lium is also required for the ocular surface to act as a barrier against invading organisms, as well as to provide a smooth surface for visual clarity. The clinical data available to date show that both AM sheets and carrier-free sheets can restore the epithelium for more than 1 year,[7,9] which would not be possible without the restoration of progenitor cells.

Another major benefit of carrier-free cell sheets is the surgical technique, which does not require the use of sutures for donor fixation. The mechanisms involved may be multiple, however, Nishida et al.[8] show that intact basement membrane substrates and adhesion molecules may play a major role. We have confirmed the presence of β1 integrin in the carrier-free group, which may have aided the carrier-free sheets in remaining on the ocular surface without sloughing off. In contrast, AM sheets require sutures for transplantation, and ingrowth of cells was observed under the AM carrier in several cases. These results show that attachment of cell sheets to the underlying stroma is stronger with carrier-free sheets during the early postoperative stage. Furthermore, the method we describe for engineering carrier-free sheets is different from previous approaches involving temperature-responsive dishes and does not require any specialized equipment or high levels of technical expertise.

The design of our study made use of rabbits with denuded epithelium, including the limbal area. We did not take into account any damage to the underlying stromal tissue, which is sometimes observed in clinical cases after severe chemical and thermal burns. The conclusions drawn from our study therefore should be interpreted as being based on epithelial sheet transplantation in situations with relatively intact stromal tissue. The AM is rich in basement membrane components since the amnion itself supports epithelial cells in the uterus. The use of an AM carrier may therefore have benefits in cases with extensive damage and inflammation in the underlying stroma.

There are still several issues to be resolved before the generalization of epithelial sheet surgery. The manufacture of stratified epithelial sheets requires the use of 3T3 feeder cells and culture-grade serum. Although adverse effects have not been reported, xeno-free techniques should be pursued. Similarly, the choice of whether to use carriers or not requires elucidation. Our data clearly show that cell sheets engineered without carriers reconstruct host tissue nearly to its original state as early as 1 week after surgery. Further refinements in surgical technique and quality control of cultured sheets should expand the therapeutic indications for tissue-engineered cell sheet transplantation.

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Hypoxia Enhances the Expansion of Human Limbal Epithelial Progenitor Cells In Vitro

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Purpose. To demonstrate the effects of hypoxia on proliferation and differentiation of human limbal epithelial cells in vitro.

Methods. Primary human limbal epithelial cells were harvested from the rim of donor corneas. Colony-forming efficiency (CFE) and cell proliferation were observed in standard (20% O_2) or hypoxic (2% O_2) culture conditions. Cell cycle, forward scatter (FSC) and side scatter (SCC) of cells were analyzed by flow cytometry. Proliferating cells were also observed by pulse labeling (2 hours) with BrdU and Ki67 staining. Apoptosis was detected by TUNEL assay. Isolated colonies were examined by immunohistochemistry against K15, p63, involucrin, and K3. Involucrin expression was also analyzed by Western blot analysis.

Results. Both CFE and proliferation of limbal epithelial cells was significantly enhanced in hypoxia. Flow cytometry revealed a higher fraction of hypoxic cells in the G_0/G_1-phase and fewer cells in the S-phase, compared with normoxia. However, there was no difference in the uptake of BrdU during a 2-hour pulse, suggesting that hypoxic colonies contained rapidly cycling cells. Apoptotic cells were sparse in both groups, and hypoxic cells showed lower FSC compared with normoxic cells. Although there was no difference in the staining pattern of K15, p63, and Ki67, cells cultivated in normoxia expressed higher levels of the differentiation markers involucrin and K3. Significantly higher involucrin expression was also observed by Western blot.

Conclusions. Hypoxic culture (2%) enhances proliferation while inhibiting differentiation of limbal epithelial cells in vitro. (*Invest Ophthalmol Vis Sci.* 2007;48:3586–3593) DOI: 10.1167/iovs.07-0077

Recent advances in the study of limbal epithelial stem cells include the use of cell sorting to isolate the so-called side-population (SP) cells in the limbus,[1,2] which is based on a method first described for isolating bone marrow stem cells.[5] However, there are still no reports of expanding SP cells as an undifferentiated population in vitro, suggesting that this method is not effective in isolating progenitor cells for further ex vivo expansion. Hayashi et al.[4] recently reported the expression of N-cadherin in patches of basal limbal epithelial cells (LECs), which may be used to isolate stem cells by cell sorting, since N-cadherin is a membrane surface protein.

However, from the point of view of regenerative medicine, the primary goal is to enhance the amount of progenitor cells that can be expanded ex vivo, regardless of the method of primary isolation. The availability of a serum-free, low-Ca^{2+} medium has significantly improved the yield of cells with high proliferative potential.[5] It is well known that both Ca^{2+} and serum triggers differentiation in several lines of epithelial cells.[6–9] Using irradiated or mitomycin-treated 3T3 feeder cells is another method used to isolate colonies of limbal progenitor cells.[10,11] The amniotic membrane with or without an intact epithelial layer was shown to maintain cocultivated epithelial cells in a less-differentiated state,[12–14] suggesting that the microenvironment surround the cells is crucial in maintaining an undifferentiated state. However, the use of feeder cells and amniotic substrates is considered a "black box" that introduces several uncharacterized factors to the culture system. Using xenogenic cells may also pose ethical problems when transplanting epithelial sheets cocultured with 3T3 feeder cells.

Recently, the use of hypoxic incubation was reported to enhance progenitor cells in the bone marrow,[15] neural cells,[16] and epidermal keratinocytes.[17,18] We therefore hypothesized that hypoxia can also be used to induce immature cells to expand from the limbus. Although the cornea is exposed to atmospheric oxygen, it is possible that lower oxygen levels are maintained in the limbal stem cell environment. Recently, oxidative stress was shown to suppress quiescence of stem cells in the bone marrow,[19] further indicating that hypoxia may be beneficial for stem cells in general. In the present study, low levels of O_2 (2%) induced the selective proliferation of undifferentiated LECs. Hypoxic cells express lower levels of differentiation markers and form larger colonies, suggesting that hypoxia may help maintain progenitor cells during ex vivo expansion of cultivated epithelial cell sheets.

Materials and Methods

Tissue Preparation and Cell Culture

Human LECs were isolated from the limbus of eye bank corneas after the central buttons were used for transplantation. Iris, endothelium, and conjunctiva were surgically removed from corneal limbus, and the limbus was treated with 2.5 U /mL Dispase II (Roche, Basel, Switzerland) in F12/DMEM at 4°C overnight. The epithelium was separated from the stroma with a cell scraper and dispersed in 0.05% trypsin EDTA at 37°C for 30 minutes. LECs were suspended in serum-free low-Ca^{2+} medium (defined K-SFM; Invitrogen, Carlsbad, CA) consisting of 10 ng/mL human recombinant EGF (Invitrogen), 100 ng/mL cholera toxin (Calbiochem; Merck KGaA, Darmstadt, Germany), antibiotics, and growth supplement supplied by the manufacturer. Unless indicated otherwise, 5×10^4 LECs were seeded in 25-cm^2 flasks. Flasks were cultured either in 5% CO_2 at 37°C as a normoxic control or in hypoxia (2% O_2 and 5% CO_2 at 37°C) using an N_2/CO_2 multigas incubator (APM-30D; Astec, Fukuoka, Japan). Medium was changed every 3 to 4 days.

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CFE and Cell Proliferation

LECs were inoculated in 60-mm dishes at 1000 cells/dish and cultured for 10 to 14 days. Cultured cells were stained with rhodamine B (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 30 minutes. Colony-forming efficiency (CFE) was calculated as number of colonies/number of inoculated cells. Five independent experiments were performed.

LEC proliferation was observed in 24-well plates initially seeded with 2500 cells per well. Two wells in each condition were fixed with 70% ethanol every 2 days for up to 24 days. Medium was changed every 2 days. After all wells were fixed, the plates were stained with eosin for 1 hour. Images were scanned, and eosin-stained area was measured using Scion Image software (Scion Corp., Frederick, MD). Four independent experiments were performed.

Flow Cytometry

LECs (5×10^4 cells) were cultured in 25-cm^2 flasks for 12 days in normoxia and hypoxia, as just described, and dispersed by enzyme treatment (37°C, 10 minutes; TrypLE Express; Invitrogen). For forward scatter (FSC) and side scatter (SSC) analysis, the cells were resuspended in 0.1% sodium azide in PBS and analyzed by flow cytometry (EPICS XL; Beckman Coulter, Hialeah, FL). For cell-cycle analysis, the cells were resuspended in a solution containing 4 mM sodium citrate (pH 7.6; Wako), 0.2% Nonidet P-40 (Calbiochem), and 50 μg/mL propidium iodide (Wako). After incubation on ice for 30 minutes, the cell suspensions were treated with 250 μg/mL RNase A (Fermentas, Hanover, MD) for 15 minutes at 37°C to remove double-stranded RNA. Cells were measured by flow cytometry at an excitation wavelength of 488 nm. Data analysis was performed with commercial software (FlowJo; Tree Star, Inc., Ashland, OR).

BrdU Labeling

LECs (10^4/well) were cultured in gelatin-coated two-well chamber slides for 7 days, and BrdU (final, 10 μM) was added to the culture for 2 hours. After fixing with methanol at RT for 10 minutes, cells were treated with 1 N HCl at RT for 1 hour, and BrdU was detected as described later. Thirty randomly selected clones in each group were photographed, and the percentage of BrdU$^+$ cells in each colony was calculated. Four independent experiments were performed.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from LECs cultured for 10 to 14 days by using the SV total RNA isolation system (Promega Corp., Madison, WI), and cDNA was synthesized by using AVM reverse transcriptase XL (Takara; Bio Inc., Tsu, Japan). The same amount of cDNA was amplified by PCR (GeneAmp 9700; Applied Bioscience, Inc, [ABI], Foster City, CA) and the following primer pairs: GAPDH mRNA, forward (5′-GACCACAGTCCATGCCATCAC-3′), and reverse (5′-TCCACCACCCTGTTGCTGTAG-3′); involucrin mRNA, forward (5′- TGTTCCTCCTCCAGTCAATACC-3′), and reverse (5′-TCCCAGTTGCTCATCTCTCTTG-3′); K3 mRNA, forward (5′-GACAATAATCGTTCCCTGG-3′), and reverse (5′-TTGCGGTAGGTGGCGATCT-3′); K15 mRNA, forward (5-GAGAACTCACTGGCCGAGAC-3′), and reverse (5-GGGACGTTTCTCCTGCAATA-3′); ΔNp63α mRNA, forward (5-CTGGAAAACAATGCCCAGAC-3′) and reverse (5-ATCGCATGTCGAAATTGCTC-3′).[20] PCR products were analyzed by agarose gel electrophoresis.

Western Blot Analysis

Cell pellets were dissolved with lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40) and homogenated. Samples were incubated for 40 minutes at 4°C, and then centrifuged at 15,000 rpm for 30 minutes at 4°C. Protein concentration of the supernatant was determined by the DC protein assay (Bio-Rad Laboratory, Hercules, CA). All samples were then diluted in 2× sample buffer, containing 100 mM Tris-HCl (pH 6.8), 4% SDS (Invitrogen), 20% Glycerol (Wako), and 12% 2-mercaptoethanol (Wako), and boiled. Ten micrograms of each sample were loaded on a 10% Bis-Tris gel (Novex NuPAGE; Invitrogen) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were blocked with 5% skim milk (Difco Laboratories, Detroit, MI), 1.5% normal donkey serum, and PBS for 60 minutes at room temperature. Membranes were reacted with an anti-involucrin antibody (SY5; Abcam, Cambridge, UK) and β-actin (mabcam 8226; Abcam) for 60 minutes at room temperature. After three washes in Tris-buffered saline with Tween 20 (TBST), donkey biotinylated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added for 30 minutes at room temperature. Protein bands were visualized by the avidin-biotin complex (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA), with diaminobenzidine (DAB; Vector Laboratories) as substrate. The plot profile of the bands was analyzed with the NIH image 1.63 software (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

Immunostaining

LECs (5×10^3 cells/ well) were cultured in gelatin-coated, four-well chamber slides and fixed with 2% paraformaldehyde (PFA, Wako) for the immunostaining of involucrin, K3, p63, with cold acetone for Ki67, or with cold methanol for K15. PFA-fixed cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) (involucrin, K3,

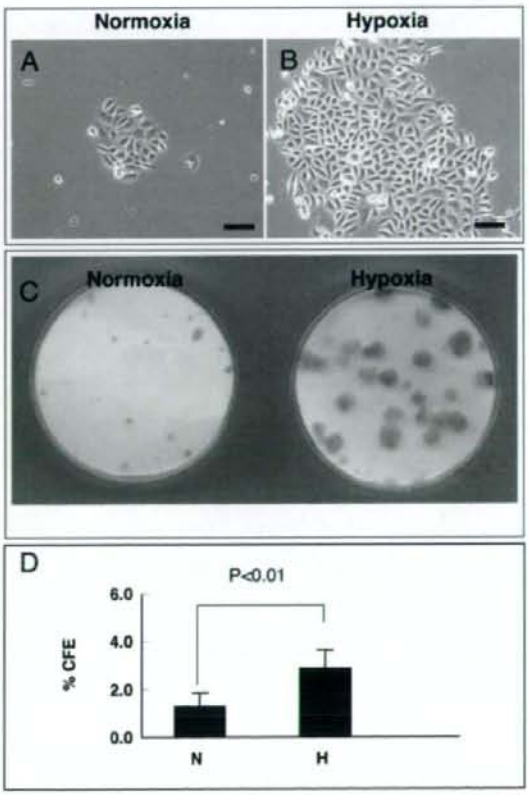

FIGURE 1. CFE in normoxic and hypoxic cultures. LECs seeded at clonal densities formed colonies in both normoxia (**A**) and hypoxia (**B**) without the use of feeder cells. Individual colonies were larger in hypoxia (**C**), and CFE was statistically higher in the hypoxia group than in the normoxia control (**D**: $n = 5$, Student's t-test). Scale bars, 100 μm.

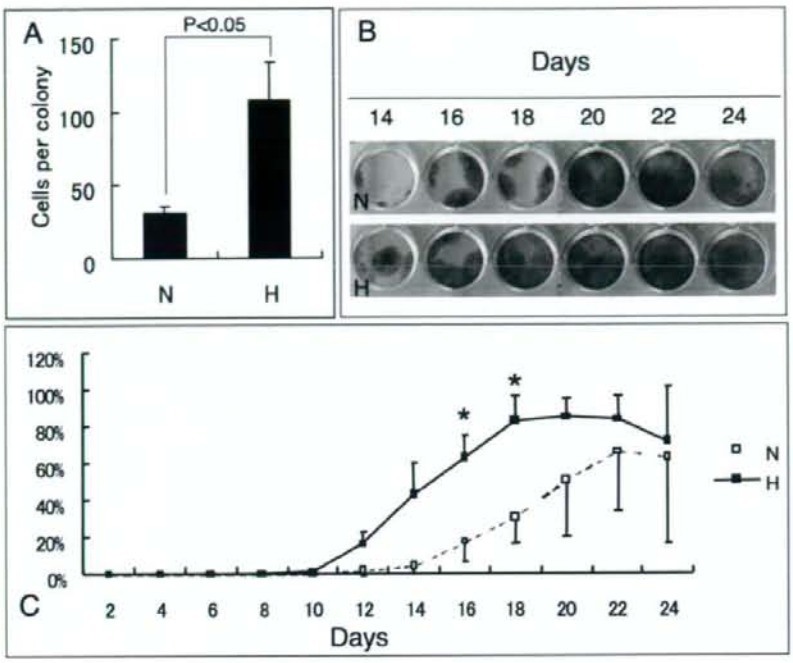

FIGURE 2. Enhanced cell proliferation in hypoxia. LEC proliferation was enhanced in hypoxia, with larger initial colonies (**A**) and rapid expansion of cells within the culture dish (**B**). The average area covered by proliferating cells was significantly higher in hypoxia by day 16 until the cells reached confluence (**C**: $n = 4$, $^*P < 0.05$ Mann-Whitney test). N, normoxia; H, hypoxia.

p63). After background staining was blocked with 10% normal donkey serum, the cells were treated with the following monoclonal primary antibodies: anti-K3 (clone AE5; Progen, Heidelberg, Germany), anti-involucrin (SY5, YLEM, Rome, Italy), anti-Ki67 antigen (MIB-1; Dako, Glostrup, Denmark), anti-p63 (4A4; Calbiochem), anti-K15 (LHK15; Laboratory Vision, Fremont, CA), and anti-BrdU (Chemicon International, Temecula, CA). The cells were then treated with Alexa Fluor 488– or 555–conjugated secondary antibodies (Invitrogen) or rhodamine (Jackson ImmunoResearch)- or Cy3 (Chemicon)-conjugated secondary antibodies. The nuclei were counterstained with 4′,6′-diamino-2-phenylindole (1 mg/mL, DAPI; Dojindo Laboratories, Tokyo, Japan) or TO-PRO-3 (Invitrogen).

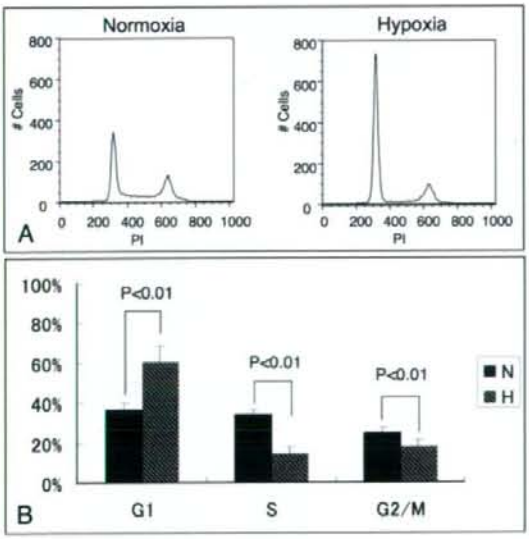

FIGURE 3. Cell-cycle profile of cultured LECs. Flow cytometry showed a prominent peak of G_0/G_1 cells in the hypoxia group, whereas normoxic cells were distributed throughout the cell cycle (**A**). The average ratio of cells in the G_0/G_1-phase was significantly higher in the hypoxia group, whereas cells in the S- and G_2/M-phase were significantly higher in the normoxic group (**B**: $n = 6$, Student's t-test). N, normoxia; H, hypoxia.

TUNEL Assay

The terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick-end labeling (TUNEL) technique was performed to detect apoptosis (In situ cell death detection kit; TMR red; Roche Diagnostics, Indianapolis, IN). Chamber slides were fixed with 4% paraformaldehyde (Wako) for 1 hour and permeabilized in 0.1% Triton X-100, 0.1% sodium citrate at 4°C for 2 minutes. The slides were incubated with TUNEL reaction mixture for 1 hour at 37°C. After washing, the slides were counterstained with DAPI (1 mg/mL; Dojindo Laboratories).

RESULTS

CFE and Cell Proliferation

Figure 1 shows colony formation by limbal epithelial cells in normoxia and hypoxia after 10 to 14 days. Normoxic colonies (Fig. 1A) were smaller compared with colonies formed in hypoxia (Fig. 1B). In addition to colony size, colony-forming efficiency was significantly higher in hypoxic cultures (Fig. 1C). Hypoxia yielded higher colonies at all seeding densities investigated in a preliminary experiment (Supplementary Fig. S1 and Methods, online at http://www.iovs.org/cgi/content/full/48/8/3586/DC1). We further examined whether hypoxia caused an increase in cell proliferation rate. Despite the same initial seeding density, hypoxic colonies had a significantly higher number of cells per colony after 7 days (Fig. 2A). When

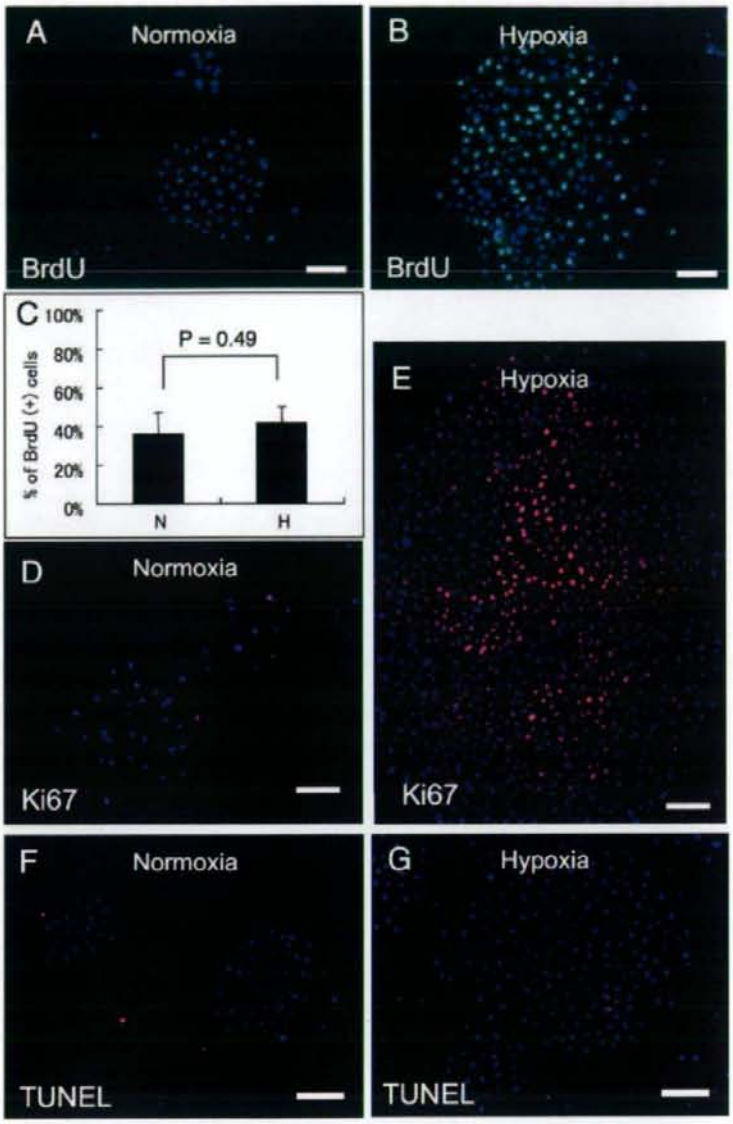

FIGURE 4. Cell proliferation and apoptosis markers. Although colonies cultured in hypoxia were constantly larger than those raised in normoxia, the ratio of $BrdU^+$ cells (*green*) after a 2-hour pulse was similar in both groups (**A**–**C**). Similarly, there was no difference in the ratio of $Ki67^+$ (*red*) cells (**D**, **E**). $TUNEL^+$ apoptotic cells (*red*) were sparse in both groups during the growth phase (day 12) of culture (**F**, **G**). Nuclei counterstained with TO-PRO-3 (**A**, **B**) and DAPI (**D**–**G**). Scale bars, 100 μm.

the area covered by epithelial cells was used as a measure of cell proliferation, we found that hypoxic cultures reached confluence faster than normoxic cultures (Fig. 2B) and that the average area covered by the cells was significantly higher after 16 days, until the cells became confluent (Fig. 2C).

Cell Cycle Analysis

We examined the cell-cycle profile of LECs cultured in hypoxia to determine whether the increased proliferation can be explained by a higher ratio of dividing cells. Flow cytometry of cells in the proliferative stage (12 days) showed that hypoxic cells were predominantly in the G_0/G_1-phase of the cell cycle, whereas the number of cells in the DNA-replicating stage (S-phase) was more evident in normoxic cells (Fig. 3A). The higher number of cells in the S-phase and fewer cells in the G_0/G_1-phase in normoxia was statistically significant compared with hypoxia (Fig. 3B).

Cell Proliferation Markers and Apoptosis

To explain the paradoxical results of flow cytometry, we pulse labeled cells for 2 hours to observe the total number of dividing cells during the period. The ratio of $BrdU^+$ cells was not only similar, but also tended to be higher in the hypoxia group (Figs. 4A–C). When cells were stained with the proliferation marker Ki67, both normoxic (Fig. 4D) and hypoxic (Fig. 4E) colonies were positive, especially in cells toward the center of the colony. This trend was more evident in hypoxic cells, which constantly produced larger colonies. However, the ratio of $Ki67^+$ cells within the entire colony was not different between both groups. To rule out the possibility of increased apoptosis

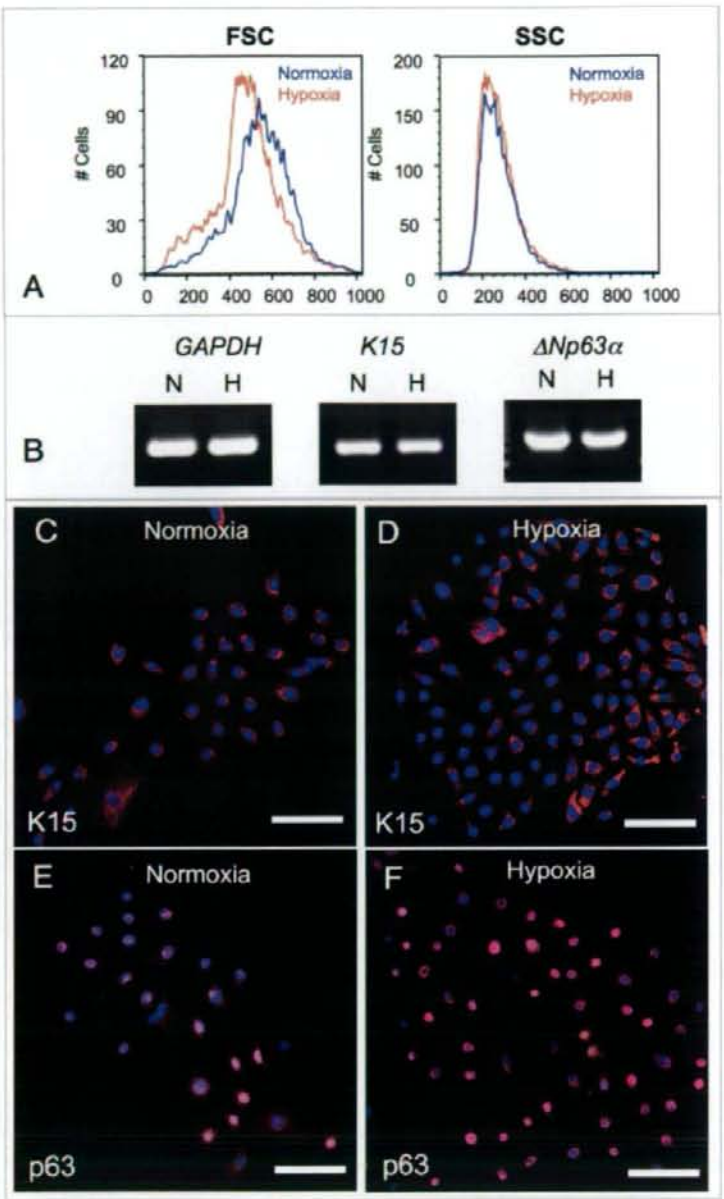

FIGURE 5. Expression of progenitor markers. Flow cytometry showed that hypoxic cells had slightly lower FSC, whereas SSC was similar (**A**). RT-PCR (**B**) and immunocytochemistry (**C–F**) revealed that the expression of the progenitor markers K15 (*red*) and p63 (*red*) were similar in both groups. (**C–F**) Nuclei counterstained with TO-PRO-3. Scale bar, 100 μm.

in normoxia as the reason for smaller colonies and slower proliferation, we performed TUNEL staining of colonies cultured for 12 days. However, cells undergoing apoptosis were sparse in both groups during this phase of proliferation (Figs. 4F, 4G). There was also no difference in PI-positive dead cells during earlier phases of cell culture (data not shown).

Phenotype of Limbal Epithelial Cells in Hypoxia

Differentiation of LECs cultivated in hypoxia and normoxia were compared. Flow cytometry revealed that hypoxic LECs were smaller, as shown by lower FSC in cells cultivated in hypoxia (Fig. 5A). Although there was no significant difference in FSC, the average cell density was significantly higher in hypoxic colonies due to the presence of enlarged cells scattered in normoxic colonies (Supplementary Fig. S2 and Methods http://www.iovs.org/cgi/content/full/48/8/3586/DC1). There was no difference in the expression of the progenitor markers K15 and p63 when examined by RT-PCR (Fig. 5B) and immunocytochemistry (Figs. 5C–F). However, the differentiation markers involucrin and K3 showed stronger staining in normoxic colonies (Figs. 6A, 6C) compared with hypoxic colonies (Figs. 6B, 6D). The difference was confirmed by RT-PCR

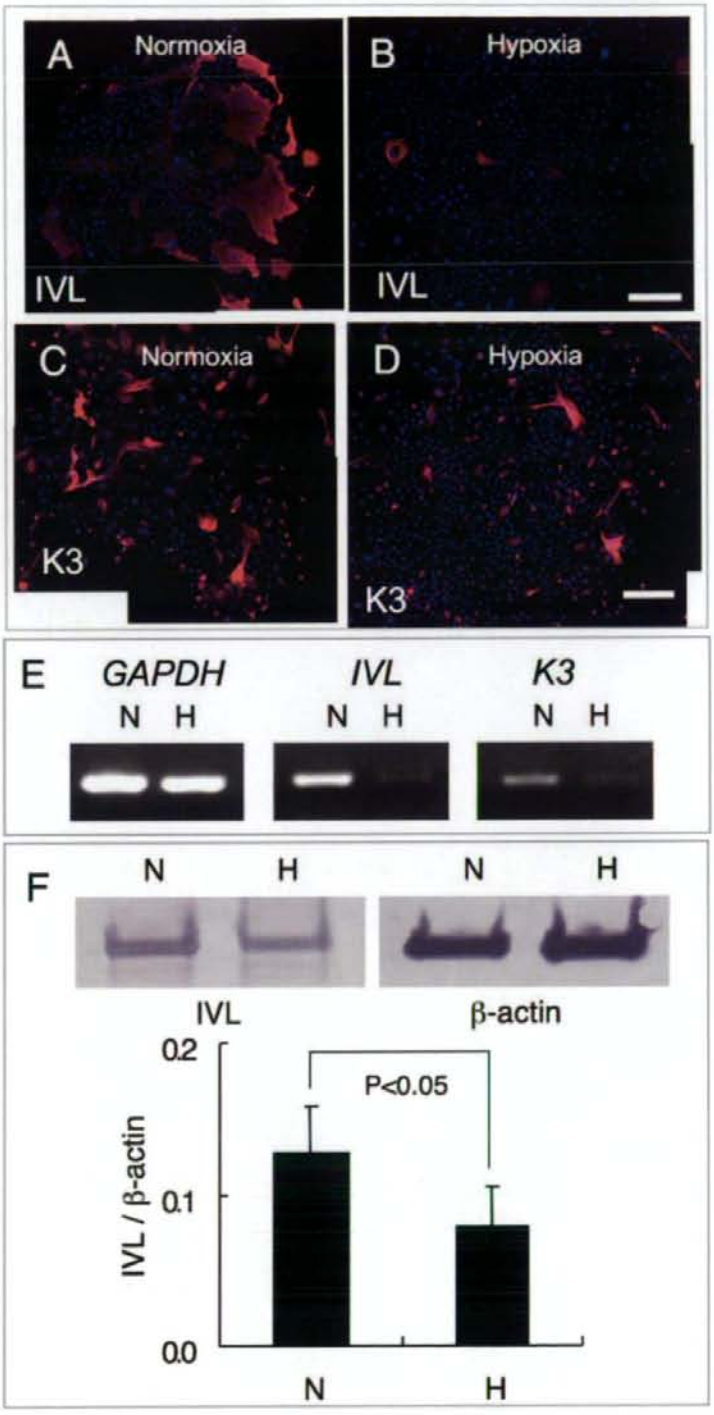

FIGURE 6. Expression of differentiation markers. Normoxic colonies expressed higher levels of the differentiation markers involucrin (*red*) (**A**) and K3 (*red*) (**C**) compared with hypoxic colonies (**B**, **D**). The difference was also confirmed by RT-PCR for involucrin and K3 (**E**) and by Western blot for involucrin (**F**). Semiquantitative analysis of Western blots showed that involucrin expression by normoxic cells was significantly higher in normoxia ($n = 6$, Student's t-test). (**A**–**D**) Nuclei were counterstained with DAPI. Scale bar, 200 μm.

(Fig. 6E) and by quantitating protein expression of involucrin by Western blots (Fig. 6F). These results show that cells cultured in normoxia are driven toward differentiation.

Discussion

Maintaining limbal progenitor cells in an immature state in vitro is vital in the study of limbal stem cell biology and for cultivating transplantable epithelial cell sheets. Recently, several studies on tissue stem cells have used hypoxic culture conditions to propagate cells in an immature state. Reduced oxygen was shown to enhance the survival of central nervous system (CNS) stem cells.[21] Hypoxia not only promoted proliferation of CNS stem cells, but also reduced apoptosis.[16] The effects of hypoxia on epithelial cells was reported in epidermal keratinocytes cultured in 2% O_2 that showed increased colony-forming ability[18] and increased population doubling producing a larger yield of epithelial cells to form epithelial sheets.[17]

We hypothesized that hypoxia may also be effective in expanding limbal epithelial progenitor cells. As expected, culturing primary LECs in 2% O_2 without feeder cells produced larger colonies than did culturing in normoxia (Figs. 1A, 1B). Furthermore, CFE was higher in hypoxic cultures (Figs. 1C, 1D). We further observed the effects of hypoxia on cell proliferation by seeding primary LECs at higher densities, and found that cell growth was enhanced in hypoxia giving rise to larger initial colonies that reached confluence earlier than normoxic cultures (Fig. 2).

A clear enhancement of cell proliferation prompted us to examine the cell cycle profile of cultured LECs by flow cytometry. Of interest, we found that a significantly higher number of cells were in the S- and G_2/M-phases during normoxia, and that most of the cells in hypoxia were in the G_0/G_1-phase (Fig. 3). This observation seems to contradict the notion of a higher proliferation rate in hypoxia, since flow cytometry results indicated a larger number of cells undergoing cell division in normoxia. Because flow cytometry only shows the state of cells at a specific time point, we pulse labeled cells with BrdU for 2 hours, to identify the cells entering the cell division cycle during this time frame. There was no difference in $BrdU^+$ cells between both groups, with a tendency toward a higher ratio in hypoxia (Figs. 4A–C). There was also no difference in the expression of the proliferation marker Ki67 (Figs. 4D, 4E), as well as apoptotic cells shown by TUNEL staining (Figs. 4F, 4G). The results suggest that the larger colonies and increased cell proliferation in hypoxia is due to the presence of rapidly cycling cells and explains why more cells were in the nondividing G_1-phase of the cell cycle in hypoxia at a specific time point, whereas the number of BrdU-labeled cells during a 2-hour pulse was the same.

The next step of the study was to examine the differentiation status of cells in both conditions. Although flow cytometry suggested that hypoxic cells were slightly smaller than normoxic cells (Fig. 5A), there was no difference in the expression of the progenitor markers K15 and p63 examined by RT-PCR (Fig. 5B) and immunocytochemistry (Figs. 5C–F). However, normoxic cells expressed higher levels of the expression markers involucrin and K3 (Fig. 6). Normoxic colonies also had a significantly lower cell density due to the presence of scattered large cells (Supplementary Fig. S2). These results clearly show that hypoxic conditions maintain LECs in a more undifferentiated state. Because primary LECs were from the same donor source for each experiment, we can assume that the initial density of stem cells was the same. An increase in rapidly proliferating, undifferentiated cells in hypoxia may indicate that lower oxygen levels facilitate the proliferation of transient amplifying (TA) cells.

The mechanisms involved in the maintenance of progenitors during hypoxia are still not clear. One of the major intracellular regulators during hypoxia is hypoxia-inducible factor (HIF1)-α, and several reports have already suggested a role for HIF1-α in the inhibition of adipocyte differentiation during hypoxia.[22] HIF1-α was also shown to interact with Notch-responsive promoters during Notch activation in hypoxia to block neuronal and myogenic differentiation.[23] However, Notch seems to induce differentiation in keratinocytes,[24] suggesting that several pathways exist in the maintenance of the undifferentiated state in hypoxia. Perhaps oxygen at atmospheric levels alone may be a source of reactive oxygen species that may drive stem cells from a quiescent state as was shown in bone marrow stem cells.[19]

We have shown that limbal epithelial progenitor cells can be efficiently expanded in serum-free, feeder-free medium in hypoxic conditions. This protocol may have an impact on the way ex vivo expansion is performed in the future. For example, maintaining progenitor cells in a less differentiated state may allow the engineering of transplantable epithelial sheets from even a single cell source. One question that remains to be clarified is whether hypoxia can maintain quiescent stem cells during cultivation. Although further studies are needed resolve this question, similar observations found in various other cell types suggest that a hypoxic microenvironment is a key component of the epithelial stem-cell niche.

Acknowledgments

The authors thank Mifuyu Ishiwata, Tomomi Sekiguchi, and Fumito Morito for their technical assistance and the staff of the Cornea Center Eye Bank for administrative support and Nobuhito Goda and Makoto Suematsu (Department of Biochemistry, Keio University School of Medicine, Tokyo, Japan) for use of their hypoxic culture chamber.

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Stratified epithelial sheets engineered from a single adult murine corneal/limbal progenitor cell

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Abstract

The limbal region of the adult cornea contains stem cells which are ultimately responsible for regeneration of the corneal epithelium during wound repair. However, primarily-isolated murine corneal/limbal epithelial cells rapidly senesce on plastic in a serum-free low [Ca^{2+}] medium, suggesting only transit amplifying cells are promoted. We developed a novel expansion method by seeding at a low cell density (<500 cells/cm^2) and prolonging each culture time beyond the lifespan of transit amplifying cells (4 weeks). Expanded cells were uniformly small, negative to K12 keratin, but positive for p63 nuclear staining, and could be subcultured beyond 100 passages. After limiting dilution, one clone (TKE2) was selected that exhibited single cell clonal expansion with a doubling time of 34.2 hrs, and had normal karyotyping, but no anchorage-independent growth. A single cell could be continually expanded to a confluent monolayer on denuded amniotic membrane and became stratified by exposing to the air-medium interface. The resultant stratified epithelium expressed K14 keratin, involucrin, connexin 43 and p63, but not K12 keratin or Pax 6. However, expression of K12 could be up-regulated by increasing extracellular calcium concentration and addition of foetal bovine serum (FBS) at P12, but less so at P85. Therefore, this murine limbal/corneal epithelium-derived progenitor cell line still retained the plasticity for adopting corneal lineage differentiation, could be useful for investigating limbal niche cues that may promote corneal epithelial fate decision.

Keywords: cornea • epithelium • stem cell • regenerative medicine • culture • senescence and growth

Introduction

Stem cells (SCs) with extensive proliferative potential are crucial for maintaining the homeostasis of a given tissue. Although SCs hold considerable promise for treating a number of diseases in regenerative medicine, availabilityof SCs in sufficient quantities remains a key obstacle to overcome, and until now has relied on *ex vivo* expansion, which is a task dependent on isolation, preservation and proliferation of SCs in an *in vitro* environment.

The corneal epithelium is unique in that its SCs are exclusively located in the basal layer of the limbus (between the cornea and the conjunctiva), while transit amplifying cells (TACs) are located in the basal to suprabasal layers of the limbal epithelium and the entire corneal epithelium. [1] This unique anatomic enrichment at the limbus allows one to gain an easy access to these adult somatic SCs [2], which have the smallest cell size [3] and a long cell cycle [4], do not express K3/K12 keratins [1, 5] and connexin 43 [6], but preferentially express p63 [7], Bcrp1/ABCG2 [8] or N-cadherin [9]. As a result, the SC-containing limbal epithelium has higher clonogenecity on 3T3 fibroblasts feeder layers [10, 11].

Despite a variety of transgenic mice have been available, studies of murine limbal/corneal epithelial SCs have met a greater challenge. Toward this goal, we reported a method to successfully isolate viable mouse corneal/limbal epithelial sheets, of which subsequent growth and differentiation is greatly influenced by extracellular calcium concentration ([Ca^{2+}]) and the presence of foetal bovine serum (FBS). [12] Even if cultured at a high density in keratinocyte serum-free defined medium (KSFM) containing 0.07 mM [Ca^{2+}] and supplemented with growth-promoting agents, cells reached confluence in 1 week and could only be subcultured at 1:3 splits for up to 2–3, suggesting only TACs were expanded [12].

[#]Proprietary Interest: SCGT and TK have filed a patent based on this work.

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Table 1 Sources of primary antibodies

Antigens	Category	Clone	Dilution	Method	Source
PCNA	Mouse monoclonal	PC10	1:50	IHC	DAKO*
p63	Mouse monoclonal	4A4	1:50	IHC	DAKO
Pan-cytokeratin	Mouse monoclonal	Mixed‡	1:100	IF	Sigma**
Cytokeratin K12	Goat polyclonal	L15	1:20	IF	SantaCruz†
Cytokeratin K14	Mouse monoclonal	B429	1:100	IF	Abcam††
Pax6	Rabbit polyclonal	NA	1:100	IF	Chemicon‡‡

‡Mixed clone: C-11, PCK-26, CY-90, KS-1A3, M20 and A53-B/A2.
*Carpinteria, CA. **St. Louis, MO. †Santa Cruz, CA. ††Cambridgeshire, UK. ‡‡Temecula, CA.

Herein, we demonstrated that it was possible to preferentially encourage expansion of limbal epithelial progenitor cells, characterized by small cell size, negative K12 keratin expression, and strong nuclear p63 expression, when the culturing time was extended to 4 weeks (*i.e.* beyond the TAC's lifespan) and when the seeding density was lowered to minimize any paracrine influence from TACs. As a result, clonal initiation and continuous expansion was achieved for more than 100 passages. Such expanded progenitor cells exhibited single cell clonal growth, could be used to engineer a stratified epithelium, and upon increasing extracellular calcium concentration and adding FBS a small proportion of cells expressed K12 keratin. The significance of this as-yet-unrecognized culturing method to isolate and expand murine limbal/corneal progenitor cells is discussed.

Materials and methods

Reagents

Tissue culture plastic wares were purchased from Becton Dickinson (Lincoln Park, NJ, USA). Amphotericin B, Dulbecco's modified Eagle's medium (DMEM), F-12 nutrient mixture (F12), Defined Keratinocyte-SFM (KSFM), FBS, phosphate-buffered saline (PBS), TripLE® and 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco-BRL (Grand Island, NY, USA). Dispase II powder was from Roche (Indianapolis, IN, USA). Other reagents and chemicals including bovine serum albumin (BSA), cholera-toxin, dimethyl sulfoxide, hydrocortisone, insulin, mouse epidermal growth factor (EGF), sorbitol, Hoescht 33342 and fluorescein-conjugated (FITC) secondary antibodies were from Sigma (St. Louis, MO, USA). Optimal cutting temperature (OCT) compound was from Sakura Finetek (Torrance, CA, USA). Isotype mouse IgG1 and rabbit IgG were purchased from Dako Cytomation and Jackson ImmunoResearch Laboratories (West Grove, PA, USA), respectively. Rhodamine-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories and Chemicon International Inc. Live/Dead Assay® was from Molecular Probes (Eugene, OR, USA). Penicillin and streptomycin was from Wako (Osaka, Japan). The SV total RNA isolation system was from Promega (Madison, WI, USA). Avian myeloblastosis virus (AVM) reverse transcriptase XL was from Takara, Bio (Shiga, Japan). All primary antibodies used in this study are summarized in Table 1.

Isolation of murine corneal/limbal epithelial sheets

CD-1 albino mice of more than 3 weeks old (Charles River, Boston, MA, USA) were handled according to the Association for Research in Vision and Ophthalmology (ARVO) guidelines for animal care. Mouse corneal/limbal epithelial sheets were isolated in the same manner as recently reported [12]. In brief, more than 200 eye globes were enucleated by forceps, washed profusely in PBS, stored in KSFM and then transported at 4°C within 24 hrs to the laboratory. These eyes were digested at 4°C for 18 hrs in KSFM containing 15 mg/ml dispase II and 100 mM sorbitol. KSFM contained 0.07 mM [Ca^{2+}] and was supplemented with 10 ng/ml EGF and 10^{2-10} M cholera toxin. Subsequently, each mouse eye was held in place by suction applied to the posterior pole using a transfer pipette and was gently shaken in KSFM to loosen the ocular surface epithelial sheet.

Culture manipulation

Single cells obtained from the above corneal/limbal epithelial sheets by 0.25% trypsin/1 mM EDTA in HBSS for 10 min. followed by vigorous pipetting were seeded at a density of 20,000 cells per cm^2 on plastic containing KSFM. In 1 week, cells reached confluence and were subcultured by trypsin/EDTA at 1:3 split to Passage 1 (P1) cultures. At this point, cells were subcultured at 1:3 split either in 1 week as previously reported [12] or in 4 weeks, that is, 3 weeks beyond confluence. Cells subcultured in the latter manner could continually be passaged, and at P3, the average cell size (μm^2) was monitored by phase contrast micrography weekly for 100 randomly selected cells using Image J (NIH, Bethesda, MD, USA), and the total cell number was determined in triplicate by haemocytometry during the 4 week course. At P4, cells were also seeded at a density of 500, 5000 or 50,000 cells per cm^2 and cultured for 4 weeks ($n = 5$). Cell viability was measured by Live/Dead Assay®, and Hoescht 33,342 staining.

Immunostaining

To determine the cornea-type epithelial differentiation, immunofluorescence staining to K12 keratin was performed as previously reported [12].

Table 2 Primers used for RT-PCR

Primer	Sequence (5′ → 3′)	Product size (bp)
Keratin 10	GGCTCTGGAA GAATCAAACT ATGAGC	167
	GGATGTTGGC ATTATCAGTT GTTAGG	
Keratin 12	CGGGAGTGGTATGAAGCA	188
	CATTCTGAAGTCGTCGGC	
Keratin 14	CCCCTCCACGTGGAGATTCA	1417
	CCTGCAGATGGATAAGAGGG	
Pax 6	AGTTCTTCGC AACCTGGCTA	500
	TGAAGCTGCT GCTGATAGGA	
Involucrin	CAGGACATGCTAGTACCACAGG	883
	GTGTCCGGTTCTCCAATTCGTG	
Connexin 43	CCTTCTTGCTGATCCAGTGGTAC	154
	ACCAAGGACACCACCAGCAT	
GAPDH	ACCACAGTCCATGCCATCAC	452
	TCCACCACCCTGTTGCTGTA	

To determine the status of epithelial progenitor cells including SCs, we performed immunohistochemistry to p63 using clone 4A4, which recognizes all six p63 isotypes [13], similar to what has been reported by Pellegrini *et al.* [7] Immunostaining to detect nuclear expression of proliferating cell nuclear antigen (PCNA) was used to evaluate the proliferative potential. Immunofluorescence staining to K14 keratin and Pax 6 besides aforementioned marker p63 and K12 keratin was also performed in stratified epithelial sheets generated from a single cell. Substitution of primary antibody with PBS served as negative controls. Images were photographed with a NikonTE-2000U Eclipse epi-fluorescent microscope (Nikon, Tokyo, Japan).

Clonal Assay in KSFM and on 3T3 feeder layers

To determine whether the newly devised cultivation method of a prolonged culturing time and a lower seeding density could maintain SCs, we seeded primary and cells subcultured to P4, P8 and P12 at a density of 40 cells per cm^2 in KSFM for 4 weeks. The clonal growth visualized by crystal violet staining, colony forming efficiency, colony size and cell sizes in the central and peripheral area of the colony were analysed and compared in triplicate to those established by seeding at the same density on mitomycin C-treated 3T3 feeder layer as previously described [14].

The doubling time of cells was measured by counting the asynchronously growing cells at day 7.

Soft agar colony assay

To determine whether expanded cells were transformed, P23 cultures were trypsinized and washed to generate single cell suspensions and seeded as 1×10^3 cells/ 24-well flat-bottomed plates using a two-layer soft agar system in a volume of 1000 μl/well as previously described [15]. Clonal growth was compared to that of 3T3 fibroblasts as the negative control and that of a retinoblastoma cell line (YB67) (kindly provided by Dr. Chia-Yang Liu, Cincinnati, OH) as the positive control.

Engineering of stratified epithelial sheets from a single cell

P20 cells were subjected to limiting dilutions in order to achieve single cell clonal growth using 96 wells in KSFM. One of these clones, designated as TKE2, was treated by TripLE® for 10 min., rendered into single cells, and cultured in KSFM on EDTA-denuded amniotic membrane fastened to a culture insert as reported [16]. The culture was submerged in KSFM until confluence, switched from KSFM for 1 day to the supplemental hormonal epithelial medium (SHEM), made of equal volumes of DMEM/F12 containing bicarbonate, 10 ng/ml human EGF, 5 μg/ml insulin, 100 ng/ml cholera toxin, 15% FBS, 70 μg/ml penicillin and 140 ng/ml, and exposed to the air-medium interface for 1 week with

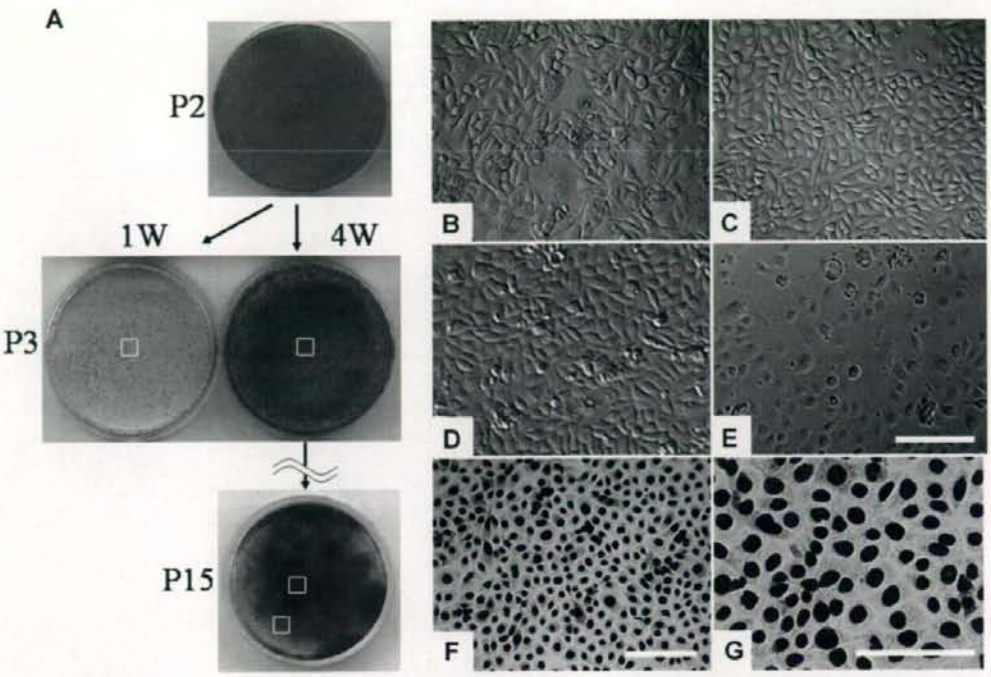

Fig. 1 Growth potential promoted by prolonging the culturing time. Primary (P0) cells seeded at a high density of 20,000 per cm^2 reached confluence in 1 week in keratinocyte serum-free defined medium (KSFM). When subcultured at 1:3, cells at Passage 1 (P1) became confluent in 1 week, and similarly subcultured to P2 for 1 week (1 W) revealing a mixture of small and large cells **(B)**. In contrast, cells were predominantly small if cultured for 4 weeks (4 W) before subculturing **(C)**. P2/4 W cultures subcultured at 1:3 did not reach confluence in 1 W (**A**, P3/1 W), but reached confluence in 4 W (**A**, P3/4 W). P3/1 W cultures could not be subcultured. In contrast, P3/4 W cultures could continually be subcultured if each culture was grown for 4 weeks (**A**, 4 W). Their growth was more clonal after P5. Cells in the centre of the clone (**A**, P15, inset) were uniformly small and compact **(D)**, while cells in the periphery (**A**, P15, inset) were also small but less compact **(E)**. Nuclear staining to PCNA was uniformly positive in more than 95% of P15 cells (**F** and **G**). Bars represent 100 μm.

mitomycin C (MMC)-treated 3T3 fibroblasts feeder layers pre-seeded on the plastic to promote stratification.

RT-PCR

Total RNA was isolated from expanded cells, stratified epithelial sheets, mouse skin and mouse corneal epithelium using the SV total RNA isolation system according to the manufacturer's recommendations, and generated cDNA using oligo(dT) priming and AVM reverse transcriptase XL by incubation of a 25 μl mixture at 41°C for 1 hr. RT-PCR was performed by containing oligonucleotide primers specific to each gene (Table 2) in 1 μl cDNA in a total reaction volume of 50 μl and amplified at 95°C for 30 sec. at 53°C for 30 sec. at 72°C for 20 sec. (20 cycles) using the Takara EX Taq DNA polymerase (Takara). Using glyceraldehyde-3-phosphate dehydrogenase (GADPH) as an internal control, PCR amplified products were separated by electrophoresis on a 1.5% agarose gel. Table of used primers.

Results

Prolonged culturing time preferentially preserved small epithelial cells

As reported previously [12], primary cultures (P0) seeded at 20,000 cells per cm^2 in KSFM reached confluence in 1 week. When subcultured at 1:3 splits, passage 1 (P1) cells reached

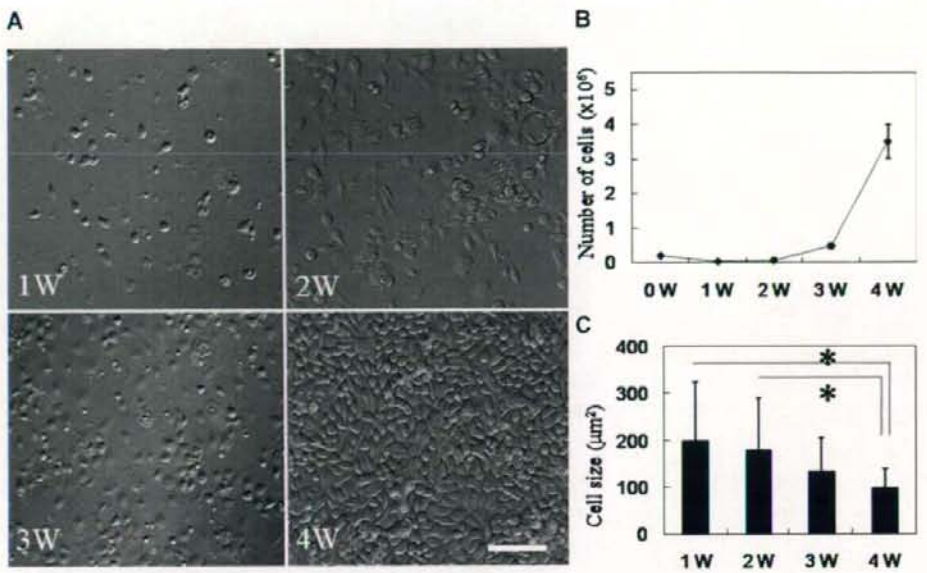

Fig. 2 Cell morphology and size affected by prolonging the culturing time. In P3 cultures, cells were mostly of an intermediate size with large squamous cells and 30–40% confluence at one week (**A**, 1 W), and mostly large squamous cells and 40–50% confluent at 2 weeks (**A**, 2 W). However, some small cells appeared with 60–70% confluence at 3 weeks (**A**, 3 W), and were mostly uniformly small and near confluence at 4 weeks (**A**, 4 W). The total cell number increased more dramatically after 3 weeks (**B**). The average cell size decreased steadily from 1 to 4 weeks (**C**). Bar represents 100 μm.

confluence again in 1 week. P2 cells subcultured for 1 week (1 W) revealed a mixture of small and large cells, and could not be subcultured at P3. However, P2 cultures consisted of predominantly small cells if cultured for 4 weeks (4 W) (Fig. 1). Furthermore, P3/4 W cultures could be continually subcultured for at least 100 passages if each passage was maintained low-seeding density. Importantly, clonal growth was observed after P4, and nuclear staining to PCNA was uniformly positive in more than 95% of cells (Fig. 1), indicating a highproliferative activity. To determine whether such small cells were selectively preserved when the culturing time was prolonged to 4 W, we measured the total cell number and the cell size weekly during the 4 weeks of P3 cultures. After 1 week, cells were heterogeneous and consisted of large cells with a prominent cytoplasm and small cells with a scanty cytoplasm (Fig. 2A, 1 W). The proportion between small cells to large cells was increased by the second week (Fig. 2A, 2 W) and the third week (Fig. 2A, 3 W). Notably, most cells were small by the 4th week (Fig. 1A, 4 W). The total cell number dramatically increased after the third week (Fig. 2B). The average cell size, however, decreased steadily from 1 W to 4 W as larger cells desquamated from the dish (not shown). Although most small cells remained in a monolayer at the end of 4 weeks, cells showed stratification and spontaneous desquamation in some areas. Such desquamated small cells still retained proliferative capacity when transferred to another dish in KSFM (not shown).

Further enrichment of small epithelial cells by lowering the seeding density

Small epithelial cells were selectively promoted by prolonging the culturing time to 4 weeks (Fig. 2), at the time when large differentiated cells had desquamated *via* senescence. Therefore, we speculated that small epithelial progenitor cells could be further selected by lowering the seeding density, which decreased the proportion of large differentiated cells to small cells. In P4 cultures, cells seeded at 50,000 cells per cm^2 degenerated into cell debris after 1 week of culturing (Fig. 3A). Hoescht 33342 staining revealed pronounced nuclear fragmentation suggestive of apoptosis (Fig. 3D), and the Live and Dead Assay showed marked cell death (Fig. 3G). Cells seeded at 5000 cells per cm^2 showed some spindle cells mixed with small cells (Fig. 3B, indicated by *), which had less fragmented nuclei (Fig. 3E), and fewer dead cells (Fig. 3H). In

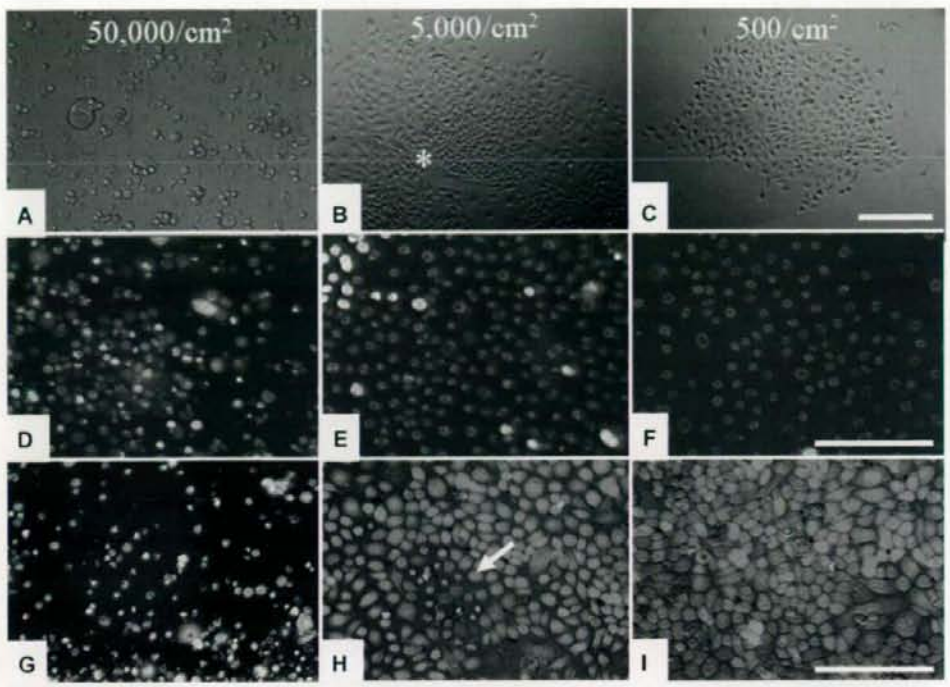

Fig. 3 Morphology (top), nuclear fragmentation (middle) and Live and Dead Assay (bottom) at different seeding densities. In P4 cultures, cells seeded at 50,000 cells per cm^2 degenerated into cell debris after 1 week of culturing **(A)**. This change was associated with pronounced nuclear fragmentation shown by Hoescht 33,342 staining **(D)**, and by marked cell death **(G)**. Cells seeded at 5000 cells per cm^2 showed some spindle cells mixed with small cells (**B**, indicated by *). Some cells showed fragmented nuclei **(E)**, and there were patches of dead cells (**H**, indicated by arrow). Cells seeded at 500 cells per cm^2 showed uniform small cells **(C)** without fragmented nuclei **(F)** or dead cells (**I**). Bars represent 50 μm.

contrast, cells seeded at 500 cells per cm^2 were uniformly small (Fig. 3C) without fragmented nuclei (Fig. 3F) or dead cells (Fig. 3G). These results indicated that small epithelial progenitor cells were indeed preferentially enriched by lowering the seeding density.

Epithelial differentiation at different seeding densities

Because cells seeded at higher densities contained a heterogeneous population of small and large cells (Fig. 3), we wondered whether these large cells consisted of more differentiated cells. To resolve this issue, immunostaining was performed with antibodies against K12 keratin, a marker for corneal-type epithelial differentiation [5], and p63, a transcription factor specific for epithelial progenitor cells [7] in the above P4 culture. At the density of 50,000 cells per cm^2, 42.3 ± 7.8% of cells cultured for 1 week were positive for K12 keratin in the cytoplasm (Fig. 4A), and 59.3 ± 7.4% of them were positive for p63 in the nucleus (Fig. 4D). Large cells tended to be positive for K12 keratin and negative for p63. At a density of 5000 cells per cm^2, 34.7 ± 7.1% of cells were positive for K12 keratin (Fig. 4B), while 81.7 ± 5.0% of cells were positive for p63 (Fig. 4E). In contrast, at a density of 500 cells per cm^2, nearly all cells were negative for K12 expression (Fig. 4C), but uniformly positive for p63 expression (Fig. 4F) Cells at a low-seeding density (500 cells/cm^2) had significantly lower K12 and higher p63 expression than those at intermediate and high seeding densities (5000 and 50,000 cells/cm^2, respectively) (both $P < 0.01$). These results indicated that cell differentiation was promoted by at a high seeding density, which explained in part why lower seeding density further enriched epithelial progenitor cells.

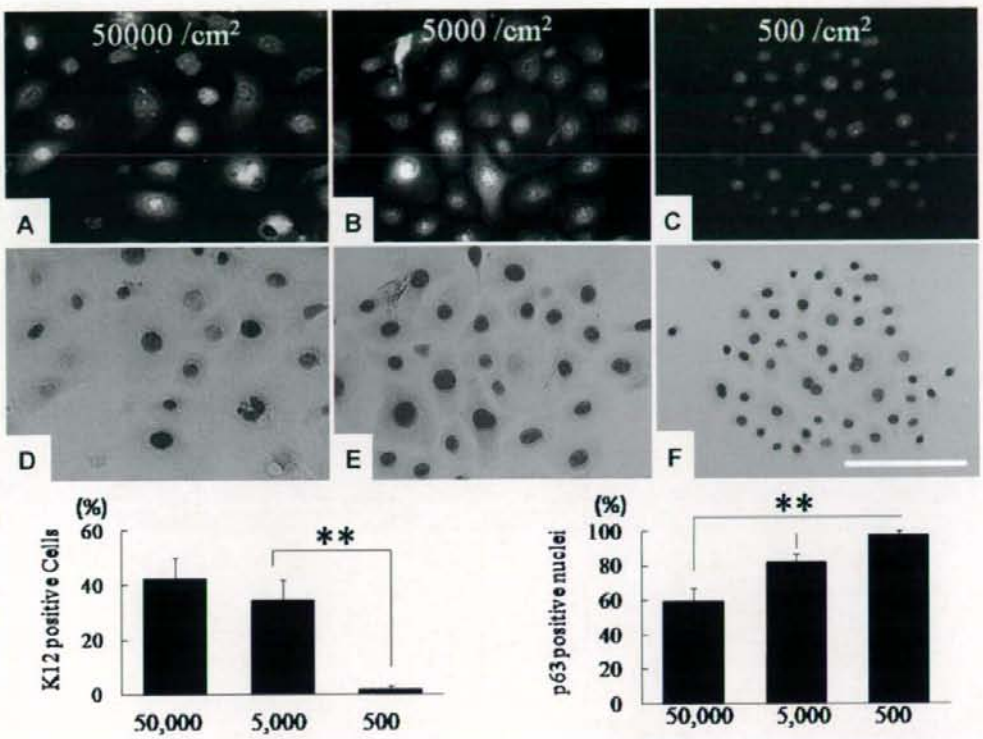

Fig. 4 Expression of K12 Keratin (top) and p63 (bottom) at different seeding densities. In the above P4 culture (Fig. 3), immunostaining was performed with antibodies against K12 keratin and p63. At 50,000 cells/cm^2, ~40% of cells cultured for 1 week were positive for K12 keratin in the cytoplasm **(A)**, and ~60% of them were positive for p63 in the nucleus **(D)**. Large cells tended to be positive for K12 keratin and negative for p63. At 5000 cells/cm^2, less cells were positive for K12 keratin (B), while more cells were positive for p63 **(E)**. In contrast, cells seeded at 500 cells/cm^2 were negative for K12 expression but uniformly positive for p63 expression **(F)**. PI revealed nuclear size was not different between K12-positive and negative cells **(D)**. Bar represents 50 μm. When the seeding density was decreased from 50,000 to 500 cells/cm^2, K12 positive cells decreased from 42.3% to 1.7%, while p63 positive cells increased from 59.3% to 97.7% ($^{**}P < 0.01$).

Clonal growth by lowering the seeding density and prolonging the culturing time

To further confirm that small cells were indeed progenitor cells, we compared their clonal growth in KSFM by lowering the seeding density and prolonging the culturing time simultaneously. P0 cells after isolation were seeded at the density of 40 cells/cm^2 on plastic in KSFM and on mitomycin C-arrested 3T3 fibroblast feeder layer. Large clones with a smooth contour resembling holoclones [17] wereformed in both KSFM (Fig. 5A) and 3T3 fibroblast feeder layer (Fig. 5B). Cells in both the centre and the periphery of the clone formed in KSFM were uniformly smaller (Fig. 5C and E, respectively). In contrast, cells in the centre were large and squamous but in the periphery were small in the clone formed on 3T3 fibroblast feeder layer (Fig. 5D and F, respectively). The colony-forming efficiency was 0.27 ± 0.25% in KSFM, which was significantly fewer than 2.4 ± 0.5% in 3T3 fibroblast feeder layer (Fig. 5G, $P < 0.001$). In contrast, the average diameter of colonies formed in KSFM was 7.0 ± 4.2 mm, which was significantly larger than 1.3 ± 1.4 mm in 3T3 fibroblast feeder layer (Fig. 5H, $P < 0.05$).

To further determine whether small cells expanded during continuous passages in KSFM still possessed progenitor cell status, cells subcultured to P4, P8 and P12 were seeded at a density of 40 cells/cm^2 on plastic in KSFM, and compared to those seeded in

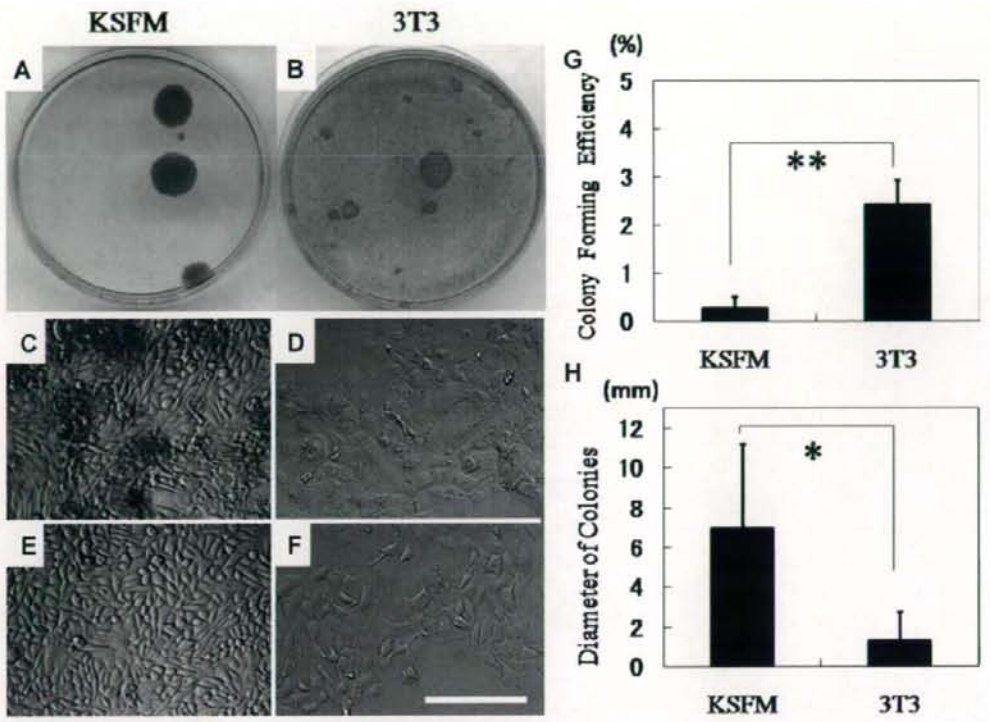

Fig. 5 Clonal growth of primary murine corneal/limbal epithelial cells. P0 clonal cultures were established by seeding 40 cells/cm^2 on plastic in KSFM and in supplemental hormonal epithelial medium (SHEM) with mitomycin C-treated 3T3 fibroblast feeder layers, and cultured for 4 weeks. Clones in KSFM **(A)** visualized by crystal violet staining were fewer but larger than those in 3T3 feeder layers **(B)**. Cells in KSFM were uniformly smaller than those in 3T3 feeder layer at central (**C** and **D**, respectively) and peripheral areas (**E** and **F**, respectively). Clones formed in KSFM cultures were fewer (**G**, $^{**}P < 0.001$) but larger (**H**, $^{*}P < 0.05$). Bar represents 100 μm.

SHEM containing 3T3 fibroblast feeder layer. After 4 weeks of culturing, colonies visualized by crystal violet were found in both culturing systems. However, fewer but larger round colonies were consistently observed in KSFM than in 3T3 fibroblast feeder layers for these three subpassages (Fig. 6A). In KSFM, cells remained uniformly small (Fig. 6B), while cells on 3T3 fibroblast feeder layer were initially small but rapidly enlarged to squamous and elongated cells (Fig. 6C and D). Large P12 squamous cells on 3T3 fibroblast feeder layer expressed more K12 keratin (Fig. 6E), contained a lower percentage of p63 nuclear positive cells (Fig. 6F), and had larger irregular nuclei (counterstained with Hoescht 33342) (Fig. 6G) than cells in colonies formed in KSFM (see Fig. 5 for comparison). These results suggested that clonal growth of expanded epithelial progenitor cells were supported better by KSFM than by 3T3 fibroblast feeder layers.

Normal differentiation induced by increasing [Ca^{2+}] and adding serum at P12

Previously, we noted that an increase of [Ca^{2+}] to 0.9 mM and addition of 5% FBS in KSFM restored expression of K12 keratin by large squamous epithelial cells in P2 cultures [12]. To make sure that the aforementioned expansion of small cells still retained the capability of adopting normal epithelial differentiation, we raised [Ca^{2+}] to 0.9 mM and/or added 5% FBS for 2 days in P12 cultures. In the control culture containing KSFM alone, cells expanded up to P12 remained uniformly small (Fig. 7A), did not express K12 keratin (Fig. 7E) and uniformly expressed p63 in the nucleus (Fig. 7I). An increase of [Ca^{2+}] to 0.9 mM rendered them into large squamous cells (Fig. 7B), of which some expressed K12 keratin (Fig. 7F), and lost p63 nuclear

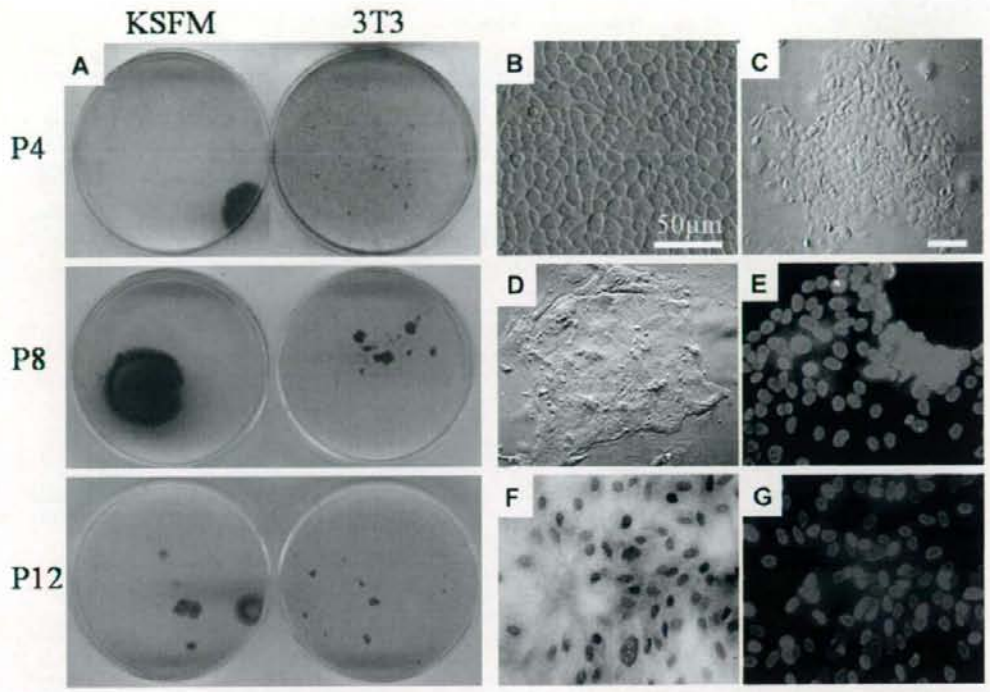

Fig. 6 Clonogenicity of expanded small cells during continuous passage. To further analyse clonogenicity, cells expanded at P4, P8 and P12 were seeded at 40 cells/cm^2 on plastic in KSFM or in SHEM containing 3T3 fibroblast feeder layers, and cultured for 4 weeks. Clones in KSFM visualized by crystal violet staining were fewer but larger than those in 3T3 fibroblast feeder layers **(A)**. In KSFM, cells remained uniformly small **(B)**, while cells on 3T3 fibroblast feeder layer were initially small **(C)** but rapidly enlarged to squamous and elongated cells **(D)**. P12 large squamous cells on 3T3 fibroblast feeder layer expressed more K12 keratin **(E)**, contained a lower percentage of p63 nuclear positive cells **(F)**, but larger irregular nuclei (counterstained with Hoescht 33342) **(G)** than cells in KSFM (cf. Fig. 7E and I). Micrographs of **C** and **D** were taken at same magnification, while the rest were taken at higher magnification. Bars represent 100 μm.

staining (Fig. 7J). Addition of 5% FBS also rendered them into large squamous cells (Fig. 7C), which expressed K12 keratin (Fig. 7G) and lost p63 nuclear expression (Fig. 7K). An increase of [Ca^{2+}] to 0.9 mM and addition of 5% FBS synergistically produced larger squamous cells (Fig. 7D), which expressed more K12 keratin (Fig. 7H), and further lost p63 nuclear staining (Fig. 7L). Besides an increase in the cell size, increased [Ca^{2+}] and/or addition of FBS also significantly increased the nucleus size. Collectively, these data indicated that small epithelial cells were indeed p63-expressing progenitor cells that retained K12 keratin expression upon appropriate stimulation by an increase of [Ca^{2+}] and/or addition of FBS at P12.

Single cell clonal expansion

P20 cells could successfully generate colony formation by limiting dilution on day 14 with the colony-forming efficiency around 3–4% without feeder layers. Although cell size, morphology and colony formation were similar as shown (Fig. 8, above), growth rate of those cells was different among cultures obtained by limiting dilutions (Fig. 8, below left). But there tended to be two growth patterns with either high or low proliferation. The mean doubling time of these clones was 31.3 hrs, but was 34.2 hrs for one of the clones, designated as TKE2 at day 7. The soft agar assay performed in TKE2 did not reveal any anchorage-independent growth

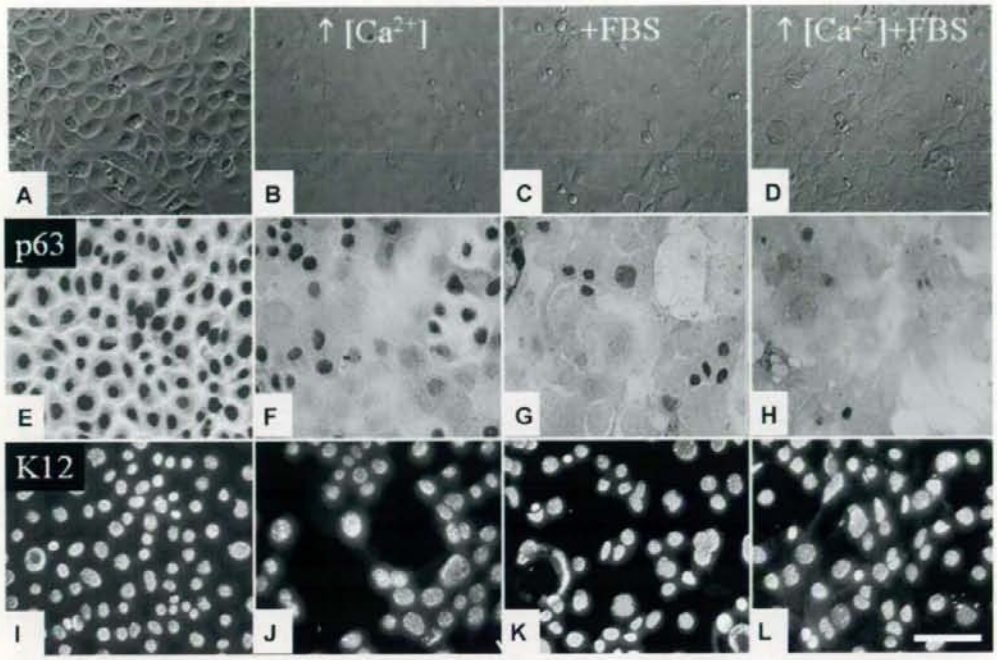

Fig. 7 Normal differentiation promoted by increased [Ca^{2+}] and addition of FBS during clonal expansion. Cells expanded up to P12 remained uniformly small **(A)**, uniformly expressed p63 in the nucleus **(E)**, and did not express K12 keratin **(I)**. Increasing [Ca^{2+}] to 0.9 mM rendered them into large squamous cells **(B)**, of which some lost p63 nuclear staining **(F)**, and expressed K12 keratin **(J)**. Addition of 5% foetal bovine serum (FBS) also rendered them in to large squamous cells **(C)**, which lost p63 nuclear expression **(G)** and expressed K12 keratin **(K)**. Increasing [Ca^{2+}] to 0.9 mM and addition of 5% FBS synergistically produced more large squamous cells **(D)**, which lost more p63 nuclear staining and increased the nuclear size **(H)**, and expressed more K12 keratin **(L)**. Bar represents 50 μm.

when compared to the positive colony formations in a retinoblastoma cell line (1.1%), and to the negative control using Swiss-3T3 fibroblasts (Fig. 8, right below, $n = 3$).

Single cell-generated stratified epithelial sheets

P20 TKE2 clone was expanded until confluence in KSFM on denuded amniotic membrane fastened on an insert as previously described [16], and then induced into marked stratification with 5–7 layers by exposure to the air-medium interface (Fig. 9, right). Immunostaining showed that basal to suprabasal cell layers were positive to p63 and K14 keratin, but negative to K12 keratin and Pax 6 Fig. 9, right). RT-PCR further confirmed that cells in such epithelial sheets indeed expressed K14 keratin and DNp63, but not K10 keratin, K12 keratin and Pax6 (Fig. 9, left). As compared to positive expression of connexin 43 and involucrin in both normal corneal and epidermal epithelia, TKE2 stratified epithelial sheets also expressed both connexin 43 and involucrin, suggesting that progenitor cells could exhibit differentiation. These results collectively indicated that *in vitro* engineered stratified epithelial sheets adopted a basal cell phenotype of stratified epithelium but has not turned on normal corneal differentiation or abnormal epidermal differentiation.

Plasticity into corneal differentiation

To determine whether TKE2 cells still retained the plasticity into corneal differentiation at late passage, we cultivated cells (P85) under four different conditions, that is, KSFM, KSFM containing 0.9 mM [Ca^{++}], KSFM containing 5%FBS, KSFM containing both 0.9 mM [Ca^{++}] and 5%FBS. Using P12 cells, immunstaining showed that cells remained uniformly small, uniformly expressed p63 in the nucleus, but did not express K12 keratin (Fig. 7).

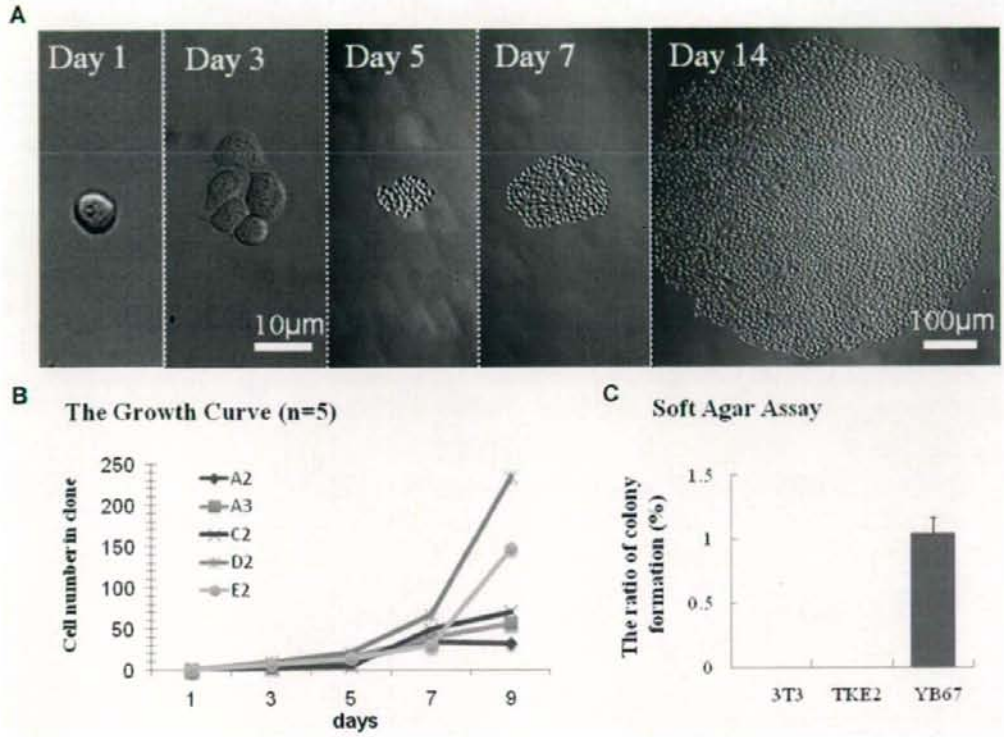

Fig. 8 Single cell clonal growth of P20 cells. Single P20 cells can be plated on 96-well plate containing KSFM by limiting dilution method. 3.4% of the wells showed clonal growth with small cells **(A)**. Cells were counted in five different clones over 1 week and revealed a different growth (Clone D2 and E2 had exponential growth). Among those clones, E2 clone were selected by small uniformed cell shape and proliferation with doubling time of 34.2 hrs **(B)**, and did not show colony formation in soft agar when compared to the positive control of YB67 cells, which showed 1.1% colony formation, and the negative control of 3T3 cells **(C)**.

However, an increasing [Ca^{2+}] to 0.9 mM or addition of 5% FBS, especially both rendered them into large squamous cells, of which some lost p63 nuclear staining, and began to express K12 keratin (Fig. 7). Using P85 cells, RT-PCR revealed that the same experimental maneuver caused a decline in expression of DNp63 but an increase in that of β1-integrin and TGF-βRII when cells enlarged in size and differentiated. Under these conditions, expression of Cx43 maintained while no discernable K12 expression was noted (Supplemental Fig. A). However, immunostaining of late-passage cells (P85) revealed strong K12 expression in a small population (Supplemental Fig. B), and RT-PCR analysis further demonstrated expression of OCT3/4, KLF4 and K14, markers of progenitor epithelial cells (Supplemental Fig. C). The results collectively explained why TKE2 cell could differentiate into K12 expressing cells in SHEM, and still possessed the plasticity to differentiate into a normal corneal epithelial phenotype in KSFM, especially under increasing [Ca^{++}] and addition of FBS.

Discussion

Compared to cells of other species, murine keratinocytes and corneal/limbal epithelial cells are known to be extremely difficult to culture. Previously, we established that growth and differentiation of murine corneal/limbal epithelial cells are susceptible to increased [Ca^{2+}] and addition of FBS [12]. When they were cultured at a high density in serum-free KSFM containing 0.07 mM [Ca^{2+}] and supplemented with growth-promoting agents, confluence was