ABCG2-Dependent Dye Exclusion Activity and Clonal Potential in Epithelial Cells Continuously Growing for 1 Month from Limbal Explants

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PURPOSE. To determine changes in ABCG2-transport-dependent dye exclusion in outgrowths from limbal explants.

METHODS. Human or rabbit limbal strips were deposited onto inserts. Over a month, the segments were twice transferred to new inserts. Fresh tissue (FT) cells, obtained by sequential dispase-trypsin digestion and the cells growing from the explant cultures, were characterized for ABCG2-dependent efflux by flow cytometry using a newly identified substratum, JC1. Rabbit cells were sorted into JC1-excluding (JC1^{low}) and main (JC1^{main}) cohorts and seeded with feeder 3T3 cells to determine colony formation efficiency (CFE).

RESULTS. The JC1^{low} cells were all Hoechst 33342-excluding cells and vice versa, establishing the physical equivalence between JC1^{low} and the side population (SP). JC1^{low} cell content was reduced by three ABCG2-specific inhibitors: FTC, Ko143, and glafenine. JC1^{low} percentiles for the fresh human and rabbit cells were 1.4% and 4.1% and CFEs for rabbit JC1^{low} and JC1^{main} were 1.2% and 5.3%. In contrast, the respective JC1^{low} percentiles in the first and second outgrowths were 19.5% and 27.4% and 25.8% and 32.5%, and the rabbit JC1^{low} and JC1^{main} CFEs were 12.3% and 0.9%. Thus, although in FT the contribution of the JC1^{low} cohort to the CFE is minimal, in the explant culture the phenotype incorporates >80% of the CFE.

CONCLUSIONS. ABCG2-dependent dye exclusion undergoes a large expansion in explant culture and becomes associated with a high CFE. The transport increase is more pronounced at late outgrowth times, suggesting permanence of stem cells within the explant. (*Invest Ophthalmol Vis Sci.* 2011;52:4330–4337) DOI:10.1167/iovs.10-5897

The limbal zone contains the majority of the stem cells (SCs) for the limbal–corneal epithelial lineage.¹ Consequently, the limbus plays a critical role in the survival of this vision-essential lineage. Epithelium and/or limbal zone loss, due to

disease, trauma, or congenital deficiencies, results in visual decrease or vision loss,^{2,3} and reintroduction of epithelial cells derived from a contralateral eye pre-expanded in vitro can reestablish a fully functional lineage,^{4–8} provided the engrafted cell population incorporates cells retaining the SC/progenitor phenotype.⁹ One indicator of the stem/progenitor cell phenotype is the expression of the multidrug resistance protein ABCG2/BCRP1, a xenobiotic transporter expressed at high levels within subpopulations of adult SCs of multiple lineages.^{10,11} ABCG2 is a very efficient efflux transporter of the supravital DNA binding dye Hoechst 33342; thus, cells with the highest levels of functional ABCG2 exclude the dye. Due to concentration-dependent bathochromic features of ABCG2, the dye exclusion results in a characteristic dual-wavelength emission flow cytometry pattern known as a side population (SP). This feature has allowed the isolation of viable stem and progenitor cells by flow cytometry from multiple lineages and organs, including cells from the limbal and conjunctival epithelia.^{12–20} We demonstrated that the SP cells identify with cells that before isolation have been in the slow cycling state that is unique to tissue SCs.^{17,21} SP cells obtained from fresh adult tissue show a very low propensity to start proliferating in clonal conditions. This failure to proliferate may reflect the immediate transition of these quiescent cells from the nurturing in vivo environment to an in vitro environment lacking the conditions for proper activation of proliferation.^{16,17,19,21}

One common approach to generate therapeutic epithelial cell populations for ocular surface reconstruction is based on the explant method, where cells outgrow into a suitable biological or synthetic substratum from a small tissue segment.^{22–26} Retention of SC-associated features in these explants has undergone extensive characterization. In outgrowth over an amniotic membrane, ABCG2 is well expressed in the proximity of the outgrowth and decreases in intensity toward the margin of the culture, whereas keratin 3, a marker of differentiation, displays the opposite distribution.²⁶ These characterizations notwithstanding, many questions about the origin of the outgrowth population and the explant's biological dynamics have not been fully addressed. In particular, given the slow cycling/quiescent features of somatic adult tissue SCs they need to become activated to contribute to the outgrowth population. It is not clear what the contribution of bona fide SCs to the outgrowth is, vis--vis the contribution of other proliferative limbal epithelial cells. In particular, the unique niche localization of SCs also raises questions about the permanence or survival of the SC phenotype within the explant proper, which is usually not included with the cell populations used for corneal regeneration.

To start addressing these questions we have now investigated the ability of limbal explants to generate outgrowths over prolonged time periods, and the relative density of cells exhibiting a high level of ABCG2 transport. In these studies we have replaced Hoechst 33342, a DNA binding dye that displays

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substantial toxicity, with JC1, a new ABCG2 substratum that binds to mitochondria instead of nuclei and shows minimal toxicity. The results show that (1) limbal explants could continuously give rise to outgrowths for at least 1 month; (2) JC1^{low} cells are as much as 10- to 15-fold more frequent in these outgrowths than in the cell population isolated from fresh tissue (FT); (3) the percentage of JC1^{low} cells is higher in the second outgrowth round than that in the first one; and (4) unlike the cells isolated from FT, the JC1^{low} cells isolated from the outgrowths display a colony formation efficiency (CFE) that is 14-fold higher than that of the JC1^{main} cells and incorporate the majority of the colony-forming cells within the outgrowths. The potential implications of these findings for the application of limbal explant methodology to ocular surface reconstruction are discussed.

MATERIALS AND METHODS

Tissue Procurement

Donor human (Hu) corneas and conjunctivas (CNJs) from unidentifiable cadavers were obtained through the National Disease Research Interchange (Philadelphia, PA). The Institutional Review Board determined that use of these tissues does not constitute research on human subjects. Tissue acceptance criteria included (1) the donor ranging in age from 30 to 69 years at death; (2) the donor was not maintained on a ventilator before death or subjected to two cycles of chemotherapy during his/her lifetime; (3) tissue harvest occurred within 12 hours of death; and (4) the donor tested negative for human immunodeficiency virus, hepatitis B or C, Epstein-Barr virus, and syphilis. Corneas showing substantial central corneal epithelial desquamation on arrival, an indication of postmortem surface drying, were not used. Rabbit corneas and conjunctivas were excised from the eyes of 2-2.5 kg rabbit cadavers (approximately 3 months of age) obtained from local abattoirs soon after kill.

Under a horizontal sterile-air-flow hood and with the help of a dissecting microscope, corneas were initially split into equal quarters, the conjunctival edge of each quarter was dipped in 1% trypan blue (Sigma, St. Louis, MO) to stain the conjunctival stroma, and all remnants of conjunctiva were carefully removed using conjunctival scissors. After scleral tissue was trimmed away, 0.5-mm-wide limbal strips were cut away from each corneal quarter with the use of a sharp blade.

Enzymatic Preparation of Single-Cell Epithelial Populations from Fresh Tissues

The protocol to generate single epithelial cell suspensions directly from the limbus and conjunctiva has been described.^{17,27} Briefly, the quarter-cornea limbal strips or quartered conjunctivas were incubated for 18 hours in 5 mg/mL neutral protease (Dispase II; Roche, Nutley, NJ) made in Hepes-buffered Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 1:1 nutrient mixture (hbD/F-12; Invitrogen, Carlsbad, CA) under side-to-side tilting motion, to induce spontaneous separation of intact epithelial sheets. These sheets were then collected and digested in trypsin-EDTA for 25 minutes under slow orbital mixing. Trypsin activity was stopped with 1 volume of DMEM (Invitrogen) + 10% fetal bovine serum (FBS; Invitrogen), the cell suspension was spun down, and the cells were resuspended in D/F-12 + 5% FBS. These steps are schematically described in the right branch of the adjacent flow chart (Fig. 1).

Epithelial Explant Culture and Enzymatic Preparation of Single-Cell Populations

For explant culture all or most of the limbal strips generated from a corneal pair were cut into 2- to 4-mm-long segments and deposited, epithelial side up, on 0.4- or 1- μ m-pore polyethylene terephthalate (PET) membrane inserts (Falcon; Becton Dickinson) for six-well plates. Typically, each experiment involved between 9 and 12 initial explants.

FIGURE 1. Schematic description of protocols used to compare JC1^{low} content in the cells outgrown from three consecutive rounds of explant culture (*left*) or cells isolated from tissue (*right*).

The inserts were pre-equilibrated in 2.3 mL medium for the previous 24 hours. After 72 hours of culture at the air-liquid interface, after confirming the presence of an encircling island of outgrowing cells, and every 48 to 72 hours thereafter, the culture solution was replaced with 3 mL medium. Human cells were cultured in supplemented human epithelial medium (SHEM) containing (per liter) 950 mL D/F-12; 50 mL FBS complemented with 5 μ g each of insulin, transferrin, and selenium; 10 ng cholera toxin; 5 ng epidermal growth factor; and 28 mg phosphoethanolamine. Rabbit cells were cultured in 84% D/F-12 + 16% FBS. All media additionally contained a penicillin-streptomycin mix. Whenever a source is not indicated the chemical was purchased from Sigma.

For continued cell production from the explant, after 11 to 12 days for human or 8 to 10 days for rabbit (at which point outgrowths were 3-4 cm wide but had not yet reached the edges of the insert), six of the explants were selected based on proper outgrowth development. The selected cultures were incubated overnight in calcium-free D/F-12 + 3% FBS ([Ca²⁺] $\approx$ 0.06 mM) to loosen cell-cell attachments. The PET membrane was then dismantled, explants were cut away using a microscalpel (if the removed material included attached underlying PET membrane, the latter was separated by a slight agitation in PBS), and repositioned, epithelial-side-up in a new insert, to extend the explant culture beyond the initial round. After the transfer of the explant, outgrown cells were fully dissociated into single cells by incubation in trypsin for 5 to 10 minutes. In most experiments the trypsinized cells from all six inserts were pooled together and after neutralization, trituration, centrifugation, and resuspension in D/F-12 + 5% FBS were replated for overnight culture. Transferred explants were cultivated for an additional 8 or 9 days. The entire process of explant transfer and outgrown cell collection and overnight reculture was then repeated, thereby generating a second recultured, outgrown cell population. After an additional 8- to 9-day explant culture period, a third recultured explant outgrown population was harvested and recultured overnight. In most cases, explants were discarded at this point. This repetitive explant transfer, outgrowth cell harvest, and overnight reculture are schematically described in Figure 1.

Flow Cytometry

All recultured cells, from either fresh tissues or explant outgrowth, were made at seeding cell densities of approximately 10,000 to 20,000 attached cells/cm², using amounts of cells that took into account the fact that only 25% to 30% of cells isolated from the stratified FT

attached, whereas 60% to 80% of the outgrowth do so. After overnight incubation, the medium was complemented with 0.25 $\mu\text{g}/\text{mL}$ JC1 (Axxora, San Diego, CA) for 45 minutes. The attached cells were washed twice with PBS, released by a 2- to 3-minute trypsinization, spun down, resuspended in ice-cold Hanks' balanced salt solution + 4% FBS + 1 $\mu\text{g}/\text{mL}$ propidium iodide (PI; Invitrogen) solution (fluorescence-activated cell sorting buffer), and subjected to flow cytometry for analysis and/or sorting. Modifications to this basic protocol included addition of either (1) one of three ABCG2-specific inhibitors: FTC²⁸ (Sigma), Ko143^{29,30} (Tocris, Ellisville, MO), or glafenine³¹ (Fisher, Fair Lawn, NJ); (2) the ABCG2/MDR inhibitor MK571³² (Fisher); or (3) the ABCB/MDR and the ABCB1/MDR1 inhibitor ivermectin.³³ For the simultaneous study of JC1 and Hoechst 33342 (Invitrogen) exclusion, the latter dye was added to the dye incubation 45 minutes before the addition of JC1, after which flow cytometry was performed (Accuri 6; Accuri, Ann Arbor, MI; Accuri data were analyzed using the instrument software; or Influx; BD, Sparks, MD; data from the Influx cytometer were processed using FCS Express [Toronto, Ontario, Canada]). Fluorescent emissions from UV excitation were collected at 450 nm (Hoechst blue) and 692 nm (Hoechst red); emissions from the 488-nm laser were collected at 531 nm (green) and 572 nm (red) for JC1 and at 610 nm for PI. Aggregated (elevated forward scatter width parameter) and dead (PI^{high}) cells were excluded from the analysis.

Colony Formation Efficiency

Sorted rabbit cells were seeded in triplicate on culture vessels preseeded with lethally irradiated 3T3 fibroblasts. The culture was continued for up to 14 days with medium replacements at days 4, 7, 10, and 12. Cultures were fixed in cold methanol and stained with triphenylmethane dye (Coomassie Brilliant Blue R). Stain images were captured using an office scanner.

Histology

Human and rabbit explant cultures were fixed at various times in 4% paraformaldehyde solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Live explant cultures were observed in an inverted phase-contrast microscope. Images in each case were captured using a digital camera (Nikon D-50).

Toxicity Studies

SV40 immortalized human corneal epithelial cells (svHCECs)³⁴ were generously provided by Araki Sasaki (Ideta Eye Hospital, Kumamoto City, Kumamoto, Japan). To estimate dye washout time, stained cells were washed twice with PBS and either trypsinized for 10 minutes immediately afterward ($t = 0$) or returned to culture for 2 or 9 hours before trypsinization. The average dye remaining in the cells at 2 and 9 hours was determined from the emission intensity at these times relative to the intensity at $t = 0$. Each measurement was performed in duplicate using 10,000 cells per count. To assess dye toxicity, the svHCECs were seeded at low density in six-well plates in four replicates; 6 hours later the cells were treated with either JC1 or Hoechst 33342, extracellular dyes were washed away, and the cells were cultured for 72 hours. Cells were then trypsinized for 45 minutes in 2 mL solution to ensure complete cell-cell dissociation. Suspension of the cells was complemented with 100 μL buffered 10% formalin and cell numbers were accurately determined using the flow cytometer cell-counting software (Accuri 6).³⁵

To determine the effect, if any, of JC1 on clonogenic cells, recultured rabbit cells obtained from a first explant culture round were incubated with JC1 as described earlier, trypsinized, and seeded on feeder cells in duplicate using two untreated cultures as controls. After 14 days, the cultures were stained and compared for CFE.

RESULTS

JC1 Staining Generates an ABCG2-Dependent SP Equivalent to That Generated by Hoechst 33342 with Very Low Toxicity

JC1, an argon laser (488 nm)-excitable dye, accumulates in the inner mitochondrial membrane, where it displays a concentration- or aggregation-dependent bathochromic shift in emission.³⁶ When freshly isolated limbal or conjunctival human or rabbit epithelial cells are stained with this dye at selected concentrations and incubation times and subjected to analysis by flow cytometry, green versus red (531 vs. 572 nm) emission plots include a cell small cohort displaying reduced staining and a higher green/red ratio than that of the general cell population (Figs. 2A, 2B). In these features, the JC1^{low} cohort resembles the SC-rich, Hoechst 33342-excluding (Hoechst^{low}) cohort observed in these same cells in blue versus red (405 vs. 670 nm) emission plots generated by UV excitation, and known as the SP.^{10,11,17,19} Experiments in which cells were simultaneously stained with both dyes demonstrate that the Hoechst^{low} and JC1^{low} populations incorporate the same cells (Figs. 2C, 2D). Additionally, the fact that the size of JC1^{low} is markedly reduced by three different ABCG2 inhibitors used previously in studies with Hoechst, Ko143 (Figs. 3A, 3D), glafenine (Figs. 3B, 3E), and FTC (Figs. 3F, 3H, 3I), provides strong support for the notion that the JC1^{low} phenotype reflects the activity of the stem cell-associated ABCG2 transporter. Furthermore, neither 20 μM MK571³¹ (Fig. 3J) nor 10 μM ivermectin (not shown)³² reduced the JC1^{low}. These agents have been shown to respectively inhibit the activity of ABCG2/MDR and ABCB1/MDR1. Thus, these alternative multidrug resistance transporters could not be responsible to any measurable degree for the generation of JC1^{low}. Finally, it should be noted that, although the studies presented in Figure 3 were made with high JC1^{low}-content cells derived from explant outgrowths (see following text), they have also been confirmed in cells obtained from FT.

JC1 Toxicity

To our knowledge, the toxicity of JC1 has not been previously evaluated. Thus, to assess its potential use for the isolation of viable ABCG2-rich cells, we studied the effect of the dye incubation conditions used earlier to identify ABCG2^{high} cells on overall cell survival. JC1 had minimal or no effect on either the cell proliferation of svHCECs (Fig. 4A) or the CFE of rabbit limbal cells (Fig. 4B). In contrast, the Hoechst 33342 exposure had substantial detrimental effects on the proliferation of svHCECs (Fig. 4A), consistent with several recent reports on Hoechst toxicity in multiple cell types.³⁷⁻³⁹ A slow washout rate may be a critical component of this Hoechst toxicity (Fig. 4C), which may be further aggravated during cell sorting by exposure of the Hoechst-DNA complex to UV (405 nm) radiation.⁴⁰ Since, in addition, the use of JC1 has several practical advantages related to the limited availability of UV laser-equipped flow cytometers, most of the experiments in this study were performed with the mitochondrial dye.

Content of JC1^{low} Cells in Freshly Isolated Limbal Epithelial Cells

Freshly isolated human and rabbit limbal cells subjected to overnight reculture as described in Figure 1 contained, respectively, $1.4\% \pm 0.9\%$ ($n = 3$) and $4.1\% \pm 1.5\%$ ($n = 5$) JC1^{low} cells. These percentiles are similar to the SP percentiles previously reported from the studies with Hoechst 33342.^{17,19,27,40,41}

FIGURE 2. JC1 dual-emission plots and the reciprocal relationships between JC1^{low} and Hoechst 33342 SP cohorts. (A, B) JC1 plots of freshly isolated rabbit conjunctival and human limbal epithelial cells, respectively. The JC1^{low} and JC1^{main} domains are indicated. (C, D) Plots in freshly isolated rabbit limbal epithelial cells simultaneously incubated with 4 μ g/mL Hoechst 33342 for 90 minutes and with 0.25 μ g/mL JC1 for the last 45 minutes. The *insert* in (C) shows the location of the Hoechst SP in the JC1 plot, as indicated by the *intraframe arrow*. The *insert* in (D) shows the location of the JC1^{low} cells in the Hoechst 33342 emission plot. It is visually obvious that the two dye-exclusion cohorts identify the same set of cells. The difference in JC1^{main} shape between (B) and (C) results from a spectral alteration induced by Hoechst. The source of this effect is unknown.

Gross Morphologic Features of Explant Cultures over the Synthetic PET Membrane

Figures 5A and 5B show representative H&E-stained cross-sections of limbal explant cultures, from a first round of human and a second round of rabbit tissue, respectively. On the explant itself, epithelial stratification was preserved and basal cells within the Palisades remained very small. Stratification decreased toward the edges of the explant and the cultures became mostly single layered away from the explant. In addition, the cells also infiltrated under the explant, adhering to the PET membrane (Fig. 5A) or to both membrane and explant underside (Fig. 5B) matrix. Representative phase-contrast micrographs of the human and rabbit explant cultures at various stages of outgrowth development are shown in Figures 5C-I. At this morphologic level we could not observe marked differences in either the rate of growth or the morphology of the outgrown cells between the different rounds of explant from either species. On the other hand, it was apparent that, with time, the explant itself underwent gradual loss of morphologic features and size suggestive of matrix degradation (Figs. 5F, 5H) and the frequency of contamination of the epithelial outgrowth by fibroblasts increased. Occasionally, when the explant decay events proceeded at a slower pace we were able to harvest up to five consecutive outgrowths, the last one after 45 days of continuous explant culture.

Content of JC1^{low} Cells in Cells Outgrown from Limbal Explants in Sequential Stages

To determine ABCG2 activity in the explant cultures, attempts were initially made to stain these cultures with JC1 in situ. However, subsequent dissociation of the outgrowths by direct transfer to trypsin-EDTA required as much as a 40-minute incubation under vigorous agitation, a treatment that caused both stain fading and cell damage. In addition, it was difficult to excise the explant for further culture without causing physical

stress to the outgrowth cells adjacent to it. Thus, we developed the protocol described earlier in the Materials and Methods section (Fig. 1), where cell-cell contacts are initially weakened by culturing in low-calcium medium, thereby shortening the time needed for enzymatic treatment and facilitating the removal of the explant for further culture. The trypsinized outgrowth cells were cultured overnight at low density to allow for metabolic recovery, as previously implemented with limbal and conjunctival cells recovered from FT.²⁷ Use of low seeding densities during the reculture period prevents promotion of cellular differentiation via cell-cell contacts, ensures that cells have complete and equal access to the staining dye, and facilitates cell recovery by a brief 2- to 3-minute trypsinization. Seeding the cells at higher densities yielded very similar results but total cell yields were lower due to formation of floating cell aggregates that prevent attachment and the establishment of groups of cells firmly attached to one another, which necessitated increased trypsinization times to generate cell suspension free of aggregates. Approximately 90% of the attached cells showed a cytokeratin staining pattern (K14+/K12- or K14+/K3-) consistent with basal, undifferentiated epithelial origin (not shown).

A pilot study was performed to assess the effect of time and/or culture status on the content of JC1^{low} cells. A single quarter arc of human limbus incorporating prominent Palisades of Vogt was split into six nearly identical segments and cultured. After visually confirming that all six explants displayed a similar size and distribution by day 10, two of the explants were immediately processed, two explants were maintained for 4 more days, and the remaining two explants were continued until day 18, approximately 4 days past visual confirmation of culture confluence, before starting the same processing protocol. SP yields for all three groups were 23.3%, 20.0%, and 7.2%, respectively. The lower percentile of the latter group is likely to reflect accelerated differentiation. Thus,

FIGURE 3. Representative images of *green/red* JC1 emission plots of cells derived from explant culture outgrowths at various culture rounds and the effect of ABCG2 transporters on the magnitude of the JC1^{low} cohort. (A–C) Plots of pooled human explant culture outgrowths from a single set of six explants after the first 10 days of culture (A), after 12 days and an additional 8 days after transference to a new insert (B), or for 12 days followed twice by 8 days of culture (C). (D, E) Plots for the populations shown in (A) and (B) when the JC1 incubation solution contained 25 μ M Ko143 (D) or 10 μ M glafenine (E). (F) Composite showing the highest JC1 levels observed (in a second round of rabbit limbal explant) and the reduction of JC1^{low} content by 10 μ M FTC. (H–J) Plots from cell harvested from a first round explant culture incubated with JC1 in control condition (H) or in the presence of 20 μ M FTC (I) or 20 μ M MK 571 (J).

the final protocol used was designed to avoid the formation of confluent cultures.

JC1^{low} levels in the outgrowths from the first, second, and third culture stages were: 19.3 ± 4.4 (6), 27.4 ± 4.8 (6), and 13.7 ± 3.5 (3) for human and 25.8 ± 6.4 (6), 32.5 ± 5.0 (6), and 18.4 ± 3.6 (3) for rabbit explant outgrowths, respectively.

These percentiles along with those from FT cells are graphically displayed in Figure 6. The increases in JC1^{low} percentiles in the second explant round with respect to the first one were statistically significant. JC1^{low} percentiles diminished in the third round. Figures 3A–C show flow cytometry plots for the three rounds of a single human experiment.

Experiments were also performed using central corneal tissue. Recent studies suggest that this area also incorporates SCs with regenerative properties.⁴² When using approximately 1-mm-wide biopsies we either failed to establish cell growth altogether or obtained only small outcrops of large and flat cells, with the morphologic phenotype of differentiated corneal cells ($n = 6$ for human). Since some degree of growth was attained when using full quarters of central cornea, to quantify the relative growth potential of the corneal zone, we compared the yield of six limbal biopsies with that of six central corneal quarters, all generated from two corneas obtained from a single 35-year-old donor. After 12 days of culture, cells were harvested from all explants. A 45-minute trypsinization step was used to attain full cell-cell dissociation, and cells were then pooled and immediately counted by flow cytometry. The limbal tissue yielded an average of 746,000 cells/per explant strip; the corneal tissue generated only 126,800 cells/quarter corneas. Limiting the count to PI negative (live) cells the equivalent counts were 689,300 and 89,700, respectively. Since the epithelial area of these quarters is six- to eightfold larger than that of the explanted limbal segments, it appears that the epithelial cell growth potential of the limbal zone is at least 20-fold greater than that of the cornea. Furthermore, very few corneal-derived cells attached during the overnight reculture period (Fig. 1), indicating that the yield from the cornea consisted mainly of cells that have already substantially advanced along the differentiation path. In the case of rabbit, the full corneal quarters did yield explants similar to those obtained from narrow limbal biopsies and the explanted population was found to incorporate 17% of JC1^{low} SP cells ($n = 6$). However, once transferred to new inserts to continue the culture, these explants failed to generate further outgrowth.

FIGURE 4. Dye washout and toxicity assays. (A) Rate of dye washout in svHCEC cultured cells. Amounts of JC1 (■) and Hoechst 33342 (●) were determined according to the decrease in emissions at 531 and 405 nm, respectively. (B) Effect of JC1 on rabbit CFE. Five hundred rabbit outgrowth cells from first culture explant round seeded in each flask. *Top flask* is a control; the *bottom flask* is JC1 treated. (C) Relative cell numbers present in svHCEC 72-hour cultures in control condition, or after exposure to JC1 or Hoechst 33342 under the respective staining conditions described in Materials and Methods. * $P < 0.05$, $n = 4$).

FIGURE 5. Morphologic features of the explant culture. (A) H&E stain of a first-round human explant culture. Cut was made perpendicular to the larger axis of the explant. The *insert* shows selected features at higher magnification. (B) Phase-contrast micrograph of a human explant after 6 days of culture. After a 1- to 2-day initial delay, the cells are growing rapidly. At the edges (*white arrow* in *insert*, $\times 2$) the cells are elongated in perpendicular to the expansion axis. (The homogeneously distributed $0.1\text{-}\mu\text{m}$ *white spots* represent the phase-contrast image of the PET membrane pores.) Explanting cells in direct contact with the substratum form a homogeneous mosaic (*left insert*, $\times 2$). The white protrusions in the proximity of the biopsy (*dual black arrowheads*) seem to result from outgrowth of cells over the existing basal cell layer. (C) H&E stain of a second round rabbit explant culture. Cut was made in parallel to the larger axis of the explant. Three higher magnifications of selected zones are included *inserts*. (D, E) Phase-contrast micrographs of rabbit explant cultures from a first (D, E), third (F, G), and fourth (H, I) explant culture rounds, respectively.

CFE of JC1^{low} Cells Isolated from FT and Limbal Explant Outgrowths

Similar to the previously observed low clonogenicity of SP cells vis-à-vis that for non-SP cells,^{17,19} the rabbit JC1^{low} cells obtained from FT limbal displayed substantially lower CFEs than those of the matching JC1^{main} cells (Figs. 7A, 7B). In contrast, the CFE of rabbit JC1^{low} sorted from the outgrowths of the first (Figs. 6C, 6D) and the second (Figs. 7E, 7F) rounds of explant culture were much higher than those of the JC1^{main} cells; that is, not only did the percentage of JC1^{low} cells increase dramatically but there was a large increase in the CFE of these cells, causing an inversion of the JC1^{low}/JC1^{main} CFE relationship.

FIGURE 6. Percentage of JC1^{low} cells in cell populations derived from fresh tissue (FT) or from the outgrowths from the first (1st), second (2nd), and third (3rd) rounds of explant culture. (A) Rabbit. (B) Human. * $P < 0.05$, $n = 6$.

When cultured for 14 days, the larger clones grew to approximately 2-cm diameter (Figs. 7E, 7F). Under microscopic examination we calculated that these large colonies contain a minimum of 200,000 cells, a number that requires 21 cell doublings (approximately one doubling every 14 to 16 hours). CFEs for the JC1^{low} and JC1^{main} cells for four independent first culture round experiments were $12.3\% \pm 4.0\%$ and $0.85\% \pm 0.64\%$ ($P < 0.01$), respectively. JC1^{low} accounts for 25.8% of this rabbit cell population (Fig. 6). Thus, the JC1^{low} cell cohort contains the majority ($>83\%$) of the rabbit cells able to initiate clonal growth under feeder cell support.

DISCUSSION

Various different approaches are currently used to expand limbal populations. In one approach, cells are grown from limbal stroma-epithelial explants as outwardly radiating cultures.^{22–26} The limbal stromal component is thought to provide niche-like functions that help to maintain progenitor properties in the outgrowing cells proximal to the explants.²⁷ In this study, we have used the exclusion of JC1, a substratum that effectively replaces Hoechst 33342 in the identification of cells with high level of ABCG2-dependent efflux transport and generates a cell-segregated cell cohort very similar to that generated by exclusion of the DNA binding dye, generically known as the SP. Culturing was performed over permeable synthetic substrata to avoid any confounding effects of more complex biological supports to the phenotype of the growing

FIGURE 7. Colony formation by rabbit cells sorted according to JC1 stain. (A and B) 9-day colonies from cells isolated from fresh limbal tissue. (C and D) 12-day colonies from cells isolated from a first round explant. (E and F) 14-day colonies from cells isolated from a second explant culture round. A, C, and D: JC1^{low} cells. B, D, and E: JC1^{main} cells. Bar, 2 cm.

cells and using an explant transfer approach (Fig. 1) that facilitated the extension of the culture for over a month. Bradshaw et al.⁴³ used a similar approach to study the transduction of corneal epithelial progenitor cells by a retroviral vector.

The dependence of the JC1^{low} phenotype on ABCG2 transport was confirmed by the effect of multiple specific inhibitors on the extent of dye exclusion. The relationship of the exclusion phenotype to the stem/progenitor cell status was confirmed by the ability of the sorted cells to generate large colonies requiring >20 population doublings. Further studies should determine how these JC1^{low} cells relate to bona fide stem cells as defined by more rigorous criteria, such as the sequential clonal yield test introduced by Barrandon and Green⁴⁴ for the definition of keratinocyte stem cells.

Our investigation led to four main findings. First, a large fraction of the explanted cells exhibit the JC1^{low} phenotype. In light of the previous results regarding SP cell content in human, rabbit, and pig ocular surface epithelia, and the typical low percentiles reported for this phenotype elsewhere, this was an unexpected result. To our knowledge, the JC1^{low} percentiles reported in this study greatly exceed the SP percentiles reported for any other normal mammalian cell populations. Since the JC1^{low} cells display high clonogenicity, this fact suggests that the explant-outgrowth culture model provides favorable conditions for stem/progenitor replication. However, since a straightforward relationship between high clono-

genicity and long-term repopulating ability has been recently called into question,^{45,46} the validity of this suggestion warrants further examination.

Second, JC1^{low} cells from the explant outgrowths cells have a much larger CFE than that of the homonymous cells isolated from FT (Fig. 7). The difference may reflect the biological status of the JC1 excluding cells in vivo and in these cultures. In vivo, SP stem cells are in a slow cycling/quiescent state¹⁷ that may hinder the ex vivo activation,^{17,27,41} whereas they are actively proliferating in the outgrowths. A comparable observation of CFE augmentation by incubation in an in vitro niche-like environment was made for bone marrow SP cells.¹⁶ Another possibility, however, is that the very conditions of the explant culture used induce the overexpression of ABCG2, independently of the SP status of the originating cells. In either case the relationship between native and explant culture expanded cells with the JC1^{low} phenotype, in terms of actual long-term regenerative capacity,⁴⁶ remains to be examined.

Third, the percentile of SP cells in the outgrowths increased over time, at least temporarily. This observation raises the possibility that bona fide or “core” epithelial SCs do not migrate out of the explant, but remain in their natural niche environment, supporting an outward migrating progeny. The SP percentile will increase because in the initial growth stage transient amplifying cells can be expected to be the main source of outgrowing cells, but as these cells wane, activated SCs are expected to become the dominant cell source. In that case, culture conditions that prevent the observed decay of explant features may provide means for extended production of cells for autologous regenerative procedures.

Fourth, the SP population comprises the majority of the clonogenic cells present in the outgrowth. Thus, assuming that indeed the regenerative potential rests primarily on cells exhibiting high ABCG2-dependent dye exclusion, flow cytometry of JC1, Hoechst 33342, or any other useful transporter substrata could be used to determine the relative regenerative capacity of cultured limbal populations.

Finally, it should be noted that the cellular features within the limbal explant itself have not been fully examined. If the cells with the highest reproductive capacity remain trapped in the explant, the outgrowths may not incorporate the cells with maximal regenerative capacity.

References

- Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol.* 1986;103:49–62.
- Chen JJ, Tseng SC. Abnormal corneal epithelial wound healing in partial-thickness removal of limbal epithelium. *Invest Ophthalmol Vis Sci.* 1991;32:2219–2233.
- Huang AJ, Tseng SC. Corneal epithelial wound healing in the absence of limbal epithelium. *Invest Ophthalmol Vis Sci.* 1991;32:96–105.
- Kenyon KR, Tseng SC. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology.* 1989;96:709–722.
- Tsai RJ, Tseng SC. Human allograft limbal transplantation for corneal surface reconstruction. *Cornea.* 1994;13:389–400.
- Tsubota K, Satake Y, Ohshima M, et al. Surgical reconstruction of the ocular surface in advanced ocular cicatricial pemphigoid and Stevens-Johnson syndrome. *Am J Ophthalmol.* 1996;122:38–52.
- Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet.* 1996;349:990–993.
- Kolli S, Ahmad S, Lako M, Figueiredo F. Successful clinical implementation of corneal epithelial stem cell therapy for treatment of unilateral limbal stem cell deficiency. *Stem Cells.* 2010;28:597–610.

9. Rama P, Matuska S, Paganoni G, Spinelli A, De Luca M, Pellegrini G. Limbal stem-cell therapy and long-term corneal regeneration. *N Engl J Med*. 2010;363:147-155.
10. 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.
11. 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.
12. Lassalle B, Bastos H, Louis JP, et al. "Side population" cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. *Development*. 2004;131:479-487.
13. Murayama A, Matsuzaki Y, Kawaguchi A, Shimazaki T, Okano H. Flow cytometric analysis of neural stem cells in the developing and adult mouse brain. *J Neurosci Res*. 2002;69:837-847.
14. Ono M, Maruyama T, Masuda H, et al. Side population in human uterine myometrium displays phenotypic and functional characteristics of myometrial stem cells. *Proc Natl Acad Sci USA*. 2007;104:8700-8705.
15. Hoshi N, Kusakabe T, Taylor BJ, Kimura S. Side population cells in the mouse thyroid exhibit stem/progenitor cell-like characteristics. *Endocrinology*. 2007;148:4251-4258.
16. Goodell MA, Rosenzweig M, Kim H, et al. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat Med*. 1997;3:1337-1345.
17. 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.
18. 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.
19. Umemoto T, Yamato M, Nishida K, Yang J, Tano Y, Okano T. Limbal epithelial side population cells have stem cell-like properties, including quiescent state. *Stem Cells*. 2006;24:86-94.
20. 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.
21. Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell*. 1989;57:201-209.
22. Ti SE, Anderson D, Touhami A, Kim C, Tseng SC. Factors affecting outcome following transplantation of ex vivo expanded limbal epithelium on amniotic membrane for total limbal deficiency in rabbits. *Invest Ophthalmol Vis Sci*. 2002;43:2584-2592.
23. Wang DY, Hsueh YJ, Yang VC, Chen JK. Propagation and phenotypic preservation of rabbit limbal epithelial cells on amniotic membrane. *Invest Ophthalmol Vis Sci*. 2003;44:4698-4704.
24. Kim H-S, Song XJ, de Paiva CS, Chen Z, Pflugfelder SC, Li D-Q. Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explant and single cell cultures. *Exp Eye Res*. 2004;79:41-49.
25. Li W, Hayashida Y, He H, Kuo CL, Tseng SC. The fate of limbal epithelial progenitor cells during explant culture on intact amniotic membrane. *Invest Ophthalmol Vis Sci*. 2007;48:605-613.
26. Kolli S, Lako M, Figueiredo F, Mudhar H, Ahmad S. Loss of corneal epithelial stem cell properties in outgrowths from human limbal explants cultured on intact amniotic membrane. *Regen Med*. 2008;3:329-342.
27. Akinci MA, Turner H, Taveras M, Wolosin JM. Differential gene expression in the pig limbal side population: implications for stem cell cycling, replication, and survival. *Invest Ophthalmol Vis Sci*. 2009;50:5630-5638.
28. Rabindran SK, Ross DD, Doyle LA, Yang W, Greenberger LM. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. *Cancer Res*. 2000;60:47-50.
29. Mattsson P, Pedersen JM, Norinder U, Bergström CA, Artursson P. Identification of novel specific and general inhibitors of the three major human ATP-binding cassette transporters P-gp, BCRP and MRP2 among registered drugs. *Pharm Res*. 2009;26:1816-1831.
30. Kwatra D, Vadlapatla RK, Vadlapudi AD, Pal D, Mitra AK. Interaction of gatifloxacin with efflux transporters: a possible mechanism for drug resistance. *Int J Pharm*. 2010;395:114-121.
31. Zhang Y, Byun Y, Ren YR, Liu JO, Laterra J, Pomper MG. Identification of inhibitors of ABCG2 by a bioluminescence imaging-based high-throughput assay. *Cancer Res*. 2009;69:5867-5875.
32. Gekeler V, Ise W, Sanders KH, Ulrich WR, Beck J. The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. *Biochem Biophys Res Commun*. 1995;208:345-352.
33. Dupuy J, Alvinerie M, Ménez C, Lespine A. Interaction of anthelmintic drugs with P-glycoprotein in recombinant LLC-PK1-mdr1a cells. *Chem Biol Interact*. 2010;186:280-286.
34. Araki-Sasaki K, Ohashi Y, Sasabe T, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci*. 1995;36:614-621.
35. Wang Z, Reinach PS, Zhang F, et al. DUSP5 and DUSP6 modulate corneal epithelial cell proliferation. *Mol Vis*. 2010;16:1696-1704.
36. Smiley ST, Reers M, Mottola-Hartstorn C, et al. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc Natl Acad Sci USA*. 1991;88:3671-3675.
37. Kallestad KM, McLoon LK. Defining the heterogeneity of skeletal muscle-derived side and main population cells isolated immediately ex vivo. *J Cell Physiol*. 2010;222:676-684.
38. Chu K, Lee S. Revisiting calcein AM: alternative tool for identifying dye-effluxing cancer stem cells? *Cancer Biol Ther*. 2009;8:2206-2208.
39. Liu WH, Qian NS, Li R, Dou KF. Replacing Hoechst33342 with rhodamine123 in isolation of cancer stem-like cells from the MHCC97 cell line. *Toxicol In Vitro*. 2010;24:538-545.
40. Purschke M, Rubio N, Held KD, Redmond RW. Phototoxicity of Hoechst 33342 in time-lapse fluorescence microscopy. *Photochem Photobiol Sci*. 2010;9:1634-1639.
41. Akinci MA, Turner H, Taveras M, et al. Molecular profiling of conjunctival epithelial side-population stem cells: atypical cell surface markers and sources of a slow-cycling phenotype. *Invest Ophthalmol Vis Sci*. 2009;50:4162-4172.
42. Majo F, Rochat A, Nicolas M, Jaoudé GA, Barrandon Y. Oligopotent stem cells are distributed throughout the mammalian ocular surface. *Nature*. 2008;456:250-254.
43. Bradshaw JJ, Obritsch WF, Cho BJ, Gregerson DS, Holland EJ. Ex vivo transduction of corneal epithelial progenitor cells using a retroviral vector. *Invest Ophthalmol Vis Sci*. 1999;40:230-235.
44. Barrandon Y, Green H. Three clonal types of keratinocyte with different capacities for multiplication. *Proc Natl Acad Sci USA*. 1987;84:2302-2306.
45. Strachan LR, Scalapino KJ, Lawrence HJ, Ghadially R. Rapid adhesion to collagen isolates murine keratinocytes with limited long-term repopulating ability in vivo despite high clonogenicity in vitro. *Stem Cells*. 2008;26:235-243.
46. Strachan LR, Ghadially R. Limiting dilution analysis of murine epidermal stem cells using an in vivo regeneration assay. *Methods Mol Biol*. 2010;585:421-432. n