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, availability of 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 keratocyte 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].

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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 [Ca2+] and was supplemented with 10 ng/ml EGF and 102 ± 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 cm² 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 continuously be passaged, and at P3, the average cell size (μm²) was monitored by phase contrast microscopy 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 cm² 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, immunoflourescence staining to K12 keratin was performed as previously reported [12].

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.
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 cm² 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 × 10³ 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

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Table 2 Primers used for RT-PCR

| Primer       | Sequence (5' → 3')                  | Product size (bp) |
|--------------|-------------------------------------|-------------------|
| Keratin 10   | GGCTCTGGCAA GAATCAAAC ATGAGC        | 167               |
|              | GGATTTGAG ATTATCGTT GTTGG           |                   |
| Keratin 12   | CGGGAGTGTGATGAAAGCA                 | 188               |
|              | CATTCTGAAGTGCATGTCGGC               |                   |
| Keratin 14   | CCCCTCCACGTTGGAGATTCA               | 1417              |
|              | CCTGCAGATGGATAAGAGGG                |                   |
| Pax 6        | AGTTCTTGAC AACCTGGCTA               | 500               |
|              | TGAAGCTGCT GCTGATAGGA               |                   |
| Involutrin   | CAGGACATGTGACGGACCACAGG             | 883               |
|              | GTGTCGGTTCTCCAATTCGCG              |                   |
| Connexin 43  | CCTTCTTGATGACGTGAGGGTAC            | 154               |
|              | ACCAAGGACACCCACACCAT               |                   |
| GAPDH        | ACCAAGCTCCATGGCATCAC                | 452               |
|              | TCCACCACCCTGTGCTGTA                |                   |
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 AVN 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 cm² in KSFM reached confluence in 1 week. When subcultured at 1:3 splits, passage 1 (P1) cells reached
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 high-proliferative 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. 2A, 4 W). The total cell number dramatically increased after 3 weeks (B). The average cell size decreased steadily from 1 to 4 weeks (C). Bar represents 100 μm.

**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 cm² 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 cm² 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
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.
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/cm² on plastic in KSFM and on mitomycin C-arrested 3T3 fibroblast feeder layer. Large clones with a smooth contour resembling holoclones [17] were formed 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 ± 0.002% in KSFM, which was significantly lower 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/cm² on plastic in KSFM, and compared to those seeded in
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.

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 expression (Fig. 7I).
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 \([\text{Ca}^{2+}]\) 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 \([\text{Ca}^{2+}]\) 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 \([\text{Ca}^{2+}]\) 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.
when compared to the positive colony formations in a retinoblas-
toma cell line (1.1%), and to the negative control using Swiss-3T3
fibroblasts (Fig. 8, right below, n/H110053).

Single cell-generated stratified epithelial sheets

P20 TKE2 clone was expanded until confluence in KSFM on denud-
ed 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 pro-
genitor 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, immunostaining
showed that cells remained uniformly small, uniformly expressed
p63 in the nucleus, but did not express K12 keratin (Fig. 7).
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 \(\beta\)-integrin and TGF-\(\beta\)RII 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\(^{2+}\)] 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
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.
expression. Such abnormal terminal differentiation is further ev-
etually turn on abnormal epidermal type differentiation 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 spec-
ulate 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 find-
ing 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 layers are 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, presum-
ably via vitamin A [12]. In this study, we noted that cells in P4 cul-
tures still expressed K12 keratin at high seeding densities (Fig. 4), but
did not express K10 keratin (not shown). Expression of K12 ker-
atin by RT PCR was eventually lost in P52 cells (Fig. 9), but that
defined by immunostaining could still be up-regulated in P12 cul-
tures 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 prolif-
eration and differentiation in response to these extracellular stim-
uli, 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
clon: 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 epithially 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 Dnpp63, and by negative expres-
sion 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)
(Supplemental 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].

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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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