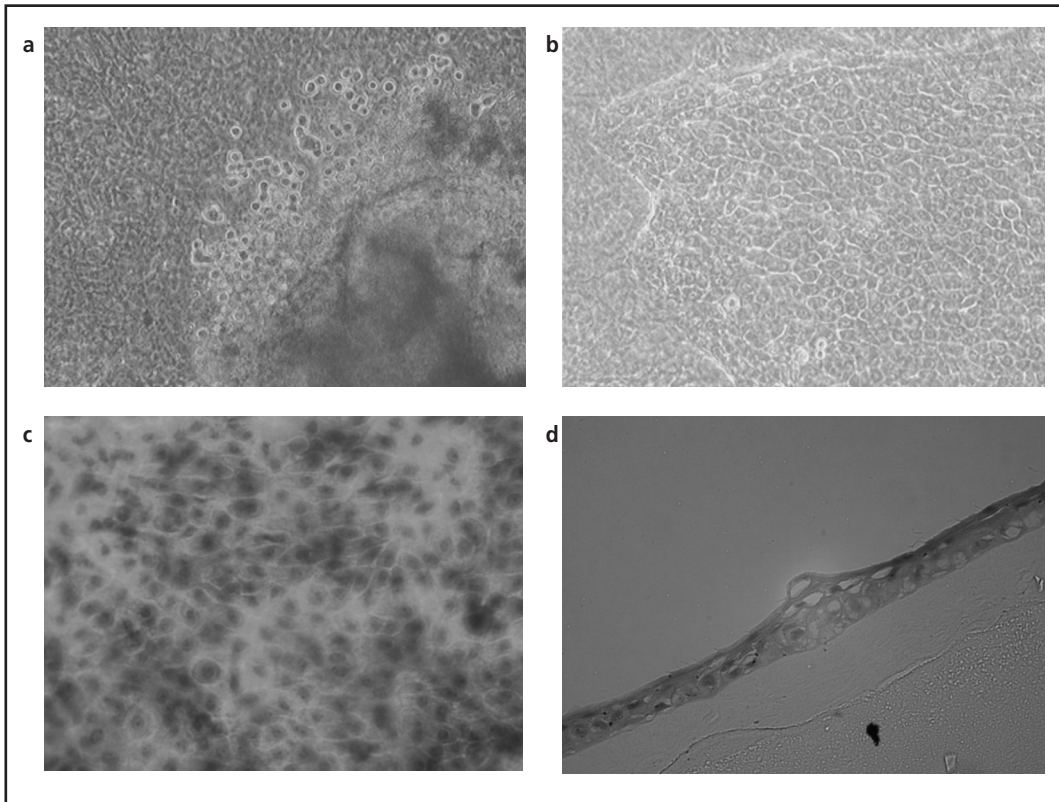
Fig. 1: Morphological evaluation of cultivated limbal epithelial cells. (A) Cluster of round cells were observed under phase contrast microscope on day 2 at the edge of the explants. Background seen was amniotic membrane, AM; (B) A closely packed compact monolayer of polygonal cells with a growing age (arrows) was seen on day 7; (C) Hematoxylin & eosin (H&E) whole mount staining of a confluent layer of expanded limbal epithelial cells at day 14; all magnification 100 x. (D) Paraffin section (4μm) of 3-week cultivated limbal epithelium was stained with H&E. One-three layers of stratified limbal epithelium were observed (magnification 400 x).
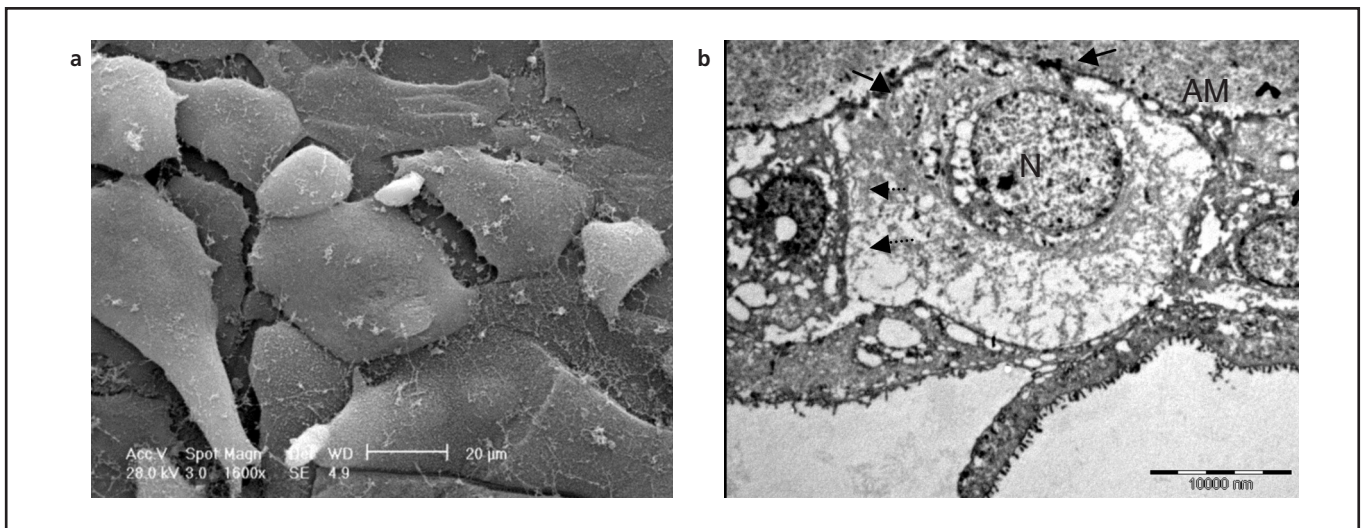
Fig. 2: Ultra structure of cultivated limbal epithelium. (A) Scanning electron micrograph showed multilayer of flat polygonal epithelial cells of the cultivated epithelium. Desquamating cells were observed on the surface of epithelium. (B) When analyzed by transmission electron microscope, basement membrane material was evident, and the basal cells were attached to the denuded AM by hemidesmosomes (black arrows). Desmosomal (dotted arrows) junctions were seen between neighboring basal cells. Nucleolus (N) was seen in the nucleus.
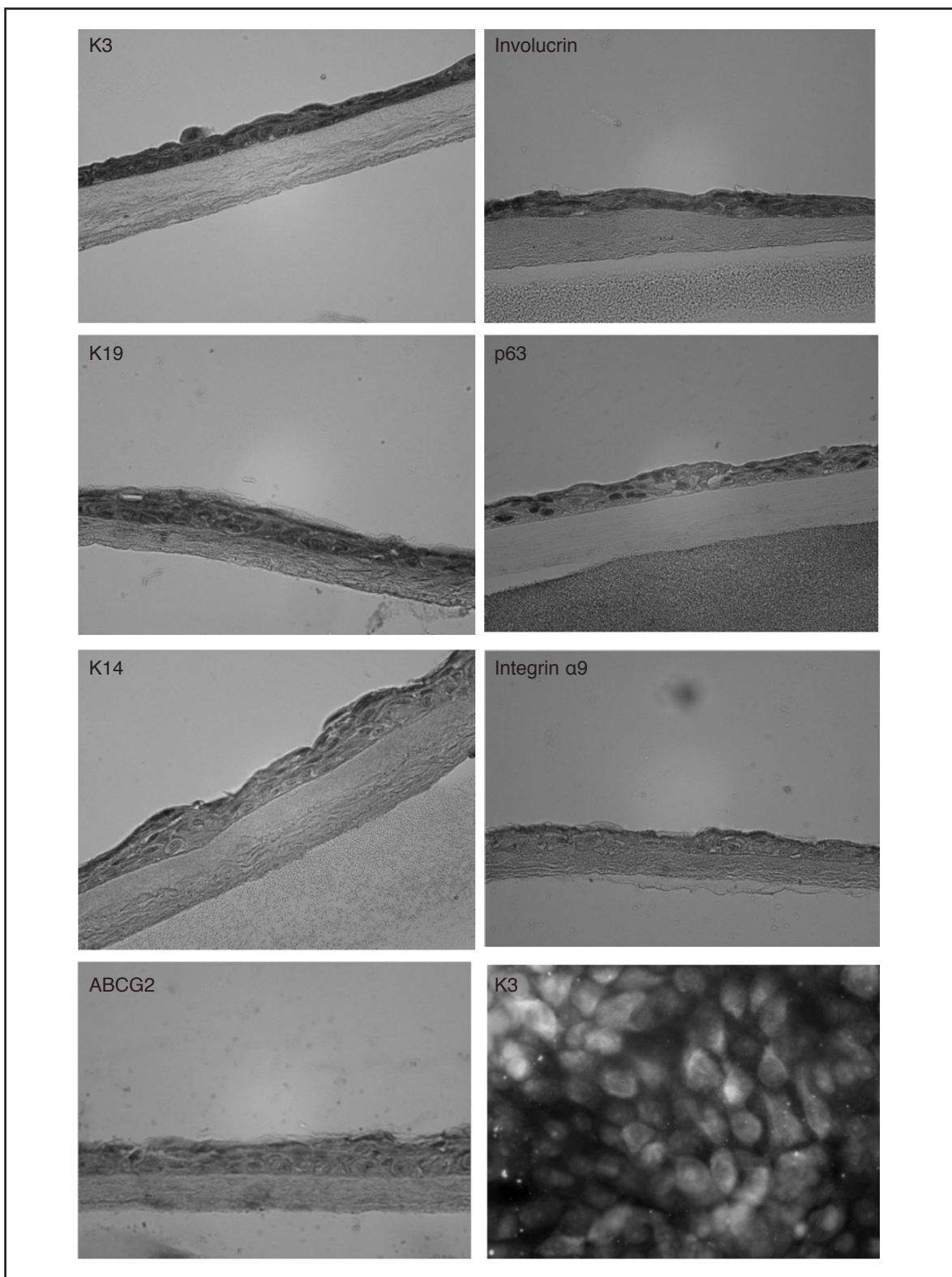
Fig. 3: Immunohistochemical staining of paraffin sections of 3-week cultivated limbal epithelial cells using a panel of antibodies against K3, K14, K19, p63, ABCG2 and integrin α9. (A) Cells were strongly positive for cornea differentiation marker i.e. K3 (magnification 200 x). (B-D) The cultivated cells were also partially positive for involucrin, K19 (basal layer only) and p63 (basal layer only) (magnification 200 x). (E-G) Cells were totally negative for K14 (magnification 400 x), Integrin α9 and ABCG2 (magnification 200 x). (H) Immunofluorescence staining with anti-K3 showed positive result especially at the edge of cells growth (magnification 100 x).
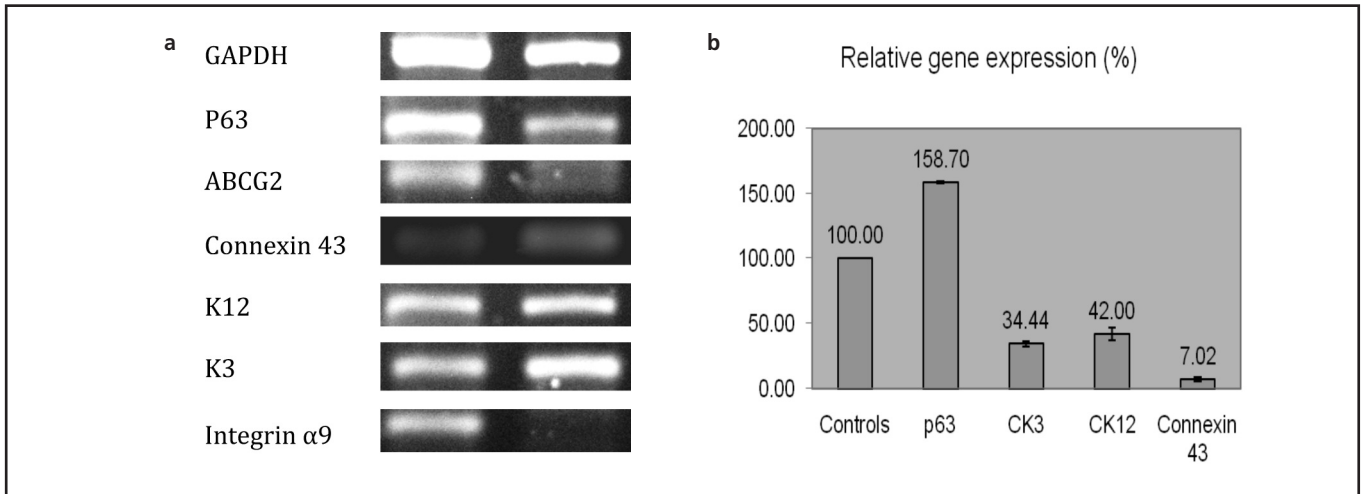
Fig. 4: (A) Real-time RT-PCR products for a panel of genes (ABCG2, p63, K3, K12, connexin 43, integrin α9 and GAPDH as internal control) were loaded into agarose gel for visualization. Genes amplified from limbal biopsy were served as reference. (B) Bar chart showing means and standard deviations (error bars) of relative gene expression (%) of cultivated limbal epithelial cells as compared to limbal biopsy.

animal feeder cell layer is not required, which makes this method simpler and safer compared to others. Besides, this method is relatively cheaper as cell culture inserts are not required. Mitotic figures and multiple nucleoli were often seen indicating that the cells were actively proliferating. Moreover, electron microscopy confirmed the formation of normal looking desmosomes and hemidesmosomes. From the cell morphology and nuclear cytoplasmic ration it would appear that most of the cells in the sheets were differentiating cells rather than stem cells. From published studies[18,29,30], it appears that this does not make a difference to the clinical outcome. It is also not clear whether 5-6 layers of stratified epithelium confers any advantage over sheets with 2-3 layers of cells.

To date, no single specific limbal stem cells marker has been identified[31]. However, a panel of markers has been proposed, which may help to identify a sub-population of cells that may contain limbal stem cells. We studied our cultivated cells using such a panel of markers, which comprised of differentiation-associated markers (K3, K12, connexin 43 and involucrin) and putative stem cell-associated markers (p63, K19, K14 ABCG2 and integrin α 9). While most putative SC markers label the majority of limbal basal cells and, therefore, may not distinguish SC from progenitor cells; only ABCG2 was strictly confined to small clusters of basal cells in the limbal epithelium. ABCG2 therefore appears to be the most useful cell surface marker for the identification and isolation of limbal SC[20, 32]. Our results showed that the cultivated cells were positive for K3, both by PCR and immunostaining. The cultivated cells were also partially positive for involucrin, K19 and p63 (only basal layer). However the cells were totally negative for K14, integrin α9 and ABCG2. These results revealed a mixture of progenitor cells or known as transit amplifying cells and differentiated limbal epithelial cells being expanded during the culture. Since ABCG2 and integrin α9 were not detected by immunohistochemical staining but detected by real-time RT-PCR method, we

conclude that the cultivated cells may be expressing very low levels of ABCG2 and integrin α9. Our real-time RT-PCR results showed that all the genes were expressed by the cultivated cells. Up regulation of p63, compared to expression in limbal epithelium, was noted in the cultivated cells where as gene expression of K3 and K12 was down regulated. These results were similar to that reported by Liu *et al*.[32].

Six culture showed sign of contamination when the medium changed to yellowish and turbid. The contamination probably occurred from the culture environment as the media and amniotic membranes, when individually tested, were free from contamination. Most of the contaminations can be avoided by proper handling of the cultures in a clean room facility which conforms to Good Manufacturing Practice (GMP) standards. The study emphasizes the risk of contaminated cultured products and all protocols must include evaluation for contamination prior to release for clinical use. Mycoplasma contamination was not noted in any culture. This may relate to the fact that only primary cultures were studied. Multiple passages could increase the risk of mycoplasma contamination. As we did not use 3T3 murine fibroblasts, or any other cell line in our culture system, a major risk associated with the use of animal cells was avoided. We feel that our protocol may be the way forward with ex vivo explants, given its reduced risks. It is suggested that fetal bovine serum used for clinical trials should be sourced from Australia or New Zealand herds, a practice that is consistent with guidelines issued by European Medicines Evaluation agency (EMEA) and Therapeutic Goods Administration (Australia), for managing the risk of transmissible spongiform encephalopathy (TSE) from bovine products. However, use of autologous serum from patient or serum free media for culturing limbal epithelium has been reported as well[33]. Cultivated limbal epithelial cells transplantations have been conducted since 1997 by Pellegrini *et al*[11]. In all the clinical trials that had been followed up for more than one year, tumour formation has

not been reported[18]. Therefore, the findings indicate that there is no risk of tumour formation with transplanted *ex vivo* expanded limbal epithelium.

There is no universally accepted standard protocol for the production of cultivated limbal epithelium. Each centre has its own protocol with subtle or significant variations from others. Different culture methods and media might produce limbal epithelial cells at different stages of maturation and phenotype which could determine clinical outcomes. Future research will help to establish the best culture method and media for producing the highest quality of limbal epithelium for transplantation. A clean room facility which conforms to GMP standards is a prerequisite. Use of media free of animal derivative is also desirable.

CONCLUSION
Our results showed that the limbal epithelial cells sheets were successfully generated by using the adapted method. The expanded limbal epithelial cells possessed the characteristic of progenitor and mature phenotypes. The bioengineered cell sheets were reasonably safe and have the potential to be used in future clinical trial study.

REFERENCES
1. Davenger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. Nature 1971; 229: 560-1.
2. Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64 K corneal keratin in vivo and in culture. J Cell Biol 1986; 103: 49-62.
3. Tseng SC. Concept and application of limbal stem cells. Eye 1989; 3: 141-57.
4. Dua HS, Miri A, Alomar T, Yeung AM, Said DG. The role of limbal stem cells in corneal epithelial maintenance: testing the dogma. Ophthalmology 2009; 116: 856-63.
5. Majo F, Rochat A, Nicolas M, Jaoude GA, Barrandon Y. Oligopotent stem cells are distributed throughout the mammalian ocular surface. Nature 2008; 456: 250-55.
6. Chen JJ, Tseng SC. Abnormal corneal epithelial wound healing in partial-thickness removal of limbal epithelium. Invest Ophthalmol Vis Sci 1991; 32: 2219-33.
7. Huang AJ, Tseng SC. Corneal epithelial wound healing in the absence of limbal epithelium. Invest Ophthalmol Vis Sci 1991; 32: 96-105.
8. Dua HS, Joseph A, Shanmuganathan VA, Jones RE. Stem cell differentiation and the effects of deficiency. Eye 2003; 17: 877-85.
9. Puangsricharern V, Tseng SCG. Cytologic evidence of corneal diseases with limbal stem cell deficiency. Ophthalmology 1995; 102: 1476-85.
10. Holland EJ, Schwartz GS. The evolution of epithelial transplantation for severe ocular surface disease and a proposed classification system. Cornea 1996; 15: 549-56.
11. Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, de Luca M. Long-term restoration of damaged corneal surface with autologous cultivated corneal epithelial epithelium. Lancet 1997; 349: 990-93.
12. Lindberg K, Brown ME, Chave HV, Kenyon KR, Rheinwald JG. In vitro propagation of human ocular surface epithelial cells for transplantation. Invest Ophthalmol Vis Sci 1993; 34: 2672-9.
13. Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface diseases. Cornea 2000; 19: 421-6.
14. Kozuimi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. Ophthalmology 2001; 108: 1569-74.
15. Tseng SC, Meller D, Anderson DF et al. Ex vivo preservation and expansion of human limbal epithelial stem cells on amniotic membrane for treating corneal diseases with total limbal stem cell deficiency. Adv Exp Med Biol 2002; 506: 1323-34.
16. Gruterich M, Espana EM, Toushami A, Ti SE, Tseng SC. Phenotypic 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.
17. Tan DT, Ang LP, Beuerman RW. Reconstruction of the ocular surface by transplantation of a serum-free derived cultivated conjunctival epithelial equivalent. Transplantation 2004; 77: 1729-34.
18. Sangwan VS, Matalia HP, Vemuganti GK et al. Clinical outcome of autologous cultivated limbal epithelium. Indian J Ophthalmol 2006; 54: 29-34.
19. 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.
20. Schlotzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. Exp Eye Res 2005; 81: 247-64.
21. Schwab IR, Johnson NT, Harkin DG. Inherent risks associated with manufacture of bioengineered ocular surface tissue. Arch Ophthalmol 2006; 124: 1734-1740.
22. Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. Stem Cells 2004; 22: 355-66.
23. Fatima A, Sangwan VS, Iftekhar G, Reddy P, Matalia H, Blasubramanian D, Vemuganti GK. Technique of cultivating limbal derived corneal epithelium on human amniotic membrane or clinical transplantation. J Postgrad Med 2006; 52: 257-61.
24. De Paiva CS, Chen Z, Corrales RM, Pflugfelder SC, Li DQ. ABCG2 transporter identifies a population of clonogeneic human limbal epithelial cells. Stem Cells 2005; 23: 63-73.
25. Lim MN, Lau NS, Chang KM, Leong CF, Zakaria Z. Modulating multidrug resistance gene in leukaemia cells by short interfering RNA. Singapore Med J 2007; 48: 932-8.
26. Pfaffl MW, Horgan GW, Dempfle I. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002; 30: e36.
27. Meller D, Pires RTF, Tseng SCG. Ex vivo preservation and expansion of human limbal epithelial stem cells on amniotic membrane cultures. Br J Ophthalmol 2002; 86: 463-71.
28. Kozuimi N, Cooper L, Fullwood N, *et al*. An evaluation of cultivated corneal limbal epithelial cells, using cell-suspension culture. Invest Ophthalmol Vis Sci 2002; 43: 2114-21.
29. Pauklin M, Steuhl KP, meller D. Characterization of the corneal surface in limbal stem cell deficiency and after transplantation of cultivated limbal epithelium. Ophthalmology 2009; 116: 1048-56.
30. Harkin DG, Barnard Z, Gillies P, Ainscough SL, Apel AJG. Analysis of p63 and cytokeratin expression in a cultivated limbal autograft used in the treatment of limbal stem cells deficiency. Br J Ophthalmol 2004; 88: 1154-8.
31. Chee KYH, Kicic A, Wiffen SJ. Limbal stem cells: the search for a marker. Clin Exp Ophthalmol 2006; 34: 64-73.
32. Liu S, Li J, Wang C, Tan D. Human limbal progenitor cell characteristics are maintained in Tissue Culture. Ann Acad Med Singapore 2006; 35: 80-86.
33. Lekhanont K, Choubtum L, Chuck RS, Sa-ngiampornpanit, Chuckpaiwong V, Vongthongsri Anun. A serum- and feeder-free technique of culturing human corneal epithelial stem cells on amniotic membrane. Mol Vis 2009; 15: 1294-302.