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Title:

Characterisation of human limbal side population cells isolated using an optimised protocol from an immortalised epithelial cell line and primary limbal cultures.

Running title: **Limbal SP cells from optimised protocol**

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ABSTRACT

The challenges in limbal stem cell biology largely remain in the process of identification, isolation and expansion of these adult corneal epithelial stem cells of the eye. Due to the absence of specific limbal stem cell markers, identification and isolation of putative limbal stem cells become a complicated task. The side population assay is an isolation method that utilises the ability of stem cells to efflux the DNA-binding dye Hoechst 33342 (or other vital dyes) combined with dual wavelength flow cytometry and is a valuable strategy to enrich for limbal stem cells. This assay has been used to successfully identify stem/ progenitor cell populations in a variety of tissues and cell lines. Here we optimise this assay to identify SP cell populations in both primary human limbal epithelial cultures and in an established human corneal epithelial cell line. The limbal SP fraction showed higher expression of *ATP-binding cassette sub-family G member 2* (ABCG2), Δ Np63 – a common limbal stem cell marker and the stem cell marker Sox2 compared to non-SP cells (NSP).

Keywords: Hoechst 33342, limbal stem cells, side population, FACS

1.0 INTRODUCTION

The cornea forms the clear tissue at the front of the eye that enables the transmission of light to the back of the eye for visual perception. The corneal epithelium that covers the corneal surface is maintained by stem cells located at the periphery of the cornea, in a region known as the limbus. Characterisation of limbal stem cells remains challenging in part due to the absence of specific limbal stem cell markers. One of the ways to isolate an enriched population of putative stem cells is by utilising ATP-binding cassette (ABC)-transporter mediated efflux of a DNA-binding dye such as the most commonly used Hoechst 33342. A seminal work by Goodell, *et al*, demonstrated a population of stem cell enriched cells from within the haematopoietic cells of the mouse, these were isolated using high-speed dual wavelength flow cytometry analysis combine with vital dye efflux [1].

Hoechst 33342 is a fluorescent dye which binds to DNA in live cells. When excited by ultra violet light, Hoechst 33342 has an emission peak at 450nm which can be detected in what is commonly referred to as the Hoechst Blue Filter, in this case a 450/50 band pass filter.

Hoechst dye also shows a secondary emission defined as Hoechst Red detected in this case by a 675nm long pass filter. This appears to be partially dependent on the DNA conformation. As the intensity of the dye signal in the Blue channel is proportional to the DNA content of the cell this can be used to detect the cell cycle status. SP is detected as a discrete “tail” attached to the G0/G1 subpopulation in a bivariate plot of these two signals.

In order to determine that this “tail” of cells are indeed SP cells confirmation of the SP phenotype is necessary. This can be determined using an ABC-transporter inhibitor which needs to be added to the cells (from the same cell preparation) to prevent dye efflux. Some of

the ABC-transporter inhibitors responsible for preventing vital dye efflux are verapamil which blocks ABCB1 efflux activity and partially blocks ABCG2 efflux, cyclosporine A which inhibits ABCC1 and fumitremorgin C that is a specific inhibitor for ABCG2 [2].

Transporters are trans-membrane proteins which facilitate entrance of nutrients into cells or the release of toxic products into external environment [3-5]. The largest and most important family of membrane transport proteins are the ABC transporters. The ABC genes include the *ATP-binding cassette sub-family B member 1 (ABCB1)*, *ATP-binding cassette sub-family G member 2 (ABCG2)* and *ATP-binding cassette sub-family C member 1 (ABCC1)* genes.

The combination of SP profile with cell surface phenotyping can lead to further functional characterization of putative stem cell or cancer stem cell populations such as cellular metabolic activity, viability (through simultaneous use of a vital dye such as Propidium iodide), proliferative capacity, and molecular markers. Evidence on characterisation of limbal stem cells by limbal SP is accumulating. Previous investigations in both human and animals showed limbal SP cells had features consistent with stem cells [6-10]. Studies of ABCG2 expression at mRNA and protein levels have provided convincing evidence that ABCG2 is the main transporter implicated in determining the limbal SP phenotype [6,7,9,11].

Here we describe the methodology for the identification and isolation of corneal epithelial SP in both primary human limbal epithelial cultures (LEC) and in a human telomerase-immortalised corneal epithelial cell line (HTCEC).

2.0 ETHICS

Cadaveric limbal tissues were obtained from UK Eye Bank. Experiments were conducted in accordance to the local and international guidelines of the Tenets of Helsinki.

3.0 MATERIALS

a. LEC

Isolation and cultivation techniques of limbal epithelial sheets are based on keratinocyte culture system by Rheinwald and Green [12-14] and described in many reports [15-18]. The cells are propagated in a co-culture system with murine 3T3-J2 fibroblast feeder layer (a kind gift from Professor Fiona Watt, King's College, London, United Kingdom).

b. HTCEC

Our stock of HTCEC line [19-21] was generously contributed by Dr James Jester and Winston Kao, of the University of Cincinnati, Cincinnati, Ohio, USA. We successfully expanded HTCECs and maintained this cell line in feeder- and serum-free conditions.

c. Hanks balanced salt solution (HBSS) medium was prepared as follows; 500 ml HBSS [Sigma] containing 2% fetal bovine serum (FBS) [Lonza], and 5 ml of 5,000 units of penicillin/streptomycin [Gibco].

d. Verapamil [Sigma Aldrich]

Verapamil is a general calcium channel inhibitor, which inhibits efflux of drugs at the ATP-dependent pumps e.g. Hoechst 33342 dye in SP discrimination assay. Verapamil is made into a 5 mM stock solution in H₂O and stored frozen at -20⁰C, once thawed it can be stored at 4⁰C for 1 month and is used at 20 – 40 μM concentration.

e. Fumitremorgin-C [Axxora]

Fumitremorgin C (FTC) is a specific inhibitor of ABCG2. FTC is diluted in DMSO to make a 10 mM stock and stored at -20°C and is used at a final concentration of 5 µM concentration.

f. Hoechst 33342 [Sigma]

25 mg Hoechst 33342 in powder form was dissolved in 25 ml H₂O to a final concentration of 1 mg/ml, filter sterilized and aliquots frozen at -20°C. Once thawed Hoechst was kept at 4°C for 1-2 months.

g. Propidium iodide (PI)

25 mg PI powder was dissolved in 2.5 ml H₂O to a final concentration of 10 mg/ml, filter sterilised and aliquots were stored at -20°C. Once thawed vials were kept at 4°C.

4.0 METHODS

a. LEC sample preparation

In brief, limbal epithelial sheets can be produced by enzymatic or non-enzymatic digestion of limbal tissues. We used serial trypsinisation of limbal tissues using trypsin/EDTA [Gibco] to obtain a single cell suspension. The cell pellet was re-suspended in epithelial medium. This process of trypsinisation of the limbal pieces and centrifugation of the resulting cell suspension was repeated for a further three times using the same limbal tissue. The resulting limbal cell suspensions were pooled together. After performing a count of the viable cells, 30,000 viable limbal epithelial cells in epithelial medium were added to the 2 cm² tissue culture wells containing irradiated 3T3 fibroblasts.

On Day 10, cells from limbal epithelial culture were detached from a tissue culture well by first adding 1 ml of Ethylenediaminetetraacetic acid (EDTA) for 3 minutes to remove any residual feeder cells, and subsequently adding 1 ml of 0.05% trypsin-EDTA and placed in a CO₂ incubator at 37°C for 3 minutes. To inactivate trypsin, 1 ml of medium containing 10% Fetal Bovine Serum (FBS) [Lonza] was added to the well subsequently. Suspension was centrifuged and cell pellet was resuspended in Hank's medium.

b. HTCEC sample preparation

Medium was first removed and cultures irrigated with Dulbecco's Phosphate Buffer Saline (DPBS) [Sigma]. Cells in a 75 cm² tissue culture flask were then treated with a cell dissociation buffer – enzyme free, Hank's based [Gibco] in a CO₂ incubator at 37°C for 20 minutes and then rinsed with PBS. 5 ml 0.05% Trypsin-EDTA was then added and incubated for another 3 minutes. The steps as described above were repeated.

c. Isolation of SP cells

Cells were counted manually using an improved Neubauer haemocytometer [Reichert], 1.0 x 10⁶ cells/ml were resuspended in Hank's media and placed into micro centrifuge tubes [Fisher] to be stained with either Hoechst 33342 dye alone or with Hoechst dye in the presence of ABC-transporter inhibitors. Prior to addition of Hoechst the cells were incubated in the presence of inhibitor for 30 minutes in a 5% CO₂ incubator at 37°C in a MACsMix rotator [Miltenyi Biotec]. After this, Hoechst dye was then added to all tubes as described above.

All reactions were terminated by placing the contents of the tubes in a 15 ml centrifuge tube [Fisher] containing 10 ml ice cold Hank's/FBS medium. The tubes were then centrifuged at

2,500 rpm for 5 minutes and cells resuspended with 500µl Hank's medium and passed through a 70µm pore size cell strainer [BD Falcon] and stored on ice prior to FACS analysis.

d. Flow Cytometry Analysis and Fluorescence Activated Cell Sorting

Flow Cytometric Analysis and Cell Sorting used either an LSR2 SORP analyser or a FACSAria IIu sorter [Becton Dickinson]. Both machines used identical filter sets and laser configurations. Propidium iodide (PI) was added immediately prior to analysis at a final concentration of 2µg/ml. Initial gating was designed to exclude non viable cells, these were defined as PI positive and were detected using 488nm excitation and a 610/20 emission filter (data not shown). SP cells were identified on a bivariate plot showing Hoechst Blue (355nm excitation, 450/50 emission) versus Hoechst Red (355nm Excitation, 675LP emission). For analysis, at least 50,000 events were collected. For sorting SP and NSP, cells were collected into either culture media for molecular characterisation and cell culture or directly onto microscope slides for immunofluorescence studies.

e. Immunofluorescence staining

50 to 100 HTCEC SP and NSP cells were sorted directly onto glass slides. Slides were fixed with cold methanol [BDH Laboratory Supplies] by incubation at -20 °C for 20 minutes and then washed with PBS. Cells were permeabilised in 0.3% (v/v) Triton X-100 [Fisher Scientific] in PBS for 10 minutes and then washed again with PBS. Non-specific binding sites were blocked with 5% normal goat serum [Invitrogen] in PBS for 30 minutes in a humid chamber in the dark. Cells were then incubated with rabbit polyclonal anti-human ABCG2 antibody in 1:100 dilutions [Novus Biologicals] for 1 hour in the dark in a humid chamber, and then washed with PBS three times, 5 minutes. Cells were incubated with secondary antibody; goat anti-rabbit FITC conjugated in 1:25 dilutions [Jackson Immunology Research

Laboratory], for 30 minutes in a humid chamber in the dark. Following this, cells were again washed with PBS (three times, 5 minutes), and mounted in vectashield anti-fading media containing DAPI [Vector Laboratories], cover slipped and examined using an Axioplan F microscope [Carl Zeiss]. Immunofluorescence staining for Δ Np63 and Sox2 follow the same methods as described above. Mouse anti-human Δ Np63 (Clone 4A4) primary antibody was purchased from Santa Cruz Biotechnology and rabbit polyclonal anti-human Sox 2 antibody from Abcam. In the case of Δ Np63 slides, vectashield without DAPI was used. Negative controls were cells incubated without addition of primary antibody, but with secondary antibody only.

f. Image quantification

Under 20X magnification, the collection site for cells on the slides (stained as mentioned in section e) were arbitrarily divided into 2 – 3 quadrants. For each quadrant, all cells were selected for image quantification using image quantification software [Image J version 1.44]. The means ($\pm$ standard deviation) of signal intensities were averaged and analysed to compare the means of signal intensities between SP and NSP cell fractions, using independent t-test [IBM SPSS Statistics Version 16]. *P* value of < 0.05 was taken as a significant difference in means between two groups.

g. Polymerase chain reaction (PCR)

A standard polymerase chain reaction was performed using a PCR master mix [Promega]. PCR was performed on this final reaction mixture for 30-35 cycles. Denaturation was set at 94°C for 30 minutes and elongation took place at 72°C for 1 minute at primer pair specific annealing temperature, and maintained at 4°C. The sequence of primers [Invitrogen] were: 1) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Forward primer: *GCACCGTCAAGGCTGAGAAC*, Reverse primer: *GCCTTCTCCATGGTGGTGAA*, 2)

ABCG2: Forward primer: *GCGTGCTGTGCCCACTCAAA*, Reverse primer: *AGCATGTGCACGGTGCGTTC*, and 3) ABCB1 Forward primer: *GACGTCATCGCTGGTTTCGAT*, Reverse primer: *TCATTTCTGCTGTCTGCATTGT*. The final PCR products were either stored at +4°C or electrophoresed on an agarose gel.

5.0 RESULTS

Optimisation of limbal SP (LSP) protocol

Due to the limitations associated with primary limbal tissues and its culture conditions which involve co-culture expansion with animal-sourced feeders or amniotic membrane, and often limited cell numbers, optimisation for LSP was done using the HTCEC line. An algorithm of optimisation steps for LSP is presented in Figure 1.

Hoechst concentration: To determine the optimal Hoechst concentration cells were incubated in the presence of a range of concentrations from 0.5 µg/ml to 3.0 µg/ml. When using the lowest concentration of Hoechst dye at 0.5 µg/ml, the cells can be seen in the SP gate (2A), however the phenotype of these cells could not be confirmed in the presence of the inhibitor verapamil (2B). In the presence of the highest concentration of 5 µg/ml of Hoechst, no SP cells can be detected and cell viability is severely reduced at 47% (2C), and in the presence of verapamil (2D) further cell death occurs (54%). In the presence of 3 µg/ml of Hoechst dye, an SP population of 0.2% could be detected with cell death of 37% (2E), furthermore the efflux ability of these cells could be blocked in the presence of verapamil (2F).

Incubation time: To determine the impact of time of cell contact in the presence of Hoechst dye cells were incubated at 1×10^6 /ml in 3 μ g/ml Hoechst dye for 45 and 90 minutes (Figure 2G and 2H). At 90 minutes incubation time no SP cells could be detected and cell death had increased to 64% (2G) compared with cell death of 51% using an incubation time of 45 minutes (2H).

Limbal Side population assays

Using our optimised protocol, SP of 0.8% was detected, the phenotype of which was confirmed on addition of Verapamil (Figure 3). The SP assays on limbal epithelial cultures gave a range of between 0.1 to 0.8% with the final SP phenotype being confirmed by either verapamil or fumitremorgin C inhibition.

The ability of both primary limbal epithelial SP and HTCEC to efflux Hoechst dye could be inhibited by the addition of verapamil or fumitremorgin C alone. This inhibition was either complete or partial, which was the same for both cell line and primary epithelial cultures, but partial inhibition is a phenomenon more frequently associated with primary epithelial cultures, an example is given in Figure 4A, 4B and 4C. In the presence of 3 μ g/ml of Hoechst dye, an SP population of 0.4% could be detected (4A), which was partially inhibited by verapamil, as noted in the SP gate (4B), but total cell loss was evident in the SP gate on addition of fumitremorgin C (4C).

When a partial inhibition was observed we hypothesised this was due to there being more than one ABC transporter being responsible for the SP phenotype. The presence of both ABCG2 and ABCB1 transporter genes was observed in both primary limbal epithelial cells and

HTCEC, as evident by polymerase chain reaction (Figure 4D and 4E) and ABCG2 expression on immunocytochemistry (Figure 4F and 4G).

Using our optimised limbal SP protocol we sorted SP and NSP cells of the HTCEC directly onto slides and stained them with antibodies to ABCG2, Δ Np63 and Sox2. Using Image J software combined with microscopy, the signal intensity for each marker was quantified. For ABCG2, Δ Np63 and Sox2, the expression of all three markers was significantly higher in the SP versus NSP cells. The means in signal intensity were significantly different between the SP and NSP groups ($P < 0.05$) for ABCG2 expression ($P= 0.026$), Δ Np63 ($P=0.007$) and Sox2 ($P=0.02$). The statistics for image quantification are presented in Table 1. Results of immunofluorescence staining are shown in Figure 5 for Δ Np63 expression in SP (A) and NSP (B), ABCG2 in SP (C) and NSP (D) and Sox2 in SP (E) and NSP (F) .

6.0 DISCUSSION

The side population assay has been used widely to identify cells which exhibit stem cell activities and are present in various normal tissues i.e. umbilical cord blood, skeletal muscle [22], kidney, liver, pancreas [23], cardiac tissue [24], mammary glands, lung, brain and cancer tissue and cell lines [25]. SP phenotypes have also been identified in cancers such as lung carcinoma [26], ovarian cancers [27], breast cancers [25], and other malignant tumours [28], (reviewed in [29]).

In a critical appraisal for SP methods, Golebiewska et al listed critical parameters to ensure reproducibility and standardisation of SP data [30]. Among these parameters were Hoechst

dye concentration, cell concentration, Hoechst incubation or contact time, stable incubation conditions, and concentrations of inhibitors. Several optimisation of SP protocols for murine cardiac progenitors [31,32] and bone marrow, muscle and skin [33] have been reported. The optimisation steps combined with appropriate sorter equipped with suitable laser for vital dye of choice are essential to obtain a high resolution SP FACS data plot and to allow for discrimination between the SP and NSP fractions of cells.

Cumulative data from previous SP protocols showed differences exist between various tissues of the same species, or the same tissues from different species. Furthermore, human tissue sourced SP cells often required different Hoechst concentration and contact times from those of their animal counterparts. For example, in murine bone marrow and skeletal muscle assay, 5 µg/ml Hoechst dye was used, while in liver, spleen and kidney, 10 µg/ml of dye was used [34]. The SP assays for murine cardiac progenitor cells, has been reported to use between 1 – 5 µg/ml of Hoechst dye and a 90 minute incubation period [31,32]. However, a study in a human developing heart used 1.25 µg/ml of Hoechst dye and a 60 minutes incubation time. In the field of limbal corneal SP, a noticeable variation among species was also observed. As examples, in the pig – Hoechst concentration of 5 µg/ml for 90 minutes was used [35] and rabbit – 3 µg/ml for 90 minutes [10].

While SP cells have been identified in the limbal cell population in previous investigations, the conditions used have primarily been based on those originally described for mouse bone marrow SP [1]. Reported studies mentioned above suggest that modifications of protocol due to species-to-species variation, culture conditions and other variables, is required to obtain a distinct SP cell data plot. The need for an optimised protocol for human limbal SP studies is to enable better separation between SP and NSP fractions, better cell viability and enable

direct comparisons to be made between studies of human limbal SP cells between different laboratories.

The optimal inhibitor for use in the SP assay for determination of limbal SP may be variable when using patient samples perhaps due to variation in ABC transporter expression. This has been reported previously in SP cell populations taken from breast tumours [25]. We observed complete or partial inhibition when using verapamil. Incomplete inhibition by verapamil is not exclusive to limbal cells, it has also been observed in the SP assays of murine skin and muscle [33], alluding to the presence of more than one type of transporters being present in these tissues.

Limbal SP cells have been shown to be enriched for stem cell activities such as increased colonyforming efficiency and high proliferative capacity [6,7,35]. Colony forming efficiency is considered a stem cell characteristic in other adult stem cells such as retinal stem cells [36], bone marrow stem cells [37], and dental pulp stem cells [38].

SP cells have previously been reported to be present in conjunctival and limbal epithelial cell cultures but absent from the corneal epithelium in both rabbit [6,9] and human [6]. Whereas in the rat, SP cells have been shown to be present in both limbal and central cornea [9] suggesting species variations as this contradicts findings from rabbit and human limbal SP studies. While a number of studies have demonstrated the presence of limbal SP cells in both animal and human limbus and have provided evidence that these SP cells have some stem cell characteristics, few have looked at optimisation of the SP assay.

Budak and colleagues [6] commented that optimisation of their protocol for limbal SP cells (rabbit and human) was initially done on rabbit cells and from the results of these they concluded what the optimal Hoechst dye concentration and incubation time would be for the human cells. Whereas de Paiva and colleagues [7] used both freshly isolated and primary

human cell cultures as a source of limbal SP cells but did not optimise the SP cell assay, instead they based their SP protocol on a method previously used to isolate SP cells from hematopoietic stem cells. Watanabe and colleagues [39] also reported on the identification of human limbal SP cells from human limbus but again did not report on optimisation of the SP assay used. However they did use a Hoechst concentration of 3µg/ml and an incubation time of 90 minutes. In this study we have optimised our SP cell assay using human cells and concluded that a Hoechst concentration of 3µg/ml was optimal but that an incubation time of 45 minutes rather than 90 minutes resulted in a lower rate of cell death.

Functional role of ABCG2 in limbal stem cells have been explored in a study of oxidative stress in corneal epithelial cells [40]. Although ABCG2-knock out mice have morphologically normal corneal epithelium, they were susceptible to oxidative stress and prone to hypoxic cell death induced by Mitoxantrone. This supports the role of ABCG2 in maintaining the limbal progenitors and stem cell pool.

Using the SP assays, limbal epithelial cells were enriched for stem cells by showing presence of extremely low side scattering and slow cycling cells [6], which is similar to flow cytometric analysis of murine keratinocyte stem cells and rat conjunctival stem cells [41-43].

Studies of ABCG2 expression at mRNA and protein levels have provided convincing evidence that ABCG2 is the main transporter implicated in determining the limbal SP phenotype [6,7,9,11]. Again however in studies of human limbal SP cells while ABCG2 expression has been reported to be upregulated in SP cells versus NSP cells, this has been focused on gene expression [7,39]. Several studies have used immunohistochemical staining to examine tissue sections to show the location of ABCG2 expressing cells in the human eye and have reported that ABCG2 positive cells can be found in the limbal epithelial basal layer [7,11,39], however this does not prove they are stem cells.

de Paiva and colleagues also selected out ABCG2-positive and ABCG2-negative cells using an anti-ABCG2 antibody combined with flow cytometry from their primary limbal cell cultures [7]. They showed that these cells expressed ABCG2 and Δ Np63 mRNA whereas the ABCG2-negative cells did not, moreover the positive expressing cells had a great colony forming efficiency than the negative cells, suggesting that within the ABCG2-positive population there were stem cells. Moreover they also selected SP and NSP cells from the primary human limbal cell cultures and showed that these SP cells showed upregulated expression of ABCG2 and Δ Np63 mRNA and that these SP cells had a greater colony forming efficiency than NSP; however they did not look at protein expression for either of these markers. In our study we not only looked at protein expression for ABCG2, Δ Np63 and Sox2 in SP cells and NSP cells from the HTCEC, we also quantitated the level of expression, this is important as NSP cells can also express some level of all these markers.

Rabbit limbal SP contained a high proportion of cells with a smaller diameter than the NSP cells [10]. The same findings were observed in skeletal muscle SP, where the SP cells had a relative small diameter and a high nuclear to cytoplasmic ratio [22]. Cell size and a high nuclear-to-cytoplasmic ratio are parameters that have been used to describe stem cell populations, including epidermal stem cells [44] and epithelial stem cells [42].

Clearly these are evidence that SP cells are isolated in the limbus and ABCG2 being the main transporter involved in the SP phenotype. SP sorted cells are also enriched for limbal stem cells and important players in limbal stem cell maintenance.

There are critical parameters that can challenge the design of experiments and interpretation of results of an SP assay. It is crucial to obtain a single cell suspension with limited cellular damage because the assay is based on active physiological process which depends on

viability and protein integrity at the cellular membrane. This poses a challenge when processing tissues using enzymatic digestion which is potentially damaging to the cells. When an established or stable cell line is used, factors that needed to be looked into are the cell density, nutrient used, serum or oxygen levels [45], therefore it is advisable to keep to the same culture conditions. It is also important to maintain similar cell concentration/number of cells to gather the equilibration of the dye with the intracellular components.

Confirmation that the cells identified are SP cells is important due to issues that can be caused by under or over staining therefore the inhibitor and its concentration need to be validated. Evidence has shown that variable inhibitors are involved in SP phenotypes in cancer cells where the expression of ABC-transporter inhibitor varies with pathological states i.e. epithelial-mesenchymal transition [46], cancer subtypes and progression of disease [4,25].

Although the cell count of 1.0×10^6 cells/ml is the standard cell concentration, it is particularly important when doing a profile on primary cultures to do adequate gating paying attention to single, viable and nucleated cells. Enucleated cells from highly vascularised tissues such as brain and heart will influence Hoechst dye equilibration, thus further steps may be needed to distinguish them such as utilising red cell lysis systems [32], or setting up an additional gate for erythrocyte exclusion. Since Hoechst dye stains all DNA regardless if cells are alive or dead, dead cell discrimination is an important step in flow cytometry analysis. Appropriate gating for cell debris and dead cells should be integrated into the analysis.

For a consistent temperature control, Hoechst incubation should be carried out under stable temperature control we suggest the use of a 37°C incubator using a gentle shaker or rotator.

It is also important to note that to prevent further efflux of Hoechst from cells after staining is complete, all activities post staining and prior to analysis should be performed at 4°C.

The advantage of SP which is a functional assay performed on viable cells, is it enables subsequent *in vitro* and *in vivo* functional characterisation to be performed e.g. further characterisation using stem cell markers [31]. In the case of limbal SP and NSP the two cell fractions may be further characterised using polymerase chain reaction [7,39] or immunofluorescence studies, or propagated for proliferative studies such as colony forming efficiency [7] or spheroid formation [47,48].

To our knowledge this report is the first presentation of detailed optimisation methods for SP assays both in primary human corneal epithelial cultures and a human telomerase-immortalised corneal epithelial cell line. We also show that SP cells isolated from the HTCEC line using this protocol, have significantly elevated expression of ABCG2, Δ Np63 and Sox2 above that displayed by the NSP cell fraction, supporting the use of this protocol for identification and separation of a stem cell enriched cell fraction of limbal cells. The use of established cell lines as sources of SP cells have been reported previously [25,27,28,49]. While the percentage of SP cells is relatively small in the HTCEC line, stem cells are in many systems rare cells, however it was also demonstrated that as few as 500 rabbit limbal SP cells were able to regenerate cornealike epithelium which was p63-positive in a 3-D culture system [50].

The convenient use of an established cell line surpasses the difficulty in the isolation and identification of limbal stem cells from primary cultures and enables studies to be performed when valuable primary tissue is unavailable. We also provide evidence of expression of mRNA for another ABC transporter ABCB1, in both the HTCEC and primary limbal cell

cultures which may have a role to play in the limbal SP cell phenotype. Further studies will be needed to determine if this is the case.

7.0 CONCLUSION

In the past 17 years since Goodel, *et al* demonstrated SP in murine haematopoietic stem cells, accumulating evidence has shown this assay to be useful for functional characterization of putative stem cells and cancer stem cells, especially in the absence of specific molecular markers. There are many dynamic factors involved in the assay, such as the metabolic nature of the dye, the integrity of cell membrane and viability of the cell populations, which may result in technical difficulty and compromise data analysis.

The sensitivity of the assay to different species and cell types also makes standardisation of SP protocols mandatory to ensure reliability and reproducibility of SP data. This will ultimately result in better interpretation of SP results and enable more accurate comparison between data generated in multiple studies.

Conflict of interest:

Authors do not have actual or potential conflict of interest including any financial, personal or other relationships with other people or organisations that could inappropriately influence, or be perceived to influence our work.

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FIGURE LEGENDS

Figure 1. Optimisation steps for side population assay of corneal epithelial cells,

**pre-optimised in previous literature (Rabindran et al, 2000)*

Figure 2. Representative FACS data plot showing SP protocol optimisation. Hoechst red on the horizontal axis and Hoechst blue on the vertical axis, gating indicates SP cells. Cells incubated with 0.5 µg/ml of Hoechst (A), cells from the same preparation but with the addition of VP (B). Cells stained with 5 µg/ml of Hoechst (C) and cells from the same preparation but with the addition of VP (D) Cells stained using Hoechst concentration of 3 µg/ml, note the presence of SP cells in the gated region (E) and absence of cells in the SP gate in the presence of VP, (F). Cells incubated with 3 µg/ml of Hoechst at 45 minutes (G) and 90 minutes (H) showing increased cell death. *Abbreviations: SP – side population, VP - Verapamil*

Figure 3. Representative FACS data plot shows the presence of SP cells in primary limbal epithelial using a Hoechst concentration of 3 µg/ml and 45 minutes incubation time (A) and cells of the same preparation but with the addition of Verapamil (B). Note the absence of SP cells in the gated region. *N=11*

Figure 4. FACS Images of primary limbal epithelial cell cultures showing the presence of SP cells in primary limbal epithelial cultures (N=3). Hoechst red on horizontal axis and Hoechst blue on vertical axis, Note the presence of 0.4% SP obtained with a Hoechst concentration of 3 µg/ml (A). Cells from the same preparation but in the presence of Verapamil (B) and in the presence of Fumitremorgin C (C). Transcriptional expression of ABCG2 and ABCB1 in human telomerase immortalised corneal epithelial cells (HTCEC) (D) and primary limbal epithelial culture (E). Product size for ABCG2= 143 bp, ABCB1=116 bp, GAPDH=160 bp. Immunohistochemical analysis for ABCG2 expression in unsorted cells of HTCEC 20x magnification (F) and in primary limbal epithelial cultures (G) Note ABCG2 staining is localised to a holoclone as indicated by white arrow, in (G) 10X magnification.

Figure 5. Representative immunofluorescence images for expression of Δ Np63 in SP (A) and NSP (B), ABCG2 in SP (C) and NSP (D), and Sox2 in SP (E) and NSP (F) cells of the HTCEC line. *Antibody-FITC = green, cell nuclei DAPI = blue. Size bar = 20 μ m .*

Table 1. Image quantification analysis showed significant difference in mean signal intensities in SP and NSP fractions of the HTCEC cell line

Table 1. Image quantification analysis of immunofluorescence studies from SP-sorted cells

Markers		Means	($\pm$) SD	<i>P</i> value
ABCG2	SP	17.26	5.7	0.025
	NSP	13.14	4.4	
Δ Np63	SP	36.32	3.7	0.000
	NSP	28.09	2.5	
Sox 2	SP	33.15	3.9	0.180
	NSP	23.76	5.8	

Abbreviations: ABCG2 = *ATP-binding cassette sub-family G member 2*, SP = side population, NSP = Non-side population

Uninhibited

+ Verapamil

+ FTC

D

E

F

G

SP

NSP

Δ Np63

ABCG2

Sox 2