Ex vivo preservation and expansion of human limbal epithelial stem cells on amniotic membrane cultures

D Meller, R T F Pires, S C G Tseng

Background/aim: Amniotic membrane (AM) transplantation effectively expands the remaining limbal epithelial stem cells in patients with partial limbal stem cell deficiency. The authors investigated whether this action could be produced ex vivo.

Methods: The outgrowth rate on AM was compared among explants derived from human limbus, peripheral cornea, and central cornea. For outgrowth of human limbal epithelial cells (HLEC), cell cycle kinetics were measured by BrdU labelling for 1 or 7 days, of which the latter was also chased in primary cultures, secondary 3T3 fibroblast cultures, and in athymic Balb/c mice following a brief treatment with a phorbol ester. Epithelial morphology was studied by histology and transmission electron microscopy, and phenotype was defined by immunostaining with monoclonal antibodies to keratins and mucus.

Results: Outgrowth rate was 0/22 (0%) and 2/24 (8.3%) for central and peripheral corneal explants, respectively, but was 77/80 (96.3%) for limbal explants (p <0.0001). 24 hour BrdU labelling showed a uniformly low (that is, less than 5%) labelling index in 65% of the limbal explants, but a mixed pattern with areas showing a high (that is, more than 40%) labelling index in 35% of limbal explants, and in all (100%) peripheral corneal explants. Continuous BrdU labelling for 7 days detected a high labelling index in 61.5% of the limbal explants with the remainder still retaining a low labelling index. A number of label retaining cells were noted after 7 day labelling followed by 14 days of chase in primary culture or by 21 days of chase after transplantation to 3T3 fibroblast feeder layers. After exposure to phorbol 12-myristate 13-acetate for 24 hours and 7 day labelling, HLEC transplanted in athymic mice still showed a number of label retaining basal cells after 9 days of chase. HLEC cultured on AM were strongly positive for K14 keratin and MUC4 and slightly positive in suprabasal cells for K3 keratin but negative for K12 keratin, AMEM2, and MUC5AC. After subcutaneous implantation in athymic mice, the resultant epithelium was markedly stratified and the basal epithelial cells were strongly positive for K14 keratin, while the suprabasal epithelial cells were strongly positive for K3 keratin and MUC4, and the entire epithelium was negative for K12 keratin and MUC5AC.

Conclusions: These data support the notion that AM cultures preferentially preserve and expand limbal epithelial stem cells that retain their in vivo properties of slow cycling, label retaining, and undifferentiation. This finding supports the feasibility of ex vivo expansion of limbal epithelial stem cells for treating patients with total limbal stem cell deficiency using a small amount of donor limbal tissue.

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tem cells (SC) of the corneal epithelium have been found to be located exclusively at the limbus—that is, the anatomical junction between the cornea and the conjunctiva. Limbal epithelial SC are the ultimate source of regeneration of the entire corneal epithelium under both normal and injured states (reviewed in Tseng1). Studies of the epithelial phenotype have shown that the limbal basal epithelium does not express corneal epithelial specific keratin 3’ or keratin 12.2 Studies of the cell cycle kinetics have shown that some portions of the limbal basal epithelial cells are slow cycling3 and label retaining.4 Other studies have further confirmed that limbal epithelial SC have a greater growth potential in explant cultures5 and higher clonogenicity when co-cultured on 3T3 fibroblast feeder layers6,7 and that their proliferative potential is resistant to tumour promoting phorbol esters.8,9

When the SC containing limbal epithelium is partially10 or totally11–13 damaged, the corneal surface is invariably covered by ingrowing conjunctival epithelial cells with goblet cells, and the corneal stroma becomes vascularised with chronic inflammation. These pathological signs signify a process of conjunctivalisation and constitute the basis for the diagnosis of a number of corneal disease with limbal SC deficiency14 (for reviews see Tseng1 and Tseng and Sun15). Transplantation of an autologous or allogeneic source of limbal epithelial SC is necessary to restore vision and the normal corneal surface in these diseases (for reviews see Tseng16 and Holland and Schwartz17).

Previously, preserved human amniotic membrane (AM) has been transplanted as a substrate together with transplantation of limbal epithelial SC for treating these limbal SC deficient corneas.18–27 Interestingly, we noted that AM transplantation alone is sufficient to restore the corneal surface in which the limbus has been partially damaged,28,29 suggesting that AM may help expand the residual limbal epithelial SC. Recently, clinical29–31 and experimental31 studies have also shown that ex vivo expanded limbal epithelial cells on AM are capable of restoring the corneal surface with limited SC deficiency. We provide here additional evidence that AM might be an ideal matrix for ex vivo preservation and expansion of limbal epithelial SC. The significance of this finding for improving clinical efficacy of transplanting limbal epithelial SC is further discussed.

MATERIALS AND METHODS

Animals

All procedures were performed according to the ARVO statement for the use of animals in ophthalmic and vision research. Balb/c athymic mice, aged 6–10 weeks, were purchased from Charles River Laboratories (Wilmington, NC,
USA). The mice were housed conventionally under temperature, humidity, and light (12 hour light cycle; lights on at 7 am) controlled conditions in filter covered cages and were kept on standard chow and water ad libitum. Before surgery, mice were anaesthetised by intramuscular injection of 14 mg/kg ketamine and 7 mg/kg xylazine and were killed by cervical dislocation.

Materials

Dulbecco’s modified Eagle’s medium (DMEM), HEPES-buffer, trypsin-EDTA, amphotericin B, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Dispase II and FITC conjugated and affinity purified goat anti-mouse IgM antibody were obtained from Boehringer Mannheim (Indianapolis, IN, USA). The IgG monoclonal antibody AE5, recognising the 64 kD keratin K3 was purchased from ICN (Costa Mesa, CA, USA). The IgM monoclonal IgG antibody 15H10 directed against MUC4 was a kind gift from Jacques Bara, MD (Paris, France). The FITC conjugated goat anti-mouse IgG and IgM antibodies adsorbed with human serum proteins, the mouse monoclonal IgG antibody 19M1 directed against MUC5AC was a kind gift from Kermit Carraway, PhD (University of Miami, FL, USA). The mouse monoclonal IgG antibody 19M1 directed against MUC5AC was a kind gift from Jacques Bara, MD (Paris, France). The FITC conjugated goat anti-mouse IgG and IgM antibodies adsorbed with human serum proteins, the mouse monoclonal IgG antibody against 5 bromo 2’ desoxy-uridine 5’monophosphate (BrdU), BrdU, gentamicin, hydrocortisone, dimethyl sulphoxide, amphotericin B, insulin transferrin sodium selenite media, and phorbol 12 myristate 13 acetate (PMA) were all from Sigma Chemical Company (St Louis, MO, USA). The mouse monoclonal antibodies AK2 to K12 keratin and AMEM2 to mucosal epithelial membrane associated glycosylalyx were of the IgM class and developed in our laboratory. The mouse monoclonal antibody against keratin K14 was from Novocastra (Burlingame, CA, USA). The Vectastain Elite ABC Kit for mouse IgG was obtained from Vector Laboratories (Burlingame, CA, USA). The tissue culture plastic plates (six well) were from Becton Dickinson (Lincoln Park, NJ, USA). Culture plate inserts used for fastening AM were from Millipore (Bedford, MA, USA). The anti-mouse lymphocyte serum was purchased from Accurate Chemicals Co (Westbury, NY, USA).

Expant cultures on amniotic membrane

Human tissue was handled according to the tenets of the Declaration of Helsinki. Corneoscleral tissues from human donor eyes, aged 30–50 years, were obtained from the Florida Lions Eye Bank (Miami, FL, USA). The tissue was rinsed three times with DMEM containing 50 µg/ml gentamicin and 1.25 µg/ml amphotericin B. After careful removal of excessive scera, iris, corneal endothelium, conjunctiva and Tenon’s capsule, the remaining tissue was placed in a culture dish and exposed for 5–10 minutes to Dispase II (1.2 U/ml in Mg2+ and Ca2+ free Hanks’ balanced salt solution (HBSS)) at 37°C under humidified 5% carbon dioxide. Following one rinse with DMEM containing 15% FBS, the tissue was subdivided by a trephine into three portions—that is, the central cornea (7.5 mm in diameter), the peripheral cornea (7.5 mm to 1 mm within the limbus), and the limbus (the remainder). Each of these three portions was then cut into cubes of approximately 1 × 1.5 × 2.5 mm by a scalpel.

Preserved human AM was kindly provided by Bio Tissue (Miami, FL, USA), and fastened onto a culture insert as recently reported.16 On the centre of AM a tissue cube was placed (Fig 1A) and cultured in a medium described by Jumblatt and Neufeld15 made of an equal volume of HEPES buffered DMEM containing bicarbonate and Ham’s F12, and supplemented with 5% FBS, 0.5% dimethyl sulphoxide, 2 ng/ml mouse EGF, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 0.5 µg/ml hydrocortisone, 30 ng/ml cholera toxin, 5% FBS, 50 µg/ml gentamicin, and 1.25 µg/ml amphotericin B. Cultures were incubated at 37°C under 5% carbon dioxide and 95% air and the medium was changed every 2–3 days while the extent of each outgrowth was monitored with a phase contrast microscope.

BrdU labelling

When the outgrowth reached 5–8 mm in diameter, explant cultures were incubated with a fresh medium containing 10 µM BrdU for 1 (n = 20 for the limbus and n = 13 for the peripheral cornea) or 7 days (n = 10 for the peripheral cornea). Some limbal cultures (limbus: n = 4) after 7 day labelling were chased for 14 days by switching to the BrdU free medium. A separate experiment was carried out by transplanting an AM sheet of 6 mm diameter which contained the HLEC outgrowth with 7 day BrdU labelling, to a secondary culture (n = 10), which had been laid down with a mitomycin C treated 3T3 fibroblast feeder layer prepared in a conventional manner,17 and then chased for 14 days. All samples were fixed in cold methanol.
and processed for immunostaining. In a subset of experiments, HLEC on AM (n = 7) were treated for 24 hours with 1.0 µg/ml PMA, and then continuously labelled with BrdU for 7 days. Four cultures were processed directly for immunostaining, while the remaining three were subcutaneously transplanted in nude mice (see below) and chased for 9 days in vivo.

Subcutaneous implantation in athymic Balb/c mice

Because our preliminary experiments showed that subcutaneous implantation in athymic Balb/c mice showed a striking non-specific inflammatory response with macrophages around the abdominal rectus muscle, we found it necessary to tame such inflammation by intraperitoneal injections of 0.5 ml anti-mouse lymphocyte serum 37–39 1 day before and 2 days after implantation.

After the skin covering the rectus abdominis was undermined to expose an area measuring approximately 1.5 cm × 1.5 cm, HLEC cultured on AM for 2–3 weeks were implanted subcutaneously onto the fascial surface of the muscle, and the skin flap was then closed with a running, coated Vicryl 7-0 suture (Ethicon, Somerville, NJ, USA). Postoperatively, gentamicin ointment was applied once daily on the wound. A firm subcutaneous nodule formed during an 8 day period was excised together with the surrounding skin and the muscle for histology and immunostaining.

Immunostaining

For BrdU labelling, the flat mount preparation of the epithelial outgrowth on AM was used directly, and the tissue from athymic mice was subjected to frozen sections of 6 µm thickness in OCT (Tissue Tek, Sakura FineTEK, Torrance, CA, USA). After the sample was air dried, rehydrated for 5 minutes in PBS, treated with 2N HCl at 37°C for 60 minutes to denature DNA, and neutralised in boric acid (pH 8.5) for 20 minutes, incorporated BrdU was detected by immunostaining with a mouse anti-BrdU antibody followed by a Vectastain Elite Kit which was processed according the recommendations of the manufacturer. The slides were finally counterstained with eosin. Under 400× magnification, positive nuclei were counted among the total nuclei within the entire field, and a total of two to four fields were counted per specimen. The labelling index was expressed as the number of positively labelled nuclei/the number of all nuclei × 100%.

For other immunostaining work, sections were preincubated with 0.3% hydrogen peroxide to eliminate endogenous peroxidase, and with goat serum (1:100) to prevent non-specific staining. After rinsing twice for 5 minutes with PBS, they were incubated with each of the following mouse monoclonal antibodies with respective dilution: AE5 (1:100), AK2 (1:500), K14 (1:40), AMEM2 (1:300), MUC4 (1:400), and MUC5AC (1:100). After twice washing with PBS for 5 minutes, they were incubated with an FITC conjugated secondary antibody (goat anti-mouse IgG at 1:100 for K14, AE5, MUC4, and MUC5AC or goat anti-mouse IgM at 1:100 for AK2 and AMEM2). After two additional PBS washes, sections were mounted with an anti-fade solution and analysed with a Zeiss Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany).

Transmission electron microscopy

Selected specimen were fixed in 2% glutaraldehyde and 1% formaldehyde and processed for conventional transmission electron microscopy. Samples were rinsed in 0.1 M phosphate buffer (pH 7.3), postfixed in 1% osmium tetroxide, and embedded in Epon. Semithin sections were stained with 1% methylene blue, 1% Azure II, and 1% borax. Ultrathin sections were cut and conventionally stained with uranyl acetate and...
Figure 3  Immunostaining of keratins K3 (A, B), K12 (C, D), and K14 (E, F), and MUC4 (G, H) in the normal limbal (A, C, E, and G) and corneal (B, D, F, and H) epithelium. K3 keratin and K12 keratin were expressed by the suprabasal limbal epithelium (A, C) and the full thickness of the central corneal epithelium (B, D). K14 keratin was expressed strongly in basal and suprabasal limbal (E) and predominantly basal corneal epithelium (F). MUC4 was expressed in the full thickness of the limbal (G) and corneal epithelium (H). The dotted line (A, C, and E) indicates the border between basal limbal epithelium and stromal tissue. Magnification: 400× (A–H).

RESULTS

Epithelial morphology

The rate of epithelial outgrowth was slow for the first week, but became rapid from then on and frequently reached a size of 2–3 cm in diameter in 2–3 weeks. The basal epithelial cells of the outgrowth of HLEC in 1 week was uniformly small, cuboidal, and with an areal ratio of close to 1:1 between the nucleus and the cytoplasm (Fig 1B–D), similar to that of conjunctival epithelial cells grown in the same culture system. The outgrowth reached confluence—that is, the limit of the membrane fastened to a 35 mm insert, in 4–5 weeks. As with conjunctival epithelial cultures, the resultant epithelial sheet of HLEC cultures remained as two to four cell layers and had scanty cytoplasm in the basal cells (Fig 2A). Transmission electron microscopy showed that occasionally there were hemidesmosomes anchored in a rudimental to partially developed basement membrane of AM (Fig 2B), a finding similar to that was recently reported. The outgrowth of HLEC cultured on AM showed full thickness staining to K14 keratin after 13 days (Fig 4C) and 21 days of culturing, when occasionally a stratified epithelium developed. MUC4 was found in vivo throughout the whole limbal (Fig 3G) and corneal (Fig 3H) epithelial layer. HLEC cultured on AM showed full thickness positive labelling with MUC4 after 13 and 21 days of culturing (Fig 4D). MUC5AC recognises conjunctival goblet cell stained mucins and stains conjunctival goblet cells in vivo. MUC5AC did not stain any cells cultured on AM (not shown). AE5 antibody was also positive for limbal suprabasal epithelial cells (Fig 3C) and for the full thickness of the corneal epithelium (Fig 3D), but negative for the conjunctival epithelium in vivo. HLEC on AM were negative for AK2 (Fig 4B). K14 keratin was expressed in the basal and suprabasal cell layers of the conjunctival (not shown), limbal (Fig 3E) and peripheral corneal epithelium, but was predominantly in the basal epithelial cells of the central corneal epithelium (Fig 3F).

Differentiation of human limbal epithelial cells after subcutaneous grafting into Balb/c athymic mice

To promote epithelial differentiation, we transplanted AM with HLEC outgrowth into the subcutaneous tissue of the abdomen in seven athymic Balb/c mice for 8 days. Excluding one dying of bleeding, four of the remaining six mice (66.6%) showed graft survival evidenced by the formation of a stratified epithelium resembling a normal limbal epithelium in vivo. Figure 5 depicts the stratified epithelium of a representative sample consisting of one layer of small round to cuboidal basal epithelial cells, three to four layers of wing cells, and verify their antigenic epitopes, we first stained normal ocular surface epithelia in vivo. As reported, AE5 antibody, which recognises K3 keratin, stained the suprabasal limbal epithelium (Fig 3A) and the full thickness of the central corneal epithelium (Fig 3B), but not the conjunctival epithelium (not shown). AE5 antibody stained suprabasal HLEC cultured on AM for 13 or 21 days (Fig 4A). Immunostaining for K12 keratin by AK2 was also positive for limbal suprabasal epithelial cells (Fig 3C) and for the full thickness of the corneal epithelium (Fig 3D), but negative for the conjunctival epithelium in vivo. HLEC on AM were negative for AK2 (Fig 4B). K14 keratin was expressed in the basal and suprabasal cell layers of the conjunctival (not shown), limbal (Fig 3E) and peripheral corneal epithelium, but was predominantly in the basal epithelial cells of the central corneal epithelium (Fig 3F).

The differences in outgrowth rate between explant cultures from the limbus, peripheral, and central cornea were analysed with Fisher’s exact test. Data from the proliferation assay were analysed by paired or unpaired Student’s t test, as appropriate. A p value of less than 0.05 was considered significant.

Epithelial phenotype

To examine the epithelial phenotype, we immunostained with a panel of monoclonal antibodies to mucins and keratins. To lead citrate, and finally examined with a Philips EM 420 electron microscope (Philips, Eindhoven, Netherlands).
three to four layers of flattened superficial layers (Fig 5A). The staining by AE5 antibody was negative in the basal cell layer, but positive in suprabasal and superficial epithelial cells (Fig 5B), a pattern identical to that of the normal human limbal epithelium. The staining by AK2 antibody was weakly positive in the superficial epithelial layers (not shown). The staining to K14 keratin was positive throughout the entire stratified epithelium including the basal cell layer (Fig 5C). MUC4 was diffusely expressed by all cell layers (not shown), a pattern also resembling that of the normal limbal epithelium in vivo. Staining for MUC5AC and by AMEM2 antibody was all negative (Fig 5D). Taken together, these data support the notion that subcutaneous transplantation in nude mice promotes epithelial stratification with proper differentiation into a phenotype resembling the normal limbal epithelium.

**Proliferation of ex vivo expanded HLEC**

There were no outgrowths (0%) out of 22 central corneal explants tested, while there was some outgrowth in two out of 24 (8.3%) peripheral corneal explants. In contrast, abundant outgrowth was noted in 77 out of 80 (96.2%) limbal explants \((p < 0.0001, \text{Fisher's exact test})\). These results suggest that AM preferentially supports the outgrowth of epithelial progenitor cells derived from the limbus.

To determine the cell cycle, we labelled the S phase with BrdU, an analogue of thymidine, for 24 hours in 2–3 week old cultures. In all limbal explants tested, 35% of them had a mixed pattern with areas of a moderate high labelling index \((\text{mean} 44.3\% \pm 15.3\%, n = 7)\) (Fig 6A and 6B), while the majority (65%) had a uniformly low labelling index \((3.3\% \pm 3.3\%, n = 13)\) (Fig 6C and 6D) \((p<0.0001, \text{unpaired } t\text{ test})\). In contrast, the outgrowth from all (100%) peripheral corneal explants had a uniformly high labelling index \((61.7\% \pm 2.2\%, n=2)\) (Fig 6E and 6F), indistinguishable from that shown in Figure 6A and 6B. These results indicated that 24 hour BrdU labelling predominantly labelled rapid cycling progenitor cells in the peripheral cornea. The patchy pattern of high labelling index in some limbal explants might be caused by the inclusion of peripheral corneal transient amplifying cells. To confirm that the low labelling index of the limbal outgrowth was indeed a result of slow cycling progenitor cells but not because of post mitotic differentiated cells, we labelled a total of 13 limbal explants continuously for 7 days. The results showed that eight of them \((61.5\%)\) showed a high labelling index of 75.5% \((10.9\%)\) (Fig 6G and 6H), while the remaining five \((38.5%)\) still showed a low labelling index of 10.01% \((10.8%)\) \((p<0.0001, \text{unpaired } t\text{ test})\). This result indicated that the majority of limbal epithelial explants had a slower cell cycle, and some of them had a cell cycle length longer than 7 days.

**Figure 4** Immunostaining of keratins K3 (A), K12 (B), and K14 (C), and MUC4 (D) of HLEC grown on AM cultures. K3 keratin was expressed by suprabasal cell layers (A). K12 keratin was not expressed (B). K14 keratin was expressed by all cell layers (C). MUC4 was also expressed by all cell layers (D). The dotted line (A) indicates the extent of HLEC cultured on AM. Magnification: 40x (A–D).

**Figure 5** HLEC on AM after subcutaneous implantation in athymic Balb/c mice. (A) Haematoxylin and eosin staining showed a markedly stratified epithelium with cuboidal basal epithelial cells. (B) K3 keratin was not expressed by the basal layer, but was strongly expressed by all suprabasal and superficial epithelial cells. (C) K14 keratin was expressed by all layers. (D) Negative control. The dotted line indicates the upper border of the stratified epithelium. Magnification: 200x (A); 400x (C, D).
After a continuous labelling for 7 days and a chase for 14 days, HLEC showed a uniformly high labelling index (94.7%, 3.5%), n=4) (Fig 6I and 6J). In a separate experiment, we transplanted 6 mm or 7 mm diameter sample of HLEC laden AM, which had been labelled with BrdU for 7 days, onto 3T3 feeder layers. We continued to detect BrdU retaining nuclei after 21 days of chase in this secondary outgrowth (Fig 7). These results showed that HLEC on AM had a number of label

Figure 6  Cell cycle analysis using BrdU labelling. After 24 hours of labelling (1d BrdU), 35% of all limbal explants tested had a mixed pattern with areas of relatively high labelling index (A, B), while the remaining 65% had a low labelling index (C, D). Arrows indicate those positive nuclei. All peripheral corneal explants had a uniformly high labelling index (E, F). After a continuous labelling for 7 days (7d BrdU), 61.5% of primary HLEC cultures showed a high labelling index (G, H), but the remainder still had a low labelling index (not shown). These 7 day labelled cultures were chased for 14 days, the label index remained high (I, J). Magnification: 100x (A, C, E, G, and I); 200x (B, D, F, H, and J).
retaining cells after prolonged chase, a property further confirming their slow cycling nature. In a subset of experiments, HLEC on AM were treated for 24 hours with 1.0 µg/ml PMA, and labelled continuously with BrdU for 7 days. Phase contrast microscopy revealed that there was increased cellular desquamation after the treatment with PMA (Fig 8A). These changes were observed in some areas leading to a total loss of adherent cells and exposure of the underlying AM stroma, while in other areas adherent cells were less affected. Continuous desquamation was still noted 3 days after treatment (Fig 8B). Remaining adherent cells proliferated to form a cohesive sheet after 7 days (Fig 8C), when they were continuously labelled with BrdU. A high labelling index was noted with BrdU positive cells either as single cells scattered randomly or close to one another in a patch (Fig 8D and 8E), supporting the notion that they were PMA resistant progenitor cells. These cells and AM were transplanted to the subcutaneous space in athymic Balb/c mice and chased for 9 days. The resultant stratified epithelium showed that the majority of label retaining cells were located at the basal cell layer in the stratified epithelium. Arrows indicate the basally labelled nuclei. Magnification: 80× (D); 100× (F); 200× (A, E, and G); 400× (B, C).

**Figure 7** Label retaining cells after transplantation to 3T3 feeder layers. Secondary outgrowth derived from a 6 mm or 7 mm diameter sample of HLEC laden AM on a 3T3 feeder layer showed numerous label retaining cells after prolonged BrdU labelling for 7 days and 21 days of chase. Magnification: 40× (A); 200× (B).

**Figure 8** Labelling retaining cells after PMA treatment. (A) This phase contrast micrograph reveals increased desquamation leading to denuded AM in some areas of HLEC outgrowth 1 day after PMA treatment. (B) Continuous desquamation was still noted 3 days after PMA treatment. (C) A cohesive monolayer of compact epithelium was noted 7 days after PMA treatment. (D, E) After continuous BrdU labelling for 7 days, a number of cells were labelled on the epithelial sheet. (F, G) After BrdU labelling for 7 days, subsequent implantation into nude mice, and chase for 9 days, the majority of BrdU labelled cells remained in the basal cell layer in the stratified epithelium. Arrows indicate the basally labelled nuclei. Magnification: 80× (D); 100× (F); 200× (A, E, and G); 400× (B, C).
AM cultures preferentially permitted outgrowth from limbal explants, of which the progenitor cells retained the slow cycling property and their proliferative capacity was not inhibited by PMA treatment. After continuous labelling by BrdU, these progenitor cells still retained labels following a prolonged period of chase in vitro and in vivo.

**DISCUSSION**

In this study we presented a colossus of experimental evidence to support the hypothesis that AM cultures preferentially preserve and expand limbal epithelial SC.

The basal cell layer of HLEC grown on AM was uniformly small, compact, and cuboidal, and had a scanty cytoplasm, poor formation of desmosomal junctions, and rarely developed hemidemosomes (Figs 1 and 2). The expression of corneal specific keratins 3 and 12 was positive on the suprabasal layers. To prove that these basal epithelial cells are indeed undifferentiated, we transplanted the outgrowth together with AM into the subcutaneous environment of nude mice, a manoeuvre previously used to examine SC function of the intestinal epithelial SC and isolated ocular surface epithelial cells by promoting proper differentiation. As a result, the transplanted HLEC was markedly stratified and exhibited a phenotype resembling that of the normal limbal epithelium in vivo (Fig 5)—that is, the lack of expression of keratins 3 and 12 in the basal epithelial layer. These data indicate that the progenitor cells of HLEC are indeed preserved during the expansion by AM culture.

The outgrowth from limbal explants was preferentially promoted when compared to those derived from the peripheral cornea and central cornea. As a matter of fact, the central cornea did not yield any outgrowth. The lack of supporting progenitor cells from the central cornea was different from those reported using the plastic culture or 3T3 fibroblast feeder layers, of which both permitted some growth from the human central corneal epithelium. Therefore, it is tempting to speculate that AM cultures are more selective for supporting the growth of limbal progenitor cells. Using a similar AM culture, Koizumi et al., however, reported that rabbit central corneal explants still generate some outgrowth although to a lesser extent than limbal explants. We attribute such a discrepancy to the difference in the species used because the rabbit central corneal epithelium is known to be more proliferative and retain labels after treatment with such a tumour promoter.

After xenotransplantation to nude mice, the resultant stratified epithelium on AM showed negative staining to K3 keratin in the basal epithelium, and to K12 keratin in both the basal and suprabasal epithelium. The former was identical to what has been reported in the limbal epithelium in vivo, whereas the latter was different. Our preliminary study in rabbits showed that the additional negative staining in the suprabasal epithelium was a result of hyperproliferation in the limbal epithelium. This notion was supported by the finding that the staining for K14 keratin was basal and suprabasal in the resultant epithelium on AM following xenotransplantation while the staining of K14 keratin was only basal in the limbal epithelium in vivo. Further studies are needed to elucidate the exact mechanism of this different staining pattern.

Previously, epithelial SC have been expanded by co-cultured 3T3 fibroblasts. Such ex vivo expanded epithelial SC have been used to reconstruct skin following burns and in eyes with total limbal SC deficiency. The fact that limbal epithelial SC can be preserved and expanded by AM cultures without the inclusion of 3T3 fibroblasts reduces the potential risk of using a mouse derived cell line in human trials. Such ex vivo expanded limbal SC on AM also make it easier to transfer to the recipient eye during surgery. The clinical efficacy of this new approach of transplanting limbal epithelial SC for treating corneal diseases has been reported in a short term study in rabbits and clinical patients with unilateral partial or total limbal SC deficiency. This new approach provides an advantage over the conventional limbal conjunctival autograft by reducing the risks to the donor eye inasmuch as a small biopsy of approximately less than 1.5 mm², but not too large strips of the limbal tissue, will be removed.

In conclusion, strong experimental evidence has been gathered to support the hypothesis that AM cultures preferentially preserve and expand limbal epithelial SC that retain their in vivo characteristics of undifferentiation, slow cycling, label retaining, and resistance to a phorbol ester tumour promoter. Studies to elucidate how such a culture system achieves this novel action may help unravel the secret how the “stemness” of epithelial SC is maintained. This culture system may serve as a first step towards engineering various epithelial tissues and developing new therapeutics targeted at epithelial SC in the future.
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Proprietary interest: SCGT has obtained a patent for the method of preparation and clinical uses of amniotic membrane.

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