Connexin 43 Expression and Proliferation of Human Limbal Epithelium on Intact and Denuded Amniotic Membrane

Martin Grueterich, Edgar Espana, and Scheffer C. G. Tseng

**PURPOSE.** Stem cell (SC)–containing limbal basal epithelium and transient amplifying cell (TAC)–containing corneal basal epithelium lie on different mesenchymal matrices. The gap junction protein connexin 43 (Cx43) is absent in the limbal basal epithelium but is present in the corneal basal epithelium, suggesting that the expression of Cx43 denotes SC differentiation into TACs. Amniotic membrane (AM) can expand limbal epithelial progenitor cells in vivo and in culture for subsequent corneal surface reconstruction. In this study, the modulation of Cx43 expression, gap junction intercellular communication (GJIC), and proliferative activity of ex vivo expanded human limbal epithelial (HLE) cells on intact and denuded AM was investigated.

**METHODS.** HLE cells were expanded on intact (i.e., remaining devitalized amniotic epithelium) or epithelially denuded AM (EDTA-treated). Cx43 expression and 24-hour 5-bromo-2′-deoxyuridine-5′-monophosphate (BrdU) labeling index (percentage) were determined by double immunostaining. GJIC was investigated by a scrape-loading dye transfer assay. In a subset of cultures Cx43 and K3 keratin as well as BrdU-retaining nuclei were analyzed in the stratified epithelium obtained 5 days after subcutaneous transplantation in NIH bg-nu-xidBR mice of AM cultures continuously labeled with BrdU for 7 days.

**RESULTS.** The outgrowth rate, overall, was significantly higher on EDTA-treated AM than on intact AM (P < 0.05). Cx43 was expressed in 12.4% ± 14.5% (n = 5) on intact and 57.5% ± 18.2% (n = 5) on EDTA-treated AM (P < 0.05). The BrdU labeling index was 2.4% ± 0.9% (n = 5) for the intact AM group, which was significantly less than 22.5% ± 8.2% (n = 5) for EDTA-treated AM (P < 0.05). BrdU-labeled cells did not express Cx43. The dye transfer assay revealed reduced GJIC on both AM-cultured groups compared with the control culture on plastic (P < 0.002). GJIC on intact AM (17%) was reduced compared with that on EDTA-treated AM (27%; P = 0.42). After xenotransplantation, the basal layer of the stratified epithelium was Cx43 and K3 keratin negative and retained BrdU on intact AM, resembling characteristics of the limbal basal epithelium in vivo. In contrast, that of EDTA-treated AM was Cx43 and K3 keratin positive without BrdU retention, resembling characteristics of the corneal epithelium in vivo.

**CONCLUSION.** These data indicate that denudation of the devitalized amniotic epithelium to expose its basement membrane might be a microenvironmental cue to promote TAC differentiation. The model system described herein is ideal for future exploration of the exact mechanistic operation in the microenvironmental niche that maintains the “stemness” of limbal SCs as well as in the signal that promotes corneal TAC differentiation. (Invest Ophthalmol Vis Sci. 2002;43:63–71)

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(i.e., retaining devitalized AM epithelium) is a more favorable microenvironment for the expansion of SC-containing limbal epithelium than is EDTA-denuded AM (i.e., exposed AM basement membrane) with respect to the preservation of a Cx43-negative and GJIC-deficient phenotype. This notion is further supported by phenotypic characteristics of the expanded cell population after the induction of stratification through xenotransplantation into nude mice. The potential clinical significance of these two culture conditions for limbal epithelial cell expansion is further discussed.

**Materials and Methods**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM), HEPES buffer, gelatin, dispase II, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). The mouse monoclonal IgG antibody against β3- and β2-deoxyuridine-5’-monophosphate (Brdu) and Disperse II were obtained from Roche Molecular Biochemicals (Indianapolis, IN). The FITC-conjugated goat anti-mouse IgG and IgM antibodies adsorbed with human serum proteins, gentamicin, hydrocortisone, dimethyl sulfoxide, cholera-toxin, insulin-transferrin-sodium selenite media supplement, lucifer yellow, rhodamine-dextran, EDTA, dianeminobisidine (DAB), propidium iodide, and Triton X-100 were all from Sigma Chemical Co. (St. Louis, MO). An ABC kit (Vectastain Elite) for mouse and rabbit IgG and the mounting medium (Vectorstain) were provided by Vector Laboratories (Burlingame, CA). The mouse monoclonal anti-Cx43 antibody was from Chemicon (Temecula, CA), and the polyclonal antibody against Cx43 was from Zymed (South San Francisco, CA). The IgG monoclonal antibody AE5, recognizing the 64-kDa keratin K3 was purchased from ICN (Costa Mesa, CA). The tissue culture plastic plates (six-well) were from Becton Dickinson (Lincoln Park, NJ). Culture plate inserts used for fastening AM were from Millipore (Bedford, MA).

**Human Tissue Preparation**

Human tissue was handled according to the Declaration of Helsinki. Conjunctival tissues from human donor eyes were obtained from the Florida Lions Eye Bank (Miami, FL) immediately after the central corneal button had been used for corneal transplantation. The tissue was rinsed three times with DMEM containing 50 µg/mL gentamicin and 1.25 µg/mL amphotericin B. After careful removal of extracellular sclera, irid, and corneal endothelium, the remaining tissue was placed in a culture dish and exposed to Dispase II (1.2 U/mL in Mg²⁺ and Ca²⁺-free Hanks’ balanced salt solution) at 37°C under humidified 5% CO₂ for 5 to 10 minutes. After one rinse with DMEM containing 10% FBS, the scleral rim was trimmed to obtain limbal tissue cubes of approximately 1 × 1.5 × 2.5-mm size.

**Human Limbus Cultures on Amniotic Membrane**

Preserved human AM was kindly provided by Bio-Tissue (Miami, FL). AM was preserved according to the method described by Lee and Tseng. Briefly, AMs derived from cesarean section placentas were rinsed in PBS containing 100 U/mL penicillin with 0.2 mg/mL streptomycin and stored in a solution of 50% DMEM and 50% glycerol at −80°C for at least 3 months. With this preservation method, both amniotic epithelium and stromal mesenchymal cells lose their viability and proliferative capacity. After thawing at room temperature, AM with the epithelial side facing up was fastened onto a culture insert, as previously reported. Fifty percent of the membranes used for limbal cultures were treated with 0.1% sterile EDTA solution for 30 minutes and then gently scrubbed with an epithelial scrubber (Amoils Epithelial Scrubber; Innova, Innovative Eximer Solutions, Inc., Toronto, Ontario, Canada), to remove the amniotic epithelium without breaking the underlying basement membrane. With this method, 90% to 100% of the epithelium could be removed. On the center of the AM, an explant was placed and cultured in a medium made of an equal volume of HEPES-buffered DMEM containing bicarbonate and Ham’s F12. The medium was supplemented with 0.5% dimethyl sulfoxide, 2 ng/mL mouse epidermal growth factor (EGF), 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium, 0.5 µg/mL hydrocortisone, 30 ng/mL cholera toxin A subunit, 5% FBS, 50 µg/mL gentamicin, and 1.25 µg/mL amphotericin B. Cultures were incubated at 37°C under 5% CO₂ and 95% air, and the medium was changed every 2 to 3 days. Each time the medium was changed, the outgrowth area was measured. When HLE cultures had almost reached confluent growth, defined as 270° of the circular outgrowth reaching the plastic culture ring or no further growth noticed after 4 weeks of culture, they were subjected to a semiquantitative dye transfer assay or incubated with 10 µM BrdU for 24 hours followed by fixation in cold methanol for immunostaining.

**Xenotransplantation**

All procedures were performed according to the ARVO Statement for the use of Animals in Ophthalmic and Vision Research. NIH bg-nu/sidBR mice, which have no thymus-derived T-cells, T-independent B lymphocytes, and natural killer cells, aged 6 to 10 weeks were purchased from Charles River Laboratories (Wilmington, CA). The animals were housed under temperature-, humidity-, and light- (12-hour light cycle; lights on at 7:00 AM) controlled conditions in filter-covered cages in a laminar flow-equipped room and kept on standard chow and water ad libitum. Before surgery, animals were anesthetized with intramuscular injection of 0.1 mL ketamine (35 mg/kg) and xylazine (5 mg/kg). Nearly confluent HLE cultures on intact and EDTA-treated AM were labeled with BrdU for 7 days to label rapid and slow-cycling cells and transplanted to the subcutaneous plane of the abdomen of NIH bg-nu/sidBR mice for a chase of 5 days, during which time only slow-cycling cells will retain the label. The mice were killed by cranio-cerebral dislocation after intramuscular injection of 0.3 mL ketamine (35 mg/kg) and xylazine (5 mg/kg). The tissue, including implanted AM, was removed and embedded in optimal cutting temperature (OCT) compound for cryosectioning. Twelve cultures (six per condition) were transplanted.

**Immunostaining**

Frozen sections of 3 µm obtained from specimens after xenotransplantation were fixed in cold methanol for 20 minutes at −20°C followed by a 10-minute incubation in 0.1% Triton X-100 in PBS. After three rinses with PBS for 7 minutes each and preincubation with 5% BSA to block nonspecific staining, sections were incubated with a rabbit polyclonal anti-Cx43 (1:200), AE5 (mouse anti-K3; 1:100), or mouse anti-Brdu (1:1000) monoclonal antibody for 45 minutes. After three washes with PBS for 15 minutes, the sections were incubated with an FITC-conjugated secondary antibody (goat anti-rabbit or anti-mouse IgG at 1:200) for 45 minutes. After three additional PBS washes (15 minutes each), they were mounted with an anti-fade solution (Vectashield, Vector Laboratories, Burlingame, CA) and analyzed with a fluorescence microscope (Axiovert; Carl Zeiss, Oberkochen, Germany). For Brdu and Cx43 double-labeling, confluent cultures were incubated with 10 µM Brdu and the same culture on the same coverslips for 24 hours. These cultures on AM were prepared as flatmount samples. After samples were air dried, rehydrated in PBS for 5 minutes, treated with 2 N HCl at 37°C for 45 minutes to denature DNA and neutralized in boric acid (pH 8.5) for 20 minutes, incorporated Brdu and Cx43 expression were detected by immunostaining with a mouse anti-Brdu antibody (1:1000) and a mouse anti-Cx43 antibody (1:200) followed by an ABC kit (Vectastain Elite) protocol (DAB-peroxidase staining). Samples were counterstained with hematoxylin. Under magnification of ×400, positive nuclei were counted among the total nuclei within the entire field, and a total of 16 fields were counted per specimen. The labeling index for Brdu was expressed as the number of positively labeled nuclei/the number of all nuclei × 100. If all cells in one ×400 field expressed Cx43, we defined it as 1 unit of Cx43 expression. That is, if 50% of cells expressed Cx43, 0.5 unit was defined. We counted...
and reported their mean iodide.

cytoplasm.

basement membrane in EDTA-treated AM (Fig. 1F). The amniotic

whereas all HLE cells had direct contact with the amniotic

cells on top of a layer of amniotic epithelial cells (Figs. 1E, 4E),

most of the area of intact AM, we could identify expanding HLE

Outgrowth Rate

The outgrowth area of 10 HLE cultures was photographed and measured each time the culture medium was changed, until

100 fields per sample for a total of 10 samples (n = 5 per condition) and reported their mean ± SD. Counterstaining on nude mice specimens to analyze BrdU retaining cells was performed with propidium iodide.

**Semi-quantitative Dye Transfer Assay**

We used the scrape-loading dye transfer assay originally described by El-Fouly et al. and Troso et al. For a positive control, we cultured HLE cells from an explant on plastic dishes for 14 days. HLE cells on plastic or AM were rinsed with sterile PBS. One milliliter lucifer yellow plus rhodamine-dextran (0.5 mg/ml) in PBS was added to the culture dish. A sterile scalpel blade was applied with gentle pressure to cut the cells. Six scrape lines were placed in different areas per culture. Dishes were left in a dark room for 3 minutes. Cells were rinsed extensively with PBS to prevent high background fluorescence. Cultures were fixed in 4% formalin and epifluorescence was examined using a fluorescence microscope (Axiophot; Zeiss) equipped with a UV light source. A rhodamine filter set was used to identify the red color of the primary loaded cells along the scrape line (absorbency 555 nm, emission 580 nm). Fluorescence filter sets were used to detect green fluorescence of lucifer yellow, which was transferred through gap junctions (absorbency 428 nm, emission 536 nm). We analyzed a total number of 54 scrape lines (6 scrape lines per culture for three separate cultures per condition; intact AM, EDTA-AM, control). The percentages of the entire length of all six scrape lines per culture were measured when we observed dye transfer more than four cell rows away from the initially loaded cells.

**Statistical Analysis**

A Greenhouse-Geisser corrected test of interaction between groups and the linear time component was used to evaluate the statistical significance between intact and EDTA-treated AM groups. The Mann-Whitney test was applied to compare the means of the cell density in both conditions. Data from the proliferation as well as from the Cx43 expression assay was analyzed by an unpoled variance approximate t-test. An ANOVA was applied to analyze the dye transfer assay. The Fisher exact test was used to compare samples with and without label-retaining basal cells after xenotransplantation. P < 0.05 was considered statistically significant.

**RESULTS**

**General Morphologic Features**

Figure 1A shows a typical circular cell outgrowth of HLE cells on intact AM. The explant was surrounded by a cell layer termed the major outgrowth area. The leading edge of the outgrowth showed stratified cells composed of migrating HLE cells and devitalized amniotic epithelial cells. The leading edge was much smoother in the EDTA-treated group from which the amniotic epithelium had been removed before culture (Fig. 1B). Phase-contrast microscopy showed that the cell morphology of expanded HLE was small, compact, and uniform on both intact (Fig. 1C) and EDTA-treated AM (Fig. 1D), with the latter being more uniform than the former. Cross-section analysis showed that most HLE on intact AM (E) migrated on top of the devitalized AM epithelial cells (arrows), whereas HLE on EDTA-treated AM (F) had direct contact with the amniotic basement membrane. Bar, (C, D) 100 μm; (E, F) 50 μm.

**Outgrowth Rate**

The outgrowth area of 10 HLE cultures was photographed and measured each time the culture medium was changed, until the cultures reached near confluence in 3 to 4 weeks. HLE cultures grown on EDTA-treated AM showed a significantly higher outgrowth rate than those on intact AM and reached almost confluent growth after 12 days (P < 0.05; Fig. 2). Cultures on EDTA-treated AM started to grow on day 2, whereas those on intact AM began to grow on day 4. The growth rate, measured by the slope of the curve, was identical for both culture conditions between days 4 and 8. However, the growth rate increased from days 8 to 12 for cultures on EDTA-treated AM, whereas that of the intact AM group showed a slight decrease.

**Cx43 Expression and Cell Cycle Analysis**

After 3 to 4 weeks of culturing on intact AM, the majority of expanded HLE did not express Cx43, regardless of the final outgrowth area's being 70% or 90% or completely confluent. HLE expressing Cx43 were calculated as 12.4 ± 14.5-positive units on intact AM, which was significantly lower than 57.5 ± 18.2-positive units of the EDTA-treated AM (Figs. 3A–C; P < 0.05). Positive Cx43 staining appeared in a punctate pattern, which was confined to the cell membrane of adjacent cells, compatible with the formation of gap junction channels (Fig. 3A–C).
In both groups Cx43 expression was found in localized areas predominantly adjacent to the explant or randomly scattered among the expanded cells without any preferred location.

To correlate Cx43 expression with the proliferative activity at the same time, we labeled the S phase of the cell cycle with BrdU, a thymidine analogue, for 24 hours in nearly confluent cultures, and performed double immunostaining. The labeling index was low, in the range of $2.4\% \pm 0.9\% (n = 5)$ for the intact AM group (Fig. 4A), which was significantly lower than the $22.5\% \pm 8.2\% (n = 5)$ in the EDTA-treated AM group (Fig. 4B; $P < 0.05$, Fig. 4C). Because the AM used in this study did not contain vital epithelium or fibroblasts, BrdU could have been incorporated only by expanded HLE cells. Areas with high BrdU uptake were found predominantly near the explant or at the leading edge of the outgrowth and were devoid of Cx43 expression (Fig. 4A, inset). To confirm that the nonlabeled cells were indeed slow cycling and not postmitotic differentiated cells, we continuously incubated a set of six cultures with BrdU for 6 days. As shown in Figure 4D, the BrdU-labeling index increased to $62\% \pm 9.5\%$. This result indicated that most of the expanded HLE cells on intact AM were indeed slow cycling. BrdU detection using immunostaining with FITC as a fluorescence probe further allowed visualizing HLE nuclei that incorporated BrdU (Figs. 4E, 4F; horizontal, open arrow) and amniotic epithelial cells (Fig. 4E, vertical arrow) at the same time. Expanded HLE growing on top of amniotic epithelial cells (Fig. 4E) showed an overall lower BrdU incorporation compared with areas where HLE expanded on EDTA-denuded AM had direct contact with the amniotic basement membrane (Fig. 4F).

**Semiquantitative Dye Transfer Assay**

To evaluate whether immunohistochemically detected Cx43 was indeed assembled into functioning gap junction channels, we performed a semiquantitative dye transfer assay using the previously described scrape-loading technique. HLE cells expanded on intact AM did not show any dye transfer from the scraped area to the adjacent cells in most of the scrape lines performed (Figs. 5A, 5B). When semiquantitated, HLE cells cultured on intact AM showed dye transfer to adjacent cells in $17\%$ of the total length of 18 scrape lines (three cultures and six scrapes per sample). When the same sample was subjected to subsequent immunostaining, we did not detect any Cx43 expression in these areas (Fig. 5C). However, in those areas that revealed focal GJIC (Figs. 5D, 5E), we noted positive expression of Cx43 (Fig. 5F, punctate staining). HLE cells expanded on EDTA-treated AM showed a similar result, except that we noted a slightly increase of communicating areas, amounting to $27\%$ of the total length of 18 scrape lines ($P = 0.42$). As a positive control, we also scrape loaded the outgrowth of HLE grown on plastic and found $94\%$ of the total length of 18 scrape lines showing dye transfer to adjacent cells (Figs. 5G, 5H). The positive GJIC correlated with positive expression of Cx43 of
the same area (Fig. 5I). This amount of GJIC was significantly higher than amounts in both AM groups ($P < 0.002$).

**Cx43 and K3 Keratin Expression and BrdU Retention after Xenotransplantation**

To determine whether the increase of the BrdU-labeling index and Cx43 expression of HLE monolayer on EDTA-treated AM represents real phenotypic differentiation into TACs, we transplanted AM with expanded HLE as a composite graft (n = 12; 6 per group) into the subcutaneous plane of NIH bg-nu-xidBR mice to promote stratification and differentiation after the AM-cultured cells had been labeled continuously with BrdU for 7 days. In addition, we also looked for the expression of keratin K3, which is known to be positive in the suprabasal layers of the limbus and the full thickness of the corneal epithelium but is absent in the limbal basal epithelium.$^{1-4}$ Five days later, the resultant epithelium was stratified to an average of five cell layers in both groups. Basal cells were small and compact, whereas the more superficial cells appeared more flat and squamous (Figs. 6A, 6B). It should be noted that the existence of devitalized amniotic epithelial cells could not be discerned anymore as shown in Figure 6A. Expression of Cx43 was absent throughout the entire epithelium on intact AM (Fig. 6C, the inset shows the positive control of murine epidermis of the same specimen). Basal cells, but not suprabasal cells, on EDTA-treated AM expressed Cx43 punctate staining at the cell borders (Fig. 6D), resembling the phenotype of the corneal basal epithelium in vivo (Fig. 6D, inset). Expression of keratin K3 was absent in the basal layer on intact AM, but was expressed in the suprabasal and superficial layers of the same sample (Fig. 6E). In contrast, the stratified epithelium on EDTA-treated AM showed keratin K3 expression throughout all cell layers (Fig. 6F). BrdU label–retaining cells were detected in the basal layer in all samples of the intact AM group (6/6; Fig. 6G), but not in the EDTA-treated samples (0/6; Fig. 6H; $P = 0.0022$, Fisher exact test). Because the amniotic epithelium and fibroblasts were devitalized and showed no proliferative activity after the applied method of preservation,$^{18}$ the basal BrdU-labeled cells were judged to be derived from HLE cells.

**DISCUSSION**

In this study we provided strong experimental evidence that the monolayer of HLE cells cultured on intact and EDTA-denuded AM expressed different phenotypes in Cx43 expression, GJIC, and proliferative activity. More Cx43 expression and a higher proliferative activity were promoted when HLE were grown in direct contact with the amniotic basement membrane (e.g., EDTA-denuded AM). After xenotransplantation to promote stratification and further differentiation, ex-
pansion on top of the devitalized amniotic epithelium (e.g., intact AM) promoted a Cx43-negative, keratin K3-negative and label-retaining basal epithelial phenotype, resembling that of the limbal basal epithelium in vivo. In contrast, EDTA-denuded AM promoted a Cx43-positive, keratin K3–positive, and nonlabeled basal epithelial phenotype, resembling that of the corneal basal epithelium in vivo. Collectively, these data support the hypothesis that intact AM preferentially preserves and expands limbal epithelial progenitor cells, whereas EDTA-denuded AM promotes corneal TAC differentiation.

Our data show that HLE expanded on intact AM had a significantly slower outgrowth rate than those expanded on EDTA-denuded AM (Fig. 2). This difference was due to a delayed onset of growth of cultures on intact AM and an increase of the growth rate on EDTA-treated AM in the late-culture phase. This finding was consistent with the data published by Koizumi et al.,15 using rabbit limbal and corneal explants. Because 3T3 fibroblast feeder layers were used in their coculture system 15 but not in ours, we thus speculate that the fibroblast feeder layer does not play a major role in yielding such a difference in proliferative activity. One may speculate that such a different growth rate is caused by a mechanical resistance of a firmly adhered amniotic epithelium against expanding HLE that had to grow over them (Figs. 1E, 1F). However, another alternative explanation may be that HLE’s proliferative activity was lower on intact AM, as shown by a significantly lower BrdU-labeling index on intact AM than on EDTA-denuded AM (Fig. 4). Koizumi et al.,15 as well as Meller et al.,14 from our laboratory, demonstrated desmosomal structures between HLE and the underlying amniotic epithelium, using electron microscopy. We have recently conducted further analyses and noted that both integrins α6 and α3 were not expressed, whereas integrins β4 and β1 were expressed by HLE in contact with devitalized amniotic epithelial cells. This was in great contrast with the positive expression of integrins α6β4 and α3β1 by HLE when growing on denuded amniotic basement membrane (Grueterich et al., manuscript in preparation). This information strongly suggests that HLE uses different adhesion complexes when growing on intact versus EDTA-treated AM.

We further confirmed that the lower labeling index of HLE on intact AM was a result of a slow cell cycle and not of postmitotic differentiation, in that continuous BrdU labeling for 6 days caused a 30-fold increase in the labeling index (Fig. 4D). It should be noted that the BrdU labeling index was calculated at different time points, because both culture conditions grew at different growth rates. However, we believe this concern would not have affected the interpretation of our data because the same growth condition was used for both groups during the labeling experiment. Immunofluorescent visualization of incorporated BrdU of HLE nuclei confirmed that the number of BrdU-positive nuclei was indeed higher in areas where amniotic epithelial cells were absent than in those where they were present (Fig. 4E, 4F). We thus conclude that HLE expanded on intact AM maintains a slower cell cycle—one feature of epithelial SCs in vivo.

FIGURE 5. Scrape-loading dye transfer assay. Primary injured cells were identified by red fluorescence of rhodamine-dextran, which cannot be transferred to neighboring cells through gap junctions because of its high molecular weight. Communicating cells were identified by lucifer yellow, which can be transferred to neighboring cells through gap junctions. Using this method, most of the analyzed areas on intact AM showed no GJIC (A, B), when the corresponding area revealed no Cx43 expression by counterimmunostaining (C). Some localized areas of HLE cells on either intact or EDTA-treated AM showed GJIC (D, E). The corresponding area showed positive expression of Cx43 (F). In contrast, marked GJIC to adjacent cells was noted in the control of HLE cells cultured on plastic (G, H). The corresponding area showed positive Cx43 expression (I). Bar: (A, B, D, E, G, H) 100 μm; (C, F, I) 50 μm.
finding was also consistent with previous findings by Meller et al. (Ref. 14 and manuscript submitted).

The next natural question is whether the rapid cell cycle and the higher outgrowth rate of HLE on EDTA-denuded AM represent a rapid self-renewal of limbal progenitor cells or their actual differentiation into TAC. Matic et al.8 first proposed the theory that the absence of Cx43-containing gap junctions and GJIC is a mechanism by which SCs maintain their “stemness” in their specialized microenvironment, and expression of Cx43 activates GJIC that is needed for corneal TAC synchrony. We thus examined the expression of Cx43 and GJIC and noted that HLE cells expanded on intact AM were largely devoid of Cx43, a phenotype resembling that of the SC-containing basal limbal epithelium in vivo (D, inset). The positive control staining of Cx43 was found in mouse epidermis within the same sample that expressed abundant Cx43 (C, inset). K3 keratin expression was negative in the basal epithelium on intact AM (E), whereas positive in basal epithelium on EDTA-treated AM (F). When HLE cells were continuously labeled with BrdU for 7 days before xenotransplantation, BrdU-retaining cells could be exclusively identified in some areas of the basal layer (green fluorescence) on intact AM (G), but not in EDTA-treated AM cultures (H). The red fluorescence indicates nuclear staining with propidium iodide. * indicates the basement membrane. Bar, 100 μm.

Positive GJIC areas of the EDTA-denuded AM group were larger than those of the intact AM group (27% vs 17%), but this difference was not statistically significant and was not consistent with the aforementioned 10-fold difference in Cx43 expression. There are several possible reasons for this discrepancy. First, Cx43 detected by immunostaining had not yet assembled into connexon associations between neighboring cells, which are essential for gap junction conductance. Second, it is not known whether gap junctions in corneal and limbal epithelium also form heterotypic junctions (i.e., a gap junction channel composed of different Cx subtypes). If this were the case, other Cxs might respond differently to the two culture conditions described herein, which may explain why we found no difference in GJIC, even though Cx43 expression was 10 times higher in cultures on EDTA-treated AM.

To further explore the cell population derived with our culture system, we transplanted expanded HLE on AM as a composite subcutaneous graft in immunocompromised mice to promote stratification and differentiation. In addition to the nude gene, which results in an absence of thymus-derived...
T-cells, these mice have two other mutations important in regulating the function of the immune system. They are X-linked immune defect (xid), which affects the maturation of T-independent B-lymphocytes, and beige (bg), in which the homozygote is devoid of natural killer cells that are cytotoxic to tumor cells in vitro. In both conditions a nicely stratified epithelium was found with a relatively small and compact basal cell layer. The presence of the devitalized amniotic epithelium could not be discerned underneath the stratified HLE any longer, suggesting their partial or complete disintegration, as proposed by others.23 Our results showed that the basal layer of the resultant stratified epithelium on intact AM did not express Cx43, in contrast to HLE on EDTA-treated AM (Figs. 6C, 6D). In addition, we found that keratin K3 was absent in the basal layer on intact AM, whereas the basal layer in the EDTA-treated group expressed keratin K3 (Figs. 6E, 6F). Moreover, BrdU label-retaining nuclei were found in the basal layer of the epithelium on intact AM, but not at all in EDTA-treated AM cultures (Figs. 6G, 6H). That the amniotic epithelium and fibroblasts are devitalized and do not have any proliferative activity after the present method of preservation18 shows that the basal BrdU-labeled cells are derived from HLE. Collectively, these data indicate that a rapid cell cycle and positive expression of Cx43 and keratin K3 were promoted when HLE grew directly on the amniotic basement membrane, strongly supporting the notion that this culture condition promotes TAC differentiation. This interpretation, however, cannot be extrapolated to the method used by Koizumi et al.15,22 in which ST3 fibroblasts feeder layers are routinely included. Nevertheless, in another report,13 they mentioned that corneal differentiation is promoted, but failed to provide evidence that limbal epithelial progenitor cells are actually preserved. Future studies are needed to show that denudation of the amniotic epithelium to expose the amniotic basement membrane is an important microenvironmental “cue” in promoting TAC differentiation. This hypothesis can be tested by transplanting ex vivo expanded HLE in a rabbit model of limbal SC deficiency. This hypothesis can be tested by transplanting ex vivo expanded HLE in a rabbit model of limbal SC deficiency. The presence of the devitalized amniotic epithelium could not be discerned underneath the stratified HLE any longer, suggesting their partial or complete disintegration, as proposed by others.23 Our results showed that the basal layer of the resultant stratified epithelium on intact AM did not express Cx43, in contrast to HLE on EDTA-treated AM (Figs. 6C, 6D). In addition, we found that keratin K3 was absent in the basal layer on intact AM, whereas the basal layer in the EDTA-treated group expressed keratin K3 (Figs. 6E, 6F). Moreover, BrdU label-retaining nuclei were found in the basal layer of the epithelium on intact AM, but not at all in EDTA-treated AM cultures (Figs. 6G, 6H). That the amniotic epithelium and fibroblasts are devitalized and do not have any proliferative activity after the present method of preservation18 shows that the basal BrdU-labeled cells are derived from HLE. Collectively, these data indicate that a rapid cell cycle and positive expression of Cx43 and keratin K3 were promoted when HLE grew directly on the amniotic basement membrane, strongly supporting the notion that this culture condition promotes TAC differentiation. This interpretation, however, cannot be extrapolated to the method used by Koizumi et al.15,22 in which ST3 fibroblasts feeder layers are routinely included. Nevertheless, in another report,13 they mentioned that corneal differentiation is promoted, but failed to provide evidence that limbal epithelial progenitor cells are actually preserved. Future studies are needed to show that denudation of the amniotic epithelium to expose the amniotic basement membrane is an important microenvironmental “cue” in promoting TAC differentiation. This hypothesis can be tested by transplanting ex vivo expanded HLE in a rabbit model of limbal SC deficiency that we have recently reported.23 If this were the case, we may understand why migration of the offspring of limbal SCs to the corneal basement membrane signifies the beginning of TAC differentiation. Future studies should also be extended to investigating which amniotic basement membrane component(s)—laminin-1, laminin-5, collagen VII, and fibronectin—is responsible for upregulation of Cx43 expression and thus, TAC differentiation. In this regard, previous studies have shown that rat hepatocyte cultures25,26 and human epidermal keratinocyte cultures27 show an increase in gap junction synthesis and GJIC when exposed to certain extracellular matrix components (e.g., glycosaminoglycans, proteoglycans, and laminin-5).

Similarly, it seems equally important to delineate the role of devitalized amniotic epithelial cells in endowing HLE with a status of slow cycling and poor differentiation—that is, features resembling limbal epithelial SCs in vivo. Whether this role is a simple masking of the amniotic basement membrane or a release of cytokines from the devitalized amniotic epithelium, comparable to a feeder layer system, should be further investigated. Collectively, we believe the model system described herein is ideal for future exploration of the exact mechanistic operation in the microenvironmental niche that maintains “stemness” in limbal SCs, and in the cue that promotes corneal TAC differentiation. This new knowledge will help us understand the pathogenesis of limbal SC deficiency that develops in various ocular surface diseases and how transplantation of limbal epithelial SCs can be better defined in the future.

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