Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells.

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Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells

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Summary

When cell populations are incubated with the DNA-binding dye Hoechst 33342 and subjected to flow cytometry analysis for Hoechst 33342 emissions, active efflux of the dye by the ABCG2/BCRP1 transporter causes certain cells to appear as a segregated cohort, known as a side population (SP). Stem cells from several tissues have been shown to possess the SP phenotype. As the lack of specific surface markers has hindered the isolation and subsequent biochemical characterization of epithelial stem cells this study sought to determine the existence of SP cells and expression of ABCG2 in the epithelia of the ocular surface and evaluate whether such SP cells had features associated with epithelial stem cells. Human and rabbit limbal-corneal and conjunctival epithelial cells were incubated with Hoechst 33342, and analyzed and sorted by flow cytometry. Sorted cells were subjected to several tests to determine whether the isolated SP cells displayed features consistent with the stem cell phenotype. Side populations amounting to <1% of total cells, which were sensitive to the ABCG2-inhibitor fumitremorgin C, were found in the conjunctival and limbal epithelia, but were absent from the stem cell-free corneal epithelium. Immunohistochemistry was used to establish the spatial expression pattern of ABCG2. The antigen was detected in clusters of conjunctival and limbal epithelia basal cells but was not present in the corneal epithelium. SP cells were characterized by extremely low light side scattering and contained a high percentage of cells that: showed slow cycling prior to tissue collection; exhibited an initial delay in proliferation after culturing; and displayed clonogenic capacity and resistance to phorbol-induced differentiation; all features that are consistent with a stem cell phenotype.

Key words: Stem cells, Side population, Epithelia, Cornea, Limbus, Conjunctiva

Introduction

The vertebrate ocular surface is lined with two stratified, constantly renewing tissues, the limbal/corneal and conjunctival epithelia. These two closely related but distinct cell lineages (Kruse et al., 1990; Wei et al., 1993; Wei et al., 1996; Moyer et al., 1996), arise simultaneously from a few PAX6-positive ectodermal cells that remain in the embryonic ectodermal surface following formation of the lens vesicle by the bulk of the PAX6+ cells (Davis and Reed, 1996; Koroma et al., 1995; Moyer et al., 1996). The self-renewing nature of the limbal/corneal and conjunctival epithelium makes their long-term survival ultimately dependent on small populations of stem cells. In the limbal/corneal epithelium, SCs are exclusively situated in the basal cell layer of the limbus, the outer vascular rim at the junction between the cornea and the conjunctiva (Schemer et al., 1986; Cotsarelis et al., 1989). The epithelium covering the transparent central cornea contains no stem cells (Schermer et al., 1986; Cotsarelis et al., 1989; Wolosin et al., 2000). Several lines of evidence suggest that conjunctival epithelial stem cells are concentrated in the fornix (Wei et al., 1995; Pellegrini et al., 1999), though stem cells may also be present in other areas (Pelligrini et al., 1999; Wirtschafter et al., 1999; Chen et al., 2002; Nagasaki and Zhao, 2005).

The ability to isolate viable somatic stem cells will provide new, invaluable means to characterize these cells and use them in gene therapy protocols (Bradfute and Goodell, 2002). The identification of a number of surface markers has facilitated the routine preparation of hematopoietic stem cells (Civin, 1992; Silvestri, 1992). Identification of comparable markers in epithelial systems has remained a challenging task. An alternative method for the purification of stem cells exploits their special spectral emission pattern following staining with the DNA dye Hoechst 33342 (Hoechst). In the free state the dye exhibits a fluorescence emission peak at 465 nm (Hoechst blue). In the DNA-bound state in live cells, electronic interactions between the densely packed Hoechst molecules cause a bathochromic shift leading to a secondary emission at 675 nm (Hoechst red). Hoechst-stained bone marrow cells subjected to flow cytometry reveal a population of cells displaying very low fluorescent emission intensities and a reduced bathochromic shift. The cohort, or side population...
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Below 1% of the maximal staining) were analyzed for parametric correlations and annotated using FCExpress (DeNovo Software, Toronto, Canada).

For direct microscopic observation, human limbal cells were spun down and gently resuspended in a minimal volume of HBSS with 0.1% Trypan Blue and examined in an inverted microscope using phase-contrast and transmitted light illuminations.

**BrdU labeling in vivo**

Three 2ML2 osmotic Alzet mini pumps (Durect, Cupertino, CA) calibrated to produce a constant delivery of ~24 mg bromodeoxyuridine (BrdU)/day for 14-16 days, were implanted subcutaneously into 3 kg (3-4 months old) rabbits. Most animals were sacrificed 11 days later, when continuous steady state label pumping had proceeded for 10 days. In one case the pumps were removed after 17 days, and the animal was left undisturbed for 38 days prior to sacrifice and tissue collection. Small segments of the limbal/corneal and conjunctival tissues were fixed overnight at 4°C in 50 mM glycine, 70% ethanol, pH 3.0 and embedded in paraffin. The majority of the tissue was used to isolate the limbal and conjunctival epithelium, sort single cell suspensions by flow cytometry and to determine the BrdU content of the sorted fractions.

**BrdU detection in tissue sections**

Paraffin-embedded tissues were cut into 4-µm-thick sections, deparaffinized, rehydrated in PBS, immersed in 2N HCl for 30 minutes and digested with trypsin for 15 minutes. BrdU incorporation was revealed by indirect immunofluorescence. Sections were blocked in 5% goat serum in PBS (T-PBS). After washings, sections were stained for 1 hour with 1 µg/ml Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR), counterstained with 0.01% PI-PBS, mounted with anti-fading medium and photographed in an Olympus epifluorescence microscope equipped for green (for Alexa 488), red (for PI) and dual fluorescence image capture.

**BrdU detection in sorted cells**

Limbal and conjunctival epithelial cells were alternatively sorted according to their Hoechst 33342 staining, or light-scattering properties. Sorted cells were either cytospun onto poly-L-lysine-coated glass slides using a Shandon Cytospin3 centrifuge (Shandon, Wyman, MA) or concentrated and plated overnight on the slides for selection of adherent (i.e. basal) cells. Slides were immersed in 70% ethanol for 30 minutes, briefly air-dried, immersed in 0.07 N NaOH for 2 minutes, overlaid with FITC-conjugated monoclonal anti-BrdU antibody (Pharmingen, San Diego, CA) prepared in T-PBS for 30 minutes, counterstained with 0.01% PI-PBS and mounted and photographed as described above. BrdU-free cells were processed in parallel to serve as controls.

**ABCG2 detection in tissue sections**

Freshly dissected tissue segments were embedded in Cryomatrix™ (Shandon, Pittsburgh, PA), frozen in liquid nitrogen, cut into 8- to 10-µm-thick sections in a cryotome, collected on gelatin-coated glass slides. The sections were fixed in cold methanol, briefly air-dried, rehydrated in PBS and blocked with 5% goat serum. ABCG2 was detected by indirect immunofluorescence using one of two anti-ABCG2 monoclonal antibodies, BXP21 (Chemicon, Temecula, CA) and BXP34 (Alexis, San Diego, CA) as the primary reagent and Alexa 488 goat anti-mouse IgG as the secondary antibody. Both primary antibodies recognize internal epitopes within the C-terminus of human ABCG2 (Mallepaard et al., 2001). Stained samples were mounted and photographed as described above.

**Flow cytometry**

Pure epithelial sheets were incubated for 20-30 minutes at 37°C in trypsin. When >90% of the population consisted of single cells, the suspension was complemented with one volume of hDF-12 containing 20% fetal calf serum (FCS), cells were dispersed using a fire-polished Pasteur pipette, sequentially filtered through 40 µm and 10 µm nylon meshes, suspended at 10^6 cells/ml in hDF-12 containing 2% FCS, and incubated with 2.5 µg Hoechst (Sigma, St Louis, MO) for 45-90 minutes at 37°C under slow swirling motion. To inhibit ABC transporters, 10 µM reserpine (Sigma), 10-100 µM verapamil (Sigma) or 10 µM fumitremorgin C (a generous gift from Susan Bates, NCI, Bethesda, MD) were added 5 minutes before the addition of Hoechst. Cells were spun down, resuspended at 10^6 cells/ml in HBSS with 4% FCS and 2 µg/ml propidium iodide (PI; Roche, Indianapolis, IN) dissolved in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered 1:1 mix of Dulbecco’s modified Eagle’s medium and Ham-F12 (hDF/F-12) with moderate shaking. This results in the spontaneous release of whole epithelial sheets.

**Materials and Methods**

**Tissue procurement and processing**

Rabbit tissues were dissected from eyes obtained from local abattoirs. Human corneas and conjunctivae unsuitable for human transplant, all from unidentifiable donors, were obtained from the National Disease Research Interchange (Philadelphia, PA) within 48 hours of collection. For the cell kinetics studies (see below), 3-month-old albino New Zealand rabbits were housed in an American Association of Laboratory Animal Care-accredited facility with free access to food and water. All animal procedures complied with the USPHS policy on humane care and use of laboratory animals. For limbal dissection, corneas were quartered, and after careful trimming of conjunctival remnants, placed and surgically dissect the rabbit limbus as a discrete 0.5-0.8 mm-wide whitish outer strip and the human limbus as a discrete ~1-mm-wide zone containing the Palisades of Vogt. Tissues were incubated for 16-17 days, and after continuous steady state label pumping had proceeded for 14-16 days, were implanted subcutaneously into 3 kg (3-4 months old) rabbits. Most animals were sacrificed 11 days later, when continuous steady state label pumping had proceeded for 10 days. In one case the pumps were removed after 17 days, and the animal was left undisturbed for 38 days prior to sacrifice and tissue collection. Small segments of the limbal/corneal and conjunctival tissues were fixed overnight at 4°C in 50 mM glycine, 70% ethanol, pH 3.0 and embedded in paraffin. The majority of the tissue was used to isolate the limbal and conjunctival epithelium, sort single cell suspensions by flow cytometry and to determine the BrdU content of the sorted fractions.
SP cells were present in rabbit and human limbal and conjunctival epithelia (Fig. 1A-D and Table 1). Inclusion of the ABCG2-specific inhibitor fumitremorgin C (Rabindran et al., 2000; Robey et al., 2001; Nakanishi et al., 2003) resulted in a 80-90% reduction in the size of the SPs in both ocular surface epithelia (Fig. 1E,F), confirming that the SP phenotype primarily reflects the activity of the ABCG2 transporter (Zhou et al., 2001). Reserpine, a powerful pan-ABC transporter inhibitor (Zhou et al., 2001; Murayama et al., 2002), completely abolished SP cells (Fig. 1D,F inserts). In contrast, verapamil caused only a 20-30% decrease in rabbit conjunctival SP cells, consistent with the demonstrated lack of effectiveness of this P-glycoprotein inhibitor on ABCG2 activity (Zhou et al., 2001; Ozvegy et al., 2001).

ABCG2 is expressed in the ocular surface epithelia in discreet clusters

The expression of the fumitremorgin C-sensitive ABCG2 transporter was investigated at the mRNA and protein levels.

Results

Limbal and conjunctival epithelia contain ABCG2-dependent side populations

Initial studies with rabbit cells established the best conditions for the generation of distinguishable SP cells. From these studies we concluded that the optimal Hoechst concentration for the conjunctival epithelium, when performing 90-minute incubations, occurs at 3.0-3.5 μM. For the limbal epithelium better results were obtained by reducing the dye concentration to 2.0-2.5 μM and the incubation time to 60 minutes.

Ocular epithelial side population stem cells

Fig. 1. Hoechst plots of human and rabbit ocular surface epithelial cells. Human (A,B) and rabbit (C-F) limbal cell plots obtained using 2.0 μM dye (left-hand graphs) and conjunctival cell plots using 3.0 μM dye (right-hand graphs). (C,D) Controls. (E,F) Identical experiments to C and D, respectively, but with 10 μM fumitremorgin C added 5 minutes prior to the introduction of Hoechst. Inserts in D and F are a different experiment with rabbit conjunctiva in control condition or with 20 μM reserpine. The three main zones present in these plots, the SP, the main cohort of cells containing one copy of DNA (cells in G0-G1) and the cohort of most cells containing DNA in excess of one copy by virtue of being in the S, G2 or M phase of the cell cycle (S-G2/M) are indicated.
Real-time quantitative PCR combined with product size confirmation by agarose chromatography (a single 71 bp band; not shown) demonstrated the expression of this protein throughout the ocular surface. Overall message levels were extremely low: in the limbal epithelium ABCG2 they amounted to 1/4800 of the β-actin value. Our measurements indicated very similar message content for the conjunctival and limbal epithelia (ratio=0.96). In contrast, in the stem and SP cell-free corneal epithelium the message amounted to only 1/26.3 of the level measured in the limbal counterpart.

Immunofluorescence detected ABCG2 protein in the periphery of both limbal and conjunctival epithelia cells. Both monoclonal antibodies used generated similar patterns but only results for the clone displaying lower background, BXP21, are shown (Fig. 2). In the limbal/corneal epithelium, ABCG2 antigenicity was observed exclusively in the limbal zone. The stain occurred in cell clusters comprising basal and proximal suprabasal cells. Stain intensity was higher in the cells at the center of each cluster (Fig. 2A-C). The greatest accumulation of such clusters occurred within the Palisades of Vogt (Townsend, 1991), the area generally believed to contain the highest concentration of stem cells within the human limbus. In some of the palisades proximal to the conjunctiva, all basal and suprabasal epithelial cells appeared to be ABCG2-positive.

There was no ABCG2 stain in the corneal zone, i.e., the small amount of ABCG2 message expressed in this area did not translate into any visible protein expression (Fig. 2D). In the conjunctival epithelium, ABCG2 stain was detected at low levels in a large fraction of the basal cells throughout the tissue, though it appears to be more intense in the Goblet cell-rich areas (Fig. 2E-G).

**Ocular epithelial SP cells display physical and proliferative features consistent with a stem cell phenotype**

Analysis of the correlation between light scattering and Hoechst emission features yielded significant insight into physical features of the ocular surface epithelia SP cells (Fig. 3). FSC is generated by particles that are relatively large with respect to the wavelength of the incident light, and thus, this parameter is considered to be proportional to the size of the cell. SSC is generated by particles in the range of, or smaller than, the length of the analyzing beam. Therefore, the magnitude of this signal reflects cell cytosolic and/or cell membrane complexity. The SP cells displayed low FSC, but more significantly, extremely low SSC (LSSC; Fig. 3A). Furthermore, analysis of the correlations between light scattering/Hoechst emission patterns in the opposite direction (Fig. 3B) revealed that only a fraction (~50% in most experiments) of the LSSC cells belong to the SP. To further characterize the SP cells, we compared these cells with cells collected from the core of the main cell cohort (G0-G1, see Fig. 1). Whereas G0-G1 cells ranged in size from between 12-
cells incorporated BrdU. A substantial fraction of the conjunctival (Fig. 6A) and limbal/corneal (Fig. 6D) epithelial infusion, histology revealed that a great proportion of the basal flow cytometry cohorts. After 10 days of continuous BrdU in the conjunctival and limbal epithelia and in their respective experiments aimed at identifying slow- and fast-cycling cells (Table 2, experiment number 1) using four rabbits simultaneously, half of the cells for each epithelium were sorted according to Hoechst staining to collect SP and non-SP (taken from the center of G0-G1 cohorts) cells. The other half was sorted according to light scattering properties, to collect LSSC and non-LSSC cells (taken from the center of the main cell group; see Fig. 3). The non-SP, non-LSSC samples, representing the majority of the cells in the population, contained BrdU cells in proportions similar to those observed in the tissue sections. The SP and LSSC fractions displayed much lower percentiles of BrdU+ cells (Fig. 7A,B). In another experiment SP and non-SP (G0-G1) cells were collected from the epithelia of a single rabbit and plated overnight to isolate spontaneously adherent (i.e. basal) cells. For the conjunctiva, 87% of the adherent G0-G1 cells were BrdU+ (Fig. 7C; Table 2, experiment number 2). In contrast, only 9% of the adherent SP cells contained BrdU (Fig. 7D). The results for the limbal cells were qualitatively similar, however, the very small number of cells recovered for the SP hindered a quantitative assessment for this tissue. Overall, these results are consistent with the notion that SP cells and, in fact, the overlapping LSSC cohorts consist mostly of cells that are not rapidly cycling.

To complement these studies, we performed an experiment (Table 2, experiment number 3) conforming to the classical protocol used to identify label-retaining cells (LRCs). BrdU is infused for a prolonged period of time. This causes the labeling of all rapidly dividing cells and of the fraction of the slow-cycling cells that happen to undergo division during the label infusion interval. After a subsequent chase period, only the slow-cycling cells, which remained quiescent or underwent only 1-2 divisions, could retain a detectable concentration of label (Leblond, 1964). Thus, based on the results of the BrdU uptake experiments, we extended the BrdU infusion to the full pump life (14-16 days), to maximize the initial labeling index and chased for a period of 38 days. As expected, after this chase period BrdU+ basal cells were scarce (Fig. 6). In the conjunctival epithelium, prominently stained cells were present in the Goblet cell-rich palpebral and fornical zones (Fig. 6B,C). Fewer BrdU-retaining basal cells were observed in the Goblet cell-free palpebral conjunctiva (Fig. 6B), or in the bulbar area (Fig. 6E). In the limbal epithelium, BrdU label was present in a fraction of the basal cells (Fig. 6E). Cells displaying various levels of staining were observed within the suprabasal strata in both epithelial lineages, in particular near the epithelial surface, suprabasal cells were also stained, reflecting the continuous stratifying activity of these tissues. BrdU+ cells were identified within the sorted populations (Fig. 7E-H). Percentiles of BrdU+ derived from these images are gathered in Table 2. In one experiment (Table 2, experiment number 1) only 4% of the SP cells were BrdU+ (Fig. 7G; Table 2, experiment number 2). In contrast, only 9% of the adherent SP cells contained BrdU (Fig. 7D). The results for the limbal cells were qualitatively similar, however, the very small number of cells recovered for the SP hindered a quantitative assessment for this tissue. Overall, these results are consistent with the notion that SP cells and, in fact, the overlapping LSSC cohorts consist mostly of cells that are not rapidly cycling.

Several experiments were performed to examine the presumptive stem cell status of the SP cells. Slow or infrequent cycling, is one of the most unique features of adult stem cells (Leblond, 1964; Bickenbach, 1981; Cotsarelis et al., 1989; Pavlovitch et al., 1991; Morris and Potten, 1994; Wei et al., 1995; Fortunel et al., 1998; Lehrer et al., 1998; Braun et al., 2003; Tumbar et al., 2004). Accordingly, we performed experiments aimed at identifying slow- and fast-cycling cells in the conjunctival and limbal epithelia and in their respective flow cytometry cohorts. After 10 days of continuous BrdU infusion, histology revealed that a great proportion of the basal conjunctival (Fig. 6A) and limbal/corneal (Fig. 6D) epithelial cells incorporated BrdU. A substantial fraction of the

Fig. 3. Reciprocal correlations between Hoechst emission and light scattering features in ocular surface cells. (A) Human limbal epithelium. The scatter plot for all the cells (All cells) shows a main cohort and a small group of cells (LSSC) displaying very low SSC values. Note that ~50% of the SP cells are LCCS cells and that some of the LSSC cells are not SP cells. (B) Representative experiment of the distribution of LSSC cells within the Hoechst plot in the rabbit conjunctiva. Note that the LSSC cells (zone highlighted with a gray mask) distribute equally between the SP and G0-G1 zone. Axis denominations are provided in capital italics within the frames.

Fig. 4. Phase-contrast micrographs of human limbal epithelial G0-G1 and SP cells. The non-SP cells were collected from the center of the G0-G1 spot. Bar, 30 µm.

Fig. 5. Hoechst and scatter plots of human corneal cells. Note the complete absence of SP and the paucity of LSSC cells.
indicating that label disappearance during the 6-week chase interval reflects not its only dilution through multiple cell divisions but also loss through cell exfoliation. Consistent with the tissue immunostaining patterns, cytometer-sorted conjunctival epithelial G0-G1 fraction, which represents the majority of the cell population, contained very few BrdU+ cells (Fig. 7E). In contrast, 22% of the cells in the conjunctival SP sample were BrdU-rich (Fig. 7F; Table 2, experiment number 3). With respect to the study of the limbal cells, we needed to consider that when dealing with a small number of cells (such as in the case of the limbus) the multiple steps involved in Hoechst analysis leads to substantial cell attrition. Thus, given the high degree of overlap between SP and LSSC cohorts and the similarity of BrdU uptake rates observed for both populations (Table 2), we chose to sort limbal cells according to light scattering into LSSC and non-LSSC cohorts, thereby circumventing the Hoechst loading step. Results were similar to those obtained for the SP/non-SP-based conjunctival cell
developments.
Comparison (Fig. 7G,H; Table 2). Only the LSSC population contained a substantial percent of BrdU+ cells.

Further tests of cycling rate were based on the proliferative behavior of the LSSC and SP cells immediately after transference to the in vitro environment. We reasoned that cells that exhibit slow or infrequent cycling in vivo might require a longer time to initiate proliferation in culture than cells that are rapidly cycling. Indeed, rabbit LSSC cells (about half of which are SP cells; Fig. 3), contained a high proportion of the cells that remained quiescent during the first 72 hours after seeding (Fig. 8). In contrast, the great majority of the cells in the much larger general population began to proliferate within the first 48 hours (or, alternatively, may have detached from the plate during this period). Similar results were obtained using conjunctival epithelial cells (data not shown). The cultured CFDA–SE-loaded cells were also sorted according to their Hoechst emission features and analyzed for CFDA fluorescence retention (Table 3). Sixteen hours after seeding, when few cells have completed a round of cell division, the CFDA-SE fluorescence of the average SP cell amounted to about 80% and 70% of that for the G0-G1 or the S-G2/M cells respectively. This initial intensity ratio is consistent with the smaller mass of the SP cell. However, after another 48 hours of culture, the fluorescence of the non-SP cells underwent a fourfold reduction, whereas the fluorescence of the SP cells had decreased by only one half. As loss of fluorescence primarily reflects cell division, these studies indicate the slow-cycling nature of the SP cells during the early post-seeding period.

In addition to the studies of cycling rate, we assessed the colony-forming efficiency (CFE) of SP and non-SP cell fractions. Freshly trypsinized limbal and conjunctival rabbit epithelial cells displayed CFEs of between 6-9% after 14 days of culture (based on at least ten experiments in each case). Incubation with Hoechst did not change CFEs. Cell processing through the flow cytometer has only a moderate effect (CFE decreased from 5.3 to 4.2% for the limbal sample; n=2). The CFE for the selected limbal cell G0-G1 fraction, 5.1% (n=3),

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Conditions</th>
<th>Tissue</th>
<th>SP</th>
<th>G0/G1</th>
<th>LSSC</th>
<th>non-LSSC</th>
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<tr>
<td>1</td>
<td>10-day infusion</td>
<td>Limbal-cs</td>
<td>13</td>
<td>57</td>
<td>11</td>
<td>51</td>
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<tr>
<td></td>
<td></td>
<td>Conjunctival-cs</td>
<td>15</td>
<td>47</td>
<td>14</td>
<td>41</td>
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<td>2</td>
<td>10-day infusion</td>
<td>Conjunctival-pl</td>
<td>9</td>
<td>87</td>
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</tr>
<tr>
<td>3</td>
<td>&gt;14-day infusion</td>
<td>Limbal-cs</td>
<td>22</td>
<td>&lt;1</td>
<td>18</td>
<td>&lt;1</td>
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<tr>
<td></td>
<td>38-day chase</td>
<td>Conjunctival-cs</td>
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Cs and pl indicate cytopun and plated cells, respectively. All percentiles are based on counting at least 240 cells.

Fig. 8. CFDA-SE retention of freshly plated limbal epithelial cells as a function of their SSC. The time elapsed since plating is indicated within each plot. The decrease in CFDA-SE fluorescence primarily reflects cell division. The general population of adherent cells exhibits large decreases in fluorescent intensity in the 24-48, 48-72 and 72-96 hour intervals whereas the great majority of the LSSC cells seem to retain most of the initial fluorescence for the first 72 hours. The experiment shown is representative of two experiments with limbal and two experiments with conjunctival epithelial cells.

Fig. 9. Clonogenic growth of rabbit limbal G0-G1 and SP cells on 3T3 feeder cells. G0-G1 cell collection was restricted to cells having an FSC range comparable to that of the SP cells. Colonies from G0-G1 (A-C) and SP cells (D-F) were generated from 2000 plated cells after 11 days (A,B) or after 14 days (C,D) of culture. PMA (100 µM) was included in these cultures for 4-72 hours post-plating interval. Note that essentially all colony formation by the G0-G1 cells is arrested whereas a substantial number of colonies grow from the SP cells, and the SP culture contains a substantial number of microcolonies (mc). Microscopic examination showed that most of these colonies consist of small, morphologically undifferentiated cells. (C,F) Fate of microcolonies. Dishes were complemented with 400 limbal epithelial cells and treated with PMA as described for B and E. After 14 days, colonies were present only in the SP dish. These large fast-growing colonies were removed by swiping (clear cell-free areas marked by asterisks on the grayish background generated by the 3T3 cell mat) and the culture was continued for two more weeks. The microcolonies continued to grow to form new large colonies.
was similar to that of the whole population (Fig. 9A). Considering such results, the CFE for rabbit SP cells grown in FCS-hD/F12 was poor, amounting to 2.9±1.2% (n=3) and 3.6±2.0% (n=4), for the limbal (Fig. 9D) and conjunctival epithelia, respectively. Nevertheless, cells within almost all colonies derived from the SP fraction remained small and highly compact for the first 14 days of culture, features associated with undifferentiated cells. In contrast, many of the G0-G1-derived colonies ceased to grow after 8-10 days in culture and developed large irregularly shaped cells indicative of a more differentiated phenotype. To obtain some insight on the proliferative capacity of the SP-derived colonies, three large clones from the rabbit limbal SP culture were serially cultivated using porous inserts containing a layer of feeder 3T3 cells (Wolosin et al., 2000). We terminated this serial cultivation when a cumulative number of about 10^9 cells were cultivated when microcolonies grew to form tightly packed colonies of small cells. The number of these late colonies was far greater than the number of the PMA-resilient fast growing colonies (Fig. 9F). The PMA-treated G0-G1 dishes failed to generate any such slow-developing colonies (Fig. 9C).

**Discussion**

Cells displaying an SP phenotype were initially identified in bone marrow as a small cohort of blood progenitors displaying stem cell features (Goodell et al., 1996). We demonstrate in this report that (1) cells with the SP phenotype are present in the conjunctival and limbal/corneal epithelial lineages; (2) within the limbal/corneal epithelium, SP cells are present in the cells from the limbal zone, where the stem cells reside (Schemer et al., 1986; Cotsarelis et al., 1989; Lehrer et al., 1998), but absent from the stem cell-free corneal epithelial domain; (3) at least 50% of the SP cells display a light-scattering phenotype characterized by an extremely low SSC; (4) the ocular surface SP cells display features consistent with adult stem cells; and (5) the LSSC cells display the same stem cell-associated properties as the SP cells. These findings represent a substantial step towards the isolation and characterization of the stem cells present within the ocular anterior surface epithelium.

Our conclusion on the stem cell nature of the isolated ocular surface epithelial SP and LSSC cells is based on a multifaceted examination of the properties of these SP cells. The strongest and most significant evidence is provided by the observation that the SP and LSSC cohorts contain a high proportion of slow-cycling cells, the most recognized feature of stem cells in self-renewing adult tissues. The results of label uptake during the 10-day infusion and label retention after a 14–16-day infusion followed by an extended label chase are consistent. They show that in these populations slightly more than 1% of the cells undergo cell division each day (e.g. in the conjunctival SP, 15% of the cells incorporate label during the 10-day infusion and 22% of the cells incorporated and retained label following the 14–16-day infusion). Thus, we can conclude that in these nearly mature rabbits the average SP and LSSC cell cycles once every 2–3 months.

The manner in which the SP and LSSC cells behaved when placed in culture provided further evidence that these cells are slow-cycling. Relatively quiescent stem cells frequently require a longer time to initiate proliferation upon transference to a cell culture environment when compared with cells that are rapidly dividing (Oostendorp et al., 2000; Ladd et al., 1997; Punzel et al., 2002). These cells remained quiescent for at least the first 72 hours after seeding, whereas the non-SP and non-LSSC cells began to divide within 24–48 hours. Taken together, these results establish the slow-cycling nature of the limbal and conjunctival epithelial SP cells as well as the LSSC cell populations. The extremely low light side-scattering phenotype is indicative of minimal intracellular complexity or ‘primitiveness’, which is a property consistent with the highly undifferentiated nature of stem cells.

Other major properties ascribed to stem cells from stratified epithelia are their ability to initiate clonal growth and yield colonies consisting of small cells that have a long survival time, known as holoclones (Barrandon and Green, 1987; Wei et al., 1993; Rochat et al., 1994; Pelligrini et al., 1999; Pelligrini et al., 2001; Papini et al., 2003), and the capacity of these cells to survive and clonally proliferate following exposure to PMA.

**Table 3. Yields and CFDA fluorescence in the various cohorts of cultured rabbit limbal cells**

<table>
<thead>
<tr>
<th>Time in culture</th>
<th>% viable cells</th>
<th>CFDA fluorescence*</th>
<th>SP/G1 x100</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 hours</td>
<td>3.4</td>
<td>93.4</td>
<td>12.6</td>
</tr>
<tr>
<td>CFDA fluorescence*</td>
<td>312</td>
<td>389</td>
<td>463</td>
</tr>
<tr>
<td>64 hours</td>
<td>0.93</td>
<td>41.9</td>
<td>24.8</td>
</tr>
<tr>
<td>CFDA fluorescence*</td>
<td>222</td>
<td>110</td>
<td>132</td>
</tr>
</tbody>
</table>

*Arbitrary relative units.
Ocular epithelial side population stem cells

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References


transfected with the breast cancer resistance protein. *Cancer Res.* 60, 47-50.


