Breast Cancer Resistance Protein–Mediated Efflux of Androgen in Putative Benign and Malignant Prostate Stem Cells

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Abstract

Malignantly transformed stem cells represent a potential common nidus for the primary cancer and the recurrent cancer that arises after treatment failure. Putative prostate stem cells and prostate tumor stem cells in benign and malignant human prostate tissue, in primary human prostate xenografts, and in the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model of prostate cancer, are defined by expression of breast cancer resistance protein (BCRP), a marker of pluripotent hematopoietic, muscle, and neural stem cells, and by an absence of androgen receptor (AR) protein. Inhibition of BCRP-mediated efflux of dihydrotestosterone by novobiocin or fumitremorgin C in a rat prostate progenitor cell line that expresses BCRP and AR mRNAs, but minimal AR protein, results in stabilization and nuclear translocation of AR protein, providing a mechanism for lack of AR protein in BCRP-expressing stem cells. In both benign and malignant human prostate tissue, the rare epithelial cells that express BCRP and lack AR protein are localized in the basal cell compartment, survive androgen deprivation, and maintain proliferative potential in the hypoxic, androgen-deprived prostate. Putative prostate tumor stem cells that express BCRP but not AR protein in TRAMP are the source of a BCRP-negative and AR-negative, Foxa2- and SV40Tag-expressing, transit amplifying compartment that progresses to the poorly differentiated carcinomas that arise rapidly after castration. Therefore, BCRP expression isolates prostate stem/tumor stem cells from the prostate tissue microenvironment through constitutive efflux of androgen, protecting the putative tumor stem cells from androgen deprivation, hypoxia, or adjuvant chemotherapy, and providing the nidus for recurrent prostate cancer. (Cancer Res 2005; 65(15): 6640-50)

Introduction

Adult tissue stem cells represent a logical target for malignant transformation, suggesting that tumors can be modeled as stem cell–fed lineages composed of “malignant stem cells and their differentiated progeny admixed to form a caricature of the tissue of origin” (1). Consequently, treatment regimes designed to reduce the bulk of a tumor or eliminate the proliferative compartment, but not target the phenotypically distinct tumor stem cell, ignore the etiology of the neoplasm and cannot be curative (2). In the prostate, stem cells and their transformed derivatives are anticipated to be localized in the basal cell compartment, with their progeny capable of differentiation along basal epithelial, secretory epithelial, and neuroendocrine cell lineages (3, 4). Consistent with their anticipated pleiotropic potential, putative prostate stem cells have been reported to express cytokeratins and lineage markers characteristic of both basal and secretory epithelial cells (5), and, like prostate basal and neuroendocrine cells, but unlike secretory epithelial cells, to be resistant to androgen deprivation–induced apoptotic death (3, 4). Prostate cancer, therefore, provides a unique model system for characterization of the role of a tumor stem cell as the nidus of a primary prostate cancer, and the recurrent cancer that emerges after failure of hormonal therapy, because the bulk of the “quasi-differentiated” tumor cell component can be removed selectively by androgen deprivation at any point during disease progression, without damage to tissue architecture or to the putative tumor stem cell.

Adult stem cells, independent of tissue of origin, have been proposed to express genes associated with immortalization and with resistance to apoptosis, phenotypic characteristics associated with unlimited potential for tissue renewal (2, 6). In contrast, breast cancer resistance protein (BCRP/ABCG2), whereas a marker expressed consistently by adult stem cell populations that possess pluripotentiality and long-term repopulation capability, is of unknown mechanistic significance to the stem cell phenotype (7). BCRP/ABCG2 is a member of the ATP-binding cassette (ABC) transporter family associated initially with resistance of cancer cells to chemotherapeutic agents. Subsequently, BCRP-mediated efflux of Hoechst 33342 was shown to define the pluripotent stem cell containing “side population” of bone marrow, skeletal muscle, and neural tissue (7). Recently, BCRP was also shown to transport steroids, estrone sulfate, and 17β-estradiol sulfate; the efflux of these substrates, as well as chemotherapeutic agents, such as mitoxantrone and topotecan, was inhibited competitively by estrogen, antiestrogens, progesterone, cholesterol, and DHEA sulfate (8, 9).

A primary challenge for prostate cancer researchers has been the lack of appropriate models to examine, within an intact prostate microenvironment, the events that culminate in the transition to recurrent prostate cancer. Of particular importance is modeling the role of androgen receptor (AR)–mediated signaling in the prostate stem cell, tumor stem cell, and their progeny, in the regulation of proliferation and induction of differentiation, in their sensitivity to androgen deprivation–induced apoptosis, and in the transition of androgen-regulated growth to androgen-independent growth in response to androgen deprivation therapy. Cunha et al. (10) showed that an endodermal primordium that lacks AR gave rise to an adult prostate epithelium under the inductive influence of a stromal compartment with functional AR; however, the

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differentiated function of the epithelium was compromised without AR-mediated gene transactivation. In contrast, expression of AR in the endodermal primordial cells, under the inductive regulation of a mesenchymal compartment that lacked AR function, could not direct formation of prostate glandular structures. Consequently, AR expression may not be requisite for a putative prostate stem cell, suggesting a phenotype for the stem cell separate from the secretory epithelial cell, a hypothesis consistent with the observation that the prostate stem cell is not sensitive to androgen deprivation–induced apoptotic death (4). The role of the stem cell in prostate development is also supported by tissue rescue studies in which urogenital sinus isolated from embryos lacking basal cells (p63−/−) transplanted beneath the kidney capsule develops into prostate glandular tissue with luminal and neuroendocrine cells that is capable of regeneration after castration in response to androgen administration (11). The rapid repopulation of an involuted prostate epithelium by residual stem cells that survive androgen deprivation in response to exogenous androgen, the immediate expression of AR protein with commitment of the stem cell progeny to differentiation to the secretory lineage, and the rapid reappearance of AR in the absence of androgen in prostate cancer cells that survive androgen deprivation, shows the complex nature of regulation of AR function.

We have characterized the response to androgen deprivation of putative prostate stem cells and tumor stem cells, and their progeny, in multiple in vivo model systems, each uniquely suited to address a specific aspect of their role in the transition to recurrent prostate cancer. In a human prostate primary xenograft model developed in our laboratory, the response of putative prostate stem cells and tumor stem cells to androgen deprivation was characterized in xenografts transplanted into athymic nu/nu mice (12, 13). Prostate stem/progenitor cells in the xenografts survived androgen deprivation and maintained pluripotentiality, as shown by their capacity to generate progeny that differentiated along multiple lineages in response to inductive microenvironmental signals (13). Prostate stem/progenitor cells in xenografts maintained in castrate hosts differentiated preferentially along a neuroendocrine cell lineage in response to androgen deprivation, and differentiated along a secretory epithelial lineage only in response to induction with exogenous androgen (13). In contrast, putative prostate tumor stem cells in xenografts maintained in castrate hosts differentiated preferentially along a secretory epithelial lineage in the androgen-deprived prostate tissue microenvironment (13). The SV40Tag-driven transgenic adenocarcinoma of the mouse prostate (TRAMP) provides a model of the role of the prostate stem cell in the nidus of recurrent prostate cancer, the neuroendocrine-like poorly differentiated carcinomas that develop rapidly after castration in the androgen-deprived prostate tissue microenvironment that causes the adenocarcinomas to cease growth and involute (14).

The current study results from our observation that putative stem cell–driven responses of the prostate epithelial compartment in human patients, in primary xenografts from these patients, and in the prostates of TRAMP mice, were correlated with the presence of small foci of cells that lacked AR protein. These morphologically and phenotypically distinct foci were evaluated for expression of a panel of markers anticipated to be characteristic of adult stem cells. BCRP was the only marker that was expressed consistently by a subpopulation of cells within the foci that lacked expression of AR protein. This study shows that BCRP-expressing putative prostate stem/progenitor cells were present in surgical specimens of benign and malignant human prostate; that the stem cells/tumor stem cells were maintained in primary xenografts established from these surgically resected prostate tissues; that the stem cells survived androgen deprivation in both human patients undergoing androgen deprivation therapy and in human prostate xenografts maintained in castrate hosts; and that BCRP-expressing cells were the nidus of the poorly differentiated (recurrent) prostate cancer that arose in TRAMP after androgen deprivation. Finally, constitutive efflux of androgen by BCRP resulted in the absence of AR protein in cells that expressed AR mRNA, whereas inhibition of BCRP efflux by novobiocin or fumitremorgin C resulted in stabilization and nuclear translocation of AR protein, suggesting a novel mechanism for posttranslational regulation of AR function mechanistically associated with expression of the stem cell marker, BCRP.

**Materials and Methods**

**Cell lines.** Rat prostate progenitor cell lines (RPE, DP2, DP3, and DP4) were established from regenerating prostates of Fischer-344 rats and were maintained in prostate growth media (15) supplemented with 2% fetal bovine serum (FBS; HyClone, Logan, UT) without androgen supplementation. For analysis of AR expression, RPE cells (5 × 10^5 cells/chamber) were incubated in the presence or absence of 3 mmol/L dihydrotestosterone and/or 50 μmol/L fumitremorgin C (Sigma, St. Louis, MO) for 14 hours, fixed in 10% formalin for 5 minutes, and evaluated by immunocytochemistry as described previously (15) utilizing polyclonal anti-AR (Upstate, Lake Placid, NY) and biotinylated goat anti-rabbit antibody (Vector, Burlingame, CA). A subpopulation of RPE cells was selected for resistance to mitoxantrone (Mx-RPE) by continuous culture in increasing concentrations of mitoxantrone (0.2-4 μmol/L; Sigma). For analysis of BCRP-mediated efflux of androgen, Mx-RPE cells (2 × 10^6 cells/well) were preincubated with 0, 5, or 10 mmol/L novobiocin or 10 mmol/L fumitremorgin C (gift of Drs. Robert Robey and Susan Bates, National Cancer Institute, Bethesda, MD) for 20 minutes, and were incubated with 3 mmol/L [3H]dihydrotestosterone (Amersham Biosciences, Buckinghamshire, United Kingdom) with/without novobiocin or fumitremorgin C for 2 hours. Cultures were washed with PBS, cells were lysed with 2 N NaOH, the lysate was neutralized with 2 N HCl and added to ScintiVerse (Fisher,Suwanee, GA), and retained [3H]dihydrotestosterone was quantified by liquid scintillation counting. For analysis of Hoechst 33342 efflux, Mx-RPE cells (5 × 10^6 cells/chamber) were incubated for 20 minutes with Hoechst 33342 (0.1 μg/mL; Sigma) in the presence of 0, 5, or 10 mmol/L novobiocin or 10 μmol/L fumitremorgin C. Hoechst retention was evaluated by fluorescence microscopy.

**Human specimens.** All human prostate specimens were excess tissue harvested at the time of radical prostatectomy or needle biopsies harvested during androgen deprivation therapy, in accordance with NIH guidelines for use of human subjects, with approval by the Institutional Review Board at University of North Carolina.

**Models of benign prostate and prostate cancer.** All experiments using laboratory animals were in accordance with Institutional Animal Care and Use Committee and NIH guidelines. Human prostate primary xenografts were established as described previously (13). TRAMP mice were transgenic F1 males (C57BL/6 TRAMP +/+) by FVB; ref. 14). Twelve-week-old TRAMP mice wereimplanted with 2 pumps for 2 weeks with Alzet Minipumps (Durect Corp., Cupertino, CA) containing 200 μL of bromodeoxyuridine (BrdUrd;60 μg/mL; Sigma). Two days after pump removal, mice were castrated, or sham castrated, and prostates harvested at 0, 1, 2, 4, 7, and 14 days postcastration/sham castration (five mice per group).

**Immunohistochemistry.** Prostate tissue from surgical specimens, human prostate primary xenografts, and TRAMP mice was processed, and immunohistochemistry was done as described previously (13). Tissue specimens were incubated with primary antibodies: polyclonal anti-AR (Upstate); rat monoclonal anti-BCRP (Bxp-53; Caltag Laboratory, Burlingame, CA; ref. 16); rabbit polyclonal antisynaptophysin (Zymed Laboratories, South San Francisco, CA); mouse monoclonal anti-BrdUrd (Sigma); rabbit polyclonal anti-Ki-67 (Novocastra Laboratories, Newcastle upon Tyne,
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United Kingdom); mouse monoclonal anti-SV40Tag (BD PharMingen, San Diego, CA); mouse monoclonal anti-p63 (Santa Cruz, Santa Cruz, CA); mouse monoclonal 34βE12 anti–high molecular weight cytokeratin (Enzo Diagnostics, Farmingdale, NY); goat polyclonal anti-Foxa2 (HNF3; Santa Cruz); mouse monoclonal anti–smooth muscle α-actin (Sigma); or rabbit polyclonal anti–α-methylacyl-CoA racemase (AMACR/P504S; Biocare Medical, Walnut Creek, CA). Biotinylated secondary antibodies (Vector) were utilized, and immunoreactive targets were detected using the Vectastain Elite ABC immunoperoxidase kit and 3,3'-diaminobenzidine, Nova Red (Vector), TrueBlue (KPL, Gaithersburg, MD), or ABC Alkaline Phosphatase Kits I or III (Vector). Incubation without primary antibody and tissue specimens harvested from animals not injected with BrdUrd served as negative controls for immunohistochemical studies; mouse small intestine and ventral prostate were included on each blot as positive controls for each experiment.

Enhanced chemiluminescence detection system (Pierce, Rockford, IL). Rat horseradish peroxidase (Amersham Biosciences) were detected using an antibodies for AR (Calbiochem, San Diego, CA), BCRP (Bxp-21; Chemicon, Walnut Creek, CA). Biotinylated secondary antibodies (Vector) were

phoresed in 4% to 12% Bis-Tris gels (Invitrogen, Carlsbad, CA). Proteins were identified by AMACR (racemase) staining of adjacent serial sections.

Reverse transcription-PCR analysis. Total RNA was isolated with the RNaseasy kit (Qiagen, Chatsworth, CA). Reverse transcription-PCR (RT-PCR) was done with the Advantage RT-PCR kit using primers specific for: GDPDH (BD Biosciences Clonetech, Palo Alto, CA), rat AR (17), and rat BCRP (forward primer: 5′-AGTCCGGAAAACAGCTGAGA-3′; reverse primer: 5′-CCATCACAACGTCATCTTG-3′). PCR conditions consisted of 40 cycles of 1 minute of denaturation at 95°C, 1 minute of annealing at 56°C, and 90 seconds of primer extension at 72°C. PCR reactions containing RNA, but without the reverse transcription reaction, served as negative controls for each RT-PCR experiment. Rat ventral prostate, small intestine, and colon RNA were used as positive controls for each experiment.

Immunoblot analysis. RPE cells and rat tissues were homogenized on ice in lysis buffer [150 mmol/L NaCl, 1% Nonidet P-40, 0.5% Deoxycholic acid, 0.1% SDS, 50 mmol/L Tris-HCl (pH 8.0), 0.4 mmol/L EDTA (pH 8.0), 10% Glycerol] containing a cocktail of protease inhibitors (Complete Mini; Roche, Indianapolis, IN). Homogenates (50 μg of protein) were electro-phoresed in 4% to 12% Bis-Tris gels (Invitrogen, Carlsbad, CA). Proteins were electroblotted to Hybond nitrocellulose membranes (Amersham Biosciences), and proteins of interest were immunodetected using primary antibodies for AR (Calbiochem, San Diego, CA), BCRP (Bxp-21; Chemicon, Temecula, CA), and actin (Santa Cruz). Secondary antibodies conjugated to horseshadish peroxidase (Amersham Biosciences) were detected using an enhanced chemiluminescence detection system (Pierce, Rockford, IL). Rat small intestine and ventral prostate were included on each blot as positive controls for BCRP and AR expression.

Statistical analysis. The percentage of BCRP-expressing cells/total epithelial cells was determined based on a minimum of three ×200 microscopic fields, where at least 1,000 total epithelial cells were counted, and the percentage of BCRP-positive cells was averaged for multiple patients or TRAMP mice. The percent of BCRP-expressing cells/gland was calculated by quantitation of the epithelial compartment from all identifiable glands per section, and means calculated for each patient or TRAMP mouse specimen. Means, SE, and one-way nonparametric ANOVA tests were done using Instat software (GraphPad, San Diego, CA). AR protein levels in immunoblots were evaluated and normalized to actin controls using ImageJ Software (18). Immunohistochemistry for AR expression and Hoechst 33342 fluorescence were analyzed using Optimas software (Media Cybernetics, Silver Springs, MD).

Results

Breast cancer resistance protein-expressing cells in human prostate tissue and primary xenografts. Putative prostate stem cells were identified in histologic sections of surgically resected human prostate tissue as rare, isolated cells that expressed BCRP (BCRP+) and lacked AR protein (AR−; Fig. 1A). Based on the expectation that prostate stem/precursor cells would express basal cell markers, human prostate tissue was analyzed for BCRP+ cells that coexpressed the prostate basal cell markers p63 (Fig. 1B) and/or high molecular weight cytokeratin (Fig. 1C). Although the BCRP+ cells were contained within the p63/high molecular weight cytokeratin expressing basal cell compartment and were localized proximal to the basement membrane, BCRP+ cells did not coexpress p63 or high molecular weight cytokeratin, suggesting strongly that prostate stem/precursor cells are not a subset of the basal cell population. In addition, BCRP+ cells did not coexpress synaptophysin (Fig. 1D) or smooth muscle α-actin (Fig. 1E), demonstrating that BCRP+ cells are neither neuroendocrine cells nor a component of the stromal compartment. Therefore, a minimal phenotype of human prostate stem cells is proposed as BCRP+, AR protein-negative (AR−), p63-negative (p63−), and synaptophysin negative (Syn−). BCRP+/AR−/p63−/Syn− cells comprised 1.04% of the epithelial cells in benign human prostate glands, and were observed at a comparable frequency (0.57%) in glands containing prostate cancer (Fig. 1F; Table 1); prostate cancer was identified with AMACR in serial sections (AMACR staining not shown). In surgically resected human prostate tissue specimens, 35.7% of prostate glands contained at least a single BCRP+ cell. The primary xenografts established by s.c. transplantation of benign and cancer-containing human prostate surgical specimens into androgen-supplemented immunocompromised hosts contained comparable proportions of glands with BCRP+ stem/precursor cells (24.4%) after 1 month of maintenance in an androgenic environment.

Response of BCRP+ cells to androgen deprivation. The proportion of glands that contained BCRP+/AR−/p63−/Syn− cells were comparable in xenografts harvested from hosts after 1 month of establishment in an androgenic environment (24.4%), and xenografts harvested from hosts after 1 month of maintenance in an androgenic environment, castration, and maintenance for an additional 1 month postcastration in an androgen-deprived environment (29.5%). BCRP+/AR−/p63−/Syn− putative stem cells in human prostate histologic specimens were observed as isolated cells, with only rare glands (0.2%) containing multicellular foci of BCRP+ cells, in contrast to the BCRP+/AR−/p63−/Syn− cells. Furthermore, the proportion of the glands (20.4%) in xenografts after 1 month of establishment in intact hosts contained foci of BCRP+ cells. In the primary xenografts, the proportion of glands that contained isolated BCRP+ cells in the surgical specimen (&lt;0.005 comparing the three groups). The cells of the BCRP+ foci were AR− (Fig. 1G), p63− (Fig. 1H), and Syn− (data not shown), demonstrating that they were not secretory epithelial, basal epithelial, or neuroendocrine cells. Stimulation of the residual epithelial cell compartment in the involuted glands with exogenous androgen (for 48 hours) after 30 days of androgen deprivation induced focal proliferative activity in the epithelial compartment (13). The proliferative active cells (Ki67+) seemed to be immediate progeny of surviving BCRP+ cells because they were clustered adjacent to the BCRP+/AR−/p63−/Syn−/Ki67− putative stem cells (Fig. 1I) that survived castration. The loss of BCRP expression in the Ki67+ cells suggests entrance of the progeny of stem cells into the transit/amplifying compartment.

We previously reported that the prostate tumor stem cell compartment in the human prostate xenografts survived...
androgen deprivation, and maintained proliferative capability, by demonstration of a proliferative response to administration of exogenous androgen (13). Consequently, we investigated whether BCRP+ prostate tumor stem/precursor cells survive hormonal therapy in advanced prostate cancer patients. In a single patient for whom serial biopsy specimens after the initiation of androgen deprivation therapy were available, there was evidence of survival and possible expansion of the putative tumor stem cell compartment after 1 month of hormonal therapy (Fig. 1K). In contrast, BCRP+/AR−/p63− cells were observed as rare, isolated cells in a biopsy specimen harvested from an advanced prostate cancer patient undergoing hormonal therapy (Fig. 1J and K). Needle biopsies harvested from TRAMP mice and xenografts harvested after 30 days of androgen deprivation and 2 days of dihydrotestosterone stimulation immunostained for BCRP (blue) and AR (red). BCRP+ cells in “recurrent” tumors in castrate TRAMP mice. Adenocarcinoma of the prostate in TRAMP mice arises as early as 8 weeks of age and is characterized by tumor cells that uniformly express high levels of AR and that regress upon castration. In contrast, poorly differentiated neuroendocrine-like carcinomas in TRAMP mice are characterized by expression of the neuroendocrine marker synaptophysin, and an absence, or weak and heterogeneous, expression of AR. Neuroendocrine-like carcinomas are rare in young TRAMP mice, but arise rapidly in mice that are castrated between 12 and 14 weeks of age (14, 19). In 14-week-old TRAMP mice that showed multiple well-established AR+ adenocarcinomas per gland, BCRP+/AR−/p63− cells represented 0.68% of the epithelial cells in the highly cellular prostate glands, with potential in the absence of androgen, and, therefore, could represent a nidus for recurrent growth.

**BCRP+ cells in “recurrent” tumors in castrate TRAMP mice.**

Adenocarcinoma of the prostate in TRAMP mice arises as early as 8 weeks of age and is characterized by tumor cells that uniformly express high levels of AR and that regress upon castration. In contrast, poorly differentiated neuroendocrine-like carcinomas in TRAMP mice are characterized by expression of the neuroendocrine marker synaptophysin, and an absence, or weak and heterogeneous, expression of AR. Neuroendocrine-like carcinomas are rare in young TRAMP mice, but arise rapidly in mice that are castrated between 12 and 14 weeks of age (14, 19). In 14-week-old TRAMP mice that showed multiple well-established AR+ adenocarcinomas per gland, BCRP+/AR−/p63− cells represented 0.68% of the epithelial cells in the highly cellular prostate glands, with
38.6% of glands containing at least one BCRP+ cell (Table 1). The BCRP+ putative tumor stem cells were localized to foci of AR− cells in glands of the ventral prostate, the site of origin of the greatest number of poorly differentiated carcinomas that arise after castration. Importantly, the frequency of BCRP+ cells was comparable in intact (2.0%) and castrated (1.8%) TRAMP mice (between 1 and 14 days postcastration), suggesting the AR− foci that contained the BCRP+ tumor stem cells were preexisting and not induced by androgen deprivation. However, cells in AR−/SV40Tag+/foci in castrate or intact TRAMP mice are 9-fold (P < 0.001) more likely to be proliferatively active (Ki67+) and express synaptophysin, compared with cells in the AR− foci in intact mice, suggesting that androgen deprivation accelerates, and/or selects for, progression of the tumor stem cell–driven foci that were refractory to androgen deprivation–induced involution.

In castrate TRAMP mice, the cells in the AR− foci that contain the putative tumor stem cells differ morphologically from the adenocarcinoma cells that express AR cytoplasmically (because the host is castrated) and that are SV40Tag−/Ki67− (Fig. 2A–C). Expression of SV40Tag in the AR− foci in the androgen-deprived prostate is regulated potentially by the transcriptional regulatory protein Foxa2 (HNF3β; ref. 20), a member of the forkhead homeobox gene family (Fig. 2D). Foxa2 was expressed consistently in the AR−/SV40Tag+/Ki67− foci in prostates of both castrated (Fig. 2D–F) and intact TRAMP mice (data not shown). The BCRP+/AR− foci (Fig. 2F–J) arise rapidly postcastration (1 day; Fig. 2G) independent of AR-expressing, well-differentiated adenocarcinomas, possibly representing the nidus of the neuroendocrine-like carcinomas (Fig. 2J and K) that progress rapidly postcastration, but that also emerge eventually in intact TRAMP mice.

Our hypothesis that BCRP+ cells represent tumor stem cells in TRAMP mice is supported by the demonstration that BCRP+/AR− cells behave as label-retaining cells, a stem cell characteristic. BCRP+ cells that were prelabeled by incorporation of BrdUrd during a 2-week pulse before castration retained BrdUrd label for 2 weeks after castration (Fig. 2H and I). In contrast, adenocarcinoma cells in the same prostates that also were prelabeled during the pulse period proliferated repeatedly during the chase period, diluting the incorporated BrdUrd to levels below detection. The role of BCRP+ putative tumor stem cells as the nidus of poorly differentiated neuroendocrine-like carcinomas (recurrent cancer) was shown directly in a neuroendocrine-like carcinoma harvested from a castrated TRAMP animal that contained large focal areas of proliferatively active, BCRP+/AR−/Sy+/Ki67+ cells (Fig. 2J and K). The predictable progression of neuroendocrine-like carcinomas in TRAMP mice, in contrast to the limited expansion of the stem cell foci in human xenografts of benign prostate, suggests the activation of a potent mechanism to replace androgen-mediated signaling in castrate TRAMP mice, possibly related to Foxa2-mediated activation of the SV40Tag transgene.

**Breast cancer resistance protein–mediated efflux of androgen from prostate progenitor cells.** We propose BCRP-mediated efflux of androgen in putative prostate stem cells as the mechanism for maintenance of the prostate stem cell phenotype, and that may be associated with their insensitivity to androgen-mediated differentiation and androgen deprivation–induced apoptotic cell death. Constitutive efflux of androgen would block ligand-dependent activation and stabilization of AR, preventing transactivation of AR-regulated genes. The role of BCRP-mediated efflux of androgen (dihydrotestosterone) in maintenance of the phenotype of putative prostate stem cells was investigated indirectly using novobiocin, an inhibitor of BCRP-mediated efflux (8, 21). The rat prostate progenitor cell line RPE (15) expressed BCRP mRNA at levels comparable with rat small intestine and colon, and expressed AR mRNA at levels comparable with rat ventral prostate (Fig. 3A). However, RPE cells contained little detectable AR protein when cultured in 2.0% FBS (Fig. 3B and C), although they expressed substantial levels of AR mRNA. Consequently, the RPE cell line was utilized to examine the role of BCRP-mediated efflux of androgen in the regulation of the AR axis. Incubation of RPE cells with dihydrotestosterone, as a control, with novobiocin, or with novobiocin plus dihydrotestosterone, all resulted in the

<table>
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<th>Prostate model</th>
<th>BCRP+/AR−/p63− cells / total epithelial cells</th>
<th>Glands containing BCRP+/AR−/p63− cells / total glands</th>
<th>Glands containing BCRP+/AR−/p63− foci / total glands</th>
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</thead>
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<tr>
<td>Human non-PCA prostate surgical specimens</td>
<td>1.04 ± 0.23 (n = 10)</td>
<td>35.7 ± 5.6 (n = 10)</td>
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<td>Human PCA surgical specimens</td>
<td>0.57 ± 0.22 (n = 6)</td>
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<td>ND</td>
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<td>Human prostate primary xenografts (50-d posttransplantation)</td>
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<td>24.4 ± 7.6 (n = 7)</td>
<td>20.4 ± 7.9 (n = 7)</td>
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<tr>
<td>Postcastrate human prostate primary xenografts</td>
<td>ND</td>
<td>29.5 ± 9.7 (n = 11) ³</td>
<td>32.2 ± 4.2 (n = 34)³</td>
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<tr>
<td>Intact TRAMP</td>
<td>0.68 ± 0.22 (n = 6)</td>
<td>38.6 ± 7.6 (n = 6)</td>
<td>2.0 ± 0.41 (n = 28)</td>
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<tr>
<td>Postcastrate TRAMP</td>
<td>ND</td>
<td>ND</td>
<td>1.8 ± 0.4 (n = 23)</td>
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Abbreviations: PCA, prostate cancer; n, sample number; ND, not determined.

*Values are represented as a percentage.

¹P < 0.005 comparing human prostate non-prostate cancer surgical specimens, intact human prostate primary xenograft, and postcastrate human prostate primary xenografts.

²Xenografts in hosts 30 days postcastration.

³Xenografts in hosts 1 to 14 days postcastration.
stabilization and nuclear translocation of AR protein as visualized by immunocytochemistry (Fig. 3B). Inhibition of BCRP-mediated transport by novobiocin alone resulted in a 2.4-fold increase in the level of nuclear-localized AR in RPE cells, compared with a 2.8-fold increase in response to incubation with dihydrotestosterone, and a 4.7-fold increase in response to incubation with dihydrotestosterone plus novobiocin (n > 200 cells/group, P < 0.0001). Immunoblot analysis of RPE cell lysates confirmed that novobiocin-mediated inhibition of BCRP function resulted in stabilization of intracellular AR protein, resulting in a 7-fold increase in response to incubation with dihydrotestosterone plus novobiocin (n = 8, P < 0.0001). The more modest increase in AR stabilization measured by digital image analysis of immunocytochemistry compared with the much larger increase measured by immunoblot analysis reflects the limited dynamic range of the digital imaging technology, not a biologically significant difference between the experimental end points.

Novobiocin and fumitremorgin C were compared in RPE cells as inhibitors of BCRP-mediated efflux of Hoechst 33342, the marker utilized to identify the side population phenotype of stem cells. Fumitremorgin C is a specific inhibitor for BCRP-mediated transport. In contrast, whereas novobiocin inhibits BCRP selectively among the family of ABC cassette transporters, novobiocin is also a nonspecific inhibitor of cellular ATPases. Novobiocin was utilized in these studies in spite of the lack of specificity because fumitremorgin C is extremely neurotoxic, limiting use in vivo, whereas novobiocin is a widely utilized antibiotic with well-tolerated toxicity in humans. Inhibition of BCRP-mediated transport in RPE cells by incubation with 5 or 10 mmol/L novobiocin resulted in a 1.2- and 1.45-fold increase in retention of Hoechst 33342, respectively, as determined by fluorescence digital image analysis (Fig. 4). Inhibition of BCRP-mediated transport with 10 μmol/L fumitremorgin C resulted in a 2.3-fold increase in nuclear Hoechst 33342 compared with the DMSO control (Fig. 4), confirming that BCRP is responsible for a majority of Hoechst.

Figure 2. BCRP expression in putative prostate tumor stem cells during the emergence of neuroendocrine-like carcinomas in the TRAMP model. A to C, serial sections of ventral prostate from a TRAMP animal 4 days postcastration immunostained for (A) AR (brown), (B) SV40Tag (brown), and (C) Ki67 (blue) and synaptophysin (pink). D to F, serial section of ventral prostate from a TRAMP animal 14 days postcastration immunostained for (D) Foxa2 (brown), (E) SV40Tag (brown), and (F) BCRP (blue) and AR (red). G, ventral prostate from a TRAMP animal 1 day postcastration immunostained for BCRP (blue) and AR (red) and (H) BrdUrd (blue) and synaptophysin (red). J and K, serial sections of neuroendocrine-like carcinoma in prostates harvested 14 days postcastration immunostained for (J) BCRP (blue) and AR (red), and (K) Ki67 (blue) and synaptophysin (pink). Black arrowheads, AR+/SV40+/Syn+/Ki67+/Foxa2+ foci (transit/amplifying populations) postcastration. Black arrows, BCRP+/AR+ putative tumor stem cells. Red arrows, BCRP+ foci. Red arrowheads, location of BCRP+ cells in the matched serial section (D, E, I, and K). Bar, 20 μm.
efflux in RPE cells. The moderate increase in nuclear Hoechst 33342 dye in RPE cells with inhibited BCRP function reflects a combination of the limited dynamic range of digital imaging technology and the constitutive expression of MDR-1 in the RPE cells (data not shown). MDR-1 also effectively transports Hoechst 33342 dye; however, novobiocin and fumitremorgin C inhibit only the BCRP-mediated component of Hoechst efflux, not MDR-1 function (8, 21).

BCRP-mediated efflux of androgen (dihydrotestosterone) was evaluated directly in a subline of RPE. Mx-RPE is selected by continuous culture in increasing levels of mitoxantrone, a prototypical substrate for BCRP transport (8). Mitoxantrone resistance in cell lines selected by the same protocol is usually associated with increased expression of BCRP due to gene amplification (22). Inhibition of the BCRP-mediated efflux of [3H]dihydrotestosterone in Mx-RPE cells by 5 or 10 mmol/L novobiocin or by 10 μmol/L fumitremorgin C resulted in 2.4-, 5.5-, and 3.3-fold increases in intracellular levels of [3H]dihydrotestosterone, respectively (Fig. 4), demonstrating that androgen is transported from Mx-RPE cells specifically by BCRP. In contrast to the RPE cells that express BCRP, three rat prostate epithelial cell lines established in our laboratory (DP2, DP3, and DP4) do not express BCRP, and only DP3 expresses AR mRNA (Fig. 3A). Consequently, the DP3 cell line was utilized as a control to identify alternative mechanisms for intracellular accumulation of [3H]dihydrotestosterone in the absence of BCRP-mediated efflux, particularly mechanisms related to collateral effects of the ATPase inhibitory activity of novobiocin or to ligand-independent stabilization of AR protein. The DP3 cell line that lacked endogenous BCRP-mediated transport accumulated 3-fold higher concentrations of [3H]dihydrotestosterone than the Mx-RPE cells in the absence of novobiocin or fumitremorgin C. Novobiocin had no effect on the level of accumulation of intracellular [3H]dihydrotestosterone in the DP3 cell line, suggesting that BCRP and not other ABC transporters or ATPase-dependent molecules, was the principal mechanism of cellular efflux of dihydrotestosterone. Constitutive BCRP-mediated efflux of androgen, therefore, resulted in prostate progenitor cells that lacked AR protein, whereas expressing AR mRNA, and loss/inhibition of BCRP function, allowed rapid cellular accumulation of dihydrotestosterone with stabilization and nuclear localization of AR protein.

Discussion
This study has identified a consistent phenotype for a putative prostate stem cell and tumor stem cell in human clinical prostate specimens, primary xenografts of human prostate tissue, the TRAMP mouse prostate, and in a rat prostate progenitor cell in culture. The definitive phenotypic characteristic of putative prostate stem cells, expression of BCRP and lack of AR protein (BCRP+/AR−), was linked mechanistically to the maintenance of the prostate stem cell in the stem cell state through the demonstration that BCRP mediated the constitutive efflux of dihydrotestosterone, preventing function of the AR-mediated androgen axis. Constitutive transport of androgen would provide a posttranslational regulation of AR level and function, blocking AR-mediated exit from the stem cell compartment and commitment to differentiation.

BCRP was initially identified in chemotherapy-resistant cancer cells (including prostate cancer) as a cellular defense mechanism...
responsible for efflux of multiple types of unrelated chemotherapeutic agents, such as mitoxantrone, camptothecin-derived and indol carbazole topoisomerase I inhibitors, and methotrexate (8, 21, 23, 24). More recently, BCRP was shown to either transport, or to be competitively inhibited by, multiple sterols, steroids, estrogens, antiestrogens, sulfated estrogens, and DHEA sulfate (8, 9). In addition, BCRP is the ABC cassette transporter that characterizes the side population from bone marrow that contains the c-kit+/Sca-1+/Lin−/CD34− hematopoietic stem cells that provide long-term repopulation of the bone marrow of sublethally irradiated mice (25–27). Analysis for side populations in multiple tissues has identified BCRP+ stem/progenitor cells in bone marrow, muscle (satellite skeletal muscle cells), and neural tissue that show pluripotentiality when transplanted to multiple organ sites (7, 26, 27). However, BCRP is expressed in several differentiated cell types, including placental syncytiotrophoblasts; epithelium of small intestine, colon, ovary, kidney, heart, ducts, and lobules of breast; liver canalicular cells; brain; desmoplastic stroma; inflammatory cells; and venous and capillary endothelium (8, 9, 24, 28). In the current study, immunohistochemical staining of BCRP expression in endothelial cells of the prostatic vasculature provided a reproducible internal control. The proportion of BCRP+ cells in human prostate epithelium quantitated in situ was higher than the BCRP+ side population in human bone marrow (0.05%) or lung (0.03-0.07%), measured by flow microfluorometry (26, 29). The elevated proportion of BCRP+ cells relative to differentiated epithelial cells in the human prostate enumerated in situ, compared with the lower estimate of the side population quantitated by flow cytometry in disaggregated tissues, could reflect both the inclusion of multiple nonepithelial cell populations in the non–side population compartment in flow analyses, reducing the apparent representation of stem cells, and that the human prostate specimens were harvested from the peripheral zone, the portion of the prostate anticipated to be enriched in cells capable of repopulating the prostatic epithelium. However, the consistency of the number of BCRP+ putative stem cells observed in surgical specimens of human prostate, human prostate primary xenografts, and the prostates of TRAMP animals suggests that these cells are present normally in human and rodent prostate epithelium.

The BCRP+/AR− stem/precursor cell was shown to survive androgen deprivation, and to not only retain proliferative potential but to undergo limited focal expansion in xenografts of benign prostate tissue maintained in the androgen-deprived microenvironment of castrate hosts. However, there was no evidence of a repopulation of the epithelial compartment after 1 month of androgen deprivation. In fact, in human xenografts of benign prostate tissue, a small proportion of the immediate progeny of the stem cell seemed to differentiate preferentially along a neuroendocrine lineage in the absence of androgen, and differentiated along a secretory epithelial lineage only under stimulation of exogenous androgen (13). In contrast, androgen deprivation did not prevent the progeny of prostate tumor stem cells from differentiating preferentially along a secretory epithelial lineage in the absence of androgenic signaling in our primary xenografts of prostate cancer, in other currently available xenograft models of prostate cancer (CWR22, LAPC, or LuCaP; ref. 30), and in human prostate cancer patients undergoing hormonal therapy (31). However, the mechanism responsible for the increased cellular proliferation, stabilization of AR protein with up-regulation of AR-mediated transactivation, and commitment to differentiation along the secretory epithelial lineage in the absence of androgen is unknown. This study suggests that the neuroendocrine-like carcinomas that progress rapidly after castration in TRAMP mice provide a valuable model of the cellular origin of the androgen-independent expansion of recurrent prostate cancers in advanced prostate cancer patients. Our studies suggest that the neuroendocrine-like, poorly differentiated carcinomas that arise rapidly after androgen deprivation are derived from latent tumor stem cell–fed foci. However, it has been proposed that an adenocarcinoma cell may lose expression of differentiation markers and acquire more stem/progenitor cell properties, representing a cell with tumor-initiating potential (32). Tumor stem cells survive androgen deprivation, and the foci are independent of, not a progression from, the well-differentiated adenocarcinomas that result from AR-mediated SV40Tag transgene expression in secretory epithelial cells growing in an androgenic environment. Unexpectedly, the AR−/SV40+/p63−/Syn+ foci were found to retain expression of the SV40Tag transgene in the absence of the androgen-mediated signaling that normally regulates transcription of its probasin promoter (33). The AR− foci were examined for alternative potential inducers of the AR-driven, probasin promoter–regulated transgene, such as progesterone receptor, glucocorticoid receptor, and estrogen receptor α, but their expression was not detected within the foci (data not shown). However, cells in the AR−/SV40+/p63−/Syn+ foci were observed to express Foxa2 (HNF-3β), a homeobox gene critical in determining endodermal cell fate during development (34), including formation of prostate buds in early stages of organogenesis (35). Foxa2 expression in the NE-like carcinomas in TRAMP was comparable to the pattern of Foxa2 expression in the NE carcinomas in the 12T-10 LADY model (20). Foxa2 represents a potential alternative to androgen as a transcriptional regulator of the SV40Tag transgene in the early poorly differentiated cancers in that the probasin promoter contains HNF-3-binding sites, and probasin can be transcriptionally activated by Foxa2 in the absence of androgen (20). Molecular phenotyping of these two tumor types in TRAMP showed marked differences between the well-differentiated adenocarcinomas and the poorly differentiated neuroendocrine-like carcinomas for expression of signaling molecules and differentiation markers, proliferation, apoptosis, vessel density, and androgen responsiveness, supporting our hypothesis that in TRAMP, adenocarcinomas are not the precursor of the poorly differentiated neuroendocrine-like carcinoma (14, 36–41). In fact, the well-differentiated adenocarcinomas may suppress expansion of the AR−/SV40+/p63−/Syn+/Foxa2+ foci in intact TRAMP animals, and the castration-induced regression of the AR+ adenocarcinomas may allow progression of the AR−/SV40+/p63−/Syn+/Foxa2+ foci derived from the BCRP+ tumor stem cell. The general lack of coexpression of BCRP with Foxa2 and synaptophysin, except in early foci of poorly differentiated tumors, suggests that BCRP+/Foxa2+/Syn+ cells may represent an intermediate population. The lack of coexpression of Foxa2 and synaptophysin with either BCRP or AR suggests that Foxa2+/Syn+ cells may represent a transit/amplifying compartment between the BCRP+ putative tumor stem cells and the AR-expressing “differentiated” tumor epithelial cells. However, whereas characterization of the mechanism(s) that regulate expression of SV40Tag in castrate TRAMP mice in the absence of androgenic signaling may be of relevance to our understanding the reappearance of AR function in the transition to recurrent prostate cancer in humans, the transforming function of the SV40Tag in the putative TRAMP tumor stem cell may result from a TRAMP-specific mechanism(s).
response to microenvironmental signaling that facilitates the stochastic progression of androgen-insensitive prostate tumor stem cells to poorly differentiated "recurrent" tumors.

Apoptotic death of secretory epithelial cells in response to androgen deprivation is a consequence not only of a direct effect of loss of androgenic signaling to the epithelial cells, but also of changes in the prostate stroma and prostate vasculature (42–44). The prostatic vasculature undergoes vasoconstriction, decreased blood flow, and endothelial cell apoptosis within 24 hours following androgen ablation (43–46). Hypoxic conditions can be shown in the rat prostate microenvironment as early as 2 days after castration, accompanied by a significant increase in hypoxia-inducible factor-1α (HIF-1α) protein (47). In human prostate xenografts and TRAMP prostate, BCRP+/AR+/p63+/Syn+ stem/precursor cells survived androgen deprivation and the hypoxia that resulted from vascular regression. Recently, BCRP was shown to enhance survival of hematopoietic stem cells through activation of the hypoxic response mechanism, with the resultant increased level of HIF-1α protein capable of inducing BCRP gene expression with an expansion of the side population (48). In addition to hypoxia, mitogens, such as insulin, IGF-I, and IGF-II, or activated HER2/neu can induce expression of HIF-1α and loss of the tumor suppressor genes PTEN, p53, or VHL can result in posttranslational stabilization of HIF-1α protein (49), supporting the potential for constitutive regulation of HIF-1α function in tumor cells by mechanisms beyond induction by hypoxia. Consequently, survival and focal expansion of BCRP+ prostate tumor stem cells in an androgen-deprived human prostate may reflect a combination of HIF-1α–mediated protection from hypoxia and HIF-1α–mediated transcriptional up-regulation of BCRP, mitogens, and cell survival factors, in contrast to the driving role of SV40Tag in TRAMP.

The cellular origins of prostate cancer and recurrent prostate cancer are unknown. Figure 5 presents a model for the role of BCRP in the response of prostate stem cells to the prostate tissue microenvironment in primary prostate cancer arising in an androgen-stimulated prostate, and in recurrent prostate cancer in an androgen-deprived prostate. BCRP expression maintains the stem cell compartment via constitutive efflux of androgen, with exit of the progeny of the stem cell into the transit/amplifying compartment associated with the loss of BCRP expression and stabilization of AR protein and AR-mediated transactivation. Progeny of the stem cell/tumor stem cell are under the inductive influence of the prostate microenvironment, with AR-mediated signals directing differentiation of both benign and malignant progeny along a secretory epithelial lineage. The probability of progression of the progeny of the tumor stem cell into a clinically significant malignancy is determined by a balance between the loss of differentiation-inducing signaling from the prostate tissue microenvironment...
with age, and the nature of the transforming genetic changes. Therefore, many individual patients may die of other causes before the loss of tissue-organizing/tumor-suppressing signaling allows progression of the latent cancer. Alternatively, perturbation of tissue homeostasis by environmental factors may accelerate the degradation of tissue-organizing signaling, precipitating the emergence of a clinical cancer in advance of the age-related loss of suppressive signaling. In the androgen-deprived prostate, the progeny of benign stem cells do not transit the transit/amplifying compartment; however, the progeny of tumor stem cells can transit the transit/amplifying and acquire proliferative potential. Development of the poorly differentiated carcinomas in TRAMP seems to be driven by SV40Tag expression in the progeny of the tumor stem cell. In contrast, in advanced prostate cancer in humans, tumor stem cells survive hormonal therapy and are stimulated to proliferate, and their progeny to enter and transit the transit/amplifying compartment by alternative autocrine/paracrine signaling pathways (such as the HIF-1α pathway), by mitogenic products of neuroendocrine cells, and/or by the low level of dihydrotestosterone present in the androgen-deprived prostate (50), setting the stage for failure of hormonal therapy.

BCRP expression as an integral component of the prostate stem/progenitor cell phenotype provides new insights into the pathogenesis of prostate cancer. Androgen deprivation therapy of advanced prostate cancer must be viewed as a double-edged sword; while producing marked symptomatic improvement, it may predispose to recurrent growth by favoring population expansion from BCRP+/AR+ tumor stem cells. Further, BCRP expression by prostate tumor stem cells suggests why mitoxantrone is a prototypical substrate for BCRP expression by prostate tumor stem/progenitor cells and eliminates the nulid of recurrent prostate cancer, and use of inhibitors of BCRP in combination with hormonal, chemotherapeutic, or angiogenic therapies would target all cellular components of prostate cancer.

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References


Breast Cancer Resistance Protein–Mediated Efflux of Androgen in Putative Benign and Malignant Prostate Stem Cells
