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

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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 epithelia. 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 ($\leq 11 \mu\text{m}$ in diameter) than did the SSEA4⁺ population. The expression levels of the putative LSC markers ABCG2, $\Delta\text{Np}63\alpha$, 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 Ophthalmol Vis Sci.* 2011;52:6315–6320) DOI: 10.1167/iov.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.^{1–3} 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 $\Delta\text{Np}63\alpha$ 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-

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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/KSFM.

SSEA4⁺ and SSEA4⁻ cells were separated by using a magnetic bead kit (Dynabeads; Invitrogen) according to the manufacturer's instruction. 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/KSFM. 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 was changed every 3 days, and the cells were cultured for 14 to 25 days.

To prepare growth-arrested feeder layers, subconfluent 3T3 cells were incubated with 10 μ g/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 3×10^4 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 20 $\times$ phase objective of a phase-contrast microscope by digital camera (Insight SPOT; Diagnostic Instruments, Inc; Sterling Heights, MI). Cell diameter was measured by using Image-J 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.

Immunofluorescent Staining

The human sclerocorneal tissues were embedded in OCT (Sakura Finetek, Torrance, CA) on dry ice. Seven-micrometer cryosections were obtained (model CM3050S 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 μ g/mL Hoechst 33342 (Invitrogen) for 15 minutes. The slides were then washed with PBS five times and mounted in aqueous mounting medium (Fluoromount; Sigma-Aldrich). Images were obtained under 10 $\times$ and 25 $\times$ 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 (RNeasy 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 $2^{-\Delta\Delta C_t}$ 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

TABLE 1. Primary and Secondary Antibodies

Protein	Company	Host	Species Reactivity
SSEA4	R&D Systems, Minneapolis, MN	Mouse	Human/mouse
Alexa Fluor 546 IgG	Invitrogen, Carlsbad, CA	Donkey	Mouse
Alexa Fluor 488 IgG	Invitrogen	Goat	Mouse

TABLE 2. RT-PCR and qRT-PCR Primers

Gene	Direction	Primer Sequence
<i>SSEA4</i> synthase	Forward	TGGACGGGGCACAACCTTCATC
	Reverse	GGGCAGGTTCTTGGCACTCT
<i>ABCG2</i>	Forward	AACCTGGTCTCAACGCCATC
	Reverse	GTCGGGTGCTCCATTATC
Δ Np63 α	Forward	TCCATGGATGATCTGGCAAGT
	Reverse	GCCCTTCCAGATCGCATGT
<i>K12</i>	Forward	CCAGGTGAGTCAAGGTAGAA
	Reverse	CCTCCAGGTTGCTGATGAGC
<i>K14</i>	Forward	GACCATTGAGGACCTGAGGA
	Reverse	ATTGATGTCGGCTTCCACAC
N-cadherin	Forward	GAGGAGTCAGTGAAGGAGTCA
	Reverse	GGCAAGTTGATTGGAGGGATG
<i>GAPDH</i>	Forward	CGACCACTTTGTCAAGCTCA
	Reverse	AGGGGTCTACATGGCAACTG

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 CFEs. *P* ≤ 0.05 was considered statistically significant.

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.¹⁷ The presence of SSEA4 synthase is an indicator of SSEA4 synthesis.²² As determined by RT-PCR, SSEA4 synthase mRNA was present in both corneal and limbal epithelia (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 that were scattered across the limbal epithelium (Figs. 1D, 1E). Expression was seen mostly in the suprabasal limbal epithelial cells and to a lesser extent in some of the basal cells within the 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 subsequent phenotypic analysis.

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.^{23,24} 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* < 0.0001 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.^{25,26} 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

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).

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 38.1% ± 3.1% were SSEA4⁺. * $P < 0.05$ between SSEA4⁻ and SSEA4⁺ populations ($n = 6$).

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 epi-

thelial 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 expressed SSEA4 (Fig 1D).

The human limbal basal cells have been reported to be small, with an average diameter of $10.1 \pm 0.8 \mu\text{m}$ (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 vivo^{28–30} 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 ($\leq 11 \mu\text{m}$) than the SSEA4⁺ population strongly suggests that the SSEA4⁻ population has a larger proportion of LSC/progenitor cells. This hypothesis is further supported by results of the phenotypic studies. The SSEA4⁻ population has higher expression levels of the three putative stem cell markers, ABCG2 $\Delta\text{Np}63\alpha$ and K14 and a lower expression

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 $\leq 11 \mu\text{m}$, whereas 1.7% ± 0.6% of SSEA4⁺ cells had the same diameter ($P < 0.001$, $n = 6$).

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, ΔNp63α, 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 SSEA4⁺ populations, ** $P < 0.05$ between SSEA4⁻ and unsorted populations.

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,¹⁷ which are expressed in preimplantation human embryos and embryonic stem cells (hESCs).¹⁸ 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.³⁴ 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.³⁵ Interestingly, SSEA4 is also expressed on some highly differentiated cells such as erythrocytes¹⁷ and

functional dorsal root ganglion neurons.³⁶ 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 cell³⁷ and a 60-kDa protein in hESCs have been identified.³⁴ 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⁻ limbal epithelial cells, 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.

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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. * $P \leq 0.05$ between the SSEA4⁻ and SSEA4⁺ populations. ** $P \leq 0.05$ between the SSEA4⁻ and unsorted populations.

References

- Schlotzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. *Exp Eye Res.* 2005;81:247-264.
- Pellegrini G, Rama P, Mavilio F, De Luca M. Epithelial stem cells in corneal regeneration and epidermal gene therapy. *J Pathol.* 2009;217:217-228.
- Ahmad S, Kolli S, Lako M, Figueiredo F, Daniels JT. Stem cell therapies for ocular surface disease. *Drug Discov Today.* 2010;15:306-313.
- Tseng SC. Regulation and clinical implications of corneal epithelial stem cells. *Mol Biol Rep.* 1996;23:47-58.
- Lehrer MS, Sun TT, Lavker RM. Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. *J Cell Sci.* 1998;111(Pt 19):2867-2875.
- Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol.* 1986;103:49-62.
- Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell.* 1989;57:201-209.
- Dua HS, Shanmuganathan VA, Powell-Richards AO, Tighe PJ, Joseph A. Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. *Br J Ophthalmol.* 2005;89:529-532.
- Shortt AJ, Secker GA, Munro PM, Khaw PT, Tuft SJ, Daniels JT. Characterization of the limbal epithelial stem cell niche: novel imaging techniques permit in vivo observation and targeted biopsy of limbal epithelial stem cells. *Stem Cells.* 2007;25:1402-1409.
- Majo F, Rochat A, Nicolas M, Jaoude GA, Barrandon Y. Oligopotent stem cells are distributed throughout the mammalian ocular surface. *Nature.* 2008;456:250-254.
- Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet.* 1997;349:990-993.
- Tsai RJ, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med.* 2000;343:86-93.
- Rama P, Matuska S, Paganoni G, Spinelli A, De Luca M, Pellegrini G. Limbal stem-cell therapy and long-term corneal regeneration. *N Engl J Med.* 2010;363:147-155.
- de Paiva CS, Chen Z, Corrales RM, Pflugfelder SC, Li DQ. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells.* 2005;23:63-73.
- Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De Luca M. Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci U S A.* 2005;102:9523-9528.
- Alison MR, Islam S. Attributes of adult stem cells. *J Pathol.* 2009;217:144-160.
- Kannagi R, Cochran NA, Ishigami F, et al. Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. *EMBO J.* 1983;2:2355-2361.
- Henderson JK, Draper JS, Baillie HS, et al. Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. *Stem Cells.* 2002;20:329-337.
- Gang EJ, Bosnakovski D, Figueiredo CA, Visser JW, Perlingeiro RCR. SSEA-4 identifies mesenchymal stem cells from bone marrow. *Blood.* 2007;109:1743-1751.
- Barraud P, Stott S, Möllgård K, Parmar M, Björklund A. In vitro characterization of a human neural progenitor cell coexpressing SSEA4 and CD133. *J Neurosci Res.* 2007;85:250-259.
- Nakatsu MN, Ding Z, Ng MY, Truong TT, Yu F, Deng SX. Wnt/ β -catenin signaling regulates proliferation of human cornea epithelial stem/progenitor cells. *Invest Ophthalmol Vis Sci.* 2011;52:4734-4741.
- Saito S, Aoki H, Ito A, et al. Human alpha2,3-sialyltransferase (ST3Gal II) is a stage-specific embryonic antigen-4 synthase. *J Biol Chem.* 2003;278:26474-26479.
- Angello JC, Pendergrass WR, Norwood TH, Prothero J. Proliferative potential of human fibroblasts: an inverse dependence on cell size. *J Cell Physiol.* 1987;132:125-130.
- Watt FM, Green H. Involucrin synthesis is correlated with cell size in human epidermal cultures. *J Cell Biol.* 1981;295:738-742.
- Kurpakus MA, Maniaci MT, Esco M. Expression of keratins K12, K4 and K14 during development of ocular surface epithelium. *Curr Eye Res.* 1994;13:805-814.
- Barnard Z, Apel AJ, Harkin DG. Phenotypic analyses of limbal epithelial cell cultures derived from donor corneoscleral rims. *Clin Exp Ophthalmol.* 2001;29:138-142.
- Hayashi R, Yamato M, Sugiyama H, et al. N-cadherin is expressed by putative stem/progenitor cells and melanocytes in the human limbal epithelial stem cell niche. *Stem Cells.* 2007;25:289-296.
- Romano AC, Espana EM, Yoo SH, Budak MT, Wolosin JM, Tseng SC. Different cell sizes in human limbal and central corneal basal epithelia measured by confocal microscopy and flow cytometry. *Invest Ophthalmol Vis Sci.* 2003;44:5125-5129.
- Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells.* 2004;22:355-366.
- Barrandon Y, Green H. Cell size as a determinant of the clone-forming ability of human keratinocytes. *Proc Natl Acad Sci U S A.* 1985;82:5390-5394.
- 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.
- Izumi K, Tobita T, Feinberg SE. Isolation of human oral keratinocyte progenitor/stem cells. *J Dent Res.* 2007;86:341-346.
- Calenic B, Ishkitiev N, Yaegaki K, et al. Magnetic separation and characterization of keratinocyte stem cells from human gingiva. *J Periodont Res.* 2010;45:703-708.
- Brimble SN, Sherrer ES, Uhl EW, et al. The cell surface glycosphingolipids SSEA-3 and SSEA-4 are not essential for human ESC pluripotency. *Stem Cells.* 2007;25:54-62.
- Yamashita T, Wada R, Sasaki T, et al. A vital role for glycosphingolipid synthesis during development and differentiation. *Proc Natl Acad Sci U S A.* 1999;96:9142-9147.
- Dodd J, Solter D, Jessell TM. Monoclonal antibodies against carbohydrate differentiation antigens identify subsets of primary sensory neurones. *Nature.* 1984;311:469-472.
- Katagiri YU, Kiyokawa N, Nakamura K, et al. Laminin binding protein, 34/67 laminin receptor, carries stage-specific embryonic antigen-4 epitope defined by monoclonal antibody raft. *Biochem Biophys Res Commun.* 2005;332:1004-1011.