Low-Calcium Serum-Free Defined Medium Selects for Growth of Normal Prostatic Epithelial Stem Cells

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Abstract

Stage-specific differentiation markers were used to evaluate the cellular composition and the origin of nonimmortalized (PrEC) and immortalized (PZ-HPV7, CA-HPV10, RWPE-1, and 957E/hTERT) human prostate cell lines. These studies documented that immortalized and nonimmortalized prostate epithelial cells established and maintained in low (i.e., <300 μmol/L) Ca2+ serum-free defined (SFD) medium were all derived from normal nonmalignant prostate tissues and contain CD133+/ABCG2+/α2β1+/p63+/PSCA+/AR+/PSA+ prostate stem cells. In these cultures, prostate stem cells are able to self-renew and generate two distinct cell lineages: the minor proliferatively quiescent neuroendocrine lineage and the major transit-amplifying cell lineage. Subsequently, CD133+/ABCG2+/α2β1+/p63+/PSCA+/AR+/PSA+ transit-amplifying cells proliferate frequently and eventually mature into proliferatively quiescent CD133+/ABCG2+/α2β1+/p63+/PSCA+/AR+/PSA+ intermediate cells. Such proliferatively quiescent intermediate cells, however, do not complete their full maturation into CD133+/ABCG2+/α2β1+/p63+/PSCA+/AR+/PSA+ luminal-secretory cells in low Ca2+ SFD medium. Addition of universal type I IFN and synthetic androgen (R1881) to culture medium resulted in up-regulation of a limited number of divisions (i.e., amplifications) before transiting a maturation process of terminal differentiation (refs. 1, 9; Fig. 1). These transit-amplifying cells do not express AR protein and are dependent for proliferation but not survival on AR signaling in the stroma (9, 15). In contrast to stem cells, transit-amplifying cells obligatorily express the basal epithelial marker p63 (16, 17) as well as other basal markers, such as cytokeratins 5 and 14 (3, 5, 16, 17), Jagged-1, and Notch-1 (17, 18). Transit-amplifying cells express very low levels of luminal cytokeratins 8 and 18 (3, 5, 6, 19) and stain negative for prostate luminal-secretory cell markers, such as NKX3.1, AR, prostate-specific antigen (PSA), or hK2 (15–19). Thus, prostate transit-amplifying cells too can be identified based on their unique molecular CD133+/ABCG2+/α2β1+/p63+/PSCA+/AR+/PSA+ expression profile (Fig. 1).

Although prostate stem cells possess high self-renewal capacity, they proliferate infrequently to renew themselves and simultaneously generate progeny for two distinct prostate epithelial cell lineages. The first and much less frequent lineage commitment is differentiation into proliferatively quiescent neuroendocrine cells (7, 13). The second and much more common lineage commitment involves differentiation into transit-amplifying cells that undergo a limited number of divisions (i.e., amplifications) before transiting a maturation process of terminal differentiation (refs. 1, 9; Fig. 1).

Introduction

The normal human prostate is composed of a stratified epithelium that is functionally organized in stem cell units (refs. 1–11; Fig. 1). Recently, the nature of prostate stem cells has become a subject of intense investigation. In particular, Hudson et al. showed that ~0.5% of epithelial cells isolated from normal human prostate tissue attach rapidly to type I collagen-coated dishes and possess the highest clonogenic ability of any prostate cell subtype when grown in low (i.e., <300 μmol/L) Ca2+ serum-free defined (SFD) medium (4). In an effort to further characterize these putative stem cells, the authors documented that they are derived from the basal layer of prostatic epithelium (4). Collins et al. showed that such rapid attachment is due to high expression of α2β1 integrins by these putative stem cells (6). Richardson et al. further showed that when cells are directly isolated from prostate tissue and passaged in low (i.e., <100 μmol/L) Ca2+ SFD medium, CD133+/α2β1+ coexpressing putative stem cells in these cultures have the highest clonogenic ability and the longest proliferative life span in comparison with CD133+ cells (10). Huss et al. reported that prostate stem cells also express the ABC transporter G2 isotype (i.e., ABCG2) protein but not androgen receptor (AR) or p63 proteins (11). The p63+/AR− status of prostate stem cells is consistent with the observations that embryonic epithelial cells from the urogenital sinus of either AR or p63 knockout mice still undergo prostatic organogenesis and glandular renewal when transplanted in combination with wild-type urogenital sinus mesenchyme into the kidney of nude mice (12–14). In summary, the prostate stem cells can be identified based on their unique molecular CD133+/ABCG2+/α2β1+/p63+/PSCA+/AR+/PSA+ expression profile (Fig. 1).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).
express AR mRNA but not AR protein (16, 17). These CD133<sup>-</sup>/ABCG2<sup>-</sup>/α<sub>4</sub>β<sub>1</sub>/p63<sup>+</sup>/PSCA<sup>-</sup>/PSA<sup>-</sup> intermediate cells then migrate upward to form the luminal-secretory layer, where they begin to express AR protein at high levels (20, 21). Ligand occupancy of this AR induces proliferative quiescence and completion of differentiation into PSA<sup>+</sup> secretory cells (ref. 9; Fig. 1).<sup>4</sup> Luminal-secretory cells constitute the terminal maturation stage of hierarchically expanding transit-amplifying cells and thus form quantitatively the major epithelial cell type in the gland, although they are proliferatively quiescent (22). Unlike their proliferating precursors, luminal-secretory cells depend on stromally derived andromedins for survival; hence, androgen ablation or specific inactivation of AR function in prostate stroma induces apoptosis of these cells (1, 9, 23).

To efficiently study prostatic epithelial differentiation and carcinogenesis, well-characterized in vitro human cell line models are needed. Indeed, nonimmortalized early-passage normal human prostatic epithelial cells, termed PrEC, can be purchased from Cambrex BioSciences (East Rutherford, NJ). In addition, Weijerman et al. (24) and Bello et al. (25) used the human papillomavirus (HPV) 18 DNA to immortalize human prostate epithelial cells from normal prostate to establish the serially passagable PZ-HPV7 and RWPE-1 cell lines, respectively, whereas Weijerman et al., using the same technique, also immortalized prostate epithelia from human prostate cancer tissue establishing the permanent CA-HPV10 cell line. In addition, Yasunaga et al. immortalized the 957E/hTERT cell line via retroviral transduction of a human telomerase (hTERT) subunit into epithelial cells from a patient with hereditary prostate cancer (26). In determining the cellular origin of these lines, it is important to consider that all of the above nonimmortalized and immortalized lines were established in low (i.e., <300 μmol/L) Ca<sup>2+</sup> SFD medium minimally containing bovine pituitary extract and epidermal growth factor. Such low Ca<sup>2+</sup> SFD medium selects against prostate cancer cells while allowing basal layer-derived normal epithelial cells to grow out (18). This observation is consistent with why none of the aforementioned normal or cancer-derived lines are tumorigenic when inoculated in nude mice (18, 24–26).

In present studies, the expression of stage-specific differentiation markers (Fig. 1) as well as morphologic and growth characteristics were evaluated in the nonimmortalized PrEC and immortalized RWPE-1, PZ-HPV7, CA-HPV10, and 957E/hTERT lines to define their cellular composition and to resolve whether these cultures contain prostate epithelial stem cells. Resolving these issues is important to clarify how each of these lines can be used appropriately for the in vitro studies of both normal prostatic epithelial differentiation and prostatic carcinogenesis.

**Materials and Methods**

**Materials.** The synthetic androgen R1881 was purchased from Perkin-Elmer (Boston, MA). Universal type I IFN was obtained from PBL.
Biomedical Laboratories (Piscataway, NJ), DU145, LNCaP, PC3, and CA-HPV10 human prostate cancer lines, PZ-HPV7 and RWPE-1 immortalized prostate nonmalignant lines, and SV40-immortalized WPMY-1 prostate stromal cells (27) were purchased from the American Type Culture Collection (Rockville, MD). CWR22Rv1 line (28) was generically provided by Dr. J.W. Jacoberger (Case Western Reserve University, Cleveland, OH). The 95T6/hTERT, 4C-2B, LAPC-4, and MDA-PC-2B lines were obtained as described previously (18). The DuCaP (29) and VCaP (30) lines were generously provided by Dr. K. Pienta (University of Michigan, Ann Arbor, MI). The E066A line (31), was generously provided by Dr. S. Koockehpour (Louisiana State University, New Orleans, LA). All of the above lines, except the 95T6/hTERT, LARC-4, MDA-PC-2B, RWPE-1, CA-HPV10, and PZ-HPV7, were serially passaged in RPMI 1640 (total Ca2+ is 650 ± 10 μM/L) containing 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA; ref. 18). The LAPC-4 and MDA-PC-2B cells were grown in serum-containing medium (total Ca2+ is >700 μM/L) as described previously (18). The RWPE-1, PZ-HPV7, CA-HPV10, and 95T6/hTERT cell lines were grown in keratinocyte serum-free medium (total Ca2+ is 75 ± 2 μM/L) supplemented with bovine pituitary extract and recombinant epidermal growth factor (Invitrogen Life Technologies; ref. 18). PrEC cells were purchased from Cambrex BioSciences and grown in PrEGM SFD medium (total Ca2+ is 260 ± 14 μM/L) containing all provided supplements (18). All cells were grown in 5% CO2, 95% air humidified incubator at 37°C.

**In vitro growth assays.** Cell growth was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay from Promega Corp. (Madison WI) as described previously (15, 18). Real-time Taqman reverse transcription-PCR quantitation of AR and PSA expression. The Taqman primers and probes for human AR and human kallikrein-3 (i.e., PSA) genes were purchased from Applied Biosystems (Foster City, CA). Total RNA was extracted with Qiagen RNeasy Mini kit (Valencia, CA) according to the manufacturer’s instructions. The reverse transcription was carried out using appropriate Taqman reagents and 5 μL of this reaction mixture were used for the subsequent polymerase reaction. The Taqman reverse transcription was carried out on a Bio-Rad iCycler (Hercules, CA). Each reverse transcription-PCR experiment included a standard curve of known copy number using full-length wild-type cDNA of either AR or PSA subcloned into the TA cloning vector (Invitrogen Life Technologies). AR and PSA mRNA levels were determined and expressed as copy number per microgram of total RNA for each cell line.

**Western blotting.** Western blots were carried out on cell lysates equivalent to 106 cells per lane as described previously (15, 18). Rabbit polyclonal AR, PSCA, and GATA-2 and mouse monoclonal ΔNp63 isoform antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal TAp63 isoform antibody was purchased from CellSignaling (Beverly, MA). Mouse monoclonal β-actin antibody was purchased from Sigma (St. Louis, MO).

**Immunocytochemistry for AR, p63, PSCA, and chromogranin A.** Immunocytochemistry was done as described previously (18) using the antibodies described for Western blotting as well as mouse monoclonal chromogranin A antibody (Calbiochem, La Jolla, CA).

**Measurement of PSA production.** Total PSA in the culture medium was analyzed using the Hybritech ELISA assay on the Beckman Access Immunoassay System (Beckman Coulter, Inc., Brea, CA) as described previously (27). This assay has a limit of detection of 0.1 ng PSA/ml medium.

**Flow cytometry and time-lapse microscopy.** One million cells were suspended in wash buffer [PBS, 0.5% bovine serum albumin (BSA), 2 mM/L EDTA] and antibody incubation was conducted for 10 minutes on ice. Subsequently, the cells were washed and analyzed by a LSR flow cytometer using CellQuest software (BD Biosciences, Franklin Lakes, NJ). Counts (105) were obtained in three independent experiments and the percentage of positive cells was determined by comparison with isotype control antibody staining results. Cells were labeled using one or more of the following antibodies: mouse monoclonal phycoerythrin (PE)–conjugated CD133 antibody (Miltenyi Biotech, Auburn, CA), mouse monoclonal FITC-conjugated ABCG2 antibody (Chemicon), mouse monoclonal PE/Cy5-conjugated β1 integrin (BioLigand), or a rabbit-polyclonal PSCA (Santa Cruz Biotechnology) followed by a goat anti-rabbit IgG FITC (Santa Cruz Biotechnology). Multichannel analyses for β1 integrin, CD133, and ABCG2 triple-positive cells were conducted by gating the β1 integrin and ABCG2 cells for the expression of CD133. Time-lapse microscopy was done on a TE-2000 Nikon (Melville, NY) microscope with a 37°C heated stage in a 5% CO2 chamber (Live Cell NEVE Product Group). Images were captured every 20 minutes using a Cool Snap ES CCD camera (Princeton Instruments, Trenton, NJ).

**Isolation of CD133+ cells.** CD133+ cells were isolated by fluorescence-activated cell sorting (FACS) using FACS area flow cytometer. Specifically, 3 × 106 PrEC cells were incubated with PE-conjugated CD133 mouse monoclonal antibody and then sorted. Alternatively, CD133+ cells were isolated using magnetic cell isolation per the manufacturer's instructions (Miltenyi Biotech). Briefly, 1 million PrEC cells were labeled with CD133 microbeads, incubated on ice for 30 minutes, and washed with provided buffer (PBS, 0.5% BSA, 2 mM/L EDTA). Cells were isolated by passage through a series of two magnetic columns and washed and the CD133+ cells were cytopsins onto slides for staining or grown in culture.

**Results**

Nonimmortalized PrEC cultures contain stem cells that undergo limited differentiation in vitro. PrEC cultures obtained commercially are supplied at passage 2 and, although not immortalized, can be serially propagated for up to 10 passages before becoming proliferatively quiescent (15–17). These cultures are established and maintained in low (i.e., <300 μM/L) Ca2+ SFD medium (18). To investigate whether these PrEC cultures contain stem cells, multivariable gated flow cytometry was used to evaluate the percentage of PrEC cells expressing CD133+, ABCG2+, and α2β1 integrin stem cell makers alone and in combination. These results document that essentially all (i.e., 98.9 ± 0.1%) of the PrEC cells express high levels of the β1 and α2 integrin subunits (i.e., ααββ, Supplementary Fig. S1A). A minor subset (i.e., 6.47 ± 0.46%) of these ααββ cells also express the ABCG2 transporter protein and an even smaller subset (i.e., 1.30 ± 0.18%) of these cells coexpress all three CD133+/ABCG2+/ααββ stem cell markers (Supplementary Fig. S1A and B). When the PrEC cultures reach an ~2/3 confluent state, 8.37 ± 0.54% of PrEC cells express the intermediate cell marker PSA; however, none of the CD133+/ABCG2+/ααββ stem cells was observed to express PSCA protein as expected (Supplementary Fig. S1A). Both the low (i.e., 1.3%) frequency of these CD133+/ABCG2+/ααββ stem cell markers and their ability to be serially passaged in low Ca2+ SFD medium are consistent with these cells being prostate stem cells.

To further characterize the molecular phenotype of these CD133+/ABCG2+/ααββ/PSA− stem cells, p63 protein expression was evaluated because prostate stem cells are p63− (13, 14). To do this, CD133+ cells were isolated by FACS from exponentially growing PrEC cultures. These isolated cells when analyzed immunocytochemically were >80% negative for p63 expression and consistently small. Furthermore, the above immunocytochemical staining revealed the presence of an occasional cell in mitotic telophase in which one of the daughter cells is p63+, whereas the other daughter cell expresses nuclear p63 (Fig. 2A). These results are consistent with one of the daughter cells remaining a CD133+/p63− stem cell (arrow) with the other daughter becoming a CD133+/p63+ transit-amplifying cell.

The p63 gene is a definitive marker for prostate transit-amplifying cells (33). The p63 gene can undergo alternative splicing to generate a series of molecular weight variants, including the α, β, and γ isoforms, of the TA or AN families of p63 (ref. 34; Fig. 2B). When the entire PrEC culture is subjected to p63 expression...
Stem Cells in Prostatic Epithelial Cell Cultures

Figure 2. A, ΔNp63 immunocytochemical staining (magnification, ×100) of CD133+/p63+ magnetic bead-selected stem cells (arrow). B, p63 gene can be alternatively spliced to generate a variety of splicing variants known as α, β, and γ isoforms of the TA or ΔN families of p63. C, expression of ΔNp63 and TAp63 proteins in human prostate normal and malignant cell lines. Actin was evaluated as a loading control. D, expression of AR, ΔNp63, PSCA, and actin (as a loading control) in the denoted lines.

analysis by Western blot, all three of the ΔNp63 isoforms are detected, with the 66-kDa α isoform being the most prominent one (Fig. 2C). However, these cultures do not express detectable levels of any TAp63 isoforms (Fig. 2C). At passage <6, the vast majority (i.e., >90%) of PrEC cells are small in size and express nuclear ΔNp63 protein as detected by immunocytochemistry (Fig. 3A), documenting that the prostate transit-amplifying cells comprise the overwhelming majority (90%) of PrEC culture. The p63− population accounts for ∼10% of PrEC culture and can be morphologically classified into large, small, and elongated/neuroendocrine-type cells. As documented above, small CD133+/p63+ putative stem cells account for ∼1% of total PrEC culture and are able to give rise to small p63− transit-amplifying cells. Evaluation of the remaining p63− cells revealed that the large p63− cells are the most predominant (i.e., ∼8% of all cells in PrEC culture) of the three p63− cell types (Fig. 3B). These large cells also consistently express PSCA protein as documented by flow cytometric analysis in Fig. 3D. Such morphology and p63+/PSCA− molecular expression profile are consistent with intermediate cell phenotype. The remaining subset of the p63− population consists of elongated/neuroendocrine cells (Fig. 3C) that express chromogranin A (Supplementary Fig. S2D). Because neuroendocrine cells are proliferatively quiescent and are derived from stem cells (7, 13), their detection is further indicative of the presence of stem cells in PrEC cultures.

To further validate the stem cell behavior of the CD133+/p63− cells, PrEC cultures were subjected to CD133-immunoconjugated magnetic bead enrichment to isolate CD133+ stem cells. These cells were plated at low density and monitored by time-lapse microscopy. These CD133+ cells attach and are initially nonmotile forming cell clusters (Supplementary Fig. S2A). Eventually, however, after several rounds of division, a subset of progeny becomes highly motile and acquires the ability to migrate apart before undergoing cell proliferation (Supplementary Fig. S2B). As these colonies expand in low Ca2+ SFD medium, <10% of small cells undergo maturation into large nonproliferative intermediate cells, whereas ∼1% to 3% of these cells mature into elongated/neuroendocrine-type cells (Supplementary Fig. S2C and D). Flow cytometric analysis documented that although the large intermediate cells are proliferatively quiescent, the level of PSCA expression per cell, the percentage of PSCA+ cells, and the cell size of PSCA+ cells increase over time in cultures but never exceed 15% to 20% even when the cultures are maintained for >10 days as shown in Fig. 3D. These results document that PrEC CD133+/p63−/PSCA+ stem cells give rise to both neuroendocrine and p63−/PSCA−/AR−/PSA−/PSA− transit-amplifying cell lineages in vitro, where subsequently a limited subset of transit-amplifying cells are able to further mature into p63−/PSCA−/AR+/PSA−/PSA− large intermediate cells. In turn, newly formed proliferatively quiescent PrEC intermediate cells do not complete their maturation into p63+/PSCA+/AR+/PSA+/PSA+ luminal-secretory cells in low Ca2+ SFD medium as documented by detection of only a low level of AR and PSA mRNA expression (Table 1). Consistent with these cells not completing their maturation, no AR protein (Fig. 2D) or PSA protein expression was detectable by Western blot (Fig. 5D) and ELISA assay, respectively, even when the cultures were allowed to age for >2 weeks.

Malignant versus nonmalignant nature of immortalized human prostatic epithelial cell lines established in low Ca2+ SFD medium. Previously, Dalrymple et al. documented that low (i.e., <300 μmol/L) Ca2+ SFD medium does not allow for continuous proliferation of any tested (i.e., n = 8) human prostate cancer cell lines (18). These results raise the question of what type of prostatic epithelial cells grow out when cultures from malignant prostate tissues are established and maintained in such low Ca2+ SFD medium.
medium. This is particularly critical because in establishing permanent lines from malignant tissues, it is often assumed that the outgrowing population will be of malignant phenotype. Indeed, both the CA-HPV10 and 957E/hTERT cell lines were initially established in culture from such malignant tissues using low (i.e., <100 μmol/L) Ca\(^{2+}\) SFD medium and the cultured cells subsequently were immortalized to produce permanent “prostate cancer cell lines” (24, 26). Likewise, both RWPE-1 and PZ-HPV7 cells were established and immortalized using this low (i.e., <100 μmol/L) Ca\(^{2+}\) SFD medium but from supposedly normal prostate tissues (24, 25). However, because prostate cancers are often multifocal and diffuse, the nonmalignant nature of these latter two immortalized lines requires verification and vice versa.

The expression of ΔNp63α protein can be used to verify malignant versus nonmalignant nature of the immortalized cell lines because this protein is not expressed by prostate cancer cells (33). As shown earlier, normal nonimmortalized transit-amplifying cells within PrEC cultures express ΔNp63 isoforms (Fig. 2C and D). Similarly, the RWPE-1, PZ-HPV7, CA-HPV10, and 957E/hTERT immortalized lines all express detectable levels of ΔNp63 isoforms (Fig. 2D). In contrast, none of a series of 11 human prostatic cancer lines (PC3, DU145, LNCaP, C4-2B, MDA-PC-2B, LAPC-4, E006AA, DuCaP, VCaP, CWR22Rv1, and CWR22R1) express any of the ΔNp63 isoforms and only the LNCaP line and its derivative (C4-2B) express a detectable level of the TAp63 isoform (Fig. 2C). These results document that all four of the immortalized nontumorigenic lines tested (RWPE-1, PZ-HPV7, CA-HPV10, and 957E/hTERT) are derived from normal prostate basal epithelial cells but not prostate cancer cells.

**Cellular heterogeneity of immortalized normal human prostate cell lines established in low Ca\(^{2+}\) medium.** Because, RWPE-1, PZ-HPV7, CA-HPV10, and 957E/hTERT human cell lines are derived from normal, not malignant, immortalized basal cells, this raised the question of whether any of these lines contain immortalized normal stem cells. To resolve this matter, the lines were analyzed by multivariable flow cytometry for the presence of CD133+/ABCG2+/α2hHi-coexpressing stem cells. This analysis showed that all of the lines contain a detectable but variable

![Figure 3. Normal human prostate epithelial PrEC cultures are heterogeneous in cellular composition.](cancerres.aacrjournals.org)
immortalized lines in addition to CD133+ stem and neuroendocrine PSCA, but not AR, proteins. These results confirm that all of the amplifying cells as observed by time-lapse microscopy and intermediate cells derive from proliferating small p63+/PSCA+ transit-amplifying cells with a minor fraction of CD133+/ABCG2+/α3β3II-coexpressing stem cells (Supplementary Fig. S1). In particular, PZ-HPV7 line had the highest (i.e., 1.75 ± 0.13%) percentage of triple-positive cells. Furthermore, all studied cultures were documented to contain neuroendocrine cells (Fig. 4D), which is indicative of the presence of stem cells. Further molecular evaluation of these cultures documented that PZ-HPV7 cultures were heterogeneous with respect to cellular composition consisting of small p63+ transit-amplifying and p63- stem cells as well as p63+/PSCA+ large intermediate cells (Supplementary Fig. S3). Cell cultures of the other three (957E/hTERT, CA-HPV10, and RWPE-1) lines predominantly contained small p63+/PSCA+ transit-amplifying cells with a minor fraction (i.e., ≤10%) of large p63+ /PSCA+ intermediate cells (see Fig. 4A and B for examples of 957E/hTERT cultures). These p63+/PSCA+ late intermediate cells derive from proliferating small p63+ transit-amplifying cells as observed by time-lapse microscopy and subsequent immunocytochemistry (Fig. 4A-C). Western blot evaluation of p63, PSCA, and AR expression supports the above immunocytochemistry findings. As documented in Fig. 2D, all of the immortalized lines were documented to express ΔNp63α and PSCA, but not AR, proteins. These results confirm that all of the immortalized lines in addition to CD133+ stem and neuroendocrine cells also contain p63+ transit-amplifying cells, a subset of which can mature into PSCA+ intermediate cells but not into AR+/PSCA+ luminal-secretory cells. This latter point is further supported by the observation that all of the four immortalized lines (RWPE-1, PZ-HPV7, CA-HPV10, and 957E/hTERT) as well as the nonimmortalized PrEC cells express low levels of AR mRNA (Table 1). The levels of AR mRNA expression in these normal lines are 10^3 to 10^4 lower than those of prostate cancer cells and 10^2 lower than those of normal prostate stromal (e.g., WPMY-1) cells (Table 1). Furthermore, lack of PSA protein expression (Fig. 5D) and detection of only low PSA mRNA levels (Table 1) in these normal prostate epithelial cells is consistent with the absence of AR protein expression and corresponding lack of differentiation.

<table>
<thead>
<tr>
<th>Human cell line</th>
<th>mRNA copies/μg RNA</th>
<th>AR</th>
<th>PSA</th>
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<tbody>
<tr>
<td>PrEC (normal prostate)</td>
<td>3.2 ± 0.4 × 10^3</td>
<td>2.6 ± 0.2 × 10^3</td>
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<td>PZ-HPV7 (normal prostate)</td>
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<td>5.8 ± 0.7 × 10^3</td>
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<td>CA-HPV10 (normal prostate)</td>
<td>2.1 ± 0.3 × 10^3</td>
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<tr>
<td>RWPE-1 (normal prostate)</td>
<td>6.4 ± 0.8 × 10^3</td>
<td>5.6 ± 0.7 × 10^3</td>
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<tr>
<td>957E/hTERT (normal prostate)</td>
<td>2.0 ± 0.3 × 10^3</td>
<td>4.1 ± 1.0 × 10^3</td>
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<tr>
<td>DU145 (prostate cancer)</td>
<td>ND*</td>
<td>ND</td>
<td></td>
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<tr>
<td>PC3 (prostate cancer)</td>
<td>ND</td>
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<tr>
<td>LNCaP (prostate cancer)</td>
<td>1.1 ± 0.2 × 10^7</td>
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<td>C4-2B (prostate cancer)</td>
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<td>5.6 ± 0.3 × 10^6</td>
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<td>LAPC-4 (prostate cancer)</td>
<td>5.8 ± 0.5 × 10^6</td>
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<td>CWR22Rv1 (prostate cancer)</td>
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<td>MDA-PG-2B (prostate cancer)</td>
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<tr>
<td>6S (prostate stroma)</td>
<td>1.3 ± 0.1 × 10^5</td>
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<tr>
<td>WPMY-1 (prostate stroma)</td>
<td>3.5 ± 0.2 × 10^5</td>
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<td></td>
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</table>

*Not detectable; <10^3 copies/μg RNA.

Table 1. Expression of AR and PSA mRNA in various human cell lines

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<td>PZ-HPV7 (normal prostate)</td>
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<td>RWPE-1 (normal prostate)</td>
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<td>5.6 ± 0.3 × 10^6</td>
<td></td>
</tr>
<tr>
<td>LAPC-4 (prostate cancer)</td>
<td>5.8 ± 0.5 × 10^6</td>
<td>3.0 ± 0.3 × 10^7</td>
<td></td>
</tr>
<tr>
<td>CWR22Rv1 (prostate cancer)</td>
<td>4.1 ± 0.3 × 10^6</td>
<td>6.1 ± 0.1 × 10^6</td>
<td></td>
</tr>
<tr>
<td>MDA-PG-2B (prostate cancer)</td>
<td>1.0 ± 0.9 × 10^6</td>
<td>4.2 ± 0.4 × 10^6</td>
<td></td>
</tr>
<tr>
<td>6S (prostate stroma)</td>
<td>1.3 ± 0.1 × 10^5</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>WPMY-1 (prostate stroma)</td>
<td>3.5 ± 0.2 × 10^5</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Real-time PCR analysis of AR and PSA mRNA expression in a series of prostate lines. ARHi/PSAHi prostate cancer lines and ARLo/PSA+ prostate stromal lines were used as positive controls for comparison with the nonimmortalized and immortalized normal human prostate cell lines. AR- PC3 and DU145 cells were used as negative controls for AR and PSA mRNA expression.

Effect of Notch-1 signaling on differentiation of intermediate cells into luminal-secretory cells. We have documented previously that in PrEC cells grown in low Ca^2+ SFD medium Notch signaling is constitutively active even in the absence of cell contact (i.e., receptor-ligand interaction; ref. 18). This phenomenon is explained by the ability of γ-secretase in such low Ca^2+ medium to proteolytically process Notch-1 receptor, thereby producing the active 110-kDa cleaved Notch transcription factor (18). Hey-1 is a direct transcriptional target of this cleaved Notch fragment and

Can IFN and/or androgens induce intermediate cells to mature into luminal-secretory cells in low Ca^2+ SFD medium? Androgen stabilizes AR protein from degradation (32). No androgen is present routinely in standard low Ca^2+ SFD medium used for growing PrEC or the other four immortalized human prostatic nonmalignant cell lines. This raises the question of whether providing exogenous androgen to the medium would induce the expression of a sufficient AR protein to induce maturation of intermediate cells into luminal-secretory cells. Thus, expression of AR and PSA proteins as well as cell growth were evaluated in the presence or absence of 1 nmol/L of the synthetic androgen R1881. Treatment of cells with androgen did not result in detectable expression of AR protein in any of the normal prostate epithelial cell lines (Fig. 5A). In contrast, both the CWR22Rv1 human prostate cancer cells and the WPMY-1 immortalized human prostate stroma cells up-regulated AR protein in response to R1881 (Fig. 5A). Furthermore, R1881 treatment did not simulate the growth of RWPE-1, PZ-HPV7, CA-HPV10, and 957E/hTERT cells (Fig. 5B). In contrast, a significant growth response was observed in androgen-sensitive, AR+ CWR22Rv1 human prostate cancer line but not in the AR+ WPMY-1 human prostate stromal line (Fig. 5B). The latter lack of response is consistent with the previous demonstration that the growth of normal human prostate stromal cells was not stimulated by androgens (35).

It has been recently reported that the addition of IFN to low Ca^2+ SFD medium induces PrEC cells to increase their level of AR protein expression (36). To confirm this, RWPE-1 cells were exposed for 24 hours to 1,000 units/mL universal type I IFN in the presence or absence of added androgen (i.e., 1 nmol/L R1881) and AR protein levels were determined by Western blot (Fig. 5C). These results documented that RWPE-1 cells are able to up-regulate their expression of AR protein; however, the absolute level of expression was still >10-fold lower than that of normal human prostate stromal cells (i.e., WPMY-1 cells; Fig. 5C), which in turn express 10^2 lower levels of AR protein than prostate cancer cells (e.g., CWR22Rv1; Fig. 5A). When medium from R1881- and IFN-treated RWPE-1 cells was screened for PSA protein by ELISA assay, no expression was detected. These results document that the addition of IFN to low Ca^2+ SFD culture medium allows intermediate cells to enhance their expression of AR protein but does not induce full differentiation of these cells toward the PSA+ luminal-secretory phenotype. Notably, these findings are consistent with previous reports of RWPE-1 cells being able to undergo limited differentiation and acinar formation in three-dimensional Matrigel cultures in the presence of added androgen (37, 38). Such ability of RWPE-1 cells to undergo acinar differentiation is further suggestive of the presence of prostate stem cells in these cultures.
thus a downstream effector of the Notch signaling pathway (39). The Hey-1 gene encodes a basic helix-loop-helix protein, which forms a Hey-1/NCoR/Sin3/HDAC1 repressor complex and downregulates the expression of a series of genes, including the family of GATA transcription factors (39, 40). Furthermore, in addition to repressing GATA-2 and GATA-3 expression transcriptionally, Hey-1 also physically complexes with GATA transcription factors, thereby blocking their transactivation activity (41). Hey-1 also was able to efficiently repress AR transcriptional activity by binding to the AF1 domain of the receptor (42). These findings are significant because GATA-2 and GATA-3 transcription factors in concert with AR regulate prostate epithelial differentiation (9, 43). In particular, PSA enhancer, in addition to numerous AR-binding sites, also contains six GATA-binding sites, which are essential for proper AR-regulated transactivation of PSA (43). Thus, Hey-1 prevents the expression of critical GATA- and AR-regulated genes and therefore blocks differentiation of transit-amplifying cells into intermediate cells and later into AR+/PSA+ luminal-secretory cells. Consistent with this hypothesis is a recent observation that transit-amplifying cells that down-regulate Notch-1 mRNA, and thus Hey-1 protein expression, in turn up-regulate expression of AR and PSA luminal-secretory differentiation markers (17). As documented in Figs. 3D and 5A, only a small (i.e., <15%) fraction of transit-amplifying cells mature into intermediate cells and no maturation into luminal-secretory phenotype is observed in PrEC or four immortalized normal prostatic epithelial cell lines grown in low Ca²⁺ SFD medium. To elucidate whether such lack of differentiation is due to continuous Notch-1/Hey-1 signaling, we evaluated expression of these markers and GATA-2 transcription factor in these lines. Western blot analysis documents that Notch-1/Hey-1 signaling is active in all of the studied normal epithelial lines because both the cleaved 110-kDa Notch-1 fragment and its downstream effector Hey-1 are expressed. Interestingly, none of the studied normal prostate lines expressed GATA-2 protein, which is consistent with the ability of Hey-1 to repress expression of GATA-2 and subsequent differentiation. As documented in Fig. 5D, Hey+/GATA-2− normal human prostate epithelial cells do not express PSA prostatic differentiation marker. In contrast, all AR+/PSA+ human prostate cancer (DuCaP, VCaP, LNCaP, C4-2B, LAPC-4, MDA-PC-2B, CWR22Rv1, and CWR22R1) lines that are grown in

![Figure 4](image-url)

**Figure 4.** A, Immunocytochemical ΔNp63 and PSCA staining (magnification, ×60) of 957E/hTERT cells. Time-lapse phase-contrast (magnification, ×20) monitoring of 957E/hTERT culture documented that large PSCA+ intermediate cells are proliferatively quiescent, whereas otherwise viable and mobile. For instance, two large intermediate cells (arrows) were monitored from day 1 (B) to day 4 (C) of the time-lapse experiment and were documented not to divide. In contrast, small cells in 957E/hTERT culture proliferated continuously. D, Phase contrast (magnification, ×80) of neuroendocrine cells (arrows) in 957E/hTERT, CA-HPV10, and RWPE-1 cultures.
high (i.e., >600 μmol/L) Ca\(^{2+}\) serum-containing medium failed to express Hey-1 repressor protein, which is consistent with their advanced PSA\(^{+}\) differentiation status (ref. 44; Table 1; Fig. 5). Notably, AR\(^{+/0}\)/PSA\(^{-}\)/DU145 and PC3 prostate cancer lines that are also grown in high (i.e., >600 μmol/L) Ca\(^{2+}\) serum-containing medium showed continued expression of Hey-1. These findings validate that although all evaluated prostate cancer lines express GATA-2 protein at variable levels, only in PC3 and DU145 cells GATA-2 transcriptional activity is repressed by Hey-1; as a consequence, these cells do not express detectable levels of PSA mRNA and protein (Table 1; Fig. 5D) or other prostate differentiation markers (e.g., hK2 and PSMA; ref. 27). Thus, in low Ca\(^{2+}\) SFD medium, Notch-1/Hey-1 signaling is playing an important role in preventing efficient differentiation of prostate transit-amplifying cells toward the luminal-secretory phenotype.

**Discussion**

The present studies characterized the origin and cellular composition of normal nonimmortalized human PrEC prostate...
epithelial cells and immortalized human PZ-HPV7, CA-HPV10, 957E/hTERT, and RWPE-1 prostate epithelial cell lines, all of which were established and maintained in low (i.e., <300 μmol/L) Ca²⁺ SFD medium. Using appropriate molecular markers, all of the above cell lines were documented to be derived from normal prostate tissues and to contain CD133⁺/ABC2⁺/ανβ₃⁺/p63⁻ epithelial stem cells that give rise to both neuroendocrine and CD133⁻/p63⁻/PSCA⁻ transit-amplifying epithelial cell lineages. In human prostate, these p63⁻ transit-amplifying cells undergo a limited number of proliferations before a subset of these cells matures into CD133⁻/p63⁻/PSCA⁻ intermediate cells. Such intermediate cells, however, do not efficiently mature into CD133⁻/p63⁻/PSCA⁻/AR⁻/PSA⁻ luminal-secretory cells in culture in the low Ca²⁺ SFD medium. Even supplementation of medium with androgens and IFN, which up-regulate AR protein expression, failed to induce PSA expression and cell differentiation. This inability to completely differentiate into luminal-secretory cells has been shown to be due to Notch-1 signaling pathway induction of Hey-1 effector protein expression. Such Hey-1 expression down-regulates GATA-2 expression and blocks its function, thus preventing full differentiation.

Understanding the cell of origin and composition of the available primary and immortalized prostate cell lines is critical for studying the processes of normal epithelial differentiation and prostatic carcinogenesis. Identification of the cell of origin for prostate carcinogenesis is presently unresolved. A critical question that requires our attention is whether this initiating prostate cancer cell is a transformed genetically unstable stem cell or whether it is one of its more differentiated progeny (45–47). A growing body of evidence suggests that, once initiated, such a genetically unstable cell behaves as a "cancer stem cell" (47). The defining characteristic of such a "cancer stem cell" is its retention of unlimited self-renewal while allowing a subset of its progeny to mature down an aberrant differentiation/maturation pathway toward the luminal-secretory cell phenotype. Coupled with such an aberrant maturation process, these more differentiated malignant progeny retain only a limited ability to proliferate. Thus, under this scenario, the continuous malignant growth (i.e., clonal expansion) is maintained by the proliferation of cancer stem cells and not the progeny that undergo limited proliferation before terminal differentiation. Theoretically, such "cancer stem cells" can be derived as a result of transformation of an undifferentiated normal stem cell and/or from any of its more differentiated progeny (i.e., neuroendocrine, transit-amplifying, intermediate, and secretory cells). Effective therapy must eradicate malignant cells with unlimited clonogenic expansion ability (i.e., cancer stem cells) within the cancer population regardless of whether such cancer stem cells are derived from undifferentiated normal stem cells or its more differentiated progeny.

Resolving the specific cell of origin for prostate cancer is critical to appropriately define rational targets for therapeutic interventions, because there are major differences in the growth-regulatory pathways for stem cells versus their more differentiated progeny (48). For example, the targeting of AR axis is the most obvious point in question. In prostatic cancer, there are molecular changes that convert the AR axis from a tumor suppressor of normal prostatic epithelial growth to a gain of function tumor oncogene directly stimulating prostate cancer cell growth (9). If prostate cancer stem cells are derived from transformed normal stem cells in which AR is not expressed, then such androgen stimulation occurs via enhancing the growth not of the cancer stem cells but of their AR⁺ differentiated progeny. In such circumstances, androgen ablation would inhibit growth of these more differentiated progeny but not of the cancer stem cells and thus would not be curative no matter how complete is the extent of ablation (48). This would explain why current standard of care "complete androgen ablation" has failed to be curative against prostate cancer (49).

Recently, additional evidence suggesting that cancer stem cells are androgen and AR independent was put forth by Collins et al., where they documented that "prostate cancer stem cells" express CD133⁻ and ανβ₃⁺ and are derived from normal stem cells and thus do not express AR protein (50). Although such findings are intriguing, it is important to point out that Collins et al. study used low Ca²⁺ SFD medium to grow such "prostate cancer stem cells" and did not report on the p63 expression of the CD133⁻/ανβ₃⁺ "prostate cancer stem cells" or their progeny. Thus, there is a strong possibility that as described before for CA-HPV10 and 957E/hTERT cells, which were derived from contaminating normal, not malignant, CD133⁻/ABC2⁺/ανβ₃⁺ prostate stem cells, a similar unexpected selection may have occurred in the Collins et al. study. Although it is possible that CD133⁻/ABC2⁺/ανβ₃⁺ "prostate cancer stem cells" do exist, this must be documented functionally by their ability to generate p63⁺ growing cancers when xenografted into nude mice. In summary, the current study provides critical knowledge on the cellular origin and biological heterogeneity due to in vitro differentiation of several available prostate epithelial cell lines and documents that low Ca²⁺ SFD medium selects for growth of normal prostate epithelial stem and transit-amplifying cells. Such knowledge should enable and facilitate the studying of prostate epithelial differentiation and prostatic carcinogenesis.

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Low-Calcium Serum-Free Defined Medium Selects for Growth of Normal Prostatic Epithelial Stem Cells

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