A Subset of Human Limbal Epithelial Cells With Greater Nucleus-to-Cytoplasm Ratio Expressing High Levels of p63 Possesses Slow-Cycling Property

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Purpose: The purpose of this study was to evaluate the subset of limbal epithelial cells with greater nucleus-to-cytoplasm (N/C) ratio expressing high levels of p63 for their slow-cycling property, a characteristic feature of stem cells (SCs).

Methods: Limbal and peripheral corneal explant cultures were pulse labeled with 5-5-bromo-2′-deoxyuridine (BrdU) for 5 days, followed by a period of 3-week chase. Cultured explants were cryosectioned and stained for BrdU. The epithelial cells in the outgrowth and those remaining on the explant were isolated and subjected to cytospin and double immunostaining for BrdU and p63, followed by identification of label-retaining cells (LRCs) and quantification of p63 expression using confocal microscopy.

Results: A distinct population of small cells with large N/C ratio expressing high levels of p63 retained the BrdU label after 21-day chase. Further, this population of LRCs, negative for the differentiation marker K3, was observed in the epithelial outgrowth of limbal but not in that of peripheral cornea. LRCs were seen to migrate along the cut edge of limbal explants in culture and were also observed as clusters of small cells in the outgrowth, which contained cells with the ability to form holoclone colonies.

Conclusions: These results demonstrate that the small cells with large N/C ratio and high levels of p63 have BrdU label retaining slow-cycling property, thus confirming that these 2 parameters in combination may serve as a precise marker for identification and quantification of ex vivo–expanded limbal SCs. This method would be useful to standardize the optimal culture conditions that can maintain and expand SCs for therapeutic applications.

Key Words: p63 expression, N/C ratio, slow-cycling property, limbal epithelial stem cells

Attempts have been made to identify corneal epithelial stem cells (SCs) using positive or negative expression of several markers. However, no single positive molecular marker is described to distinguish SCs from other corneal epithelial cells.[1–3] More definitive methods for the identification of SCs are based on their functional properties. Highest clonogenecity and proliferative potential were observed among the limbal epithelial SCs, which gave rise to "holoclones," whereas "paraclone" and "meroclone" colonies, derived from transient amplifying cells of the corneal epithelium, possessed limited proliferative capacity.[4] The slow-cycling property is another well-established characteristic of epithelial SCs. Tritiated thymidine or 5-5-bromo-2′-deoxyuridine (BrdU) was found to be retained in a population of limbal basal cells after pulse labeling followed by chase, thereby confirming the slow-cycling nature of SCs.[5,6] The slow-cycling property of SCs has also been demonstrated in vitro.[7–9] Although several studies[7,9] indicated that label-retaining cells (LRCs) are considered to be SCs, the phenotype of such cells has not been well characterized. Moreover, there is a need for determining the proportion of slow-cycling LRCs in a given ex vivo–expanded human limbal epithelium.

We have earlier demonstrated that the 2 parameters, high expression of nuclear protein p63 and a greater nucleus-to-cytoplasm (N/C) ratio, identified a subset of limbal epithelial cells with SC phenotype.[10] This approach was confirmed by 2 recent studies: one on the basis of high level of expression of p63 in combination with cell size/diameter[11] and another by observing cells, strongly positive for p63 with high N/C ratio in the limbal crypt or SC "niche" structure.[12] The present study extends the aforementioned findings to demonstrate that the small cells in the limbus with large N/C ratio and high levels of p63 have the slow-cycling label-retaining property, thus indicating the significance of these 2 parameters in combination as a precise SC marker.

MATERIALS AND METHODS

Dulbecco's Modified Eagle's Medium (DMEM), propidium iodide (PI), bovine serum albumin, mouse immunoglobulin (Ig)G_1, mouse IgG_{2a}, mitomycin C, BrdU, Nutrient mixture F-12 (Ham), epidermal growth factor, insulin, transferrin, sodium selenite, hydrocortisone, choleratoxin A, gentamicin, and penicillin–streptomycin were purchased from Sigma-Aldrich (St Louis, MO); dimethyl sulfoxide and amphotericin B were purchased from Invitrogen-GIBCO BRL (Grand Island, NY); fetal bovine serum was purchased from HyClone (Logan, UT), trypsin was purchased from

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Amresco (Solon, OH); Dispase II, monoclonal mouse anti-BrdU was purchased from Roche Diagnostics (Indianapolis, IN); mouse anti-connexin 43 (IgG_1) and streptavidin–fluorescein isothiocyanate (FITC) was purchased from BD Biosciences (San Diego, CA); biotinylated goat anti-mouse Igs, fluorescent mounting medium, and endogenous biotin blocking system were purchased from DAKO (Glostrup, Denmark); mouse monoclonal antibody against p63 protein (clone 4A4, IgG_{2a}) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA); streptavidin–Alexa Fluor 633 was purchased from Molecular Probes (Eugene, OR); mouse anti-cytokeratin 3 (AE5) was a generous gift of Dr T.T. Sun (New York School of Medicine, NY); cover glass (22 × 22 mm Nr.1) was purchased from Menzel-Glaser (Braunschweig, Germany); and plastic ware was purchased from Nunc (Roskilde, Denmark).

Human Tissue Preparation

Human tissue was handled according to the tenets of the Declaration of Helsinki. Three enucleated cadaver globes (collected within 4 hours of death and used within 24 hours) and 5 corneoscleral rims (stored in McCarey–Kaufman medium for 24 hours) were obtained from the Rotary Aravind International Eye Bank at Aravind Eye Hospital. Cadaver corneoscleral button was excised from fresh globes (donor age ranging between 60 and 75 years), which had no prior ocular surgery, trauma, or disease, and procured within 5 hours after death. Sterile cotton tip was used to mechanically remove the underlying endothelium. Peripheral cornea was separated from the limbal rim using a scalpel under the stereomicroscope.[10]

Limbal and Corneal Explant Cultures

Limbal and peripheral corneal explants (2–3 mm) from donor eyes were cultured to generate the epithelial sheet. Each explant was allowed to attach in a 35-mm plastic dish, with its epithelial side up by placing in a drop of supplemented hormonal epithelial medium (SHEM) and incubating at 37°C for 20 minutes. The explants were then submerged in SHEM and cultured for 3 weeks at 37°C, 5% CO_2, and 95% humidity. The medium was changed every 2 days. The growth was monitored under inverted phase contrast microscope (Nikon Eclipse TS100, Japan). The ex vivo–expanded epithelial cells in the outgrowth were isolated by treating with 0.25% trypsin at 37°C for 30 minutes in 5% CO_2. To obtain epithelial cells remaining on the cultured explants, they were treated with Dispase II for 20 minutes, followed by 0.25% trypsin for 30 minutes in 5% CO_2. The enzyme activity was terminated using DMEM containing 10% fetal bovine serum. Cells were washed twice with DMEM, and viability was determined by trypan blue dye exclusion method.

To determine for the presence of cells with the potential to develop holoclone colonies, cells isolated from the outgrowth of 21-day limbal explant cultures were grown on mitomycin-treated 3T3 fibroblasts feeder layer.[4,13] The colonies were examined under phase contrast microscope.

BrdU Label–Retaining Assay

BrdU label–retaining property was evaluated in ex vivo–generated epithelial cells[9] in relation to 2-parameter analysis.[10] Limbal and peripheral corneal explants were cultured for 5 days in SHEM containing 10μM BrdU. The pulse-labeled explant cultures were switched to BrdU-free medium and chased for 21 days. The epithelial outgrowth and cryosections of explants from 5- and 21-day cultures were immunostained for BrdU to detect the pulse-labeled and slow-cycling LRCs. Cytospin smears of epithelial cells isolated from the outgrowth and the remaining explant were double immunostained for BrdU and p63, as described earlier.[10] The first immunostaining was visualized using streptavidin–FITC, and the second with streptavidin–Alexa Fluor 633 at a dilution of 1:1000. In addition, smears were double immunostained for K3 (differentiation marker) and BrdU. In these preparations, cells were also assessed for N/C ratio, BrdU label retention, and K3 expression. PI was used as a DNA counterstain. Corresponding isotype controls (mouse IgG_1, IgG_{2a}) instead of primary antibodies were maintained.

Confocal Microscopy

Image acquisition, quantification of p63 nuclear protein, and measurement of nuclear and cellular areas were carried out as described previously.[10] Fluorescence Z-stack images (1 μm) were captured with a laser scanning microscope (Leica AOBS-TCS SP2, Heidelberg, Germany). The excitation (band width) for FITC ranged from 496 to 535 nm using 488 argon laser; for PI, from 560 to 600 nm using 598 He–Ne laser; and for Alexa Flour 633, from 610 to 725 nm using 633 He–Ne lasers. The aforementioned parameters were used for image acquisition of 100 epithelial cells for each assay in cytospin smears of all the groups.

Analysis

N/C ratio for 100 cells in each group was obtained. Two-parameter (N/C ratio and mean amplitude for p63) analysis was carried out as described earlier.[10] Statistical analysis was performed using nonparametric test for 2 independent samples using Mann–Whitney test.

RESULTS

Nature of Cells Pulse Labeled With BrdU in Relation to 2 Parameters

Before characterization of BrdU LRCs in 21-day cultures, it was required to ascertain the distribution of pulse-labeled proliferating cells. Therefore, the limbal explant cultures after pulse labeling for 5 days were cryosectioned and immunostained for BrdU. At this point, the epithelial outgrowth was minimal. As shown in Fig. 1, 2–3 layers of epithelial cells were present on the explant, with several BrdU-positive cells in both basal and suprabasal layers.

Analysis of LRCs in Relation to 2 Parameters

To evaluate the limbal epithelial cells for label-retaining property, limbal explant cultures were pulse labeled with BrdU for 5 days, followed by 21-day chase, and then, the epithelial cells from the outgrowth were isolated, double immunostained for p63 and BrdU, and subjected to 2-parameter analysis as described earlier.[10] The scatter plot (Fig. 2) shows the

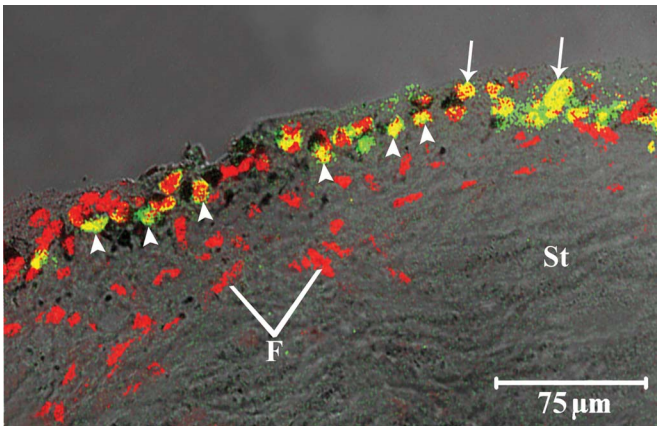

FIGURE 1. Distribution of epithelial cells pulse labeled with BrdU on the limbal explant cultures. Limbal explant cultures were pulse labeled with BrdU for 5 days, cryosectioned, and then processed for immunostaining for BrdU. Confocal overlay images of BrdU (green) and PI (red) along with transmitted light image are shown. Note the presence of 2–3 layers of epithelial cells on the stroma of limbal explant cultures, with several BrdU-positive cells, which appeared yellow because of merging of green and red. Arrowheads indicate BrdU-positive pigmented cells in the basal layer of cultured limbal explant; arrows indicate BrdU-positive limbal suprabasal cells. F, fibroblast; St, PI-stained stromal cells.

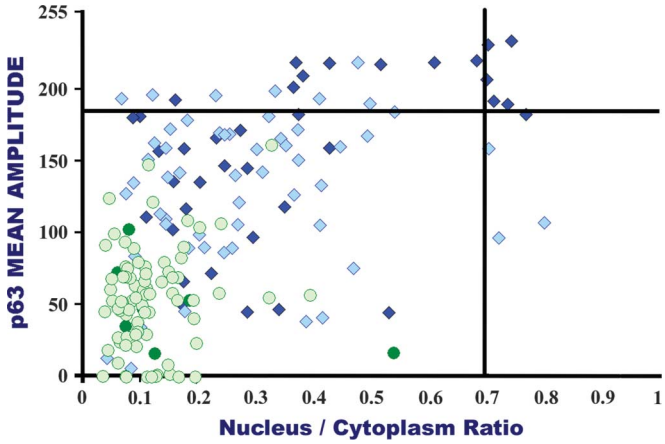

FIGURE 2. Scatter plot of 1 of the 3 representative experiments for p63 expression levels and N/C ratio in relation to their LRC property. Limbal explant cultures were pulse labeled with BrdU for 5 days, followed by 21-day chase. Cytospin smears of isolated cells from the outgrowth were double immunostained for p63 and BrdU. The rationale for the separation of scatter plot into 4 quadrants was described earlier.[10] ◆ and ◆ represent limbal outgrowth cells positive and negative for BrdU, respectively. ● and ○ represent peripheral corneal outgrowth cells positive and negative for BrdU, respectively. Note that the subset of cells with high expression of p63 (mean amplitude > 185) and N/C ratio greater than 0.7 located in UR quadrant was derived only from limbal outgrowth. BrdU-positive cells were also present in the UL and LL quadrants derived from limbal cultures. Cells from the peripheral corneal cultures were located only in the LL quadrant. Only a few of them were BrdU positive.

distribution of 100 cells into 4 quadrants. The distinct population of cells with N/C ratio above 0.7 and high p63 expression (mean amplitude above 185) located in the upper right (UR) quadrant were observed in the outgrowth of limbal explant cultures but not in that of peripheral cornea. Further, LRCs were observed among the UR quadrant population, with a labeling index of 85% on the explant and 76.7% in the outgrowth (Table 1). The cells in the UR quadrant from cultured epithelium, though larger (110.5 ± 26.1 μm^2) in size compared with those in the native limbal epithelium (88.7 ± 16.9 μm^2),[10] did meet the criterion of N/C ratio in our 2-parameter analysis. In other words, LRCs in the UR quadrant were small primitive cells with a greater N/C ratio expressing high levels of p63 (Figs. 2, 3; Table 1), thereby suggesting that these were cells with slow-cycling property. Additional experiments revealed the presence of 2 populations of LRCs: one with high N/C ratio (>0.7), negative for differentiation marker K3, and the other with low N/C ratio (<0.7), positive for K3 (Fig. 4). The former corresponds to

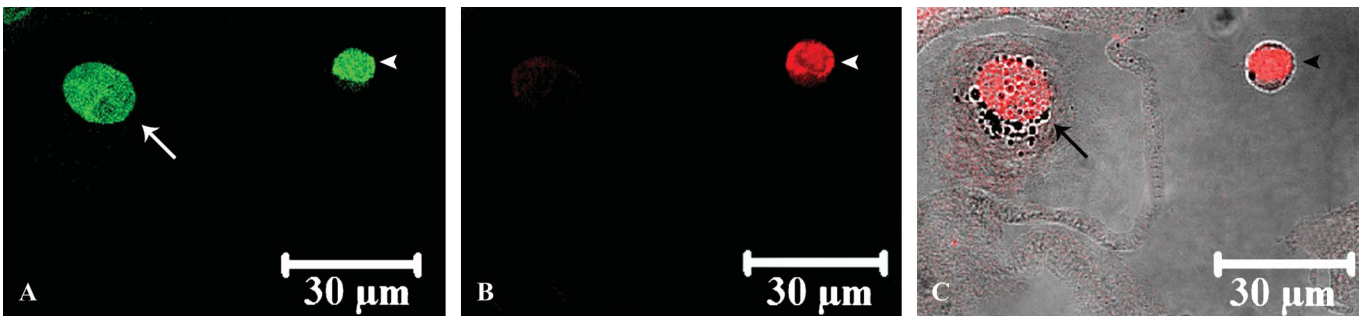

FIGURE 3. Characteristics of LRCs in cultured limbal epithelial cells in relation to 2 parameters. Limbal explant cultures were pulse labeled with BrdU, followed by 3-week chase as given in (Fig. 2). A, Confocal image after 2-dimensional average reconstruction showing 2 cells positive for p63 (green). A small cell (arrowhead) with high levels of p63 (mean amplitude = 197) and a high N/C ratio (>0.7) from UR quadrant is shown. A large cell (arrow) with a low N/C ratio (<0.7) and high levels of p63 (mean amplitude = 194) from UL quadrant is also shown. B, The same field as in (A), showing BrdU-positive LRC (red, arrowhead) and the large cell negative for BrdU. C, Overlay image of transmitted light and PI (red) for cells in (A).

TABLE 1. Profile of Epithelial Cells Based on 2-Parameter Analysis in Relation to Label-Retaining Property

BrdU	UR	UL	LR	LL
Limbal epithelium on explant				
% Of cells	5.3 ± 1.3	16.7 ± 6.0	0–3	77.0 ± 6.9
Labeling index	85 ± 13.2	70 ± 18.5	*	52.5 ± 18.6
Cell area (μm^2)	99.3 ± 48.8	663.4 ± 449.4	—	1041 ± 755.0
N/C ratio	0.8 ± 0.1	0.3 ± 0.2	—	0.2 ± 0.1
Limbal epithelial outgrowth				
% Of cells	4.6 ± 1.7	16.3 ± 7.4	0–3	76.9 ± 7.9
Labeling index	76.7 ± 8.3	64.3 ± 13.2	*	26.6 ± 8.7
Cell area (μm^2)	110.5 ± 28.1	858.1 ± 676.5	—	1070.9 ± 856.8
N/C ratio	0.7 ± 0.03	0.3 ± 0.2	—	0.2 ± 0.1
Peripheral corneal epithelium on explant				
% Of cells	No cells	No cells	No cells	100
Labeling index	—	—	—	21.3 ± 18.9
Cell area (μm^2)	—	—	—	973.1 ± 646.6
N/C ratio	—	—	—	0.2 ± 0.1
Peripheral corneal epithelial outgrowth				
% Of cells	No cells	No cells	No cells	100
Labeling index	—	—	—	7.2 ± 6.3
Cell area (μm^2)	—	—	—	1349.1 ± 934.1
N/C ratio	—	—	—	0.2 ± 0.1

Data are expressed as mean percent ± SD of 3 cultures:

$$Labeling\ index = \frac{No.\ BrdU\text{-}labeled\ cell\ in\ a\ quadrant}{Total\ number\ of\ cell\ in\ that\ quadrant} \times 100.$$

Cell area and N/C ratio given were for the BrdU-positive cells.
*No cells were labeled for BrdU in this quadrant.
BrdU, 5-5-bromo-2′-deoxyuridine.
UR, upper right; UL, upper left; LR, lower right; LL, lower left; quadrants of Figure 2.

cells in UR quadrant with slow-cycling property and the latter to the cells in upper left (UL) and lower left (LL) quadrants positive for differentiation marker. For comparison, the cultures of peripheral corneal cells were analyzed, and the outgrowth consisted of mainly large differentiated cells (LL quadrant), wherein 21.3 ± 18.9% on the explant and 7.2 ± 6.3% in the outgrowth were BrdU positive (Table 1).

Migration of LRC in Limbal Explant Cultures

It was required to determine whether SCs in the limbal explants migrate onto the culture dish and whether slow-cycling LRCs were present in the outgrowth. Fig. 5A shows the migration of clusters of pigmented cells from the limbal explant, possibly representing the movement of pigmented SCs to form the outgrowth. Further, the presence of LRCs in the basal layer of limbus in 5-day culture along its cut edge suggested the migratory pattern of SCs (Fig. 5B). To understand the distribution of slow-cycling LRCs, the limbal epithelial outgrowth was immunostained for BrdU. As shown in Fig. 6, the clusters of BrdU-positive small cells in addition to a few individual BrdU-positive large cells were observed in the outgrowth, indicating that the former represents a group of slow-cycling SCs and latter differentiated cells retaining BrdU. Moreover, certain cells in the outgrowth of limbal explant cultures were shown to have the ability to form the holoclone colonies (Fig. 7).

DISCUSSION

In Vitro Method to Assess the Label-Retaining Property of Limbal Epithelial Cells

We have used limbal explant culture method to elucidate the slow-cycling property of the subset of epithelial cells with a greater N/C ratio expressing high levels of p63. To demonstrate the slow-cycling property of the ex vivo–expanded epithelial cells, it was required to identify the distribution and proportion of proliferating cells in cultures pulse labeled with BrdU. Using the method of Kim et al,[9] we demonstrated the presence of BrdU-positive actively proliferating cells on the limbal explants, after 5 days of pulse labeling. At this early stage, when the epithelial outgrowth was minimal, BrdU-positive cells were observed in its basal and suprabasal layers of the explant. Moreover, the same culture conditions were found suitable to assess LRC in ex vivo–expanded epithelium, as described by others.[8,9]

We have also analyzed the pulse-labeled proliferating cells on the basis of 2 parameters (data not shown). Among the distinct population with high level of p63 expression combined with greater N/C ratio in the limbal explant culture, about 70% were actively proliferating cells after pulse labeling and the remaining BrdU negative. Both BrdU-positive cells (10–14 μm in diameter) and BrdU-negative cells (9–10 μm in diameter) were negative for differentiation marker K3. The former were in the S phase of cell cycle at the time of addition

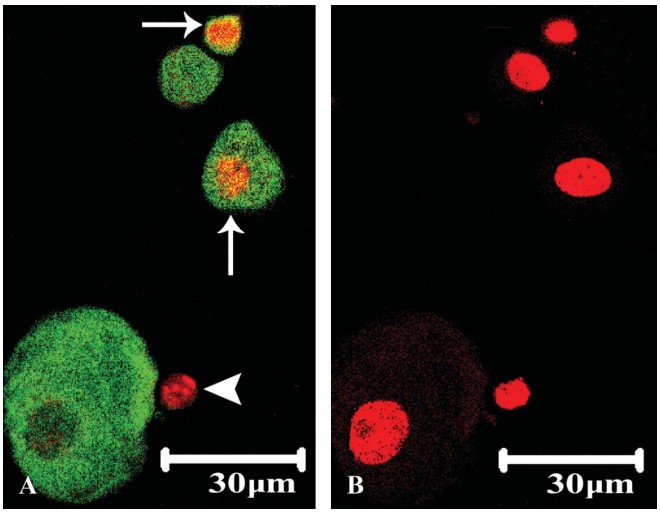

FIGURE 4. Characteristics of LRCs in the cultured limbal epithelial cells in relation to K3 expression and N/C ratio. A, Cytospin smear double immunostained for BrdU (red) and K3 (green). Note that a label-retaining small cell (arrowhead) was negative for K3 having high N/C ratio (0.7343). Among the K3-positive cells, 2 (arrows) were positive for BrdU, having low N/C ratio (0.526 and 0.321). B, Same field as in (A), showing nuclei (red) counterstained with PI.

of the label, and the later were much smaller in size and more primitive, representing the nondividing quiescent population. The presence of a small proportion of quiescent cells has been demonstrated in vitro with characteristics of primitive side population like cells.[14] Overall, both BrdU-positive pulse-labeled cells and quiescent cells observed in the present study met with the criteria of adult limbal SCs.[6,14]

Label-Retaining Cells

Our results presented in Figs. 2–4 and Table 1 clearly indicated the presence of 2 populations of LRCs in 21-day cultures. One consisted of large differentiated cells positive for differentiation marker, observed in UL and LL quadrants. The presence of LRCs expressing differentiation markers has also been reported in the mouse limbus and cornea. These α9 integrin–positive LRCs were considered to be the progeny of SCs that retained the BrdU label as they take up the path of differentiation during wound healing.[15] Another study in the skin reported the presence of LRCs expressing the differentiation markers $\alpha6^{bri}CD71^{dim}$.[16] Thus, the 2-parameter analysis coupled with LRC assay revealed that not all LRCs are slow-cycling adult epithelial SCs.

Another population of LRCs represents slow-cycling cells, characterized by high levels of p63 and a large N/C ratio, and they were negative for differentiation marker. Such cells were observed in the limbal explant cultures but not in those of peripheral cornea. Several reports indicated varying proportion of LRCs in vitro on the basis of Fluorescence Activated Cell sorting of $Cx43^{dim}$ expression (3.5%)[13] or cell size (11%).[17] These discrepancies may be because of the use of a single marker. However, our 2-parameter analysis is far more stringent SC identification tool, wherein we demonstrated that about 4% (in the UR quadrant) of total limbal epithelium in 3-week cultures was true slow-cycling LRCs with SC phenotype. Collectively, the study confirms that high expression of p63 and large N/C ratio were useful for the identification and quantification of the SC content in the ex vivo–generated epithelium.

Two major methods of ex vivo expansion of limbal epithelial cells have been described. One method was the cultivation of isolated limbal epithelial cells on amnion along with the mitomycin-treated 3T3 fibroblast as feeder layer.[18,19] The other method was the cultivation of limbal explants, including the substantial amount of stroma.[7,9,20] Using such epithelial sheet from limbal explant cultures, successful reconstruction of the corneal surface has been achieved.[20–22] Though the corneal epithelial SCs are not known to migrate outside limbus in vivo, we have demonstrated in the present study the migration of LRC from the explant (Fig. 5) and the presence in the outgrowth of limbal explant cultures of small cells with high level of p63 expression and a greater N/C ratio, having slow-cycling label-retaining property and with the ability to form holoclone colonies (Fig. 7). We have also

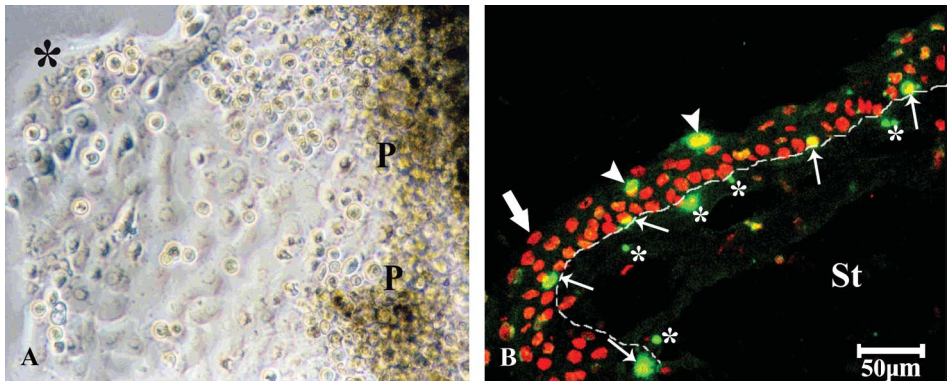

FIGURE 5. A, A phase contrast image of 3-day limbal explant culture, showing small outgrowth of rounded and flattened cells (asterisk) on the dish and clusters of pigmented cells (P) migrating from the limbal explant along the cut edge. Explant is located toward the right of pigmented cells. Scale = ×100. B, Location of BrdU LRCs in cryosections of cultured limbal explant, which were pulse labeled with BrdU for 5 days, followed by 21-day chase. Z-stack images were enlarged and analyzed to define the border (dotted line) between the epithelium and the stroma. Confocal overlay image showing BrdU LRCs (green) in the limbal basal layer (arrows) and also in the suprabasal layers (arrowhead). Note the presence of BrdU-positive LRCs in the basal layer along the cut edge of the limbal explant; nucleus counterstained with PI (red) shown. Yellow color was because of overlay of cells in green and red. Thick arrow indicates 3 layers of epithelium on the stroma. Asterisks indicate a few stromal fibroblasts positive for BrdU. St, stroma.

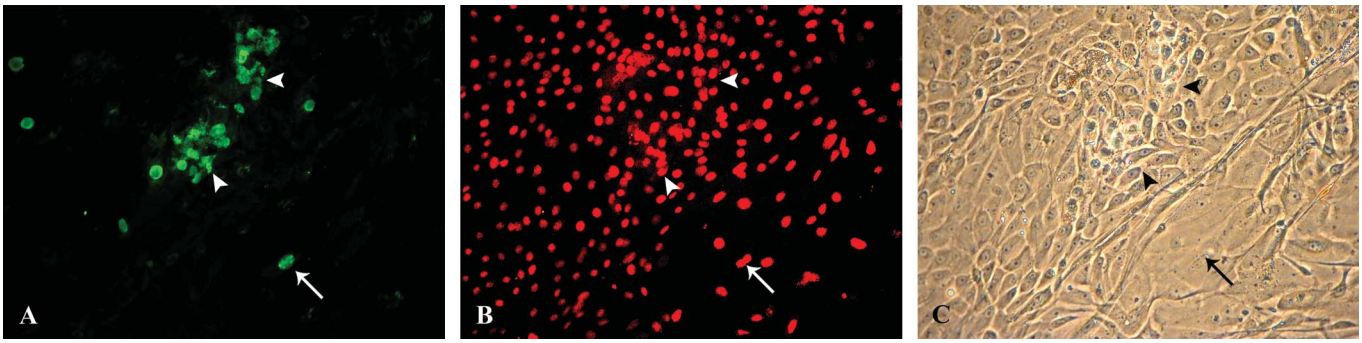

FIGURE 6. Distribution of LRCs in the outgrowth of limbal explant cultures. Limbal explant cultures were pulse labeled with BrdU for 5 days, followed by 21-day chase. A, Epithelial cells in the outgrowth showing BrdU-positive (green) cells in 2 clusters (arrowheads) of small cells and a few labeled large cells (arrow). B, PI (red) nuclear counterstained image of (A). C, Phase contrast image of the same region showing small and large cells. Magnification = ×200.

shown that sequential reexplanting of limbal tissue gave rise to fresh epithelial sheet at least 3 times, thus indicating the presence of SCs on the stroma during the period of culture (data not shown). Therefore, the simple method of limbal explant culture system would be extended to generate SC-rich epithelial sheet possibly through the influence of human limbal stromal cells in the xenobiotic-free culture medium.

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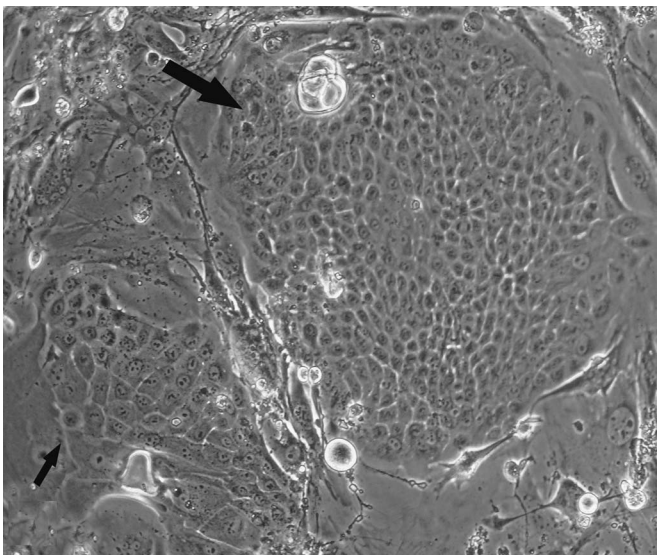

FIGURE 7. Phase contrast image of 5-day colonies generated from the epithelial outgrowth of 21-day limbal explant culture. Note a large colony of small cells with compact morphology (thick arrow) representing a holoclone and a small wrinkled colony (thin arrow) with large cells representing a merolone. Scale = ×100.

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ERRATUM

In the article "Novel Use of Fibrin Glue in the Treatment of Conjunctivochalasis," which appeared in volume 27 of *Cornea* on pages 950-952, the corresponding author's e-mail address was printed incorrectly. The correct e-mail address is ebrod073@uottawa.ca.

Brodbaker E, Bahar I, Slomovic AR. Novel use of fibrin glue in the treatment of conjunctivochalasis. *Cornea*. 2008;27:950-952.