STEM CELLS AND DEVELOPMENT
Volume 18, Number 10, 2009

DOI: 10.1089/scd.2008.0302

# Hoechst 33342 Side Population Identification Is a Conserved and Unified Mechanism in Urological Cancers

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Mutation within the adult human stem cell (SC) compartment has been proposed as a factor in the initiation and promotion of carcinogenesis. Isolation of these cancer stem cells (CSCs) has proven difficult, limiting their subsequent phenotypic, functional, and genetic characterization. We have used the Hoechst 33342 dye efflux technique to isolate an epithelial side population (SP) from genitourinary (GU) cancers, which is enriched for cells with SC traits. With informed consent, samples were taken from patients with primary tumors and undergoing surgery for prostatic (CaP), invasive bladder transitional cell (TCC), and renal cell carcinomas (RCC). Single cell epithelial suspensions were extracted from these and incubated with Hoechst 33342. Hoechst SP/non-SP profiles were then generated by flow cytometry using standardized protocols. SP/non-SP cell cycle status was established by Hoechst 33342 and Pyronin Y staining. Immunocytochemistry staining was performed for markers suggested as stem markers as well as lineage-specific markers. Functionality was determined using colony-forming assays and long-term monolayer culture. A characteristic verapamil-sensitive SP was isolated from all 3 urological malignancies and represented 0.57% ± 0.11% (CaP), 0.52% ± 0.49% (TCC), and 5.9% ± 0.9% (RCC) of the total epithelial population. Cell cycle analysis showed that the SP had enhanced numbers of cells in $G_0$ as compared to the total cell population (CaP 12.4% ± 3.2 vs. 3.8% ± 1.0, RCC 23.2% ± 3.4 vs. 1.8% ± 0.9, and TCC 28.5% ± 4.9 vs. 4% ± 1.3). Immunocytochemistry demonstrated an increased expression of proliferative and putative stem markers within the SP fraction. Cultures confirmed significant enhancement of colony-forming ability and proliferative capacity of the SP fraction. A characteristic SP enriched for stem-like cells has been isolated from the 3 most common urological malignancies. This provides strong evidence that Hoechst 33342 efflux is a conserved and unified mechanism in GU cancer.

## Introduction

The adult tissue-specific stem cell (SC) is believed to be present in most human tissues and is defined by the specific properties of self-renewal, clonogenicity, and pluripotentiality [1–3]. SCs are thought to play a key role in organogenesis as well as the homeostasis and repair of tissues. Despite their proliferative potential, SCs usually remain quiescent within their niche, constituting only a small fraction of the total cellularity of a tissue [4]. By the process of asymmetric division these primitive cells produce an identical copy of themselves, thus maintaining their own status, as well as a daughter cell that divides into numerous intermediate cell types termed "committed progenitors" or the transit-amplifying population (TAP). Whilst having a limited phenotypic potential, the TAP expands the numbers of cells for which the differentiation pathway is now already determined and is responsible for generating the population of terminally differentiated cells of the mature tissue.

The attribute of self-renewal, in combination with their intrinsic growth potential, makes SCs relevant to carcinogenesis [5,6]. The cancer stem cell (CSC) theory has gained popularity and it is postulated that CSC arise from the direct mutation of normal SCs or that dedifferentiation of the TAP leads to its constituent cells regaining the properties of self-renewal [7,8]. Accurate identification and isolation of such CSCs would afford the opportunity to determine the

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underlying mechanisms of disease, with the ultimate aim of developing novel diagnostics, prognostics, and targeted treatments.

Previously, the expression of cell surface markers has been used as the method of SC and CSC enrichment. Individual tissues often display specific markers, though expression is not consistent between tissues and no one common marker has been identified to date. Much effort has therefore been made to identify enriched SC populations based on their physical and physiological properties. One such mechanism for SC isolation is to exploit a cytoprotective mechanism possessed by noncommitted SCs. SCs express ATP-binding cassette (ABC) and multidrug resistance (MDR) efflux pumps and thus are able to remove toxic metabolites and xenobiotics from the interior of the cell. This survival advantage is clearly beneficial to the organ or tissue as it is the SC that would survive and be able to repair and repopulate an injured tissue. In the case of CSCs, these cells would potentially have greater resistance to therapy, a feature that could predispose to disease resistance and/or recurrence. The DNA-binding dye Hoechst 33342 is affected by this process, with stem-like cells retaining the ability to efflux this chemical using the ABC/MDR mechanism. Cells able to efflux Hoechst 33342 therefore appear to the bottom left corner of a dual parameter fluorescence-activated cell sorting (FACS) analysis as a Hoechst $33342^{low}$ side population (SP) [9]. Goodell et al. [10] developed this technique to identify a population of cells from the mouse hematopoietic system that was able to actively efflux cytotoxic agents (such as the Hoechst 33342 dye) as well as showing increased proliferative and self-renewal capacity. Subsequent work (reviewed by Challen and Little [11]) has identified an SP in various human solid tissues including prostate [12,13], breast [14], skeletal muscle [15,16], intestinal tract [17], liver [18], and brain [19].

We have applied the Hoechst 33342 dye efflux technique to the 3 most common urological malignancies, prostate, bladder, and renal cancers, to determine whether SP cells could be identified and their functional phenotype characterized.

## Materials

All materials were sourced from Sigma-Aldrich (Poole, UK) apart from: RPMI 1640 and fetal calf serum (FCS) (Cambrex BioSciences, Verviers, Belgium), collagenase type 1 and trypsin (Lorne Laboratories, Twyford, UK), Hanks' balanced salt solution (HBSS), HEPES, Keratinocyte Serum-Free Media, Dulbecco's Modified Eagle's Medium (DMEM), insulin-transferrin-selenium, Hoechst 33342 (Invitrogen, Paisley, UK), and Optiprep (Axis-Shield Diagnostics Limited, Dundee, UK). Individual culture media was prepared for each tissue type using the following recipes. Prostate carcinoma [20]; cholera toxin (100 ng/mL), GM-CSF (1 µg/mL), leukocyte inhibitory factor (2 ng/mL), and SC factor (20 mg/mL), added to keratinocyte serum-free media treated with 0.005 µg/mL of human epithelial growth factor, 0.05 mg/mL of bovine pituitary extract, antibiotics, and antimycotics (100 IU/mL penicillin G sodium, 100 µg/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B in 0.85% saline). Renal cell carcinoma (RCC); equal volumes of DMEM and Ham's F12 media with insulin (200 µg/L), transferrin (100 µg/L), selenium (25 nM), hydrocortisone (18.2 µg/L), epidermal growth factor (1 µg/L), ethanolamine (10 mM), *O*-phosphorylethanolamine (1.4 mg/L), triiodothyronine (0.68 µg/L), sodium pyruvate (0.5 mM), HEPES (10 mM), bovine serum albumin (BSA) (0.02%), and FCS (5%). Transitional cell carcinoma (TCC); Ham's F12 medium with 1% fetal bovine serum, $5 \times 10^{-8}$ M sodium selenite, $10^{-4}$ M ethanolamine, 2.7 mg/mL D-(+)-glucose, 2 mM L-glutamine, $10^{-4}$ M nonessential amino acids, 10 µg/mL insulin, 5 µg/mL transferrin, 1 µg/mL hydrocortisone, and antibiotics and antimycotics (100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B in 0.85% saline). Wash buffer was made from phosphate-buffered saline (PBS) with 1 mM EDTA and 1% BSA. Hoechst buffer consisted of HBSS with 10% FCS, 2% D-glucose, and 1% HEPES.

All samples were analyzed and sorted using a Becton Dickinson FACSVantage SE Flow Cytometer. A BroadPass 424/44 filter was used to measure Hoechst red along with a 675/20 BroadPass filter to measure Hoechst Blue (Omega Optical, Brattleboro VT).

## Methods

### *Sample preparation*

Samples were collected with full ethical approval from consenting patients undergoing channel transurethral resection of the prostate (TURP) for adenocarcinoma of the prostate, radical cystectomy for TCC, and radical nephrectomy for RCC. Eleven specimens from each malignancy were obtained for analysis. Samples were finely diced and digested overnight in type 1 collagenase solution (200 U/mL in RPMI 1640 media with 2% FCS). Representative samples were sent to confirm each diagnosis histologically. The samples were washed with wash buffer, resuspended in RPMI 1640, and differentially centrifuged (360*g*, 1 min, no brake) to separate the epithelial cells from the fibroblast and stromal cells.

### *Hoechst 33342 staining and fluorescence-activated cell sorting (FACS)*

Live cells were isolated using Optiprep density gradient medium (60% iodixanol [w/v] in water) and suspended in Hoechst buffer at a concentration of $1 \times 10^6$ cells/mL. CD45-FITC (1:500) and 5 µM Hoechst 33342 dye were added and the cell suspension incubated on a shaker-plate (5 Hz) at 37°C for 90 min. An identical sample was prepared with the addition of 50 µM of verapamil. A control sample was also prepared using mouse IgG-FITC (1:500) and 5 µM Hoechst 33342. After 75 min, Pyronin Y (0.5 ng/mL) was added to the samples used for cell cycle analysis. These samples were then returned to the shaker-plate and incubated for a further 15 min.

The samples were then washed in ice-cold Hoechst buffer before being placed on ice and taken for immediate analysis on the FACSVantage Flow Cytometer. The live, $CD45^{-ve}$ SP cells were identified, isolated, and collected in Hoechst buffer, along with live, $CD45^{-ve}$ non-SP (NSP) cells for use as a comparative control.

### *Immunocytochemistry*

After FACS sorting, live $CD45^{-ve}$ SP and NSP cells were centrifuged at 800*g* for 5 min and resuspended in PBS to a concentration of $10^4$ cells/mL. Ten microliters of this cell

suspension were placed into each of 12 individual wells on a poly-L-lysine-coated slide. The cells were fixed in 4% formaldehyde and permeabilized in methanol at −18°C. Cells were blocked with 10% serum and 0.3% $H_2O_2$ and stained with primary antibodies against putative SC markers, lineage markers, or with isotype control antibodies (see Table 1). Primary antibodies were labeled with biotinylated secondary antibody and avidin DH/biotinylated horseradish peroxidase complex (Vectastain EliteABCkit; Vector Labs, Burlingame, CA). Cells were developed by the addition of DAB substrate for 5 min and counterstained with hematoxylin. Slides were then counted with an Olympus BX51 microscope with a 40× objective lens.

### *Long-term monolayer culture*

Following FACS sorting, cells were placed in Falcon $T_{25}$ tissue culture flasks in appropriate media as described in the Materials section. Forty-eight hours prior to the cells being seeded, STO cells were irradiated with a single fraction of 50 Gy and seeded in the Falcon $T_{25}$ culture flask at a density of $10^6$ cells/flask.

The cells were then incubated in 5% $CO_2$ in air at 37°C and cultured until confluent. The number of cells in each flask was counted prior to a 1 in 4 split and recultured in $T_{25}$ tissue culture flasks. This was repeated until proliferation ceased, at which point "duration in culture" and "number of serial passages" were recorded.

### *Colony-forming efficiency assays*

During FACS sorting, live $CD45^{-ve}$ SP cells were placed into individual wells of a 96-well plate, containing a feeder layer of irradiated (50 Gy) STO cells and 150 μL of growth media. A corresponding plate of NSP cells was also prepared. The plate was then placed in 5% $CO_2$ in air at 37°C and left for 7 days, following which 100 μL of the growth media was discarded from each well and replaced with fresh media. After a further 7 days, the plates were removed and colonies (>32 cells) counted.

### *Statistical analysis*

A 2-tailed paired *t*-test was used to determine statistical significance during analysis. A significance level of $P < 0.05$ was used.

## Results

### *Identification of a side population (SP) in Hoechst 33342 flow cytometry profiles*

The flow cytometer defined gates to ensure the required populations were analyzed. First, the live population was gated with the characteristic dead cells (small cell size and low cell granularity) appearing to the left of a plot of forward against side scatter (Fig. 1A). To ensure there was no contamination by SCs from coexistent lymphocytes, the pan-leukocyte marker CD45 was used and the second gate applied to exclude the $CD45^{+ve}$ population (Fig. 1B). The cytoprotective ABC and MDR efflux pumps that remove toxic metabolites and xenobiotics from the interior of the cell are inactivated by verapamil. The accurate definition of the SP tail is confirmed by its verapamil sensitivity (Fig. 1C). Characteristic Hoechst 33342 red/blue flow cytometry profiles have been successfully produced for $CD45^{-ve}$ cell populations from all 3 genitourinary malignancies. The SP tail contains those cells that are able to efflux the Hoechst 33342 dye, and as such display low red and blue fluorescence on a red/blue dual parameter plot. The SP comprised 0.57 ± 0.11%, 0.52 ± 0.49%, and 5.9 ± 0.9% of the total epithelial populations of carcinoma of the prostate (CaP), TCC, and RCC, respectively (Fig. 1D). The $CD45^{-ve}$ SP and NSP populations were subsequently collected for phenotypic and functional analysis.

### *Phenotype of SP and NSP populations*

With the exception of the immunocytochemical markers β-Catenin and Cleaved Notch in TCC, and PAX-2 in CaP, all other putative SC markers were enriched in the SP compared with the NSP (Table 2). The markers p21 and

Table 1. The Antibodies Used for Immunocytochemistry Staining of the Cells Obtained From Each Tissue, Along With Respective Controls and Secondary Antibodies

| *Antibody* | *Host animal* | *Dilution* | *Secondary antibody* | *Control* |
|---|---|---|---|---|
| p21 | Mouse | 1:10 | Rabbit anti-mouse | Mouse IgG |
| p27 | Mouse | 1:40 | Rabbit anti-mouse | Mouse IgG |
| Beta-catenin | Rabbit | 1:50 | Swine anti-rabbit | Rabbit IgG |
| Cleaved notch | Rabbit | 1:50 | Swine anti-rabbit | Rabbit IgG |
| Musashi-1 | Mouse | 1:2000 | Rabbit anti-mouse | Mouse IgG |
| Pax-2 | Rabbit | 1:500 | Swine anti-rabbit | Rabbit IgG |
| Notch-1 | Goat | 1:50 | Rabbit anti-goat | Goat IgG |
| Controls | | | | |
| IgG | Mouse | 1:100 | Rabbit anti-mouse | |
| IgG | Rabbit | 1:1000 | Swine anti-rabbit | |
| IgG | Goat | 1:1000 | Rabbit anti-goat | |
| Secondary antibodies | | | | |
| Rabbit anti-mouse | | 1:40 | | |
| Swine anti-rabbit | | 1:40 | | |
| Rabbit anti-goat | | 1:40 | | |

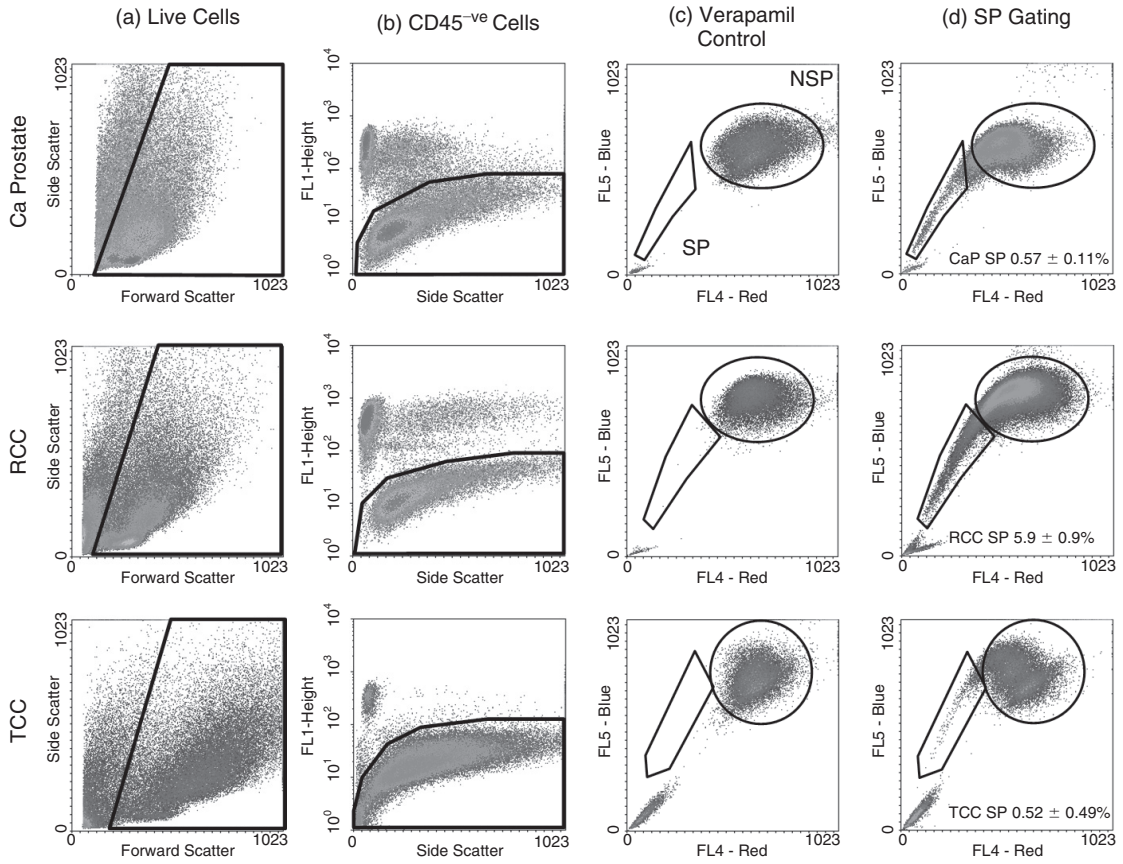


**FIG. 1.** Flow cytometry density plots demonstrating the process of gate definition and the identification of live cells (a), CD45$^{-ve}$ selection (b), verapamil-sensitive SP region identification (c), and SP and NSP epithelial cell populations (d) isolated from carcinoma of the prostate (CaP), renal cell carcinoma (RCC), and transitional cell carcinoma (TCC).

Musashi-1 (Msi-1) were significantly raised in RCC and CaP and were close to significance in TCC though the level of $P < 0.05$ was not reached. The SP was significantly enriched for p27 and PAX-2 in RCC and TCC. Whilst the SP was significantly enriched for Sonic hedgehog (Shh) in only TCC, the marker was highly expressed in the other 2 tissues and significance levels were close to the $P < 0.05$ level. None of the individual markers were significantly enriched in the SP

Table 2. The Differential Expression of Immunocytochemical Stem Cell Markers in the SP and NSP Isolated From Each of the Three Genitourinary Malignancies

| | *RCC* | | | | | *CaP* | | | | | *TCC* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *SP* | | *NSP* | | | *SP* | | *NSP* | | | *SP* | | *NSP* | | |
| | *Mean (%)* | *SD (%)* | *Mean (%)* | *SD (%)* | *P* | *Mean (%)* | *SD (%)* | *Mean (%)* | *SD (%)* | *P* | *Mean (%)* | *SD (%)* | *Mean (%)* | *SD (%)* | *P* |
| p21 | 48 | 5 | 16 | 4 | 0.005* | 44 | 6 | 25 | 5 | 0.022* | 9 | 3 | 2 | 0 | 0.063 |
| p27 | 79 | 8 | 44 | 16 | 0.018* | 18 | 6 | 11 | 3 | 0.210 | 12 | 1 | 4 | 2 | 0.039* |
| Beta-catenin | 79 | 12 | 55 | 10 | 0.186 | 50 | 6 | 16 | 5 | 0.010* | 55 | 19 | 53 | 6 | 0.828 |
| Cleaved notch | 80 | 11 | 12 | 8 | 0.004* | 31 | 4 | 11 | 3 | 0.032* | 36 | 9 | 52 | 3 | 0.136 |
| Msi-1 | 4 | 1 | 0 | 1 | 0.009* | 4 | 1 | 1 | 1 | 0.035* | 10 | 2 | 4 | 3 | 0.170 |
| PAX-2 | 62 | 7 | 20 | 2 | 0.009* | 33 | 10 | 33 | 6 | 0.920 | 55 | 15 | 17 | 6 | 0.044* |
| Shh | 72 | 12 | 36 | 15 | 0.064 | 34 | 6 | 16 | 4 | 0.089 | 58 | 15 | 22 | 7 | 0.018* |
| Notch 1 | 63 | 10 | 33 | 14 | 0.143 | 41 | 9 | 16 | 5 | 0.071* | 1 | 2 | 0 | 0 | 0.422 |

The mean percentage of cells positive for the various markers, as compared with the relevant isotype control antibodies, is shown along with the standard deviation. The level of significance for the differential expression between SP and NSP is reported. *Significant *P* values.

Abbreviations: CaP, carcinoma of the prostate; RCC, renal cell carcinoma; TCC, transitional cell carcinoma; SP, side population; NSP, non-side population.

across all 3 tissue types. Furthermore, variation in the absolute expression of markers is evident between different tissue types (Table 2).

### *Functional assessment of SP characteristics*

Four samples from each tumor type were selected for functional analysis to determine the SP's potential for colony-forming ability and proliferation capacity compared to non-SP cells. Compared with the NSP, the SP demonstrated a statistically significant 6.42, 5.77, and 4.21-fold enhancement in colony-forming ability: CaP ($P < 0.001$), RCC ($P < 0.001$), and TCC ($P = 0.004$), respectively. The duration of culture was significantly greater in the SP in all 3 cell types; cultures lasted 2.70, 1.60, and 3.28 times longer in the SP of CaP ($P = 0.018$), RCC ($P = 0.033$), and TCC ($P = 0.002$). A significantly increased number of serial passages was possible with the SP successfully being passaged 2.88 times more than NSP cells in CaP ($P < 0.001$), 3.14 times in RCC ($P < 0.001$), and 3.72 times in TCC ($P = 0.039$). (Data summarized in Table 3.)

### *Cell cycle analysis*

Four samples from each tumor were used for the determination of cell cycle status. Flow cytometry plots of Hoechst Blue (FL-5) against Pyronin Y (FL-2) were constructed. Each of the plots reveal 2 columns of cells representing the normal (n) and twice normal (2n) DNA copy number evident just prior to cytokinesis. The cells in $G_0$ sit below the horizontal line drawn from the lowest extremity of the 2n column across to the *y*-axis and therefore are defined as cells with a normal (diploid) DNA copy number and low RNA content. Analysis was performed to determine the number of cells in $G_0$ in the entire CD45$^{-ve}$ cell population (Fig. 2A) compared with the SP tail (Fig. 2B). Results showed that the SP from all 3 malignancies had enhanced numbers of cells in $G_0$ (CaP 3.8% ± 1.0 of total vs. SP 12.4% ± 3.2, $P = 0.01$, RCC 1.8% ± 0.8 of total vs. SP 23.2% ± 3.4, $P = 0.002$, and TCC 4% ± 1.3 of total vs. SP 28.5% ± 4.9, $P = 0.003$).

Table 3. Results of the Functional Analysis of the Live, CD45-Depleted SP and NSP

| | *SP* | | *NSP* | | *Fold* | |
|---|---|---|---|---|---|---|
| | *Mean* | *SD* | *Mean* | *SD* | *enhancement* | *P value* |
| Colony-forming ability (%) | | | | | | |
| CaP | 34.0 | 6.9 | 5.3 | 1.0 | 6.43 | <0.001 |
| RCC | 27.1 | 2.3 | 4.7 | 1.3 | 11.78 | 0.004 |
| TCC | 16.0 | 2.8 | 3.8 | 1.0 | 4.21 | 0.004 |
| Duration of culture (days) | | | | | | |
| CaP | 73 | 10.2 | 27 | 9.8 | 2.70 | 0.018 |
| RCC | 67 | 12.1 | 42 | 4.7 | 1.60 | 0.033 |
| TCC | 56 | 7.2 | 17 | 3.6 | 3.29 | 0.002 |
| Number of cell passages | | | | | | |
| CaP | 8.8 | 1.7 | 2.8 | 0.9 | 3.14 | <0.001 |
| RCC | 9.5 | 1.9 | 3.3 | 1.3 | 2.88 | <0.001 |
| TCC | 9.3 | 2.6 | 2.5 | 2.0 | 3.72 | 0.039 |

The mean stem cell characteristics of colony-forming ability, duration of culture, and total number of cell passages are reported with standard deviation (SD) for each of the 3 tissues types. For each tissue type, the mean result for both SP and NSP cells, along with the fold enhancement between the populations and the *P* value generated from a Student's two-tailed *t*-test.

Abbreviations: CaP, carcinoma of the prostate; RCC, renal cell carcinoma; TCC, transitional cell carcinoma; SP, side population; NSP, non-side population.

## Discussion

The traditional stochastic theory of cancer suggested that any cell within a tumor population had the potential ability to proliferate extensively and induce carcinogenesis (reviewed by Ward and Dirks [21]). The life span of such cells is comparatively low and it seems unlikely that a differentiated cell would survive long enough to receive the necessary number of mutations required for carcinogenesis and tumor formation. Many investigators now favor the SC theory of cancer [7]. The relative quiescence and prolonged life span of the stem population means the cells are exposed to multiple potential carcinogenic events and therefore are more likely to develop oncogenic mutations, which are maintained by asymmetric division and consequently long-term self-renewal [22,23]. Furthermore, the ability of SCs/CSCs to produce a number of phenotypically distinct lineages offers explanation for the observed heterogeneity of tumors. Failure to treat the mutated CSC adequately results

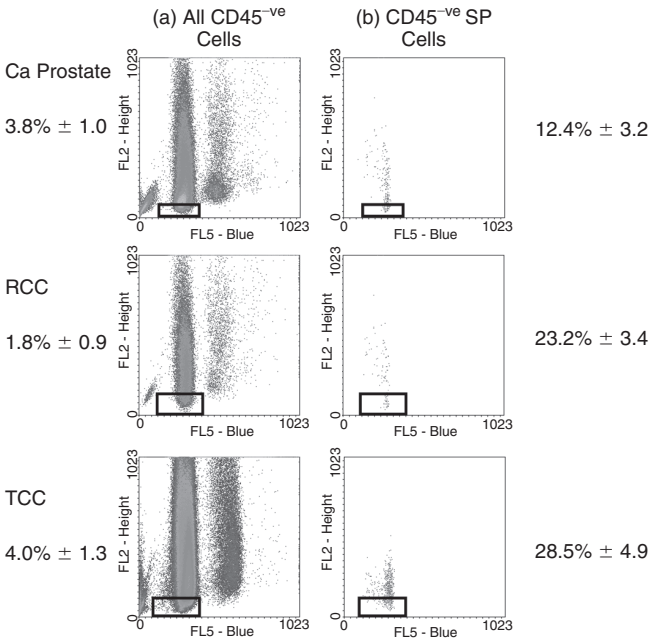


**FIG. 2.** Flow cytometry plots illustrating cell cycle analysis for each of the 3 tissue types. The (a) plots demonstrate flow cytometry plots of Hoechst 33342 Blue (FL-5) against Pyronin Y (FL-2) for the entire live CD45$^{-ve}$ cell population. Low levels of RNA (low Pyronin Y staining) are indicative of quiescent cells in $G_0$ phase and are highlighted by the box. The (b) plots show the CD45$^{-ve}$ SP cells only. Again the box in this column denotes the quiescent cells. The number of cells in $G_0$ is expressed as a percentage of the total cell number for that particular population and the SP and total live CD45$^{-ve}$ percentages are then compared.

in a persistent potential for disease recurrence as the CSC has the ability to repopulate the entire tumor.

Identification and subsequent isolation of the rare SCs/CSCs have proven difficult. Cell surface antigens represent one method of SC detection and isolation. However, no single marker has been elucidated to date that can be used consistently through different tissue types. As can be seen from our results, although the SP is significantly enriched for many of the putative markers in 1 or 2 tissue types, there was no consistent elevation of any single marker in all tissue types. For example, CD133 has been proposed as a marker for prostatic SCs [20], though other studies have suggested that CD133 is not present in bladder mucosal cells [24], so it would appear it cannot be used universally. The markers we have used to phenotype the SP cells have been previously proposed to be elevated in enriched stem-cell-like populations, but it is clear that no one marker can be used in all tissues.

Recent work has identified a population of $CD44^+/\alpha2\beta1^{high}/CD133^+$ cells from prostate cancer tissue [20] and these cells have been shown to have increased tumorigenic properties consistent with CSC-like behavior. However, other groups have demonstrated that $CD44^+/\alpha2\beta1^{high}/CD133^+$ prostate cells behaved similarly to $CD44^+/\alpha2\beta1^{low}/CD133^+$ cells [25], casting doubt on the role of α2β1 as a marker for SCs. Equally, some work has suggested that whilst $CD133^+$ cells from renal tissue are more proliferative than $CD133^-$ cells and have greater colony-forming potential, they did not display the significant self-renewal potential expected of SCs [26]. Work from our group has also shown the $CD133^+$ cells can be cultured from selected $CD133^-$ cells in renal tissue [27], suggesting that even if CD133 is a marker for SCs in prostate cells, it probably represents a more mature cell in renal tissue.

At present there is no single, cross-tissue SC marker and for many of the markers proposed in individual tissues there are doubts as to whether they are markers of SCs or instead markers of early progenitor cells. For this reason our group looked to explore different methods of identifying putative stem populations. The Hoechst 33342 dye efflux assay in genitourinary (GU) tissue allows the isolation of an enriched population with similar characteristics to those gained from the hematological system [10]. The fundamental methodological principal is that the greater the ability of a cell to efflux toxic metabolites (including Hoechst 33342 dye), the more primitive its lineage. It is hypothesized that those cells at the distal SP tail, which are the most efficient at effluxing the Hoechst 33342 dye, may represent a SC-enriched population. We have demonstrated that side populations can be obtained from both normal and cancerous solid tissue from the human GU tract. Furthermore, these SP cells can be isolated and cultured, when they show a generally quiescent population with the potential for proliferation.

Recent work has shown that SP cells isolated from a variety of cancer cell lines, including several cells from prostate cancer, display similar properties of enrichment for stem-like properties [28]. However, this group did demonstrate that cells lacking lineage markers and ABCG2 (1 of the transmembrane glycoproteins thought to be responsible for the efflux of the Hoechst 33342 dye) showed greater expression of Notch-1, SMO, and Oct-4, had greater self-renewal potential, had greater proliferative potential but were slower cycling than $Lin^-/ABCG2^+$ cells. This would appear contradictory as it suggests that cells that efflux Hoechst 33342 are not SCs whereas SP cells (which have effluxed Hoechst 33342) are enriched for SCs. It is known though that ABCG2 is not solely responsible for the efflux of Hoechst 33342 (indeed, only a fraction of the SP cells express ABCG2) with other ABC transporters, such as MDR-1, MRP-1, and ABCA2 also playing an important role. It would appear likely that the $ABCG2^-$ cells within the SP use other ABC transporters to efflux Hoechst 33342, hence their $Hoechst^{low}$ state. It is recognized that the SP is heterogeneous, so although the lack of ABCG2 expression cannot be used solely to identify SCs, it may be useful to further enhance the SP for cells with stem-like characteristics.

Our group successfully applied the Hoechst 33342 dye efflux assay to solid tumors by investigating the prostate [12,13] and we now have extensive experience of the technique in determination of the SP in both normal and malignant epithelial populations. We have previously reported functional and phenotypic characterization of the prostatic SP, demonstrating enrichment for cells with SC characteristics and have, with others, gone on to apply this methodology to normal human renal cell populations isolated from RCC patients [29,30]. We have also subsequently identified an SP profile in a bladder cell population [31]. An important feature of this work is not only the demonstration that the SP profile enriches significantly for cells with proliferative potential or that a large proportion of these cells are in the $G_0$ phase, but also that this property is a conserved feature of all 3 of the most common genitourinary malignancies.

The Hoechst 33342 dye efflux technique is a useful assay in the isolation of putative SCs and acts to support the SC theory of cancer within such malignancies. One concern raised about the Hoechst 33342 dye efflux technique is that Hoechst 33342 is cytotoxic, so cells which are having high intracellular Hoechst 33342 concentrations may display poor performance in culture due to poor cellular viability due to the cytotoxic effects of the Hoechst 33342. We, and others using the Hoechst efflux technique [28], have found that cells treated with Hoechst 33342 have similar viability to untreated cells. We have demonstrated using primary human cell populations in vitro that the SP is enriched for cells with the characteristics of clonogenicity, enhanced proliferative capacity, and long-term self-renewal. We acknowledge that further work is required using in vivo systems to demonstrate asymmetric division and serial tumor repopulation studies in animals. However, ultimately the Hoechst 33342 dye efflux assay provides strong evidence that the SP process is a unified mechanism in normal GU tract tissue and their associated cancers, and as such may play an important role in the development of novel diagnostic, prognostic, and treatment strategies in these cancers.

## Conclusion

A distinct subpopulation enriched for SC characteristics has now been isolated from the 3 most common urological malignancies. In vitro, these cells have enhanced growth potential, have a high proportion of cells in $G_0$, and they are enriched for known stem markers. Although these findings require further validation in in vivo systems, they do provide further evidence that the CSC may reside within this population and that the multidrug resistance pathways, which underpin the Hoechst 33342 dye efflux technique,

potentially provide new therapeutic targets for the treatment of these important cancers.

## Acknowledgments

The authors would like to thank Dr. Morgan Blaylock, Jeff Barry, and Mike Hughes of the Flow Cytometry Facility at the Paterson Institute for Cancer Research for all their technical support and assistance. We would also to thank the Urological Surgeons of The Christie NHS Foundation Trust, Salford Royal Hospitals NHS Foundation Trust, and University Hospitals of South Manchester NHS Foundation Trust for patient recruitment and sample collection.

## Author Disclosure Statement

No competing financial interests exist.

## References

1. Till JE and EA McCulloch. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 14:213–222.
2. Becker A, EA McCulloch and J Till. (1963). Cytological demonstration of the clonal nature of spleen colonies from transplanted mouse cells. Nature 197:452.
3. Potten CS and LG Lajtha. (1982). Stem cells versus stem lines. Ann N Y Acad Sci 397:49–61.
4. Weissman IL. (2000). Stem cells: units of development, units of regeneration, and units in evolution. Cell 100(1):157–168.
5. Al-Hajj M and MF Clarke. (2004). Self-renewal and solid tumor stem cells. Oncogene 23(43):7274–7282.
6. Pardal R, MF Clarke and SJ Morrison. (2003). Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 3:895–902.
7. Reya T, SJ Morrison, MF Clarke and IL Weissman. (2001). Stem cells, cancer, and cancer stem cells. Nature 414(6859):105–111.
8. Jordan CT, ML Guzman and M Noble. (2006). Mechanisms of disease—Cancer stem cells. N Engl J Med 355(12):1253–1261.
9. Bunting KD. (2002). ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells 20(1):11–20.
10. Goodell MA, K Brose, G Paradis, AS Conner and RC Mulligan. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183(4):1797–1806.
11. Challen GA and MH Litle. (2006). A side order of stem cells: the SP phenotype. Stem Cells 24:3–12.
12. Bhatt RI, MD Brown, CA Hart, PE Gilmore, VA Ramani, NJ George and NW Clarke. (2003). Novel method for the isolation and characterisation of the putative prostatic stem cell. Cytometry Part A 54A(2):89–99.
13. Brown MD, PE Gilmore, CA Hart, JD Samuel, VA Ramani, NJ George and NW Clarke. (2007). Characterization of benign and malignant prostate epithelial Hoechst 33342 side populations. Prostate 67:1384–1396.
14. Welm B, F Behbod, MA Goodell and JD Rosen. (2003). Isolation and characterization of functional mammary gland stem cells. Cell Prolif 36:17–32.
15. Gussoni E, Y Soneoka, CD Strickland, EA Buzney, MK Khan, AF Flint, LM Kunkel and RC Mulligan. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 401:390–394.
16. Bachrach E, S Li, AL Perez, J Schienda, K Liadaki, J Volinski, A Flint, J Chamberlain and LM Kunkel. (2004). Systemic delivery of human micro-dystrophin to regenerating mouse dystrophic muscle by muscle progenitor cells. Proc Natl Acad Sci (USA) 101:3581–3586.
17. Haraguchi N, H Inoue, F Tanaka, K Mimori, T Utsunomiya, A Sasaki and M Mori. (2006). Cancer stem cells in human gastrointestinal cancers. Hum Cell 19:24–29.
18. Wulf GG, KL Luo, KA Jackson, MK Brenner and MA Goodell. (2003). Cells of the hepatic side population contribute to liver regeneration and can be replenished by bone marrow stem cells. Haematologica 88:368–378.
19. Kim M and CM Morshead. (2003). Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis. J Neurosci 23:10703–10709.
20. Collins AT, PA Berry, C Hyde, MJ Stower and NJ Maitland. (2005). Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 65(23):10946–10951.
21. Ward RJ and PB Dirks. (2007). Cancer stem cells: at the headwaters of tumor development. Annu Rev Pathol 2:175–189.
22. Morrison SJ and IL Weissman. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity 1:661–673.
23. Morrison SJ, AM Wandycz, HD Hemmati, DE Wright and IL Weissman. (1997). Identification of a lineage of multipotent hematopoietic progenitors. Development 124:1929–1939.
24. Jászai J, C Fargeas, M Haase, L Farkas, W Huttner and D Corbeil. (2008). Robust expression of Prominin-2 all along the adult male reproductive system and urinary bladder. Histochem Cell Biol 130(4):749–759.
25. Patrawala L, T Calhoun-Davis, R Schneider-Broussard and D Tang. (2007). Hierarchical organization of prostate cancer cells in xenograft tumors: the CD44+alpha2beta1+ cell population is enriched in tumor-initiating cells. Cancer Res 67(18):6796–6805.
26. Bussolati B, S Bruno, C Grange, S Buttiglieri, MC Deregibus, D Cantino and G Camussi. (2005). Isolation of renal progenitor cells from adult human kidney. Am J Pathol 166(2):545–555.
27. Oates JE, BR Grey, SK Addla, CA Hart, VA Ramani, MD Brown and NW Clarke. (2008). CD133 is a marker of early progenitor cells but not cancer stem cells in Renal Cell Carcinoma (abstract). BJU Int 101(s5):1–56.
28. Patrawala L, T Calhoun, R Schneider-Broussard, J Zhou, K Claypool and D Tang. (2005). Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. Cancer Res 65(14):6207–6219.
29. Inowa T, K Hishikawa, T Takeuchi, T Kitamura and T Fujita. (2008). Isolation and potential existence of side population cells in adult human kidney. Int J Urol 15:272–275.
30. Addla S, M Brown, C Hart, V Ramani and N Clarke. (2008). Characterization of the Hoechst 33342 side population from normal and malignant renal epithelial cells. Am J Physiol Renal Physiol 295:680–687.
31. Oates JE, BR Grey, JD Samuel, SK Addla, MD Brown and NW Clarke. (2007). Hoechst 33342 stem cell identification is a conserved and unified mechanism in urological cancers. BJUI 99(s4):2–15.

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Received for publication October 7, 2009
Accepted after revision March 4, 2009
Prepublished on Liebert Instant Online March 4, 2009