Acute Wound Healing in the Human Central Corneal Epithelium Appears to Be Independent of Limbal Stem Cell Influence

Chuan-Yuan Chang, Colin R. Green, Charles N. J. McGhee, and Trevor Sherwin

Purpose. In the adult cornea, epithelial cells are maintained by limbal stem cells (LSCs) that cycle slowly and give rise to transient amplifying (TA) cells. These migrate centripetally, differentiate outward to the surface, and are then lost by desquamation. This study was conducted to analyze the contribution of human central corneal epithelial cells toward corneal epithelial regeneration.

Methods. A human corneal organotypic culture model was used to assess corneal healing in vitro in 12 matched cornea pairs. Two types of ablation were studied: (1) A ring-shaped, transepithelial, excimer laser (193 nm) ablation, of 7 mm outer diameter and 3 mm inner diameter, to a depth of 80 μm—sparing the central and peripheral corneal epithelium; and (2) an ablation pattern identical to that in (1) with ablation of the limbal epithelium in addition. Corneal healing was followed using time-lapse dark-field microscopy for up to 12 hours, and the corneas were analyzed by using immunohistochemical markers for cell proliferation and stem cells.

Results. In the donut model, corneal epithelial repair originated from both the limbus and the central corneal epithelium with the average rate of epithelial recovery from the center being similar to the rate from the periphery (0.06 ± 0.01 mm/h vs. 0.07 ± 0.03 mm/h, $P = 0.44$). When the area of recovery was calculated relative to original edge circumferences, the central epithelial rate tended to be faster than the peripheral (0.06 ± 0.02 mm^2/mm/h vs. 0.04 ± 0.01 mm^2/mm/h, $P = 0.04$). Similar rates in epithelial recovery were identified in centripetal and centrifugal directions in both the donut and donut+limbus ablation models. Central epithelial cell density increased 36% over the control cornea within 12 hours after surgery, but there was no change at the periphery. Cell proliferation, assessed using Ki67 and BrdU labeling, was observed across the entire cornea. Expression of the putative stem cell markers p63 and ABCG2 was clearly evident in the basal layer of the limbus. However, weaker labeling was also observed in the central epithelium. Connexin 43 (Cx43), a differentiation marker, was mainly absent in the normal untreated limbal basal cells, but more Cx43-positive cells were labeled in the basal layer of the limbus after wounding.

Conclusions. After wounding, the capacity for epithelial cell proliferative and migration appears to be as active in the central cornea as in the periphery/limbus. Central and peripheral epithelial recovery remains equal even after ablation of the limbus. Central human corneal epithelial cells are therefore capable of corneal epithelial regeneration, at least in the first 12 hours after wounding. (*Invest Ophthalmol Vis Sci.* 2008;49:5279–5286) DOI:10.1167/iovs.07-1260

A natural turnover of human corneal epithelial cells takes place wherein superficial cells are shed from the corneal surface by constant desquamation (Z component) and replaced from a population of stem cells that reside in the basal limbal region and continue to cycle slowly throughout life. Their daughter cells, the transient amplifying (TA) cells, migrate centripetally (Y component) into the basal layer of the corneal epithelium and differentiate into the upper layers of the corneal epithelium (X component) to become postmitotic cells. This mechanism of corneal maintenance, the X, Y, Z hypothesis,[1] has been widely accepted and combined with the general belief that corneal epithelial stem cells principally reside in the basal layer of the highly specialized and protected limbal niche.[2,3] The radial structure of the limbus is also known as the palisades of Vogt. Limbal stem cells (LSCs) are a distinct subpopulation of cells that are undifferentiated or poorly differentiated, slow cycling, small, and highly proliferative.[4–7] In general, they serve as the reserve for cell proliferation, maintaining a balance between cell production and cell loss.[1]

The central corneal epithelium is generally believed to have no stem cell or stem cell–like epithelial cells and therefore to have little proliferation capacity compared with peripheral epithelial cells and LSCs.[8–10] Lehrer et al.[11] showed that TA cells of the peripheral cornea can divide multiple times during homeostasis, whereas those of central cornea can probably divide only once; however, the number of times both TA cells can replicate is increased in response to corneal wounding. Moreover, human limbal epithelium has been shown to grow better in culture, followed by peripheral epithelium and the most slowly growing, central corneal epithelium.[12,13] Studies have also shown that side population (SP) cells are present in the limbus, but not in the epithelium of the central cornea.[8–10] SP cells are isolated on the efflux of Hoechst 33342 dye, by using fluorescence-activated cell sorting (FACS; BD Biosciences, San Diego, CA) and the SP phenotype is shared by stem cells in various tissues and species, including hematopoietic stem cells, where the phenotype was first identified.[14] Immunohistochemistry studies[8–10,15–18] have shown that putative stem cell markers such as p63 and ABCG2 are expressed only in the limbus, but not in the rest of the corneal epithelium. P63 is a transcription factor belonging to the p53 family, unlike p53 which is a tumor-suppressor gene. p63 is normally expressed in the nuclei of keratinocytes with proliferative potential, including skin, cervix, prostate, and cornea.[19] ABCG2 is an ATP-binding cassette transporter that acts as a molecular determinant of SP phenotype cells in a wide range of organs.[20] Finally, proposed differentiation markers such as Cx43 and cytokeratin 3/12 (K3/12) are absent in the limbal basal layer, since stem cells are undifferentiated, leading to the suggestion that Cx43 is a negative limbal stem cell marker.[15]

From the Department of Ophthalmology, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand.

Supported in part by The New Zealand Lottery Board, The New Zealand Marsden Fund, and the Save Sight Society of New Zealand.

Submitted for publication September 27, 2007; revised March 2 and April 3, 2008; accepted September 18, 2008.

Disclosure: **C.-Y. Chang**, None; **C.R. Green**, None; **C.N.J. McGhee**, None; **T. Sherwin**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Trevor Sherwin, Department of Ophthalmology, University of Auckland, Private Bag 92019, Auckland, New Zealand; t.sherwin@auckland.ac.nz.

TABLE 1. Donor Details

Donors	Age	Sex	Postmortem Delay	Cause of Death
A	83	F	38 h	Multiorgan failure due to sepsis after laminectomy
B	81	M	40 h	Bronchopneumonia
C	87	F	14 h	Advanced old age
D	60	F	26–34.5 h	Cardiotoxicity completing chemotherapy for breast cancer
E	77	M	13 h	Leukemia
F	75	F	Unknown	Intracerebral hemorrhage
G	67	M	25–30 h	Unknown

Corneas were excised from the globe of these donors and then ablated ex vivo, followed by the time-lapse microscopy experiments.

The purpose of this study was to investigate the direction of wound healing after a paracentral corneal epithelial ring or "donut" ablation and to evaluate the regenerative capability of central epithelial cells with or without retention of the limbus. Although Thoft and Friend[1] considered the Y component to be an ongoing, slow, centripetal cell movement that occurs even in the absence of an acute epithelial defect, many ophthalmic researchers appear to confuse this hypothesis of homeostasis with wound-healing responses, assuming that LSCs and TA cells will be induced after acute wounding[11,21,22] and will migrate centripetally to repair the wound.[5,22,23] Our results demonstrate that the central corneal epithelium is capable of self-regeneration and repair and that LSCs may in fact not respond immediately to an acute wound. The later strongly suggests that there may be a threshold of injury for LSCs to become active.

METHODS

Materials and Reagents

Phosphate-buffered saline (PBS) was prepared from PBS tablets (OXOID PBS; Unipath Ltd., Basingstoke, Hampshire, UK). The following were used: mouse monoclonal antibody anti-p63 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-BCRP (also known as anti-ABCG2) antibody (BXP21) (Calbiochem, San Diego, CA), anti-connexin 43 (Sigma-Aldrich, St. Louis, MO), nuclear antigen Ki67 (Immunotech, Marseille, France), bromodeoxyuridine (BrdU) and anti- BrdU (Invitrogen-Molecular Probes, Eugene, OR), fluorescein Alexa-488 conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Invitrogen-Molecular Probes), fluorescein Cy3 conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), reduced-serum medium (Opti-MEM I; Invitrogen-Gibco, Grand Island, NY), OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA), and slides (Superfrostplus; Menzel-Gleser, Braunschweig, Germany). Fetal calf serum (FCS) and goat serum were both obtained from Invitrogen-Gibco. Antifade medium (Citifluor) was purchased from Agar Scientific (Stansted, UK).

Human Corneal Tissue Preparation and Excimer Laser Ablation

Approval for all human tissue-based research was obtained from the Northern X Regional Human Ethics Committee. All research procedures were developed in accordance with the Declaration of Helsinki. Fresh, matched pairs of cadaveric human corneas were obtained from donors sourced through the New Zealand National Eye Bank (Auckland, NZ). Corneas used in this study were unsuitable for transplantation because the cause of death was unknown or there was underlying systemic disease in the donor (see donor details in Table 1). Corneas were excised from the globe by cutting around the limbus to leave a 2- to 3-mm scleral margin and transported in New Zealand National Eye Bank medium (Eagles MEM+2% FCS). The corneas were then ablated ex vivo with an excimer laser (217 Zyoptix 100; Bausch & Lomb, Rochester, NY), to remove the corneal epithelium and the stroma to a total combined depth of 80 μm. A metal mask was held in a support stand overlaying the corneoscleral button, placed epithelium uppermost (Fig. 1), during laser ablation. A donut-shaped laser ablation of 7-mm outer diameter and 3-mm inner diameter was performed, leaving a masked central epithelial island of 3-mm diameter (Fig. 2A) separated from the periphery and the limbus (Fig. 2B). In two corneas, the limbal area including the stem cell repository was also removed by ablation after the donut ablation had been performed. An experienced surgeon manually navigated the laser and performed 2.0-mm overlapping limbal ablations using 100 laser pulses per ablation. The overlapping circular limbal ablations resulted in a continuous, slightly scalloped ablation edge at the limbus with total predicted depth of 80 μm (Fig. 3).

After ablation, the corneoscleral buttons were cultured in the reduced-serum medium (Opti-MEM I; Invitrogen-Gibco) containing 5% FCS, L-Glutamine, and antibiotic PSN with the medium maintained at a high level to keep the entire cornea moist at all times. During incubation, corneal regrowth was filmed using dark-field time-lapse photography. At the end of 12 hours, the corneas were fixed in 4% fresh paraformaldehyde for 1 hour at 4°C or fresh frozen in liquid nitrogen. Unablated corneas were used as control specimens for normal cell activity.

Time Lapse Microscopy and Image Analysis

Epithelial recovery in cultured corneas was observed with dark-field microscopy (Wild, Heidelberg, Germany) with digital images taken every 5 minutes throughout the 12-hour incubation period with a digital camera and software (NIS-Elements BR2.30 software and a Digital Sight DS-U1 camera; Nikon, Tokyo, Japan). All image analyses were performed by using the Image Processing and Analysis function of Java in Image J (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). For the donut model, epithelial migration distance was measured along four equally distributed, straight lines. These were marked around the cornea creating lines A–D extending radially from the center of the cornea. Distance mea-

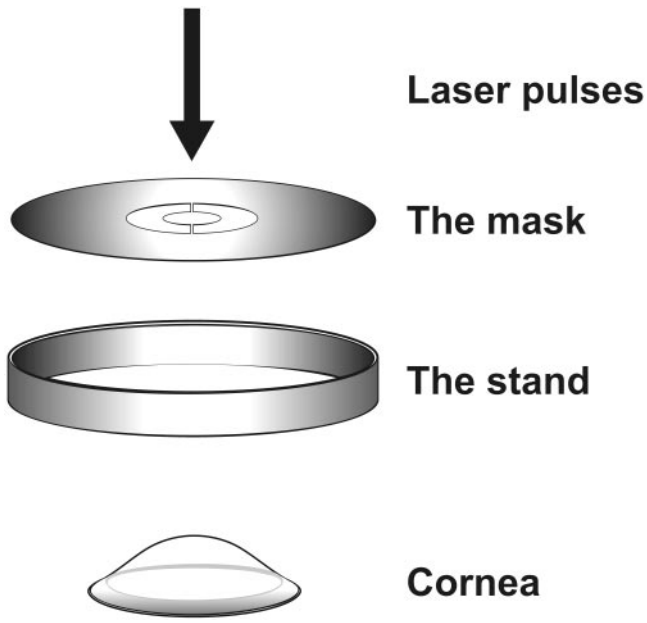

FIGURE 1. Donut- or ring-shaped laser ablation masking system set up. A human cornea is placed beneath a circular stand used to hold the mask. The area without the metal masking (i.e., donut shape) is ablated with an excimer laser.

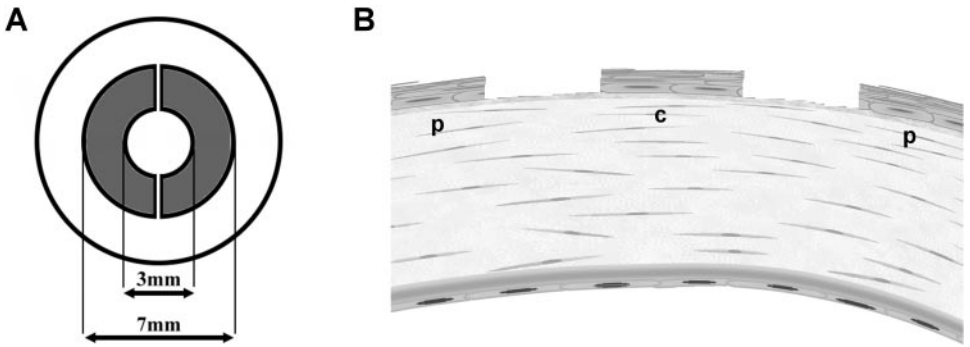

FIGURE 2. The donut-shaped laser ablation zone on a human cornea. (**A**) Two-dimensional diagram shows the pattern of the metal mask in Figure 1. The ablation zone (*gray*) has a 7-mm outer diameter and a masked center of 3-mm diameter. The ablation is set to a depth of 80 μm, to ensure ablation of all epithelial layers and minimal anterior stroma. The remainder of the cornea (*white*), being masked, is left with its epithelium intact. (**B**) A cartoon cross-section view of a donut-lasered human cornea. The corneal surface has a central island of epithelium (c) separated by the ablation zone from the peripheral epithelium (p), which is still in contact with the limbus.

surements were taken separately from the center outward and the periphery inward along these four defined lines, with the original marked boundaries used as baselines. To measure the regrowth area, we marked the original ablation boundary on each image and measured the area from the original boundary to the newly defined regrowth edge. The regrowth area was then divided by inner or outer circumferences to obtain the rate of epithelial recovery based on the number of cells contributing to regrowth. The total number of cells was counted per 120 μm length along the central or peripheral epithelium in histologic sections. For the donut+limbus ablation model, because the limbus was ablated manually, a standardized circumference from which to calculate the rate of epithelial recovery in area per unit length of circumference was not available. However, distance measurements were performed as for the donut model, and in this model measurements of epithelial recovery were not only taken from the donut center outward and the donut periphery inward, but also from the peripheral corneal epithelium at the junction of the limbal ablation outward to the ablated limbal region. Distance measurements were taken along the four defined lines using the original marked boundaries as baselines at every 4-hour time point.

Immunohistochemistry

After time lapse recording, the corneas were cut in half before being mounted in OCT compound (Tissue-Tek; Sakura Finetek) and frozen in liquid nitrogen. Corneal cryosections were cut 10 to 16 μm in thickness and immediately mounted on electrostatic slides (Superfrost Plus; Menzel-Gleser). Surrounding OCT was removed by washing the slides in PBS before staining. Nonspecific binding was blocked with 10% goat serum-PBS for 1 hour at room temperature. The slides were then incubated with the monoclonal mouse antibodies against p63 (1:200), ABCG2 (1:25), cell proliferation marker Ki67 (1:100), or polyclonal antibody against Cx43 (1:1000) overnight at 4°C. After the specimens were rinsed in PBS three times for 15 minutes each, they were incubated with Alexa-488 or Cy3-conjugated secondary antibodies for 2 hours at room temperature and washed three times for 15 minutes each in PBS before counterstaining with DAPI at 0.1 μg/mL for 10 minutes. Finally, the slides were mounted in antifade medium (Citifluor; Agar Scientific) and assessed using a confocal laser scanning microscope (TCS SP2; Leica Microsystems, Heidelberg, Germany) or a macroview zoom microscope (MVX10; Olympus Microsystems, Melville, NY).

BrdU Cell Proliferation Analyses

Cell proliferation was assessed by BrdU incorporation. BrdU is a synthetic thymidine analogue that becomes incorporated into a cell's DNA during the S-phase of the cell cycle. After corneal laser ablation as described earlier, the corneas were placed into a 10- to 50-μM BrdU-containing solution of complete reduced-serum medium (Opti-MEM I; Invitrogen-Gibco) for a 12-hour incubation. After labeling, the corneas were washed three times in BrdU-free complete reduced-serum medium and allowed an additional 15-minute incubation after washes. They were subsequently rinsed once in PBS before fixing in 4% paraformaldehyde for 1 hour at 4°C. Thereafter, corneas were embedded in OCT and 10- to 16-μm sections cut. Sections were then washed in PBS and permeabilized in ice-cold 70% methanol for 10 minutes. HCl pretreatment (2 N) was used to denature DNA for 30 minutes at room temperature, followed by three 10-minute washes. The sections were neutralized using 0.1 M borate buffer (pH 8.5) for 20 minutes at room temperature. They were again washed three times in PBS, 10 minutes each, and then transferred to a 10% normal goat serum blocking solution. The slides were processed for immunofluorescence with primary mouse anti-human BrdU at 1:20 dilution overnight at 4°C and

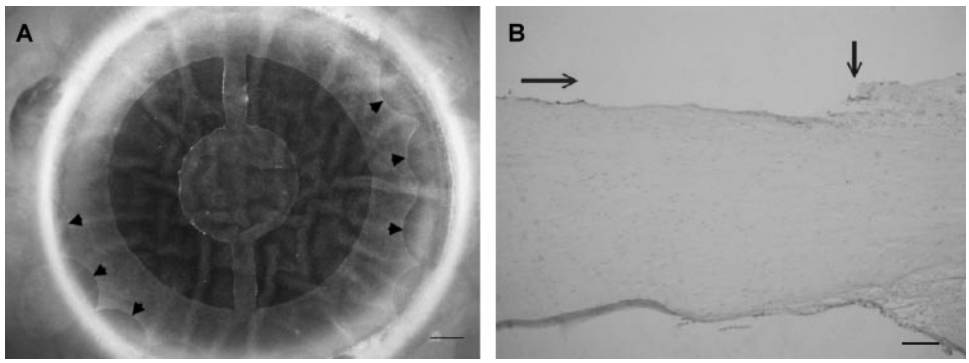

FIGURE 3. Donut+limbus ablation of a human cornea. (**A**) A dark-field microscopic image showing a clear donut-shaped ablation boundary in the center and a scalloped ablation edge at the limbus (*arrowheads*). (**B**) A cross section of the corneal limbal region revealed histologically with H&E stain after limbal ablation and 12-hour culture recovery. The limbus was clearly ablated (*vertical arrow*) and epithelial cells had started to migrate back centrifugally from the periphery toward the limbus (*horizontal arrow*). Scale bar: (**A**) 1 mm; (**B**) 0.15 mm.

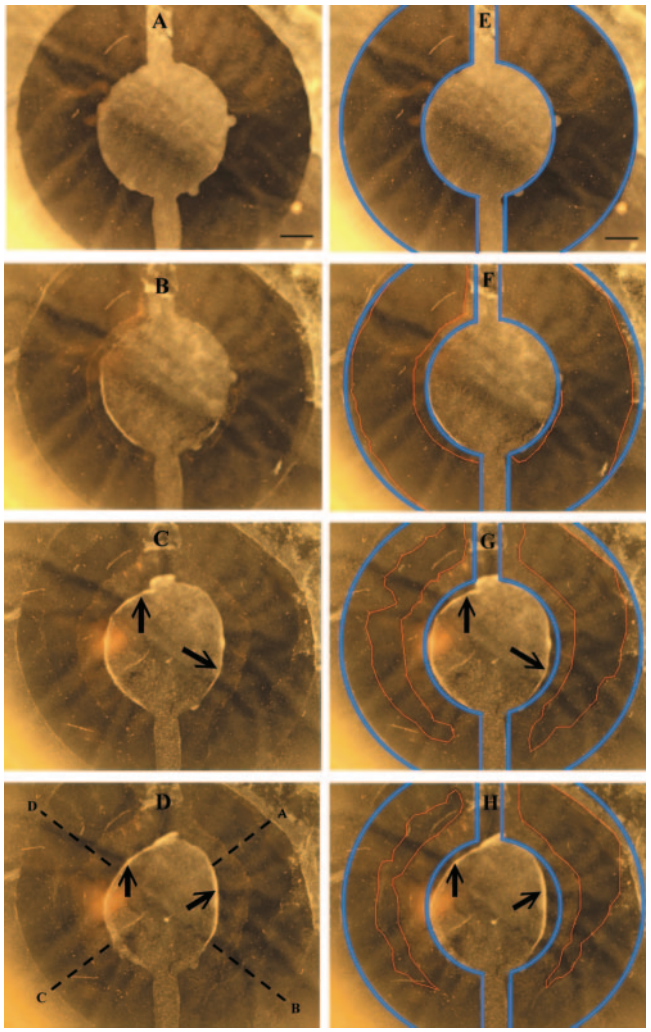

FIGURE 4. Dark-field microscopy showing human corneal epithelial recovery at different time points after a donut laser ablation. (**A**) At 0 hours, a clear donut-shaped ablation boundary was seen. (**B**) At 4 hours, cells from the center and the limbus/periphery had started to migrate across the lesion from both directions. Lighter colored regrowth zones were visible adjacent to the original ablation boundary. (**C**) At 8 hours, the cells had migrated farther both centripetally and centrifugally. (**D**) By 12 hours, cell growth from the two directions had nearly joined across the ablation zone in some regions. In (**C**) and (**D**), *arrows* indicate die-back of the superficial epithelial layers from the original laser ablation boundary. Equally distributed radial spokes were used to calculate the distance measurements (**D**, *dotted lines*). (**E–H**) Identical images to those in (**A–D**), but with original ablation boundary marked in *blue* and newly defined regrowth edge marked in *red*. Movie S1 showing 12 hours human corneal epithelial recovery after a donut ablation is available online at http://www.iovs.org/cgi/content/full/49/12/5279/DC1. Scale bar: 0.75 mm.

secondary goat anti-mouse Cy3 at 1:400 dilution for 2 hours at room temperature.

RESULTS

Direction of Corneal Epithelial Healing

The epithelial recovery of seven donut-ablated corneas that underwent excimer laser paracentral donut and donut+limbus ablations was filmed with consistent results. Corneal epithelial recovery in our donut ablation model is highlighted in Figure 4 at the 0-, 4-, 8-, and 12-hour time points. By 4 hours, epithelial cells started to regrow from both the peripheral and central ablation edges, although the original donut ablation edge remained well demarcated (Fig. 4A). This bidirectional growth was unexpectedly seen throughout the 12-hour incubation period, with the central epithelium appearing to recover as quickly as the peripheral epithelium. The superficial layers of the preexisting epithelium, before laser ablation, died back over time (Fig. 4, arrows). However, this die-back did not influence analysis, because we used the original ablation boundary for all measurements, and our interest focused on the advancing edges of new epithelial growth. In the donut+limbus ablation model, the epithelial cells not only regrew from both the donut periphery inward and the donut center outward but also from the periphery of the limbus ablation back toward (centrifugally) the limbus. This tridirectional growth was clearly seen at the 4-hour time point and continued subsequently (data not shown).

Rate of Epithelial Recovery

To investigate the rate of epithelial recovery in the donut model, four equally distributed lines were marked as shown in Fig. 4 D, and regrowth distance measurements were made separately along each line from the peripheral edge inward and the central edge outward at hourly time points. Faster epithelial regrowth was observed from the central epithelial cells along line A, whereas the remaining three lines showed faster epithelial growth from the periphery (Fig. 5A). The average rate of epithelial recovery from the center is similar to the rate from the periphery (0.06 ± 0.01 mm/hr vs. 0.07 ± 0.03 mm/hr, $P = 0.44$), showing no statistically significant difference between the two. Since there is a large difference in the number of cells contributing to repair based on the circumference of the inner and outer ablation edges, the rate of epithelial recovery was also quantified on the basis of regrowth area per unit circumference (Fig. 5B) and the central epithelium demonstrated a faster growth rate per unit area circumference than peripheral epithelium in five cornea samples (0.06 ± 0.02 mm^2/mm/hr vs. 0.04 ± 0.01 mm^2/mm/hr, $P = 0.04$).

The rate of epithelial recovery in the donut+limbus ablation model was measured, as in the donut model, on the basis of regrowth distance in two cornea samples. Measurements were made centrifugally from the center of the donut, centripetally from the periphery of the donut, and centrifugally from the periphery toward the limbus (Fig. 5C). The average rate of epithelial recovery from the center is similar to the rate from the periphery to the center and the rate from the periphery toward the limbal region (0.06 ± 0.02 mm/h vs. 0.05 ± 0.02 mm/h vs. 0.04 ± 0.01 mm/h, $P > 0.05$), showing no statistically significant difference between any of the three. Overall, we noted that the corneal epithelium tended to speed up its regrowth rate after 4 hours in ex vivo cultivation and then started to slow after 10 hours.

Potential for Cell Proliferation

To study the cells' proliferative ability, two matched pairs of corneas were examined after 12 hours of in vitro corneal healing. Normal, nonablated control corneas were used in each pair. The number of cells per unit length of sectioned tissue was calculated at the corneal periphery and center where there was a 36% increase in central epithelial cell density compared with control tissue (57 ± 6.84 cells/120 μm vs. 46 ± 4.22 cells/120 μm, $P = 0.0009$; Fig. 6A) at 12 hours after laser ablation. There was no change in epithelial cell density at the periphery (42 ± 6.30 cells/120 μm vs. 42 ± 3.76 cells/120 μm, $P = 1.000$; Fig. 6B) yet staining with Ki67 revealed that the cells were dividing across the whole corneal epithelium after wounding (Figs. 7A–C). Five human corneas were labeled with Ki67 and quantified. In fresh (noncultured) control, nonab-

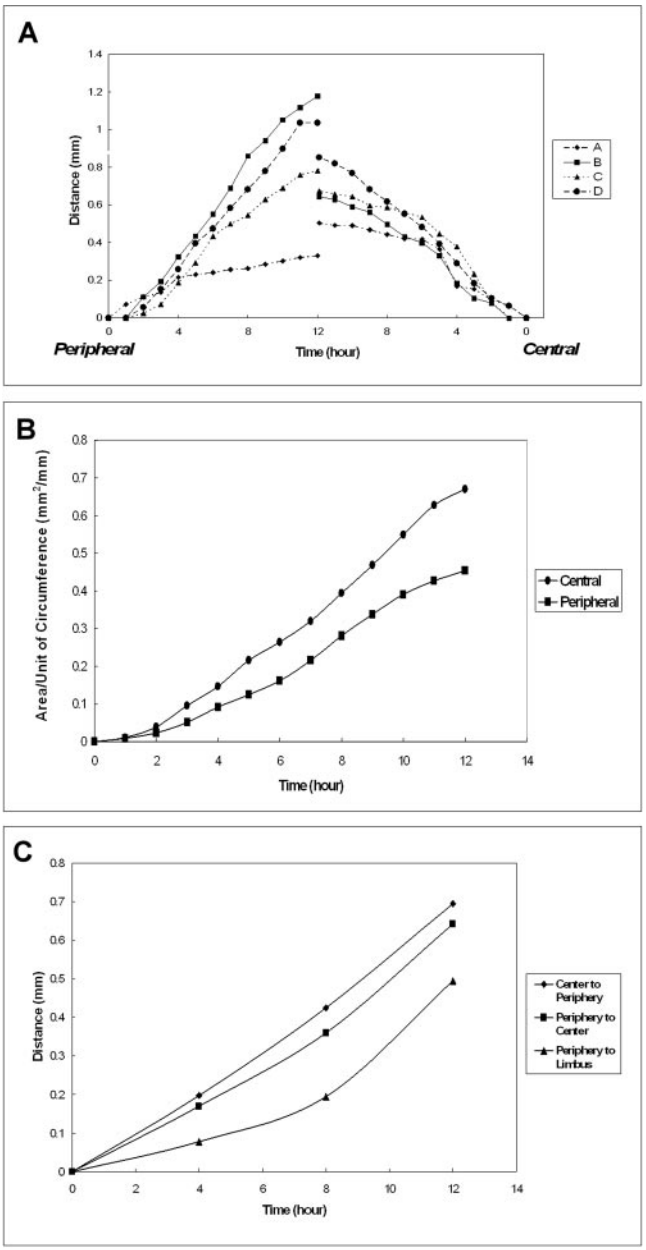

FIGURE 5. Rate of epithelial recovery across wounded human corneas. (**A**) Rate of epithelial recovery in distance covered with regrowth distance measured centripetally from the periphery and centrifugally from the center (donut model). All measurements were made from the marked original ablation boundary along four equally spaced lines, as indicated in Figure 4D. (**B**) Rate of epithelial recovery in area/unit length of circumference (donut model). Regrowth area was again measured from the original ablation boundary. Rate of growth from the center was calculated by dividing the growth area by the inner circle circumference length with rate of growth from the periphery calculated as peripheral growth area divided by the outer circumference length. (**C**) A graph showing the average rate of epithelial recovery in distance migrated in the absence of LSCs (donut+limbus ablation model). The average rates were measured along four equally distributed lines and averaged for cells from the donut center migrating centrifugally, from the donut periphery migrating centripetally, and from the periphery of the limbal ablation centrifugally toward the ablated limbal region.

lated human corneas, approximately half as many Ki67-positive cells were seen in the central epithelium (6–7 cells/mm) compared with the peripheral epithelium and limbus (12–14 cells/mm). In comparison, nonablated control human corneas cultured for 12 hours had no Ki67-positive cells in the central epithelium, retained a similar number of positive cells in the peripheral epithelium, but had almost 2.5 times as many positive cells in the limbus (32 cells/mm). In contrast, in donut-ablated corneas at 12 hours after surgery, there were up to 57 positive cells/mm in the central epithelium, with almost every second cell in the center staining with the Ki67 cell proliferation marker. Meanwhile, there was an almost 3-fold increase in the number of proliferating epithelial cells at the periphery (i.e., cells from the peripheral leading edge to the epithelial–limbal junction) and a 1.5-fold increase in the limbus after wounding. The number of Ki67-positive cells in the center is similar to the periphery (46.3 ± 10.5 cells/mm vs. 42.1 ± 6.3 cells/mm, $P = 0.447$). Of note, whereas basal cells at the periphery and in the center were both BrdU and Ki67 positive, dividing cells in the limbus were restricted to the suprabasal layers (possibly early TA cells; Fig. 7C). These were also confirmed using BrdU labeling (Fig. 7D).

Expression of p63, ABCG2, and Cx43

To reveal what types of cells were involved during the healing process, we labeled human corneal sections to locate cells expressing p63, ABCG2, or Cx43. We observed p63 expression not only in the basal layer of the limbus where stem cells reside (Fig. 8B), but also in the central epithelium, albeit to a lesser extent. The staining of p63 within the central epithelium was not confined in the basal layer, appearing in most layers to some extent (Fig. 8A).

ABCG2, a universal stem cell marker, was observed in clusters of cells in the basal layer of the epithelium. Figure 8D shows a high-magnification image of one of the positive clusters, suggesting ABCG2 was immunolocalized to the cell membrane of basal and some suprabasal epithelial cells in the human limbus of wounded corneas. We also identified patches of ABCG2 labeling in most layers of the central epithelium although to a lesser density (Fig. 8C). In summary, both p63 and ABCG2 labeling showed high intensity in the basal layer of the limbus, where stem cells are thought to be located, but positive signals were also identified within layers of central epithelium.

Cx43 is a gap junction protein involved in direct cell-to-cell communication and is used as a differentiation marker in the cornea. Cx43 labeling showed strong expression in the corneal and limbal suprabasal epithelial cells, but the basal cells of the limbal epithelium were essentially negative, with just a few weakly positive cells (Fig. 8E, arrows). However, more Cx43 positive cells were labeled in the basal layer of the limbus after wounding (Fig. 8F). Normal Cx43 levels in the periphery are demonstrated in Figure 8G. A decrease in Cx43 expression was observed at the very edge of the wound (Fig. 8H, arrow) where cells are differentiating into a migratory phenotype to close the wound, but Cx43 expression was upregulated in the epithelium a short distance away from the wound edge (Fig. 8I) where increased proliferation is required.

DISCUSSION

LSCs are believed to be the primary source of corneal epithelium.[4,7] They have an unlimited proliferative capacity and give rise to TA cells that have limited proliferative capacity. TA cells then migrate centripetally and differentiate into terminally differentiated cells, which are shed from the surface. There is now a large body of evidence leading to a belief that not only do LSCs undergo continual self-renewal to maintain the corneal epithelium, but they are also responsible for epithelial tissue repair and regeneration in an adult cornea[7,23,24] and the regeneration process is thought to be faster in response to wounding

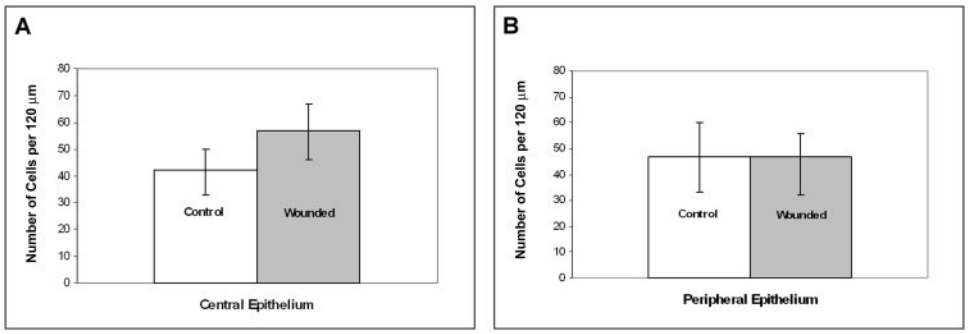

FIGURE 6. Total number of cells per unit length of epithelium for central and peripheral epithelium. Cells were counted on H&E-stained human corneal sections. (**A**) Cell density had increased 36% in the central epithelium at 12 hours after surgery. (**B**) There was no change in cell density between normal and postwound corneal peripheral epithelium.

compared with corneal epithelial homeostasis. To date, no direct methods have been unequivocally established to identify the corneal stem cells because of the lack of specific molecular markers, although a variety of putative stem cell markers have been proposed in the past 10 years. Because LSCs have such a great potential in the clinic and for research, they have attracted much attention, sometimes to the neglect of the potential of epithelial cells outside the limbus.

In the present study, we performed donut-shaped excimer laser epithelial ablations on human corneas, with or without the preservation of LSCs. It is widely expected that corneal repair or regeneration comes from the limbus, but these models allowed us to examine the proliferative ability in the central epithelium. In the donut model, central epithelial cells grew centrifugally and, as might be expected, cells from the limbus, and periphery grew centripetally during our ex vivo incubations. The recovery rate from the center was faster than that from the limbus (0.06 ± 0.02 mm^2/mm/h vs. 0.04 ± 0.01 mm^2/mm/h, $P = 0.04$). During the repair, die-back from the outer and upper epithelial layers of the laser wound edge was observed (Fig. 4, arrows) but this did not affect accurate measurements, since the original ablation boundary was used as a starting point for each measurement. We believe the die-back occurred only in the superficial layers, since clear forward cell migration was evident in the bottom layers of the epithelium as seen in time-lapse recording. This observation suggests that proliferation took place mainly in the basal and suprabasal layers of the epithelium, as reinforced by the Ki67 and BrdU labeling. In control (nonablated) tissues, cells in the periphery and the limbus were dividing for epithelial homeostasis, but were dividing less in the central epithelium. The culturing period and condition tend to induce proliferation in limbal cells, but no changes in central and peripheral cells. However, acute cell proliferation responses were seen in the center, the periphery, and the limbus after injury, suggesting that the central epithelium retains some level of regenerative ability. This would be consistent with Lehrer et al.[11] who noted that central TA cells can shorten the cell cycle time and proliferate more times in response to an topical application of TPA within the first 24 hours, although they still suggest that the bulk of epithelial recovery is from the limbus. This in vivo animal model strengthens the reliability of our human in vitro explant model. Combining the cell proliferation results and the 36% increase in the number of central epithelial cells per unit length after wounding, it is clear that epithelial recovery is supported by cell proliferation, not simply spreading or sliding of existing cells. The migration of proliferated and differentiated cells in corneal wound repairs is entirely consistent with results obtained in other tissues.[25–28] In the limbus itself, the data suggest that LSCs located in the basal layer of the limbus did not proliferate and may not therefore play a role in acute corneal epithelial regeneration for at least the first 12 hours after wounding.

Our p63 labeling results concur with those of Dua et al.,[29] suggesting p63 was not only strongly expressed in the basal and some suprabasal layers of the limbus, but also expressed in layers of the central epithelium. In addition, we noted exactly the same pattern found in a rodent model[4]—a gradient of p63 signaling across the basal corneal epithelium with the highest signal intensity in the limbus, followed by the peripheral cornea and finally the central cornea. Thus, p63 is no longer considered a limbal stem cell marker, and recent studies on p63 isoforms indicate that only the ΔNp63α isoform is necessary for maintenance of the proliferative potential of LSCs. This isoform has been detected in the basal-to-intermediate layers of the limbus only.[30,31] However, ΔNp63 has been found to be abundantly expressed in corneal pannus (i.e., conjunctiva-derived epithelium) removed from patients with total LSC deficiency, again raising doubts as to its validity as a LSC marker.[32] Similarly, with ABCG2, we identified patches of ABCG2 expression in the basal and suprabasal layers of the limbal epithelium, but weak positive ABCG2 signals were also observed in layers of the central epithelium. Inconsistent with our findings, previous studies[8,9,17] showed no signs of ABCG2 expression in the central epithelium. Surprisingly, Chen et al.[33] showed that there is a new population of MHC class II–positive small slow-cycling cells with a large nuclear-cytoplasmic (N/C) ratio that expresses ABCG2 in the limbal epithelial basal layer, and Wolosin et al.[34] observed nonepithelial cells in limbal SP population. Whenever a new protein is found that is located in the limbal basal epithelium, researchers have tended to link it to LSCs or raise the possibility of using it as a potential LSC marker—for example, Notch-1,[35] and N-cadherin.[36] Accumulating all these results, the exact location of LSCs remains to be fully ascertained owing to the unreliability of these widely used putative stem cell markers. Whether stem cell–like cells remain in the central epithelium should be investigated further.

Cx43 is a gap junction protein that plays a role in cell–cell communication. It is known that gap junction–mediated intercellular communication is linked to the regulation of cellular growth, development, differentiation, and homeostasis. Thus, regenerating epithelium may also reflect the involvement of direct cell–cell communication in corneal wound healing. In the present study, the number of Cx43-labeled cells was increased in the basal layer of the limbus after wounding. In normal human cornea, Cx43-negative limbal basal cells were interspersed with a few positively labeled basal cells indicating that those positive cells could be early TA cells. The increase in Cx43-positive cells in response to damage may be explained if some LSCs have responded to injury signals and evolved into early TA cells, or if early TA cells have differentiated into late TA cells. We believe it will be more likely to be the latter, since we identified cells proliferating only in the suprabasal layer of the limbus with Ki67 and BrdU labeling at 12 hours after surgery, not in the basal layer where stem cells supposedly reside. Cx43 was downregulated at the very wound edge and was upregulated in nearby epithelium where the cells reverted into a proliferation phenotype for regeneration. The finding

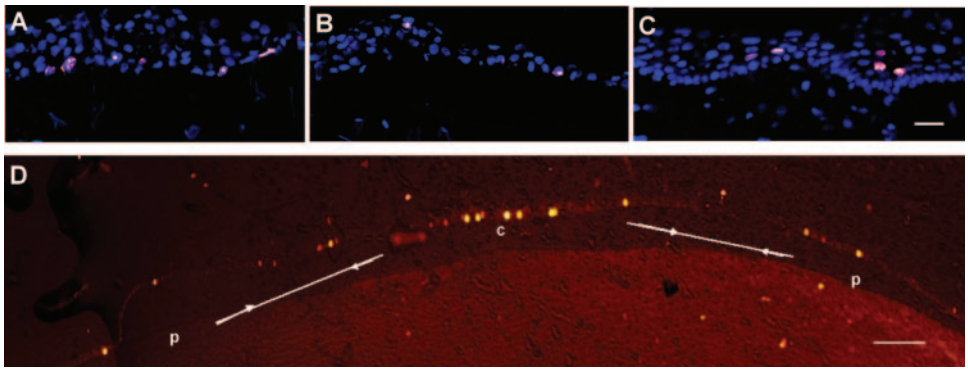

FIGURE 7. Cell proliferation across the human cornea. (**A–C**) Results from a cell proliferation marker, Ki67, labeling dividing cells in the central (**A**), peripheral (**B**), and limbal (**C**) regions at 12 hours after surgery, with DAPI counterstain showing cell nuclei. (**D**) A confocal image of BrdU label across the corneal epithelium 12 hours after wounding. Cell proliferation had clearly taken place in the center (c), in the regrowing epithelial monolayer (*white lines*), and in the peripheral epithelium (p). *Arrowheads*: direction of epithelial recovery and cell migration. Scale bar, (**A–C**) 30 μm; (**D**) 0.4 mm.

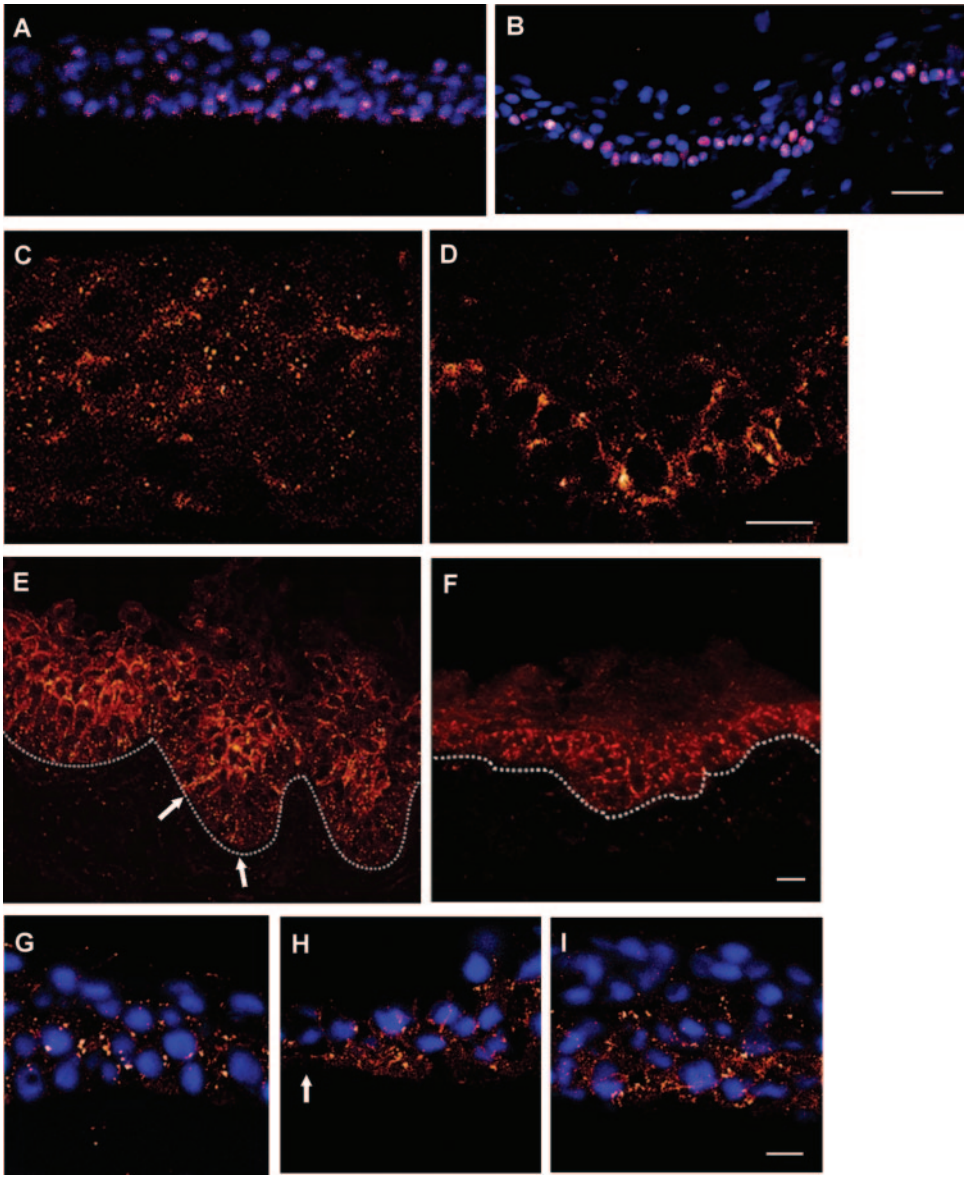

FIGURE 8. Immunohistochemical analysis of human corneal sections. (**A**, **B**) p63; (**C**, **D**) ABCG2; and (**E–I**) Cx43. (**A**, **B**, **G–I**) DAPI counterstain, to show cell nuclei. p63 expression was observed in the central epithelium (**A**), although brighter p63 expression was present in the basal layer of the limbus (**B**) where stem cells reside. Another stem cell-associated marker, ABCG2, was seen in the central epithelium (**C**), albeit to a lesser extent than in the limbal basal cells (**D**). Labeling with the suggested cell differentiation marker Cx43 revealed clusters of Cx43-negative limbal basal cells (**E**) interspersed between weakly positive basal cells (**E**, *arrows*). After wounding, more Cx43-labeled cells in the basal layer of the limbus (**F**) were seen. *Dotted line*: basal lamina. Low Cx43 expression was present at the migrating wound front (**H**, *arrow*) with upregulation in the proliferating epithelium back from the wound edge (**I**), compared with normal levels in the periphery (**G**). Scale bar: (**A**, **B**) 40 μm; (**C**, **D**, **G–I**) 15 μm; (**E**, **F**) 25 μm.

was consistent with our previous experimental results on skin[37] and as previously reported for the cornea.[38]

The idea of central epithelial cells being fully capable of corneal epithelial regeneration in the donut model was further confirmed by our donut+limbus ablation model. The removal of the limbal epithelium did not have any significant effect on the rate of epithelial recovery. When the two models were compared, similar recovery rates from the center outward and from the periphery inward were seen, indicating that the observed corneal epithelial recovery is independent of LSCs at least in the first 12 hours after injury in our explant model. Of interest, similar growth rates from the edge of the peripheral ablation back toward the limbus was observed in the absence of limbal stem cells. In addition, there was no correlation observed between the rate of epithelial recovery and the donor variability (age, sex, postmortem delay or cause of death).

Our key findings are that after wounding, cell proliferation and migration in the center of the cornea is just as vigorous as in cells proliferating and migrating from the limbus and periphery. Central human corneal epithelium cells appear to be fully capable of corneal epithelial regeneration, whereas LSCs do not respond to acute injury within at least in the first 12 hours.

Acknowledgments

The authors thank the New Zealand National Eye Bank for supplying the human corneas; Jane McGhee for assistance with excimer laser corneal ablations; and the Eye Institute, Auckland, for unrestricted access to their laser facility for these experiments.

References

1. Thoft RA, Friend J. The X, Y, Z hypothesis of corneal epithelial maintenance. *Invest Ophthalmol Vis Sci*. 1983;24:1442–1443.
2. Dua HS, Shanmuganathan VA, Powell-Richards AO, Tighe PJ, Joseph A. Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. *Br J Ophthalmol*. 2005;89:529–532.
3. Shanmuganathan VA, Foster T, Kulkarni BB, et al. Morphological Characteristics of the Limbal Epithelial Crypt. *Br J Ophthalmol*. 2007;91(4):514–519.
4. Chee KY, Kicic A, Wiffen SJ. Limbal stem cells: the search for a marker. *Clin Exp Ophthalmol*. 2006;34:64–73.
5. Dua HS, Azuara-Blanco A. Limbal stem cells of the corneal epithelium. *Surv Ophthalmol*. 2000;44:415–425.
6. Romano AC, Espana EM, Yoo SH, Budak MT, Wolosin JM, Tseng SC. Different cell sizes in human limbal and central corneal basal epithelia measured by confocal microscopy and flow cytometry. *Invest Ophthalmol Vis Sci*. 2003;44:5125–5129.
7. Boulton M, Albon J. Stem cells in the eye. *Int J Biochem Cell Biol*. 2004;36:643–657.
8. Watanabe K, Nishida K, Yamato M, et al. Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. *FEBS Lett*. 2004;565:6–10.
9. Umemoto T, Yamato M, Nishida K, et al. Rat limbal epithelial side population cells exhibit a distinct expression of stem cell markers that are lacking in side population cells from the central cornea. *FEBS Lett*. 2005;579:6569–6574.
10. Budak MT, Alpdogan OS, Zhou M, Lavker RM, Akinci MA, Wolosin JM. Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. *J Cell Sci*. 2005;118:1715–1724.
11. Lehrer MS, Sun TT, Lavker RM. Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. *J Cell Sci*. 1998;111:2867–2875.
12. Ebato B, Friend J, Thoft RA. Comparison of central and peripheral human corneal epithelium in tissue culture. *Invest Ophthalmol Vis Sci*. 1987;28:1450–1456.
13. Ebato B, Friend J, Thoft RA. Comparison of limbal and peripheral human corneal epithelium in tissue culture. *Invest Ophthalmol Vis Sci*. 1988;29:1533–1537.
14. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med*. 1996;183:1797–1806.
15. Chen Z, Evans WH, Pflugfelder SC, Li DQ. Gap junction protein connexin 43 serves as a negative marker for a stem cell-containing population of human limbal epithelial cells. *Stem Cells*. 2006;24:1265–1273.
16. de Paiva CS, Chen Z, Corrales RM, Pflugfelder SC, Li DQ. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells*. 2005;23:63–73.
17. Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells*. 2004;22:355–366.
18. Schlotzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. *Exp Eye Res*. 2005;81:247–264.
19. Pellegrini G, Dellambra E, Golisano O, et al. p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci U S A*. 2001;98:3156–3161.
20. Zhou S, Schuetz JD, Bunting KD, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med*. 2001;7:1028–1034.
21. Park KS, Lim CH, Min BM, et al. The side population cells in the rabbit limbus sensitively increased in response to the central cornea wounding. *Invest Ophthalmol Vis Sci*. 2006;47:892–900.
22. Davanger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature*. 1971;229:560–561.
23. Collinson JM, Morris L, Reid AI, et al. Clonal analysis of patterns of growth, stem cell activity, and cell movement during the development and maintenance of the murine corneal epithelium. *Dev Dyn*. 2002;224:432–440.
24. Charukamnoetkanok P. Corneal stem cells: bridging the knowledge gap. *Semin Ophthalmol*. 2006;21:1–7.
25. Laplante AF, Germain L, Auger FA, Moulin V. Mechanisms of wound reepithelialization: hints from a tissue-engineered reconstructed skin to long-standing questions. *FASEB J*. 2001;15:2377–2389.
26. Buck RC. Cell migration in repair of mouse corneal epithelium. *Invest Ophthalmol Vis Sci*. 1979;18:767–784.
27. Garlick JA, Taichman LB. Fate of human keratinocytes during reepithelialization in an organotypic culture model. *Lab Invest*. 1994;70:916–924.
28. Jansson K, Kratz G, Haegerstrand A. Characterization of a new in vitro model for studies of reepithelialization in human partial thickness wounds. *In Vitro Cell Dev Biol Anim*. 1996;32:534–540.
29. Dua HS, Joseph A, Shanmuganathan VA, Jones RE. Stem cell differentiation and the effects of deficiency. *Eye*. 2003;17:877–885.
30. Kawasaki S, Tanioka H, Yamasaki K, Connon CJ, Kinoshita S. Expression and tissue distribution of p63 isoforms in human ocular surface epithelia. *Exp Eye Res*. 2006;82:293–299.
31. Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De Luca M. Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci U S A*. 2005;102:9523–9528.
32. Espana EM, Di Pascuale MA, He H, et al. Characterization of corneal pannus removed from patients with total limbal stem cell deficiency. *Invest Ophthalmol Vis Sci*. 2004;45:2961–2966.
33. Chen W, Hara K, Tian Q, Zhao K, Yoshitomi T. Existence of small slow-cycling Langerhans cells in the limbal basal epithelium that express ABCG2. *Exp Eye Res*. 2007;84:626–634.
34. Wolosin JM, Budak MT, Akinci MA. Ocular surface epithelial and stem cell development. *Int J Dev Biol*. 2004;48:981–991.
35. Thomas PB, Liu YH, Zhuang FF, et al. Identification of Notch-1 expression in the limbal basal epithelium. *Mol Vis*. 2007;13:337–344.
36. Hayashi R, Yamato M, Sugiyama H. N-Cadherin is expressed by putative stem/progenitor cells and melanocytes in the human limbal epithelial stem cell niche. *Stem Cells*. 2007;25:289–296.
37. Franke S. *Regulating Gap Junction Communication to Reduce Scarring in Skin Wound Repair*. Auckland, New Zealand: Department of Anatomy with Radiology, The University of Auckland; 2005.
38. Ratkay-Traub I, Hopp B, Bor Z, Dux L, Becker DL, Krenacs T. Regeneration of rabbit cornea following excimer laser photorefractive keratectomy: a study on gap junctions, epithelial junctions and epidermal growth factor receptor expression in correlation with cell proliferation. *Exp Eye Res*. 2001;73:291–302.