SSEA4 Is a Potential Negative Marker for the Enrichment of Human Corneal Epithelial Stem/Progenitor Cells

Thuy T. Truong,1 Kyle Huynh,2 Martin N. Nakatsu,1 and Sophie X. Deng1

PURPOSE. To examine the expression of stage-specific embryonic antigen-4 (SSEA4) in the epithelium of the human ocular surface and characterize SSEA4+ and SSEA4− limbal epithelial cells.

METHODS. SSEA4 expression in the human cornea and limbus was examined by RT-PCR and immunohistochemistry. SSEA4+ and SSEA4− cells were then separated by using magnetic beads. The phenotypes of these two cell populations were evaluated on the basis of cell size, clonogenic assay, and expression of putative limbal stem cell (LSC) and corneal epithelial differentiation markers.

RESULTS. SSEA4 was expressed in all layers of the corneal and anterior limbal epithelium. Discrete clusters of SSEA4+ cells were present in the central and posterior limbal epithelia. SSEA4+ cells accounted for an average of 40% of the total limbal epithelial cells. The SSEA4+ population contained five times more small cells (≤ 11 μm in diameter) than did the SSEA4− population. The expression levels of the putative LSC markers ABCG2, ΔNp63α, and cytokeratin (K)14 were significantly higher in the SSEA4+ population than in the SSEA4− population. The SSEA4− cells also expressed a significantly higher level of N-cadherin, but a lower level of the differentiation marker K12. The colony-forming efficiency in the SSEA4− population was 25.2% (P = 0.04) and 1.6-fold (P < 0.05) higher than in the unsorted population and the SSEA4+ population, respectively.

CONCLUSIONS. SSEA4 is highly expressed in differentiated corneal epithelial cells, and SSEA4− limbal epithelial cells contain a higher proportion of limbal stem/progenitor cells. SSEA4 could be used as a negative marker to enrich the isolation of LSCs. (Invest Ophtalmol Vis Sci. 2011;52:6315–6320) DOI: 10.1167/iovs.11-7518

It has been widely accepted that the homeostasis of the corneal epithelium is maintained by a small subpopulation of limbal stem cells (LSCs) that localize at the basal layer of the limbus, a narrow zone circling the cornea and bordering it from the bulbar conjunctiva.1–5 Limbal basal epithelial cells are not homogeneous, but consist of diverse cell populations including LSCs, transient amplifying cells, and terminally differentiated cells, among which LSCs are found in a very small number, usually less than 10%.4–7 Although several studies have proposed the potential locations of the LSC niche, such as limbal crypts and focal stromal projections,8,9 to date, the exact location and spatial arrangement of the LSCs and their niche are unknown. Furthermore, Majo et al.10 recently proposed that the limbus may not be the only location of corneal epithelial progenitor cells and that the epithelium in the central cornea may also contain corneal epithelial progenitor cells. The heterogeneous cell population and unknown location of corneal stem/progenitor cells in the limbal region highlight the importance of searching for molecular markers, especially cell surface markers, to serve as tools, not only to identify stem cells in situ but also to efficiently isolate LSCs for ex vivo expansion for transplantation, a procedure that effectively treats limbal stem cell deficiency (LSCD).11–13 Among many molecules that have been proposed as markers of LSCs, ATP-binding cassette subfamily G member 2 (ABCG2) and ΔNp63α are the most often used to identify the stem cell population.14,15 In addition, other stem cell properties could be used to help identify the stem cell population. These include small cell size, high proliferative and clonogenic potential in vitro, and functional tissue regeneration.1,16

Stage-specific embryonic antigen-4 (SSEA4) is a globo-series carbohydrate core structure of glycoproteins.17 It has been commonly used as a pluripotent human embryonic stem cell marker18 and has been used to isolate mesenchymal stem cells19 and enrich neural progenitor cells.20 Expression of SSEA4 on the ocular surface has not been fully investigated. In the present study, we found that, in contrast to the high level of uniform expression of SSEA4 in differentiated corneal epithelial cells, this antigen is expressed only in clusters of limbal epithelial cells. Further characterization of SSEA4− limbal epithelial cells showed that this population contains a higher proportion of limbal stem/progenitor cells than do the unsorted and SSEA4+ cells.

METHODS

Human Sclerocorneal Tissue

Human sclerocorneal tissues of healthy donors were obtained from the Lions Eye Institute for Transplant and Research (Tampa, FL), the Tissue Bank International (Baltimore, MD), and the San Diego Eye Bank (San Diego, CA). Experimentation on human tissues complied with the Declaration of Helsinki. The experimental protocol was evaluated and approved by the University of California, Los Angeles Institutional Review Board. The age of the donors ranged from 17 to 62 years. For the purpose of RNA isolation, the death-to-preservation time was less than 7 hours. The tissues were either snap frozen on dry ice on procurement or preserved in RNA stabilizer (RNALater; Ambion Inc., Austin, TX) at 4°C. For immunohistochemistry, the death-to-preserva-
tion time was less than 8 hours and time to tissue processing was less than 5 days. For the purpose of cell culture, the limbal rim was collected from the unused portion of healthy donor tissues immediately after cornea transplantation.

Isolation of Limbal Epithelial Cells

Human limbal epithelial cells were isolated from corneoscleral rims after the central cornea button was used for transplantation, as previously described.21 The residual iris tissue, endothelium, conjunctiva, and Tenon’s capsule were removed. The tissues were treated with 2.4 U/ml of Dispase II (Roche, Indianapolis, IN) in keratinocyte serum-free medium (KSFM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 37°C for 2 hours. The limbal stromal layer was removed, and the limbal epithelium layer was treated with a 0.25% trypsin/1 mM EDTA solution (Invitrogen) until a single-cell suspension was obtained, and enzymatic activity was stopped by adding an equal volume of 10% FBS/KSF.

SSEA4+ and SSEA4− cells were separated by using a magnetic bead kit (Dynabeads; Invitrogen) according to the manufacturer’s instructions. Briefly, single limbal epithelial cells were incubated with anti-SSEA4 antibody for 15 minutes. After unbound antibodies were removed by washing with 10% FBS/KSFM, the cell suspension was incubated with the beads and subsequently exposed to a magnet. The bead-bound cells were collected as the SSEA4+ population, and the remaining suspension was the SSEA4− population. The cell suspension, incubated with the anti-SSEA4 antibody and beads but not exposed to a magnet, was the unsorted population. A small portion of each cell population was seeded on collagen I-coated chamber slides (Laboratory-Tek; Nunc, Rochester, NY) and cultured for 2 days in 10% FBS/KSF. The slides were then fixed and immunostained for SSEA4 expression to evaluate separation efficiency.

Primary Limbal Epithelial Cell Culture

The limbal epithelial cells isolated as described above were seeded on growth-arrested mouse NIH 3T3 cells (American Type Culture Collection [ATCC], Manassas, VA) at a density of 100 and 300 cells/cm² for clonogenic assays in 10% FBS/KSFM containing bovine pituitary extract and epidermal growth factor (2 ng/mL). The KSFM growth medium consisted of 150 cells in each cell population was measured for each experiment.

Clonogenic Assay

Cells in duplicate culture dishes were fixed with 4% PFA and stained with 10 μg/mL mitomycin C (Sigma-Aldrich, St. Louis, MO) for 2 hours at 37°C and then trypsinized and subcultured in Dulbecco’s modified Eagle’s medium (DMEM; ATCC) supplemented with 10% FBS at a density of 3 × 10⁴ cells/cm².

Cell Size Analysis

To measure cell diameter, the single-cell suspension was introduced into a phase hemocytometer chamber. The cells were photographed through a 20X phase objective of a phase-contrast microscope by digital camera (Insight SPOT; Diagnostic Instruments, Inc; Sterling Heights, MD). Cell diameter was measured by using ImageJ software (developed by Wayne Rasband; National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). A minimum of 150 cells in each cell population was measured for each experiment. Six experiments were performed.

Table 1. Primary and Secondary Antibodies

<table>
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<tr>
<th>Protein</th>
<th>Company</th>
<th>Host</th>
<th>Species Reactivity</th>
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<tbody>
<tr>
<td>SSEA4</td>
<td>R&amp;D Systems, Minneapolis, MN</td>
<td>Mouse</td>
<td>Human/mouse</td>
</tr>
<tr>
<td>Alexa Fluor 546 IgG</td>
<td>Invitrogen, Carlsbad, CA</td>
<td>Donkey Mouse</td>
<td></td>
</tr>
<tr>
<td>SSEA4</td>
<td>Invitrogen</td>
<td>Goat</td>
<td>Mouse</td>
</tr>
<tr>
<td>Alexa Fluor 488 IgG</td>
<td>Invitrogen</td>
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<td>Mouse</td>
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Immunofluorescent Staining

The human sclerocorneal tissues were embedded in OCT (Sakura Finetek, Torrance, CA) on dry ice. Seven-micrometer cryosections were obtained (model CM3505S cryostat; Leica Microsystems, Wetzlar, Germany) and stored at −80°C. Tissue cryosections or cell culture slides were fixed in 4% paraformaldehyde (PFA) for 15 minutes and permeabilized (except for SSEA4) by washing three times for 5 minutes with 0.3% Triton X-100 (Sigma-Aldrich) in phosphate-buffered saline (PBS). Fixed slides were incubated with appropriate 10% blocking serum (Jackson Immunoresearch Laboratories, West Grove, PA) in PBS for 30 minutes, washed three times with 1% BSA/PBS, and incubated with primary antibodies for 1 hour at room temperature. The slides were then washed three times with 1% BSA/PBS and labeled with the appropriate secondary antibody for 1 hour at room temperature. The primary and secondary antibodies are listed in Table 1. The nuclei were labeled with 4 μg/mL Hoechst 33342 (Invitrogen) for 15 minutes. The slides were then washed with PBS five times and mounted in aqueous mounting medium (Fluromount; Sigma-Aldrich). Images were obtained under 10× and 25× objectives with a fluorescence microscope (Carl Zeiss, Inc., Oberkochen, Germany).

RNA Extraction and Quantitative RT-PCR

Limbal epithelial cells were lysed and homogenized by using a spin column (QIAshredder; Qiagen; Valencia, CA). Total RNA was extracted (RNaseasy Micro and Mini Kits; Qiagen). The quantity and quality of the total RNA were assessed with a spectrophotometer (NanoDrop 1000; NanoDrop, Wilmington, DE) and a bioanalyzer (model 2100; Agilent Technologies; Santa Clara, CA). Only RNA with minimal degradation was used for subsequent experiments.

Total RNA was reverse transcribed (Superscript II RNase H2 reverse transcriptase; Invitrogen) according to the manufacturer’s recommendations. The relative abundance of transcripts was detected by quantitative (q)RT-PCR (Brilliant SYBR Green qRT-PCR Master Mix on an Mx3000p real-time PCR system; Stratagene, La Jolla, CA). Cycling conditions were as follows: an initial denaturing step of 5 minutes at 94°C and 40 subsequent cycles of amplification in which each cycle consisted of 15 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C. To generate a dissociation curve after the amplification cycles, we incubated each sample at 95°C for 1 minute followed by a melting curve program (55–99°C with a 5-second hold at each temperature). The fluorescence intensity of each sample was acquired during the execution of the melting curve program and normalized in relation to that of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase. The average value of the triplicates from each transcript was used for comparison. The relative changes in gene expression from the qRT-PCR results were analyzed by the 2−ΔΔCt method. At least three independent experiments were performed, and a total of three donors were used for qRT-PCR. To allow for direct comparison among independent experiments, the ratios of the absolute expression values of the SSEA4+ and SSEA4− population to those of the unsorted population were used. The primers used for qRT-PCR and RT-PCR are listed in Table 2.

Clonogenic Assay

Cells in duplicate culture dishes were fixed with 4% PFA and stained with 2% rhodamine B (Sigma-Aldrich). Colonies were counted under a dissecting microscope. Colony-forming efficiency (CFE) was calculated...
**SSEA4, a Negative Marker of Limbal Stem Cells**

To evaluate the isolation efficiency, the separated cells were examined by immunohistochemistry for their expression of SSEA4. As shown in Figure 2B, the unsorted population contained both SSEA4⁺ and SSEA4⁻ cells. After separation with magnetic beads, all cells from the SSEA4⁺ population stained positively for SSEA4 expression (Fig. 2C). As expected, the SSEA4⁻ population did not express SSEA4 (Fig. 2D). The SSEA4⁻ and SSEA4⁺ cells accounted for 59.5% ± 5.7% and 38.1% ± 3.1% (P < 0.05) of the total population of freshly isolated primary limbal epithelial cells, respectively (n = 6; Fig. 2E). There was a loss of 2.3% ± 3.9% cells during the separation process. These results confirmed the high efficiency of the separation method and purity of these two cell populations.

**SSEA4⁻ Limbal Epithelial Cells Contained a Higher Proportion of Small Cells**

Stem cells and progenitor cells tend to be smaller than differentiated cells. We next examined the size of cells in the SSEA4⁺ and SSEA4⁻ populations. As shown in Figure 3, the diameter of limbal epithelial cells ranged from 8 to 30 μm. Interestingly, the cells in the SSEA4⁻ population had a mean diameter of 19 to 21 μm, whereas those of the SSEA4⁻ population were skewed to the left. We found that 10.8% ± 3.1% of SSEA4⁺ cells and 1.7% ± 0.6% of SSEA4⁻ cells had a diameter ≤11 μm (P < 0.001). Forty-seven percent of SSEA4⁻ cells had a diameter between 12 and 17 μm, whereas 47% of SSEA4⁻ cells had a larger diameter, between 18 and 23 μm (Fig. 3). These findings show that most SSEA4⁺ cells were smaller than the SSEA4⁻ cells.

**The SSEA4⁻ Population Was Less Differentiated**

To confirm that SSEA4⁻ cells were indeed less differentiated, we first examined the mRNA expression levels of the putative LSC markers, ABCG2, ΔNp63α, and K14 and of the mature corneal epithelial marker K12 by qRT-PCR. The SSEA4⁻ population expressed 2.3- and 1.3-fold higher levels of ABCG2 than did the SSEA4⁺ and the unsorted populations (P < 0.001 and P = 0.007, respectively; Fig. 4). The expression level of ΔNp63α was also highest in the SSEA4⁻ population. K14 is another marker of less differentiated limbal epithelial cells. Again, its expression level was highest in the SSEA4⁻ population, moderate in the unsorted population, and lowest in the SSEA4⁺ population. The difference was statistically significant between the SSEA4⁻ and SSEA4⁺ populations (P = 0.03). In contrast, comparison of the expression levels of K12 showed no significant differences between the populations.

**RESULTS**

**SSEA4 Expression in Human Corneal and Limbal Epithelia**

SSEA4 is a carbohydrate epitope of glycoproteins expressed during early development and is a marker of human embryonic stem cells. The presence of SSEA4 synthase is an indicator of SSEA4 synthesis. As determined by RT-PCR, SSEA4 synthase mRNA was detected in both the corneal and limbal epithelia. (Figs. 1A, 1B). Expression was seen mostly in the suprabasal cell clusters that were scattered across the limbal epithelium (Fig. 1A). SSEA4 was uniformly present in all layers of the corneal epithelium at a very high level detected by immunohistochemistry (Figs. 1B, 1C), but was detected only in discrete cell clusters. This expression pattern suggested that SSEA4 expression might be more specific to the differentiated corneal epithelial cells. To test this hypothesis, SSEA4 and SSEA4⁺ populations were separated for further phenotypic analysis.

**Table 2. RT-PCR and qRT-PCR Primers**

<table>
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<tr>
<th>Gene</th>
<th>Direction</th>
<th>Primer Sequence</th>
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<tr>
<td>SSEA4</td>
<td>Forward</td>
<td>TGGACGGGCGACAATTCA</td>
</tr>
<tr>
<td>ABCG2</td>
<td>Forward</td>
<td>GGGCAAGCTGGGTGAGTCGTCGTC</td>
</tr>
<tr>
<td>ΔNp63α</td>
<td>Forward</td>
<td>GCCACCTCTGACGATCGCA</td>
</tr>
<tr>
<td>K12</td>
<td>Forward</td>
<td>GGCAAGTTGATTGGAGGGATG</td>
</tr>
<tr>
<td>K14</td>
<td>Forward</td>
<td>AGGGGTCTACATGGCAACTG</td>
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<tr>
<td>N-cadherin</td>
<td>Reverse</td>
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<tr>
<td>GAPDH</td>
<td>Forward</td>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGGGGTCTACATGGCAACTG</td>
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by dividing the number of colonies by the number of cells seeded per dish.

**Statistical Analysis**

Student’s t-test was performed to analyze the ratios for gene expression analysis, cell size distributions, and CFUs. P ≤ 0.05 was considered statistically significant.

**GAPDH**

**Cornea**

**Limbus**

**SSEA4 synthase**

**GAPDH**

**Cornea Limbus**

**SSEA4 synthase**

**GAPDH**

**Cornea Limbus**

**SSEA4**

**Nucleus**

**Figure 1.** Expression of SSEA4 synthase mRNA (A) and SSEA4 (B–E) in the human cornea and limbus. (A) SSEA4 synthase mRNA was detected by RT-PCR in both the corneal and limbal epithelia. (B–E) Detection of SSEA4 by immunohistochemistry in the human corneal and limbal epithelia. SSEA4 was uniformly present in all layers of the corneal epithelium (B, C), but only in clusters of the limbal epithelium (D, E).
that the SSEA4− population had a significantly lower expression level (0.3) than did the unsorted cell population (1.0, P < 0.0001) and the SSEA4− population (1.7, P < 0.0001). N-cadherin is expressed by the limbal stem/progenitor cells in their niche.27 Interestingly, the SSEA4− cells expressed a significantly higher level of N-cadherin than did the SSEA4+ population (P < 0.0001). Again these findings suggest that the SSEA4− population contains a higher proportion of stem/progenitor cells.

SSEA4− Population Has a Greater Clonogenic Potential In Vitro

When the clonogenic assay was performed to assess the CFE of the three different cell populations, the SSEA4− cells generated a 25.2% higher CFE than the unsorted population (P = 0.04) and a 2.6-fold higher CFE than the SSEA4+ cells (P < 0.05, Fig. 5).

DISCUSSION

In the present study, we used a simple but efficient method to isolate and characterize SSEA4− and SSEA4+ limbal epithelial cells. Like K12-negative and connexin 43-negative cells, SSEA4+ cells are located in the limbus but not in the cornea. In addition, only a subset of the basal limbal epithelial cells reside in the limbal basal layer.28,29 A large number of studies have shown the close correlation between the cell size and the proliferation and differentiation potential of keratinocytes. The smallest cells possess stem cell properties both in vivo28–30 and in vitro.30,31 This property is seen in the epithelia of the epidermis,30 ocular surface,28 and oral mucosa.32,33 Our observation that the SSEA4− population contained five times more small cells (<11 μm) than the SSEA4+ population (P < 0.001, n = 6).

The human limbal basal cells have been reported to be small, with an average diameter of 10.1 ± 0.8 μm (range, 8.5–12.2 μm), and are the smallest cells of the corneal and limbal epithelia reside in the limbal basal layer.28,29 A large number of studies have shown the close correlation between the cell size and the proliferation and differentiation potential of keratinocytes. The smallest cells possess stem cell properties both in vivo28–30 and in vitro.30,31 This property is seen in the epithelia of the epidermis,30 ocular surface,28 and oral mucosa.32,33 Our observation that the SSEA4− population contained five times more small cells (<11 μm) than the SSEA4+ population (P < 0.001, n = 6).

**Figure 2.** Separation of the SSEA4+ and SSEA4− populations of the limbal epithelial cells using magnetic bead sorting. (A) The negative control (i.e., unsorted cells that were not incubated with primary antibody) showed no labeling by the secondary antibody alone. (B) The population of unsorted limbal epithelial cells contained SSEA4+ cells and SSEA4− cells. (C, D) After sorting, the SSEA4+ population contained only SSEA4− cells (C), and the SSEA4− population contained only SSEA4+ cells (D). Many SSEA4+ cells showed squamous morphology (arrows). (E) Quantitation of the SSEA4+ and SSEA4− cell populations. SSEA4+ cells accounted for 59.5% ± 5.7% of the total population of freshly isolated limbal epithelial cells, and 58.1% ± 3.1% were SSEA4−. *P < 0.05 between SSEA4+ and SSEA4− populations (n = 6).

**Figure 3.** Difference in sizes of cells in the SSEA4+ and SSEA4− populations. The SSEA4− population contained a larger proportion of small cells than the SSEA4+ population: 10.8% ± 3.1% of SSEA4− cells had a diameter ≤11 μm, whereas 1.7% ± 0.6% of SSEA4+ cells had the same diameter (P < 0.001, n = 6).
level of the corneal epithelial maturation marker K12. In addition, N-cadherin, which is expressed in limbal stem/progenitor cells in the stem cell niche, is expressed at a significantly higher level in the SSEA4 population. The SSEA4 cells contain more differentiated corneal epithelial cells. These observations further support the notion that the SSEA4 population contains a larger number of stem/progenitor cells. The SSEA4 population is heterogeneous and whether it also contains stromal cells and/or melanocytes should be further investigated.

SSEA4 is an epitope on related glycosphingolipids with globo-series carbohydrate core structures, which are expressed in preimplantation human embryos and embryonic stem cells (hESCs). The anti-SSEA4 antibody recognizes the globo-series carbohydrate core, but not the protein itself. Although SSEA4 has been used as a surface marker of human embryonic carcinoma cells, human embryonic germ cells, and hESCs, its function is largely unknown. The expression of SSEA4 is tightly regulated during preimplantation development and on hESCs, but this expression is not crucial for regulation of pluripotency. Depletion of glycosphingolipids, including SSEA4, appears to be dispensable for the undifferentiated state, but is required for cellular differentiation or survival of differentiated lineages during development. Interestingly, SSEA4 is also expressed on some highly differentiated cells such as erythrocytes and functional dorsal root ganglion neurons. Therefore, SSEA4 has been suggested as a versatile carbohydrate antigen, and care should be taken when using this marker to evaluate or isolate stem cells. The alternative functional candidate would be the glycoprotein(s) carrying the SSEA4 epitope, not the glycolipid. A laminin-binding protein on a mouse ES cell and a 60-kDa protein in hESCs have been identified. Whether the glycoprotein carrying the SSEA4 epitope is the same in the corneal epithelial cells as in the hESCs and its function in development remains to be determined.

In summary, our study shows the SSEA4 expression pattern on the ocular surface epithelium. Mature corneal epithelial cells have a higher expression level of SSEA4 than the less differentiated limbal epithelial cells. We developed a fast and efficient method to isolate the SSEA4 population, a population that contains a higher proportion of limbal stem/progenitor cells. These findings suggest that SSEA4 might be a marker for maturation of the corneal epithelium and could be used to enrich the limbal stem/progenitor cell population.

Acknowledgments

The authors thank Debra Farber for use of the real-time PCR system.

Figure 4. Relative mRNA levels of putative stem cell and differentiation markers evaluated by qRT-PCR. The SSEA4 population expressed higher levels of putative stem/progenitor cell markers (ABCG2, ΔNp63a, K14, and N-cadherin) and a lower level of differentiation marker (K12) than did the SSEA4 and unsorted populations. *P < 0.05 between SSEA4 and SSEA populations. **P < 0.05 between SSEA4- and unsorted populations.

Figure 5. Colony-forming efficiency of the SSEA4+ and SSEA4- cell populations. (A) SSEA4+ cells had a significantly higher colony-forming capacity than did the SSEA4- population and the unsorted population. (B) Representative photos of colonies from the SSEA4+, SSEA4-, and the unsorted populations seeded at 100 or 300 cell/cm². *P ≤ 0.05 between the SSEA4- and SSEA4+ populations. **P ≤ 0.05 between the SSEA4- and unsorted populations.
References


31. de Paiva CS, Pflugfelder SC, Li DQ. Cell size correlates with phenotype and proliferative capacity in human corneal epithelial cells. Stem Cells. 2006;24:368–375.


