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Limbal Epithelial Side-Population Cells Have Stem Cell–Like Properties, Including Quiescent State

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ABSTRACT

Corneal epithelial (CE) stem cells are believed to reside in the basal layer of the limbal epithelium but remain poorly understood due to the lack of an accepted in vivo reconstitution assay as well as definitive markers for epithelial stem cells. It has been reported that side-population (SP) cells with the ability to efflux the DNA-binding dye Hoechst 33342 have stem cell–like properties and that the SP phenotype accurately represents a quiescent and immature stem cell population in the adult bone marrow. In the present study, we investigated whether SP cells isolated from the limbal epithelium have stem cell–like properties. SP cells, separated by fluorescence-activated cell sorting, comprise approximately 0.4% of all limbal epithelial cells and have markedly higher expression of the stem cell markers ABCG2, Bmi-1, and nestin but no expression of markers for differentiated CE cells compared with non-SP cells. Cell-cycle and telomerase activity analyses revealed that SP cells are growth arrested and reside in the quiescent state. Moreover, limbal epithelial SP cells did not demonstrate proliferative capabilities under typical in vitro epithelial cell culture conditions using 3T3 feeder layers. These findings present the possibility that quiescent limbal epithelial SP cells may represent an extremely immature stem cell population compared with currently defined epithelial stem or progenitor cells. STEM CELLS *2006;24:86–94*

INTRODUCTION

Adult tissue-specific stem cells have the unique ability for self-renewal and govern the maintenance of their tissue of origin. These stem cells, which reside in specialized niches, are vital in sustaining long-term repopulation of the specific tissue via progression through a series of increasingly differentiated cells. Adult stem cells are particularly resistant to various stresses, and the proper regulation of the stem cell niche is required in maintaining their immature, undifferentiated form. Of all adult lineages, hematopoietic stem cells (HSCs) are the most well-established, as there is significant knowledge and insight into the factors and characteristics that are necessary for the regulation of their self-renewal and differentiation. HSCs previously have been shown to be both quiescent and antiapoptotic, meeting the criteria for tissue-specific adult stem cells [1]. This noncycling state is believed to protect HSCs from external stresses and allows for the maintenance of their self-renewal. It has recently been demonstrated that the single characteristic that

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most accurately represents quiescent HSCs is the side-population (SP) phenotype [1, 2]. SP cells have the unique ability to efflux the DNA-binding dye Hoechst 33342 via the ATP-binding cassette transporter G2 (ABCG2), a member of the multiple-drug resistance family of membrane transporters [3–6]. Bone marrow SP cells have the capability for the long-term multilineage reconstitution of the hematopoietic system [2, 7], and it is believed that these SP cells represent quiescent HSCs more accurately than HSCs characterized by other means. In addition, SP cells have been isolated from various other adult tissues, including liver [8, 9], skeletal muscle [10], and lung [11], demonstrating that this phenotype with the existence of ABCG2 and the ability to efflux Hoechst 33342 may represent a common feature of all adult stem cells.

Corneal epithelial (CE) stem cells are thought to reside in the basal layer of the limbus [12, 13], the transitional zone between the cornea and the peripheral bulbar conjunctiva. CE stem cells maintain the ocular surface by generating transient amplifying (TA) cells that migrate, proliferate, and differentiate to replace lost or damaged CE cells [14–16]. Whereas traditional methods use colony-forming assays or pulse-chase experiments of labeled thymidine, the lack of both definitive markers and an established in vivo reconstitution assay still remain serious obstacles in the unequivocal identification of not only CE stem cells but also other epithelial stem cells. Recently we have demonstrated that the limbal epithelium contains SP cells that express ABCG2 [17]. It is believed that these cells may allow for the separation of CE stem cells from their more differentiated progeny. We therefore investigated the properties of limbal epithelial SP cells to determine whether they possess stem cell–like properties. Our results demonstrate that limbal epithelial SP cells closely resemble HSCs and strongly suggest that these cells allow for the first insights into a true epithelial stem cell population.

Materials and Methods

Cell Preparation

Corneoscleral rims were obtained from New Zealand white rabbits. Limbal tissues were obtained with scissors, and 2.0-mm-diameter portions of the central corneas were obtained by trephination. Excised tissues from the limbus and central corneas were treated with Dulbecco's modified Eagle's medium (DMEM) containing 250 U/ml dispase II (Godo Shusei, Tokyo, Japan, http://www.godo.jp) at 37°C for 1 hour. Epithelial cells were then separated under a dissecting microscope and treated with 0.25% trypsin/1 mM EDTA solution (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) for 20 minutes at 37°C to create single-cell suspensions from the limbal epithelium and corneal epithelium, and enzymatic activity was stopped by adding an equal volume of DMEM containing 10% fetal bovine serum (FBS) (Moregate BioTech, Queensland, Australia, http://www.moregatebiotech.com).

Hoechst 33342 Exclusion Assay Using Fluorescence-Activated Cell Sorting

Limbal epithelial and CE cells were resuspended at a concentration of 10^6 cells/ml in staining medium (DMEM containing 2% FBS and 10 mM HEPES). Cell suspensions were incubated in staining medium containing 3 μg/ml Hoechst 33342 (Sigma, St. Louis, http://www.sigmaaldrich.com) for 90 minutes at 37°C. For inhibition experiments, 50 μM of either R(+)-verapamil (Sigma) or tryprostatin-A (Alexis Biochemicals, Carlsbad, CA, http://www.alexis-corp.com) was added to the staining medium 30 minutes before the addition of Hoechst 33342. After staining, cells were washed two times with Dulbecco's phosphate-buffered saline (PBS) containing 2% FBS and 1 mM HEPES and then resuspended. Before analysis and cell sorting, propidium iodide (Sigma) was added at a final concentration of 2 μg/ml to distinguish between live and nonviable cells. Analysis and cell sorting were performed using a dual-laser fluorescence-activated cell sorter (FACS) (EPICS® ALTRA FACS analysis system, Beckman Coulter, Fullerton, CA, http://www.beckmancoulter.com). Hoechst 33342 was excited at 350 nm using a UV laser, and fluorescence emission was detected through 450-nm band-pass (BP) (Hoechst blue) and 675-nm long-pass (Hoechst red) filters, respectively. Propidium iodide was excited at 488 nm (argon ion laser), and fluorescence emission was detected through a 610-nm BP filter.

Gene Expression Analysis

After sorting and isolation of SP and non-SP (NSP) cells from the limbal epithelium and viable CE cells from central corneas, total RNA was obtained from 10,000 SP, NSP, and CE cells using ISOGEN (Nippon Gene, Tokyo, Japan, http://www.nippongene.com) according to the manufacturer's suggested protocol. After treatment with DNase I (Nippon Gene), single-stranded cDNA was created with the Superscript First-Strand System for reverse transcription–polymerase chain reaction (RT-PCR) (Invitrogen) and used as PCR templates. Primer pairs and TaqMan MGB probes labeled with 6-carboxyfluorescein (FAM) at the 5′-end and nonfluorescent quencher at the 3′-end were designed with Assay-by-Design (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). Quantitative PCR was performed with an iCycler iQTM Real-Time Detection System (Bio-Rad, Hercules, CA, http://www.bio-rad.com). Thermocycling programs consisted of an initial cycle at 50°C for 2 minutes and 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Negative con-

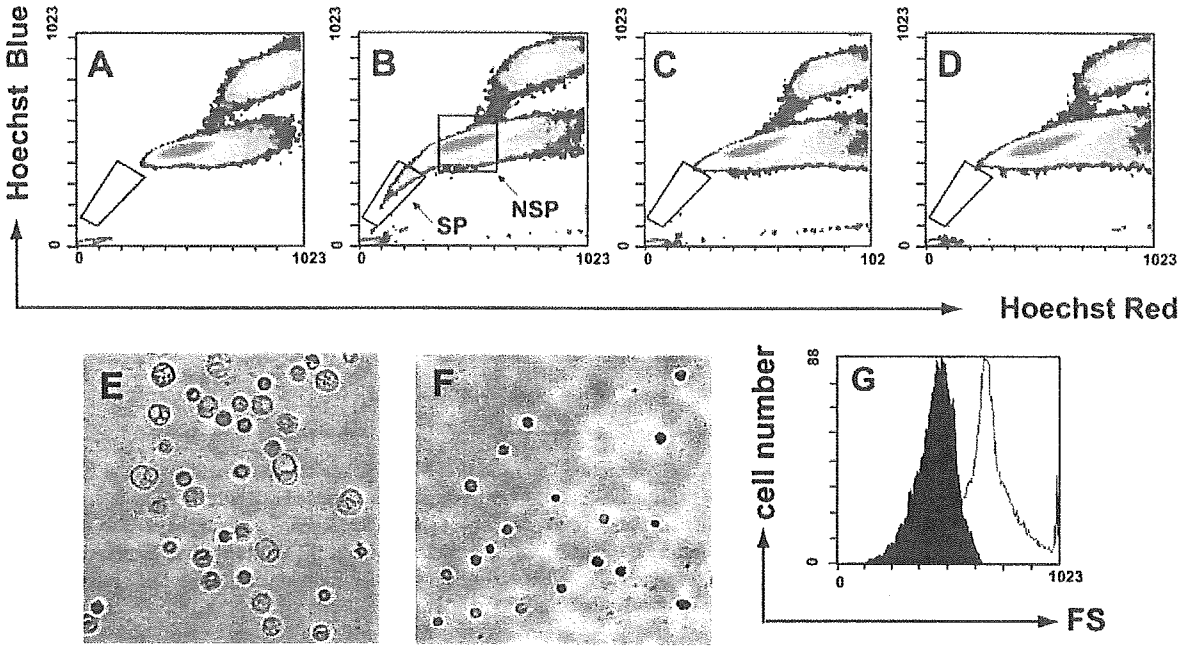

Figure 1. Hoechst 33342 staining in rabbit limbal and corneal epithelial cells. Epithelial cells were removed from the limbus and cornea and analyzed for Hoechst 33342 efflux by fluorescence-activated cell sorting (FACS). Side-population (SP) cells were detected in epithelial cells after Hoechst 33342 staining from the cornea (**A**) and the limbus (**B**). In the density plot of (**B**), the cells denoted by each enclosed area were regarded as SP cells or non-SP (NSP) cells for further characterization. Limbal epithelial cells were pretreated with each transporter inhibitor, verapamil, an inhibitor of MDR (**C**), or tryprostatin-A (TPS-A), a specific inhibitor of ABCG2 (**D**), before staining with Hoechst 33342. After isolation, NSP cells (**E**) and SP cells (**F**) were centrifuged onto glass slide slips and observed with a phase-contrast microscope (magnification ×100). Forward scatter (FS) by FACS was analyzed in SP (black) and NSP (white) cells to examine cell size in each fraction (**G**).

trols using non–reverse transcribed total RNA as template strands were performed for all experiments. All assays were run in duplicate for more than four individual samples. mRNA expression levels were normalized with the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To represent expression levels of individual mRNAs, we used an mRNA expression index that divided the value of the specific gene copies by the value for GAPDH. To compare mRNA expression between SP and NSP cells, the Mann-Whitney rank-sum test was applied and statistics were calculated using SigmaStat 2.0 (SPSS, Chicago, IL, http://www.spss.com).

Colony-Forming Assay

Feeder layers were prepared by seeding of mitomycin C–treated NIH-3T3 cells at a density of 2 × 104 cells/cm^2, and SP, NSP, or all viable limbal epithelial cells were seeded at a density of 1,000 cells per 60-mm dish (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). After culture for approximately 10 days at 37°C, colonies were fixed with 10% formalin (Wako Chemicals, Osaka, Japan, http://www.wako-chem.co.jp/english) and stained with 2% rhodamine B (Sigma), and the entire dish surfaces were scanned under a dissecting microscope.

Cell-Cycle Analysis

Limbal epithelial SP cells and total limbal epithelial cells were pelleted by centrifugation and resuspended in a solution containing 4 mM sodium citrate (pH 7.6), 0.2% Nonidet P-40, and 50 μg/ml propidium iodide. After incubation on ice for 30 minutes, cell suspensions were treated with 0.25 mg/ml RNase A for 15 minutes at 37°C to remove double-stranded RNA. Cells were finally analyzed by flow cytometry at an excitation wavelength of 488 nm.

Telomerase Activity

Using the TRAPeze XL telomerase Detection Kit (Chemicon, Temecula, CA, http://www.chemicon.com), telomerase activity was investigated in SP, NSP, and corneal epithelial cells according to the manufacturer's suggested protocol. Briefly, 10,000 SP, NSP, or CE cells were pelleted by centrifugation and resuspended in CHAPS XL lysis buffer. The reaction mixtures containing the cell lysates were then incubated for 60 minutes at 37°C and subjected to PCR. PCR consisted of 30 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute. Reaction mixtures were then subjected to electrophoresis on a 10% polyacrylamide gel. After electrophoresis, the gel was stained with SYBR Green I

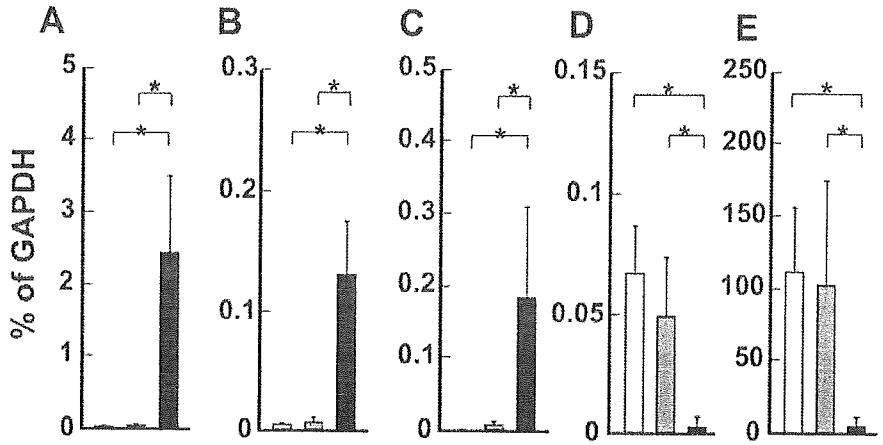

Figure 2. Quantification of mRNA in limbal epithelial side-population (SP) and non-SP (NSP) cells and corneal epithelial (CE) cells. Total RNA was extracted from limbal epithelial SP cells, NSP cells, and CE cells after fluorescence-activated cell sorting and subjected to real-time quantitative reverse transcription–polymerase chain reaction. Relative expression of the selected genes was normalized to that of GAPDH for each sample. mRNA expression of ABCG2 (**A**), Bmi-1 (**B**), nestin (**C**), CK 3 (**D**), and CK 12 (**E**) is shown. Expression levels were determined from SP cells (black bar), NSP cells (gray bar), and CE cells (white bar) for each individual mRNA. Data represent the mean value from four to six samples. Error bars indicate the standard deviation ($*p < .01$).

(Molecular Probes, Eugene, OR, http://probes.invitrogen.com) and the image was recorded with Typhoon (Amersham Biosciences, Piscataway, NJ, http://www.amersham.com).

Results

Limbal Epithelium Contains a Side Population with Cells of Smaller Size

To determine whether SP cells were present in either the limbal or corneal epithelium, both epithelial cell types were isolated from the eyes of New Zealand white rabbits and subjected to Hoechst 33342 dye efflux assay. SP cells were nearly undetected in corneal epithelial cells (Fig. 1A; 0.01% gated cells), similar to results in human eyes [17]. In contrast, a distinct population with decreased Hoechst 33342 blue/red fluorescence was detected in limbal epithelial cells (Fig. 1B; 0.40% gated cells), also similar to previous results with human limbus [17]. Hoechst 33342 efflux was antagonized by both verapamil, an inhibitor of Hoechst 33342 dye transport, and tryprostatin A, a specific inhibitor of ABCG2 (Figs. 1C, 1D) in the same fashion as previous results using different tissues of origin [8, 11, 18, 19]. SP and NSP cell fractions were collected by cell sorting as denoted in Figure 1B, and microscopic analysis (Figs. 1E, 1F) revealed that SP cells had a smaller cell size compared with NSP cells. These results were also confirmed by forward scatter analysis using FACS (Fig. 1G).

Limbal Epithelial SP Cells Express Stem Cell–Related Genes

To examine whether SP cells have stem cell–like phenotypes, gene expression analyses of SP and NSP cells from the limbus

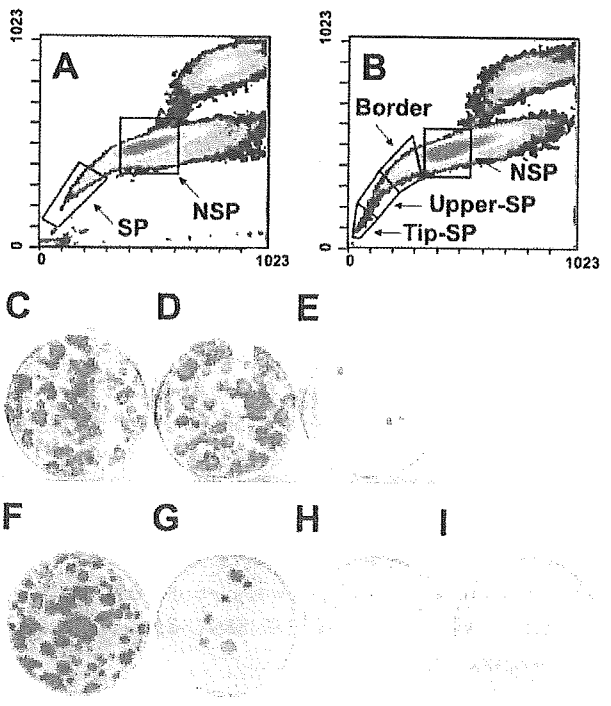

Figure 3. Colony-forming assay with limbal epithelial side-population (SP) cells and non-SP (NSP) cells. Limbal epithelial cells were separated by fluorescence-activated cell sorting into SP and NSP cells (**A**) for colony-forming assays. For in-depth analysis, limbal epithelial cells were sorted into four gates: Tip-SP, upper-SP, border, and NSP cells (**B**). After cell sorting, viable limbal epithelial cells (**C**), NSP cells (**D**), or SP cells (**E**) were seeded onto mitomycin C–treated NIH-3T3 feeder layers at a density of 1,000 cells per 60-mm dish. For detailed studies, NSP cells (**F**), border cells (**G**), upper-SP cells (**H**), or Tip-SP cells (**I**) were subjected to colony-forming assays under the same conditions. Cells were cultured for approximately 10 days followed by fixation and staining with rhodamine B.

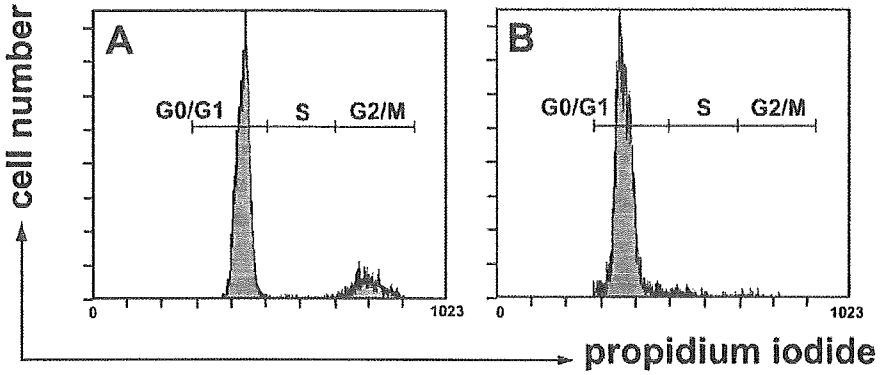

Figure 4. Cell-cycle state of limbal epithelial side-population (SP) cells. After sorting of all viable limbal epithelial cells and SP cells, cell membranes were disrupted using Nonidet P-40 followed by staining with propidium iodide. The cell-cycle phase in which each individual cell resided, from either all viable limbal epithelial cells (**A**) or SP cells (**B**), was detected by flow cytometry.

as well as CE cells were conducted using real-time quantitative RT-PCR. ABCG2, which is required to induce the SP phenotype; Bmi-1, necessary for the self-renewal of HSCs and neural stem cells [20–24]; and nestin, an intermediate filament specifically expressed in neural stem cells [25] were used as stem cell markers. Significant expression of ABCG2, Bmi-1, and nestin was detected in SP cells, but neither NSP cells nor CE cells showed expression of these genes. On the contrary, cytokeratin 3 (CK3) and cytokeratin 12 (CK12), which are known markers of differentiated CE cells [26], were significantly expressed in both NSP and CE cells but not in SP cells (Fig. 2). Additionally, results from immunostaining with anti-ABCG2 antibodies have previously revealed that ABCG2-positive cells comprise only a small portion of the human limbal epithelium, with negative staining in central corneas [17, 27].

SP Cells Are Growth Arrested in the Quiescent State

Because SP cells corresponded to a CE stem cell phenotype in location and gene expression, we investigated whether SP cells (Fig. 3A) have a strong capacity for proliferation using colony-forming assays. Unexpectedly, whereas cell fractions of all limbal epithelial cells (Fig. 3C) or NSP cells (Fig. 3D) had high proliferative capabilities, SP cells (Fig. 3E) demonstrated an extremely low colony-forming efficiency (CFE) (Table 1). Even when culture conditions were modified, such as the addition of various cytokines and growth factors, coculture with NSP cells, or isolation from wounded corneas, SP cells were still unable to form a significant number of colonies in vitro (data not shown). These results suggested that SP cells have almost no capacity for proliferation under normal in vitro culture conditions for CE cells.

To investigate the proliferative potential of limbal SP cells in greater detail, limbal epithelial cells were sorted into four gates, representing the tip-SP, upper-SP, border, and NSP cells (Fig. 3B). Results from colony-forming assays showed that whereas NSP cells (Fig. 3F) had a CFE comparable to all limbal epithelial cells, border (Fig. 3G), upper-SP (Fig. 3H), and tip-SP (Fig. 3I) cells all had negligible CFEs compared with NSP cells (Table 1). Therefore, the colony-forming ability of limbal epithelial cells is likely nearly completely derived from the NSP cell fraction.

Because SP cells demonstrated almost no proliferative capabilities in vitro, we investigated the cell-cycle state of SP cells. After sorting of SP cells and viable limbal epithelial cells, each cell fraction was permeabilized and stained with propidium iodide, followed by flow cytometric analysis. Although the cell cycle of limbal epithelial cells was promoted with active cell division, SP cells were nearly all growth arrested in G0/G1 (Fig. 4, Table 2).

Results from colony-forming assays (Fig. 3, Table 1) and cell-cycle analysis (Fig. 4, Table 2) suggested that SP cells may represent a quiescent stem cell population. To confirm this possibility, we examined telomerase activity in SP cells using

Table 1. Colony-forming efficiencies and detailed analysis of limbal epithelial cells subjected to Hoechst 33342 exclusion assays

	Viable cells (%)	CFE (%)
All	—	8.49 ± 3.03
NSP	66.1 ± 3.92	7.75 ± 2.42
SP	0.40 ± 0.19	0.38 ± 0.09
Border	2.83 ± 0.55	0.83 ± 0.15
Upper SP	0.20 ± 0.04	0.10 ± 0.10
Tip SP	0.03 ± 0.01	ND

Epithelial cells subjected to fluorescence-activated cell sorting were sorted, and the corresponding percentages of cells in each gate from Figures 3A and 3B are presented. Additionally, colony-forming efficiencies for cells from each gate are shown. Values are presented as means± standard deviations.
Abbreviations: CFE, colony-forming efficiency; ND, not determined.

Table 2. Cell-cycle state of limbal epithelial side-population cells

	G0/G1	S	G2/M
SP	96.9 ± 0.6	1.1 ± 0.3	1.6 ± 0.4
All	78.6 ± 2.5	3.2 ± 0.8	18.4 ± 1.7

Values correspond to the percentage of cells in each cell-cycle phase. Values are presented as means ± standard deviations.

the TRAP assay. It has been previously reported that quiescent stem cells have no telomerase activity but that it is upregulated in actively proliferating TA and progenitor cells in hair follicles [28], skin [29], and bone marrow [30]. Cell lysates from each cell fraction purified by FACS were reacted to lengthen telomeres, followed by amplification of the product with PCR. HeLa cells were used as a positive control, and each cell fraction was also treated with heat as a negative control. We found that both SP and CE cells had no telomerase activity, whereas NSP cells demonstrated weak activity (Fig. 5).

Discussion

Limbal Epithelial SP Cells Resemble Other Adult Tissue-Specific Stem Cells

In the present study, we demonstrated that limbal epithelial SP cells have HSC-like properties, including small size, increased expression of the stem cell markers ABCG2, Bmi-1, and nestin, and an SP phenotype. Immature cell types such as stem cells are thought to be much smaller in size compared with normal cells, and limbal epithelial SP cells were significantly smaller than NSP cells and appeared more immature and simple in cell shape, thus supporting the view that SP cells represent a CE stem cell population or, at the very least, an extremely immature cell population.

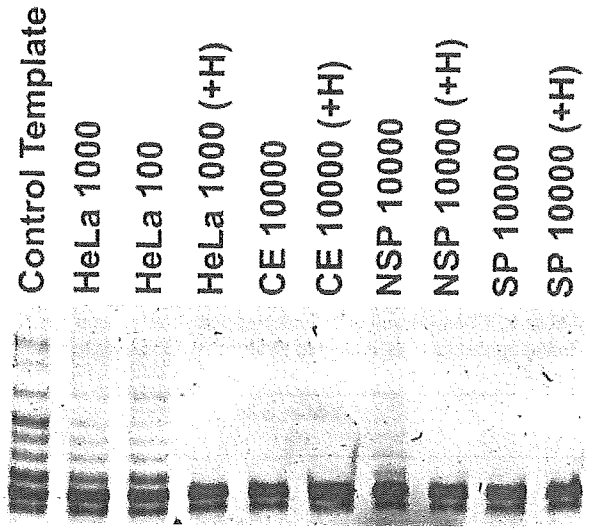

Figure 5. Telomerase activity. After cell sorting, telomerase activity was examined by the telomeric repeat amplifying protocol (TRAP) assay for each cell fraction. A control template, containing several telomeric repeats, was used at a concentration of 0.2 mol/μl. HeLa cells were selected as a positive control. Each cell lysate was also treated with heat (+H) as a negative control. After TRAP assay, each reaction mixture was subjected to polyacrylamide electrophoresis followed by staining with SYBR green I. The number of cells used for each sample is shown. Abbreviations: CE, corneal epithelial; NSP, non–side population; SP, side population.

For the SP phenotype to be indicative of stem cell–like properties, the expression of ABCG2 is required [7, 31, 32]. Because adult stem cells are known to be particularly resistant to various stresses, the expression of ABCG2 likely acts as a protective mechanism, allowing for the efflux of various soluble factors or toxins that could result in the premature differentiation of SP cells.

Bmi-1, a member of the polycomb gene family, is expressed not only in HSCs but also in neural stem cells and is vital to the self-renewal of adult stem cells [20–24]. The increased expression of Bmi-1 in limbal epithelial SP cells therefore also seems likely to be critically involved in the self-renewal of CE stem cells. Bmi-1 decreases expression of p16 and p19 [20, 21, 33], known cyclin-dependent kinase inhibitors, and increases telomerase activity [34]. However, even though Bmi-1 is believed to promote increased proliferation of cells, higher expression of Bmi-1 has been correlated with the maintenance of the stem cell phenotype. It seems that adult stem cells have high proliferative potential, but other regulatory factors act to prevent unnecessary proliferation and carefully regulate the generation of differentiated progeny.

Nestin, an intermediate filament protein and known neural stem cell marker [25], was also more highly expressed in SP cells compared with other cell fractions. Increased nestin expression has previously been detected in SP cells isolated from other tissues, such as the pancreas [19] and retina [18], and may therefore be a characteristic cytoskeletal molecule of not only neural stem cells but also other adult stem cells with the SP phenotype.

Therefore, the previously proposed environment of the basal layer, being the most isolated from the exposed ocular surface, in conjunction with the expression of the stem cell markers ABCG2, Bmi-1, and nestin, may allow for the proper maintenance and self-renewal of limbal epithelial SP cells.

SP Cells Likely Represent a Quiescent Stem Cell Population in the Limbal Epithelium

In the present study, most limbal epithelial SP cells were found to be growth arrested in G0/G1, compared with all cells isolated from the limbal epithelium. HSCs are known to be quiescent in the bone marrow niche [1, 35], and limbal epithelial SP cells therefore also resemble HSCs by being cell-cycle arrested. The

quiescent state of limbal epithelial SP cells led to the interesting finding that SP cells did not proliferate and could not create colonies in vitro, like previously defined epithelial stem cells. Whereas tip-SP cells revealed no colony formation, very low CFEs were observed for the SP, upper-SP, and border-gated cells (Table 1). These values, however, seem correlated with the purity of the SP fraction after cell sorting, because SP cells isolated by FACS inevitably contain approximately 1%–2% contamination by other cells (data not shown). Therefore, it is believed that the observed CFE is likely due to contamination by NSP cells, which account for the proliferative capabilities of all cells in the limbal epithelium. Moreover, SP cells showed lower expression of $\alpha 6$ and $\beta 1$ integrins (data not shown), which are known to be highly expressed in cells with strong proliferative capabilities [36–38].

Although a recent study reported that limbal epithelial SP cells had the ability to form colonies [39], their experimental procedures had several different conditions. In particular, the seeded cell density, UV laser intensity used for FACS, and primary cell culture before cell sorting were most likely the cause for the conflicting results. In their colony-forming assay, epithelial cells were seeded at a density that was 20 times higher than in the present study. Therefore, even though scanned images revealed dish surfaces that were nearly completely covered by proliferating cells, results actually showed significantly lower CFEs (4% for SP and 1% for NSP cells) compared with our present results (approximately 10% for NSP cells). Although these differences may be due to culture conditions that are better suited for limbal epithelial cells, we also confirmed that the proliferative capacity of limbal epithelial cells was markedly decreased, with CFEs comparable to their results, when UV laser power was greater than 50 mW (data not shown). In their study, a laser intensity of 100 mW was used, which can cause significant damage to cells during sorting and may result in diminished proliferative abilities. Additionally, the effects of cultured cells in their study, as opposed to freshly isolated cells, used for FACS in the present case, cannot be excluded. We have, in fact, observed that no SP cells were detected when primary cultured cells were sorted (data not shown), a result that supports our finding that SP cells are growth arrested in vitro.

It has previously been shown that cells that are in active proliferation, such as TA or progenitor cells, but not quiescent adult stem cells, have telomerase activity [28–30]. It is thought that upregulation of telomerase occurs upon differentiation into progenitor or TA cells to prevent the rapid depletion of telomeres during cell division. This process is transient, with telomerase activity decreasing upon the formation of fully differentiated progeny [30]. The corneal epithelium has a relatively short span of renewal, similar to bone marrow, skin, and hair follicles, and therefore the telomerase activity present in the NSP cell fraction likely corresponds to cells that are in active proliferation, such as progenitor or TA cells. Limbal SP cells that have neither telomerase activity nor expression of the differentiated cell markers CK3 and CK12 therefore seem to represent a quiescent stem cell population. NSP cells, which have increased telomerase activity and are clonogenic, likely consist of both TA and progenitor cells that are actively proliferating but not quiescent stem cells, due to both mitotic activity and expression of differentiated CE markers. Finally, CE cells, which express CK3 and CK12, are thought to be highly differentiated cells that express CK3 and CK12, in which telomerase activity has been downregulated because of the loss of proliferative potential.

Our results demonstrate that limbal epithelial SP cells have stem cell–like properties, including growth arrest in the quiescent state, thus meeting the criteria of adult stem cells. It is currently accepted that epithelial stem cells are slow cycling and demonstrate high proliferative potential. However, limbal epithelial SP cells are cell-cycle arrested and, due to their quiescent state, exhibit no in vitro proliferative capabilities. It therefore seems that previously defined epithelial stem cells via colony-forming assays or detection of label-retaining cells may not represent true stem cells, but rather immature progenitor cells. In fact, Oshima et al. [40], discussing their results on adult multipotent stem cells from hair follicles, state that these cells, with high proliferative potential, are likely generated from more immature stem cells.

To accurately identify epithelial stem cells, it seems important to meet the criteria used for other adult stem cells, such as HSCs. The current view is that epithelial stem cells, which demonstrate high proliferative capacity and slow cell cycle, give rise to TA cells, which have a limited number of cell divisions, and finally generate differentiated progeny. We now believe that limbal epithelial SP cells represent an epithelial stem cell population that resides in the quiescent state in the basal layer of the limbus. We also theorize that limbal epithelial SP cells generate progenitor or "active" stem cells that are mitotically cycling and have high proliferative potential. It is these progenitor cells that comprise the portion of the NSP fraction that have active telomerase, demonstrate a slow cell cycle, and have colony-forming abilities. Under normal epithelial cell culture conditions, these colony-forming progenitor cells can proliferate to produce more differentiated cells that can form stratified epithelial layers. CE progenitor cells may therefore be analogous to the multipotent progenitor cells of the hematopoietic system and generate TA cells that are present in the NSP cell fraction of the limbus and can also migrate toward the central cornea to produce the CE cells that express CK3 and CK12. Epithelial stem cells should therefore be defined not by markers of clonogenic or label-retaining cells but by markers of adult stem cells, such

as the SP phenotype via expression of ABCG2; Bmi-1, which regulates stem cell self-renewal; the expression of nestin found in various SP and stem cell populations; and the absence of telomerase activity, representing the cell-cycle arrest of quiescent stem cells.

Previously, we and others have shown the successful clinical transplantation of ex vivo–expanded limbal epithelial cells to human patients [41]. In such cases, the understanding of CE stem cells is of vital importance to the long-term survival of transplanted cells and may contribute to a higher quality of transplantation. However, unlike in the hematopoietic system, where there is an established and accepted in vivo reconstitution assay, the current lack of an analogous model for the corneal epithelial system makes it difficult to confirm the abilities for self-renewal and long-term maintenance of limbal epithelial SP cells. It seems likely, however, that the understanding of the mechanisms that control the proliferation and differentiation of limbal epithelial SP cells will have a significant influence on successful applications in the clinical setting.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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