Novel Method for the Isolation and Characterisation of the Putative Prostatic Stem Cell

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Background: Prostate stem cells, responsible for the development, maturation, and function of the prostate, have been implicated in the aetiology of both benign prostate hyperplasia (BPH) and prostate cancer (CaP). However, research has been hampered by the lack of a definitive stem cell marker. We have adapted the protocol for differential Hoechst 33342 uptake by hemopoietic stem cells to enable isolation of putative stem cells from the prostate.

Methods: Prostate epithelial cells isolated from prostate tissue obtained from patients with BPH after transurethral resection of the prostate were stained with Hoechst 33342. The Hoechst 33342 Red/Blue flow cytometry profile was then determined. Hoechst 33342 and Pyronin Y staining was used to determine cell cycle status.

Results: A verapamil-sensitive side population (SP) can be isolated from primary prostate tissue accounting for 1.38% ± 0.07% of prostate epithelial cells. Cell cycle analysis of this SP population revealed that the majority of SP cells are in either G0 (12.38 ± 0.31%) or G1 (63.19 ± 2.13%)


Key terms: prostate epithelial cells; Hoechst; stem cells; integrin α2; prostate cancer

Stem cells are defined as clonogenic, pluripotent, self-renewing progenitor cells that can generate one or more specialised cell types (1–3). In common with many other tissues (4,5), the prostate is believed to harbour self-renewing or “stem” cells (6–9). They are responsible for the development, maturation, and function of the prostate gland. These epithelial stem cells have been implicated in the aetiology of prostate cancer (10) and benign prostatic hyperplasia (BPH) (11).

The proliferative compartment of normal prostate resides within the basal epithelial cell layer. Approximately 70% of proliferating cells are phenotypically basal; the remaining 30% lie within the luminal secretory epithelium (12,13). The ratio of basal cells-to-secretory luminal cells is between 1:3 and 1:6 in the human prostate (14).

Controversy exists as to the exact nature and location of the prostatic stem cell, but experimental evidence suggests that it lies within the basal layer (15). Castration of male rats leads to a rapid involution of the prostate gland with only the basal epithelial cells surviving. Upon androgen replacement, the gland is restored to its normal function (16). Only the differentiated luminal cells are affected by this androgen withdrawal, suggesting that regeneration of the gland must result from proliferation and differentiation of the surviving basal cells (10).

Basal epithelial cells are capable of differentiating down various pathways and can replenish all layers within a castrated rat prostate after androgen replacement (16). Indeed basal cells retain the ability to undergo metaplasia and even undergo squamous differentiation in prostatic infarction and myoepithelial differentiation in sclerosing adenosis (9). Thus, because prostate basal epithelial cells make up the bulk of the proliferating cells and appear capable of differentiating down multiple lines, the postulated prostatic stem cells are most likely to reside in the basal compartment.

Research continues to be hampered by the lack of a universally agreed prostatic stem cell marker. Many such markers have been proposed, including prostate stem cell...
antigen (PSCA) (17), prostate specific membrane antigen (PSMA) (18–20), and phosphoprotein p32 (21–23). However, none are entirely tissue or location specific (24,25).

Integrins have been used to identify stem cells in skin (26) and testis (27). Epidermal stem cells express greater levels of integrin $\alpha_2\beta_1$ than transit amplifying cells (TAP) (28). Epithelial cells within the human prostate express integrin $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$ (29–31). Integrin $\alpha_2\beta_1$, restricted to prostatic basal cells (31), mediates adhesion of prostate epithelial cells to types I and IV collagens and to laminin I on the basement membrane (32). Putative stem cells have been shown to express higher levels of the integrin $\alpha_2$ subunit than other cells in the basal layer, and these integrin "bright" cells constitute approximately 1% of basal cells (33). Interest has focused on patterns of primary cytokeratin expression as markers for differential epithelial phenotypes. Luminal cells express cytokeratins 8 and 18 (34–37). In contrast, basal cells express mainly large molecular weight cytokeratins, namely CK4-7, 10, 14, and 15 (38,39).

In addition to cell surface markers, cell cycle status may be useful in the identification of the stem cell. Stem cells are proliferatively quiescent, residing predominantly in the $G_0$ phase of the cell cycle (40,41). Therefore, cell cycle status may be critical in defining strategies for stem cell selection. It is now possible to distinguish and isolate viable cells in $G_0$ or $G_1$ phase using a combination of the DNA and RNA binding dyes Hoechst 33342 and Pyronin Y (42).

Haemopoietic stem cells (HSC) have been the subject of considerable interest for decades and research has continued into their characteristics. In 1961, Till and McCulloch demonstrated the existence of clonogenic bone marrow precursors that give rise to multilineage haematopoietic colonies in the spleen (1). Although this finding provided evidence for the existence of the HSC, their isolation remained a problem until the development of in vitro and in vivo quantiative assays for clonogenic human stem and progenitor haematopoietic cells (43). This allowed development of distinctive marker profiles for each progenitor using monoclonal antibodies (44).

HSC can be identified by differential uptake of the vital dye Hoechst 33342. This is a fluorescent dye that binds to DNA in live cells and, therefore, is an indicator of cell cycle as it relates to DNA content. It is actively taken up by all cells (excepting red blood cells) but is actively effluxed, by means of the multidrug resistance protein (MDR1) gene product (p-glycoprotein), by primitive or stem cells (45). When analysed using flow cytometry a small population of Hoechst "low" cells termed side population (SP) cells can be defined. The SP profile is lost upon the addition of the MDR1 transporter inhibitor, verapamil. In vivo, these cells have been shown to be enriched 1,000-fold for HSC activity, which can protect murine recipients from lethal irradiation at low cell doses and contribute to both myeloid and lymphoid lineages (45). The cells have been found in multiple species, including murine, rhesus monkey, swine, and human bone marrow.

Although other strategies for the isolation and enrichment of HSC exist (44,46–48), the Hoechst SP region allows a single dye or manipulation to define a population with HSC activity that is strikingly small, homogeneous, easily identifiable and highly reproducible (45). Furthermore, the Hoechst SP region allows independent verification of cell surface characteristics, cell cycle kinetics, and the ability to collect and process cells in long-term in vitro and in vivo culture.

The aim of our study was to evaluate the Hoechst dye efflux protocol for solid tissue using the prostate as an example. We have modified the existing haematological Hoechst protocol to isolate an SP population from human prostatic epithelial cells (PEC), and subsequently investigated isolated SP cells with existing markers of stem cell phenotype, namely basal cytokeratins and integrin $\alpha_2$.

**MATERIAL AND METHODS**

**Materials**

All tissue culture medium and reagents were purchased from Invitrogen (Paisley, UK), with the exception of prostatic epithelial cell basal medium (Pr-EBM), Clonetics (Bio-Whittaker, Berkshire, UK). Bovine serum albumin (BSA), streptavidin PE-Cy5, propidium iodide, pan cytokeratin $\alpha$ (clone C-11), cytokeratin 8, cytokeratin 14 (clone CKB1), (R+-) verapamil hydrochloride, and metrizamide was from Sigma-Aldridge (Poole, UK); foetal calf serum (FCS) was supplied by Sera Labs (Sussex, UK); and Worthington collagenase type 1 and trypsin were from Lorne Laboratories, Ltd. (Twyford, UK). Hoechst 33342 was acquired from Molecular Probes (Eugene, Oregon, USA), Ber-EP4 fluorescein isothiocyanate (FITC; epithelial antigen) and rabbit anti-mouse biotinylated (RAMBO) antibodies from DAKO, Ltd. (Cambridge, UK), and streptavidin PE-Cy7 conjugate was from Caltag Laboratories (Burlingame, CA, USA). Integrin $\alpha_2$ (CD49b) and CD45 FITC were supplied by BD Pharmingen (Heidelberg, Germany).

**Methods**

**Prostatic tissue collection and culture.** Informed consent was obtained before tissue collection. Men undergoing transurethral resection of the prostate for bladder outflow obstruction arising from benign disease were studied. Prostate chips were obtained under sterile conditions. Each individual prostate chip was bisected with half being sent for histological analysis for diagnostic evaluation and the remainder used for tissue culture. Prostate epithelial cell isolation and characterisation was carried out by the method previously described (49). Briefly, by using sterile forceps and scissors, the prostate chips were chopped and placed in collagenase type I at 200 U/ml in RPMI 1640 medium with 2% v/v FCS overnight on a shaking platform at 37°C. The digest was then broken down further by shaking in 0.1% trypsin in PBS with 1% BSA and 1 mM ethylenediaminetetraacetic acid (EDTA) for 15–20 min. The cell suspension was then washed three times in PBS with 1% BSA and 1 mM EDTA before resuspending in RPMI 10% v/v FCS. Prostate epithelial cells
were separated from fibroblasts by differential centrifugation (360 g, 1 min without braking). This process produced a supernatant enriched for fibroblasts and a pellet enriched for epithelium. The epithelial cell suspension was then spun on a metrizamide gradient (1.079 g/ml), and the cells were isolated from the interface.

Ber-EP4/\alpha_2/CD45 labelling of prostate cells. Isolated prostate epithelial cells were labelled at room temperature with either anti-human integrin \alpha_2 monoclonal antibody or Ber-EP4 antibody (8 \mu g/ml in 1% BSA/PBS) for 30 min before the addition of the secondary antibody, RAMBO (2.6 \mu g/ml in 1% BSA/PBS) for 30 min. After washing with PBS, the cells were incubated for 20 min in the dark with streptavidin PE-Cy7 (20 \mu g/ml). Samples were then dual labelled with CD45-FITC (1 \mu g/ml in 1% BSA/PBS) for 30 min.

Ber-EP4/\alpha_2 and Hoechst labelling for flow cytometry. Isolated prostate epithelial cells were labelled at room temperature with anti-human integrin \alpha_2 monoclonal antibody (8 \mu g/ml in 1% BSA/PBS) for 30 min before the addition of the secondary antibody, RAMBO (2.6 \mu g/ml in 1% BSA/PBS) for 30 min. After washing with PBS, the cells were incubated for 20 min in the dark with streptavidin PE-Cy7 (20 \mu g/ml).

Hoechst staining was performed by using a modification of the protocol for HSC as described by (45). Briefly, epithelial cells were resuspended in Hoechst buffer (Hanks’ balanced salts solution, 10% FCS, 1% D-glucose, and 20mM HEPES) and warmed to 37°C. Hoechst 33342 was then added to give a final concentration of 2 \mu M and the cells incubated at 37°C for 2 h. Fifteen minutes before the end of incubation, the cells were labelled with monoclonal anti-human Ber-EP4 directly conjugated to FITC (8 \mu g/ml). The cells were then washed in ice-cold Hoechst buffer containing propidium iodide (PI) at 20 ng/ml. Fluorescence activated cell sorting analysis was performed immediately thereafter. To confirm active efflux of Hoechst 33342 by means of the MDR1 transporter, verapamil hydrochloride was added to a final concentration of 50 \mu M to the control samples before the addition of Hoechst 33342.

Flow cytometry isolation of the SP fraction. Flow cytometry was carried out using a Becton Dickinson FACS Vantage SE flow cytometer. Hoechst 33342 was excited with an argon ion, ultraviolet-enhanced laser at 350 nm, and its fluorescence was measured with a 424/44 BP filter (Hoechst BLUE) and a 675DF20 BP optical filter (Hoechst RED; Omega Optical, Brattleboro VT). A 640 LP dichroic mirror was used to separate the emission wavelengths. PI fluorescence was also measured through the 675DF20 BP (having been excited at 350 nm). A second argon ion laser was used to excite the additional fluorochrome PE-Cy7 at 488nm. PE-Cy7 was measured using a 787RDF40 (Omega Optical) filter.

Prostate epithelial cells stained with the Hoechst dye were kept ice cold during analysis on the flow cytometer by the use of a chilling apparatus. Live gates were established on forward versus side scatter and PI parameters, all PI-positive and dead cells were excluded. Cells under study were sorted by positive labelling for Ber-EP4 FITC and/or integrin \alpha_2 streptavidin PE-Cy7 before being analysed for Hoechst staining. These cells were then analysed using the Hoechst BLUE versus RED profile, with BLUE on the vertical axis and RED on the horizontal axis on a linear scale. A third gate was placed to select for the Hoechst low SP tail.

Cell cycle characterisation of SP fraction. Samples were processed as in prostatic tissue collection: epithelial cells were isolated and live epithelial cells selected after

![Figure 1](https://example.com/figure1.jpg)
FIGURE 1.
processing through a Metrizamide gradient. All epithelial fractions were resuspended in Hoechst buffer and warmed to 37°C. Hoechst 33342 was then added to give a concentration of 2 μM and incubated at 37°C for 45 min. Pyronin Y (250 ng/μl) was added to each tube, and the samples were incubated for 45 min. Monoclonal anti-human Ber-EP4 FITC (8 μg/ml) was added as appropriate 15 min before the end. After this, ice-cold Hoechst buffer was added immediately and the samples were washed then resuspended in ice-cold Hoechst buffer. The samples were analysed immediately by flow cytometry.

Flow cytometry was performed using a modification of the method described above. Cells under study were selected by positive labelling for Ber-EP4 FITC before being analysed for Hoechst and Pyronin Y staining. These cells were then analysed by plotting the Hoechst profile on the x-axis and Pyronin Y along the y-axis in a linear scale.

**Cytokeratin phenotype studies.** Samples were processed as above, divided into two fractions, and labelled with either cyto keratin 8 or 14 indirectly conjugated to PE-Cy5. Samples were then dual labelled with Ber-EP4 FITC and integrin α2 PE-CY7. Flow cytometry was performed as described and analysed on forward (FSC) and side (SSC) scatter.

**Statistical Analysis**
Statistical analysis was performed with the chi squared test. Fisher’s exact test was used when numbers analysed were less than 5.

**RESULTS**

**Isolation and Characterisation of Prostate Epithelial Cells**

Cells isolated from prostate chips from men undergoing transurethral resection of the prostate for bladder outflow obstruction arising from BPH were analysed by flow cytometry analysis to assess their phenotype. Live cells were gated (R1) on propidium iodide staining (Fig. 1a). A density plot was then constructed with forward scatter plotted along the x-axis and side scatter along the y-axis. Density plots reveal three discrete populations defined as R2, R3, and R4 (Fig. 1b). Cells lying within region R2 were extremely small, whereas the cells in R3 and R4 were larger and more comparable with each other.

The forward and side scatter profile of the cells located within R2 has a similar profile to haemopoietic lymphocytes from bone marrow. To establish the phenotype of the cells within each region, the isolated prostatic cells were labelled with the lymphocyte marker anti-CD45 and the epithelial marker Ber-EP4. Recent work by Collins et al. demonstrated that putative prostatic stem cells express integrin α2 on their surface (33). Therefore, each region was stained with integrin α2 to localise the region containing the putative stem cells.

R2 contains a population of lymphocytes as determined by their low forward and side scatter profile, positive staining for the lymphocyte marker CD45 and negative staining for either the epithelial cell marker Ber-EP4 or integrin α2. Regions R3 and R4 contain Ber-EP4-positive epithelial populations. However, we were unable to isolate a distinct population containing stem cells based on integrin α2 labeling, as both R3 and R4 cell populations contained cells that were integrin α2 positive (Fig. 1b). Because Ber-EP4 is highly conserved on epithelial cells, Ber-EP4 was used to select epithelial cell populations for further analysis, hence eliminating the lymphocyte population.

Phenotypic analysis of Ber-EP4-positive epithelial cells within regions R3 and R4 (Fig. 2) using basal (CK14) and luminal (CK8) cytokeratins demonstrated that both regions contained basal and luminal cells, with R3 being slightly enriched for luminal cells (2:9 basal:luminal in R3 compared with 1:3 in R4).

**Isolation of Prostate Stem Cells Using Hoechst 33342**

The differential uptake of the vital dye Hoechst 33342 has been used to identify HSC (45). We sought to evaluate the Hoechst 33342 dye efflux protocol for the identification of stem cells from prostate tissue. The Hoechst profile was plotted in a similar way to that published previously for haemopoietic cells (45) with Hoechst BLUE on the vertical axis and Hoechst RED on the horizontal. Figure 3a compares the predicted Hoechst 33342 profile with the profiles generated by HSC isolated from human bone marrow and Ber-EP4-positive prostatic epithelial cells derived from a BPH sample. Both bone marrow and prostate epithelia generate a Hoechst 33342 profile with a distinct side population (SP). The prostate epithelial cell profile has a greater concentration of cells within the SP population compared with the bone marrow SP population. This increased number of cells within the side population was observed in all 19 prostate samples studied. Table 1 details the percentage of epithelial cells lying within the Hoechst low SP tail for each prostate sample. On average, the prostate SP accounts for 1.38 ± 0.07% of epithelial cells (n = 19) compared with 0.14 ± 0.09% (n = 4) of cells isolated from bone marrow SP. Blocking the active Hoechst 33342 dye efflux pathway using verapamil hydrochloride, a potent inhibitor of MDR1, significantly reduces the SP population (Fig. 3b).

Enrichment for the basal integrin α2 was studied by differential staining of the tail and body of the Hoechst profile with Ber-EP4 and integrin α2. The ratio of epithelial cells (Ber-EP4 alone) to basal cells (Ber-EP4 and integrin α2) was then calculated. Table 2 details 12 samples and documents this enrichment of basal cells within the tail (mean enrichment = 218%, P < 0.005).

Hoechst 33342 profiles for regions R3 and R4 defined by FSC and SSC density plots of prostate Ber-EP4-positive epithelial cells were generated and the SP tail populations compared (Fig. 4). R3 is enriched for SP cells containing 44.5% ± 4.4% SP cells compared with 29% ± 1.34% SP cells in R4 (n = 3).
Cell Cycle Analysis of Prostate SP Cells

Cell cycle characteristics of prostate SP tail cells were evaluated by plotting Hoechst 33342 (DNA content) along the x-axis and Pyronin Y (RNA content) along the y-axis (50). This method isolates cells in G0/G1 phase by Hoechst 33342 distribution and subfractionates by RNA content into G0 (RNA low) and G1 (RNA high) as determined by Pyronin Y staining. Figure 5a shows a typical G0/G1 profile for SP cells isolated from prostate tissue, which is comparable to the G0/G1 profiles for CD34+/H11001 HSC previously generated (51). The prostate SP tail population contains both quiescent G0 cells (12.38 ± 0.31%) and cells accumulating RNA as they move into and through G1 (63.19 ± 2.12%). Plotting the G0 and G1 populations as an FSC and SSC density plot of Ber-EP4–selected prostate epithelial cells demonstrates that the G0 cells form a distinct population located within region R3 (Fig. 5b). Cells entering and progressing through G1, as determined by their increase in RNA content, formed a more dispersed population spread across R3 and R4 on the density plots.

DISCUSSION

For many years, work on the isolation of stem cells from solid tissues has lagged behind that of the haemopoietic system. This study adapts a novel technique that has been used successfully in the haemopoietic system to isolate the haematological stem cell (45). As stem cells of different tissues show certain similarities in biological behaviour, we hypothesised that they might also share certain molecular transport properties and cell kinetic characteristics. We have used the prostate as a solid tissue example to determine whether principles proven to be of value in HSC detection can be applied to other tissues.

Although numerous markers have been postulated to characterise stem cells, none are entirely tissue or location specific. The method described involves a novel use of the vital dye Hoechst 33342 to isolate both quiescent and replicating populations of stem cells. Furthermore, the isolated SP fraction is reproducible and easily identifiable. Plotting all viable cells on forward and side scatter revealed three distinct high density cell populations annotated as regions R2, R3, and R4. R2 was postulated to represent lymphocytes, and this possibility was confirmed by labelling with the lymphocyte marker CD45. Regions R3 and R4 were negative for CD45, and individual cell morphology was consistent with an epithelial origin. This finding was confirmed by their positive staining for the antibody Ber-EP4, a monoclonal antibody that reacts with a cell surface glycoprotein epitope present on human epithelial cells (52,53).

Collins et al. has demonstrated that putative prostate stem cells express integrin α2 on their surface (33). Therefore, each region was also stained with integrin α2 to localise the region containing the putative stem cells. However, we were unable to isolate a distinct population containing stem cells based on integrin α2 staining, as

![Cell Cycle Analysis of Prostate SP Cells](https://via.placeholder.com/150)
both R3 and R4 populations contained cells that were positive for integrin $\alpha_2$ (Fig. 1b). Furthermore, phenotypic analysis of Ber-EP4–positive epithelial cells within regions R3 and R4 using basal (CK14) and luminal (CK8) cytokeratins also failed to distinguish this population as both regions contained basal and luminal cells. Therefore, we went on to evaluate the Hoechst protocol for the isolation of stem cells from prostate tissue.

Results from Figure 3 showed that the prostate harbour a discrete subpopulation of cells (SP) similar to that found in human bone marrow, accounting for approximately 1% of the population. Studies on gastrointestinal and epidermal tissue reveal that the stem cell population accounts for approximately 1% of the solid tissue cell population (54,55). This figure closely matches the percentage of cells we have shown to be present in the SP fraction for PEC (Table 1). As in the haematological system (56), the Hoechst SP profile for PEC is significantly reduced when staining is performed in the presence of verapamil hydrochloride, indicating that the distinctly low

![Fig. 3. Hoechst 33342 dye efflux fluorescence activated cell sorting (FACS) profile defining the side population (SP) for bone marrow and prostate epithelia. a: Illustration of a Hoechst 33342 red/blue dye profile derived by FACS. Typical Hoechst 33342 dye profiles for bone marrow and prostatic epithelial cells are shown with the SP population. b: Hoechst 33342 dye efflux pathway blocked by the multidrug resistance protein (MDR1) inhibitor verapamil hydrochloride. Prostate epithelial cells were stained with Hoechst 33342 either in the presence or absence of 50$\mu$M verapamil hydrochloride before FACS analysis.](image)

Table 1

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<tr>
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Table 2

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*All samples were taken by transurethral resection of the prostate from patients diagnosed with benign prostate hyperplasia (BPH). The percentage of epithelial cells lying within the Hoechst 33342 red/blue low side population (SP) is shown for each patient. On average, the SP population accounts for 1.38% of epithelial cells (SEM = 0.07).

Prostate epithelial cells isolated from 12 benign prostate hyperplasia patients by transurethral resection of the prostate were labelled with anti–Ber-EP4 and anti-$\alpha_2$ integrin prior to Hoechst 33342 staining. Combination of Hoechst 33342 staining with specific epithelial (Ber-EP4) or epithelial and basal markers (Ber-EP4 and $\alpha_2$ integrin) results in enrichment of the SP population.
staining pattern of the SP cells is due to the MDR1 or MDR-like mediated efflux of the dye. Of interest, although the SP profile for PEC mirrors that found in bone marrow, the proportion of cells represented within the fraction is considerably higher than that found in the haematological system (45). Dual staining reveals that this SP fraction is highly enriched for basal integrin \( \alpha_2 \)-positive cells (Table 2).

![Fig. 4. Comparison of side population (SP) populations in regions R3 and R4. Ber-EP4-positive prostate epithelial cells were stained with Hoechst 33342. The Hoechst 33342 red/blue profiles for the forward scatter and side scatter-defined regions R3 and R4 were generated and the SP populations defined by verapamil hydrochloride sensitivity.](image)

![Fig. 5. Cell cycle characteristics for isolated prostate side population (SP) tail cells. Ber-EP4-positive prostate epithelial cells underwent simultaneous staining of DNA and RNA by Hoechst 33342 and Pyronin Y, respectively. The SP population was defined from the Hoechst 33342 red/blue profile, and their stage in cell cycle was assessed. Cells in \( G_0 \) are defined by having no more than 2n DNA and low RNA content (Pyronin Y fluorescence less than 150). Cells in \( G_1 \) have no more than 2n DNA and increasing levels of RNA (Pyronin Y fluorescence above 250). a: Prostate epithelial cell Hoechst 33342 red/blue profile displaying SP location (left panel) and the \( G_0/G_1 \) profile for the SP cells (right panel). b: Forward scatter (FSC) and side scatter (SSC) characteristics of SP cells in \( G_0 \) and \( G_1 \) compared with a FSC and SSC density plot of Ber-EP4-selected prostate epithelial cells demonstrates that the \( G_0 \) cells form a distinct population located within region R3.](image)
As with the HSC (57), cell cycle characterisation of prostate SP tail cells reveals that the SP tail population contains both quiescent G0 cells and cells accumulating RNA as they move into and through G1. G0 cells form a discrete population located within region R3, whereas those entering and progressing through G1 formed a more dispersed population spread across R3 and R4 on the density plots.

Cellular populations are highly ordered within epithelial tissues. They contain a small stem cell population that harbours the capacity for extended or prolonged growth, giving rise to progeny with more limited proliferative capacity and greater differentiation (58–60). There exists a discrete population of cells derived directly from the stem cell that are either second-order stem cells or cells with limited proliferative capacity, often termed TAP. These daughter cells are responsible for maintaining tissue balance (58,61). In the gastrointestinal system, cell types are organised whereby stem cells, TAP, and mature terminally differentiated cells occupy discrete locations within the tissue, often forming stratified layers (59). A similar hierarchical arrangement has been postulated for the prostate (10,11), but controversy remains regarding the exact nature and location of these TAP.

Foster and Ke regard the pluripotent stem cell as a basal cell that can differentiate further into a basal epithelial cell (and then into a luminal cell) or can itself differentiate down the neuroendocrine lineage and form a neuroendocrine cell (9). These basal epithelial cells, therefore, would represent a transit amplifying cell population that still resides within the basal layer. Xue et al. detail a stem cell, lying within the basal layer, that differentiates into a basal epithelial cell (62). Differentiation from this cell leads to an intermediate cell (TAP) and then after to both luminal epithelial and neuroendocrine cells. Xue et al. postulate that BPH and CaP derive secondary to an epigenetic change within the intermediate cell (62). Evidence is accumulating for the presence of such an intermediate or transit amplifying cell within the prostate with the identification of cells that coexpress both basal and luminal cytokeratins (36,63). Our results suggest the presence of a transit amplifying cell, with SP tail cells in regions R3 and R4 representing prime candidates with a part basal phenotype and a high proportion of cells in G0. These cells may be identified by the expression of integrin α2 in the basal layer, and they retain properties enabling them to efflux Hoechst 33342.

Phenotype studies were performed by cytokeratin staining of isolated epithelial cells. Dual labelling with basal and luminal cytokeratins and the basal integrin α2 place the SP cell within the basal layer, but there are at least two types of Hoechst low cells as defined by their cellular morphology and size (regions R3 and R4). We postulate that cells in R3, which are small, express basal cytokeratins, are in the quiescent cell cycle phase (G0), and fall to the extreme left of the Hoechst profile (very low), represent the putative prostatic stem cells. As mentioned above, we also postulate that SP tail cells in R3 and R4 represent an intermediate or transit amplifying cell population; the majority of these cells are in the G1 phase of the cell cycle and dual labelling studies reveal that they express an intermediate cytokeratin characteristic and also show enrichment for integrin α1. Integrin α2, therefore, is present on the putative stem cell and on the transit amplifying cell.

Future work will be required to characterise the SP cell further with respect to long-term cell culture. In vivo studies may be beneficial in this regard, comparing stem cell markers and using the Hoechst technique to confirm or reject individual marker expression for the stem cell.

In conclusion, we have shown that it is possible to isolate SP cells from primary human prostatic tissue in a way that is similar to that previously identified from haemopoietic cells, liver, lung, muscle, and brain tissue. These SP cells account for approximately 1% of the whole cell population and demonstrate enrichment for the basal cell marker integrin α2. The SP cells reveal a preponderance of quiescent G0 cells within the small epithelial cell population and can be located to a region within the integrin α2-positive basal cell layer. The above properties are consistent with a stem cell phenotype.

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LITERATURE CITED

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33. Summers JY, Heyworth CM, de Wynter EA, Chang J, Testa NG. Cord blood G(0) CD34+ cells have a thousand-fold higher capacity for generating progenitors in vitro than G(1) CD45+ cells. Stem Cells 2001;19:1005–1011.


