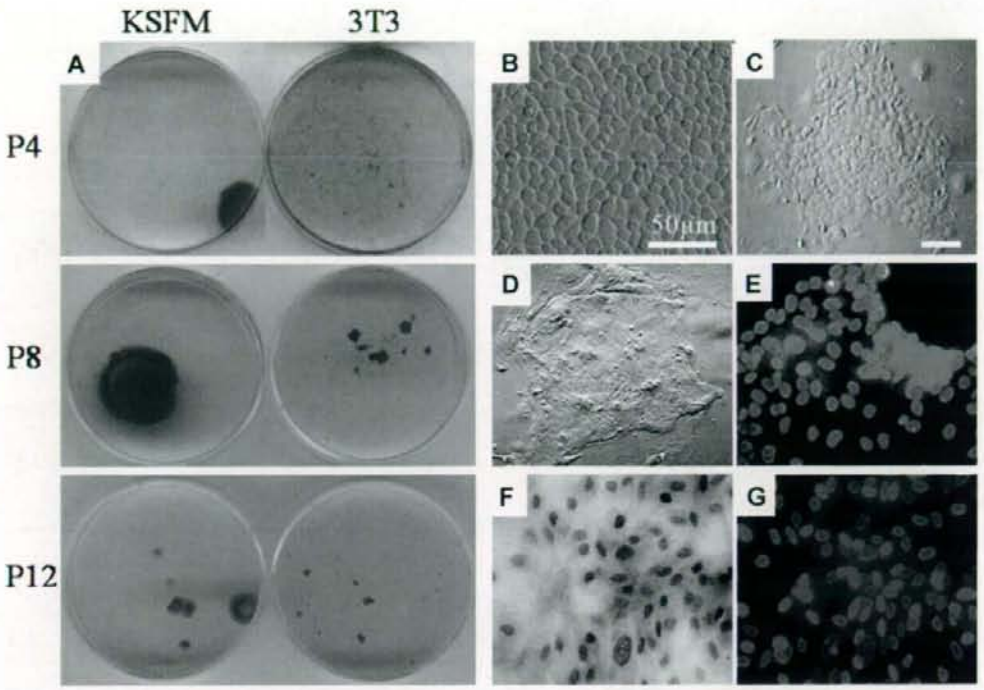

Fig. 6 Clonogenicity of expanded small cells during continuous passage. To further analyse clonogenicity, cells expanded at P4, P8 and P12 were seeded at 40 cells/cm^2 on plastic in KSFM or in SHEM containing 3T3 fibroblast feeder layers, and cultured for 4 weeks. Clones in KSFM visualized by crystal 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

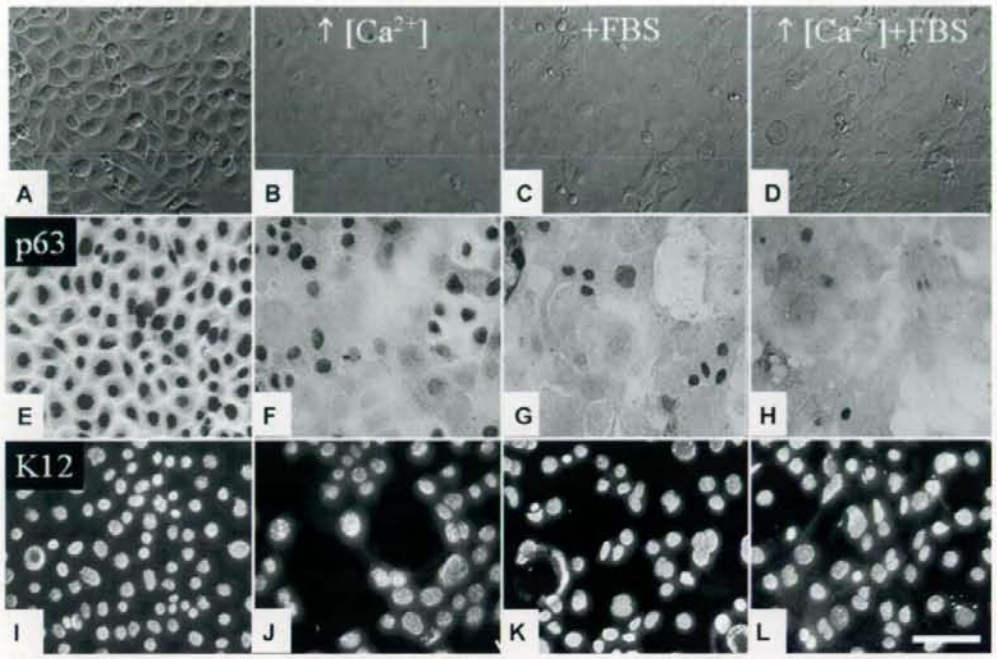

Fig. 7 Normal differentiation promoted by increased [Ca^{2+}] and addition of FBS during clonal expansion. Cells expanded up to P12 remained uniformly small **(A)**, uniformly expressed p63 in the nucleus **(E)**, and did not express K12 keratin (**I**). Increasing [Ca^{2+}] to 0.9 mM rendered them into 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).

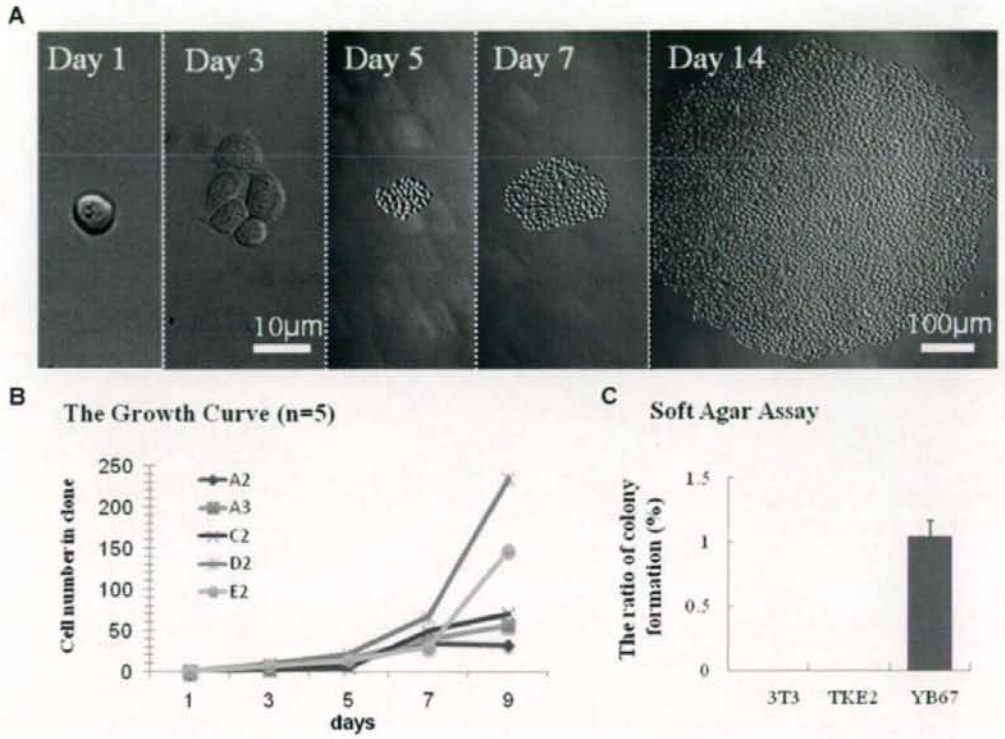

Fig. 8 Single cell clonal growth of P20 cells. Single P20 cells can be plated on 96-well plate containing KSFM by limiting dilution method. 3.4% of the wells showed clonal growth with small cells **(A)**. Cells were counted in five different clones over 1 week and revealed a different growth (Clone D2 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

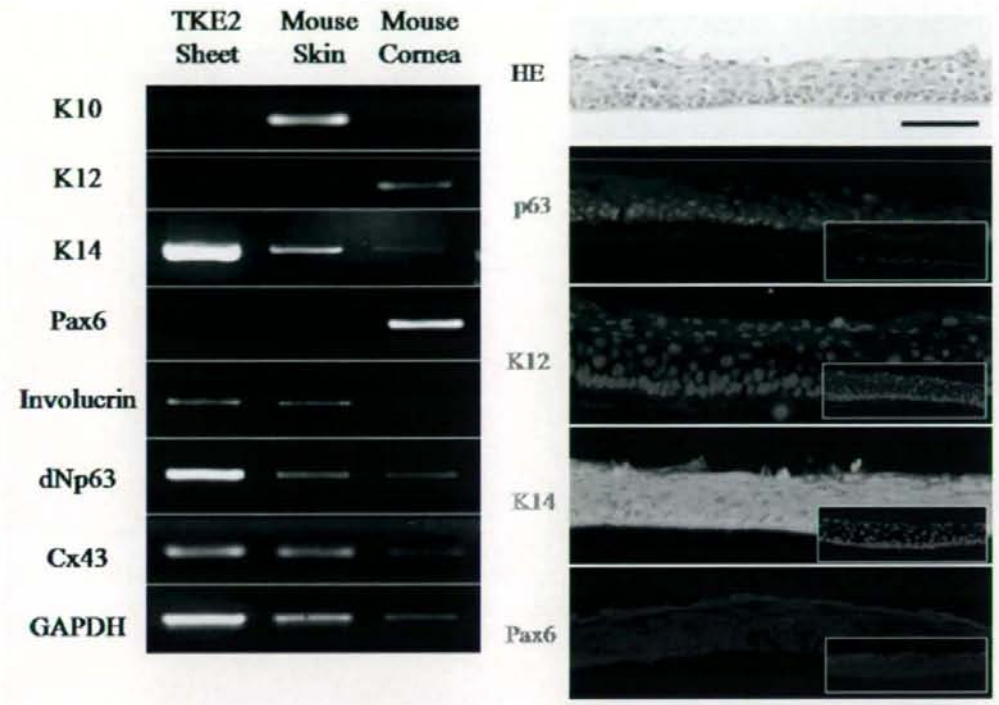

Fig. 9 Phenotypic characterization of single cell-generated stratified epithelial sheets. A stratified epithelial sheet was generated from single P52 clonally selected TKE2 cells by growing them on denuded amniotic membrane with 3T3 feeder layers on the plastic and by air-lifting technique. RT-PCR showed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

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

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

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

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

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

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

Acknowledgement

This work was supported by RO1 EY06819 and RO1EY15735 grants (SCGT) from National Institutes of Health, National Eye Institute, Bethesda, MD, a research grant from TissueTech, Inc., Grant-in-Aid for Young Scientists (B) (18791301) from the Ministry of Education, Culture, Sports, Science and Technology and Grant-in-Aid for Scientific Research (H18-regeneration-young-002) from the Ministry of Health and Welfare. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

1. **Schermer A, Galvin S, Sun T-T.** Differentiation-related expression of a major 64K corneal keratin *in vivo* and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol.* 1986; 103: 49–62.
2. **Lavker RM, Sun T-T.** Epidermal stem cells: Properties, markers and location. *Proc Natl Acad Sci USA.* 2000; 97: 13473–5.
3. **Romano AC, Espana EM, Yoo SH, Budak MT, Wolosin JM, Tseng SC.** Different cell sizes in human limbal and central corneal basal epithelia measured by confocal microscopy and flow cytometry. *Invest Ophthalmol Vis Sci.* 2003; 44: 5125–9.
4. **Cotsarelis G, Cheng SZ, Dong G, Sun T-T, Lavker RM.** Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell.* 1989; 57: 201–9.
5. **Liu C-Y, Zhu G, Converse R, Kao CW, Nakamura H, Tseng SC, Mui MM, Seyer J, Justice MJ, Stech ME.** Characterization and chromosomal localization of the cornea-specific murine keratin gene Krt1.12. *J Biol Chem.* 1994; 260: 24627–36.
6. **Matic M, Petrov IN, Chen S, Wang C, Dimitrijevich SD, Wolosin JM.** Stem cells of the corneal epithelium lack connexins and metabolite transfer capacity. *Differentiation.* 1997; 61: 251–60.
7. **Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, Ponzin D, McKeon F, De Luca M.** p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci USA.* 2001; 98: 3156–61.
8. **Watanabe K, Nishida K, Yamato M, Umemoto T, Sumide T, Yamamoto K, Maeda N, Watanabe H, Okano T, Tano Y.** Human limbal epithelium contains side population cells expressing the ATP-binding

cassette transporter ABCG2. *FEBS Lett.* 2004; 565: 6–10.

9. **Hayashi R, Yamato M, Sugiyama H, Sumide T, Yang J, Okano T, Tano Y, Nishida K.** N-Cadherin is expressed by putative stem/progenitor cells and melanocytes in the human limbal epithelial stem cell niche. *Stem Cells.* 2007; 25: 289–96.
10. **Lehrer MS, Sun TT, Lavker RM.** Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. *J Cell Sci.* 1998 10; 2867–75.
11. **Pellegrini G, Golisano O, Paterna P, Lambiase A, Bonini S, Rama P, De Luca M.** Location and clonal analysis of stem cells and their differentiated progeny in the human. *Ocular Surface.* 1999; 145: 769–82.
12. **Kawakita T, Espana EM, He H, Yeh LK, Liu CY, Tseng SC.** Calcium-induced abnormal epidermal-like differentiation in cultures of mouse corneal-limbal epithelial cells. *Invest Ophthalmol Vis Sci.* 2004; 45: 3507–12.
13. **Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dötsch V, Andrews NC, Caput D, McKeon F.** p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell.* 1998; 2: 305–16.
14. **Rheinwald JG, Green H.** Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell.* 1975; 6: 331–43.
15. **Kubota T, Koshizuka K, Williamson EA, Asou H, Said JW, Holden S, Miyoshi I, Koeffler HP.** Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both *in vitro* and *in vivo*. *Cancer Res.* 1998; 58: 3344–52.
16. **Grueterich M, Espana E, Tseng SC.** Connexin 43 expression and proliferation of human limbal epithelium on intact and denuded amniotic membrane. *Invest Ophthalmol Vis Sci.* 2002; 43: 63–71.
17. **Barrandon Y, Green H.** Three clonal types of keratinocytes with different capacities for multiplication. *Proc Natl Acad Sci USA.* 1987; 84: 2302–6.
18. **Li W, Hayashida Y, Chen YT, Tseng SC.** Niche regulation of corneal epithelial stem cells at the limbus. *Cell Res.* 2007; 17: 26–36.

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 (This link will take you to the article abstract).

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.