Characterization of Growth and Differentiation in a Telomerase-Immortalized Human Corneal Epithelial Cell Line

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Purpose. To develop and characterize a telomerase-immortalized human corneal epithelial cell line (hTCEpi) to serve as an in vitro model for studying the molecular mechanisms involved in regulating human corneal epithelial cell differentiation.

Methods. Primary cultures of human corneal epithelial cells were infected with a retroviral vector encoding human telomerase reverse transcriptase (hTERT). Infected hTCEpi cells were selected, cloned, and characterized to identify telomerase activity, proliferative capacity, karyotype, and differentiative potential in routine culture and under consecutive submerged and air-lifted conditions. Cells were evaluated to measure cell cycle kinetics (anti-Ki-67, anti-p16), stratification (phalloidin and anti-ZO-1), and differentiation (anti-K3, anti-BCL-2 and TUNEL labeling).

Results. hTCEpi cells exhibited telomerase activity, a normal karyotype and cell cycle kinetics at greater than 240 population doublings, and loss of p16 after passage 10. Air-lifting produced a well stratified epithelium (five to seven cell layers) with apical ZO-1-stained tight junctions. Submersed culture demonstrated increasing expression of stratification markers (K5/K14) with K3-corneal keratin marker expression in long-term, air-lifted culture. Anti-BCL-2 staining showed both nuclear and cytoplasmic localization with loss of nuclear BCL-2 expression in TUNEL-labeled surface epithelial cells.

Conclusions. hTCEpi cells stratify, differentiate, and desquamate similar to normal human corneal epithelium. Further study of the hTCEpi cell line may be valuable in studying the molecular mechanisms regulating corneal epithelial cell differentiation and desquamation. (*Invest Ophthalmol Vis Sci.* 2005; 46:470–478) DOI:10.1167/iovs.04-0528

The corneal epithelium is a self-renewing, stratified epithelial sheet that provides the first line of defense against invading microorganisms. The homeostatic mechanisms regulating the normal physiological renewal of the epithelial sheet, involving proliferation, differentiation, and desquamation, are vital to safeguarding this intact barrier function. The underlying molecular interactions responsible for the balance and coordination of these processes, however, are still not well defined. Cultured human corneal epithelial cell lines could provide an efficient model for the study of cellular signaling and molecular pathways regulating normal corneal epithelial cell homeostasis. Previously, cultured human cells lines have been developed for this purpose by transformation with viral oncoproteins including adenovirus E1A, the SV40 large T antigen, and HPV16-E6/E7.[1–5] The effectiveness of these virally transformed cell lines as potential research models has been hampered by both genetic instability and a lack of normal growth and differentiation properties, precluding their effective use in the study of normal epithelial cell biology.

We recently generated an immortalized human corneal epithelial cell line (hTCEpi) by infection with human telomerase reverse transcriptase (hTERT). hTERT contains the catalytic subunit for telomerase, a ribonucleoprotein enzyme normally silent in somatic cells. The site of action for telomerase are short hexameric nucleotide repeats (TTAGGG) located at the ends of chromosomes, termed telomeres.[6] In the absence of telomerase, chromosomes are unable to maintain adequate telomere length due to incomplete DNA replication mechanisms, and thus progressively shorten after each mitotic division. On reaching a critical length threshold, shortened telomeres signal replicative senescence, acting as a molecular clock for the cell.[7,8] Infection of human cell lines with hTERT leads to the activation of telomerase, preventing telomere erosion and subsequent telomere-dependent senescence.[9] Human cell lines immortalized with hTERT exhibit genetic stability, normal contact inhibition, and maintain the capacity to differentiate.[10]

In this study, we characterized gene expression profiles in a human corneal epithelial cell line with introduced hTERT to validate this cell line as a viable model for studying the molecular mechanisms involved in regulating corneal epithelial cell differentiation. In routine culture, hTCEpi cells exhibited a normal 24-hour population-doubling time, consistent with previous findings.[5] At an air–liquid interface, organotypic constructs stratified into a multilayered squamous epithelium, possessed a normal actin cytoskeleton, and expressed ZO-1, a tight-junction–associated protein located in the apical layer, characteristic of the ocular surface.[11,12] In addition, under air-exposed (air-lifted) conditions, these cells expressed keratin K3, an accepted phenotypic marker of corneal epithelial differentiation.[13] Most significantly, hTCEpi cells at an air interface exhibited apoptotic cell death of the surface cells and appeared to desquamate in vitro, analogous to the human corneal epithelium in vivo.[14,15] Taken together, these findings support the conclusion that the hTCEpi cell line will be a useful adjunct for identifying the molecular mechanism(s) mediating epithelial proliferation, differentiation, and desquamation.

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Materials and Methods

Preparation of Human Immortalized Corneal Epithelial Cells

Primary culture epithelial cells were harvested individually from 21 donor eyes (ages range, 26–67 years), over a period of 5 months. All donor corneas were obtained from the Tissue Transplant Services Lions' Eye Bank (Dallas, TX) in accordance with the provisions of the Declaration of Helsinki for research involving human tissue. Briefly, epithelial cells were isolated from the limbal region by dispase II digestion (1.2U/mL) and grown in KGM-2 medium (Clonetics-BioWhittaker, Inc., Walkersville, MD) supplemented with 200 U penicillin, 200 μg streptomycin, and 0.5 μg/mL amphotericin B (BioWhittaker, Inc.). Primary or first-passaged cells at 50% to 60% confluence were infected with an hTERT retroviral vector containing a puromycin resistant gene construct and underwent clonal selection as previously described.[16] A stable clone originating from a 62-year-old white male was selected and propagated to develop the hTCEpi cell line.

Organotypic Corneal Constructs

hTCEpi cells were routinely maintained in KGM-2 culture media (Clonetics, BioWhittaker) containing 0.15 mM Ca^{2+}. Cells were subcultured on T75 tissue culture flasks (Falcon Labware; BD Biosciences, Bedford, MA), incubated at 37°C in 5% CO_2 and passaged every 7 to 10 days. To generate organotypic constructs, 5×10^4 hTCEpi cells/cm^2 in 2 mL KGM-2 culture medium containing 1.15 mM Ca^{2+} were passed onto collagen-coated (Vitrogen; Cohesion Technologies, Palo Alto, CA) culture inserts (12-mm diameter, 3.0-μm pore size, Corning, Inc., Corning, NY) and submersed in 1 mL of the high calcium KGM-2 culture medium. The medium was changed every other day for 7 days. To induce differentiation, the medium was removed from the culture insert, exposing cells to an air–liquid interface (Fig. 1). The remaining 1 mL of medium in the culture well was changed every day during air-lifting. All cells used in air-lifted differentiation studies had undergone >100 population doublings. For measurements of cellular growth kinetics, karyotyping, and telomerase activity, cells had undergone >240 population doublings.

Telomeric Repeat Amplification Protocol

A telomeric repeat amplification protocol (TRAP) assay was performed (TRAP-eze Telomerase Detection Kit; Chemicon, Temecula, CA) as previously described.[17] TRAP activity was assessed in hTCEpi cells at early, middle, and late passages (two independent p10s, p33, p36, p63, p66) and in noninfected controls (p1 and p3). Briefly, cell lysates from 100,000 cells at each time point underwent telomerase extension followed by PCR amplification and PAGE for gel-based telomerase detection. Lysis buffer was used as the negative control. A human mammary epithelial cell line known to express telomerase served as the positive control.

Karyotype Analysis

Karyotype analysis on hTCEpi cells was performed at the University of Texas Southwestern Medical Center Cryogenic Laboratory. Cell preparation was performed as previously described.[16] For the final karyotype, all 46 chromosomes from 21 cells in metaphase were analyzed. All cells had undergone >240 population doublings.

Antibodies and Reagents

A FITC-conjugated phalloidin probe (Molecular Probes, Eugene, OR) was used to detect actin. Double-labeling with propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) was performed in all experiments to label all epithelial nuclei. The following antibodies were used for immunohistochemistry (IHC) and Western blot analysis: a mouse monoclonal anti-Bcl-2 and a rabbit polyclonal anti-BCL-2 antibody (Ancell, Bayport, MN, and Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-ZO-1 antibody (Zymed Laboratories, Inc., San Francisco, CA), the anti-keratin antibody clone AE5 (Biogenesis Inc., Kingston, NH), an anti-Ki-67 antibody to label cycling cells (Diagnostic Biosystems, Pleasanton, CA), and anti-p16 (BD Pharmingen, San Diego, CA). FITC-conjugated antibody fragments (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used for secondary antibodies. A kit was used for detection of apoptosis (ApopTag Apoptosis Detection Kit; Serologicals Corp., Norcross, GA).

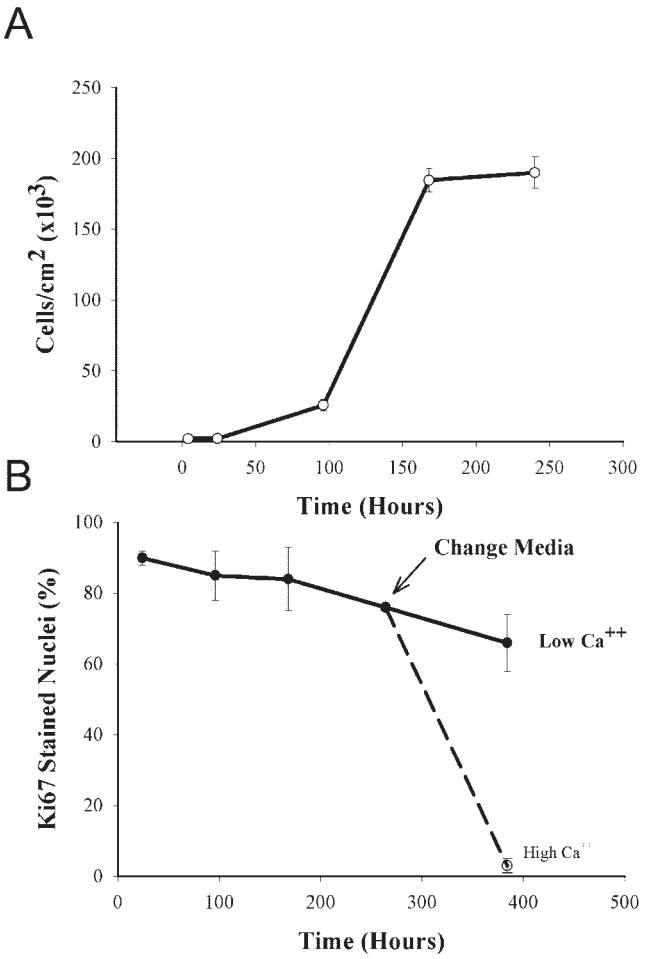

Figure 1. Cellular growth kinetics. (**A**) Cell growth in low-calcium culture conditions. During the first 96 hours, hTCEpi cells underwent 3.5 population doublings. Average population-doubling time was 24.4 hours. As cells reached confluence at 96 to 240 hours, there was a progressive reduction in cell growth. (**B**) Double-labeling with Ki-67 and PI to determine the percentage of cycling cells over time. A high percentage of cells stained with Ki67 while grown in low-calcium containing medium. Switching from low calcium to high calcium (*arrow*) dramatically reduced the number of cells staining with Ki-67.

Immunohistochemistry

hTCEpi cells suspended in low-calcium KGM-2 medium were plated at a density of 2×10^3 cells/cm^2 on collagen-coated glass coverslips (Vitrogen; Cohesion Technologies) in a 24-well culture plate (Corning Costar, Corning, NY). Cells were maintained at 37°C in 5% CO_2 and the medium was replaced every third day. For measurements of epithelial cell growth rates at 4 hours and 1, 4, 7, and 10 days, and coverslips were stained with 5 to 10 μg/mL PI. To determine the number of cycling cells, cultures were fixed in 1% paraformaldehyde and permeabilized with acetone. Cells were blocked with donkey serum at 37°C, incubated in anti-Ki-67 mouse monoclonal antibody (1:20) diluted with phosphate-buffered saline, and stained with FITC-conjugated secondary donkey anti-mouse IgG (1:100). Cells were counterstained with PI.

Coverslips were mounted on slides using 1:1 glycerol-PBS with antifade reagents.

For all other IHC, hTCEpi organotypic constructs were evaluated on tissue culture inserts after 7 days of submersed culture and 3, 7, or 10 days of air-lifted culture. Organotypic constructs were fixed with RNase free 1% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in phosphate-buffered saline (pH 7.4) for 3 minutes. Constructs were removed from inserts with a 10-mm diameter trephine, rinsed in PBS at 4°C and stained en bloc or embedded in tissue embedding medium (Leica Instruments GmbH, Nussloch, Germany) and snap frozen in liquid nitrogen for cryostat sectioning. Cryostat sections or wholemount inserts were extracted with cold acetone, washed with PBS, and blocked with 10% bovine serum albumin. Samples were then incubated in primary antibody for 1 hour at 37°C followed by secondary antibody for 1 hour at 37°C. All cells were counterstained with PI solution to label nuclei. Double-labeling experiments with TUNEL and BCL-2 were performed as previously described.[18] Wholemount constructs were imaged on a laser scanning confocal microscope (Model SP2; Leica). Cryostat sections and cells grown on coverslips were analyzed with a fluorescence microscope (Diaplan; Leitz, Wetzlar, Germany) equipped with a charge-coupled device camera (CoolSnap; Photometrics, Tuscon, AZ). For determination of population growth kinetics, five images per sample were obtained, and the number of Ki-67 cells (cycling cells) and PI positive cells (total cells) was counted. The average number of cycling cells was calculated for each coverslip, with the average of three coverslips recorded at each time point.

Protein Extraction

For collection of keratin protein, soluble proteins were first extracted with 25 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, 5 μg/mL antipain, 5 μg/mL pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 μg/mL aprotinin. The cells were sonicated for 1 minute and centrifuged at 4°C for 5 minutes. Supernatant was removed, and insoluble proteins were solubilized in a 25 mM Tris-HCl buffer containing an additional 1% sodium dodecyl sulfate (SDS) and 10 mM dithiothreitol. The cell pellet was sonicated for 30 seconds and boiled for 5 minutes. For total protein collection for BCL-2 and ZO-1, cells were solubilized in a 10 mM Tris-HCl buffer (pH 7.4) containing 1% SDS and 1.0 mM sodium orthovanadate. Cells were then sonicated for 30 seconds and boiled. Supernatant was collected after centrifugation. For total protein collection for p16, cells were solubilized in a 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$, 1 mM EDTA, 1% NP-40, 0.25 mM sodium deoxycholate, 10% glycerol, 150 mM NaCl, and 5 mM β-mercaptoethanol and mixed vigorously. The amount of protein was determined by a protein assay modified for use with thiols (Bio-Rad Laboratories, Hercules, CA).

SDS-PAGE and Western Blotting

Proteins were electrophoresed on 15% (for BCL-2), 6% (for ZO-1), 10% (for K3/K12), and 12% (for p16) polyacrylamide gels. SDS-PAGE gels were either stained with total protein stain (SYPRO Orange; Molecular Probes) or transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween-20 (TPBS). The membranes were then incubated with each primary antibody in blocking solution for 3 hours at room temperature. After they were washed with TPBS, membranes were incubated with horseradish-peroxidase–conjugated secondary antibody for 1 hour at room temperature. Protein bands were detected by enhanced chemiluminescence (ECL for ZO-1, or ECL Plus for BCL-2 Western blot detection reagents; Amersham Biosciences Corp., Piscataway, NJ). Jurkat, HeLa cervical carcinoma, and rabbit corneal epithelial cells were used as positive controls for BCL-2, p16, and K3, respectively. For comparison of telomerase activity and p16 protein levels in hTCEpi cells, parental controls and human donor tissue were used. Gels and membranes were imaged on a variable mode imager (Typhoon 9410; Amersham Biosciences Corp.).

Keratin Protein Sequencing

Insoluble protein (20 μg) extracted from 7-day air-lifted constructs were electrophoresed by 10% SDS-PAGE. Gels were stained with Coomassie blue and bands of interest were excised and digested. Proteins in each band were sequenced at the Protein Chemistry Core Facility at University of Texas Southwestern Medical Center at Dallas on a mass spectrometer (LCQ Deca XP; ThermoFinnigan, San Jose, CA) using nano-HPLC/electrospray mass spectrometry. Sequences were compared against National Center to Biotechnology Information (NCBI; Bethesda, MD) database using sonar database software (Genomic Solutions, Inc., Ann Arbor, MI).

Results

Cell Kinetics and Telomerase Activity

Measurements of cellular growth kinetics were obtained after 240 population doublings. By day 1, 24 hours after plating, the number of cells increased from $2.0 \times 10^3 \pm 0.2 \times 10^3$ cells/cm^2 to $2.2 \times 10^3 \pm 0.2 \times 10^3$ cells/cm^2, indicating a 10% increase in population growth (Fig. 1A). From 24 to 96 hours (day 4), cells underwent 3.5 population doublings ($2.2 \times 10^3 \pm 0.2 \times 10^3$ cells/cm^2 to $25.7 \times 10^3 \pm 4.0 \times 10^3$ cells/cm^2) and from 96 to 168 hours (day 7), cells underwent 2.5 population doublings (an increase from $25.7 \times 10^3 \pm 4.0 \times 10^3$ cells/cm^2 to $184.6 \times 10^3 \pm 8.3 \times 10^3$ cells/cm^2). The average population doubling time during the log growth phase was 24.4 hours, consistent with that previously established for normal human corneal epithelium.[5] By 168 hours, cells had reached 80% to 90% confluence and population growth began slowing. From 168 to 240 hours (day 10), cells reached confluence and underwent growth arrest, decreasing to only 3% cellular growth, demonstrating the retention of contact inhibition patterns characteristic of normal, epithelial cells.

To evaluate further cellular proliferation after the addition of calcium, hTCEpi cells were double labeled with Ki-67 and PI (Fig. 1B). At days 1, 4, and 7 in low-calcium culture conditions, 90% ± 2%, 85% ± 4%, and 84% ± 9% of cells were cycling, respectively. Even at 16 days in low-calcium conditions, 66% ± 8% of the epithelial cells remained positive for Ki-67 staining, despite the significant reduction in the population-doubling rate, indicating that these cells were still capable of cell division and had not exited the cell cycle. After the addition of 1.15 mM calcium at day 12, cells ceased cycling and underwent differentiation, as indicated by the decrease in the number of cycling cells to 3.0% ± 2% of the total cells present.

Telomerase Activity and Cell-Cycle Inhibitor $p16^{INK4}$

Telomerase activity was assessed at different passage times which correlated with 40 to 264 population doublings (passages 10–63). At all time points examined, hTCEpi cells had a positive TRAP assay for telomerase activity (Fig. 2). Consistent with previously reported data, normal human corneal epithelium was TRAP negative. Furthermore, cells at population-doubling 264 showed a normal karyotype (Fig. 3).

Western blot analysis of total protein lysates extracted from hTCEpi cells in low-calcium, routine culture conditions demonstrated the presence of p16 in two independent cultures at passage 10, but there was no expression at the higher passages 33, 36, 63, and 66 (Fig. 4). In total protein lysates collected from both cultured parental epithelial cells and scraped normal human donor tissue, p16 levels were undetectable by Western blot. HeLa cervical carcinoma cells, known to express p16, were used as the positive control. Western blot analysis for actin showed protein loading for each lane.

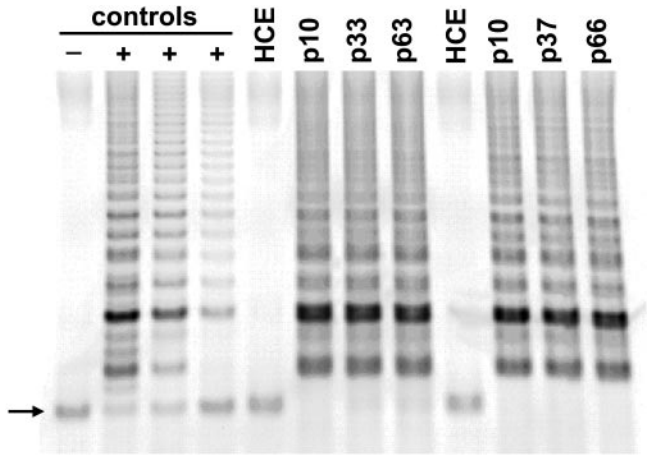

FIGURE 2. Telomerase activity. The TRAP assay was positive for telomerase activity at all passage time points for hTCEpi cells. There was no detectable telomerase activity in primary culture human corneal epithelial (HCE) cells. A 36-bp DNA ladder was used to assess telomerase activity. *Arrow*: the internal telomerase standard (ITAS) marker (internal control). Human mammary epithelial cells, HME50-5E (controls), known to express telomerase were used as a positive control. Decreasing concentrations of HME50-5E cells were loaded (*left* to *right*), demonstrated by a decrease in laddering intensity and an increase in ITAS. Lysis buffer is the negative control.

Stratification and Apical Junctions

Wholemount hTCEpi constructs were double-labeled with phalloidin-FITC and PI and evaluated at three time points (1 and 7 days submerged and 7 days air-lifted) and compared to normal human donor tissue (Figs. 5A–C). After 1 day submersed in high-calcium conditions (Fig. 5B), a confluent, a single cell layer covered the insert. After 7 days submersed in high-calcium conditions, cells appeared to multilayer into two to three cell layers (data not shown). By 7 days of air-lifted culturing (Fig. 5C), constructs had stratified into a multilayered epithelium with a morphologically distinct cuboidal basal layer, one to two polygonal wing cell layers, and one to two squamous cell layers with a visibly smooth, intact apical surface.

To confirm that the apical surface was intact, the expression and localization of the apical tight junction protein ZO-1 was evaluated. Wholemount constructs were stained en bloc using a monoclonal antibody to ZO-1 and double-labeled with PI to label cell nuclei (Figs. 5D–F). ZO-1 expression of the normal human cornea was localized to the cell boundaries in a pattern consistent with the formation of typical tight junctional complexes (Fig. 5D). After 7 days submersed in high-calcium conditions, hTCEpi constructs demonstrated the presence of a broad, indistinct ZO-1 staining pattern at the cell boundaries suggesting the formation of discontinuous junctions between surface cells (Fig. 5E). By 7 days of air-lifted culture, ZO-1 localized in a clear, linear pattern similar to the normal human cornea, suggesting the formation of continuous junctional complexes surrounding surface epithelial cells (Fig. 5F). Western blot analysis confirmed the specificity of the antibody and expression of ZO-1 in hTCEpi cells (data not shown).

Differentiation

IHC on cryostat cross-sections using an anti-keratin 3 AE5 monoclonal antibody and PI demonstrated the presence of the cornea specific keratin K3. Figure 6A demonstrates the pattern of K3 expression in the normal human cornea. Basal and suprabasal epithelial cells in normal adult human central corneal epithelium were stained by AE5 antibody. In hTCEpi cells, no K3 expression was detected after 7 days of submersed culture (Fig. 6B). At 7 days after air-lifted culture, K3 became detectable in isolated basal and suprabasal epithelial cells. Air-lifted constructs demonstrated a greater number of K3-positive cells at 10 days, suggesting that these cells are in the process of becoming terminally differentiated into corneal epithelial cells (Fig. 6C). Western blot analysis confirmed the expression of K3 in 7-day air-lifted cells, but consistent with IHC results, no expression was detected in cells in low-calcium culture or 7-day-submersed culture (Fig. 6D).

Progressive culture from plastic, to submersed, to air-lifted conditions also showed increasing expression of stratification-associated proteins K5 and K14. The largest increase was seen

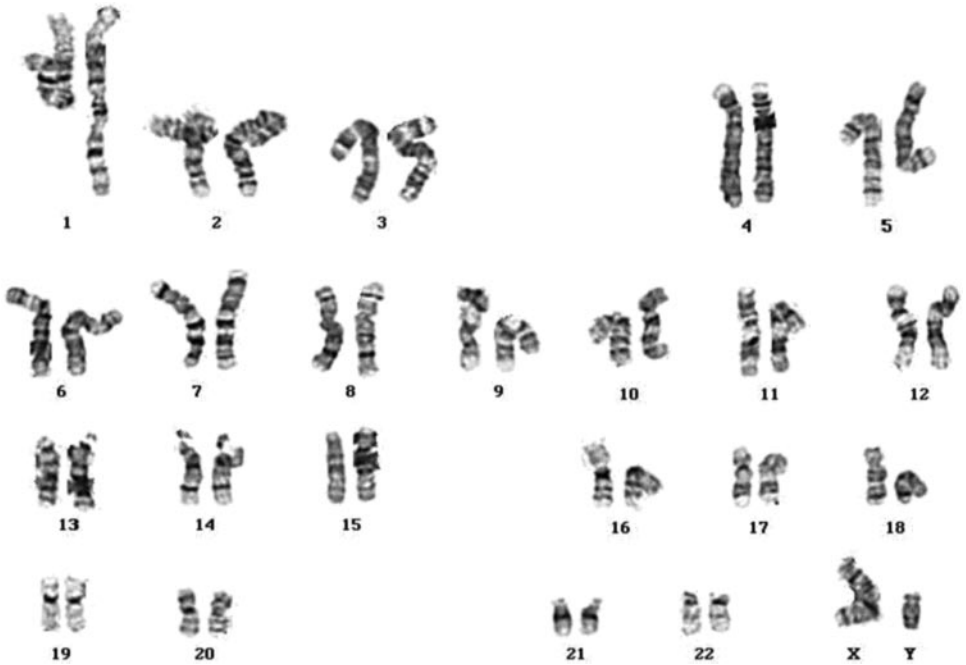

FIGURE 3. Karyotype analysis. In 21 cells examined at greater than 240 population doublings, karyotype analysis demonstrated 23 normal diploid chromosome pairs.

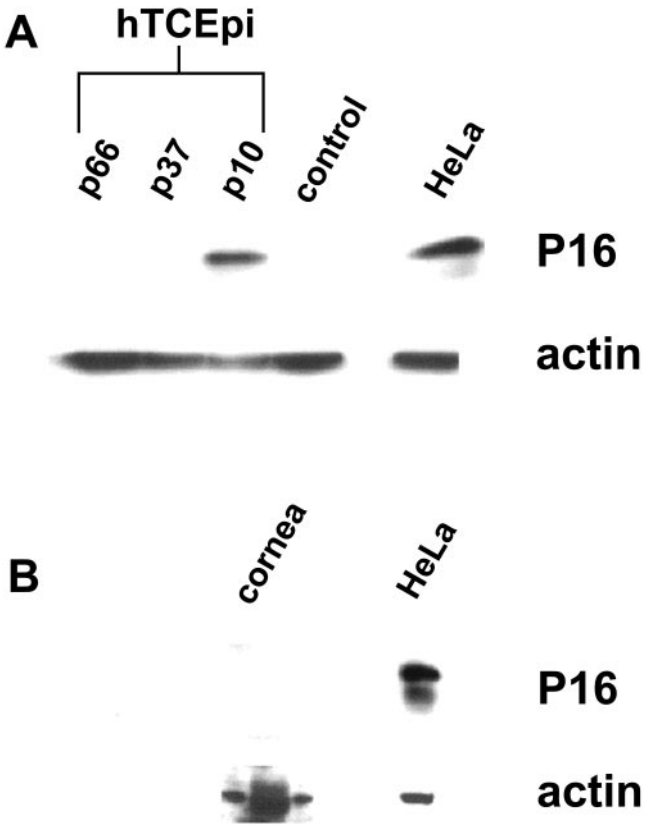

FIGURE 4. Cell cycle inhibitor $p16^{INK4}$. (**A**) Protein extracts were collected from low-, middle-, and high-passage time points in low-calcium culture conditions. p16 was present only at p10. p16 levels were undetectable in parental control p1 (control). HeLa cells were used as the positive control. Actin confirmed loading in each lane. (**B**) Cell lysates from scraped human donor tissue. Similar to primary culture epithelial cells, p16 was undetectable by Western blot analysis.

moving from plastic to submersed conditions, tapering off during air-lifting. The keratin marker for hyperproliferation (K16) remained low throughout extended culture (Figs. 6E, 6F).[19,20] Protein sequencing of the respective bands from a Coomassie blue 10% polyacrylamide gel confirmed the identity of K5, K14, and K16.

Surface Cell Shedding

Figure 7A demonstrates cytoplasmic BCL-2 expression in the normal human cornea by double-labeling with a rabbit polyclonal anti-BCL-2 antibody, which recognizes the epitope for cytoplasmic localization, and PI. A gradient of expression of cytoplasmic BCL-2 is seen with strongest expression in the basal layer and a decrease to loss of protein expression in wing and squamous cell layers. After 7 days submersed high calcium culture conditions, before the onset of epithelial differentiation, all hTCEpi cells were positive for cytoplasmic BCL-2 (Fig. 7B). Once differentiation in hTCEpi cells was induced after 10 days air-lifting (Fig. 7C), hTCEpi constructs showed a gradient pattern analogous to normal human cornea with the highest expression detected in basal epithelial cells.

Nuclear-localized BCL-2 was identified by staining with a mouse monoclonal antibody. Co-localization of PI (Fig. 7D, red) and nuclear BCL-2 (Fig. 7E, green) in 7-day air-lifted constructs detected nuclear BCL-2 expression throughout all cell layers in hTCEpi constructs (Fig. 7F). However, in the superficial layer, occasional BCL-2 negative cells were identified that were PI positive (arrow). In Figure 7G, nuclear BCL-2 expression (red) and TUNEL-labeling (7H, green) were compared. All BCL-2-positive cells were TUNEL negative and the only TUNEL-positive cell was BCL-2 negative (arrow, Fig. 7I). Previously published in vivo data in the human and rabbit report approximately 1% of the surface cells were TUNEL-positive. Consistent with these findings, occasional surface cells in the 7-day air-lifted constructs stained positive for TUNEL.[15] These findings suggest that the hTCEpi constructs undergo programmed surface cell desquamation in vitro similar to normal cornea. Western blot analysis with monoclonal antibody confirms the presence of the full-length, 26-kDa α splice variant of BCL-2 in the normal human and cultured hTCEpi corneal epithelium (Fig. 8). Also present in hTCEpi cells was a higher-molecular-mass protein at 38 kDa. This higher molecular weight band in hTCEpi cells has also been reported elsewhere in studies of normal human corneal epithelium.[18]

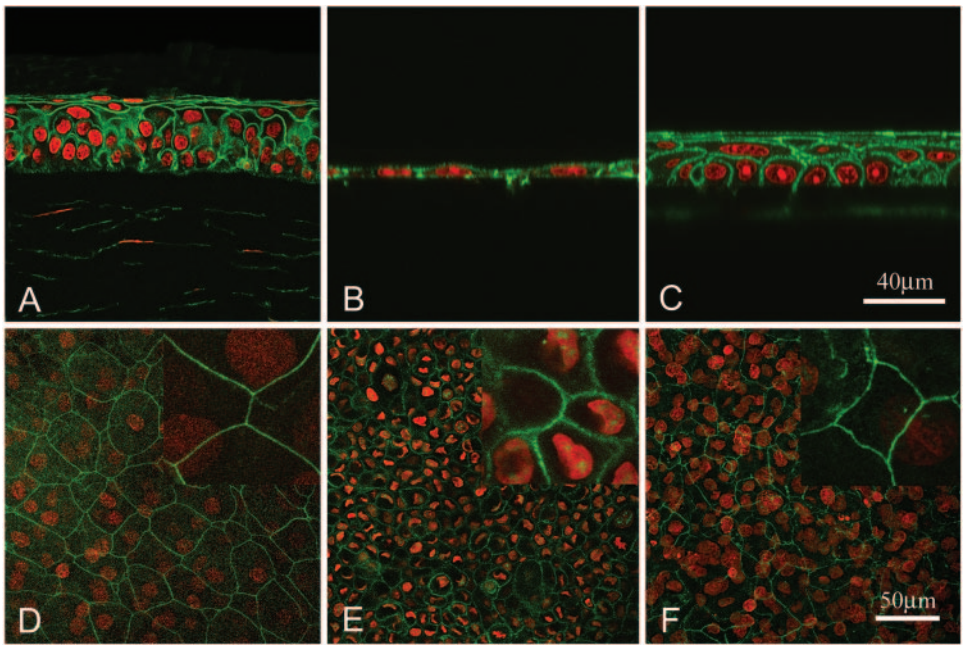

FIGURE 5. Stratification and tight junctions. (**A–C**) Double-labeling with FITC-phalloidin (*green*) and PI (*red*). (**A**) Cryostat section of normal human corneal epithelium in cross section. (**B**, **C**) Laser scanning confocal microscopy (LSCM) of cultured hTCEpi cells at 1 day submerged (**B**) and 7-day air-lifted (**C**) taken in the *x-z* plane. The 7-day air-lifted constructs differentiate into well-defined basal, wing, and squamous cell layers. (**D–F**) LSCM maximum intensity projection of normal human cornea (**D**), 7-day submerged (**E**), and 7-day air-lifted (**F**) constructs stained with anti-ZO-1 (*green*) and PI (*red*). Continuous junctions are seen in 7-day air-lifted constructs similar to those in the normal human cornea. *Inset*: 4× magnification of the original image.

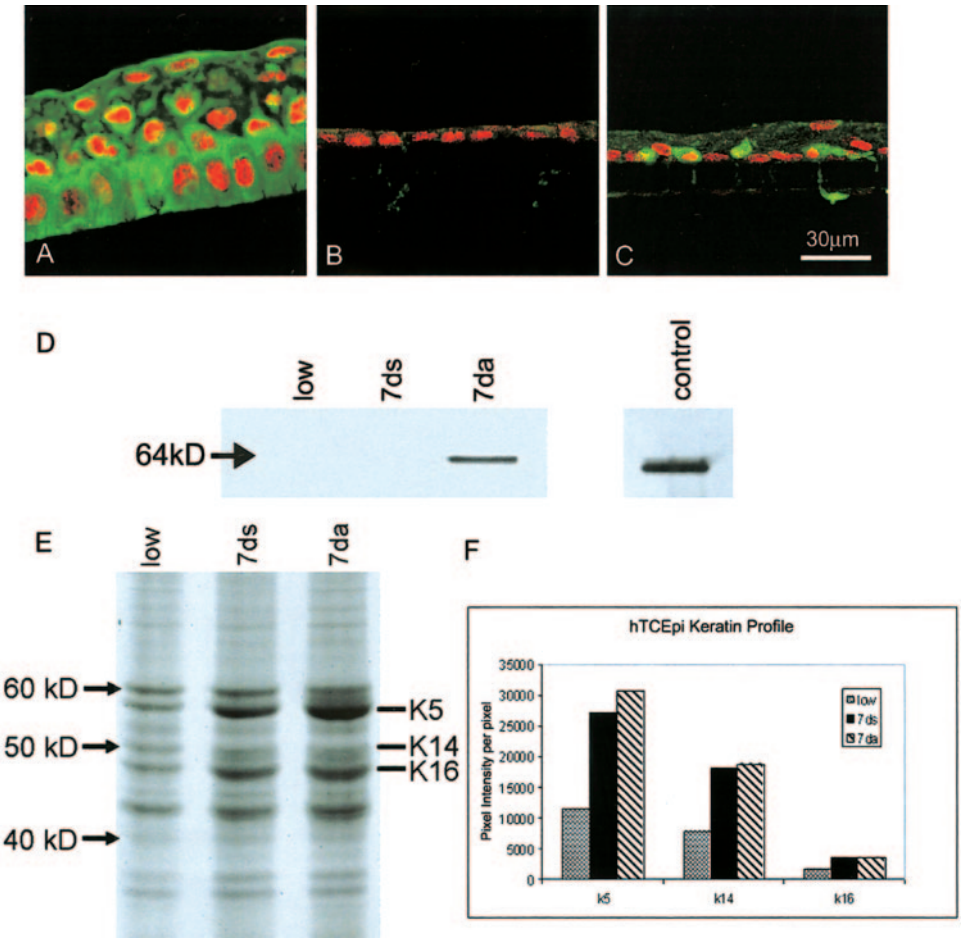

FIGURE 6. Differentiation (K3). (**A–C**) Cryostat sections double-labeled with anti-keratin K3 antibody (*green*) and PI (*red*). (**A**) Normal human corneal epithelium; (**B**) 7-day submerged construct grown in high-calcium medium; (**C**) 10-day air-lifted construct. Positive K3 expression is seen in 7- and 10-day air-lifted constructs. (**D**) Western blot for K3 confirmed the presence of the 64-kDa keratin in 7-day air-lifted culture. Rabbit corneal epithelium was used as the positive control. (**E**) Protein extracts from 3-day low calcium, 7-day submerged high calcium, and 7-day air-lifted cultures were electrophoresed through 10% SDS-PAGE and stained with a total protein dye. Air-lifted hTCEpi cells demonstrated an overall increase in the keratin markers for stratified squamous epithelial tissue (K5/K14). There was a low level of expression of the keratin characteristic of hyperproliferation (K6/K16). (**F**) Quantitative analysis of keratin markers for differentiation and proliferation. Data are representative of results in three repeated experiments.

DISCUSSION

The use of primary cultured corneal epithelial cells for studying signaling pathways and molecular interactions is hampered by a limited proliferative capacity, thus creating a need for an extended lifespan or "immortalized" cell line which retains the ability to proliferate indefinitely in culture. Previous cell lines developed for this purpose have had a combination of lack of differentiation potential, chromosomal abnormalities, and aberrant cell cycle kinetics.[1–5] This study reports the development of a telomerase-immortalized human corneal epithelial cell line by infection with hTERT alone which retains normal differentiation potential while exhibiting genetic stability. Specifically, the findings in this article suggest that the hTCEpi cell line maintains normal cell-cycle kinetics in the presence of high levels of telomerase activity; differentiates similar to human corneal epithelial cells in vivo; and under air-lifted conditions, exhibits normal patterns of surface cell desquamation.

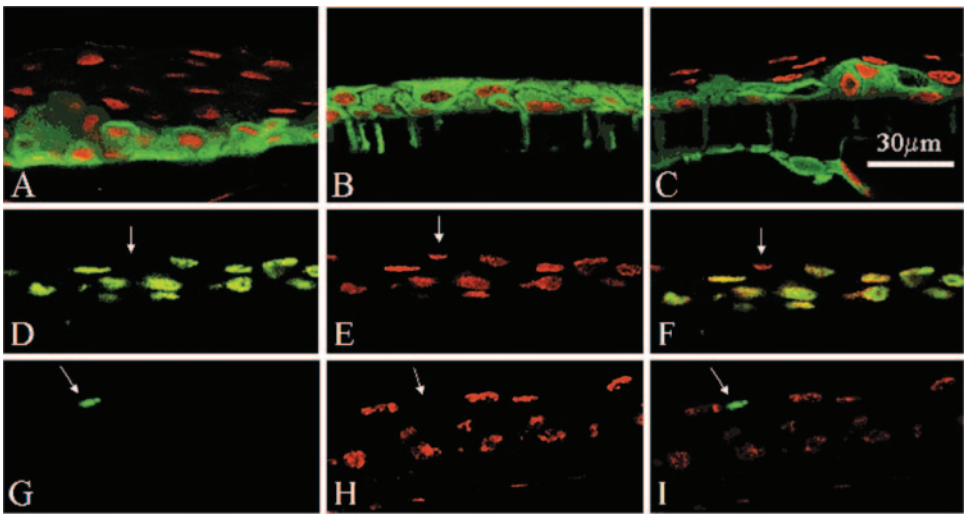

FIGURE 7. BCL-2. (**A–C**) Cryostat sections of normal human cornea (**A**), 7-day submersed (**B**) and 10-day air-lifted (**C**) hTCEpi construct stained with cytoplasmic-specific anti-BCL-2 antibody (*green*) and PI (*red*). Note that after air-lifting (**C**), a gradient of BCL-2 expression from basal to superficial cells was detected similar to the normal cornea (**A**). (**D–I**) Cryostat section of 7-day air-lifted hTCEpi construct stained with nuclear-specific anti-BCL-2 antibody (*green*) and PI (*red*). Nuclear BCL-2 expression was present throughout all cell layers, with an occasional loss of expression in superficial cells. *Arrows*: BCL-2 negative/PI-positive cell. (**G–I**) Cryostat sections of 7-day air-lifted hTCEpi construct stained with TUNEL (*green*) and BCL-2 (*red*). Note that the TUNEL-positive cell was negative for nuclear BCL-2 (*arrow*).

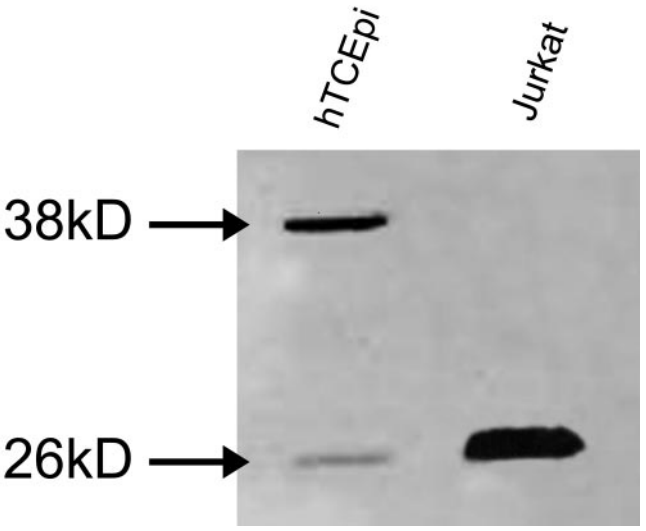

FIGURE 8. Western blot for BCL-2. Western blot analysis with a monoclonal antibody for BCL-2 demonstrated the presence of a 26-kDa band. Note the presence of a higher-molecular-mass band at 38 kDa. Jurkat cells were used as the positive control.

Cell Cycle Kinetics and hTERT

Consistent with previously established findings of telomerase activity in corneal cells, normal cultured human corneal epithelial cells did not exhibit any detectable telomerase activity.[21] The activation of telomerase seen at low, middle, and high passages suggests that hTCEpi cells have the requisite telomerase activity to circumvent crisis and proliferate indefinitely in culture. This regulated level of telomerase activity has been correlated with an increased proliferative and regenerative capacity in both stem cells and transient amplifying cells.[22,23]

Our proliferation data, collected at greater than 240 population doublings, shows that despite the addition of telomerase, hTCEpi cells in routine, low-calcium culture conditions undergo mitotic arrest on reaching confluence, suggestive of normal contact inhibition and a lack of neoplastic transformation. Furthermore, the addition of calcium to the culture medium, which mediates homotypic binding mechanisms necessary for the initiation of apical-basal polarity, initiates the signaling cascade for hTCEpi cells to exit the cell cycle and begin terminal differentiation.[24,25] Unlike the SV40 large T antigen or other oncogene-transformed immortalized cell lines that show chromosomal defects, karyotyping analysis of hTCEpi cells demonstrated 23 normal, diploid chromosome pairs.

It is interesting to note, however, that the hTCEpi cell line was developed from infection of hTERT alone. Previous reports of hTERT infection in human epithelial cells suggest that knockdown of $p16^{INK4}$ and/or p53 tumor suppressor pathways are required for cellular immortalization.[26–28] The abrogation of these cell-cycle pathways inhibits the stress induced mortality stage (M_1) thereby eradicating p16-mediated G1 growth arrest.[29] A previous study using immortalized hTERT infected human corneal epithelial cells bypassed p16 by using a dominant negative p53 and mutant Cdk 4; however, the authors reported that these cells failed to differentiate fully.[30] Alternatively, it has been reported that p16 induction can be bypassed under appropriate culture conditions.[10,31,32] To examine the role of p16 in hTCEpi immortalization, we assessed p16 expression in hTCEpi cells at early, middle, and late passages. The upregulation of p16 in early passages demonstrates that the p16 pathway is intact in hTCEpi cells. At higher passage numbers, levels of p16 become undetectable. This reduction in expression may be due to inactivation of p16 through methylation of the p16 promoter, mutation, or gene deletion as cells self-selected to emerge through the M_1 plateau phase.[33,34] Although further study is necessary to identify specifically the mechanism for p16 downregulation, the data suggest that p16 spontaneously downregulated in hTERT-immortalized cells and does not require direct abrogation.

Stratification and Differentiation

The mechanism(s) behind cellular stratification in "air-lifted" cultures are still undefined; however, it has been suggested that exposing cells to an air–liquid interface produces a more natural environment, leading to a stratified epithelial surface that is morphologically similar to that of the normal tissue.[35] IHC to evaluate cell stratification patterns, demonstrated by actin expression and localization of the apical tight junction protein ZO-1, appears to mimic those seen in the normal human cornea. Since apical corneal epithelial cells form the tight barrier responsible for protection from bacterial invasions, the smooth surface and tight junction pattern demonstrated in 7-day air-lifted constructs suggest that hTCEpi cells are exhibiting the in vivo ocular defense mechanism, and thus may represent a potential model for studying bacterial invasion at the ocular surface.

In addition to stratification, IHC revealed the presence of cytokeratin K3 in basal epithelial cells after extended air-lifted culture and was further confirmed by Western blot analysis. Moreover, analysis of keratin expression profiles showed very low levels of sequencing-confirmed keratin markers for hyperproliferation (K6/K16) and a robust increase in the keratin markers for stratification (K5/K14). This is consistent with early reports demonstrating an upregulation of K5/K14 during in vivo corneal epithelial regeneration in the rabbit, with correspondingly low amounts of K16 and K3/K12.[19] Our findings show that the increasing expression of K5/K14 tapers off in air-lifted culture, the time point at which K3 expression emerges. Although K3 is the accepted characteristic for corneal epithelial differentiation, further experiments are planned using antibodies to probe for K12, a more specific marker for corneal epithelial differentiation, in air-lifted hTCEpi cells.

Surface Epithelial Cell Desquamation

A striking feature of the air-lifted cell culture model is the expression and localization of the proto-oncogene BCL-2. Recent studies evaluating the effect of contact lens wear on epithelial homeostasis have led to the hypothesis that BCL-2 may act as a master regulator of corneal epithelial homeostasis by mediating the underlying mechanisms linking the final stages of cell shedding and apoptosis.[36] In the human corneal epithelium, cytoplasmic BCL-2 expression is strongest in the basal layer of the limbus where limbal stem cells are located.[37] This is supported by other studies reporting BCL-2 restriction to tissues such as stem cells, which are long-lived and display a high proliferative capacity.[38] Supporting this view, moving from limbus to the central cornea and from the basal to the superficial layer, cytoplasmic BCL-2 expression decreases in a gradient fashion.[18] This expression pattern of cytoplasmic localized BCL-2 thus coincides with epithelial cell terminal differentiation and cell death. In hTCEpi constructs, BCL-2 was expressed in all cells during routine culture and submersed, high-calcium conditions, but develops the characteristic "normal" gradient after air-lifting, when cells presumably begin to differentiate vertically.

Even more significant was the absence of nuclear BCL-2 with cells undergoing apoptotic cell death. Previously published results have reported the presence of nuclear localiza-

tion of BCL-2 within all corneal epithelial nuclei, which is lost before positive TUNEL-labeling.[18,36] Identical with that in the rabbit and human corneal epithelium, 7-day air-lifted hTCEpi cells double-labeled with a monoclonal BCL-2 antibody recognizing nuclear localization and TUNEL-labeling as a marker for apoptosis, clearly show a loss of nuclear expression of BCL-2 in superficial epithelial cells undergoing apoptotic cell death. This finding suggests that the pathway involving nuclear localization of BCL-2 as a potential upstream element mediating surface cell shedding within the corneal epithelium is conserved in hTCEpi cells and thus may provide a useful model for further study.

Of particular interest was the presence of a higher molecular weight form of BCL-2 detected on the Western blot. This 38-kDa band has been detected previously in the human corneal epithelium but not in our positive control or other cell lines.[18] This consistent finding suggests the possibility of a novel third splice variant or posttranslational modification, such as glycosylation for BCL-2 unique to the corneal epithelium. BCL-2 is a phosphoprotein whose function is regulated by phosphorylation at serine/threonine kinases within the flexible loop (amino acid [aa] 30-93).[39,40] Dynamic glycosylation/phosphorylation at serine/threonine sites has been demonstrated to regulate eukaryotic protein function and may play a role in mediating apoptosis at the ocular surface.[41,42]

Conclusion

In conclusion, our collective findings suggest that hTCEpi cells display normal cellular growth kinetics, express characteristic corneal phenotypic markers, and undergo cellular morphogenesis similar to their in vivo counterparts. In particular relevance to our studies, hTCEpi cells grown in organotypic culture retain cell renewal profiles which recapitulate the normal human corneal epithelium in vivo. We propose that further studies of these cells along with primary cell cultures and intact human donor tissue may help identify the underlying molecular mechanism(s) responsible for mediating corneal epithelial cell differentiation and desquamation.

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