A Rapid Separation of Two Distinct Populations of Mouse Corneal Epithelial Cells with Limbal Stem Cell Characteristics by Centrifugation on Percoll Gradient

Magdalena Krulova,1,2 Katerina Pokorna,1,2 Anna Lencova,1,3 Jan Fric,1,2 Alena Zajicova,1 Martin Filipec,2 John V. Forrester,4 and Vladimir Holan1,2

PURPOSE. To detect and isolate cells with stem cell (SC) characteristics in the limbus of the mouse.

METHODS. Limbal tissues from BALB/c mice were trypsin-dissociated and separated on the gradient Percoll (Fluka, Buchs, Switzerland). Several fractions were isolated and characterized by real-time PCR for the presence of limbal SC markers and differentiation markers of corneal epithelial cells by flow cytometry for the determination of the side-population (SP) phenotype and growth properties in vitro.

RESULTS. Cells retained in the lightest fraction (40% Percoll) and in the densest fraction (80% Percoll) of the gradient were both enriched for populations with a high expression of the SC markers ABCG2 and Lgr5 and also expressing the SP phenotype. However, the lightest fraction (representing approximately 12% of total limbal cells) contained cells with the strongest spontaneous proliferative capacity and expressed the corneal epithelial differentiation marker K12. In contrast the densest fraction (<7% of original cells) was K12 negative and contained small nonslowly proliferating cells, which instead were positive for p63. Unexpectedly, cells from this fraction had the highest proliferative activity when cultured on a 5T3 feeder cell monolayer.

CONCLUSIONS. These findings demonstrate the presence of two distinct populations of corneal epithelial cells with limbal SC characteristics, based on differential expression of the keratin-specific marker K12 and transcription factor p63, and suggest a difference in developmental stage of the two populations, with the K12 p63- population being closer to the primitive limbal SC. (Invest Ophtalmol Vis Sci. 2008;49:3903–3908) DOI: 10.1167/iovs.08-1987

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Supported by Grant KAN200528084 from the Grant Agency of the Academy of Sciences, projects 1M0506, MSM0021620806, and MSM0021620858 from the Ministry of Education of the Czech Republic; Grant 510/08/I077 from the grant Agency of the Czech Republic; and project AVOZ50520514 from the Academy of Sciences of the Czech Republic.

Submitted for publication March 6, 2008; revised April 10 and 24, 2008; accepted July 14, 2008.

Disclosure: M. Krulova, None; K. Pokorna, None; A. Lencova, None; J. Fric, None; A. Zajicova, None; M. Filipec, None; J.V. Forrester, None; V. Holan, None

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Czech Republic). The use of animals was approved by a local Ethics Committee of the Institute of Molecular Genetics, and all animals were handled in full accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation of Limbal Cells

Limbal tissue was obtained by scissor dissection of the eyes of killed mice guided by an operating microscope. Limbal tissues from 10 to 12 BALB/c mice were pooled and cut into small pieces in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO). The tissue was centrifuged (8 minutes at 250g), and the pellet was subjected to digestion with trypsin from porcine pancreas (Sigma-Aldrich). The procedure consisted in 10 trypsinization cycles (300 µL of 0.5% trypsin solution per 10 limbuses, 10 minutes incubation in 37°C). The supernatants (tissue-free solution) from each trypsinization step were harvested into an excess (50 mL) of RPMI 1640 medium with 10% fetal calf serum (FCS; Sigma-Aldrich) on ice, and the trypsinization procedure was repeated on the residual pellet. After the last trypsinization step, the harvested cell suspension was filtrated through a nylon mesh and centrifuged for 8 minutes at 250g. The pellet was resuspended in 1.2 mL of RPMI 1640 medium, and the number of cells was determined by hemocytometry.

Percoll Gradient Centrifugation

To prepare a stock solution, nine parts Percoll was mixed with one part 10× concentrated phosphate buffered saline (PBS). From the stock solution, a 40%, 50%, 60%, 70%, or 80% Percoll solution was prepared by dilution in 1× PBS. A Percoll gradient was prepared in a 10× test tube by overlaying of 1.0 mL of each Percoll dilution 80% through 40%. Finally, 1.0 mL of suspension of trypsin-dissociated limbal cells was gently overlaid on the top of the Percoll gradient. The gradient was centrifuged for 10 minutes at 300g at 4°C.

After centrifugation, the separated layers of cells on individual Percoll concentrations could be directly visualized, and individual cell layers (as well as the cell pellet) were harvested into RPMI 1640 medium with 5% of FCS and washed three times by centrifugation (8 minutes at 250g). After the last washing, the cells were resuspended in 500 µL of RPMI 1640 medium containing 10% of FCS, 10 mM HEPES buffer, antibiotics (100 U/mL of penicillin, 100 µg/mL of streptomycin) and 5 × 10⁻³ M 2-mercaptoethanol (hereinafter called complete RPMI 1640 medium). The number of cells in each fraction was then determined.

Quantitative Real-Time Polymerase Chain Reactions

The expression of genes for mouse ABCG2, Lgr5, the DeltaNp63 (p63), and K12 was determined by quantitative real-time polymerase chain reactions (L reactions included denaturation at 95°C for 3 minutes, then 40 cycles at 95°C for 10 seconds, annealing at 60°C for 20 seconds, and elongation at 72°C for 20 seconds. Fluorescence data were collected at each cycle after an elongation step at 80°C for 5 seconds.

Light-Scattering Measurements

Trypsin-dissociated unseparated limbal cells or limbal cells from individual Percoll fractions were resuspended in PBS with 5% FCS and 2 µg/mL of propidium iodide (Sigma-Aldrich) for 20 minutes at 4°C. The light-scattering properties of the cells were measured in a flow cytometer (BD LSRII; BD Biosciences, Franklin Lakes, NY), with an Argon laser (488 nm) providing the probing beam, and the FSC/SSC density plots of viable cells were generated.

Hoechst 33342 Exclusion Assay

Freshly isolated unseparated limbal cells or cells of individual fractions from a Percoll gradient were resuspended at a concentration of 1 × 10⁵ cells/mL in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 2% FCS and incubated with 5 µg/mL Hoechst 33342 (Sigma-Aldrich) dye. To determine the effect of verapamil on the Hoechst 33342 efflux, the cells were preincubated with verapamil (80 µM; Sigma-Aldrich) for 5 minutes before the addition of the Hoechst 33342 dye. After the incubation for 60 minutes at 37°C, propidium iodide (2 µg/mL) was added to exclude dead cells from the analysis, and the cells were then analyzed on a flow cytometer (FACSVantage SE; BD Biosciences), as described by Goodell et al.²⁸ Briefly, Hoechst 33342 was excited at 350 nm with a UV laser (Enterprise II-621; Coherent, Santa Clara, CA), and fluorescence emission was detected through 450-nm band-pass (Hoechst blue) and 660-nm long-pass (Hoechst red) filters.

Spontaneous Proliferation of Limbal Cells In Vitro

Unseparated limbal cells or cells from individual fractions from the Percoll gradient were diluted to a concentration of 5 × 10⁵ cells/mL in complete RPMI 1640 medium. One hundred microliters per well of cell suspension was incubated in triplicate in 96-well tissue culture plates (Nunc, Roskilde, Denmark). Cell proliferation was determined by adding 1 µCi/well of [³²P]thymidine (Nuclear Research Institute, Rez, Czech Republic) for the last 8 hours of the 96-hour incubation period. The cells were harvested (Automasch 2000 harvester; Dynatech, Burlington, MA), and [³²P]thymidine activity was determined.

Cell Proliferation on Feeder 3T3 Fibroblasts

Irradiated (150 Gy) mouse 3T3 fibroblasts were seeded as feeder cells at a concentration of 10⁵ cells/well in a volume of 50 µL of complete RPMI 1640 medium into wells of 96-well tissue culture plates (Nunc) and incubated overnight. Unseparated limbal cells or cells from individual Percoll gradient fractions (5 × 10⁵ cells in 50 µL) were then added in triplicate into wells with feeder cells. The cultures were incubated for 96 hours, [³²P]thymidine (1 µCi/well) was added for the

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
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<tbody>
<tr>
<td>GADPH</td>
<td>GGG TGT GAA CCA GGA GAA AT</td>
<td>ACA CAT TGG GGG TAG GAA CA</td>
</tr>
<tr>
<td>ABCG2</td>
<td>GCC TGG GAG TAC TTT GGA TCA</td>
<td>AAA TCC GCA GGG TGG TGG TA</td>
</tr>
<tr>
<td>p63</td>
<td>TGG AAA AGA ATG CCC AGA CT</td>
<td>CTC CTG GTC CAT GCT GTC C</td>
</tr>
<tr>
<td>Lgr5</td>
<td>CTT CAC TCG GTG CAG TGC T</td>
<td>CAG CCA GCT ACC AAA TAG GTG</td>
</tr>
<tr>
<td>K12</td>
<td>CTG TGG AGG CCT CTT TTC TG</td>
<td>ATT CCA GCT ATC CCC AAT CG</td>
</tr>
</tbody>
</table>
last 8 hours of the incubation period, and the incorporated radioactivity was determined as just described.

### Statistical Analysis

The statistical significance of differences between individual groups was calculated by Student’s t-test.

## Results

### Limbal Cell Isolation and Percoll Gradient Separation

Trypsin-dissociation of limbal tissue from one BALB/c mouse yielded on average 0.5 to 1 × 10^5 cells that were heterogeneous in both size and morphology, as determined by the light-scattering profile (Fig. 1). Accordingly, limbal cells obtained by trypsin digestion (see the Methods section) from 10 BALB/c mice were pooled and separated on the Percoll gradient. The proportion of cells retained in individual Percoll gradient fractions and the recovery of original cells are shown in Table 2. While the lightest fraction (40% Percoll) contained predominantly large and more heterogeneous cells with a smaller nucleus/cytoplasm ratio, the fraction retained on 40% Percoll (densest fraction) was enriched in small dense cells with a higher ratio nucleus/cytoplasm. The pelleted fraction contained dead cells, cell debris, and fragments of corneal cells with a higher ratio nucleus/cytoplasm. The pelleted fraction contained dead cells, cell debris, and fragments of corneal cells with a higher ratio nucleus/cytoplasm. The pelleted fraction contained dead cells, cell debris, and fragments of corneal cells with a higher ratio nucleus/cytoplasm.

### Phenotype Characterization

The expression of genes for the putative SC markers ABCG2, Lgr5, and p63 and for corneal epithelial cell differentiation marker K12 was determined by using real-time PCR in unseparated limbal cells and limbal cell fractions isolated from Percoll gradient. As demonstrated in Figure 2, both the lightest (40% Percoll) and densest (80% Percoll) fractions were enriched in cells expressing the SC markers ABCG2 and Lgr5, whereas the fraction from the middle region of the Percoll gradient had a lower expression of these markers compared to unseparated limbal cells. The marker of primitive and SCs p63 was expressed selectively in the dense (80% Percoll) cell fraction (Fig. 2). The corneal differentiation marker K12 was expressed predominantly in larger cells retained on 40% or 50% Percoll and was absent in small cells of the dense fractions (Fig. 2).

### Identification of the SP Phenotype on the Basis of the Efflux of Hoechst 33342 Dye

Using flow cytometry we first demonstrated that normal fresh mouse limbus contains a small population of cells (SP cells) that can be detected by verapamil-sensitive disappearance of a unique tail of a low Hoechst 33342 blue-red fluorescence (Figs. 3A, 3B). This population represented 2.3% to 5.4% of total mouse limbal cells. Analysis of individual fractions from the Percoll gradient showed that SP cells were enriched in light and dense fractions (40% and 80% Percoll) and were relatively decreased in cells from the intermediate fractions of the Percoll gradient (Figs. 3C–H).

Forward-scattering analysis was performed to determine the relative cell size and granularity of SP cells from unseparated total limbal cells (Fig. 4A) or of the fractions from the 40% (Fig. 4B) and the 80% (Fig. 4C) Percoll gradients. The SP cells from the 40% gradient fraction were apparently more heterogeneous with respect to granularity than were the small and more uniform SP cells in the 80% fraction.

### Growth Properties of Limbal Cell Fractions

To evaluate the proliferative capacity of the various cell fractions, we seeded the cells at a concentration of 5 × 10^3 cells/well into 96-well tissue culture plates (in a volume of 100 µL complete RPMI 1640 medium/well). Cell proliferation was determined by incorporation of [3H]thymidine. As demonstrated in Figure 5A, the 40% Percoll gradient cell fraction proliferated with a significantly higher intensity than did the unseparated limbal cells, whereas cells from the 70% or 80% fractions had very limited proliferative activity.

We also assessed the proliferative capacity of individual cell populations on irradiated 3T3 fibroblast feeder cells in 96-well tissue culture plates. The proliferative activity of limbal cells was determined according to the incorporation of radioactivity. The results are expressed as the ratio of the proliferative activity of limbal cells on a 3T3 feeder layer to the proliferation of the same cells in wells without feeder cells. As demonstrated.

### Table 2. Recovery of Original Limbal Cells and the Proportion of Cells in Individual Fractions after Percoll Gradient Centrifugation

<table>
<thead>
<tr>
<th>Exp.</th>
<th>40%</th>
<th>50%</th>
<th>60%</th>
<th>70%</th>
<th>80%</th>
<th>Bottom</th>
<th>Cell Recovery (%)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>14.2</td>
<td>17.2</td>
<td>16.3</td>
<td>22.8</td>
<td>7.2</td>
<td>8.0</td>
<td>85.7</td>
</tr>
<tr>
<td>2</td>
<td>10.4</td>
<td>17.5</td>
<td>27.1</td>
<td>15.5</td>
<td>5.1</td>
<td>8.4</td>
<td>79.8</td>
</tr>
<tr>
<td>3</td>
<td>13.0</td>
<td>11.6</td>
<td>25.1</td>
<td>17.3</td>
<td>7.7</td>
<td>9.7</td>
<td>82.4</td>
</tr>
<tr>
<td>4</td>
<td>10.2</td>
<td>13.3</td>
<td>22.1</td>
<td>21.7</td>
<td>6.1</td>
<td>4.2</td>
<td>77.6</td>
</tr>
<tr>
<td>5</td>
<td>14.6</td>
<td>16.3</td>
<td>20.0</td>
<td>20.3</td>
<td>7.1</td>
<td>8.4</td>
<td>86.7</td>
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</tbody>
</table>
and rat corneas. To date, there are no reports of isolation and expression of certain markers such as a transporter ABCG2, which are characterized by small size; a low rate of replication; located in the basal layer of the limbal epithelium. These cells, SCs for the renewal of the corneal surface epithelium are

**DISCUSSION**

was not increased over spontaneous proliferation levels with-

in Figure 5B, in this experimental setting, the strongest prolif-

erative activity was observed in the small cell population re-

in comparison to 2%–5% of cells expressing the SP phenotype

the ratio of cytoplasm to nucleus. Further analysis of this fraction showed that this population was en-

riched in cells expressing the SP phenotype (>30% of SP cells

in comparison to 2%–5% of cells expressing the SP phenotype

in the whole limbus) and the fraction also had significantly

enhanced expression of the SC markers ABCG2, Lgr5, and p63

in comparison with unseparated limbal cells. In addition, these
cells were in a nonproliferative quiescent state as demon-

strated by their very low spontaneous uptake of radioactive

thymidine in vitro. However, when cultured on a feeder cell

layer, they demonstrated considerable proliferative capacity.

All these characteristics resemble properties of limbal SC de-

scribed in human or rabbit limbal tissue.12–14,24,29

SCs for the renewal of the corneal surface epithelium are

located in the basal layer of the limbal epithelium. These cells,

which are characterized by small size; a low rate of replication;

expression of certain markers such as a transporter ABCG2,
p63, integrin α9, or K19; and by the expression of the SP

phenotype have been described in human,10,12–14 rabbit,24,29

and rat corneas. To date, there are no reports of isolation and

characterization of limbal SCs in the mouse.

We have shown that cells sharing morphologic, phenotyp-

tical, and functional characteristics with human and rabbit

limbal SC can also be found in the mouse limbus. To dissociate

limbal tissue into a single cell suspension, we compared vari-

ous enzymatic digestion protocols including dispase treat-

ment, combination of dispase, and trypsin or trypsin digestion alone.

Repeated short trypsin digestions were the optimal method,

allowing recovery of 0.5 to 1 × 10⁶ limbal cells per one BALB/c

mouse. These cells were heterogeneous with respect to the

size, granularity, and the ratio of cytoplasm to nucleus and

could be separated into six subpopulations on a 40% to 80%

discontinuous Percoll gradient. The densest fraction (80% Per-
coll), representing approximately 7% of original limbal cells,

was enriched in cells showing morphologic characteristics
described for the human and rabbit limbal SCs: small size,
dense cells, and a low ratio of cytoplasm to nucleus. Further

analysis of this fraction showed that this population was en-

riched in cells expressing the SP phenotype (>30% of SP cells

in comparison to 2%–5% of cells expressing the SP phenotype

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**FIGURE 2.** Expression of genes for SC markers ABCG2 (A), Lgr5 (B), and p63 (C), and for corneal differentiation marker K12 (D) in unseparated limbal cells and in individual fractions from a Percoll gradient. Real-time PCR was performed on unseparated limbal cells (total) and cells retained on 40%, 50%, 60%, 70%, and 80% gradients. Data are the mean ± SD of three separate experiments. The comparative C<sub>T</sub> method was used to determine the change in targeted gene expression normalized by the internal control gene GAPDH. *P < 0.05, **P < 0.01.

**FIGURE 3.** SP profile of freshly isolated unseparated mouse limbal cells and cells from the Percoll gradient fractions. Unseparated limbal cells (A, B) or cells from fraction 40% (C, D), 60% (E, F), or 80% (G, H) gradients were subjected to Hoechst 33342 exclusion assay. The cells were analyzed by flow cytometry (A–H), and the dye efflux from the SP was blocked by verapamil (B, D, F, H).
A second cell fraction showing at least some characteristics of limbal SCs was detected in the lightest cell population (40% Percoll gradient) and represented approximately 12% of the total limbal cell population. These cells expressed genes for the SC markers ABCG2 and Lgr5, and over 20% of the cells expressed the SP phenotype, a property of SCs. However, unlike the dense cell population (80% gradient) which also expressed the SP phenotype and SC markers, the light cell population was positive for corneal differentiation marker K12 and had the highest spontaneous proliferative capacity (significantly higher than unseparated limbal cells) and their proliferation response did not increase when they were cultured on a feeder cell monolayer. Thus, two separable populations of mouse limbal cells which have SC characteristics (ABCG2 and Lgr5 expression, the SP phenotype) can be obtained by centrifugation on Percoll gradient. The cells retained in the intermediate layer of the gradient (60% Percoll) had lower expression levels of SC markers and a lower percentage of cells expressing the SP phenotype than original unseparated limbal cells.

The results thus demonstrated that two distinct populations of limbal cells with SC characteristics can be isolated in the mouse. Both populations contained cells expressing the SP phenotype based on the efflux of Hoechst 33342 dye. However, forward-scattering analysis of SP cells from the top and bottom fractions showed that both populations differ in their size and granularity. The number of SP cells in the unseparated mouse limbus was 3.8% (average from five experiments) of total limbal cells, substantially higher than the number of slow-cycling corneal epithelial cells found at the mouse limbus or the number of SP cells in human, rabbit, and rat limbal epithelia, but corresponds to the number of SP cells found in the rat cornea. Although it has been shown that the SP phenotype is associated with ABCG2 expression, the corollary is not necessarily true (i.e., not all cells expressing ABCG2 exhibit the SP phenotype). The studies of Umemoto and coworkers in humans, rabbits, and rats showed that although the number of cells exhibiting the SP phenotype was less than 2% in the limbus, immunohistochemistry revealed that a larger proportion (approximately 10%) of limbal basal epithelial cells expressed ABCG2 transporter. Similarly, Budak et al. suggested the existence of a significantly higher number of ABCG2+ cells than SP cells. This discrepancy was explained by the differences in the transport activity of ABCG2.

Unseparated limbal cells (total cell population) or cells retained on 40%, 50%, 60%, 70%, and 80% Percoll gradient fractions were seeded into wells of 96-well tissue culture plates (5,000 cells/well) or into wells containing a 3T3 fibroblast feeder layer. Cell proliferation was determined by incorporation of [3H]thymidine added to the cultures for the last 6 hours of the 96-hour incubation period. The results are expressed by the counts per minute (cpm) of [3H]thymidine ([3H]Thymidine incorporation) or as the increase in the proliferation rate (Ratio) of the Percoll gradient fraction in comparison to proliferation of the same cells cultivated without the 3T3 feeder cells. Each bar represents the mean ± SE of results in three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from the control (unseparated limbal cells).
24 to 48 hours. However, when we cultured the dense cell population on a 3T3 feeder cell monolayer, they exhibited a strong proliferative activity. Similarly, de Paiva et al.\(^\text{13}\) showed that human limbal SP cells proliferate better on feeder cells than do non-SP cells. To evaluate growth properties of individual cell fractions, we cultured these cells at low cell concentrations. The cells from the light fraction formed colonies of fibroblast-like cells and their growth was enhanced in the presence of epidermal or fibroblast growth factor. On the contrary, the cells from the dense fraction did not grow in cultures without feeder cells, even in the presence of the growth factors. A similar pattern of proliferation and responsiveness to the growth factors was observed in the cells from the light fraction when cultured on a 3T3 monolayer. However, the cells from the dense fractions that did not proliferate in cultures without feeder cells, formed on the 3T3 cell monolayer colonies of spherical cells, and their growth was not significantly influenced by epidermal or fibroblast growth factor. It is possible that quiescent, slowly dividing limbal SCs (separated in the dense fraction) require a specific niche (feeder cells) to support their proliferation.

This study showed that there are two distinct populations of corneal epithelial cells with SC characteristics (expression of ABCG2 and Lgr5, an SP phenotype) that can be isolated from the mouse limbus and that Percoll gradient centrifugation is a convenient method of enriching and harvesting such cells for the study of their characteristics, growth requirements, and use to treat various limbal SC deficiencies in experimental models.

References


29. 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;24:86–90.


