

Prostate Stem Cell Antigen Is a Marker of Late Intermediate Prostate Epithelial Cells

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Abstract

Prostate stem cell antigen (PSCA, named for its strong sequence homology to the thymocyte marker stem cell antigen 2) is a cell surface antigen expressed in normal prostate and associated with human and murine prostate cancer. To begin to investigate a possible link between PSCA expression in normal prostate and prostate carcinogenesis, we characterized the phenotype and proliferative behavior of normal PSCA-expressing prostate epithelial cells (PrEC) in tissue culture. PSCA was expressed in a subset of prostate epithelial cells that coexpress basal and secretory cytokeratins. PSCA-positive cells were the direct progeny of PSCA-negative cells and were characterized by a more differentiated morphology and a slower proliferative rate than PSCA-negative cells. Although PSCA-positive cells continued to express basal cell markers such as CD44, they lost expression of the basal cell marker p63. In contrast, expression of prostate specific antigen and androgen receptor transcripts was detectable in PSCA-positive PrEC. These findings suggest that PSCA is a unique marker of an intermediate subpopulation of PrEC in transition from a basal to a terminally differentiated secretory phenotype and may be a useful marker for the study of normal and malignant prostate development.

Introduction

Normal human prostate epithelium consists of two cellular compartments containing at least three phenotypically distinct cell populations. The luminal layer contains terminally differentiated secretory cells, characterized by the expression of cytokeratins (K)8/18, androgen receptor (AR), and prostate specific antigen (PSA) (1). Secretory cells are androgen dependent and require androgen for their survival (2). The basal cell layer contains basal and neuroendocrine cells. Basal cells express high molecular weight cytokeratins (K)5/14, the putative stem cell gene *p63*, and the antiapoptotic gene *bcl-2*,

but rarely express detectable AR or PSA protein (3, 4). They are androgen independent and can survive androgen ablation (5, 6). Neuroendocrine cells are dispersed within the basal compartment and specialize in the production of chromogranin and other neuropeptides.

The relationship between the three prostate epithelial cell types is poorly understood. One hypothesis proposes that basal cells are the precursors of secretory and neuroendocrine cells (7, 8). This hypothesis is based on reports of the differential loss of secretory cells following castration and the ability of basal cells to give rise to secretory cells *in vivo* and *in vitro* on administration of androgen (9, 10). Further evidence for a precursor-progeny relationship between basal and secretory cells comes from *in vitro* and *in vivo* identification of cells in the basal and luminal compartments that are immunophenotypically intermediate (*i.e.*, transit-amplifying cells) between basal and secretory cells (11–13). Cells with mixed basal and neuroendocrine expression patterns have also been reported, implying that one phenotype might be derived from the other (7). Conversely, other investigators have suggested that basal and secretory cells may represent separate lineages, based on animal data demonstrating the persistence of both basal and secretory cells following castration and the ability of both pools of cells to proliferate in response to androgen replacement (14, 15).

The involvement of the different prostate cell types in prostate carcinogenesis remains unclear. Although prostate cancers are phenotypically and behaviorally similar in many respects to secretory cells (*e.g.*, they express PSA, AR, and are androgen dependent), recent studies suggest that prostate cancer may arise from a more immature cell type located within the basal or luminal cell layer (13, 16–18). Evidence suggestive of this hypothesis comes from the identification of basal cell-associated cytokeratins and genes in prostate cancer, particularly androgen-independent tumors (18–22). In addition, it is hypothesized that prostate cancers, like other epithelial and nonepithelial cancers, must arise from stem or progenitor cells, rather than from a terminally differentiated cell type (17). However, the identity and localization of such cell types within the prostate is not known.

The study of normal and neoplastic prostate development has been hindered by a lack of cell surface markers analogous to those used to characterize hematopoiesis. Recently, prostate stem cell antigen (PSCA) has been identified in the prostate epithelium as a GPI-anchored cell surface antigen, related to the Ly-6/Th-1 superfamily (23). Its closest homologue, stem cell antigen 2 (SCA-2), is a marker of immature thymic lymphocytes. In humans, PSCA expression is largely restricted to the prostate, bladder, and stomach. In the prostate, PSCA mRNA expression is heterogeneous and restricted predominantly to a

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subset of basal cells, although secretory staining has also been reported (23, 24). PSCA is expressed in almost all cases of high-grade prostate intraepithelial neoplasia and is overexpressed in ~40% of local and as many as 100% of bone metastatic prostate cancers (25). A murine homologue of PSCA shows a similar pattern of expression (26). In normal murine prostate, mPSCA expression is restricted to a subset of epithelial cells (~20%) and its expression diminishes with age. The percentage and intensity of mPSCA-positive cells increases dramatically in murine models of prostate cancer [e.g., transgenic adeno of the mouse prostate (TRAMP) and PTEN heterozygotes] (26–28). As in the human disease, mPSCA is widely expressed in mouse high-grade prostate intraepithelial neoplasia, the putative precursor of invasive disease, suggesting that up-regulation of PSCA is an early event in carcinogenesis. These results suggest that PSCA may mark a unique subpopulation of prostate epithelial cells associated with transformation.

The present study was undertaken to define the phenotype and growth characteristics of PSCA-positive human prostate epithelial cells, our hypothesis being that delineation of this cell type might provide new insights into normal and neoplastic prostate growth. Primary prostate epithelial cells (PrEC) were derived and passaged from cultured prostate tissue explant, and assayed for PSCA expression as well as other known prostate epithelial markers. Consistent with previous reports of primary PrEC, virtually all cells coexpress basal and secretory cell cytokeratins, suggesting that they may represent an intermediate, transit-amplifying population. A subset of these cells expresses PSCA and the percentage of PSCA-positive cells increases with the number of passages in culture. We show that PSCA-negative cells give rise to PSCA-positive ones, indicative of a precursor-progeny relationship, and that the latter have a longer doubling time than PSCA-negative cells. PSCA-positive cells have a more differentiated morphology than negative ones and tend to grow above the monolayer. Immunofluorescence and RNA expression analyses of PSCA-positive *versus* -negative cells demonstrated that PSCA-positive cells retain expression of basal cell markers such as cytokeratin (K)5/14 and CD44, but lose expression of the basal and putative stem cell marker p63. In contrast to PSCA-negative PrEC, PSCA-positive cells also had detectable levels of PSA and AR mRNA. Together, these results suggest that PSCA may mark a population of late intermediate (transit-amplifying) cells in transition to a more differentiated phenotype.

Results

PSCA Is Expressed by Subpopulation of Prostate Epithelial Cells in Culture

Previous studies in our laboratory identified PSCA mRNA expression in a subset of basal epithelial cells in human prostate. To determine if PSCA expression is retained in prostate epithelial cells *in vitro*, cultured PrEC were labeled with PSCA antibody and analyzed by flow cytometry [fluorescence-activated cell sorting (FACS)]. Typically, 5–40% of cultured prostate epithelial cells were positive for PSCA cell surface expression, suggesting that these cells represent a heterogeneous population, within which a specific subset may be identified by PSCA.

Because the percentage of PSCA-positive cells varied between different lines of commercially or surgically derived PrEC, we asked whether this subpopulation changed over time in culture. PrEC were maintained in culture, and samples from successive passages were analyzed by FACS for PSCA expression. As shown in Fig. 1A, the percentage of PSCA-positive cells increased with passage numbers (from 17% in passage 2 to 46% in passage 5). Analysis of PSCA mRNA expression by semiquantitative reverse transcription (RT)-PCR (Fig. 1B) confirmed this observation. This increasing trend was consistently observed in several different batches of PrEC, including commercially obtained cells. These results demonstrate that cultured PrEC are phenotypically heterogeneous and suggest that PSCA marks a subpopulation that changes over time.

PSCA-Positive Cells Are Derived From PSCA-Negative Ones and Have a Slow Doubling Time

The observation that PSCA positivity consistently increased over time in culture raised two possibilities. First, it is possible that PSCA-positive cells are proliferating rapidly and taking over the culture over time. Second, it is possible that PSCA-negative cells become PSCA positive as PrEC are passaged. To address this question, PrEC were sorted by flow cytometry into PSCA-positive and -negative populations (which was confirmed by repeat FACS of the sorted cells), then recultured separately. One week after reculture (postsorted passage 1), cells were harvested and analyzed for PSCA expression. As demonstrated in Fig. 1C, a percentage of PSCA-negative cells became positive and this percentage increased during further passaging (Fig. 1C). To rule out the possibility that a small population of rapidly proliferating PSCA-positive cells was contaminating the negatively sorted culture, doubling times for the two populations were calculated by cell counting. PSCA-negative cells doubled every 26–30 h, whereas PSCA-positive cells doubled every 50–60 h. To confirm this difference in proliferation, unsorted PrEC were labeled with the cell tracer dye CFSE. Samples were collected at different time points and analyzed by FACS for CFSE fluorescence and PSCA (Fig. 2A). At 24 h (Fig. 2B), there was no clear difference in the CFSE profile of the two populations. At 72 h, all the negative cells had divided as indicated by the homogeneous decrease in FL1-H fluorescence. In contrast, a significant proportion of positive cells still retained dye, indicating that a percentage of PSCA-positive cells had not divided. Dual immunofluorescent (IF) staining of PrEC with the proliferation marker Ki67 was also performed and demonstrates that cells expressing high levels of PSCA are negative or weakly positive for Ki67, whereas PSCA-negative cells are strongly Ki67 positive (Fig. 2C). These results show that PSCA-positive cells are the progeny of PSCA-negative cells. These results also demonstrate that cells strongly positive for PSCA proliferate at a slower rate than PSCA-negative cells.

PSCA-Positive PrEC Cells Have a Differentiated Morphology

The above results show that PSCA-positive cells arise from PSCA-negative ones and suggest that PSCA may mark a more

committed (*i.e.*, differentiated) lineage. Consistent with this possibility, sorted PSCA-positive cells are larger and contain more cytoplasmic vesicles than PSCA-negative cells (Fig. 3, B *versus* A). In addition, confocal microscopy demonstrated that PSCA-positive cells tended to grow above the monolayer (Fig. 4D). These results suggest that PSCA may be an early marker of prostate epithelial differentiation.

Immunofluorescent Analysis of PSCA-Positive and -Negative Cells

To characterize PSCA-positive and -negative PrEC cells, dual IF analyses were performed with previously established markers of basal and secretory cells. A majority of PrEC coexpressed both basal and secretory cytokeratins (K5/14 and K18) as previously described. By triple IF staining, PSCA was found to colocalize with a subset of K5/K14/K18-positive PrEC (Fig. 4, A and B), but was not found in rare cells that expressed K5/14 or K18 alone (Fig. 4C). As mentioned earlier, PSCA-positive cells were more common above the monolayer

(Fig. 4D) as were the rare K18 only-positive cells. These experiments identify four subpopulations of prostate epithelial cells: one that is positive only for the basal cell cytokeratins K5/14, one that coexpresses K5/14 and the luminal K18, one that coexpresses K5/14/18 and PSCA, and one that is positive only for K18.

Signoretti *et al.* (4) and Parsons *et al.* (29) have recently reported that p63 is a basal cell marker expressed by a majority of PrEC in tissue culture. They also showed that p63 is never expressed in prostate cancer. To test the hypothesis that PSCA positivity marks an early transition of PrEC from a basal to secretory cell phenotype, we stained PrEC for both p63 and PSCA. As shown in Fig. 4E, PSCA-positive cells were the only PrEC that did not express p63, suggesting that PSCA-positive cells are the earliest p63-negative cells in the prostatic epithelium. We also stained PrEC for CD44, another basal cell marker (Fig. 4F) (1). All PrEC expressed CD44, a subset of which was positive for PSCA. In contrast, the luminal secretory cell marker CD57 was not detected in PrEC (1). Collectively, these results suggest a transition in the cytokeratin and cell

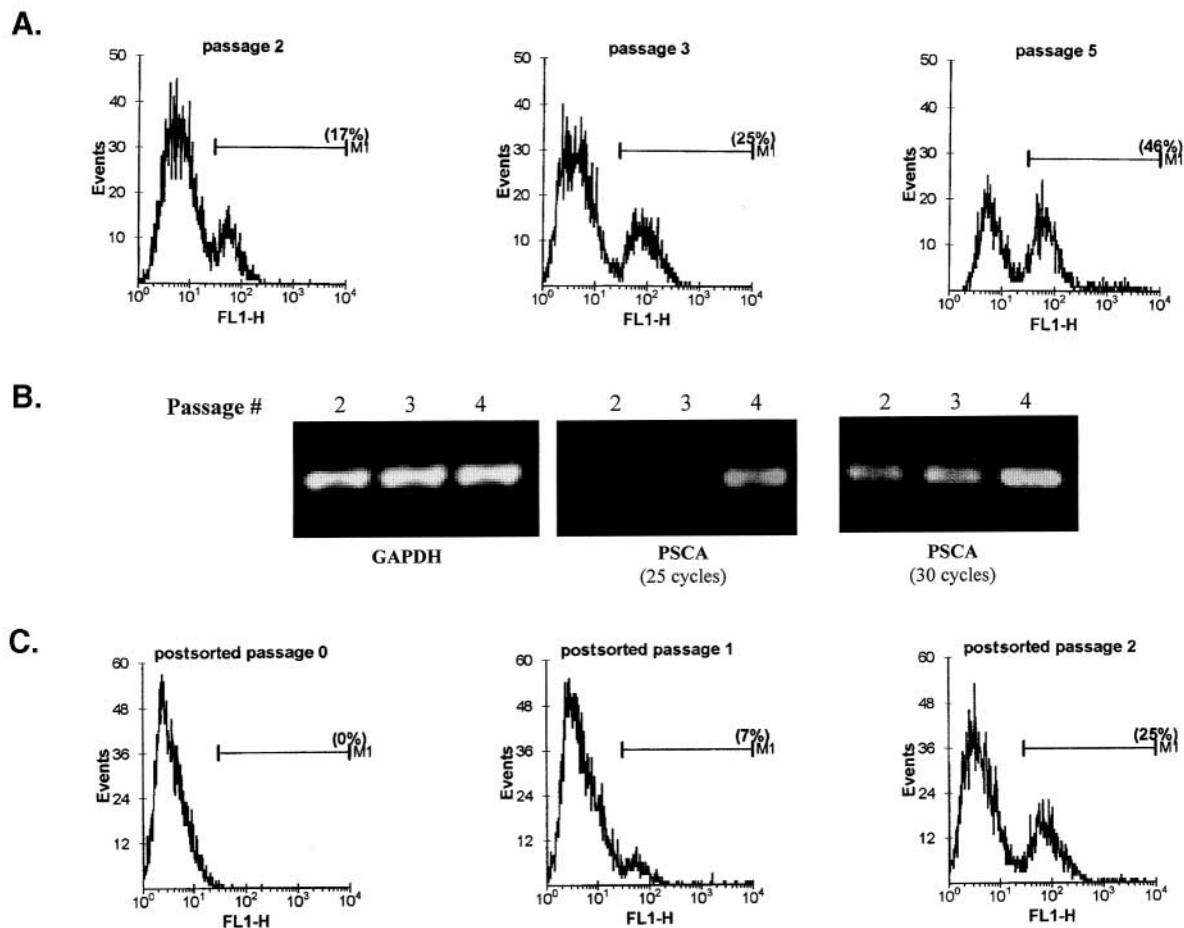


FIGURE 1. Detection of increased expression of PSCA in successive passages of PrEC in culture. **A.** FACS analysis of PrEC harvested at passage 2, 3, and 5, showing an increased percentage of PSCA-positive cells in later passages. **B.** Semiquantitative RT-PCR analysis of RNA of PrEC harvested at passage 2, 3, and 4, confirming increased PSCA expression with increased passage number. **C.** FACS analysis of postsorted PSCA-negative PrEC immediately after sorting (passage 0) and in subsequent passages, showing induction of PSCA expression over time. These experiments were each repeated in triplicate, with similar results.

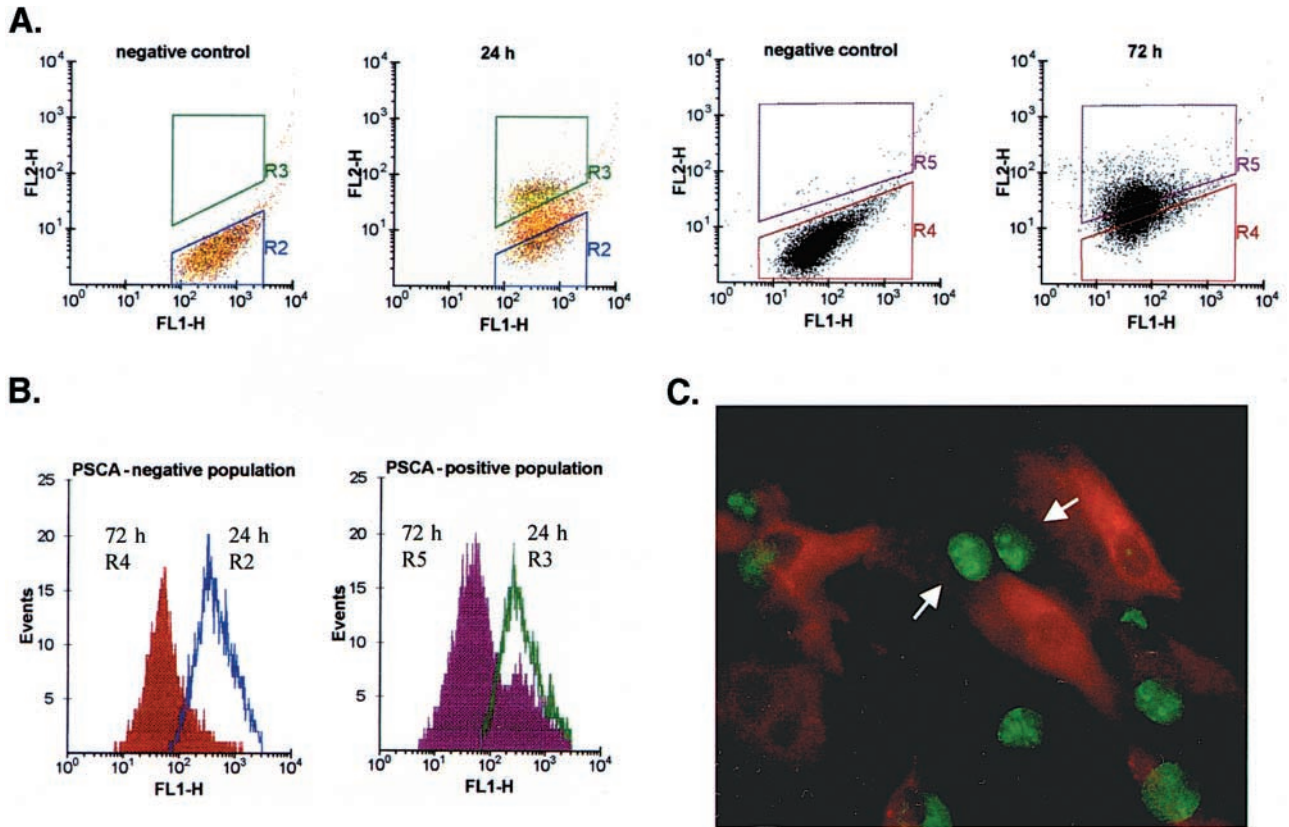


FIGURE 2. Cell division analysis of CFSE-stained PrEC over time. PrEC were stained with CFSE and cultured; at 24 and 72 h, cells were harvested and labeled with PSCA for FACS analysis. **A.** Gating of PSCA-negative and -positive populations along the FL2 channel. **B.** CFSE profile of each population over time, showing complete dilution of dye in PSCA-negative cells and retention of dye in a subset of PSCA-positive cells, indicative of slower rate of cell division. **C.** Immunofluorescent staining ($\times 400$) of PSCA (red) and Ki67 (green), showing that the two antigens do not colocalize.

surface expression pattern as PrEC progress from a basal to secretory cell phenotype. PSCA appears to mark an intermediate, transitional cell population in which pure basal cell markers such as p63 are down-regulated.

Gene Expression in PSCA-Positive Cells

To examine the phenotype of PSCA-positive and -negative cells further, the two populations were separated by flow cytometry (Fig. 5A), processed for RNA, and analyzed for selected gene expression by semiquantitative RT-PCR (Fig. 5B). GAPDH was used for normalization. As predicted, PSCA was only detected in the positively sorted population. The inverse relationship of p63 and PSCA noted above was confirmed. Similarly, CD44 was detected in both populations, although expression decreased somewhat in the positive population. This is consistent with the transition of PSCA-positive cells to a more differentiated state. Interestingly, AR and PSA mRNA were detectable at low levels in PSCA-positive cells (although AR and PSA protein were not detectable), indicating that secretory cell genes are starting to be expressed in PSCA-positive cells. We also assayed expression of *notch 1* and its ligand *jagged 1*, hypothesizing that these genes, which are associated with maintenance of a less differentiated state,

might be down-regulated as PrEC matured (30). Notch expression has also been localized to a small population of basal cells in mouse prostate (31). As seen in Fig. 5B, expression of both *notch 1* and *jagged 1* is absent in PSCA-positive cells. Together, these observations are consistent with a model in which PSCA expression is associated with a transition of PrEC to a differentiated phenotype.

Discussion

Based on cytokeratin staining, three populations of cells are identified in the present study—a small percentage that express either basal (K5/14) or secretory (K18) cell cytokeratins alone and a majority that coexpress both K5/14 and K18. Similar cytokeratin profiles of prostate epithelial cells have been reported by others both *in vivo* and *in vitro* (11, 12, 32–34). These results support a lineage relationship between basal and luminal cells. They also demonstrate that the majority of prostate epithelial cells in culture have a phenotype intermediate between “pure” basal and secretory cells (Fig. 6).

A major finding of this study is that PSCA marks a subset of “intermediate” cells expressing K5/14/18 (and the basal cell marker CD44), suggesting that intermediate cells are heterogeneous and can be stratified based on PSCA expression. PSCA-

positive intermediate cells differ morphologically, spatially, and phenotypically from PSCA-negative intermediate cells. They are larger, contain more vesicles, tend to localize above the monolayer, and express low levels of PSA and AR, all suggestive of a more differentiated stage of development than PSCA-negative intermediate cells. Similar morphologic differences among prostate epithelial cells in culture have been reported by others (32, 34). van Leenders *et al.* (34), for example, noted that cells expressing cytokeratins 5 and 18 or 18 alone emerged above the monolayer and had either an angular shape or several cytoplasmic projections, similar to the PSCA-positive and K18 only-positive cells seen in our study. These investigators proposed that basal cells lost expression of K14 and K5 sequentially as they differentiated. However, they noted that the “gradual” shifts in keratin expression in their study precluded them from discriminating between early and late

transit-amplifying cells. Our results suggest that PSCA can mark late transit-amplifying cells and will be a useful cell surface marker for isolating distinct epithelial subpopulations within the prostate.

PSCA-positive and -negative cells are distinguishable not only on morphologic and phenotypic grounds, but also on proliferative ones. These results are reminiscent of and complement studies performed by Hudson *et al.* (32), in which they performed clonogenic assays on epithelial cells obtained from prostate biopsies. These investigators identified three cell types, which could be distinguished by both phenotype and proliferative capacity. A K14-positive subpopulation formed large colonies (8–40,000 cells) in culture and doubled every 24 h. A CK8-positive subpopulation formed small colonies (~32 cells) and had virtually no proliferative capacity. A third subpopulation coexpressed K14 and K18 and had an intermediate proliferative capacity. This third population is characteristic of the majority of PrEC in our study. Our results suggest that PSCA expression can further subdivide these intermediate cells into ones characterized by higher and lower proliferative rates.

A third feature of PSCA-positive cells is that they arise from PSCA-negative cells, indicating a direct precursor-progeny relationship. Hudson *et al.* (32) showed that K14-positive cells could give rise to K18-positive cells. Similarly, van Leenders *et al.* (34, 35) and Hudson *et al.* (32) have shown that prostate epithelial cells coexpressing K5/14/18 could give rise to cells with predominant expression of K18. Taken together, these results support a stem cell model of prostate epithelial development in which luminal cells are the progeny of basal cells. In this model, a putative K5/14-positive stem cell gives rise sequentially to K5/14/18 and K5/18/PSCA⁺ transit-amplifying cells, which eventually differentiate into K18-positive luminal cells (Fig. 6).

A notable finding in this study is that the PSCA-positive population expressed PSA and AR transcripts, indicating early differentiation, but not PSA or AR protein. The lack of PSA and AR protein expression in tissue culture has been noted by other investigators and could be indicative of a failure to differentiate completely in culture. A number of investigators have speculated that the absence of PSA and AR protein is due to the absence of specific factors (*e.g.*, androgens, stroma, testosterone) in tissue culture systems (1, 36, 37). Indeed, when PrEC were propagated in matrigel, the cells formed into spheroids and AR was detectable in the lumen of the structure (32). Expression of AR protein in such three-dimensional system was also noted elsewhere (38), but only in the presence of stroma, serum, and testosterone. In this context, the PrEC cultured in this study does not represent the terminally differentiated prostate epithelium, which is marked by AR and PSA protein expression, but rather a precursor cell population capable of expressing PSCA independently of androgen and AR protein. The question of whether PrEC and PSCA expression are sensitive to androgen needs to be explored further in three-dimensional culture.

Expression of PSCA in the absence of AR protein suggests that PSCA expression is androgen independent and is consistent with studies of the PSCA promoter showing androgen-

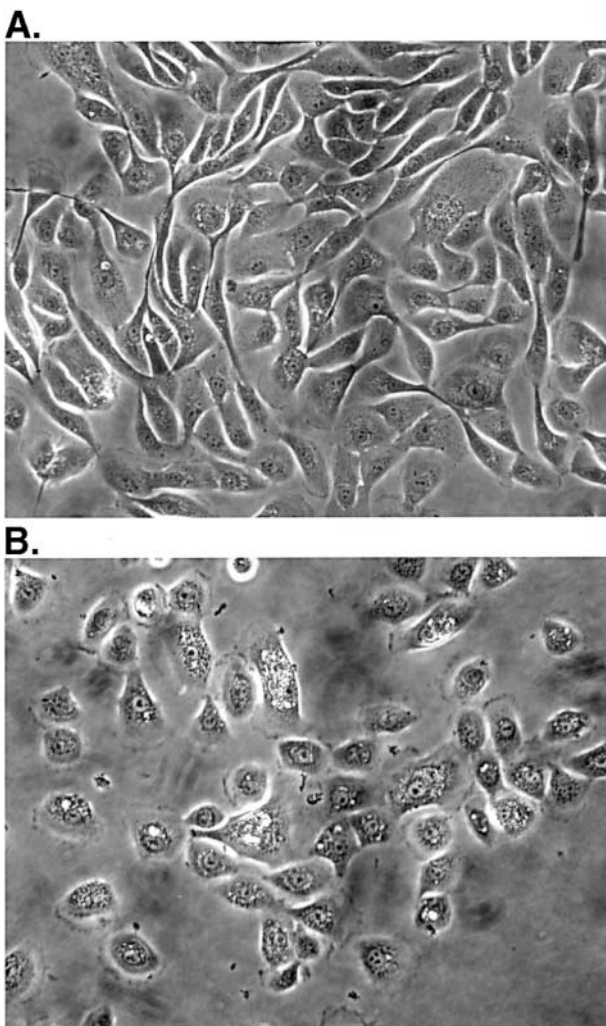


FIGURE 3. Different cell morphology of postsorted PSCA-negative (A) and -positive PrEC (B). Magnification, $\times 200$. PrEC were sorted into positive and negative populations and immediately plated. This figure shows the distinct morphology of PSCA-positive cells, with a more angulated appearance, larger size, and more abundant cytoplasmic vesicles.

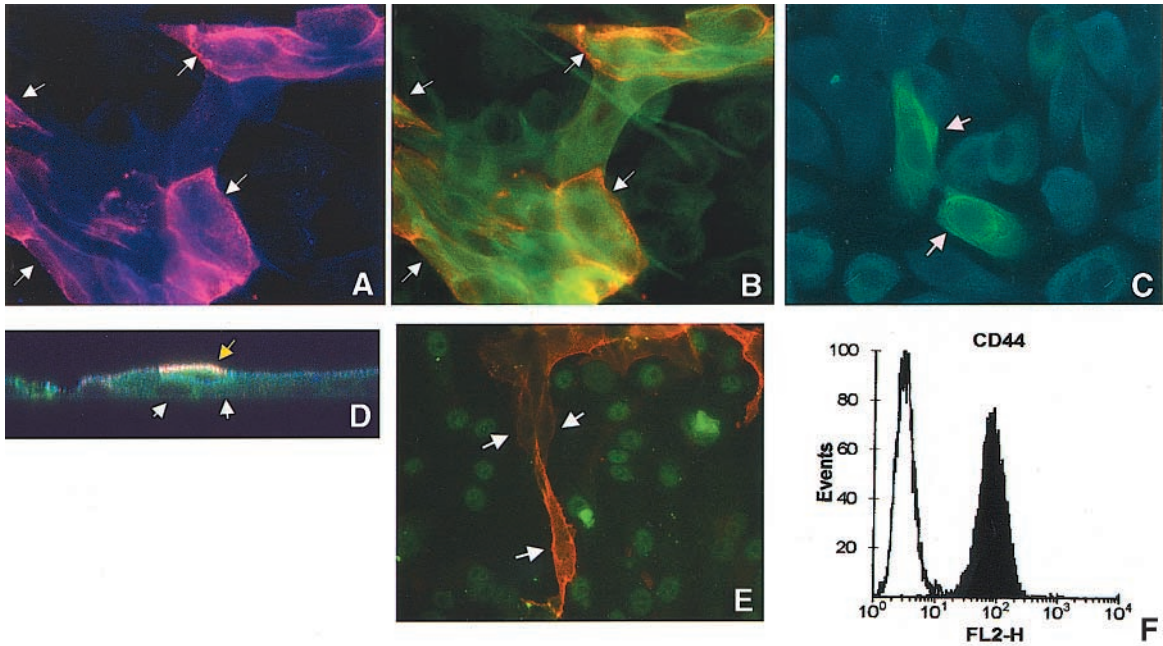


FIGURE 4. Immunofluorescent and flow cytometric analyses of PrEC. **A–D.** Triple color staining of PrEC (PSCA—red, K5/14—blue, K18—green), shown in double fields of either PSCA and K5/14 (**A**), PSCA and K18 (**B**), or K5/14 and K18 (**C**). **A** and **B.** Examples of PSCA colocalization with K5/14 (**A**) and K18 (**B**) (arrows). **C.** Examples of K5/14/18 cells that are negative for PSCA. Note the K18 only-positive cells (arrows). **D.** Confocal laser scanning of tricolor-stained PrEC on the vertical *x–z* plane (perpendicular to the plane of the cell monolayer), showing a PSCA-positive cell growing on top of the monolayer. This cell coexpresses K5/14 and K18. **E.** Dual staining of PSCA (red) and p63 (green), showing the absence of nuclear p63 staining in PSCA-positive cells. **F.** FACS analysis of PrEC, showing that the entire population is CD44 positive.

independent activity (39). At the same time, the PSCA promoter has been shown to be androgen responsive and we have recently found a functional androgen response element in the PSCA promoter (40). The ability to grow in the absence of, but respond to, androgen is a reputed characteristic of prostate transit-amplifying cells (5, 17). The androgen-independent expression of PSCA is also consistent with its expression in hormone refractory cancers (25).

Our finding that PSCA marks a late intermediate (transit-amplifying) population of prostate epithelial cells is consistent with previous studies by our group of PSCA promoter-driven green fluorescent protein (GFP) expression in transgenic mice (39). Prostatic GFP expression in these mice was associated with periods of rapid growth and differentiation. GFP expression diminished after castration, expanded following androgen replacement, and then became increasingly restricted to ductal

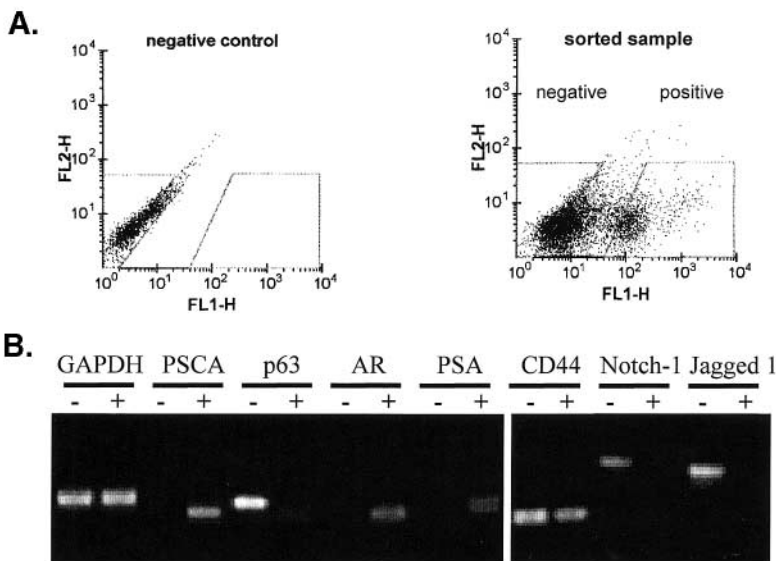


FIGURE 5. Gene expression profile of sorted PSCA-negative and -positive PrEC. **A.** PrEC were sorted by flow cytometry into positive and negative populations using the two well-separated gates. **B.** Semiquantitative RT-PCR analysis of RNA from each population, confirming adequate sorting of PSCA-positive and -negative populations and expression patterns of these two populations.

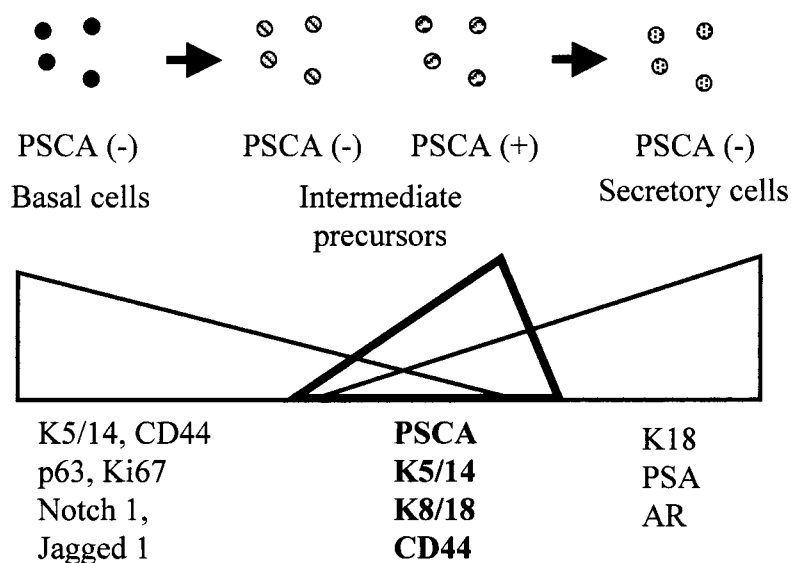


FIGURE 6. Schematic representation of PSCA expression in comparison with the expression profiles of various prostate epithelial markers.

tips once glandular growth ceased. These results suggested a model in which PSCA expression (manifested by GFP) marked a population of cells in transition between a stem cell (persisting after castration) and a terminally differentiated cell (lining the mature ducts). Similarly, GFP-positive cells in PSCA-GFP transgenic mice expressed a mixture of basal and secretory cell markers, such as CD44 and probasin, much as human PSCA-positive cells coexpress CD44 and PSA. These results complement each other and suggest that PSCA may be a useful marker for studies of prostate epithelial development in mice and men.

The relationship of PSCA-positive cells to carcinogenesis is not known. Human and murine PSCA are up-regulated in a significant percentage of prostate cancers (25, 26). Similarly, tumors resulting from the cross of PSCA-GFP and TRAMP transgenic mice express high levels of GFP compared to nontransgenic littermates (*i.e.*, those not carrying the T antigen of TRAMP mice) (39). One possibility is that PSCA expression is related to prostate tumorigenesis in general. Another possibility is that the increase in PSCA expression in cancer is related to the expansion of the PSCA-positive cell population identified in this study. In support of the latter hypothesis, PSCA-positive cells are the first identifiable population of PrEC that do not express p63. A number of groups have reported that p63 expression is lost in all prostate cancers and have proposed loss of p63 as a diagnostic marker for the disease (4, 29). Although it is possible that p63 is down-regulated on transformation, it is also possible that slowly proliferating PSCA-positive, p63-negative cells are the targets of transformation in human and murine prostate cancer. Notch expression has been reported to be absent in human prostate cancer cells and forced expression of activated *notch* inhibited the growth of various prostate cancer cell lines (31).

The hypothesis that a PSCA-positive cell population is the target of prostate transformation is consistent with reports demonstrating the presence of transit-amplifying cells (characterized by cytokeratin and c-MET expression) in prostate cancer

(13, 41). Likewise, De Marzo *et al.* (17) hypothesized that proliferating, p63-negative cells localized in the secretory cell layer or in inflammatory lesions may be the targets of transformation. Proof that PSCA is a marker of the cellular target of carcinogenesis in the prostate will require direct demonstration that these cells can be transformed. This is currently being explored in both tissue culture and transgenic model systems.

In summary, this study demonstrates that PSCA is a cell surface marker, which can distinguish among early and late transit-amplifying prostate epithelial cells in tissue culture. PSCA marks the transition of prostate epithelial cells from a highly proliferative to a more differentiated state and may identify a unique subpopulation of cells susceptible to transformation during prostate carcinogenesis.

Materials and Methods

Cell Culture

Normal prostate epithelial cells (PrEC) were obtained either commercially (Clonetics, Cambrex Corporation, Rutherford, NJ), or derived from surgical specimens. Fresh normal tissue obtained at the time of prostatectomy was finely chopped into 1 mm³ pieces in medium (RPMI supplemented with 1% FCS), washed once, then digested with collagenase type I (Life Technologies, Inc., Rockville, MD), at the ratio of 0.1 g tissue/250 unit of enzyme/ml of wash medium, for 4 h at 37°C. The cell suspension was washed twice, filtered through a 100- μ m nylon mesh, and the cells were cultured at 37°C/5% CO₂ in prostate epithelial growth medium (PrEGM, Clonetics), changing fresh medium every 3 days. This culture was designated PrEC passage 1 *in vitro*, which was maintained for 6 days before being split into the next passage. To calculate the cell doubling time (*T*) of PrEC by cell counting, the following formula was used:

$$T = 3.3(\log N - \log N_0)$$

Antibodies

Monoclonal mouse anti-human PSCA IgG1, clone 1G8, was raised in our laboratory. FITC-labeled mouse anti-human cytokeratin 18 (Cy-90), and quantum red-conjugated mouse anti-human CD57/HNK-1 (VC1.1) were obtained from Sigma (St. Louis, MO). PE-conjugated mouse anti-human CD44 and mouse anti-p63 were from PharMingen (BD Biosciences, San Diego, CA). Mouse anti-human cytokeratin 5/14 (high molecular weight, 34 β E12) was obtained from DAKO Corp., Carpinteria, CA, whereas rabbit polyclonal anti-Ki67 was from Novocastra Laboratories Ltd. (Newcastle, United Kingdom). Secondary-conjugated antibodies (goat anti-rabbit and goat anti-mouse Alexa 488, goat anti-mouse Alexa 594, and Alexa 350) were obtained from Molecular Probes (Eugene, OR).

Flow Cytometry

PrEC at 80% confluence were detached from tissue culture plates in prewarmed PBS containing 1% glucose/2 mM EDTA, washed once in PBS/1% FCS, and incubated with primary PSCA antibody 1G8 (1/100) in PBS/1% FCS for 1 h on ice. Cells were washed and incubated with secondary antibody Alexa 488 (green fluorescence) for an extra 30 min on ice. Subsequently, cells were washed and either fixed in 1% paraformaldehyde for analysis using a FACScan (Becton Dickinson, San Jose, CA), or immediately subjected to cytometric sorting into PSCA-positive and -negative populations using a FACVantage (Becton Dickinson). Cells treated with isotype-specific immunoglobulins served as controls. For direct labeling with a fluorescence-conjugated primary antibody, cells were not reincubated in secondary antibody, but fixed after washing and then subjected to FACS analysis.

For CFSE analysis, PrEC were harvested in trypsin, washed, and collected by centrifugation, then labeled with CFSE using the Vybrant CFDA SE Cell Tracer kit (Molecular Probes). The cells were incubated in 2 μ M CFSE for 15 min at 37°C, then repelleted by centrifugation, and seeded into tissue culture plates. Subsequently, at designated time points, cell samples were harvested in PBS/glucose/EDTA, labeled with 1G8, followed by PE-conjugated secondary antibody (Molecular Probes), and immediately subjected to FACS analysis.

Immunofluorescent Microscopy

Cells were seeded onto glass coverslips coated with poly-D-lysine (Becton Dickinson Labware, Bedford, MA) and grown for 2 days. For tricolor staining, cells were first fixed in 2% paraformaldehyde, stained with 1G8 for PSCA in PBS/0.2% BSA, followed by secondary antibody Alexa 594 (red). After extensive washing, cells were fixed for 5 min in 2% paraformaldehyde, then permeabilized in 0.2% Triton X-100, and stained with 34 β E12 for cytokeratin 5/14, followed by secondary antibody Alexa 350 (blue). After further washing, cells were refixed in 2% paraformaldehyde for 5 min and then stained with FITC-conjugated Cy-90 (green) for cytokeratin 18. Cells were mounted on glass slides with FluoroGuard (Bio-Rad Laboratories, Hercules, CA), and images were acquired using a fluorescent microscope Leica DM IRB/E (Meyer Instruments, Houston, TX) or a laser scanning confocal microscope LSM

310 (Carl Zeiss, Minneapolis, MN). For dual staining of 1G8 and a nuclear antigen, cells were fixed in cold methanol at -20°C for 10 min, air-dried, and incubated with 1G8, then Alexa 594 (red), followed by either Ki67 and then goat anti-rabbit Alexa 488 (green), or p63 and then goat anti-mouse Alexa 488 (green).

RT-PCR Analysis

RNA was isolated from cultured cells using the Ultraspec RNA isolation system (Biotech, Houston, TX). For RNA from unsorted PrEC, RT-PCR was performed using the GeneAmp system (Applied Biosystems, Foster City, CA). For postsorted PrEC, due to very low yield of RNA from each sorted cell population (PSCA positive and PSCA negative), each RNA sample was first amplified into double-stranded cDNA using the Smart PCR cDNA Synthesis Kit (Clontech Laboratories, Inc., Palo Alto, CA). Subsequently, PCR was performed on each cDNA pool for detection of expression of various genes. The PCR conditions are as follow: denature 95°C 40 s, cycles 95°C 40 s/60°C 40 s/72°C 40 s, end 72°C 3 min then 4°C. All primer sequences are listed below:

PSCA (25 cycles)	5' Primer 5'-TGACCATGAAGGCTGTGCTGCTT-3'
	3' Primer 5'-TCGGTGTACAGCACGTGATGTTCC-3'
GAPDH (20 cycles)	5' Primer 5'-ACCACAGTCCATGCCATCAC-3'
	3' Primer 5'-TCCACCACCCTGTGTGCTGA-3'
p63 (30 cycles)	5' Primer 5'-GTGAGCCACAGTACACGAACC-3'
	3' Primer 5'-GAGCATCGAAGGTGGAGCTGG-3'
AR (35 cycles)	5' Primer 5'-CCACAGGCTACCTGGTCTCG-3'
	3' Primer 5'-CAGCCTAGGCTCTCGCCTTC-3'
PSA (35 cycles)	5' Primer 5'-GGTGACCAAGTTCATGCTGTG-3'
	3' Primer 5'-GTGTCCTTGATCCACTTCCG-3'
CD44 (25 cycles)	5' Primer 5'-AACCTGCCGCTTTGCAAGGTGT-3'
	3' Primer 5'-TCCATCAAAGGCATTGGGAG-3'
Notch-1 (30 cycles)	5' Primer 5'-ATCGGGCACCTGAACGTGGCG-3'
	3' Primer 5'-CACGTCTGCCTGGCTCGGCTC-3'
Jagged 1 (30 cycles)	5' Primer 5'-TAGTCTGCTGCCAACCCCTGCC-3'
	3' Primer 5'-GGGGTGGACGAAGCACTGGTCGTCC-3'

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