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

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

METHODS. Corneal limbal explants of C57BL6/J mice were cultured in serum-free, low-Ca2+-containing medium supplemented with EGF and cholera toxin. Epithelial cells were subcultured at a 1:5 split until passage (P)4 and at lower densities after P4. Colony-forming efficiency, population-doubling times, and population doublings were determined. The expression of p63, keratin (K)19, and involucrin was analyzed by RT-PCR, immunocytochemistry, and Western blotting. Differentiation potential was examined by switching the medium to serum or high Ca2+-containing medium. Stratification ability was analyzed by air-lift culture.

RESULTS. Thirty of 32 (93.8%) corneal explants were successfully subcultured to P1. Cultures without cholera toxin did not proliferate past P2 (n = 12), but 55% of cultures supplemented with cholera toxin achieved P4 (n = 20). After P4, cells were stably subcultured over 25 passages. Colony-forming efficiency increased from 9.7% ± 2.6% at P5 to 29.0% ± 3.3% at P20. The cells showed cobblestone appearance and expressed p63, K19, and involucrin but were negative for K12. Serum and high Ca2+ induced differentiation, and cells cultured in DMEM/F12 with serum showed K12 mRNA expression. Stratified epithelium was formed by air-lifting.

CONCLUSIONS. With this procedure, corneal epithelial cells from a single cornea can be cultured long term and can retain the potential to differentiate and stratify. This procedure can be a powerful tool for studies that require comparison of corneal epithelial cells from normal and transgenic mice in vitro. (Invest Ophthalmol Vis Sci. 2009;50:2716–2721) DOI:10.1167/iovs.08-2139

Methods for culture and subculture of human corneal epithelial cells have been well documented.1–3 However, such has not been the case for mouse corneal epithelial cells (MCEs). Hazlett et al.5 cultured MCEs from explants but failed in subculturing the cells over three passages. Limited lifespan and insufficient cell yields hindered the application of MCEs in vitro experiments. Kawakita et al.6 reported a method to successfully isolate viable mouse corneal/limbal epithelial sheets and culture in serum-free low-Ca2+ medium. Furthermore, by prolonging the culturing time and lowering the seeding density, they successfully established a mouse corneal epithelial line.7 However, they required more than 200 eye globes to establish their stable cell line. To our knowledge, all existing methods failed in reproducing the long-term culture of MCEs from a single mouse cornea.

Using mice to study the molecular biology and physiology of the cornea has several advantages. The advent of genetic manipulation resulting in corneal phenotypes in transgenic and knockout mice provides a powerful tool to study the molecular mechanisms involved in the development and pathogenesis of diseases of the cornea. Therefore, establishing long-term culture of MCEs from a single mouse cornea becomes increasingly important to plan in vitro studies to substantiate findings from in vivo studies in these mice. Herein, we report a novel three-step culture procedure for the long-term culture of MCEs from a single mouse cornea. The initial stage uses limbal explant cultures in serum-free low-Ca2+ medium supplemented with cholera toxin, followed by subculture of corneal epithelial cells in low cell densities. The cells obtained were morphologically normal, showed the phenotype of corneal epithelial progenitor cells, and retained differentiation potential and stratification ability for more than 25 passages.

MATERIALS AND METHODS

Tissue Preparation and Cell Culture

C57BL6/J mice (CLEA Japan Inc, Tokyo, Japan), aged 8 to 10 weeks, were handled according to the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. After death, eye globes were enucleated from the mice with forceps and washed profusely in phosphate-buffered saline (PBS). Eyes from each animal were kept separate throughout the culture procedure. Corneal buttons, including the limbus, were cut from the eye and cleaned of extraneous tissue (e.g., iris, ciliary body). Primary cell culture was performed with the use of an explant culture method similar to that of Hazlett et al.5 Briefly, the button was cut in half, and each explant was plated flat, epithelium side up, on a six-well plate, one piece per well. After approximately 5 to 10 minutes to allow for attachment of the explant, serum-free low-Ca2+ medium (keratinocyte serum-free medium [KSF]; Invitrogen, Carlsbad, CA) consisting of 10 ng/mL human recombinant EGF (Invitrogen), 100 ng/mL cholera toxin (LIST Biological Laboratories Inc., Campbell, CA), antibiotics, and growth supplement supplied by the manufacturer were added. The cultures were incubated at 37°C, under 95% humidity and 5% CO2, and the medium was changed every 3 to 4 days. After approximately 10 days, the explant was carefully transferred to a new dish and cultured as described. Cells derived from passaged explants were also used for subcultures. Epithelial cells were subcultured (TrypLE Express; Invitrogen) at 1:3 splits after small cells reached subconfluence or if the colonies of small cells started to stratify before reaching subconfluence. The procedure was repeated until passage (P)4 cultures. From P5, subconfluent cells were subsequently passaged at a density of 5 × 10^5 per dish. The cultures were examined for the expression of p63, K19, and involucrin but were negative for K12. After P4, the cultures were stably subcultured over 25 passages. Colony-forming efficiency increased from 9.7% ± 2.6% at P5 to 29.0% ± 3.3% at P20. The cells showed cobblestone appearance and expressed p63, K19, and involucrin but were negative for K12. Serum and high Ca2+ induced differentiation, and cells cultured in DMEM/F12 with serum showed K12 mRNA expression. Stratified epithelium was formed by air-lifting.

CONCLUSIONS. With this procedure, corneal epithelial cells from a single cornea can be cultured long term and can retain the potential to differentiate and stratify. This procedure can be a powerful tool for studies that require comparison of corneal epithelial cells from normal and transgenic mice in vitro. (Invest Ophthalmol Vis Sci. 2009;50:2716–2721) DOI:10.1167/iovs.08-2139

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10^4 per 75-cm² flask. Cultures were incubated at 37°C, under 95% humidity and 5% CO₂, and the medium was changed every 3 to 4 days.

**Cell Cryopreservation and Thawing**

Dispersed cells were pelleted, resuspended without serum (Cell Banker-II; Mitsubishi Kagaku Iatron, Inc, Tokyo, Japan), placed in cryotubes (5 × 10⁷ to 10⁸ cells/mL), and frozen at −80°C. For cell thawing, cryotubes were partially immersed in 37°C water. Cells were harvested and plated at a density of 5 × 10⁴ per 75-cm² flask supplemented with complete culture medium, as described previously.

**CFE and Cell Proliferation**

MCEs were inoculated in 60-mm dishes at 1000 cells/dish and cultured for 10 to 14 days. Cultured cells were stained with eosin for 1 hour.

**Immunostaining**

MCEs (1 × 10⁶ cells/dish) were cultured in gelatin-coated, two-well chamber slides and fixed with 4% paraformaldehyde (PFA; Wako Ltd., Osaka, Japan). PFA-fixed cells were permeabilized with 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO). After blocking with 10% normal donkey serum, the cells were treated with the following monoclonal primary antibodies: anti-p63 (1:100, 4A4; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-keratin (K)19 (1:100; NeoMarkers for Laboratory Vision Corporation, Fremont, CA), anti-involucrin (1:100; 4A4; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-keratin (K)19 (1:100; NeoMarkers for Laboratory Vision Corporation, Fremont, CA), anti-involucrin (1:100; Co-vance, Emeryville, CA), and anti-K12 (1:100; Santa Cruz Biotechnology). Cells were then treated with Cy3-conjugated secondary antibodies (Chemicon International, Inc., Temecula, CA). Nuclei were counterstained with 4',6-diamino-2-phenylindole (1 μg/mL, DAPI; Dojindo Laboratories, Tokyo, Japan).

**Western Blot Analysis**

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

**Results**

**Establishment of Cell Lines from Single Corneas**

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

**CFE and Growth Kinetics**

CFE increased from 9.7% ± 2.6% at P5 to 29.0% ± 3.3% at P20 (mean ± SD; n = 3). PDs increased with successive passages that seemed to plateau after P12 at an average of 45.9 ± 2.4 hours (n = 6; Fig. 1E). On reaching the plateau, cells seeded at 700/cm² usually reached saturation density within 8 days after plating. They were stably subcultured though at least 25 pas-
the expression of progenitor markers p63 and K19 and the differentiation marker involucrin did not change significantly (Fig. 5) after a high concentration of Ca\(^{2+}\) was added. However, after medium was supplemented with serum, the expression of progenitor markers p63 and K19 decreased significantly, whereas the differentiation marker involucrin increased (Fig. 5). Moreover, the cells cultured in DMEM/F12 with serum did not express p63 but expressed K12 at the mRNA level (Fig. 5).

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

**DISCUSSION**

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

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

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

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**mRNA and Protein Expression Profiles**

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

**Induction of Differentiation and Stratification**

To investigate the differentiation potential of the cells, we cultured the cells in KSFM supplemented with Ca\(^{2+}\) or serum and in DMEM/F12 with serum to induce differentiation. Morphologically, the cells cultured in KSFM were homogeneously small cells (Fig. 1D), whereas cells in KSFM with high Ca\(^{2+}\) were a heterogeneous mix of small and large cells and cells in media with serum were homogeneously large and squamous (Figs. 4A-D). RT-PCR and Western blot analyses showed that

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Long-term culture of MCEs has been notoriously difficult to establish. Until now, only Hazlett et al.\(^5\) cultured MCEs for no more than three passages, and Kawakita et al.\(^7\) established a mouse corneal epithelial line but used more than 200 eye globes. To our knowledge, this is the first report to reproducibly establish long-term MCEs from a single mouse cornea. Several approaches for the primary culture of corneal epithelial cells in vitro include the cell-suspension culture and explant culture techniques.\(^10\) Each approach has its own advantages. Although cell-suspension culture can to some extent decrease
commercially available serum-free, low-Ca\textsuperscript{2+} plated counterparts. In addition, Kawakita et al.\textsuperscript{7} reported that high seeding density yielded mostly large differentiated cells, whereas Green\textsuperscript{29} and Okada et al.\textsuperscript{22} have found that cyclic AMP increases cell proliferation. Our experiment showed that cholera toxin was crucial only during stage 2 but was not required for stage 3, which suggests that dependence on cholera toxin maybe rest on the level of differentiation (i.e., limbal SCs, TACs, and terminally differentiated cells). Further investigations are necessary to clarify the role of cholera toxin and cyclic AMP in the proliferation and differentiation of primary cultured corneal epithelial cells.

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

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![Figure 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932960/) Immunochemistry of cell markers in MCEs. Stage 3 MCEs cultured in KSFM expressed progenitor markers p63 (A) at P6 and K19 (B) at P8 and the differentiation markers involucrin (IVL; C) and K12 (D) at P8. Approximately 70% of cells were positive for p63 (A) and K19 (B), and approximately 30% were positive for IVL (C). There was no cellular expression of K12 (D). Scale bars, 100 \textmu m.

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932960/) Morphology of MCEs in differentiation-inducing medium. Phase-contrast micrograph of cells at P22 cultured in KSFM with 0.9 mM Ca\textsuperscript{2+} (A) has the appearance of large cells with smaller N/C ratios compared with cells cultured in low Ca\textsuperscript{2+}. MCEs cultured in KSFM with 10% serum (B), KSFM with 0.9 mM Ca\textsuperscript{2+} and 10% serum (C), and DMEM/F12 with 10% serum (D) induce MCEs to become enlarged, showing features of cellular senescence.
that appeared homogeneously small in KSFM changed to a heterogeneous mix of small and large cells in KSFM with Ca\textsuperscript{2+} and a homogeneous mix of large and squamous cells in the other media with serum. The expression of progenitor markers p63 and K19 decreased significantly, whereas the differentiation marker involucrin increased significantly. Furthermore, MCEs cultured in DMEM/F12 with serum expressed K12 at the mRNA level, with no p63 expression. In addition, the cells had the ability to be stratified. These data suggest that Ca\textsuperscript{2+} and serum can induce MCEs to differentiate. We were unable to detect K12 by Western blot or immunohistochemistry. However, Kawasaki et al. (IOVS 2007;48:ARVO E-Abstract 2724) recently reported that the K12 gene is epigenetically regulated by methylation. Culture conditions may alter the methylation of K12, which may explain the low expression of K12 protein in our study and in previous studies on epithelial cultures.\textsuperscript{7} Established epithelial cell lines that retain their differentiation potential are valuable tools for studying gene regulation, synthesis of extracellular matrix, or response to growth factors.\textsuperscript{8}

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

References


