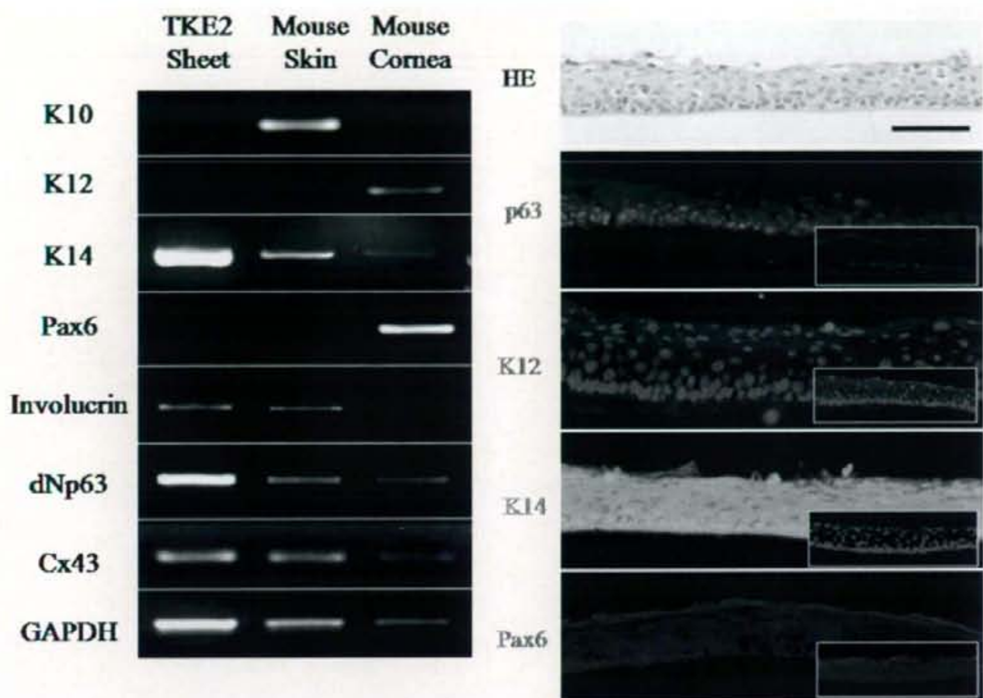

Fig. 9 Phenotypic characterization of single cell-generated stratified epithelial sheets. A stratified epithelial sheet was generated from single P52 clonally selected TKE2 cells by growing them on denuded amniotic membrane with 3T3 feeder layers on the plastic and by air-lifting technique. RT-PCR showed that this epithelial sheet expressed K14 keratin, involucrin, DNp63, but not K10 keratin, K12 keratin and Pax 6. Connexin 43 and involucrin, both normal differentiation markers for both normal skin and cornea, were also expressed in TKE2 sheets. These results was compatible with immunostaining, which showed K12 (–) and Pax6 (–) in whole layer, and K14 (+) in whole layer and p63 (+) in basal and middle layer cells. Negative controls were showed in insets. Bar represents 50 μm.

reached in 1 week. But we were disappointed to find that such cells could be subcultured at 1:3 splits only up to P2 or P3 for a total time span of 3 weeks [12]. Herein, we reported the success in expanding epithelial progenitor cells, characterized by a small cell size, negative expression of K12 keratin and positive expression of p63, that is, features known for limbal epithelial progenitors [3, 5, 7]. These progenitors continued to proliferate as evidenced by positive nuclear expression of PCNA, expand in large numbers and to be subcultured for more than 100 passages.

The above success was achieved by prolonging the culturing time to 4 weeks, that is, 3 weeks beyond confluence and passing the estimated lifespan of TAC expansion judged by our earlier report [12]. (Fig. 1) Because TACs are known to have a shorter cell cycle than limbal SCs [4], a high seeding density would have included more TACs of which the proliferation dominated the culture growth. As a result, we speculated that confluence reached by the aforementioned high-density cultures in 1 week is primarily achieved by rapid-cycling TACs [12]. When the culture period was extended beyond confluence as shown in this study, TACs eventually exhausted their proliferative potential and started degeneration and desquamation. The culture dish would then contain fewer and fewer cells, leaving the observer an impression that the growth had ceased. Nevertheless, if more time were patiently given, expansion of small epithelial progenitor cells emerged (Fig. 2). Hence, what we observed in this study bodes well with the notion that limbal SCs are slow-cycling and require a longer time to initiate expansion in the KSFM medium.

We noted that higher seeding densities led to more cell death as measured by Live/Dead Assay, more apoptosis suggested by fragmented nuclei, and larger elongated and squamous cells

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(Fig. 3), more expression of K12 keratin and less expression of nuclear p63 (Fig. 4). These results also suggested that higher seeding densities might include more TACs and terminally differentiated cells or favour such differentiation, which had invariably cumulated with an increasing culture life. We postulated that these differentiated cells might have generated a negative paracrine influence on SC expansion because the said clonal growth was inhibited by conditioned media collected from the latter cells (Hyashida *et al.* unpublished observation, 2006). Both prolonging the culturing time and lowering the seeding density made clonal growth of limbal epithelial progenitor cells possible (Fig. 1, 5 and 6). Interestingly, clones formed in KSFM were bigger and consisted of uniformly small cells as compared to those formed on conventional 3T3 fibroblast feeder layers (Fig. 5 and 6). If the large clones represent holoclones as suggested for keratinocyte SCs [17], we would speculate that KSFM is more amenable for promoting murine limbal/corneal epithelial progenitor cells than conventional 3T3 fibroblast feeder layers. This notion was also supported by the finding that cells in the center of the clones grown on 3T3 fibroblast feeder layer were large and squamous, and expressed more K12 keratin but less p63 nuclear staining (Fig. 6), consistent with a general consensus that 3T3 fibroblast feeder layer is not an ideal system to expand murine corneal/limbal epithelial progenitors.

Murine corneal/limbal epithelial cells expanded in KSFM at a high seeding density and a short culture time (*e.g.* 1 week) eventually turn on abnormal epidermal type differentiation by switching off K12 keratin expression and turning on K10 keratin expression. [12] Such abnormal terminal differentiation is further aggravated by increased [Ca^{2+}], but is reverted by FBS, presumably *via* vitamin A [12]. In this study, we noted that cells in P4 cultures still expressed K12 keratin at high seeding densities (Fig. 4), but did not express K10 keratin (not shown). Expression of K12 keratin by RT PCR was eventually lost in P52 cells (Fig. 9), but that defined by immunostaining could still be up-regulated in P12 cultures by increasing [Ca^{2+}] and/or addition of FBS (Fig. 7). Although it was small population, K12 positive cells still existed in P85 cultures (Supplemental Fig. B). Therefore, as compared to TACs, these expanded cells exhibited a clear difference in cellular proliferation and differentiation in response to these extracellular stimuli, suggesting that they adopted SC characteristics. The culturing system described herein can be used in the future to exploit the mechanism by which differentiation of SC and TAC is regulated.

Single P52 cells could exhibit clonal expansion with colony-forming efficiency of 3–4% in KSFM (Fig. 6), suggesting that not all expanded cells were kept at a progenitor status. Expanded cells had an average doubling time estimated to be 31.3 hrs at day 7 (TKE2 clone: 34.2 hrs), and continued to be uniformly small before reaching a certain clone size (*e.g.* 14 days of culture) in KSFM. They did not exhibit anchorage-independent growth (Fig. 8) or abnormal karyotyping (not shown). Single TKE2 cell-expanded progeny could be seeded on an epithelially denuded amniotic membrane to engineer a stratified epithelial sheet (Fig. 9). The resultant epithelium still retained a basal epithelial phenotype of stratified epithelia as shown by positive expression of K14 keratin and p63, especially the isoform of DNp63, and by negative expression of K12 keratin, K10 keratin and Pax 6 (Fig. 9). Because a small (less than 1%) population of late passage cells still expressed K12 expression in normal culture medium (KSFM) (Supplemenatal Fig. B), we believe this cell line could be used to search for cues in the limbal niche that may help promote the corneal lineage fate determination in the future [18].

Acknowledgement

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Supplementary Material

The following supplementary material is available for this article:

Supplementary Figure - Progenitor Status of TKE2 (P85)

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1582-4934.2008.00297.x

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A Novel NIH/3T3 Duplex Feeder System to Engineer Corneal Epithelial Sheets with Enhanced Cytokeratin 15-Positive Progenitor Populations

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ABSTRACT

Corneal epithelial cell sheets co-cultivated with feeder cells are used to reconstruct the ocular surface in stem cell–depleted eyes. The present study was conducted to investigate the optimal method of using feeder cells in the interest of preserving progenitor cells in cultivated sheets. We compared the phenotype and secondary colony forming efficiency (CFE) of cell sheets that were engineered using 3T3 feeder cells as a separate layer or as a contact layer. We also devised a novel "duplex feeder" system that consists of two separate layers of feeder cells. After cells reached confluence, cells were cultured at the air–liquid interface to allow full stratification. Stratified sheets were then analyzed using immunohistochemistry and secondary colony formation. Contact feeder cultures and duplex feeder cultures yielded epithelial sheets with small, cuboid basal cells with strong expression of keratin (K)3, K12, and K15. Furthermore, only duplex feeder layers reproduced the basal K15, suprabasal K12 limbal phenotype where epithelial stem cells reside. A similar effect was observed when cornea stroma–derived progenitor cells were used as feeder cells. Duplex feeder sheets also produced significantly more secondary colonies than cells dissociated from single layer sheets, suggesting that the duplex feeder system produces transplantable sheets with a higher yield of progenitor cells.

INTRODUCTION

CULTIVATED EPITHELIAL CELL SHEETS are used clinically for reconstructing the ocular surface in blinding diseases that destroy the corneal epithelial stem cell niche located in the limbus (reviewed in [1,2]). When chemical or thermal burns or autoimmune disorders such as ocular cicatricial pemphigoid and Stevens-Johnson syndrome severely damage the limbus, conjunctival or dermal cells replace the corneal epithelium, leading to irreversible opacification of the cornea. Transplantable epithelial sheets can be engineered using epithelial cells from various tissue sources such as the corneal limbus[3,4] or oral mucosa[5,6] using various matrixes, including the amniotic membrane,[3,7] fibrin,[8] and temperature-responsive polymers.[6,9] However, it is still unclear whether cultivated sheets can provide sufficient amounts of progenitor cells or restore the stem cell niche to its normal architecture.

The preparation of epithelial sheets requires feeder cells to expand progenitor cells and to produce stratified sheets. Feeder cells support the serial subculture of epithelial cells though several passages,[10] allow colony formation by epithelial cells,[11,12] inhibit the growth of contaminating fibroblasts,[10] and maintain human telomerase reverse transcriptase expression[13] and Sp1/Sp3 activity.[14] Using feeder cells is believed to reproduce several aspects of the stem cell

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niche, although molecular mechanisms involved in interactions between epithelial and feeder cells are unclear. Culture medium conditioned with 3T3 cells stimulates epithelial cell growth[15] but not colony formation by epithelial cells.[10] The plasma membrane of 3T3 cells or glutaraldehyde-fixed 3T3 cells has been reported to support the growth of epithelial cells seeded at a high density,[16] although epithelial cells seeded at lower clonal densities cannot form colonies when in contact with non-viable 3T3 cells.[17] Therefore, direct cell-to-cell contact and soluble factors secreted by viable feeder cells both seem to be involved in maintaining epithelial cells in an undifferentiated state.

In the current study, we sought to find an ideal method to use feeder cells to engineer a fully stratified epithelial sheet with a limbal phenotype. We compared several culture conditions using 3T3 feeder cells and found that not only did direct contact with feeder cells improve epithelial cell growth and colony formation, but also an additional feeder layer separated by a culture insert was found to produce an epithelial sheet with keratin expression patterns strikingly similar to the limbus.[18] Herein we show how this "duplex feeder system" offers the benefits of direct contact and sustained release of soluble factors that may be used as an *in vitro* model of the limbal stem cell niche in addition to transplantable cultivated sheets with enhanced progenitor populations.

MATERIALS AND METHODS

Preparation of feeder cells and human limbal epithelial cells

NIH/3T3 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured with Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS). Mouse corneal precursors (COPs) were maintained in DMEM/F12 supplemented with 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich, St. Louis, MO), 10 ng/mL fibroblast growth factor 2 (Sigma-Aldrich), B27 supplement (Invitrogen), and 10^3 U/mL leukemia inhibitory factor (Chemicon International, Inc, Temecula, CA), as previously described.[19,20] Dissociated COPs were cultured with DMEM containing 10% FBS to induce differentiation into fibroblasts. Confluent cells were treated with mitomycin C (4 µg/mL, Sigma-Aldrich) at 37°C for 2 h. Dissociated cells were cryopreserved until use. All subsequent references to feeder cells are strictly to mitomycin C–treated NIH/3T3 cells. Human limbal epithelial cells (LECs) were obtained from U.S. eye bank corneas after the central buttons were used for corneal transplantation. After removal of excess tissue, the limbus was immersed in 1.2 U dispase II (Roche Diagnostics, Basel, Switzerland) in DMEM/F12 (Invitrogen) at 4°C overnight. Epithelium was separated from the stroma and treated with 0.05 % trypsin ethylenediaminetetraacetic acid (EDTA) at 37°C for 30 min to disperse cells. LECs were seeded immediately after isolation and cultured in supplemental hormonal epithelial medium consisting of DMEM/F12, 10% FBS, 10 ng/mL EGF, 5 µg/mL human recombinant insulin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 500 ng/mL hydrocortisone (water soluble, H-0396, Sigma-Aldrich), 5 µg/mL transferrin (Sigma-Aldrich), 250 ng/mL isoproterenol hydrochloride (Wako),[21] and antibiotics. Medium was replaced 3 times per week and then every day during airlift cultures.

Colony forming efficiency and cell growth analysis

To evaluate the effect of direct cell-to-cell contact with feeder cells on colony formation by LECs, feeder cells were seeded on a 100-mm dish at a density of 2.5×10^4/cm^2. On the following day, feeder cells were removed from half of the dish using a cell scraper (Fig. 1A), and 2000 LECs were seeded on the dish. Dishes were fixed with formalin after 2 weeks and stained with 1% Rhodamine B (Wako) in distilled water. Colonies in the feeder half and feeder-free half of the dish were counted separately. Colony formation efficiency (CFE) was calculated as the number of the colonies divided by the number of cells seeded on each area (1000 cells).

To evaluate the effect of feeder contact on epithelial cell growth, feeder cells (2.5×10^5) were seeded in cell culture inserts (Transwell, cat no 3450, Corning, Corning, NY), and another 2.5×10^5 cells were seeded in the bottom of the well (Fig. 2A, right), which we have termed the "duplex feeder system." As a control, 5×10^5 feeder cells were seeded in the bottom of the well (Fig. 2A, center). On the next day, isolated LECs (4×10^4) were inoculated in each culture insert and cultured for 2 weeks. Rhodamine-stained areas were measured using the Windows version of NIH/

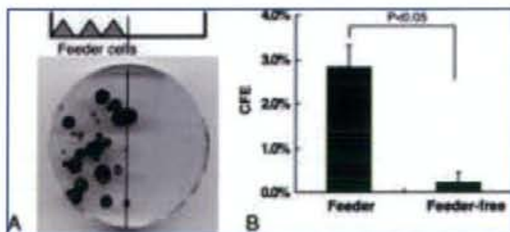

FIG. 1. Colony forming efficiency (CFE) of limbal epithelial cells. Primary limbal epithelial cells (LECs) were plated in 100-mm dishes at a density of 2×10^3/dish. (**A**) Colonies were predominantly observed in the left half of the dish, which was pre-coated with mitomycin C–treated 3T3 cells. (**B**) The higher CFE observed in LECs cultured in contact with feeder cells was statistically significant ($p<0.05$, $n=3$).

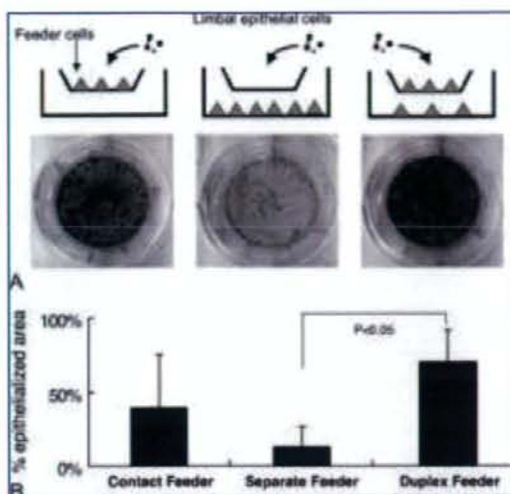

FIG. 2. The effect of duplex feeder layers on epithelial proliferation. Limbal epithelial cells (LECs) were plated in culture inserts with 3T3 feeder cells in direct contact, separated, or both (duplex feeders). (**A**) Epithelial cell proliferation after 2 weeks was greater in the duplex feeder group than in contact feeders or separate feeder controls. (**B**) The area covered by epithelium was statistically greater in the duplex feeder group ($74.3 \pm 20.4\%$) than in separate feeder controls ($n = 4$).

Image software (Scion Image, Scion Corporation, Frederick, MD).

Epithelial sheet cultivation

Stratified epithelial sheets were cultivated on cell culture inserts coated with 300 µL of fibrin (5.5 mg/mL, Bolheal, Fujisawa, Osaka, Japan), as previously described. [22] LECs (10^4/cm^2) and NIH/3T3 feeder cells or COP feeder cells were cultured as described above. Aprotinin (666 KIU/mL, Wako) was supplemented in supplemental hormonal epithelial medium to prevent the digestion of fibrin glue during culture. After epithelial cells reached confluence, cells were airlift cultured for an additional week to allow stratification.

Formalin-fixed paraffin sections of cultivated sheets were stained with hematoxylin and eosin (HE) for histological observation. For immunohistochemistry, paraffin sections were used for anti-p63 staining after antigen retrieval using heat treatment. For other antigens, fresh frozen cryosections were fixed with acetone for 5 min and reacted with primary antibodies against K3 (mouse, clone AE5, Progen, Heidelberg, Germany), K12 (rabbit, sc-25722, Santa Cruz Biotechnology, Santa Cruz, CA), K15 (mouse, LHK15, Lab Vision, Fremont, CA), and p63 (clone 4A4, Calbiochem, Merck KGaA, Darmstadt, Germany). Sections were then treated with Alexa flour 488 or 555 conjugated secondary antibodies (Invitrogen). Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (1 µg/mL, Dojindo Laboratories, Tokyo, Japan).

Secondary colony formation

To determine whether clonogenic cells remained in stratified epithelial sheets, secondary clonal growth was observed using dissociated epithelial cells. Epithelial sheets from each group were digested with trypsin EDTA for 10 to 15 min, and dissociated cells were then plated at cloning densities (10^4 cells/dish) in 100-mm dishes containing a single layer of feeder cells. After colonies were cultured for 2 weeks, cells were stained with Rhodamine B, and CFE was calculated.

RESULTS

CFE and cell growth analysis

We first confirmed colony formation by LECs seeded at a density of 2×10^3 in a 100-mm dish half coated with 3T3 feeder cells. Thus only half of the cells were in direct contact with feeders, whereas the other half were exposed only to soluble factors secreted by feeders. As shown in Figure 1, colonies were predominantly formed in the feeder-covered half (mean ± standard deviation $2.8 \pm 0.5\%$, $n = 3$), whereas only a few colonies were found in the feeder-free half ($0.2 \pm 0.2\%$) (Student t-test, $p < 0.05$). This result shows that soluble factors alone were not sufficient to initiate epithelial cell colonies.

The effect of feeder cells on epithelial cell proliferation was observed in a different experiment with LECs inoculated at a high density (10^4/cm^2). Three groups of cells were prepared, including the duplex feeder set-up along with control groups with only single feeder layers with or without direct contact. Cell proliferation was expressed as the area covered by epithelial cells after 2 weeks. Proliferation was greatest in the duplex feeder group, which covered $74.3 \pm 20.4\%$ of the culture insert ($n = 4$), which was greater than the $36.3 \pm 30.0\%$ covered by the contact feeder group and significantly greater than the $10.1 \pm 12.4\%$ covered by the separate feeder group ($p < 0.05$, Kruskal-Wallis test) (Fig. 2). Epithelial cells in the duplex feeder group reached confluence in an average of 10.1 ± 1.7 days ($n = 3$), whereas cells in the separate feeder group required 13.6 ± 3.2 days, and those in the contact feeder group required 11.3 ± 1.5 days. An additional feeder layer as a source of soluble factors seemed to enhance cell proliferation.

Epithelial sheet cultivation

We next investigated the histology of stratified sheets prepared using each feeder system. HE stains show that

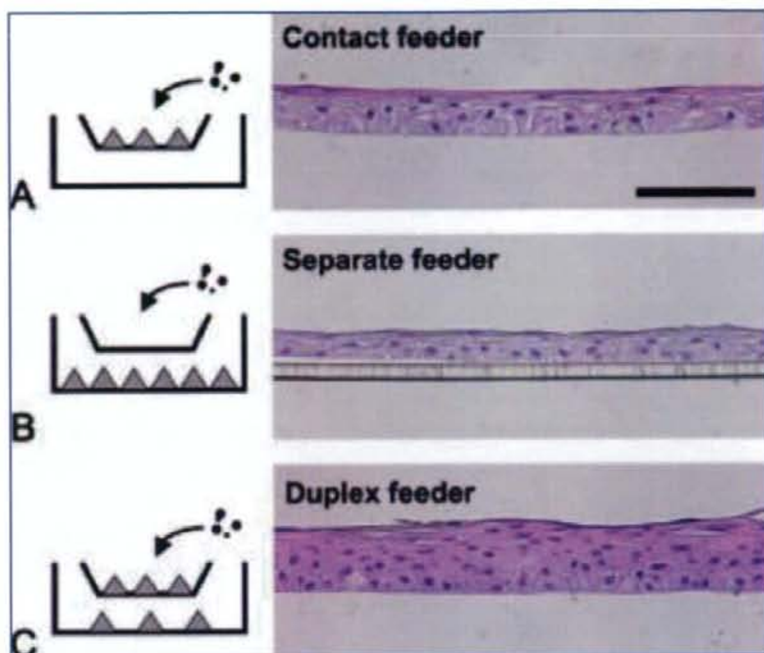

FIG. 3. Hematoxylin and eosin staining of epithelial sheets. Limbal epithelial cells (LECs) were cultured until confluence on fibrin-coated culture inserts and then allowed to stratify by airlifting. LECs formed stratified layers in the contact feeder group (**A**) and the separate feeder group (**B**). However, the duplex feeder group (**C**) constantly produced five to six layers of epithelial cells with morphology similar to that observed *in vivo*. Scale bar = 100 μm. Color images available online at www.liebertpub.com/ten.

each group produced a multilayer epithelial sheet with cuboid basal cells and flattened superficial cells (Fig. 3). Stratification was limited to two to three layers in the separate feeder group (Fig. 3B), whereas contact feeder sheets were slightly more stratified (Fig. 3A). However, the duplex feeder group produced a stratified epithelium with six to seven layers of cells with morphologies similar to those observed *in vivo* (Fig. 3C).

Differentiation of epithelial sheets was examined using immunocytochemistry against p63, K3 and K12, and K15, a keratin expressed by basal limbal epithelial cells *in vivo*. Figure 4 shows representative staining patterns observed in each group. p63 was positive in the nucleus of most cells in each group, excluding the most superficial cells (Fig. 4A–C). K3 was constantly observed in all groups, although the degree to which each layer stained positive varied in the contact feeder (Fig. 4D) and separate feeder groups (Fig. 4E). However, K3 expression was predominantly superficial in the duplex feeder group (Fig. 4F), which also showed K12 expression predominantly in the superficial layers (Fig. 4I). K12 expression was low in the contact feeder (Fig. 4G) and separate feeder (Fig. 4H) groups. K15 was also expressed in all cell sheets, although positive cells were sporadic, with no apparent pattern observed in the contact feeder (Fig. 4J) and separate feeder (Fig. 4K) sheets. On the other hand, the cuboid basal epithelial cells in the duplex feeder group were all K15 positive, with minimal staining in the superficial layers (Fig. 4L). A merged image of K12 and K15 staining shown enlarged in Figure 4M shows the organized stratification of cuboid basal K15+ cells and K12+ superficial cells, which is strikingly similar to the limbal epithelium *in vivo* (Fig. 4N). This pattern of keratin staining was highly reproducible, with the same results obtained from five independent experiments from different cell sources.

Secondary CFE

To address the question of whether cultivated sheets maintained progenitor cells, CFE was calculated using cells dissociated from each group. Clonogenic cells were abundant in epithelial cell sheets cultivated with duplex feeders, as shown by the significantly higher CFE in the duplex feeder group ($5.09 \pm 2.07\%$) than in the contact feeder ($2.09 \pm 0.82\%$, $p < 0.05$, Kruskal-Wallis test) and separate feeder ($1.46 \pm 0.91\%$, $p < 0.05$) groups (Fig. 5). Results show again that contact with feeder cells by itself was not as effective in maintaining progenitor cells during stratification.

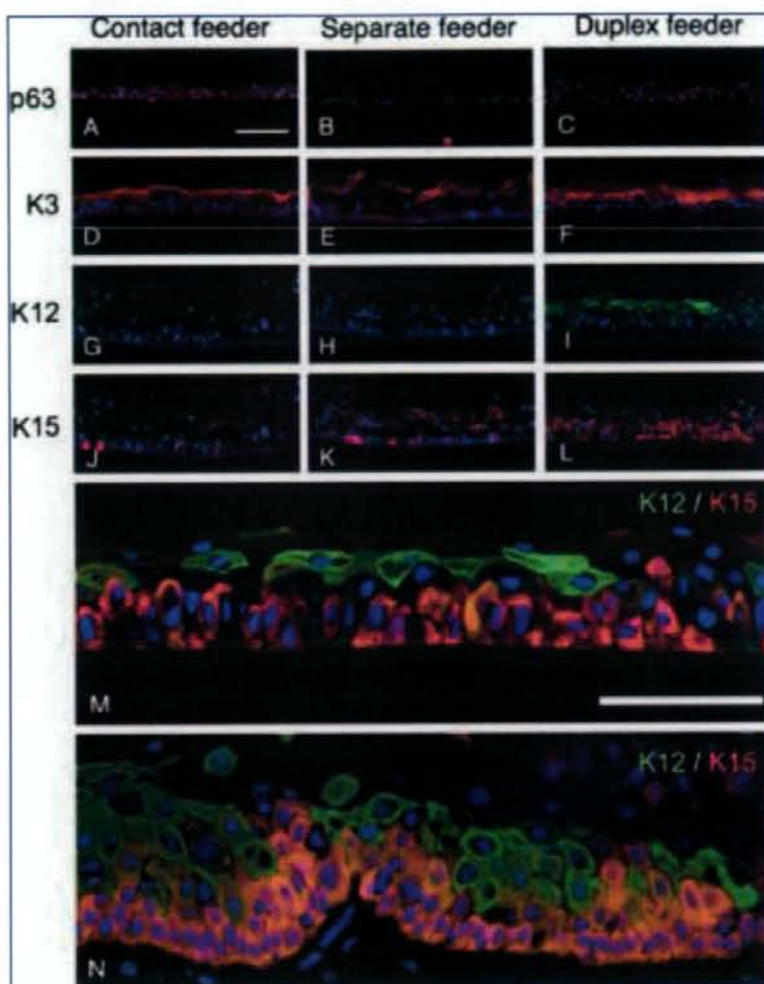

FIG. 4. Expression of p63 and keratins by stratified sheets. Epithelial sheets from the contact feeder group, separate feeder group, and duplex feeder groups were stained with anti-p63 (**A–C**), keratin (K)3 (**D–F**), K12 (**G–I**), and K15 (**J–L**), respectively. All cell sheets expressed p63, K3, and K15, whereas only duplex sheets (**I**) constantly expressed K12. Duplex sheets also showed supra-basal K3 (**F**) and basal expression of K15 (**L**), indicating that basal cells were uniformly immature. (**M**) High magnification of merged K12/K15 staining of the duplex feeder sheet is strikingly similar to the staining pattern observed in normal human corneal limbus (**N**). Bars = 100 μm.

Duplex feeder using mouse COPs

To confirm that the duplex feeder system is effective with feeder cells other than NIH/3T3 cells, fibroblasts differentiated from mouse COPs were used as feeder cells (Fig. 6). Epithelial cells cultured with duplex COP feeders (Fig. 6B) showed better stratification than cells cultured with separate COP feeders (Fig. 6A). Expression of K3 (Fig. 6C, D), K12 (Fig. 6 E, F), and K15 (Fig. 6G, H) was stronger in duplex COP feeder sheets than in separate COP feeder sheets.

DISCUSSION

The interaction between epithelial cells and mesenchymal cells via diffusible factors promotes the proliferation

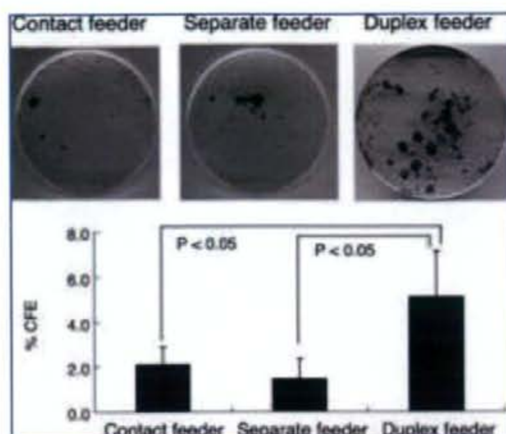

FIG. 5. Secondary colony forming efficiency (CFE) by dissociated epithelial sheets. Stratified epithelial sheets were enzymatically digested, and dissociated cells were plated in 3T3-coated dishes to calculate secondary colony formation. Average CFE in the duplex feeder group ($5.09 \pm 2.07\%$) was significantly higher than in the contact feeder group ($2.09 \pm 0.82\%$) or the separate feeder group ($1.46 \pm 0.91\%$) ($p < 0.05$, $n = 4$).

and differentiation of epithelial cells,[12,23] although conditioned medium harvested from fibroblast cultures does not support the initiation of epithelial colonies.[10,12] The results of our study are consistent with these previous reports suggesting that soluble factors alone are not sufficient to propagate colony-forming progenitor cells. Because cocultured epithelial cells enhance the production of some of these factors in a paracrine manner,[23,24] we designed our experiment so that epithelial cells not in contact with feeders were exposed to possible soluble factors secreted by interactions between feeder and epithelial cells within the same culture dish. The results showed that direct contact with feeders was still required to initiate clonal growth under these culture conditions (Fig. 1).

Epithelial cells in contact with feeders not only initiated colonies, but also showed enhanced growth, as evidenced by the growth area (Fig. 2) and shorter time required to achieve confluence. Other studies have shown that epithelial cell growth is limited without the use of feeder cells and that the number of passages is lower when epithelial cells are cultured without 3T3 feeder cells.[13,14,25] We found that an extra feeder layer in the duplex culture group further enhanced the growth rate, which can be explained by the fact that feeder cells were gradually shed from the dish

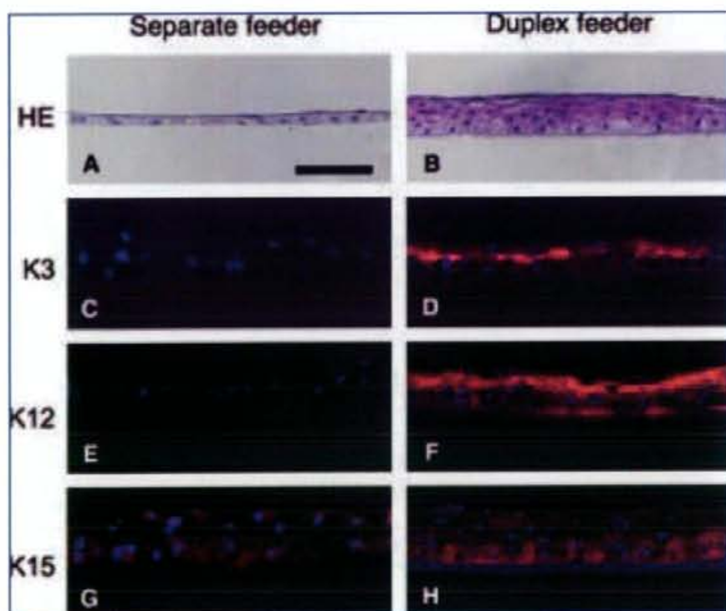

FIG. 6. The effect of a duplex feeder layer using mouse corneal precursors. Epithelial cells were cultured with fibroblasts differentiated from mouse corneal precursors (COPs) using a separate feeder (**A, C, E, G**) and a duplex feeder (**B, D, F, H**). Sections were stained with hematoxylin and eosin (**A, B**), anti-keratin (K)3 (**C, D**), anti-K12 (**E, F**), and anti-K15 (**G, H**) antibodies. A duplex feeder using other than NIH/3T3 cells also enhanced the organization and expression of differentiation keratins. Bar = 100 μm. Color images available online at www.liebertpub.com/ten.

during the expansion of epithelial cells. The additional layer probably secretes vital soluble factors required for cell growth.

Confluent sheets of cells were then cultivated at the air–liquid interface to allow stratification. Again we found that the duplex feeder system produced the most robust epithelial sheet, with features similar to the limbal epithelium *in vivo*. We recently reported that the basal limbal epithelium shows a unique expression pattern of K15 with positive cells in the basal to suprabasal layers that overlap with K12 expression.[18] Unlike K14 or K19, K15 is not found in the central cornea and can therefore be used as a marker specific to the limbus. Although basal conjunctival epithelial cells also express K15, there is no overlapping with K12 expression. The staining pattern observed in the duplex feeder cell sheets (Fig. 4M) shows that we have demonstrated that the limbal epithelial phenotype can be recreated *in vitro* using the duplex feeder system. Contact with feeder cells alone was not as effective, and cell sheet quality and staining of K12 in the contact feeder group varied between individual experiments. On the other hand, the limbus-like cell sheets cultivated with duplex feeders were highly reproducible.

The higher expression of K12 in the duplex feeder group can be interpreted as advanced differentiation in these sheets. However, lower levels of differentiation markers in the contact feeder and separate feeder sheets may be the result of poorer differentiation ability and not necessarily the maintenance of immature cells. Epithelial cells cultured under suboptimal conditions progressively lose differentiation markers,[26] suggesting that cells in the control sheets had lower proliferative and differentiation potential. HE staining of epithelial cell sheets (Fig. 3) supports this interpretation, with duplex feeder sheets having the most organized cell structures, with small, cuboid basal and superficial wing-cell-like structures. Secondary CFE assays (Fig. 5) conclusively showed that duplex feeder sheets retained more progenitor cells than controls, further supporting our hypothesis that the duplex feeder sheets can be used as an *in vitro* model for the limbal stem cell niche.

Grueterich *et al.* used the amniotic membrane (AM) as an *in vitro* model of the limbal stem cell niche.[27] They found that an AM with intact epithelium inhibited the differentiation of limbal epithelial cells, whereas denuded AM allowed the upregulation of K3 and K12. The addition of 3T3 feeder cells partially suppressed the upregulation of these differentiation markers. Intact AM epithelial cells may have acted in a similar fashion to 3T3 cells in our duplex culture system, offering direct cell-to-cell contact. We did not use AM as a substrate in this study, explaining the difference in K3/K12 expression often reported in cultivated epithelial sheets using AM carriers.[28] Our substrate was based on commercially available fibrin glue using a method reported previously.[22] The expression pattern of K12/K15 observed in the duplex feeder group more closely resembled the limbal phenotype than previous studies using AM substrates, which showed a uniform K12 staining pattern similar to the central cornea.

Although we have proposed the duplex feeder system as an *in vitro* model of the limbal stem cell niche, the remarkable reproduction of the limbal phenotype makes this technique a candidate tool for cultivating transplantable epithelial sheets. Epithelial cell sheets cultured on fibrin glue[22] or temperature-sensitive polymers[6,9] have advantages over AM sheets, such as higher transparency and rapid attachment to the ocular surface without sutures. One possible disadvantage of such carrier-free sheets is advanced differentiation, shown by the upregulation of K3.[22] Using the duplex feeder system may overcome this problem. Although using mouse-derived 3T3 feeders in direct contact with donor cells may pose ethical problems, the use of human-derived feeder cells or autologous feeder cells[29] may be an option. We have shown that fibroblasts derived from mouse COPs can also improve epithelial sheet phenotype using the duplex feeder system (Fig. 6), suggesting that other fibroblasts, including human cells, may be similarly effective. In the mean time, the duplex feeder system offers an opportunity to analyze many of the soluble factors and adhesion molecules believed to be involved in maintaining the limbal stem cell niche.

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Long-Term Culture and Growth Kinetics of Murine Corneal Epithelial Cells Expanded from Single Corneas

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Purpose. To develop a reproducible procedure for the long-term culture of corneal epithelial cells from a single mouse cornea.

Methods. Corneal limbal explants of C57BL6/J mice were cultured in serum-free, low-Ca^{2+} medium supplemented with EGF and cholera toxin. Epithelial cells were subcultured at a 1:3 split until passage (P)4 and at lower densities after P4. Colony-forming efficiency, population-doubling times, and population doublings were determined. The expression of p63, keratin (K)19, K12, and involucrin was analyzed by RT-PCR, immunocytochemistry, and Western blotting. Differentiation potential was examined by switching the medium to serum or high Ca^{2+}-containing medium. Stratification ability was analyzed by air-lift culture.

Results. Thirty of 32 (93.8%) corneal explants were successfully subcultured to P1. Cultures without cholera toxin did not proliferate past P2 ($n = 12$), but 55% of cultures supplemented with cholera toxin achieved P4 ($n = 20$). After P4, cells were stably subcultured over 25 passages. Colony-forming efficiency increased from 9.7% ± 2.6% at P5 to 29.0% ± 3.3% at P20. The cells showed cobblestone appearance and expressed p63, K19, and involucrin but were negative for K12. Serum and high Ca^{2+} induced differentiation, and cells cultured in DMEM/F12 with serum showed K12 mRNA expression. Stratified epithelium was formed by air-lifting.

Conclusions. With this procedure, corneal epithelial cells from a single cornea can be cultured long term and can retain the potential to differentiate and stratify. This procedure can be a powerful tool for studies that require comparison of corneal epithelial cells from normal and transgenic mice in vitro. (*Invest Ophthalmol Vis Sci.* 2009;50:000–000) DOI:10.1167/iovs.08-2139

Methods for culture and subculture of human corneal epithelial cells have been well documented.[1-4] However, such has not been the case for mouse corneal epithelial cells (MCEs). Hazlett et al.[5] cultured MCEs from explants but failed in subculturing the cells over three passages. Limited lifespan and insufficient cell yields hindered the application of MCEs in in vitro experiments. Kawakita et al.[6] reported a method to successfully isolate viable mouse corneal/limbal epithelial sheets and culture in serum-free low-Ca^{2+} medium. Furthermore, by prolonging the culturing time and lowering the seeding density, they successfully established a mouse corneal epithelial line.[7] However, they required more than 200 eye globes to establish their stable cell line. To our knowledge, all existing methods failed in reproducing the long-term culture of MCEs from a single mouse cornea.

Using mice to study the molecular biology and physiology of the cornea has several advantages. The advent of genetic manipulation resulting in corneal phenotypes in transgenic and knockout mice provides a powerful tool to study the molecular mechanisms involved in the development and pathogenesis of diseases in the cornea. Therefore, establishing long-term culture of MCEs from a single mouse cornea becomes increasingly important to plan in vitro studies to substantiate findings from in vivo studies in these mice. Herein, we report a novel three-step culture procedure for the long-term culture of MCEs from a single mouse cornea. The initial stage uses limbal explant cultures in serum-free low-Ca^{2+} medium supplemented with cholera toxin, followed by subculture of corneal epithelial cells in low cell densities. The cells obtained were morphologically normal, showed the phenotype of corneal epithelial progenitor cells, and retained differentiation potential and stratification ability for more than 25 passages.

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Materials and Methods

Tissue Preparation and Cell Culture

C57BL/6 mice (CLEA Japan Inc, Tokyo, Japan), aged 8 to 10 weeks, were handled according to the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. After death, eye globes were enucleated from the mice with forceps and washed profusely in phosphate-buffered saline (PBS). Eyes from each animal were kept separate throughout the culture procedure. Corneal buttons, including the limbus, were cut from the eye and cleaned of extraneous tissue (e.g., iris, ciliary body). Primary cell culture was performed with the use of an explant culture method similar to that of Hazlett et al.[5] Briefly, the button was cut in half, and each explant was plated flat, epithelium side up, on a six-well plate, one piece per well. After approximately 5 to 10 minutes to allow for attachment of the explant, serum-free low-Ca^{2+} medium (keratinocyte serum-free medium [KSFM]; Invitrogen, Carlsbad, CA) consisting of 10 ng/mL human recombinant EGF (Invitrogen), 100 ng/mL cholera toxin (LIST Biological Laboratories Inc., Campbell, CA), antibiotics, and growth supplement supplied by the manufacturer were added. The cultures were incubated at 37°C, under 95% humidity and 5% CO_2, and the medium was changed every 3 to 4 days. After approximately 10 days, the explant was carefully transferred to a new dish and cultured as described. Cells derived from passaged explants were also used for subcultures. Epithelial cells were subcultured (TrypLE Express; Invitrogen) at 1:3 splits after small cells reached subconfluence or if the colonies of small cells started to stratify before reaching subconfluence. The procedure was repeated until passage (P)4 cultures. From P5, subconfluent cells were subsequently passaged at a density of 5 ×

AQ: 1

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10^4 per 75-cm^2 flask. Cultures were incubated at 37°C, under 95% humidity and 5% CO_2, and the medium was changed every 3 to 4 days.

Cell Cryopreservation and Thawing

Dispersed cells were pelleted, resuspended without serum (Cell Banker-II; Mitsubishi Kagaku Iatron, Inc, Tokyo, Japan), placed in cryotubes (5×10^5 to 10^6 cells/mL), and frozen at −80°C. For cell thawing, cryotubes were partially immersed in 37°C water. Cells were harvested and plated at a density of 5×10^4 per 75-cm^2 flask supplemented with complete culture medium, as described previously.

CFE and Cell Proliferation

MCEs were inoculated in 60-mm dishes at 1000 cells/dish and cultured for 10 to 14 days. Cultured cells were stained with eosin for 1 hour. Colony-forming efficiency (CFE) was calculated as the percentage of colonies per number of inoculated cells. Three independent experiments were performed. Population doublings (PDs) were calculated as log 2 (D/D0), where D and D0 were defined as the density of cells at the time of harvesting and seeding, respectively. Doubling times were calculated as culture interval (hours)/PDs.

AQ: 2

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from MCEs cultured for 7 to 10 days with a commercial RNA isolation kit (RNeasy, Qiagen, Valencia, CA), and cDNA was synthesized (RevaTra Ace; Toyobo, Osaka, Japan). The same amount of cDNA was amplified by PCR (GeneAmp 9700; Applied Bioscience, Inc., Foster City, CA) for each primer pair shown in Table 1. PCR products were analyzed by agarose gel electrophoresis.

T1 AQ: 3

Immunostaining

MCEs (1×10^3cells/well) were cultured in gelatin-coated, two-well chamber slides and fixed with 4% paraformaldehyde (PFA; Wako Ltd., Osaka, Japan). PFA-fixed cells were permeabilized with 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO). After blocking with 10% normal donkey serum, the cells were treated with the following monoclonal primary antibodies: anti-p63 (1:100, 4A4; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-keratin (K)19 (1:100; NeoMarkers for Laboratory Vision Corporation, Fremont, CA), anti-involucrin (1:100; Covance, Emeryville, CA), and anti-K12 (1:100; Santa Cruz Biotechnology). Cells were then treated with Cy3-conjugated secondary antibodies (Chemicon International, Inc., Temecula, CA). Nuclei were counterstained with 4′,6′-diamino-2-phenylindole (1 μg/mL, DAPI; Dojindo Laboratories, Tokyo, Japan).

Western Blot Analysis

MCEs were dissolved with lysis buffer (M-PER; Pierce, Rockford, IL). The same amount of protein was loaded on a 10% Bis-Tris gel (Ready Gel; Bio-Rad Laboratories, Hercules, CA) and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were immunostained with primary antibodies against p63 (1:200), K19 (1:50), involucrin (1:3000), K12 (1:100), and β-actin (1:500, mabcam 8226; Abcam Inc., Cambridge, MA), respectively. After the reaction with horseradish-peroxidase–conjugated secondary antibody, protein bands were visualized by ECL (GE Healthcare, Buckinghamshire, UK) and x-ray film.

Differentiation and Stratification of Passaged Cells

MCEs were inoculated in 60-mm dishes at a density of 1.5×10^4 cells/dish and were cultured for 10 to 14 days in KSFM, KSFM supplemented with 0.9 mM Ca^{2+}, KSFM with 10% fetal bovine serum (JRH Biosciences, Inc., Lenexa, KS), KSFM with 0.9 mM Ca^{2+} and 10% serum, or DMEM/F12 (Gibco, Invitrogen) with 10% serum to induce differentiation. Cell sheets were obtained by seeding cells at a density of 3×10^5 cells/mL on cell culture inserts (Transwell, Corning, Corning, NY) in KSFM. When confluent epithelium formed, the culture medium was changed to supplementary hormonal epithelial medium (SHEM).[8] SHEM consists of an equal volume of DMEM and F12, containing 10 ng/mL human EGF, 5 μg/mL insulin (Wako), 5 μg/mL transferrin (Sigma), 0.25 μg/mL isoproterenol (Sigma), 0.5 μg/mL hydrocortisone (Sigma), and 10% serum. One day later, the medium level was lowered to expose the epithelium at the air-liquid interface with mitomycin C-treated 3T3 fibroblasts feeder layers preseeded on the plastic to promote stratification.[9] After 10 days of culture, the stratified epithelia were observed by microscopy.

Results

Establishment of Cell Lines from Single Corneas

Thirty of 32 (93.8%) cornea explants were successfully subcultured to P1. Up to 55% of P1 cells were passaged to P4 when supplemented with cholera toxin ($n = 20$), while none of the cells exceeded P2 without cholera toxin ($n = 12$). After P4, cells were stably subcultured over P25. According to the growth features of MCEs, we subjectively divided the growth process into three stages. In stage 1 (explant culture), MCEs began to grow out from the explants within 24 hours. The cells showed a cobblestone appearance, and epithelial cells near the explants stratified. We subcultured the explants by 10 days to ensure that fibroblast outgrowth was minimal. This was based on observations during preliminary experiments, when we occasionally found fibroblast growth between the 11th and 15th days of culture. Stage 2 (P1–P4) was the critical period for establishing a stable cell line. Early-passage cells revealed a heterogeneous population of small and large cells. With increasing culture time, large squamous cells underwent senescence and detached from the culture surface, whereas small cells were selectively preserved. Only on condition that small cells became subconfluent did the cells continue to proliferate for subsequent passages. Cells cultured without cholera toxin did not exceed P2 of this stage ($n = 12$; see Fig 2). Stage 3 (>P4) was the stable phase. Cells were subcultured by lowering the seeding density to induce a stable cell line consisting of uniformly small cells with typical cobblestone appearance. During stage 3, it was possible to cryopreserve cells with greater than 75% viable cells after thawing.

CFE and Growth Kinetics

CFE increased from 9.7% ± 2.6% at P5 to 29.0% ± 3.3% at P20 (mean ± SD; $n = 3$). PDs increased with successive passages that seemed to plateau after P12 at an average of 45.9 ± 2.4 hours ($n = 6$; Fig. 1E). On reaching the plateau, cells seeded at 700/cm^2 usually reached saturation density within 8 days after plating. They were stably subcultured though at least 25 pas-

F1

Table 1. Primers Used for RT-PCR

Primer	Sequence (5′→3′)	Product Size (bp)
p63	GTCAGCCACCTGGACGTATT ACCTGTGGTGGCTCATAAGG	321
Keratin 19	TGATCGTCTCGCCTCCTACT GGCTCTCAATCTGCATCTCC	356
Keratin 12	TGACGAGAGCTCATCCCTTT CCCAGGAAGGTGTAAGGTGA	380
Involucrin	CAAGACATGCTAGTACCACAGG GTGTCCGGTTCTCCAATTCGTG	883
β-Actin	TGTTACCAACTGGGACGACA TCTCAGCTGTGGTGGTGAAG	392

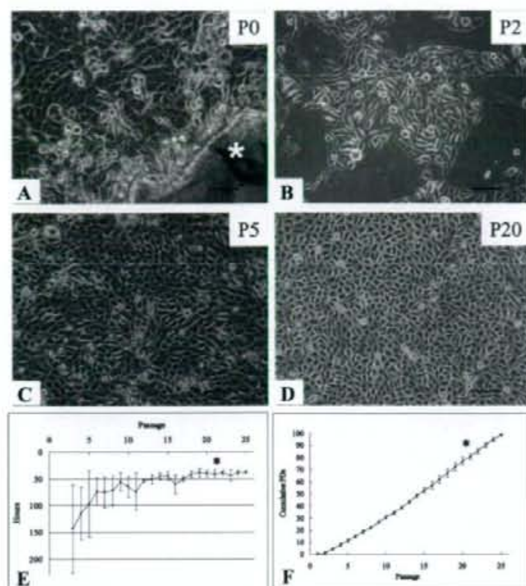

Figure 1. Phase-contrast micrograph of MCEs during serial cultures. (**A**) Primary cultures of MCEs. On day 10, cells expanding from the explant (*) had a cobblestone appearance. (**B**) Cells at P2 were a heterogeneous population of small and large cells. In the course of time, small cells proliferated and formed large colonies. Cells at P5 (**C**), P20 (**D**), and later times retained similar morphologies and a uniform cobblestone appearance by day 10. (**E**) Cell doubling times reached a plateau of approximately 50 hours after P12. (**F**) Population doubling (PDs) were constant through at least 25 passages for approximately 99 PDs. (**E**, **F**) $n = 6$; $^*n = 3$ after P20. Scale bars, 100 μm.

sages for 98.8 ± 1.0 PDs ($n = 3$; Fig. 1F) without showing signs of replicative senescence.

Cells cultured without cholera toxin ceased growth by P2 (Fig. 2C). Cell yields were significantly higher in the medium with cholera toxin in P1 (Fig. 2C; $n = 6$; $^*P < 0.05$). However, we randomly chose three cell lines in stage 3 (P18, P23, P25) and cultured them in medium with or without cholera toxin. Interestingly, we found that cholera toxin had no significant effect on cells in stage 3 in either morphology (Fig. 2D) or CFE (Fig. 2E; $P > 0.05$).

F2

mRNA and Protein Expression Profiles

RT-PCR analysis revealed that p63, K19, and involucrin were expressed; however, K12 was not detectable in cells cultured in KSFM (see Fig. 5A). Western blot analysis (Fig. 5B) confirmed these results. Immunostaining also showed approximately 70% of cells were positive for p63 and K19 and approximately 30% of cells were positive for involucrin, but none of the cells expressed K12 (Fig. 3).

F3

Induction of Differentiation and Stratification

To investigate the differentiation potential of the cells, we cultured the cells in KSFM supplemented with Ca^{2+} or serum and in DMEM/F12 with serum to induce differentiation. Morphologically, the cells cultured in KSFM were homogeneously small cells (Fig. 1D), whereas cells in KSFM with high Ca^{2+} were a heterogeneous mix of small and large cells and cells in media with serum were homogeneously large and squamous (Figs. 4A–D). RT-PCR and Western blot analyses showed that the expression of progenitor markers p63 and K19 and the differentiation marker involucrin did not change significantly (Fig. 5) after a high concentration of Ca^{2+} was added. However, after medium was supplemented with serum, the expression of progenitor markers p63 and K19 decreased significantly, whereas the differentiation marker involucrin increased (Fig. 5). Moreover, the cells cultured in DMEM/F12 with serum did not express p63 but expressed K12 at the mRNA level (Fig. 5).

F4

F5

Cells revealed marked stratification with four to six layers after exposure to the air-liquid interface for 10 days (Fig. 6A). Immunostaining showed that cells were positive for p63, K19, and involucrin but negative for K12 (Figs. 6B–D).

F6

Discussion

Long-term culture of MCEs has been notoriously difficult to establish. Until now, only Hazlett et al.[5] cultured MCEs for no more than three passages, and Kawakita et al.[7] established a mouse corneal epithelial line but used more than 200 eye globes. To our knowledge, this is the first report to reproducibly establish long-term MCEs from a single mouse cornea. Several approaches for the primary culture of corneal epithelial cells in vitro include the cell-suspension culture and explant culture techniques.[10] Each approach has its own advantages. Although cell-suspension culture can to some extent decrease

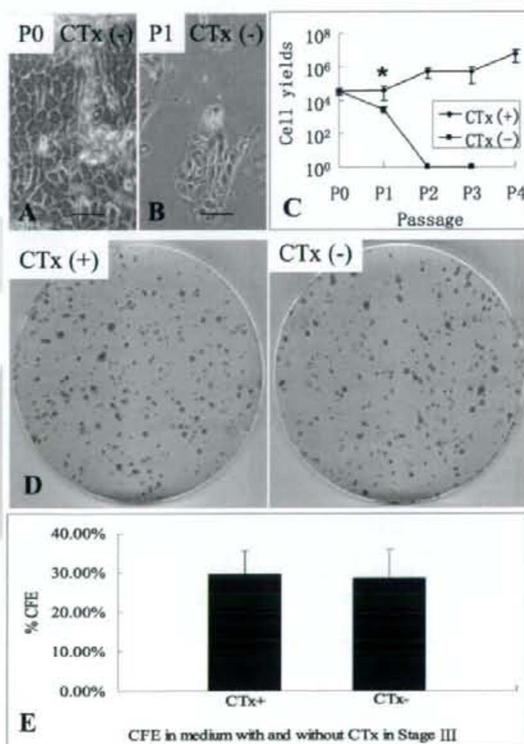

Figure 2. Phase-contrast micrograph of MCEs culture in medium with and without cholera toxin (CTx). MCEs cultured without CTx (**A**) were similar to those with CTx (see Fig. 1A) at P0. However, at P1, cells cultured without CTx (**B**) ceased growth after P2 (**C**). Cell yields were significantly higher in the medium with CTx in P1. (**C**) $n = 6$; $^*P < 0.05$. CTx had no effect on clone morphology (**D**) or CFE (**E**) after stage 3 ($n = 3$; $P > 0.05$).

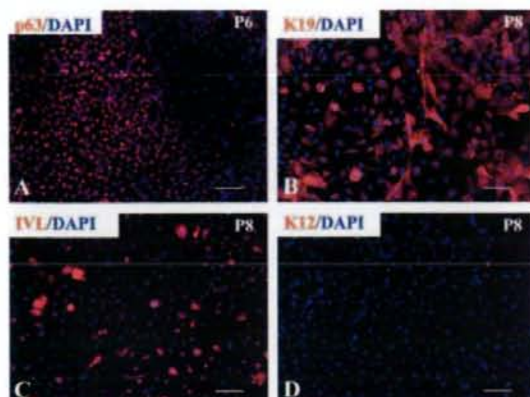

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Figure 3. Immunocytochemistry of cell markers in MCEs. Stage 3 MCEs cultured in KSFM expressed progenitor markers p63 (**A**) at P6 and K19 (**B**) at P8 and the differentiation markers involucrin (IVL; **C**) and K12 (**D**) at P8. Approximately 70% of cells were positive for p63 (**A**) and K19 (**B**), and approximately 30% were positive for IVL (**C**). There was no cellular expression of K12 (**D**). Scale bars, 100 μm.

fibroblast contamination from the stroma of explants, a large amount of tissue is required. The mouse cornea is small, and it is not practical to obtain sufficient cells from one single mouse by cell suspension. Our protocol, therefore, used the explant culture for initial expansion (stage 1) from a single cornea. The main problem during this stage is contamination by fibroblasts. Using keratinocyte growth medium and handling the explant gently when removing the tissue within 10 days of initiating the culture ensured that fibroblast outgrowth was minimal.

Subsequent stages of culture were modified according to the growth characteristics of MCEs. Stage 2 is the bottleneck that restricts the success of mouse corneal epithelial cell culture. Almost all the cell lines that extended beyond stage 2 could be successfully cultured long term. As shown in Figure 1B, the cells in stage 2 revealed a mixture of small and large cells. As culture time passed, the large squamous cells underwent senescence and detachment, whereas the small cells were selectively preserved. To maximize the number of small cells, we prolonged the culture period in this stage until the small cells reached subconfluence while disregarding the large squamous cells. In stage 3, we cultured the mouse corneal epithelial cells at a low density (5×10^4 cells per 75-cm^2 flask). With the use of human limbal epithelial cell cultures, Salehi-Had et al.[11] found that densely plated culture dishes demonstrated a decrease in the number of mitotic cells and a large number of cells with enlarged cytoplasms, low p63 expression levels, and high K3 expression levels compared with sparsely plated counterparts. In addition, Kawakita et al.[7] reported that a high seeding density yielded mostly large differentiated cells, whereas a low seeding density selectively allowed smaller cells to proliferate. These results suggest that lower seeding densities maintain progenitor cells and higher seeding densities may initiate differentiation.

Culture media were also optimized to increase progenitor cells during ex vivo expansion, enhance proliferative potential, and delay terminal differentiation. It is well known that Ca^{2+}[12,13] and serum[14,15] trigger differentiation in several lines of epithelial cells, including corneal epithelial cells.[6,16–18] Our experiment also confirmed that media supplemented with Ca^{2+} or serum induced the differentiation of MCEs. KSFM, a commercially available serum-free, low-Ca^{2+} medium, helps to maintain the basal cell phenotype of mouse epidermal keratinocytes[19] and limbal epithelial stem cells.[6] We found that KSFM was sufficient to maintain an established cell line but was not enough to establish a stable line during stage 2 of the protocol. The addition of cholera toxin, a bacterial toxin secreted by *Vibrio cholerae*, was crucial in the culture medium before stage 3. In fact, none of the mouse corneal epithelial cells could be cultured over P2 without cholera toxin in 12 attempts.

Cholera toxin catalyzes adenosine diphosphate-ribosylation of Gs protein and results in an accumulation of cellular cyclic AMP,[20–22] which is an intracellular second signaling messenger that regulates a variety of biological events, including cell proliferation and differentiation. It is known that the effect of cyclic AMP on cell proliferation is discrepant in different cells. Cyclic AMP induces cell proliferation of PC12 cells and Swiss 3T3 cells,[23,24] but it inhibits the proliferation of fibroblast cells, Rat1 cells, and NIH/3T3 cells.[25,26] In keratinocytes the effect of cyclic AMP is also a matter of controversy. Delescluse et al.[27] and Yamanishi et al.[28] have reported that cyclic AMP decreases cell proliferation, whereas Green[29] and Okada et al.[22] have found that cyclic AMP increases cell proliferation. Our experiment showed that cholera toxin was crucial only during stage 2 but was not required for stage 3, which suggests that dependence on cholera toxin maybe rest on the level of differentiation (i.e., limbal SCs, TACs, and terminally differentiated cells). Further investigations are necessary to clarify the role of cholera toxin and cyclic AMP in the proliferation and differentiation of primary cultured corneal epithelial cells.

With these modified techniques, we have cultured the cells through more than 25 passages with a high proliferative capacity and without signs of replicative senescence (Figs. 1C, 1D). Cells in stage 3 were morphologically similar and were characterized by a homogeneously small cell size and a typical cobblestone appearance, which suggests the cells maintain the progenitor cell state.[30] Immunostaining showed approximately 70% cells were positive for p63 and K19 (Fig. 3), which is in accordance with the results of RT-PCR and Western blotting (Fig. 5). The phenotype of the cells indicated that the cells may be the equivalent to corneal epithelial progenitor cells (TACs).

To investigate the differentiation potential of MCEs, we cultured the cells in KSFM supplemented with Ca^{2+} or serum and in DMEM/F12 with serum to induce differentiation. MCEs

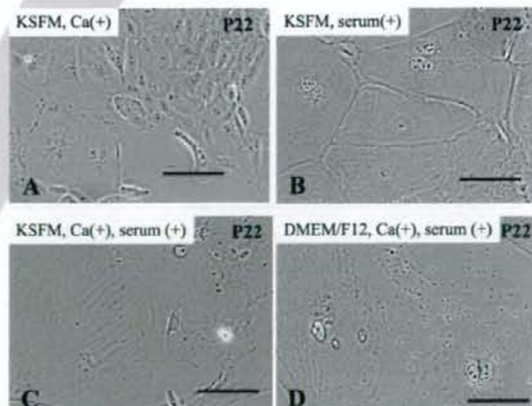

Figure 4. Morphology of MCEs in differentiation-inducing medium. Phase-contrast micrograph of cells at P22 cultured in KSFM with 0.9 mM Ca^{2+} (**A**) has the appearance of large cells with smaller N/C ratios compared with cells cultured in low Ca^{2+}. MCEs cultured in KSFM with 10% serum (**B**), KSFM with 0.9 mM Ca^{2+} and 10% serum (**C**), and DMEM/F12 with 10% serum (**D**) induce MCEs to become enlarged, showing features of cellular senescence.

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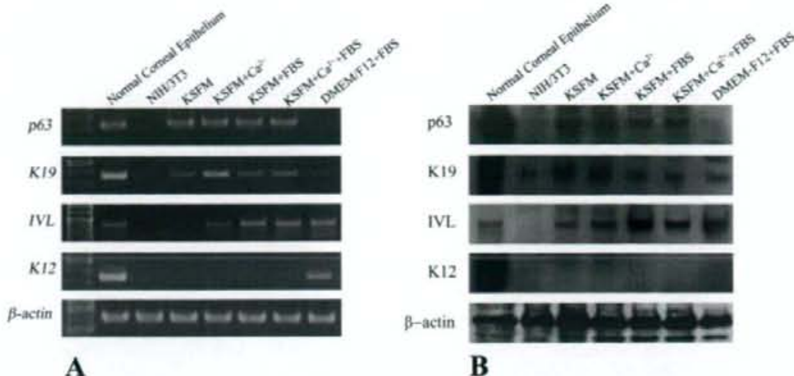

Figure 5. RT-PCR (**A**) and Western blot analysis (**B**) of p63, K19, involucrin (IVL), and K12. When P11 MCEs were induced to differentiate with the addition of Ca^{2+} and serum, IVL was upregulated while the expression of p63 and K19 decreased in DMEM/F12 with serum. K12 expression was observed by RT-PCR only. Normal mouse epithelial cells were used as positive control, and NIH/3T3 cells were used as negative control. Scale bars, 100 μm.

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that appeared homogeneously small in KSFM changed to a heterogeneous mix of small and large cells in KSFM with Ca^{2+} and a homogeneous mix of large and squamous cells in the other media with serum. The expression of progenitor markers p63 and K19 decreased significantly, whereas the differentiation marker involucrin increased significantly. Furthermore, MCEs cultured in DMEM/F12 with serum expressed K12 at the mRNA level, with no p63 expression. In addition, the cells had the ability to be stratified. These data suggest that Ca^{2+} and serum can induce MCEs to differentiate. We were unable to detect K12 by Western blot or immunohistochemistry. However, Kawasaki et al. (*IOVS* 2007;48:ARVO E-Abstract 2724) recently reported that the K12 gene is epigenetically regulated by methylation. Culture conditions may alter the methylation of K12, which may explain the low expression of K12 protein in our study and in previous studies on epithelial cultures.[7] Established epithelial cell lines that retain their differentiation potential are valuable tools for studying gene regulation, synthesis of extracellular matrix, or response to growth factors.

In conclusion, we report a reproducible procedure for the long-term culture of MCEs from a single mouse cornea using an explant culture method and a serum-free low-Ca^{2+} medium without feeder cells. MCEs after P5 were morphologically uniform, showed the phenotype of corneal epithelial progenitor cells, and retained the potential for differentiation and the ability for stratification. We propose that with specification of the passage number used (e.g., MCE-P10), this procedure can be a powerful tool for studies that require comparison of corneal epithelial cells from normal, transgenic, or knockout mice in vitro.

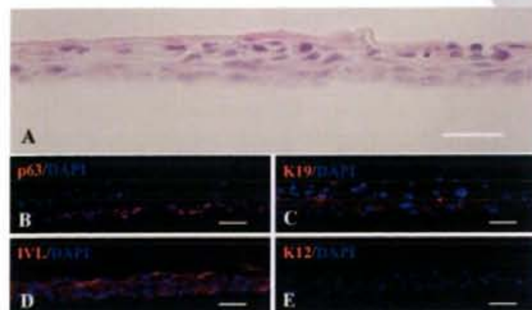

Figure 6. Stratification of MCEs at P17. Hematoxylin and eosin staining showed an epithelial sheet of four to six layers after air-lift cultures (**A**). Immunocytochemistry showed p63 (**B**) and K19 (**C**) expression in the basal and suprabasal layers, whereas involucrin (IVL) was expressed by all layers (**D**). There was no expression of K12 (**E**). Scale bars, 50 μm.

COLOR

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