P63 EXPRESSION LEVELS IN SIDE POPULATION AND LOW LIGHT SCATTERING OCULAR SURFACE EPITHELIAL CELLS

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ABSTRACT

Purpose: Because stem cells exhibit high self-renewal capacity, slow cycling, and high proliferative potential, and one of many markers postulated for epithelial stem cells, p63, is challenged by widespread expression within stem cell–free regions, we examined p63 expression in these stem cell–associated cohorts compared with their controls.

Methods: Rabbit limbocorneal cryosections, cytospun cell-sorted (by fluorescence-activated cell sorter) side population (SP) and low side scatter (LSSC) cells, and limbal epithelial cells over feeders were stained for p63 by indirect immunofluorescence. Clones were fixed and stained daily for 7 days. Image analysis measured p63 intensity, plotting it against colony size.

Results: All basal limbal cells were positive for p63, yet only 5% to 7% expressed high p63 intensities, 40% intermediate, and the majority low. Side population cells were less than 1% of total cells. The average intensity of SP staining was three times that of controls. Subpopulations displaying stemlike features exhibited highest p63 expression. Replication rates of isolated cells differed. Day 5 colonies contained 256 (16 hours/cycle) to two (96 hours/cycle) cells. Whereas all cells were positive for p63, intensity in slow-cycling cells was three to four times that in rapidly proliferating congeners. Increased cell doublings did not decrease fluorescence.

Conclusions: Results suggest that p63 concentration is maximal in stem cells and decreases with differentiation. High p63 levels seem to correlate with cells of the SP and LSSC phenotypes, indicating high cell stemness. With identification of stem cells, further studies can elucidate their use in supporting ocular surface health.

INTRODUCTION

Stem cells are generally defined as clonogenic cells capable of both self-renewal and multilineage differentiation. They exhibit high self-renewal capacity, slow cycling, and high proliferative potential. Cells exhibiting slow cycling possess several features consistent with stem cell phenotype.[1] Recently, limbal and conjunctival Hoechst 3342 side population (SP) cells were shown to exhibit many stem cell–associated features.[2] Many markers have been postulated for epithelial stem cells. One, p63, proposed for limbus,[3] is challenged by widespread expression within stem cell–free regions. During development and regeneration of a given tissue, such cells give rise to non-self-renewing progenitors with restricted differentiation potential and finally to functionally mature cells while maintaining primitive stem cells.

In the eye, corneal epithelial cells migrate centripetally from the periphery to the center of the corneal surface.[4,5] This renewal phenomenon of the corneal epithelium is attributed to stem cells that are located at the limbus.[6] Limbal basal cells contain a subpopulation of stem cells, which are characterized by high capacity of self-renewal, slow cell cycle, and high proliferative potential following wounding or placement in culture.[7-10]

Despite the crucial role of such cells in normal histologic physiology, our knowledge of their physical characteristics and the mechanisms that control their proliferation and differentiation remains limited because of their paucity. However, recent progress in cell separation technology has enabled the identification and isolation of very rare side population of cells within cell populations.[11]

Fluorescence-activated cell sorting (FACS) is useful in this regard because it is capable of sorting cells aseptically according to their size, side scatter, and fluorescence staining profiles.[12] In fact, multiple investigators have reported a variety of methods to isolate stem cells utilizing FACS.[3,12-14] One such widely used method involves the use of a flow cytometry assay to identify hematopoietic stem cells through the identification of a side population of cells displaying low Hoechst fluorescence.[3] In fact, Zhou and associates[15] demonstrated that such a side population of cells displaying low Hoechst fluorescence and low side scatter, with characteristics consistent with the stem cell phenotype, exists in multiple organs.

Despite the recently identified methods of stem cell isolation, the clear identification of stem cells remains problematic.

One such method has been the identification of p63, a transcription factor belonging to the p53 family, which plays a critical role in limbal and epidermal morphogenesis.[16-18] p63 null mice fail to develop stratified epidermis and thus die at birth as a result of a fully exposed dermis. p63 is expressed in the limbal epithelium. Using chromogenic development of p63 immunostainings, Pellegrini and associates[3] observed that in the limbus, p63 appears to be expressed in isolated basal epithelial cells. They also found that in clonal culture stem cells, p63 was detectable by Western blotting in cell populations identifiable as derived from stem cells but not in those cells representing the progeny of transient amplifying cells. These observations and the absence of correlation between p63 expression and PCNA (a marker of cell cycling activity) staining led the investigators to suggest that p63 identifies limbal stem cells.

However, this nuclear protein is not an exclusive marker for stem cells, because it has been shown to be expressed in mouse corneal and conjunctival[19] and human corneal[20] epithelial cells. In fact, when more sensitive staining methods such as immunofluorescence are used, most basal cells and many suprabasal cells fluoresce, and staining is revealed even in the basal cells of the cornea[19] (Figure 1). Such results undermine a straightforward relationship between p63 expression and cell stemness and raise the following question: What are the roles of this important developmental regulator in the limbal epithelium?
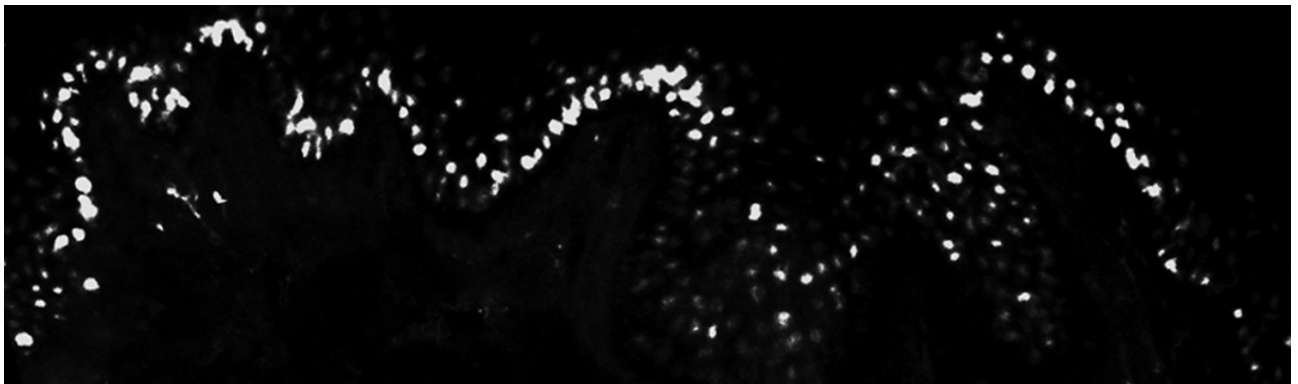

FIGURE 1

Human sections, 6 μm thick, of the Palisades of Vogt, stained for p63. Human globes were obtained and carefully trimmed. The Palisades of Vogt were dissected out and cut into 2-mm-long segments. Each was embedded, frozen, sectioned with a cryotome, and adhered onto positively charged slides, then fixed, blocked and stained for p63 by indirect immunofluorescence.

A quantitative examination of p63 levels within the human and rabbit limbal basal cells reveals great variation in p63 content (Figure 1). Thus, we wondered whether the relative level of p63 expression, rather than its absolute value, may have a role in some of the properties associated with the stem cell phenotype, including clonogenicity and rate of proliferation.

When freshly dissociated limbal epithelial cells are plated at low (clonal) density over 3T3 feeder cells, the start of proliferation of the viable adherent cells exhibits a high degree of asynchrony. Relating the delay in recruitment of proliferation to phenotypic feature of cells reveals that cells with extremely low "granularity" start their proliferation 24 to 48 hours later than most of the adherent cell population. Independently, we have demonstrated that these cells are in fact limbal stem cells.[10]

For this reason, we undertook the present study to examine the relationship between p63 levels and cells aseptically isolated utilizing FACS according to their size, side scatter, and fluorescence staining profiles.

METHODS

QUANTITATIVE HISTOLOGY

Cell Isolation

Tissues for sectioning were prepared in the stage of a dissecting microscope placed inside a horizontal laminar flow sterile hood ("clean bench") and equipped with a black cutting board and tangential illumination from freely adjustable fiberoptic-guided light source. Conjunctival remnants were carefully trimmed away from the corneoscleral tissue. The conjunctiva-free tissue was cut in four quarters and placed over the cutting board. Under these conditions, after adjusting the

illumination angle, it was possible to visualize and dissect away with single side blades the rabbit limbus as the 0.5- to 0.8-mm-wide whitish outer perimeter and the human limbus as the ~1-mm-wide zone containing the palisades of Vogt.

Immunostaining

Freshly dissected tissues were cut into 2-mm-long segments, embedded in Cryomatrix (Shandon, Pittsburgh, Pennsylvania), frozen in liquid nitrogen, and cut into 6-μm-thick sections in a Hacker cryotome. Sections were fixed in cold methanol, blocked in 5% bovine serum albumin (BSA), and overlaid with anti-p63 (clone Y4A3) primary Ab (2 μg/mL in phosphate-buffered saline [PBS]; PharMingen, BD Biosciences, San Diego, California) for 2 hours. After slides were washed in PBS three times for 5 minutes, sections were overlaid for 1 hour with Alexa 488-conjugated goat anti-mouse IgG (1 μg/mL; PharMingen) overlaid with anti-p63 (clone Y4A3) primary Ab (2 μg/mL in PBS; PharMingen) for 1 hour. After slides were washed again in PBS three times for 5 minutes, sections were overlaid and the slides were washed three times and mounted in antifading medium (Vectastain; Vector Labs Inc, Burlingame, California).

Quantitative Analysis of p63 Intensities

Cells were imaged by epifluorescent illumination, using a Zeiss Axiophot microscope (Carl Zeiss Light Microscopy, Göttingen, Germany) equipped with a 20× objective (CWD CDPlanAPO 20UV; Olympus Corporation, Inc, Japan) and a deep green filter (with a cutoff at 550 nm; Olympus Corporation). Digital images were collected using a Spot2 CCD camera and saved as TIFF files. These images were then analyzed utilizing IPLab Image Analysis software (Scanalytics, Fairfax, Virginia).

Data Analysis

The intensity of nuclear staining (relative measure of p63 content) was measured and plotted as a function of colony size by image analysis. Data were correlated and analyzed for statistical differences, utilizing the mean ± standard deviation for each of the various collection periods.[21] Two-tailed significance was established at a confidence level of $.05 \geq P \geq .95$.

SP AND LOW SIDE SCATTER EXPERIMENTATION

Cell Isolation

Tissues for enzymatic digestions were prepared in the stage of a dissecting microscope as described above with a black cutting board and tangential illumination. Limbal sections were isolated as described above. The rabbit conjunctivae were dissected to include all tissue from the mucocutaneous edge to the limbus. All dissected tissues were placed on flasks or clinical centrifuge tubes containing 5 mg/mL Dispase (Roche Diagnostics, Indianapolis, Indiana) freshly dissolved in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–buffered 1:1 mix of Dulbecco's modified Eagle's medium and Ham-F12 (hD/F-12; Gibco, Invitrogen Inc, Grand Island, New York). The flasks were rocked end to end (<20 turns per second) at 4°C overnight. The containers were inspected the following morning for the spontaneous detachment of epithelial sheets. If the detachment was only partial, or did not occur at all, tissues were agitated at greater than 200 rpm in an orbital shaker. Full separations were obtained within 15 to 30 minutes. These spontaneously released epithelial sheets were transferred to conical centrifuge tubes containing 20 mL of 0.25% Typsin·2.21 mM EDTA-4Na in calcium-, magnesium- and bicarbonate-free Hank's balanced salt solution (Mediatech, Herndon, Virginia) at 37°C for 30 minutes.

Cell Sorting

Freshly dissociated cells were counted with a hemocytometer (Fisher Scientific, Pittsburgh, Pennsylvania) and separated on the basis of forward and side-scattering properties using the MoFlo Cell Sorter (Dako Cytomation, Fort Collins, Colorado). SP and non-SP cells were gated, separated, and individually collected from the limbal cells, and low side scatter (LSSC) and non-LSSC cells were separated and individually collected from the conjunctivae.

Cytospin Preparation

Cells within each of the above described gated separations were diluted to 1×10^5 cells/mL in PBS and 200 μL cytospun onto clean positively charged slides (Fisher Scientific, Pittsburgh, Pennsylvania) using a Shandon Cytospin Centrifuge (version 3; Thermo Electron Corp, Cheshire, United Kingdom). The resulting slides were fixed in 10% buffered formalin (equivalent of 4% buffered paraformaldehyde; Fisher Chemical Co, Middletown, Pennsylvania) for 1 minute at room temperature, then slowly frozen in 100% methanol (Protein Sequencing Grade, Certified ACS reagent; Sigma-Aldrich, Inc, St Louis, Missouri) at 80°C overnight.

Immunostaining

The above cytospins were slowly brought to room temperature and allowed to air-dry (10 minutes). The slides were then detergent digested and permeabilized (Triton X-100 [Sigma-Aldrich]) for 10 minutes at room temperature, blocked in 5% BSA (room temperature), and stained for p63 using the same indirect immunofluorescence protocol previously described.

Quantitative Analysis of p63 Intensities

Cells were imaged and digitally photographed, and the resulting images were saved and analyzed by means of segmentation analysis as described above.

Data Analysis

The intensity of nuclear staining (relative measure of p63 content) was measured, and the resulting data were correlated and analyzed as described above. As before, two-tailed significance was established at a confidence level of $.05 \geq P \geq .95$.

DELAYED PROLIFERATION

Experimental Rationale

It is assumed that once a single isolated epithelial cell undergoes one cell division, it will immediately continue dividing into four cells, eight cells, and so on.

Cell Isolation

Single cell suspensions of limbal and conjunctival cells were prepared utilizing the same technique of dissection followed by dispase and trypsin digestion described above.

Clonogenic Assays

Freshly dissociated limbal and/or conjunctival cells were plated at very low density (10 to 50 cells/cm^2) in 20% FCS-hD/F12 onto coverslips containing Swiss 3T3 fibroblasts that had been incubated with 8 μg/mL mitomycin C for 3 hours within the prior 48 hours to elicit permanent cell proliferation arrest and plated at a rate of 2,000/cm^2 of culture surface. The culture medium was replaced after 3 days.

Immunostaining

Culture coverslips were fixed daily with formalin and flash frozen in methanol at –80ºC. Subsequently, coverslips from all days examined were rehydrated in PBS and stained for p63 by indirect immunofluorescence as described above.

Quantitative Analysis of p63 Intensities

Cells were imaged and digitally photographed, and the resulting images were saved and analyzed via segmentation analysis as described above.

Data Analysis

The intensity of nuclear staining (relative measure of p63 content) was measured, and the resulting data were correlated and analyzed as described above. As before, two-tailed significance was established at a confidence level of $.05 \geq P \geq .95$.

RESULTS

QUANTITATIVE HISTOLOGY

In the tissue sections, approximately 5% to 7% (rabbit conjunctiva, 6 of 89; rabbit limbus, 3 of 55: see Table 1, Figure 1) of the cells possessed distinct high stain intensities (>150, maximum of ≈210), 25% (conjunctiva, 25 of 89; limbus, 13 of 55: see Table 1, Figure 1 [conjunctiva indistinguishable from limbus]) had intermediate values (30% to 60% [75 to 150] of the maximum value), and the remainder (conjunctiva, 58 of 89; limbus, 39 of 55: see Table 1, Figure 1 [conjunctiva indistinguishable from limbus]) demonstrated staining intensities ≈10% (≈25 to 30, <75) of the maximum value. Of the intensities of the most highly stained conjunctival cells of fresh tissue, the average intensity was 203.67 ± 9.50 as compared to 91.82 ± 19.11 of the intermediate-valued cells and 51.92 ± 10.46 for the cells exhibiting the lowest staining intensities (see Table 1). For the limbal cells, the average intensity of the most highly stained cells was 193.82 ± 26.16 as compared to 99.59 ± 25.92 of the intermediate valued cells and 40.50 ± 15.02 for the cells exhibiting the lowest staining intensities (see Table 1). Whether conjunctiva or limbus, this equates to approximately a fourfold higher intensity of p63 in the highest staining subpopulation. Likewise, with an average intensity of 92.82 ± 19.41 (conjunctiva) or 99.59 ± 25.92 (limbal), the intermediately stained cells exhibited approximately a 2.5-fold higher intensity of staining over the subpopulation of cells exhibiting the lowest staining intensities.

SP and LSSC Experimentation

The p63 content of cells isolated from the conjunctival and limbal epithelia as SP cells or as LSSC cells obtained without subjecting the cells to the Hoechst loading protocol was determined. Cohorts of G_0/G_1 or non-LSSC (from the center of the scatter plot: Figure 2) were used as the respective control samples. For the limbus, we used only LSSC cells.

TABLE 1. INTENSITY OF P63 STAINING IN SNAP-FROZEN RABBIT LIMBAL AND CONJUNCTIVAL TISSUES SECTIONED, STAINED FOR P63, AND THE INTENSITY OF STAINING CALCULATED WITH IMAGE ANALYSIS

TISSUE	INTENSITY	OBSERVED RANGE*		OVERALL MEAN*	±	OVERALL SD		N
		Min	**Max**					
Conjunctiva	>200	205.07	222.32	213.70	±	12.20		2
SP/non-SP	176 – 200	198.08	199.41	198.66	±	0.62		4
	151 – 175	NA	NA	NA	±	NA		0
	126 – 150	147.36	147.36	147.36	±	NA		1
	101 – 125	113.23	119.79	116.79	±	3.34		5
	76 – 100	75.77	98.57	82.32	±	6.67		19
	51 – 75	51.41	69.4	60.32	±	6.05		31
	26 – 50	34.04	47.7	42.29	±	4.16		27
	1 – 25	NA	NA	NA	±	NA		0
							Total:	**89**
Conjunctiva	>200	210.32	210.32	210.32	±	NA		1
LSSC/non-LSSC	176 – 200	181.26	193.07	186.51	±	3.71		8
	151 – 175	151.32	172.36	161.10	±	8.37		6
	126 – 150	135.36	145.25	141.45	±	4.39		4
	101 – 125	101.23	107.79	104.98	±	3.38		3
	76 – 100	76.79	76.79	76.79	±	NA		1
	51 – 75	50.65	72.30	60.76	±	7.57		14
	26 – 50	26.30	47.69	36.78	±	7.48		14
	1 – 25	23.68	23.68	23.68	±	NA		1
							Total:	**51**
Limbus:	>200	208.73	209.12	208.93	±	0.28		2
LSSC/non-LSSC	176 – 200	NA	NA	NA	±	NA		0
	151 – 175	163.62	163.62	163.62	±	NA		1
	126 – 150	143.7	149.98	146.84	±	4.44		2
	101 – 125	123.18	123.21	123.20	±	0.02		2
	76 – 100	76.45	93.33	83.84	±	5.34		9
	51 – 75	52.85	71.2	63.79	±	7.36		9
	26 – 50	25.08	49.97	33.52	±	7.86		30
	1 – 25	NA	NA	NA	±	NA		0
							Total:	**55**

Of interest, as the averages and histograms of the observed intensities illustrate (see Table 2, results graphically depicted in Figures 3, 4, and 5), there is no overlap of the staining intensity in either conjunctival or limbal epithelial cells: even the "weakest" staining LSSC cell is more intensely stained than the most intensely stained non-LSSC. Although not shown, the histogram of the limbal cells is almost identical to that of the conjunctival. In all cases, there is a clear "lip" between the weakly stained non-LSSC cells and the intensely stained LSSC (see Figures 4 and 5).
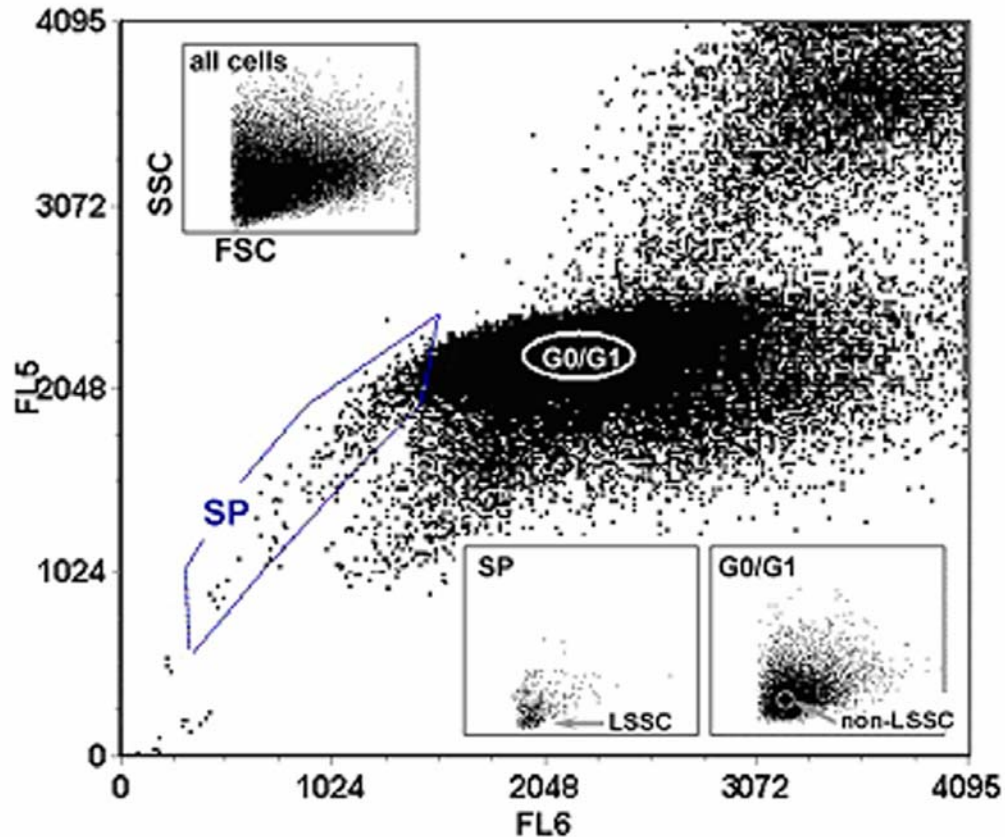
FIGURE 2

Correlations between Hoechst emission and light-scattering features in rabbit limbal epithelial cells. The main frame is a representative Hoechst 33342 plot of viable rabbit limbal epithelial cells. The insets represent the scatterplot for this cell population, for the side population (SP) subset, and for the G_0/G_1 subset marked in the Hoechst plot. Note that (a) most SP cells have very low side scatter (LSSC), and (b) the cells at the center of the G_0/G_1 Hoechst plot have SSC values higher than those of most SP cells. FSC = forward scatter.

If, as previously shown, these very low side-scattering cells are, in fact, SP, there is this same difference between the weakly stained non-SP and the intensely stained SP: an observable difference between the weakly staining non-SP and the intensely staining slow-cycling SP.

These results imply that in both conjunctiva and cornea, the subpopulation of rabbit cells exhibiting very low side scatter, and hence the SP cells, are uniformly those cells that stain the most intensely for p63.

DELAYED PROLIFERATION

As reflected in Table 3, replication rates for the isolated rabbit limbal cells were markedly different. On day 1, some colonies had already begun to develop (two cells: 18 of 56; three to six cells: 3 of 56), containing from six ($\approx 2^2$: eg, ≥ 16 hours/cycle) to two (2^1: eg, ≈ 24 hours/cycle) cells, but the majority (63%: see Table 3) had not yet undergone cell division and remained as singlets (one cell: 35 of 56). By day 4 (see Table 3), the distribution was more "normal"-appearing (in a statistical sense), and although there were still some single, undivided cells and colonies contained between 23 ($\approx 2^4$: eg, ≥ 16 hours/cycle; 2 of 26) and two (2^1: eg, ≥ 74 hours/cycle; 4 of 26) cells, a full half of the cells (26 of 52) had already undergone two mitotic cycles (3 to 6 cells: ≈ 24 hours/cycle). Twenty-four hours later (day 5: see Table 3), colonies contained between 256 (2^8: eg, ≥ 16 hours/cycle) and two (2^1: eg, 96 hours/cycle) cells.

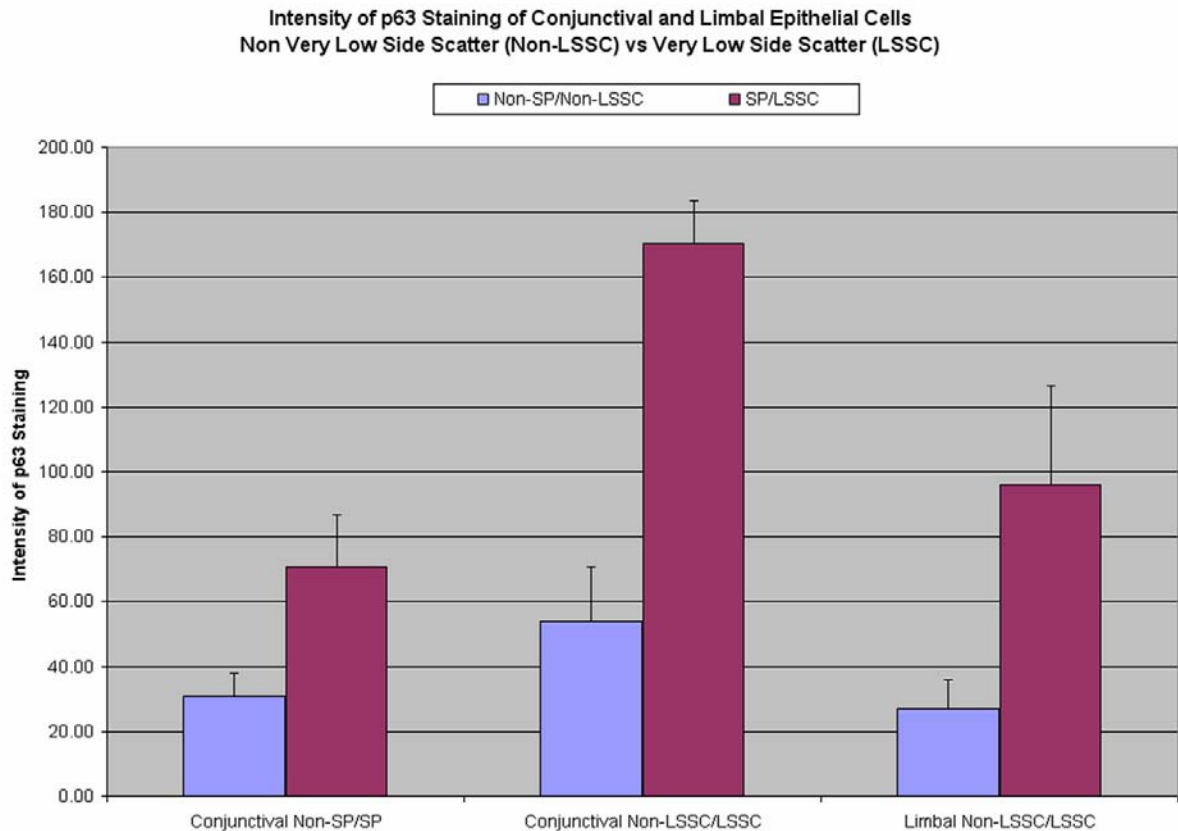**TABLE 2. MEAN ± STANDARD DEVIATION (SD) OF INTENSITY OF P63 STAINING IN SIDE POPULATION (SP), NON-SP, LOW SIDE SCATTER (LSSC), AND NON-LSSC CELLS, AND SUBSETS OF CONJUNCTIVAL AND LIMBAL RABBIT EPITHELIAL CELLS***

TISSUE	OVERALL MEAN†	±	OVERALL SD	N
Rabbit conjunctival non-SP	30.77	±	6.93	17
Rabbit conjunctival SP	70.74	±	16.02	24
Rabbit conjunctival non-LSSC	53.96	±	16.92	33
Rabbit conjunctival LSSC	170.25	±	13.21	19
Rabbit limbal non-LSSC	26.94	±	8.96	35
Rabbit limbal LSSC	95.94	±	30.54	62

*Cells were digested into individual cell suspensions (by fluorescence-activated cell sorting), cytospun, and stained for p63 by indirect fluorescence, and the amount of p63 was quantified by image analysis as described in the "Methods" section.

†Background value has been subtracted from listed intensities of p63 staining.

FIGURE 3

Intensity of p63 staining in side population (SP), non-SP, very low side scatter (LSSC), and non-LSSC subsets of rabbit conjunctival and limbal epithelial cells (as listed in Table 2). Cells were enzymatically digested into individual cell suspensions; separated by fluorescence-activated cell sorting into non-SP, SP, non-LSSC, and LSSC subsets; cytospun onto slides; then fixed, blocked, and stained for p63 by indirect fluorescence and the amount of p63 quantified by image analysis as described in the "Methods" section. Background value has been subtracted from graphically displayed intensities of p63 staining.

TABLE 3. MEAN ± STANDARD DEVIATION (SD) OF INTENSITY OF P63 STAINING IN CLONAL ASSAYS OF ISOLATED RABBIT LIMBAL CELLS DIGESTED INTO INDIVIDUAL CELL SUSPENSIONS COLLECTED AT INDICATED NUMBER DAYS AFTER INITIATION OF CULTURE

	DAY 1*				DAY 2*				DAY 3*			
NO. OF CELLS/COLONY	MEAN†	±	SD	NO. OF COLONIES	NO. OF CELLS/COLONY	MEAN†	±	SD	NO. OF COLONIES	NO. OF CELLS/COLONY	MEAN†	±	SD	NO. OF COLONIES
Background	12.03	±	0.12	12	Background	59.84	±	4.34	11	Background	59.84	±	0.73	10
1 cell	49.70	±	13.16	35	1 cell	71.32	±	4.36	6	1 cell	71.32	±	4.24	2
2 cells	40.24	±	10.29	18	2 cells	56.07	±	4.72	4	2 cells	56.07	±	1.20	2
3 to 6 cells	16.33	±	10.07	3	3 to 6 cells	24.87	±	5.72	2	3 to 6 cells	24.87	±	2.67	3
7 to 12 cells	No colonies			0	7 to 12 cells	No colonies			0	7 to 12 cells	No colonies			0
13 to 23 cells	No colonies			0	13 to 23 cells	No colonies			0	13 to 23 cells	No colonies			0
≥24 cells	No colonies			0	≥24 cells	No colonies			0	≥24 cells	No colonies			0

	DAY 4*				DAY 5*				DAY 7*			
NO. OF CELLS/COLONY	MEAN†	±	SD	NO. OF COLONIES	NO. OF CELLS/COLONY	MEAN†	±	SD	NO. OF COLONIES	NO. OF CELLS/COLONY	MEAN†	±	SD	NO. OF COLONIES
Background	12.00	±	0.02	10	Background	12.00	±	0.01	10	Background	12.58	±	0.43	10
1 cell	78.71	±	16.06	2	1 cell	119.14	±	7.97	5	1 cell	129.48	±	6.61	3
2 cells	99.83	±	10.00	4	2 cells	138.24	±	11.06	3	2 cells	148.94	±	9.61	2
3 to 6 cells	15.14	±	8.28	13	3 to 6 cells	11.78	±	7.72	4	3 to 6 cells	23.09	±	6.31	2
7 to 12 cells	13.16	±	8.08	5	7 to 12 cells	12.16	±	7.72	6	7 to 12 cells	23.35	±	7.61	4
13 to 23 cells	12.44	±	9.67	2	13 to 23 cells	14.05	±	7.72	3	13 to 23 cells	23.21	±	7.06	6
≥24 cells	No colonies			0	≥24 cells	13.84	±	9.50	1	≥24 cells	25.42	±	8.09	2

*Cell suspensions in culture with mitomycin-arrested 3T3 feeder, fixed, blocked, and stained for p63 by indirect fluorescence and the amount of p63 quantified by image analysis and separated by colony size as described in the "Methods" section.

†Background value has been subtracted from listed mean colony intensities.
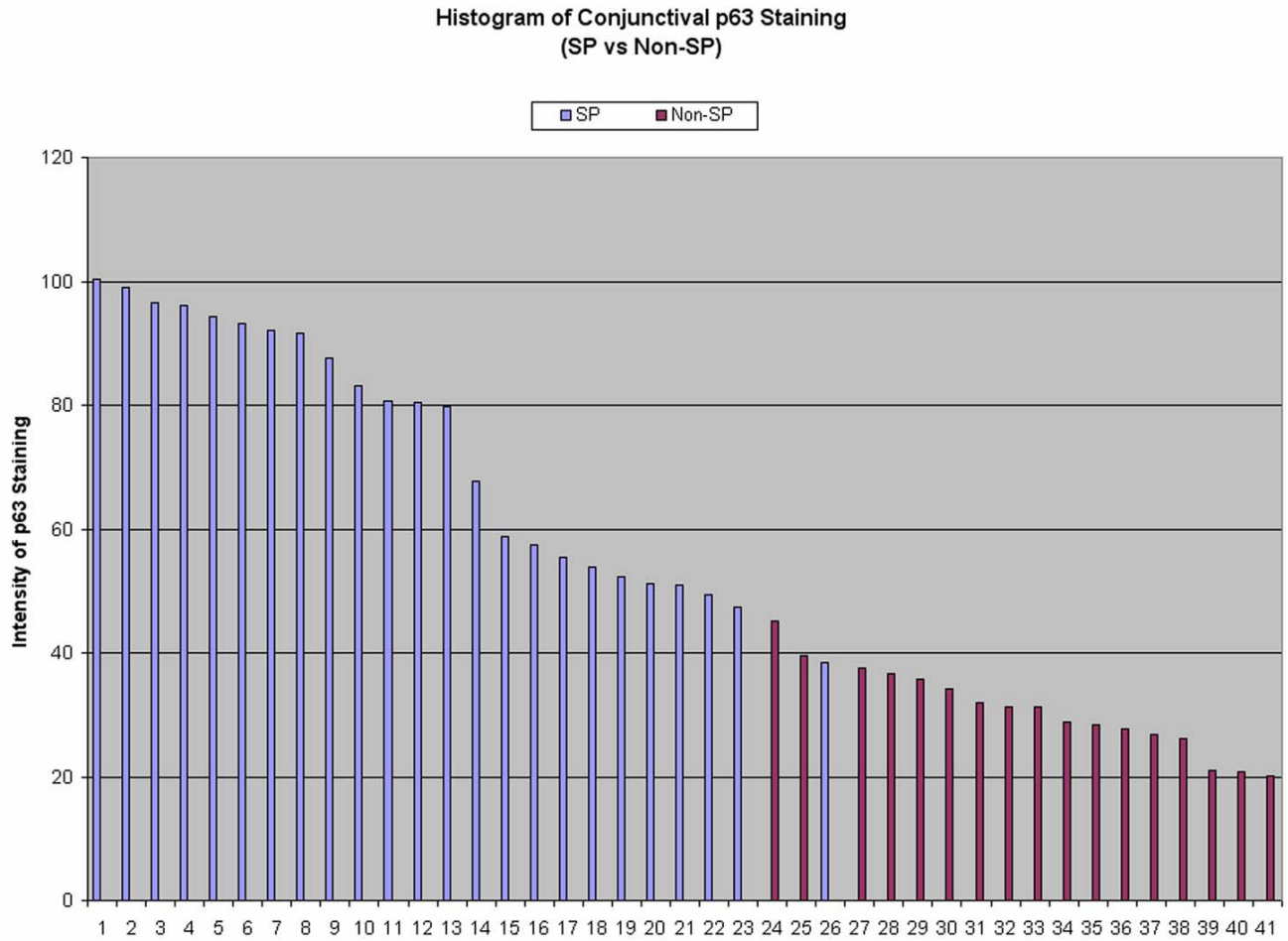
FIGURE 4

Histogram of intensities of p63 staining in individual side population (SP) and non-SP rabbit conjunctival epithelial cells. Cells were enzymatically digested into individual cell suspensions; separated by fluorescence-activated cell sorting into non-SP and SP subsets; cytospun onto slides; then fixed, blocked, and stained for p63 by indirect fluorescence and the amount of p63 quantified by image analysis as described in the "Methods" section. Background value has been subtracted from graphically displayed intensities of p63 staining.

These later cells exhibit slow cycling (or delayed proliferative start) and demonstrate several features consistent with a stem cell phenotype, including small size, low intracellular complexity, and high ability to efflux Hoechst 33342.[3] All colony cells were positive for p63. The intensity stain in the slow-cycling cells, though, was three to four times higher than in the rapidly proliferating congeners. Nuclear fluorescence intensity did not substantially decrease with the increase in cell doublings, suggesting that the difference between slow- and fast-cycling cells could not be attributed to this factor.

These results demonstrate that the subpopulation of cells that most intensely stain for p63 is slowly cycling and that those cells which most intensely stain for p63 are slow-cycling SP cells, making up approximately 5% to 7% of the overall cell population.

Histogram of Conjunctival p63 Staining
(LSSC vs Non-LSSC)
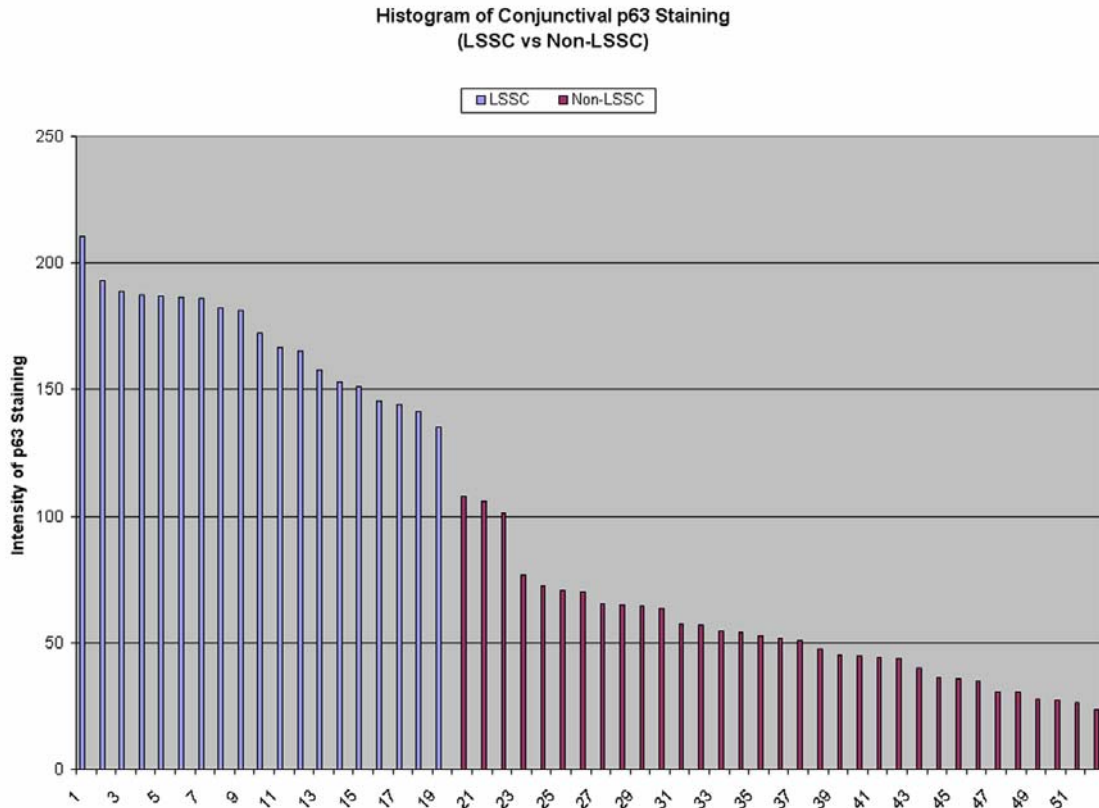
FIGURE 5

Histogram of intensities of p63 staining in individual low side scatter (LSSC) and non-LSSC rabbit conjunctival epithelial cells. Cells were enzymatically digested into individual cell suspensions; separated by fluorescence-activated cell sorting into non-LSSC and LSSC subsets; cytospun onto slides; then fixed, blocked, and stained for p63 by indirect fluorescence and the amount of p63 quantified by image analysis as described in the "Methods" section. Background value has been subtracted from graphically displayed intensities of p63 staining. Histogram of intensities of p63 in individual limbal LSSC cells indistinguishable from above (not shown).

DISCUSSION

Recently, stem cells have been the focus of intense attention as a source of cells and tissues.[22] To understand limbal stem cells or consider therapeutic use, or both, stem cells need to be easily identified and their viability and nondifferentiated state maintained. Early identification of stem cells relied on evaluating their proliferative capacity either in vitro[23] or in vivo, or both.[5,24] Cells that retain these labels over a long period (label retaining) are slow cycling, which is one known characteristic of epithelial stem cells in vivo. Stem cells with limited differentiation potentials have been identified in a number of tissues and organs, including the eye, in which they are believed to reside in the limbus.[6]

Despite the best efforts of a variety of investigators, the accurate identification of stem cells has been problematic.[24] Past efforts looked at markers in nonviable cells, which inhibited further study. Although several stem cell markers have been proposed, their role in accurately identifying stem cells is still very controversial. Basal epithelial cells of the limbus are positive for p63, ABCG2, and integrin alpha9, and negative for nestin, E-cadherin, connexin 43, involucrin, K3, and K12, with relatively higher expression of integrin beta1, EGFR, K19, and enolase-alpha.[25] Stem cells are negative for epithelial cell specific early differentiation marker AE1,[26] AE5,[6] EGFR,[27] and 14-3 3sigma.[6] Stem cells of the corneal epithelium lack connexins and metabolite transfer capacity.[28]

Although most recently p63 has been proposed to be a marker for stem cells in the limbus and skin epithelia,[1] this nuclear morphogen is not an exclusive marker for stem cells, because it has been shown to be expressed in mouse[19] and human[20,29] corneal and conjunctival epithelial cells. Despite this, it is still being actively and seriously considered as a stem cell marker.[30,31]

Although p63 has been known for some time,[32, 33] it was only recently formally described[34] and is clearly important in the biology of the stem cell[17]; obviously its mere presence cannot be construed to indicate prima facie stemness. Nevertheless, this fact does not preclude its potential use as stem cell and transient amplifying cell marker. Recently, several investigators have proposed that the level of p63, rather than its mere presence, reflects the degree of cell stemness[35] (and unpublished results), although so far there is no agreement as to whether p63 increases[35] or decreases (unpublished results) with differentiation.

Of course, as previously mentioned, p63 is not the only protein proposed as a marker for stem cells. Connexin 43, a gap junction protein required for metabolite transfer, is absent in stem cells.[28] Stem cells are also negative for epithelial cell specific early differentiation markers AE1,[26] AE5,[6, 28] and 14-3 3sigma,[6,36] as well as EGFR.[27]

If stem cells can be accurately identified and isolated, such cells will prove to be invaluable for wound healing as well as numerous other uses. The key to achieving this lies in their location: the limbus. Limbal rims are now well recognized as a great treasure for ex vivo expansion of human corneal epithelial cells, and a number of investigators are utilizing it as a source for their isolation.[24] Still, it is true that a potential problem lies in the fact that an expansion of the stem cell pool or its progeny may occur in limbal explants.[29] Although the risk of this can be abrogated or at least extremely diminished by care during the surgical isolation of the corneoscleral tissue, it is still possible that small conjunctival remnants may remain, contaminating the tissue.

Of course, this risk was completely ruled out by the use of FACS. FACS can sort out even very rare cells with great accuracy on the basis of size, side scatter, and fluorescent antibody staining profile. The problem associated with FACS lies in the time it takes for cell sorting. Even with the most current high-speed sorter, only 50,000 events per hour can currently be evaluated and separated. This number is too small for clinical use, and a way must be found to expand the speed of isolation to enable the use of this critical basic tool in the isolation of tissue-specific stem cells.

Overall, our study has demonstrated that the density of p63, not the mere presence, is correlated with the limbal stem cell population. We report on three methods to demonstrate this.

1. QUANTITATIVE HISTOLOGY

The intensity of nuclear staining (relative measure of p63 content) was quantified and analyzed in freshly dissected, snap-frozen tissues stained for p63 by indirect immunofluorescence. A 5% to 7% subpopulation of the overall tissue sections most intensely stained for p63.

We have previously shown[2] that (a) at least 50% of the side population in the ocular surface cells in either rabbit or human is characterized by very low side scattering and, conversely, that (b) when a LSSC cohort can be visually identified in scatter plots, at least 50% of those cells have the SP phenotype. Furthermore, the same studies demonstrated that LSSC and SP contained equivalent numbers of cells that have been in the slow-cycling (ie, stem) state prior to tissue collection and sorting. These results suggested that the LSSC phenotype may have an equivalent value to the SP phenotype for the isolation of populations enriched in stem cells.

When taken in context with the present results, the 5% to 7% subpopulation of the overall tissue sections mentioned above that most intensely stained for p63, constituted a range very much in agreement with the previously reported fraction of slowly cycling SP cells.

2. SP AND LSSC EXPERIMENTATION

Enzymatically digested rabbit conjunctival and limbal cells were aseptically isolated according to their size, side scatter, and fluorescence staining profiles utilizing FACS. The results from this experimentation demonstrated that both conjunctival and limbal LSSC cells expressed more than three times the intensity of p63 staining over non-LSSC with background staining removed (see Table 2, Figure 3), implying that the density of p63 antigen expressed is approximately three times higher in LSSC cells than non-LSSC cells. If, as previously shown, the majority of the side population in ocular surface cells is characterized by very low side scattering, this would indicate that the majority of these conjunctival LSSC expressing high p63 staining lie in the side population region. In addition, if the LSSC phenotype does, in fact, have an equivalent value to the SP phenotype for the isolation of populations enriched in stem cells, then this would imply that the SP cells demonstrate this same high p63 antigen expression. In other, more succinct, words, in both conjunctiva and cornea, the subpopulation of rabbit cells exhibiting very low side scatter, as well as the SP cells, are uniformly those cells that stain the most intensely for p63.

3. DELAYED PROLIFERATION

Single-cell suspensions of enzymatically digested rabbit conjunctival and limbal cells were plated at very low density onto coverslips containing Swiss 3T3 fibroblasts that had been proliferation arrested with mitomycin C and then immunofluorescently stained for p63 at various time points. The experimentation demonstrated that the majority of , if not all, limbal basal cells are p63-positive and only a small subpopulation of the entire population of limbal basal cells displays particularly high levels of expression. The proliferative start of isolated rabbit limbal cells over a 3T3 feeder layer under clonogenic conditions was determined to be highly asynchronous. In particular, "side population" stem cells displayed marked delays in proliferative start. Furthermore, day-by-day analysis of the p63 content in rabbit limbal cells grown under these same clonogenic conditions (uses cell duplets as a marker for cells that have divided within the preceding 24 hours) demonstrated that the higher the p63 content, the larger the delay in the start of proliferation. This latter result resembles the behavior of the side population cells and suggests that p63^{high} cells are also stem cells. More simply, the subpopulation of cells that most intensely stain for p63 is slow cycling, and, overall, those cells that most intensely stain for p63 are slow-cycling SP cells, making up approximately 5% to 7% of the overall cell population.

In conclusion, although the expression of p63, per se, cannot be construed to indicate cell stemness, our data suggest that the level of p63 expression seems to correlate with the degree of cell stemness. We suggest that FACS, via the side population stained with Hoechst or low side scattering, or both, can isolate a subpopulation of cells exhibiting properties highly suggestive of stem cells.

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