Molecular Vision **2005; 11:816-24** <http://www.molvis.org/molvis/v11/a97/>
Received 25 January 2005 | Accepted 22 September 2005 | Published 26 September 2005
©2005 Molecular Vision

Evidence suggesting the existence of stem cells for the human corneal endothelium

David R. Whikehart,[1] Chirag H. Parikh,[2] Alexia V. Vaughn,[1] Kathy Mishler,[1] Henry F. Edelhauser[2]

[1]*Department of Vision Sciences, Vision Science Research Center, School of Optometry, The University of Alabama at Birmingham, Birmingham, AL;* [2]*Department of Ophthalmology, Emory University, Atlanta, GA*

Purpose: The well-known reluctance of human corneal endothelial cells (HCECs) to divide has continually intrigued investigators. Related to this, the discovery of an increased endothelial cell population in the periphery of the human cornea has prompted an investigation for evidence of the existence of stem-like cells in the endothelial periphery. Showing that stem cells or transient amplifying cells may exist in the periphery might explain the origin of HCECs and indicate a source for these cells in wound repair. In addition, these cells might be of value in culturing or as a source for the synthesis of artificial corneas.
Methods: Human corneas with attached scleral rims were obtained from eye banks and were assayed for telomerase activity and BrdU (bromodeoxyridine) incorporation to determine, respectively, the presence of a stem-like cell marker and replicative activity. In the case of telomerase activity, the tissues were divided into central, intermediate and peripheral areas by the use of trephines. BrdU staining (using alkaline phosphatase bound secondary antibody) was performed on whole corneas plus scleral rims exposed to BrdU antibodies on the endothelial side whereas BrdU fluorescence (using fluorescein bound secondary antibody) was obtained from transverse sections of the these tissues by the same procedure. Some corneas were wounded to determine whether the wounded areas stimulated BrdU (by staining or fluorescence) followed by the synthesis of transforming growth factor beta (TGFβ). The latter was determined by quantitative ELISA. Rabbit corneas were also assayed for BrdU incorporation to compare their evidence of cell division with that of humans.
Results: After dividing corneas into central, intermediate, and peripheral sections, the dissected endothelial tissues exhibited positive telomerase activity in the peripheral and intermediate sections. No activity was observed in the central endothelial tissues or the limbus between the trabecular meshwork and Schwalbe's line. BrdU staining with alkaline phosphatase was occasionally observed in the wounded area's human corneal endothelial cells after wounding. When BrdU fluorescence assays were made on corneal transverse sections with fluorescein, fluorescence occurred in an area just at and adjacent to the trabecular meshwork, but was not seen at the corneal endothelium. After wounding, BrdU fluorescence extended into the corneal endothelium. TGF-β levels were increased in fluids bathing the endothelium following wounding, but the increases lagged behind the wounding event.
Conclusions: It is suggested stem-like cells may be sequestered in a niche at the junctional region where the corneal endothelial cells and the trabecular meshwork come together. These putative stem cells may supply new cells for both the corneal endothelium and the trabeculae. Evidence suggests that cells from this area migrate (perhaps as transient amplifying cells) to the endothelial periphery and, perhaps, to wounded areas of the corneal endothelium when needed. The migration may not be constant and may be age dependent.

The impaired ability of human corneal endothelial cells (HCECs) to divide, both in vivo and in culture, has been well documented in the scientific literature for a number of years [1-6]. Attempts to describe and alter this phenomenon have been difficult due to an imperfect understanding of the cell biology involved. Joyce et al. [1] have suggested that transforming growth factor-β2 (TGF-β2) may keep HCECs in a non-replicative state in the absence of contact inhibition. Paull and Whikehart [7] have also shown that p53 and TAp63 (a p53 family member) are relatively elevated in the normal central human corneal endothelium suggesting that these proteins inhibit cell-cycle promotion from the G_1 to S phase. Wilson et al. [3,4] were able to impart proliferative ability in HCECs by transfection with simian virus 40 (SV40) large T antigen [3] and E6/E7 human papilloma virus type-16 oncogenes [4]. However, this cellular intervention alters the normal expression of hypophosphorylated retinoblastoma (RB) protein needed for the expression of genes involved in cell functions [3] and, therefore, results in an abnormal cell. None of the current data provide a satisfactory explanation of why these cells fail to divide in a manner comparable to most other somatic cells. Inhibition of cell division is also more pronounced with donor age [8]. Although this impaired division, by itself, is not a threat to the normal function of the cornea to remain clear (deturgescence), it becomes a significant problem under pathological states when HCECs are lost in large numbers, as in eye bank storage, after surgery, and in the development of artificial corneas for transplant [9].

Human corneal epithelial cells (HCEP), by comparison, readily divide from a source of cells outside the cornea.

Correspondence to: David R. Whikehart, PhD, Department of Vision Sciences, School of Optometry, The University of Alabama at Birmingham, 1716 University Boulevard, Birmingham, AL, 35294; Phone: (205) 934-6749; FAX: (205) 934-6758; email: DWhikehart@icare.opt.uab.edu

Davanger and Evenson [10] first proposed that cells located in the anterior limbus acted as a source of renewed epithelial cells. Subsequent studies, as reviewed by Daniels et al. [11] and Lavker and Sun [12] showed that some cells located in the anterior limbus are stem cells which partially differentiate into transient amplifying cells while migrating centrally into the posterior epithelial layer of the cornea. For example, labeling of these cells with stem-like cell associated markers (p63, nestin, and telomerase) have heavily implicated these cells as stem cells [13].

It is currently suggested that HCECs are held in the G_1 phase of the cell cycle and cannot pass readily into the S phase prior to cell division [14]. Why this occurs remains unknown. However, this report will show that HCECs may be able to be renewed from a supply of limbal, stem-like cells located adjacent to the endothelial periphery in the posterior limbus, possibly at Schwalbe's line. Such a depot or niche of endothelial stem-like cells would be analogous to the epithelial stem cells located in the anterior limbus that supply cells to the basal epithelium as transient amplifying cells, the cells that become committed to the state of terminal differentiation [11,15].

We considered focusing our studies on the peripheral corneal endothelium and endothelial limbus after reports by Schimmelpfenning [16] and Daus et al. [17] indicated a significant increase in endothelial cell density of the peripheral corneal endothelium. This has more recently been supported by quantitative data obtained by Amann et al. [18] in which a nearly 10% average increase in cell density was recorded in the peripheral regions of the human corneal endothelium, particularly in the superior region. It was reasoned that if some of the cells in the periphery could represent stem-like cells, then the use of a stem cell marker might indicate their presence. We were further encouraged to make this investigative approach since Bednarz et al. [19] demonstrated that some HCECs in the peripheral region stained positively with BrdU indicating the synthesis of new DNA prior to cell division. This report demonstrates positive telomerase activity [20] and selective BrdU staining and fluorescence in regions of the endothelial periphery following mechanical wounding.

METHODS

All research procedures involving human corneal tissues carried out in this project were approved by the Institutional Review Boards of The University of Alabama at Birmingham and Emory University. Animals that were used in this study were treated humanely according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the procedures used on them were approved by the Institutional Animal Care and Use Committee of Emory University.

Corneal tissues: Human corneas with attached scleral rims were obtained from the Alabama Eye Bank, Georgia Eye Bank, and the Southern Eye Bank. They were stored at 5 °C in Optisol (Optisol GS, Bausch & Lomb, Irving, CA) until dissected within 24 h of receipt. No tissues were used that were 10 days past enucleation and all tissues were free of disease. New Zealand White rabbit corneas were also used and treated as in vivo tissues.

Telomerase activity: Telomerase activity was assayed by a commercial version (TRAPeze; Chemicon International, Temecula, CA) of the method described by Kim et al. [20]. The endothelial samples were taken from corneal donors of varying ages and were divided into a central area (tissue taken from the interior of an 4 mm trephine cut), a central-intermediate area (tissue taken from the interior of a 8 mm trephine cut), an intermediate-peripheral area (tissue taken from the exterior of a 4 mm trephine cut and extended to the 12 mm border of the cornea), and a peripheral area (tissue taken from the exterior of an 8 mm trephine cut and extended to the 12 mm border of the cornea). Some samples were also taken from the posterior limbus. Endothelial and limbal tissues were removed by careful stripping with fine forceps to avoid going beyond the most posterior tissues. The limbal area readily separated from the scleral tissue. The dissection of the endothelial tissues is diagrammed in Figure 1.

After dissection, the tissues from each area were separately extracted by a lysis solution containing an RNase inhibitor. Protein contents of the samples were assayed by the BCA (bicinchoninic acid; Pierce Chemical Co., Rockford, IL) dye binding assay in order to normalize the samples for later gel application. In the assay, cell extracted telomerase enzyme synthesizes telomere repeats of GGTTAG (enzyme product) onto an artificial substrate oligonucleotide from substrate NTPs in the reaction medium. These repeats were amplified by PCR (polymerase chain reaction of 30 cycles at 90 °C for 30 s and 59 °C for 30 s). The products obtained were run on a PAGE (polyacrylamide gel electrophoresis) gel at 400 V for approximately 1.5 h. Then they were stained with SYBR(r) Green (Molecular Probes, Invitrogen, Carlsbad, CA) and examined for the appearance of laddered telomere products. The following controls were routinely run with each assay: sample blank (buffer blank); primer-dimer/PCR contamination control (tests for positive PCR artifacts or carryover contamination from other samples); TSR8 or quantitation control (a control of oligonucleotide plus 8 telomeric repeats used as a reference without tissue sample); and a positive cell extract control (an extract of telomerase positive HeLa cells). Thirty kb nucleotide bands were also included with all samples as internal standards.

Alkaline phosphatase-catalyzed, bromodeoxyuridine (BrdU) labeling (AP-BrdU assays): Human corneas (35 pairs), stored in Optisol, were stained for BrdU incorporation into new DNA using a commercial procedure [21]. The human corneas had a 10 mM BrdU solution of Optisol placed onto the endothelial side of dissected corneal-scleral buttons prior to assay. These corneas were kept as organ cultures for 7 days following gentle wounding with an olive tip cannula. Tissues were stained for BrdU incorporation into new DNA by using a commercial procedure (Hoffman-La Roche, Nutley NJ) based on the antibody research of Ellwart and Dormer [21]. In brief, dissected corneas were slide mounted and fixed in ethanol at -20 °C for 20 min. The tissues were rinsed in a washing buffer before, between, and after each of the following operations. The fixed tissues were covered on the endothelial side with a 1:1000 dilution of anti-BrdU primary antibody. Next

they were covered on the endothelial side with a 1:10 dilution of anti-mouse-Ig secondary antibody coupled to alkaline phosphatase. In the last stage they were exposed to an alkaline phosphatase substrate (nitroblue tetrazolium phosphate) and examined by light microscopy. Cell division was also stimulated with epidermal growth factor (EGF) at 10 ng/ml in a total added volume of 20 ml.

Rabbit corneas were assayed due to their greater proclivity toward endothelial cell division than human cells [14]. They served, therefore, as a comparison to human corneal endothelial cell division. After light anesthetization with ketamine, xylazine, and topical 0.5% proparicane, the endothelial surface was wounded in situ by introduction of a transcleral needle to the posterior cornea. The rabbits had 100-200 μl of aqueous aspirated and then 100-200 μl of a 10 mM BrdU solution injected into the anterior chamber. This aspiration and injection occurred four times in a one week period. After euthanasia, corneal buttons with scleral rims were dissected and assayed. Tissues were stained for BrdU incorporation into new DNA as previously described for the human corneas. Rabbit corneas were also treated with epidermal growth factor, (EGF), to further stimulate cell division, at a concentration of 10 ng/ml (added in a total volume of 20 ml).

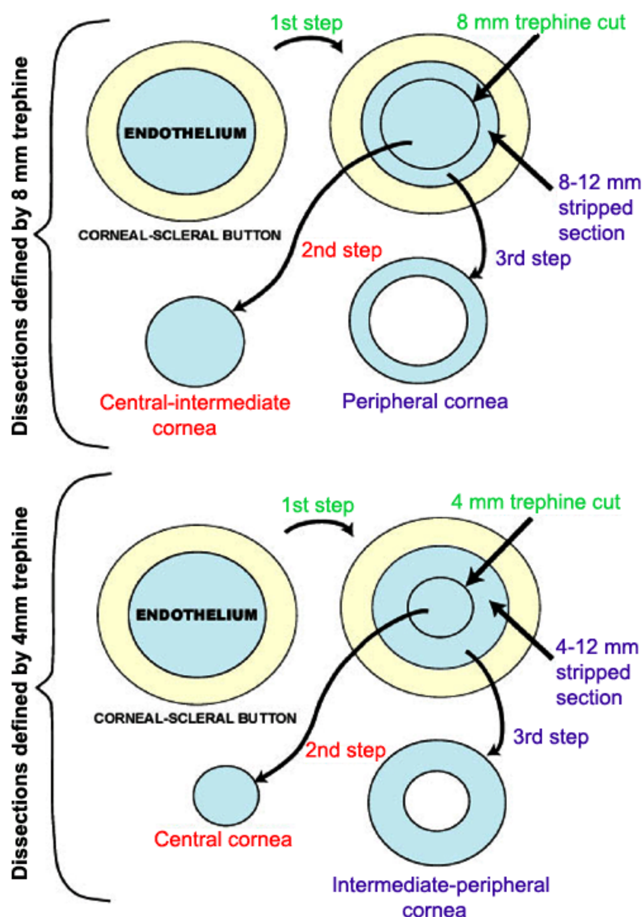

Figure 1. Flow diagram of method for dissecting corneal buttons for telomerase assays. Two types of dissections were performed: those divided by an 8 mm trephine generating central-intermediate (0-8 mm from center) and peripheral (8-12 mm from center) endothelial tissues and those divided by a 4 mm trephine generating central (0-4 mm from center) and intermediate-peripheral tissues (4-12 mm from center).

Fluorescein, bromodeoxyuridine (BrdU) labeling (F-BrdU assays): Three human cornea pairs (donor ages 30-37 years), stored in Optisol, were labeled by chemifluorescence to show incorporation of BrdU into new DNA using a commercial procedure (Hoffman-La Roche, Nutley NJ) as based on the work of Elwart and Dormer [21]. However, in this case the secondary antibody was linked to fluorescein to avoid the possibility that cellular alkaline phosphatase itself may have contributed to the final results. The BrdU reagent (10 mM) was diluted 1:10 in Optisol and 120 μl was placed on a corneal-scleral button on the endothelial side only. A fellow cornea from the same donor was exposed to Optisol without BrdU to serve as a control and both were incubated in a moist chamber at 37 °C for 48 h. Afterwards the tissues were fixed in ethanol and transverse sectioned from the sclera to mid-cornea. The tissues were slide mounted. All sections were thoroughly washed in buffer before, between, and after each of the following operations. First, the tissues were exposed for 30 min to a 1:1000 dilution of primary antibody (anti-BrdU). Next, they were exposed for 30 min to a 1:10 dilution of secondary antibody, as previously described, but coupled to fluorescein. The sections were examined under fluorescence microscopy (Leitz Aristoplan, Wetzler, Germany). Some tissues were gently, mechanically wounded in an x-shape with a dull instrument handle for 48 h prior to the assay. In the cases of wounding, fellow unwounded corneas (exposed to BrdU), served as controls.

TGF-β assays: Both rabbit and human corneas were tested for the presence of TGF-β to determine when a cytokine for the inhibition of cell division might appear following wounding. Organ cultures of human corneas were assayed over 22 days at 37 °C and 4 °C (separately) after incubation in Optisol while rabbit corneas were assayed for 4 days at 37 °C in Optisol. Each assay was performed 1-3 times as a sandwich ELISA assay with reagents based on those of R&D Systems, Inc. (Minneapolis, MN) specific for TGF-β. For each assay, 200 μl of sample (taken from the Optisol solution bathing the human and rabbit corneal endothelial tissues) was added to microplates coated with TGF-β receptor. Following incubation and washing, 200 μl of horseradish peroxidase-linked antibody was added to the plates, incubated and washed again. Tetramethylbenzadine in hydrogen peroxide was used to form a blue product that was read at 450 nm after stabilization in sulfuric acid.

RESULTS

Telomerase activity: Telomerase activity is a characteristic marker for transient amplifying cells [22]. Figure 2 shows sets of results for four areas of human corneal endothelial tissue; central endothelium (defined by endothelial cells within a 4 mm trephined boundary), central-intermediate endothelium (defined by endothelial cells within an 8 mm trephined boundary), intermediate-peripheral endothelium (defined by endothelial cells between the 4 mm trephine edge and 12 mm from

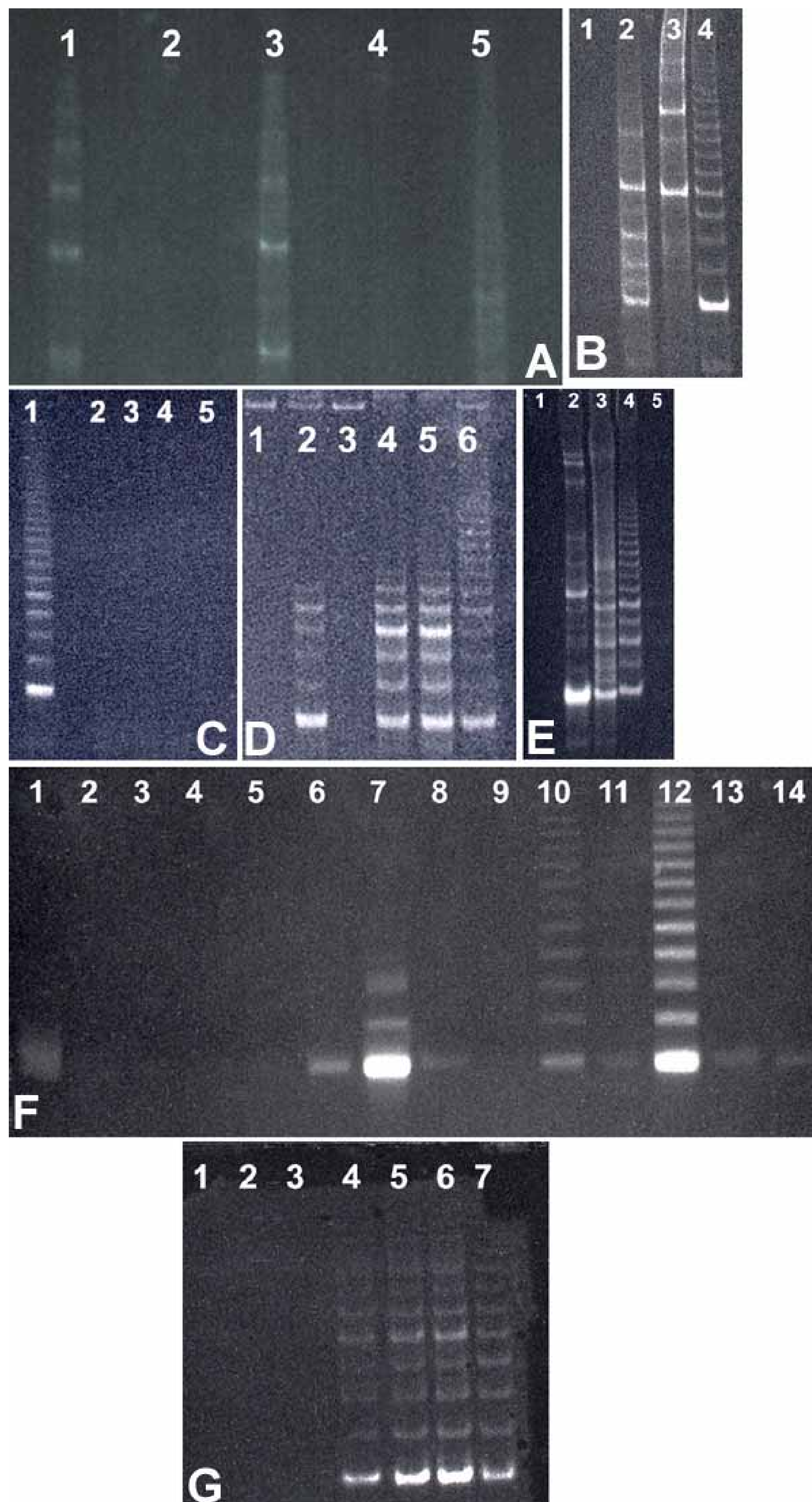

Figure 2. Telomerase activity in central, intermediate, and peripheral regions of human corneal endothelium. The figure demonstrates both the existence of telomerase activity in the peripheral endothelium (but not the central endothelium) and the elimination of contamination by Taq polymerase inhibitors in some tissue samples. The discovery of Taq polymerase inhibitors was initially a source of uncertainty in interpreting the results until its effects were eliminated by sample dilution. **A**: The initial demonstration of peripheral telomerase activity. Lane 1: HeLa cell control. Lane 2: Central-intermediate HCEC (16-year-old donor). Lane 3: Peripheral HCEC (16-year-old donor). Lane 4: Central-intermediate HCEC (66-year-old donor). Lane 5: Peripheral HCEC (66-year-old donor). **B**: This also demonstrates peripheral telomerase activity. Lane 1: Central-intermediate HCEC (55-year-old donor). Lane 2: Peripheral HCEC (55-year-old donor). Lane 3: TSR8 control. Lane 4: HeLa cell control. **C**: This panel, however, shows a complete lack of telomerase activity in peripheral endothelium as first seen in this study. Lane 1: HeLa cell control. Lane 2: Central-intermediate HCEC (26-year-old donor). Lane 3: Peripheral HCEC (26-year-old donor). Lane 4: Central-intermediate HCEC (55-year-old donor). Lane 5: Peripheral HCEC (55-year-old donor). **D**: Demonstrates the existence of a Taq polymerase inhibitor by mixing HeLa cells with HCEC cells. Lane 1: Peripheral HCEC alone (55-year-old donor from **C**). Lane 2: Peripheral HCEC mixed with HeLa cells. Lane 3: Buffer blank. Lanes 4,5: TSR8 controls. Lane 6: HeLa cells alone. **E**: Shows the existence of telomerase activity following dilution of a peripheral HCEC. Lane 1: Undiluted peripheral HCEC (23-year-old donor). Lane 2: Peripheral HCEC diluted 1:5 (same sample). Lane 3: Same diluted 1:25. Lane 4: HeLa cell control. Lane 5: Buffer blank. **F**: Demonstrates that extensive serial dilutions of central-intermediate and peripheral area HCECs only show telomerase activity in the endothelial peripheral area as taken from mixed aged donors (21-52 years). Lanes 1-5: Central-intermediate HCEC diluted 1:100, 1:400, 1:500, 1:600, and 1:800, respectively. Lanes 6-9: Peripheral HCEC diluted 1:75, 1:100, 1:200, and 1:400, respectively. Lanes 10-12: HeLa cells diluted 1:20, 1:40, and 1:1, respectively. Lanes 13,14: Buffer blanks. **G**: This panel gives no indication of central area (4 mm) telomerase activity following dilution of a 30-year-old donor. Lane 1: Buffer blank. Lane 2,3: Central HCEC undiluted and diluted 1:2, respectively. Lanes 4-6: Intermediate-peripheral HCEC undiluted, diluted 1:2, and diluted 1:10, respectively. Lane 7: HeLa cell control.

the center of the corneal endothelium), and the peripheral endothelium (defined by the 8 mm trephined edge and 12 mm from the center of the corneal endothelium). These areas have been schematically diagrammed in Figure 1.

Figure 2A shows initial results obtained from 16- and 66-year-old donors in the central-intermediate and peripheral regions. Lanes 3 and 5 show telomerase activity in the peripheral regions of the HCEC from the 16- and 66-year-old donors, respectively. Lanes 2 and 4 show an absence of telomerase in the central-intermediate regions of the respective donors. Lane 1 is a positive control obtained from HeLa cells. Figure 2B also shows positive telomerase activity in the peripheral region of a 55-year-old donor (lane 2) and negative activity in the central intermediate region (lane 1). Lane 3 is an artificial positive control (TSR8) while lane 4 is the HeLa cell positive control.

Negative findings also occurred in some peripheral regions as shown in Figure 2C for 26- and 55-year-old donors. The figure shows that there was no telomerase activity detected for both central intermediate and peripheral regions of 26- and 55-year-old donors (lanes 2, 3, 4, and 5, respectively). Lane 1 represents the HeLa cells positive control. The reason for this discrepancy became apparent after testing for the presence of a Taq polymerase inhibitor in the tissues. When donor peripheral tissues were mixed with the tissues of a positive control (Hela cells) the HeLa cell telomerase activity decreased (Figure 2D). Lane 6 shows the activity of telomerase obtained with HeLa cells while lane 2 shows the decreased activity of those same HeLa cells when the same amount of HeLa cells were mixed with the extracted tissue from the 55-year-old donor of Figure 2C. Donor tissue alone (lane 1) is negative for telomerase. Lane 3 is a buffer blank while lanes 4 and 5 are TSR8 artificial controls.

From this point we routinely diluted the tissues to determine whether a Taq polymerase inhibitor was present. Figure 2E demonstrates this for the peripheral HCEC of a 23-year-old donor. Lane 1 shows a lack of apparent activity in the undiluted tissue. Lane 2 demonstrates activity in a tissue diluted 5X with buffer while more activity is seen for the 25X diluted tissue in lane 3. Lane 4 is that activity obtained with HeLa cells while lane 5 is the buffer blank. We endeavored to

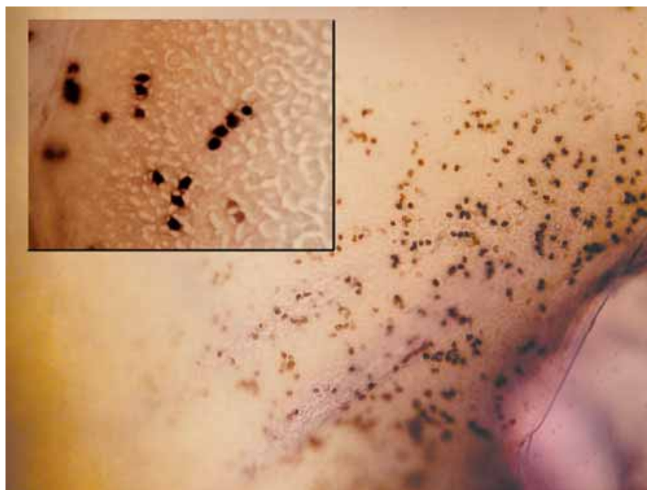

Figure 3. AP-BrdU staining of rabbit corneal endothelia at the point of wounding. BrdU staining at the periphery of a wounded rabbit corneal endothelium (the wound may be seen in the lower right corner of the large figure). The figure demonstrates that wounding induces cell division by taking up bromodeoxyuridine into new DNA. The image magnification is 27x; the magnification of the inset is 110x.

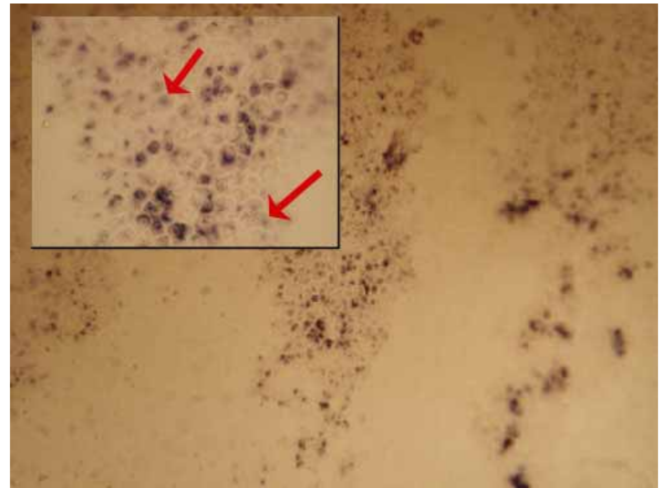

Figure 4. AP-BrdU staining of human corneal endothelia following mechanical wounding of the endothelial surface. BrdU staining was found in the vicinity of contact mechanical wounding of the endothelial surface. This demonstrates that such wounding may induce cell division in which some cells take up bromodeoxyridine into new DNA. The image magnification is 27x; the magnification of the inset is 110x. Specific nuclear staining is indicated by red arrows.

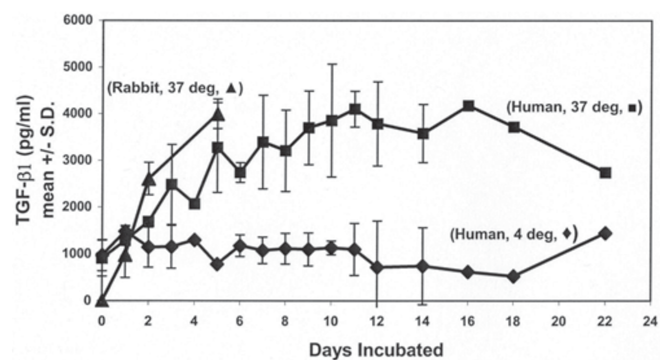

Figure 5. Levels of TGF-β1 in fluids bathing rabbit and human corneal endothelia following wounding. This figure demonstrates that levels of TGF-β, a possible agent in shutting down cell division, remain low for approximately two days following wounding in both rabbit and human CECs. This suggests that cell division in the endothelium, following wounding, is allowed to continue unchecked for at least two days. Plotted points represent either one assay or the mean of three assays. Where three assays are represented, the error bars show the standard deviations. The triangles represent rabbit data at 37 °C. The squares represent human data at 37 °C. The diamonds represent human data at 4 °C.

test both central-intermediate and peripheral activity in a donor sample of mixed ages (21-52 years) as shown in Figure 2F. The central-intermediate tissues indicated only a small amount of activity at a 1:100 dilution (lane 1) while the peripheral tissues showed a strong level of activity at the same dilution (lane 7). The usual HeLa cell control is shown in lane 12 and buffer blanks are indicated in lanes 13 and 14.

In order to be certain that no activity occurred centrally, we divided the HCEC tissues of a 30-year-old donor into central and intermediate-peripheral regions with a 4 mm trephine. The results are shown in Figure 2G. No activity was found centrally with undiluted and 1:2 diluted samples (lanes 2 and 3, respectively). Increasing bands of activity occurred in the intermediate-peripheral tissues with no dilution, a 1:2 dilution and a 1:10 dilution in lanes 4, 5, and 6, respectively. Lane 1 is a buffer blank while lane 7 represents activity from the HeLa cells. It was concluded, therefore, that there is no telomerase activity in central HCECs while some activity may be present in the peripheral region and decided activity is found in the peripheral region. This activity may be masked by a Taq polymerase inhibitor in some tissues.

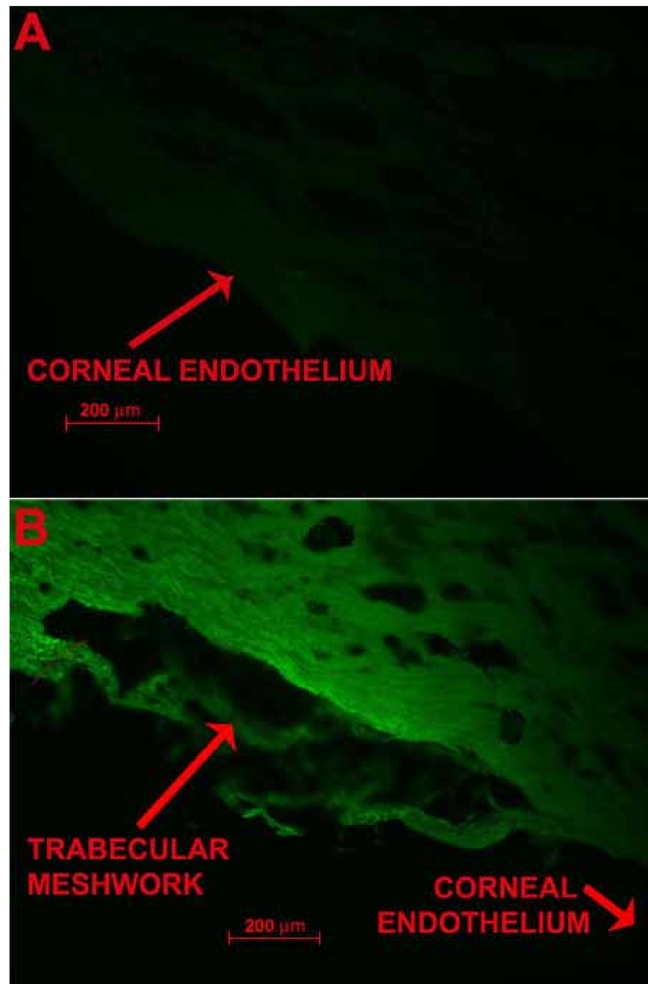

Figure 6. F-BrdU labeling of HCEC and trabecular meshwork in the absence of wounding. **A**: There is no cell division evident in this non-wounded human corneal endothelium (control). This sample is from a 37-year-old donor which was sequentially exposed to BrdU, primary, and secondary antibodies (the latter coupled to fluorescein). Only a minimal autofluorescent response occurred. The red arrow points to the location of the corneal endothelium. This figure should be compared to Figure 7C in which a wound had been made 48 h prior to fluorescent labeling of the endothelium. The comparison demonstrates how wounding gives evidence of cell division where cell division is normally not present. The BrdU labeling was continued all the way to the central endothelium. **B**: This image demonstrates the presence of cell division in the human trabecular meshwork under a non wounded (control) condition. The tissue fluorescence is evidence of cell division. Comparing this with Figure 7A suggests that cell division in the trabecular meshwork is substantially increased following wounding.

Assays were also run on extracted posterior limbal tissue strips taken beyond the 12 mm corneal border. Those assays were negative for telomerase activity (data not shown).

Alkaline phosphatase-catalyzed BrdU labeling (AP-BrdU assays): In order to determine whether cell division occurred in either the periphery of the corneal endothelium following mechanical wounding or at the site of the wound itself, corneas were tested with the AP-BrdU assay. Flat mounts of the corneas were made, treated with BrdU, primary antibody, and then stained by reacting with alkaline phosphatase-linked secondary antibody.

AP-BrdU staining occurred in the periphery of the rabbit endothelium adjacent to the location where the needle entered the anterior chamber (wound site) as shown in Figure 3. The inset of Figure 3 shows that the labeling occurred in the cell nuclei as would be expected for BrdU staining during cell division. Unwounded rabbit corneas were not tested.

AP-BrdU staining was not seen at the periphery of human corneal endothelia either with or without wounding in the absence of EGF (data not shown). When wounds were made in an X pattern (extending from the peripheral to the central cornea) in the human cornea, intracellular staining occurred in the area of the wounded tissue (Figure 4). It is noted that staining in the human cornea only occurred in 10% of the wounds (7 corneas out of 70 corneas tested) while all of the wounded rabbit corneas were stained as shown in Figure 3. When human corneal endothelial cells were stimulated with EGF following wounding, staining also appeared in the periphery (not shown).

Levels of TGFβ with wounding: A proposed mechanism for shutting down cell division, following a challenge such as wounding, may be due to the appearance of transforming growth factor β (TGFβ) [1]. It would be useful, therefore, to follow the time course of TGFβ levels subsequent to the establishment of a mechanical wound. Consequently, levels of TGFβ were measured from the Optisol media used for the rabbit and human corneas in organ culture following wounding over time (Figure 5). It can be observed that the human levels of TGFβ (at 37 °C) increased for an eleven day period following wounding. It is noted that at 7 days (the time at which BrdU staining was performed) the amount of TGFβ was at approximately 80% of the maximal level. In the human, the levels of TGFβ were at elevated levels for over two weeks. Rabbit corneal levels of TGFβ at 37 °C were initially at zero when placed in Optisol, but increased to levels compa-

rable to that of the human after a single day and continued to rise for seven days. When human corneas were kept in Optisol at 4 °C following wounding, however, the level of TGFβ did not increase significantly over 22 days indicating that a metabolic component to the generation of higher levels of TGFβ was evident.

Fluorescein, bromodeoxyuridine (BrdU) labeling (F-BrdU assays): Since alkaline phosphatase, coupled to the secondary antibody for the BrdU primary antibody, may have complicated the interpretation of the wounding experiments, we also treated human corneal-limbal tissues with BrdU histochemical assays in which fluorescein, rather than alkaline phosphatase, was coupled to the secondary antibody. Without wounding, minimal autofluorescence was observed in the corneal endothelium (Figure 6A). By way of contrast, considerable fluorescence was observed in the area of the trabecular meshwork and the posterior limbus (Figure 6B). This fluorescence was area, but not nuclei, specific.

Forty-eight hours after wounding, the endothelial layer of cells (from the periphery all the way to the central area) showed considerable fluorescence due to BrdU incorporation. For the endothelium, this is apparent in Figure 7C compared to Figure 6A. When the posterior limbus and trabecular meshwork were examined (Figure 7A), the amount of fluorescence observed was qualitatively increased over that in which no wounding occurred (Figure 6B). The fluorescence extended, without interruption, from the limbus to the corneal endothelium (Figure 7B).

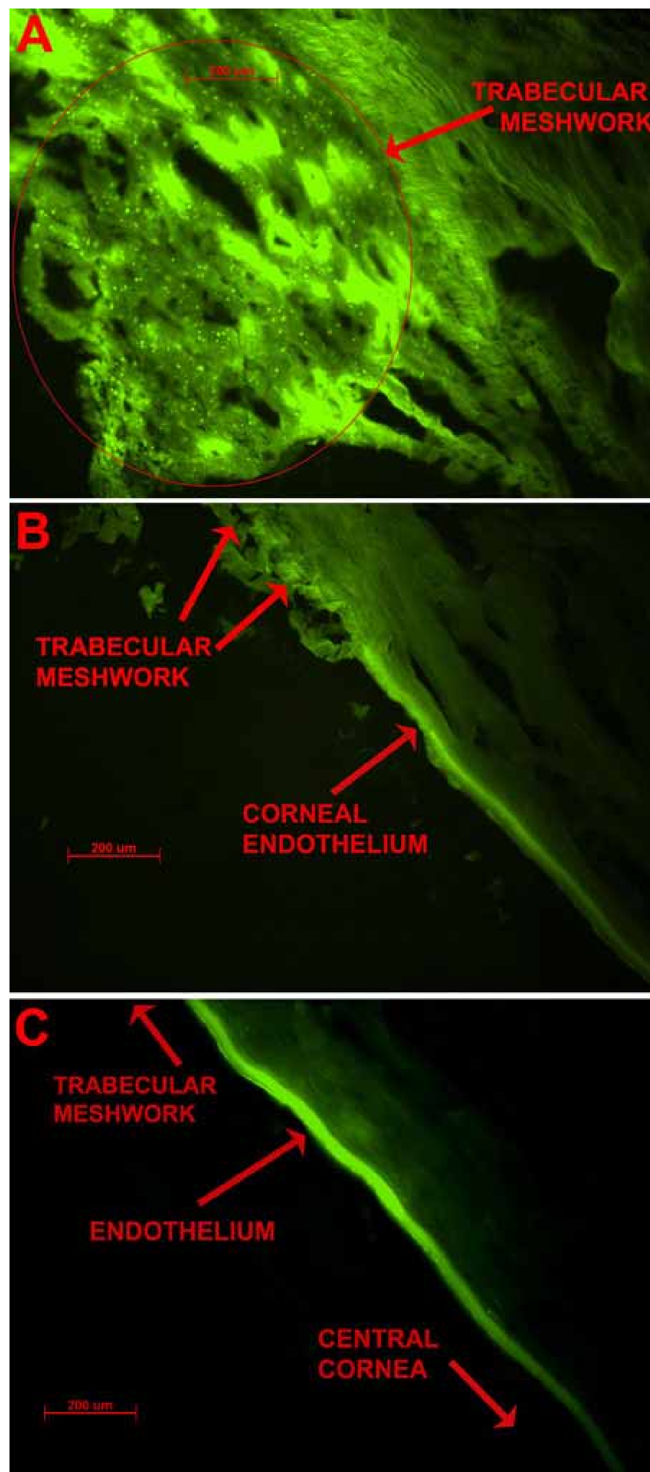

Figure 7. F-BrdU labeling of HCEC, trabecular meshwork, the corneolimbal junction (Schwalbe's line), and the peripheral corneal endothelium after a contact mechanical wound. Human corneal endothelium, trabecular meshwork, the corneolimbal junction (Schwalbe's line), and the peripheral corneal endothelium are shown 48 h after mechanical wounding. **A**: This image shows the general area of the trabecular meshwork following a contact mechanical wound to the endothelium. Although some cell division was shown here in the unwounded state (Figure 6B), this demonstrates that wounding is accompanied by a comparatively substantial increase in cell division (seen as increased green fluorescence). **B**: This image indicates that there is significant cell division in the trabecular meshwork, the peripheral corneal endothelium (all indicated by red arrows), and the area between the two anatomical regions (essentially Schwalbe's line). **C**: The image clearly shows fluorescent labeling in the area of the corneal endothelium (as indicated by the arrow) and suggests that cells in this area are involved in active division by taking up bromodeoxyuridine into new DNA. This panel should be compared with Figure 6B in which essentially no BrdU fluorescence in the control (unwounded) state can be seen. The wound was made in the fellow cornea to that of Figure 6.

DISCUSSION

Telomerase activity has been considered to be a marker for stem cells [23], but it is not found in all stem cell tissues [24]. Other investigations suggest that telomerase activity that is associated with adult stem cells may reside preferentially in transient amplifying cells, the stem cell daughters that are on the way to commitment and that, in fact, the telomerase in quiescent stem cells is repressed [22,25]. In this report, no telomerase activity in the limbal region from Schwalbe's line to the trabecular meshwork was detected. However, we have found a fluorescent marker for nestin, a neural crest stem cell marker, in the posterior limbal region of the human anterior segment in the vicinity of both the trabecular meshwork and Schwalbe's line (unpublished). Telomerase activity, then, seems to be associated with germinative cells in transition (transient amplifying cells) and cancer cells. In this study, the reaction for telomerase in the peripheral corneal endothelium was manifest (Figure 2). It may even have been present in the in-

termediate region between 4 and 8 mm from the central cornea. There was no activity manifest in the central region. Some confusing initial results that demonstrated no activity in some peripheral tissues were shown to be due to the presence of a Taq polymerase inhibitor. This would have prevented the amplification of the telomerase signal by PCR even though the activity was present. Taq polymerase inhibitors are widespread and consist of a variety of substances. The dilution procedure to detect its presence in this study is one of a variety of ways used to reveal its manifestation [26].

Other data obtained in this report (BrdU nitroblue tetrazolium staining by alkaline phosphatase and fluorescence due to fluorescein) would further suggest that putative stem cells are located in the posterior limbus between the peripheral endothelium and the trabecular meshwork. If stem cells are present in this region, they would typically represent only a small percentage of the cells in this area [27]. This would contrast sharply with the rather abundant supply of stem cells for the corneal epithelium that are known to occur in the anterior limbus [11].

The BrdU staining (AP-BrdU assay) procedure was carried out to demonstrate cellular division at the periphery of the corneal endothelium. This was initially thought to be from the presence stem cells and, in particular, as a manifest response of stem cells to wounding. In the case of rabbit corneal endothelial cells, which are known to divide with regularity, AP-BrdU staining was always seen following a mechanical wound, as shown in Figure 3. In the human corneal endothelium, staining only occurred at the wound site following wounding in 10% of the corneas tested. Such results in humans show a typical lack of propensity toward cell division even after wound stimulation. It is to be noted, however, that the method for this staining assay detects BrdU as alkaline phosphatase activity. Although this activity is indicative of dividing cells, it may also show the presence of native, cellular alkaline phosphatase activity. This activity is typical of both intracellular and cellular plasma membrane isoforms of this enzyme [28]. Interestingly, alkaline phosphatase is also indicative of stem cell presence [29]. We noted that the area beyond the endothelial periphery (the limbus) was always intensely stained whenever the AP-BrdU procedure was used. Essentially, therefore, the AP-BrdU results imply the presence of both stem cells and transient amplifying cells, but do not distinguish between them. It is also pointed out that donor age (beyond 45 years in the alkaline phosphatase-linked BrdU assays) may also have limited the human wounding response (cell division) in the corneal periphery.

BrdU fluorescence assays (F-BrdU assays) were undertaken to clarify the uncertainty of the alkaline phosphatase results since positive fluorescence could only be interpreted as coming from BrdU, that is, incorporation of new DNA into dividing cells. In the unwounded human tissue, no fluorescence in the corneal endothelium was an indication of a lack of cell division (Figure 6A). This was expected given the many reports of the tendency of a lack of cell division in unstimulated human tissue [14,30]. The area of the trabecular meshwork and its surrounding posterior limbus in the human, however, showed considerable fluorescence indicative of cell division (Figure 6B). Following wounding, increased fluorescence was seen to indicate cell division in that area (Figure 7A). There was also increased fluorescence in the area of the trabecular meshwork and posterior limbus of greater magnitude than in the unwounded condition (Figure 7B). Since this is a qualitative assay, it is not possible to determine the exact degree of cell division response. BrdU response to corneal wounding has been previously reported by Bednarz et al. [19], although that study was performed with an alkaline phosphatase based assay. The results of the current study, however, are encouraging and point more specifically toward a wound-induced cell division. It is also noted that the responses to the mechanical wounding in the F-BrdU assays were not “assisted” by the inclusion of EGF.

The more consistent indications of BrdU cellular division in the corneal endothelium with fluorescence compared to alkaline phosphatase with respect to age might be a factor as the average donor age with the fluorescence-linked assay was about 35 years compared to >45 years for the alkaline phosphatase-linked assay. Investigators have already pointed out that the limited cellular division in the corneal endothelium is facilitated by younger donors [2,4,8]. However, we also point out that the AP-BrdU assays may be more an indicator or the presence of stem cells while the F-BrdU assays may be more of an amplified signal for cell division given the previous discussion.

TGFβ has been reported to act as an inhibitor of the G_1 phase of cell division in the corneal endothelium by Joyce et al. [1]. In order to determine whether this cytokine might act in response to a wounding experiment, we measured TGFβ levels in Optisol GS bathed rabbit and human corneas. This was done following mechanical wounding. It was found that levels of TGFβ associated with human and rabbit tissues increased approximately 4 fold in 4 days following the wound and, in the case of the human, began to fall after 18 days (Figure 5). The increase in the human seems to be delayed for approximately two days. It would seem, therefore, that cellular division has at least one significant negative control component in both rabbit and human corneal endothelial cells whose activation may be delayed. This delay would be sufficient to allow reparative cell division to take place. The data also show that a basal level of TGFβ was always present in the human tissue, but absent in the rabbit until stimulated by wounding. This may indicate a continuous partial negative control of cell division in the human, but not the rabbit.

The data presented in this report (telomerase activity and BrdU-linked alkaline phosphatase activity staining) suggests that cells in the corneal endothelium may be renewed by stem-like cells located in a niche at the posterior limbus. By having such a mechanism, it is possible to minimize the loss of endothelial cells over a lifetime, due to naturally occurring cell death. However, the data further suggests that increased cell renewal may occur after trauma such as the mechanical damage shown in this report. Finally, there is evidence to show that increased cell renewal can be halted the by upregulated synthesis of TGFβ within a few days of a mechanical wound.

ACKNOWLEDGEMENTS

Presented in part at the annual meeting of the Association for Research in Vision and Ophthalmology, Fort Lauderdale, FL, May, 2002 and 2003. Supported by grants from the National Eye Institute: EY13994 (DRW) and EY00933 (HFE), an unrestricted grant from Research to Prevent Blindness (HFE) and The Eye Sight Foundation of Alabama (DRW). Dr. Parikh was supported by NEI Training Grant T32-EY07092 while Alexia V. Vaughn was supported by NEI Training Grant T35-EY07084.

REFERENCES

1. Joyce NC, Harris DL, Mello DM. Mechanisms of mitotic inhibition in corneal endothelium: contact inhibition and TGF-beta2. Invest Ophthalmol Vis Sci 2002; 43:2152-9.
2. Blake DA, Yu H, Young DL, Caldwell DR. Matrix stimulates the proliferation of human corneal endothelial cells in culture. Invest Ophthalmol Vis Sci 1997; 38:1119-29.
3. Wilson SE, Weng J, Blair S, He YG, Lloyd S. Expression of E6/E7 or SV40 large T antigen-coding oncogenes in human corneal endothelial cells indicates regulated high-proliferative capacity. Invest Ophthalmol Vis Sci 1995; 36:32-40.
4. Wilson SE, Lloyd SA, He YG, McCash CS. Extended life of human corneal endothelial cells transfected with the SV40 large T antigen. Invest Ophthalmol Vis Sci 1993; 34:2112-23.
5. Engelmann K, Bohnke M, Friedl P. Isolation and long-term cultivation of human corneal endothelial cells. Invest Ophthalmol Vis Sci 1988; 29:1656-62.
6. Baum JL, Niedra R, Davis C, Yue BY. Mass culture of human corneal endothelial cells. Arch Ophthalmol 1979; 97:1136-40.
7. Paull AC, Whikehart DR. Expression of the p53 family of proteins in central and peripheral human corneal endothelial cells. Mol Vis 2005; 11:328-34.
8. Zhu C, Joyce NC. Proliferative response of corneal endothelial cells from young and older donors. Invest Ophthalmol Vis Sci 2004; 45:1743-51.
9. Griffith M, Osborne R, Munger R, Xiong X, Doillon CJ, Laycock NL, Hakim M, Song Y, Watsky MA. Functional human corneal equivalents constructed from cell lines. Science 1999; 286:2169-72.
10. Davanger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. Nature 1971; 229:560-1.
11. Daniels JT, Dart JK, Tuft SJ, Khaw PT. Corneal stem cells in review. Wound Repair Regen 2001; 9:483-94.
12. Lavker RM, Sun TT. Epithelial stem cells: the eye provides a vision. Eye 2003; 17:937-42.
13. Seigel GM, Sun W, Salvi R, Campbell LM, Sullivan S, Reidy JJ. Human corneal stem cells display functional neuronal properties. Mol Vis 2003; 9:159-63.
14. Joyce NC, Navon SE, Roy S, Zieske JD. Expression of cell cycle-associated proteins in human and rabbit corneal endothelium in situ. Invest Ophthalmol Vis Sci 1996; 37:1566-75.
15. Wolosin JM, Wang Y. Alpha-2,3 sialylation differentiate the limbal and corneal epithelial cell phenotypes. Invest Ophthalmol Vis Sci 1995; 36:2277-86.
16. Schimmelpfennig BH. Direct and indirect determination of non-uniform cell density distribution in human corneal endothelium. Invest Ophthalmol Vis Sci 1984; 25:223-9.
17. Daus W, Volcker HE, Meysen H, Bundschuh W. [Vital staining of the corneal endothelium—increased possibilities of diagnosis]. Fortschr Ophthalmol 1989; 86:259-64.
18. Amann J, Holley GP, Lee SB, Edelhauser HF. Increased endothelial cell density in the paracentral and peripheral regions of the human cornea. Am J Ophthalmol 2003; 135:584-90.
19. Bednarz J, Rodokanaki-von Schrenck A, Engelmann K. Different characteristics of endothelial cells from central and peripheral human cornea in primary culture and after subculture. In Vitro Cell Dev Biol Anim 1998; 34:149-53.
20. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. Specific association of human telomerase activity with immortal cells and cancer. Science 1994; 266:2011-5.
21. Ellwart J, Dormer P. Effect of 5-fluoro-2'-deoxyuridine (FdUrd) on 5-bromo-2'-deoxyuridine (BrdUrd) incorporation into DNA measured with a monoclonal BrdUrd antibody and by the BrdUrd/Hoechst quenching effect. Cytometry 1985; 6:513-20.
22. Ulaner GA, Giudice LC. Developmental regulation of telomerase activity in human fetal tissues during gestation. Mol Hum Reprod 1997; 3:769-73.
23. Ouellette MM, McDaniel LD, Wright WE, Shay JW, Schultz RA. The establishment of telomerase-immortalized cell lines representing human chromosome instability syndromes. Hum Mol Genet 2000; 9:403-11.
24. Zimmermann S, Voss M, Kaiser S, Kapp U, Waller CF, Martens UM. Lack of telomerase activity in human mesenchymal stem cells. Leukemia 2003; 17:1146-9.
25. Yui J, Chiu CP, Lansdorp PM. Telomerase activity in candidate stem cells from fetal liver and adult bone marrow. Blood 1998; 91:3255-62.
26. Bourke MT, Scherczinger CA, Ladd C, Lee HC. NaOH treatment to neutralize inhibitors of Taq polymerase. J Forensic Sci 1999; 44:1046-50.
27. Szczypka MS, Humes HD. Kidney. In: Lanza R, Blau H, Melton D, Moore M, Thomas ED, Verfaillie C, Weissman I, West M, editors. Handbook of Stem Cells, Vol. 2, Adult and Fetal. New York: Elsevier Academic Press; 2004. p 475-482.
28. Moss DW. Perspectives in alkaline phosphatase research. Clin Chem 1992; 38:2486-92.
29. Park JH, Kim SJ, Lee JB, Song JM, Kim CG, Roh S 2nd, Yoon HS. Establishment of a human embryonic germ cell line and comparison with mouse and human embryonic stem cells. Mol Cells 2004; 17:309-15.
30. Joyce NC, Meklir B, Joyce SJ, Zieske JD. Cell cycle protein expression and proliferative status in human corneal cells. Invest Ophthalmol Vis Sci 1996; 37:645-55.

The print version of this article was created on 26 Sep 2005. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.