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Rat limbal epithelial side population cells exhibit a distinct expression of stem cell markers that are lacking in side population cells from the central cornea

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Abstract The side population (SP) phenotype is shared by stem cells in various tissues and species. Here we demonstrate SP cells with Hoechst dye efflux were surprisingly collected from the epithelia of both the rat limbus and central cornea, unlike in human and rabbit eyes. Our results show that rat limbal SP cells have a significantly higher expression of the stem cell markers ABCG2, nestin, and notch 1, compared to central corneal SP cells. Immunohistochemistry also revealed that ABCG2 and the epithelial stem/progenitor cell marker p63 were expressed only in basal limbal epithelial cells. These results demonstrate that ABCG2 expression is closely linked to the stem cell phenotype of SP cells.

Keywords: Corneal epithelium; Limbal epithelium; Stem cell; Side population; ABCG2

1. Introduction

Previous studies using colony forming studies and label-retaining cell assays have suggested that corneal epithelial stem cells reside in the basal layer of the limbal epithelium [1,2], which is located at the transitional zone between the central cornea and the peripheral bulbar conjunctiva. These stem cells are thought to allow for the proper renewal of the corneal epithelium by generating transient amplifying (TA) cells that migrate, proliferate, and differentiate to replace lost or damaged corneal epithelial cells [3–5]. Recently, several researchers, including our group have succeeded in the clinical transplantation of constructs generated from expanded limbal epithelial cells to treat human patients [6–11]. Additionally, because of the highly limited and well characterized localization of corneal epithelial stem cells to the limbus, the corneal epithelial system has been a model system for epithelial stem cell research [12]. However, due to the absence of definite biological markers, the unequivocal identification and isolation of epithelial stem cells within these populations remain elusive.

In 1996, Goodell et al. [13] demonstrated that mouse hematopoietic stem cells with long-term multi-lineage reconstitution abilities could be isolated as a side population (SP) based on their unique ability to efflux the DNA-binding dye Hoechst 33342. Recently, SP cells have also been identified in the hematopoietic compartments of different species [14,15], and have been isolated from various other adult tissues including the liver [16], skeletal muscle [17], brain [18], pancreas [19], and lung [20]. These findings suggest that the SP phenotype represents a common feature of adult tissue-specific stem cells. Zhou et al. [21] reported that the ATP-binding cassette transporter, ABCG2 (also known as BCRP-1 or MXR), is a molecular determinant of this SP phenotype in hematopoietic stem cells. Other studies in a wide range of organs have also indicated that the SP phenotype is largely determined by the expression of ABCG2 [16,19,20,22], a member of the multiple drug resistance (MDR) family of membrane transporters. Taken together, these previous results suggest that the SP phenotype may be an extremely useful tool for the identification of stem cells from various tissues.

Recently, we reported that both the human and rabbit limbal epithelium contains SP cells expressing ABCG2, while SP cells could not be detected in the epithelium of the central cornea [23,24]. In the present study, we investigated the presence of SP cells in both the limbal and corneal epithelium of the rat by fluorescence-activated cell sorting (FACS) and discovered that while cells with the SP phenotype could also be detected in the rat central cornea, only SP cells isolated from the limbal epithelium demonstrated a distinct expression of stem cell markers.

2. Materials and methods

2.1. Cell preparation

Corneoscleral rims were obtained from Wistar rats (8 weeks old, male) and New Zealand white rabbits (2.0 kg, male). Limbal tissues were obtained with scissors, and 2.0 mm-diameter portions of rat corneas and 8.0 mm-diameter portions of rabbit central corneas were obtained by trephination (Fig. 1). Excised tissues from the limbus and central corneas were treated with Dulbecco's modified Eagle's medium (DMEM) containing 120 U/ml dispase II (Godo Shusei, Tokyo, Japan) at 37 °C for 1 h. Epithelial cells were then separated under a dissecting microscope and treated with 0.25% trypsin/1 mM EDTA solution (Invitrogen, Carlsbad, CA) for 20 min at 37 °C, to create single cell suspensions and enzymatic activity was stopped by adding an equal volume of DMEM containing 10% fetal bovine serum (FBS; Moregate BioTech, Queensland, Australia).

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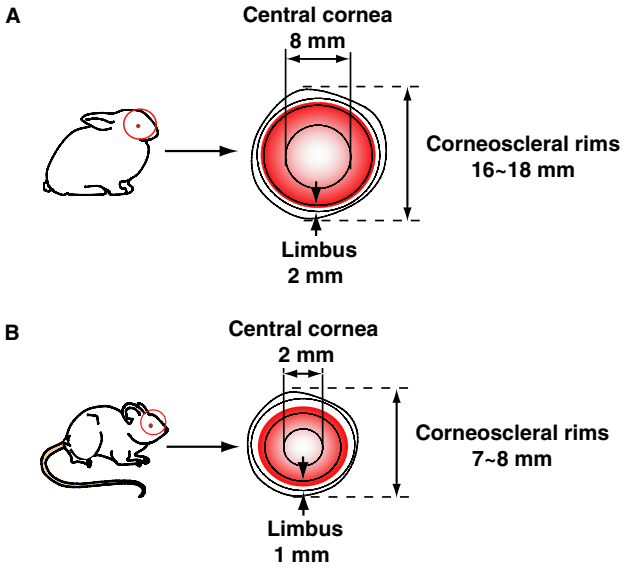

Fig. 1. Isolation of limbal and corneal tissues. (A) Approximately 16–18 mm diameter tissues including cornea, limbus and conjunctiva were isolated from New Zealand white rabbits. From these tissues, 2 mm regions of the limbus were harvested with scissors and 8 mm diameter portions of central corneas were obtained by trephination. (B) In rat tissues, 7–8 mm diameter tissues were isolated and 1 mm limbal tissues and 2 mm diameter portions of central corneas were obtained.

2.2. Hoechst 33342 exclusion assay using fluorescence-activated cell sorting

Single cells isolated from both the limbal epithelium and the corneal epithelium were subjected to FACS using previously described procedures [24].

2.3. Gene expression analysis

Gene expression analyses were conducted using real-time quantitative RT-PCR, as previously described [24].

2.4. Immunohistochemistry

Limbal and corneal tissues were fixed in 10% neutral buffered formalin (Wako Pure Chemicals, Tokyo, Japan) and routinely processed into paraffin-embedded sections. Immunostaining was then performed using the DAKO LSAB kit/HRP (DAB) (Dako Cytomation, Glostrup, Denmark). Briefly, endogenous peroxidase activity was blocked with Peroxidase Blocking Reagent, DAKO S 2001 (Dako). After incubation with 1% bovine serum albumin to block non-specific reactions, sections were incubated with either a 1/200 dilution of anti-ABCG2 antibody (5D3, MBL, Aichi, Japan) or a 1/200 dilution of anti-p63 antibody (4A4, Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 1 h at room temperature, followed by three washes with Dulbecco's phosphate buffered saline (PBS). Sections incubated identically with normal mouse IgG were used as negative controls. After incubation with horseradish peroxidase-conjugated secondary antibodies (Dako) for 30 min at room temperature, the sections were again washed 3 times with PBS. Finally, color development was performed using DAKO ENVISION kit/HRP (DAB) (Dako) and stained sections were visualized using light microscopy.

3. Results

Using FACS, a distinct population of cells with a low Hoechst 33342 blue/red fluorescence was isolated from both rabbit and rat limbal epithelial cells (Fig. 2A and E) analogously to previously reported results for both human and rabbit eyes [23–26], with dye efflux inhibited by treatment with verapamil,

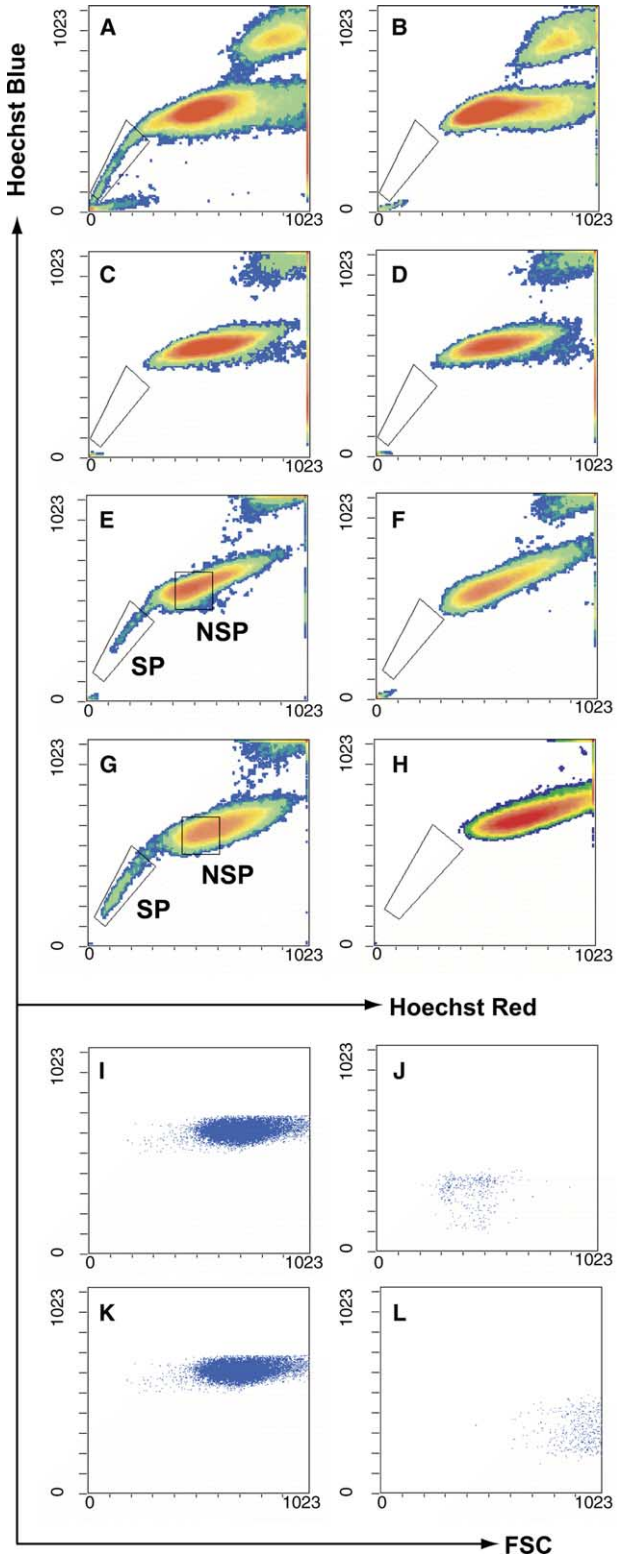

Fig. 2. Hoechst 33342 staining in rabbit and rat limbal and corneal epithelial cells. Epithelial cells were isolated for the cornea and limbus and subjected to Hoechst 33342 exclusion assay. Cells were sorted using FACS and SP cells were detected in the epithelial cells of rabbit limbus (A, B), rabbit cornea (C, D), rat limbus (E, F), and rat cornea (G, H). Dye efflux from SP cells was antagonized by verapamil (B, D, F, H). Forward scattering shows the relative cell size of limbal NSP (I), limbal SP (J), corneal NSP (K) and corneal SP cells (L) in rat. In the density plots of E and G, cells denoted by each enclosed area were regarded as SP and NSP cells for further characterization.

a known inhibitor of Hoechst 33342 dye transport (Fig. 2B and F). The frequency of 0.56% and 0.40% of gated cells for rabbit and rat, respectively, as well as elimination of the SP phenotype by verapamil, was similar to our previously reported results with human eyes [23]. In contrast, cells within the SP gate were barely detected in the corneal epithelium of rabbits (Fig. 2C). However, when epithelial cells from the rat central cornea were subjected to Hoechst 33342 exclusion assays, surprisingly, a significant cell population (4.6% of gated cells) showing the SP phenotype was detected (Fig. 2C). This frequency of rat corneal epithelial SP cells was ten times higher than from the limbal epithelium, but cells showing the SP phenotype were also blocked by treatment with verapamil (Fig. 2H). Forward scatter analyses using FACS also revealed that rat limbal SP cells (Fig. 2J) were smaller in size than both rat limbal NSP cells (Fig. 2I) and rat corneal epithelial NSP cells (Fig. 2K), but that rat corneal epithelial SP cells (Fig. 2L) were much larger than these other cell types.

SP and NSP cells isolated from both the rat limbal epithelium and corneal epithelium were then subjected to real-time quantitative RT-PCR (Fig. 3). Consistent with experimental design, significantly higher expression of ABCG2 mRNA was observed in limbal epithelial SP cells over all other cell fractions (Fig. 3A). In contrast, the expression of ABCG2 could barely be detected in rat corneal epithelial SP cells. These cell fractions were also subjected to gene expression analyses for four common stem cell markers: nestin (Fig. 3B), notch 1 (Fig. 3C), TERT and musashi 1 (data not shown). Significantly higher expression of nestin and notch 1 mRNAs were found in limbal epithelial SP cells compared to limbal epithelial NSP cells as well as both cell fractions isolated from the central corneas. However, gene expression levels of both TERT and musashi 1 were undetectable in all cell fractions (data not shown).

Finally, immunohistochemistry revealed a similar localization of the SP cell marker, ABCG2 with p63, in the rat ocular surface (Fig. 4). p63 is well-known as a marker of epithelial stem and progenitor cells, and p63 positive cells have been previously reported to be localized in the basal layer of the limbal epithelium, but not in the central cornea [27]. Our present results demonstrated that the localization of both ABCG2 and p63 were restricted to the basal layer of the limbal epithelium (Fig. 4C and E), and could not be found in any regions of the central corneas (Fig. 4D and F).

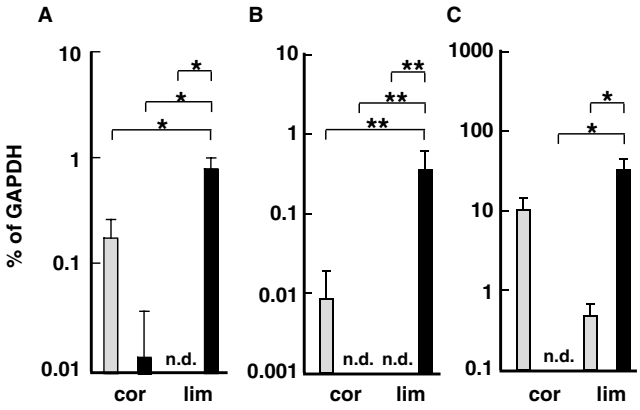

Fig. 3. mRNA analysis of stem cell marker gene expression. Total RNA was extracted from cells isolated by FACS and subjected to real-time quantitative RT-PCR. The relative gene expression of ABCG2 (A), nestin (B), and notch 1 (C) were plotted as a ratio of GAPDH gene expression. Expression levels were detected from NSP cells: gray bars, and SP cells: black bars. Data represent the mean value from three to four independent samples. Error bars indicate the SD ($^{*}P = 0.01$ and $^{**}P < 0.05$, respectively).

4. Discussion

In the present study, we report the isolation of a cell population showing the SP phenotype from rat corneal epithelium and their comparison to SP cells isolated from the limbal epithelium. The SP phenotype is now consistently attributed to various adult tissue-specific stem cells and commonly associated with the functional presence of the ATP-binding cassette transporter, ABCG2 [21,22]. The

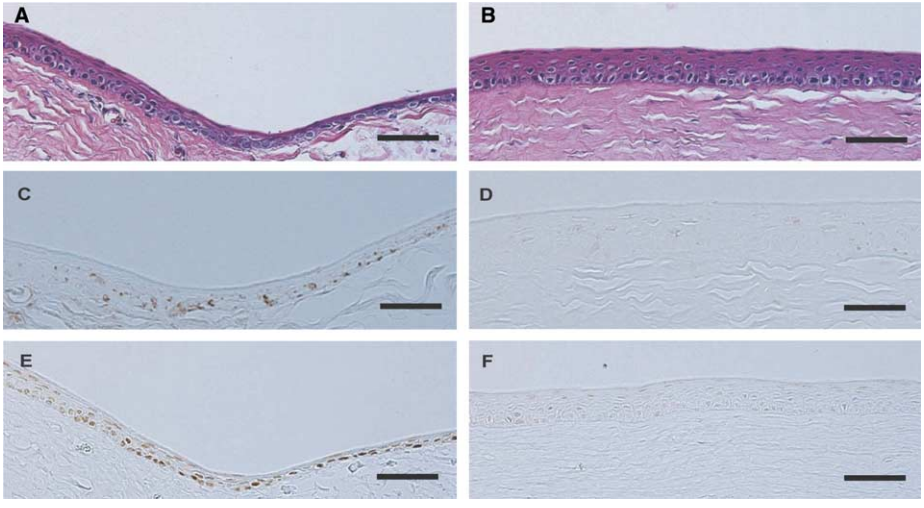

Fig. 4. Immunohistochemistry for ABCG2 and p63 in rat cornea and limbus. The distribution of cells expressing ABCG2 and p63 were examined using immunohistochemistry. Hematoxylin and eosin staining (A, B), and immunostaining with anti-ABCG2 (C, D) and anti-p63 (E, F) antibodies. Left (A, C, E) and right (B, D, F) panels represent rat limbus and cornea, respectively. Scale bars indicate 50 μm.

expression of ABCG2, which is considered a putative marker of the SP phenotype has been reported for cells isolated from numerous tissues, with these cells also exhibiting stem cell-like phenotypes [19,20,22,24]. While our results from FACS have shown that only approximately 0.5% of limbal epithelial cells exhibited the SP cell phenotype regardless of species (Fig. 2A and E) [23,24], immunohistochemistry revealed that a large portion of limbal basal epithelial cells (approximately 10% of total limbal epithelial cells) expressed ABCG2 (Fig. 4C). This apparent discrepancy may be attributed to the transport activity of ABCG2. Limbal basal epithelial cells have relatively little cytoplasmic area, and it is therefore difficult to determine whether ABCG2 molecules were localized to the plasma membranes or in the cytoplasm (Fig. 4C). Since only active ABCG2 transporter present on the cell surface can efflux Hoechst 33342 [20,28], cytoplasmic ABCG2 would be unable to contribute to the observed low Hoechst-derived fluorescence. Additionally, Mogi et al. [28] recently showed that a serine/threonine kinase, Akt, can modulate the SP phenotype by controlling the expression ABCG2, which suggests that ABCG2 function is under strict regulation.

In the present study, while limbal SP cells show higher expression of tissue-specific stem cell markers, in particular ABCG2, compared to the other cell populations examined, these differences in gene expression levels are not as great as observed for both human [23] and rabbit limbal epithelial SP cells [24]. The disparity in the present case is linked to the purity of limbal epithelial SP cells used in the present rat experiments. In the cases of human and rabbit, the central cornea does not contain SP cells that can possibly contaminate the limbal SP population. Additionally, with rat eyes, the significantly smaller size, as well as a lack of the Palisades of Vogt, means that the border between the limbus and central cornea is undefined and difficult to interpret. Therefore, the presence of SP cells in the epithelium of the central cornea inevitably leads to contamination by cells that do not show expression of stem cell markers, causing the relative expression of ABCG2, nestin, and notch 1 to be decreased in the limbal epithelial SP cell fraction that has stem cell-like properties. In contrast to rabbit and human cases, where these factors do not influence the limbal epithelial SP population, differences of approximately 100× in the expression of ABCG2, are considerably greater than the approximately 10× difference observed in the present study, which is a result of this unavoidable contamination. Regarding the relationship between ABCG2 expression and stem cell phenotypes, we have previously shown that SP cells from the mouse bone marrow have approximately 15× higher ABCG2 expression than NSP cells. However, when bone marrow SP cells were further purified for hematopoeitic stem cells by sorting of c-kit$^+$/Sca-1$^+$/Lineage cell marker$^-$ SP cells, this fraction showed 250× greater ABCG2 expression compared with NSP cells [29]. These data suggest the importance of ABCG2 expression for the identification of tissue-specific stem cells using Hoechst 33342 efflux assays.

From the rat limbal epithelium, SP cells also showed an increased expression of the stem cell markers nestin and notch 1, compared to NSP cells from both the limbal epithelium and corneal epithelium, as well as SP cells from the corneal epithelium (Fig. 3). However, even though SP cells were detected in the central cornea, this cell fraction showed significantly lower expression of these stem cell markers compared to limbal epithelial SP cells (Fig. 3).

Nestin is known as a neural stem cell marker [30], but its expression is not limited to neural tissues, and has been recently reported in pancreatic islets [19] and even in the cornea [31]. Specifically in the eyes, retinal SP cells have been reported to exhibit a high expression of nestin mRNA [32]. Notch 1 has also been frequently correlated to a stem cell-like phenotype, by delaying stem cell differentiation via the enhancement of self-renewal in both hematopoietic [33,34] and neural stem cells [35]. The higher expression of notch 1 observed in limbal epithelial SP cells suggests that these cells may indeed resemble other adult stem cells and may indicate a key role of notch 1 signaling in regulating ocular surface homeostasis through well-controlled corneal epithelial turnover. Musashi 1 is another well-recognized neural stem cell marker [36], but its mRNA expression was undetectable in all cell fractions studied, including limbal epithelial SP cells (data not shown), which may imply its specificity to neural tissues. The physiological expression of TERT is highly limited to a very specific number of cell types including proliferating stem cells, reproductive cells, and cancer cells [37,38]. In our experiments, TERT mRNA was not detected in limbal SP cells isolated from normal healthy tissues. Since adult stem cells are considered to be slow-cycling or arrested in the G0/G1 phases of the cell cycle [2,39,40], constitutive expression of TERT is unlikely to be observed in quiescent stem cells. Recent reports have shown that skin epidermal SP cells expressing ABCG2 and putative skin epidermal stem cells exhibiting slow cell cycling are two distinct cell populations [41] and we have also shown that limbal epithelial SP cells with significantly higher expression of ABCG2 represented a quiescent population with stem cell-like properties, without TERT activity [24].

While our results indicate that SP cells isolated from the rat limbal epithelium may constitute a population enriched for stem cells, when SP cells were isolated from rat central corneas, these cells had expression levels of ABCG2 that could be barely detected (Fig. 3A). Immunohistochemistry with anti-ABCG2 antibody also demonstrated that ABCG2 could not be observed in corneal epithelial cells (Fig. 4D). It was recently reported that the human epidermis contains SP cells that do not express ABCG2 [42]. These results imply that a transporter other than ABCG2 may be responsible for the observed Hoechst 33342 efflux in rat corneal epithelial cells. Furthermore, consistent with the absence of ABCG2, rat corneal epithelial SP cells also exhibited similar cell sizes to corneal epithelial NSP cells, and were larger than limbal epithelial SP cells (Fig. 2I–L). It has been noted that stem cells isolated from other tissues such as the skin and hair follicle [43–45] have smaller cell sizes than their more differentiated progeny. The SP cell frequency in rat corneal epithelium (4.6%) was also much higher than in the limbus, as well as results reported for several tissues and organs [13,16,20,23], and the presence of corneal epithelial SP cells in rat eyes were contrary to our previous results for both human [23] and rabbit [24]. Similarly, immunostaining for p63, a known epithelial stem and progenitor cell marker [27], also showed positive staining only in the limbal epithelium and not in the central cornea (Fig. 4E and F). These findings support the theory that even though SP cells were detected in the central corneas of rats, this cell population is distinct from limbal epithelial SP cells and other typical SP cells that show high expression of ABCG2 and are enriched

for tissue-specific stem cells. It is possible that other members of the ABC transporter family may be expressed in corneal epithelial SP cells allowing for the efflux of Hoechst 33342 and therefore the observed SP phenotype, but that these cells lack ABCG2 that is expressed in many tissue-specific stem cells. However, even though it seems likely that corneal epithelial SP cells lack stem cell-like properties, further analysis of the stem cell properties including studies on the capabilities for self-renewal and differentiation are required for a decisive conclusion.

In summary, we have shown that similarly to other species, rat limbal epithelial SP cells show a significantly higher expression of ABCG2 and that these cells also show increased mRNA expression of the stem cell markers nestin and notch 1. However, even with the interesting finding that SP cells may comprise a population enriched for stem cells, the presence of SP cells in the central cornea without ABCG2 expression and seemingly without stem cell-like properties, demonstrates the presence of two distinct SP cell populations in the rat ocular surface. It therefore seems increasingly necessary to confirm the expression of ABCG2 when using SP cells for stem cell research.

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