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Stem-Like Cells with Luminal Progenitor Phenotype Survive Castration in Human Prostate Cancer

MARKUS GERMANN,[a,b] ANTOINETTE WETTERWALD,[a,b] NATALIA GUZMÁN-RAMIREZ,[a,b] GABRI VAN DER PLUIJM,[c] ZORAN CULIG,[d] MARCO G. CECCHINI,[a,b] ELIZABETH D. WILLIAMS,[e] GEORGE N. THALMANN[a,b]

[a]Department of Urology, and [b]Department of Clinical Research, University of Bern, Bern, Switzerland; [c]Department of Urology, Leiden University Medical Center, Leiden, The Netherlands; [d]Department of Urology, Innsbruck Medical University, Innsbruck, Austria; [e]Centre for Cancer Research, Monash Institute of Medical Research, Monash University, Melbourne, Victoria, Australia

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ABSTRACT

Castration is the standard therapy for advanced prostate cancer (PC). Although this treatment is initially effective, tumors invariably relapse as incurable, castration-resistant PC (CRPC). Adaptation of androgen-dependent PC cells to an androgen-depleted environment or selection of pre-existing, CRPC cells have been proposed as mechanisms of CRPC development. Stem cell (SC)-like PC cells have been implicated not only as tumor initiating/maintaining in PC but also as tumor-reinitiating cells in CRPC. Recently, castration-resistant cells expressing the NK3 homeobox 1 (Nkx3-1) (CARNs), the other luminal markers cytokeratin 18 (CK18) and androgen receptor (AR), and possessing SC properties, have been found in castrated mouse prostate and proposed as the cell-of-origin of CRPC. However, the human counterpart of CARNs has not been identified yet. Here, we demonstrate that in the human PC xenograft BM18, pre-existing SC-like and neuroendocrine (NE) PC cells are selected by castration and survive as totally quiescent. SC-like BM18 cells, displaying the SC markers aldehyde dehydrogenase 1A1 or NANOG, coexpress the luminal markers NKX3-1, CK18, and a low level of AR (AR^{low}) but not basal or NE markers. These CR luminal SC-like cells, but not NE cells, reinitiate BM18 tumor growth after androgen replacement. The AR^{low} seems to mediate directly both castration survival and tumor reinitiation. This study identifies for the first time in human PC SC-/CARN-like cells that may represent the cell-of-origin of tumor reinitiation as CRPC. This finding will be fundamental for refining the hierarchy among human PC cancer cells and may have important clinical implications. STEM CELLS *2012;30:1076–1086*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Radical prostatectomy and/or local radiotherapy are the standard treatment for patients with organ-confined prostate cancer (PC). However, in 15%–33% of these patients, PC progresses as metastatic disease [1], which is treated by androgen-deprivation therapy (ADT). This treatment is initially effective, as it causes apoptotic regression of the bulk of tumor cells and/or growth arrest of residual cells [2–4]. Nevertheless, PC invariably relapses within 12–33 months as incurable, castration resistant (CRPC) [5, 6]. The emergence of castration resistant, tumor-reinitiating cancer cells is responsible for this phenomenon.

The prevailing "adaptation" model postulates that, under the pressure of the androgen-depleted condition, CRPC cells arise stochastically through genetic/epigenetic conversion of originally androgen-dependent PC cells [7]. Mechanisms of adaptation have been identified in alterations in the androgen receptor (AR) signaling, such as AR gene amplification and increased expression, AR gene mutations, alterations in AR corepressor/coactivator function, and increased intratumoral steroidogenesis [8].

The alternative "selection" model assumes that both castration-sensitive and CR cells pre-exist in PC. Castration provides selective pressure leading to apoptosis of the bulk of castration-sensitive cells but persistence of a relatively rare cell population that survive castrate levels of androgens

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Correspondence: Marco G. Cecchini, M.D., Urology Research Laboratory, Department of Clinical Research, University of Bern, Murtenstrasse 35, 3010 Bern, Switzerland. Telephone: +41-31-632-2259; Fax: +41-31-632-0551; e-mail: marco.cecchini@dkf.unibe.ch Received July 14, 2011; Revised February 21, 2012; accepted for publication February 26, 2012; first published online in STEM CELLS *EXPRESS* March 21, 2012. 1066-5099/2012/$30.00/0 doi: 10.1002/stem.1087

[3, 9]. This population may contain cells already CR for growth, while others may acquire this ability subsequently, through genetic/epigenetic events [2, 9, 10].

The epithelium of the prostate gland is highly dependent on androgens for its development and homeostasis. Castration in rodents induces rapid prostate gland involution, characterized by massive apoptosis within the luminal, secretory compartment, but persistence of a residual subpopulation of cells able to fully reconstitute the glandular epithelium after repeated cycles of long-term androgen-deprivation and androgen replacement [11]. This indicates that epithelial stem cells (SC), with self-renewing and high proliferative/differentiation potential, survive castrate levels of androgens but are dependent on intact levels of androgens for expansion and differentiation and, thus, persist as cell cycle arrested ("dormant").

PC cells with SC-like properties have been implicated in tumor initiation and maintenance [12, 13]. Several investigations have contributed to their identification in human PC cell lines [14–20], in human PC xenografts [21–24], and in clinical PC samples [20, 23, 25–27].

It has been postulated that SC-like PC cells may survive castrate levels of androgens and be the cell-of-origin of the progression to CRPC [28, 29]. CR cells have been shown to pre-exist at a low frequency in the human PC xenograft model LAPC-9 and are proposed to be SC like [2]. However, experimental evidence for an enhanced expression of SC markers in androgen-depleted PC cells in vitro and in vivo is limited [27, 30]. Importantly, the demonstration that PC cells surviving castrate levels of androgens in vivo are pre-existing cancer SC-like cells is lacking and still unresolved is their position in the hierarchy of the human prostate epithelium [12].

The relatively novel human PC xenograft model BM18 is highly castration-sensitive for survival and growth, as indicated by a nearly complete castration-induced tumor regression in vivo. It persists as androgen responsive for growth throughout prolonged periods of androgen depletion but does not develop spontaneous, androgen-independent growth reinitiation [31]. Therefore, we argued that, because of its extreme castration-induced regression, the BM18 xenograft might contain a concentrate of dormant, CRPC cells that could facilitate their characterization.

Here, we show that in the androgen-dependent BM18 xenograft model, in vivo PC cells with SC-like characteristics survive castration as dormant, express a luminal progenitor phenotype with low AR expression, and possess tumor-reinitiation potential after androgen replacement. These results strongly support the hypothesis that a first step in the progression to CRPC is the selection by castration of pre-existing PC SC-like cells.

Materials and Methods

Xenografts

BM18 xenografts were maintained by serial transplantation in male mice with severe combined immunodeficiency (SCID) male SCID mice (Charles River, L'Arbresle, France, www.criver.com), as described previously [31]. Animal protocols were approved by the Committee for Animal Experimentation and the Veterinary Authorities of the Canton of Bern, Switzerland. Castration was achieved by bilateral orchiectomy. For short-term androgen replacement, testosterone pellets (10 mg, 3-day total release time, Innovative Research of America, Sarasota, FL, www.innovrsrch.com) were implanted subcutaneously into hosts 2–36 weeks after castration. For longer periods, mice were injected i.m. every second week with testosterone (1 mg/30 μl ricin oil) (Testoviron, BayerScheringPharma, Zurich, Switzerland, www.bayer.ch). Continuous BrdU labeling (Sigma-Aldrich, Buchs, Switzerland, www.sigmaaldrich.com) from day 2 to day 16 (day of sacrifice) after castration was achieved by subcutaneous implantation of an osmotic pump (14 days release time, Alzet, Charles River), containing 6 mg BrdU (Sigma-Aldrich), dissolved in 0.9% NaCl. Excised tumors were stored in RNA later (Qiagen, Hombrechtikon, Switzerland, www.qiagen.ch) for subsequent RNA extraction. For histological procedures, tumors were embedded in Tissue-Tek (Haslab, Ostermundigen, Switzerland, www.haslab.ch) and stored at 80°C or, alternatively, fixed in Bouin's solution or 4% formalin and embedded in paraffin.

Gene Expression Analysis

RNA was extracted using RNeasy extraction kit (Qiagen, Hombrechtikon, Switzerland, www.qiagen.ch). cDNA synthesis was done using M-MLV reverse transcriptase and random hexamer primers (Promega, Dübendorf, Switzerland, www.promega.com). mRNA expression was determined by real-time PCR using human-specific TaqMan gene expression assays and an ABI-Prism 7500 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland, www.appliedbiosystems.com). Gene expression was normalized to human-specific β-actin as endogenous control. Marker gene abbreviations and corresponding gene expression assays are listed in Supporting Information Table S1.

Immunohistochemistry and Immunofluorescence

Immunohistochemical and immunofluorescent staining was carried out on deparaffinized sections or on acetone-fixed cryosections. Primary antibodies used for immunolocalization are listed in Supporting Information Table S2. Irrelevant mouse and rabbit-IgG served as negative control antibodies. For staining procedures, antibodies were diluted to the required concentrations with Dako Antibody Diluent (Dako, Baar, Switzerland, www.dako.com), supplemented with 5% goat serum. For immunohistochemistry, sections were blocked for endogenous peroxidase with 3% hydrogen peroxide. Horseradish peroxidase-polymer (Envision, Dako) and 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich) as chromogen served as detection system and sections were counterstained with Hematoxylin. For immunofluorescence, secondary antibodies conjugated with DyLight-488 or DyLight-549 (Pierce, Fisher Scientific, Wohlen, Switzerland, www.ch.fishersci.com) and counterstaining with DAPI or Hoechst 33528 (Sigma-Aldrich) were used.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism Version 4 (GraphPad Software Inc., La Jolla, USA, www.graphpad.com). Two-tailed t test was used to analyze differences in marker gene mRNA expression, parameters of tumor histomorphometry, and percentage of marker-specific cell populations.

Results

The BM18 Xenograft Is Highly Dependent on Androgens

We could confirm that BM18 xenografts show an almost complete regression in tumor volume within 14–21 days after host castration (Fig. 1A; Table 1) [31]. Growth progression in BM18 residual tumors in castrated hosts did not occur spontaneously but could be reinitiated by androgen replacement up to 7 months after castration (Fig. 1A), with full reconstitution of the original tumor morphology (Fig. 1B, 1D).

The histological aspect of the BM18 xenograft in intact hosts was characterized by densely packed, moderately differentiated pseudoacinar structures (Fig. 1B). In castrated hosts, only residual, scattered cancer cell clusters of very small size persisted within an abundant stroma (Fig. 1C). Consequently, loss in cancer cell-specific mass (−99%) exceeded the regression in general tumor volume (−93%) (Table 1).

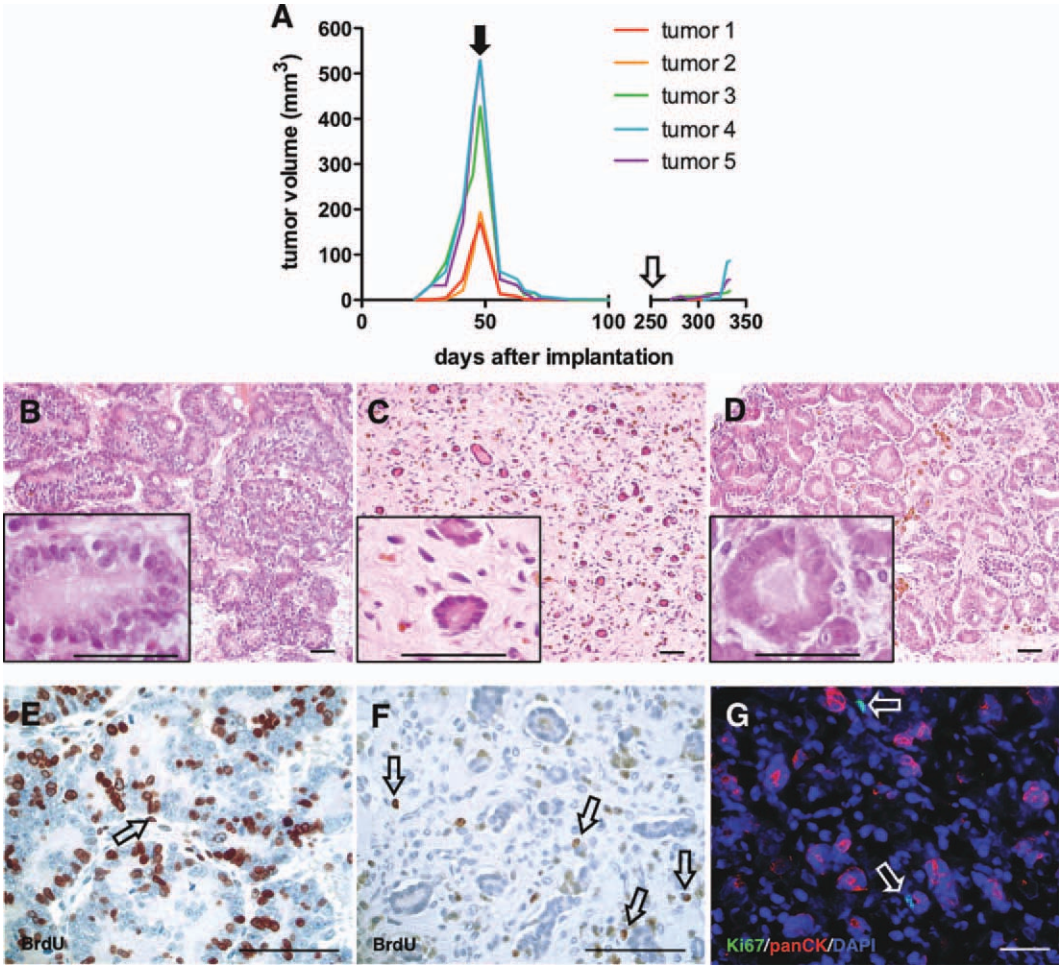

Figure 1. In BM18 xenografts, a minority of cells survives castration and has tumor-reinitiation potential. BM18 xenografts were grown subcutaneously in intact male mice with severe combined immunodeficiency (SCID) mice to an average size of 420 mm^3. Mice were subsequently castrated and, 7 months after surgery (day 250), were androgen replaced. **(A)**: Representative time course of tumor growth in different host mice. Filled arrow indicates time of castration and open arrow time of androgen replacement. H&E stained sections of paraffin-embedded BM18 tumors **(B)** before castration, **(C)** 21 days after castration, and **(D)** 7 months after castration and subsequent, additional 36 days of androgen replacement. **(E)**: BrdU incorporation in a BM18 xenograft in an intact host labeled for 2 days. **(F)**: BrdU incorporation in a BM18 xenograft in a castrated host labeled for 2 weeks following surgery. **(G)**: Immunofluorescent colocalization of Ki67 (green) and pan-CK (red) in a BM18 xenograft in a castrated host 21 days after surgery. Blue: DAPI nuclear counterstaining. Open arrows BrdU- or Ki67-positive stromal cells. Insets represent a higher magnification of selected areas. Scale bar = 50 μm. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; pan-CK, pan-cytokeratin.

CR Cells Survive As Dormant

A significant proportion of cancer cells in BM18 xenografts in intact hosts was actively cycling, as indicated by BrdU labeling (Fig. 1E) and Ki67 staining (Supporting Information Fig. S7A). Instead, all CR BM18-cancer cells lacked BrdU labeling and Ki67 staining ($Ki67^{neg}$). Few BrdU-labeled and/or Ki67-positive ($Ki67^{pos}$) cells in BM18 xenografts in castrated hosts were scattered within the stroma and lacked expression of the general epithelial marker pan-cytokeratin (pan-CK) (Fig. 1F, 1G).

CR Cells Express Markers of Either Luminal or Neuroendocrine Differentiation

It is assumed that xenografts consist of human tumor cells and mouse stromal cells [32]. However, the human stromal component of the tumor may also be xenografted and represent a confusing variable. Hoechst 33258 staining proved that all stromal cells contained in the BM18 xenograft had the punctate nuclear staining characteristic of mouse cells described by Cunha and Vanderslice [33], whereas the epithelial cancer cells had the diffuse nuclear staining characteristic of human cells (Supporting Information Fig. S1A, S1B). Therefore, human-specific real-time PCR could be used to dissect cancer cell specific from stroma-specific (mouse) gene mRNA expression.

Residual BM18-cancer cells 14 days after castration showed significantly reduced prostate-specific antigen (*PSA*), NK3 homeobox 1 (*NKX3-1*), and *AR* and increased expression of *CK18* and of the neuroendocrine (NE)-marker genes chromogranin A (*CHGA*), synaptophysin (*SYP*), and neuron-specific enolase (*NSE*), as compared to BM18-cancer cells in intact hosts. The mRNA expression for the basal cell markers *CK5*, *CK14*, and *p63* was consistently undetectable in BM18 tumors (Fig. 2).

The protein expression of prostate lineage-specific and pan-epithelial markers in BM18 xenografts of castrated hosts was compared to that in intact hosts (Table 2). BM18-cancer cells in intact hosts showed a strong cytoplasmic immunoreactivity for PSA (PSA^{high}) and CK18 ($CK18^{high}$), and all their nuclei were strongly positive for NKX3-1 ($NKX3\text{-}1^{high}$) and AR (AR^{high}) (Fig. 3A, 3C, 3E, 3G). Approximately half of BM18-cancer cells in castrated hosts showed low PSA

Table 1. Pre-existing subpopulation(s) of cells expressing the neuroendocrine marker chromogranin A ($CHGA^{POS}$) or the stem cell-like markers ALDH1 ($ALDH^{POS}$), NANOG ($NANOG^{POS}$), and CD44 ($CD44^{POS}$) are enriched in BM18 xenografts in castrated hosts

	Intact host average (± SD, *n*)	Castrated host Average (± SD, *n*)	Mean ratio of intact/castrated (Δ%)	*p* value
Tumor volume (mm^3)	346 (± 166, 5)	20 (± 14, 5)	18/1 (−93%)	<.001
Cancer cell number/total area (cells per square millimeter)	5004 (± 329, 5)	782 (± 225, 5)	6.4/1 (−84%)	<.001
Cell number/cancer cell area (cells per square millimeter)	5′633 (± 264, 3)	18′686 (± 958, 3)	1/3.31	<.001
Total cancer cell mass			100/1 (−99% ± 0.5%)	
$ALDH1^{POS}$ cancer cells/total cancer cells (%)	0.022 (± 0.012, 2)	3.646 (± 2.209, 3)	1/166	.047
$NANOG^{POS}$ cancer cells/total cancer cells (%)	0.662 (± 0.384, 2)	6.496 (± 1.858, 2)	1/10	.049
$CD44^{POS}$ cancer cells/total cancer cells (%)	0.30 (± 0.153, 2)	1.407 (± 0.768, 2)	1/5	.184
$CHGA^{POS}$ cancer cells/total cancer cells (%)	0.084 (± 0.038, 5)	3.241 (± 1.915, 4)	1/38	.007
% $ALDH1^{POS}$ cancer cells corrected for cancer cell loss	0.022 (± 0.012, 2)	0.044 (± 0.017, 3)	1/2	.1410
% $NANOG^{POS}$ cancer cells corrected for cancer cell loss	0.662 (± 0.384, 2)	0.442 (± 0.127, 2)	3/2	.522
% $CD44^{POS}$ cancer cells corrected for cancer cell loss	0.30 (± 0.153, 2)	0.096 (± 0.052, 2)	3/1	.216
% $CHGA^{POS}$ cancer cells corrected for cancer cell loss	0.084 (± 0.038, 5)	0.029 (± 0.018, 4)	3/1	.0174

Tumor volume, cancer cells per total area and per cancer cell area, total cancer cell mass, percentage of $ALDH1^{POS}$, $NANOG^{POS}$, $CD44^{POS}$, and $CHGA^{POS}$ cells were determined in BM18 xenografts from intact hosts and from hosts 3 weeks after castration.
Tumor size was measured with caliper; tumor volume was calculated by the formula: (larger diameter) × (smaller diameter)^2/2.
Cancer cell number/total area and cancer cell number/cancer cell area were determined in at least 10 random fields of 100 μm^2 each per section, two sections for each tumor xenograft. The ratio and percent variation (Δ%) of total cancer cell mass after castration were calculated by multiplying the ratios of tumor volume and cancer cell number/total area for each individual xenograft. Marker-positive cancer cells were scored in histological sections stained as described in Materials and Methods. The correction of the percentage of marker-positive cancer cells for cancer cell loss in tumors from castrated hosts was calculated by dividing the percentage of positive cells by the intact/castrated-ratio of total cancer cell mass. This was done for each tumor individually. *p* values were calculated by *t* test.
Abbreviations: ALDH1, aldehyde dehydrogenase 1; CHGA, chromogranin A

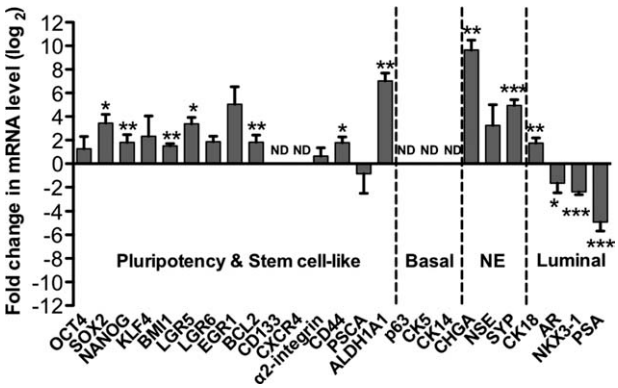

Figure 2. Castration selects stem cell (SC)-like and NE cells in BM18 xenografts. Relative mRNA expression levels of pluripotency, prostate SCs, NE, and epithelial differentiation marker genes in BM18 xenografts in castrated hosts 14 days after surgery ($n = 4$). Values are shown as mean ± SD relative to BM18 xenografts in intact hosts ($n = 4$). *, **, ***: Significantly different from BM18 xenografts in intact hosts (*, $p < .05$; **, $p < .01$; ***, $p < .001$). Abbreviations: *ALDH1A1*, aldehyde dehydrogenase 1A1; *AR*, androgen receptor; *BCL 2*, B-cell CLL/lymphoma 2; *BMI1*, BMI1 polycomb ring finger oncogene; *CHGA*, chromogranin A; *CK5*, *CK14* and *CK18*, cytokeratin 5, 14 and 18; *CXCR4*, chemokine (C-X-C motif) receptor 4; *EGR1*, early growth response 1; *KLF4*, Kruppel-like factor 4; *LGR5* and *LGR6*, leucine-rich repeat-containing G-protein-coupled receptor 5 and 6; ND, not detectable; *NE*, neuroendocrine; *NKX3-1*, NK3 homeobox 1; *NSE*, neuron-specific enolase; OCT4, octamer-binding protein 4; *PSA*, prostate-specific antigen; *PSCA*, prostate stem cell antigen; *SYP*, synaptophysin.

(PSA^{low}) expression and only a small fraction retained a degree of PSA expression equivalent to that in intact hosts (PSA^{high}) (Fig. 3B). The vast majority of them maintained nuclear NKX3-1 expression at a low level ($NKX3\text{-}1^{low}$), but a small proportion exhibited a high NKX3-1 ($NKX3\text{-}1^{high}$) expression (Fig. 3D). Virtually, all CR cells retained high CK18 expression (Fig. 3F). Most of residual cancer cells in castrated hosts showed a clear but weak AR nuclear staining (AR^{low}) and only rare cells within the cancer cell clusters were completely AR negative (AR^{neg}). All stromal cells infiltrating the tumor both in intact and castrated hosts were AR^{neg} (Fig. 3G, 3H). Consistently with the mRNA expression analysis, no protein expression of CK5, CK14, and CK19 could be detected in cancer cells of BM18 xenografts either in intact or castrated hosts (Table 2). The pan-epithelial markers EpCAM and pan-CK were strongly expressed in BM18-cancer cells with no obvious difference between xenografts in intact or castrated hosts (Table 2).

A very small proportion of BM18-cancer cells in intact hosts expressed the NE markers (NE^{pos}) CHGA and SYP, but this proportion was strongly increased in xenografts in castrated hosts (Fig. 3J, 3K; Tables 1, 2, Supporting Information Fig. S2A, S2B). NE^{pos} cells after castration were invariably all AR^{neg} (Supporting Information Fig. S2C).

CR Cells Coexpress SC-Like and Luminal Markers

CR BM18-cancer cells showed significantly increased mRNA expression of *SOX2*, *NANOG*, *BMI1* polycomb ring finger oncogene (*BMI1*), leucine-rich repeat-containing G-protein-coupled receptor (*LGR*) 5 (*LGR5*), B-cell CLL/lymphoma 2 (*BCL2*), *CD44*, and aldehyde dehydrogenase 1A1 (*ALDH1A1*). There was also an increase, although not significant, in mRNA expression of octamer-binding protein 4

Table 2. Protein expression of prostate lineage-specific, pan-epithelial, and putative stem cell markers in BM18 xenografts in intact versus castrated hosts

	Staining intensity in BM18 xenografts[a]		
	Intact host	Castrated host	Annotations
Luminal markers			
PSA	+++	+/+++	Small fraction of castration-resistant cells
NKX3–1	+++	+/+++	All castration-resistant cells
CK18	+++	+++	All castration-resistant cells
AR	+++	+	All non-NE castration-resistant cells
Basal markers			
CK5/6	Neg.[b]	Neg.[b]	
CK14	Neg.[b]	Neg.[b]	
CK19	Neg.[b]	Neg.[b]	
Pan-epithelial markers			
EpCAM	+++	+++	All castration-resistant cells
panCK	+++	+++	All castration-resistant cells
NE markers			
CHGA	++	++	All NE cells
SYP	++	++	All NE cells
SC markers			
ALDH1	++	++	Cytoplasmic staining in intact hosts, exclusively nuclear or nuclear and cytoplasmic staining in castrated hosts
ALDH1A1	++	++	
α2-Integrin	Neg.[b]	Neg.[b]	
CD44	++	++	
NANOG	++	++	
OCT4	Neg.[b]	Neg.[b]	
SOX2	Neg.[b]	Neg.[b]	
EGR1	Neg.[b]	Neg.[b]	

Antibodies are described in Supporting Information Table S2.
[a]Staining intensity was scored as follows: +: low, ++: medium, +++: high, neg.: all cells negative.
[b]Positive controls (PC sections for CK5/6, CK14, and CK19; seminoma sections for OCT4 and SOX2; sections of mouse brain for EGR1; PC-3 cell cytospins for α2-integrin) stained positive.
Abbreviations: ALDH1A1, aldehyde dehydrogenase 1A1; AR, androgen receptor; CHGA, chromogranin A; CK18, cytokeratin 18; EGR1, early growth response 1; NE, neuroendocrine; NKX3–1, NK3 homeobox 1; PSA, prostate-specific antigen; SYP, synaptophysin.

(*OCT4*), Kruppel-like factor 4 (*KLF4*), *LGR6*, and early growth response 1 (*EGR1*). Expression of α2-integrin and prostate stem cell antigen (*PSCA*) mRNA was negligible in BM18 xenografts in intact hosts and was not modified by castration. *CD133* and chemokine (C-X-C motif) receptor 4 (*CXCR4*) mRNA expression was undetectable in both conditions (Fig. 2).

In intact hosts, BM18 xenografts contained rare, isolated cells stained with an antibody specifically recognizing ALDH1, a putative SC marker [34–36]. However, the frequency of ALDH1-positive ($ALDH1^{pos}$) cells became dramatically higher after castration, and they were found either isolated or grouped within residual cell clusters (Tables 1, 2; Fig. 4A, 4B). In order to determine the exact *ALDH* isoenzyme expressed by the positive cells, we analyzed the mRNA expression of various *ALDH* family members. Expression of *ALDH1A1* only matched the proportions of positive cells (Supporting Information Fig. S3A). Furthermore, immunohistochemistry performed with an antibody specific for the ALDH1A1 enzyme isoform essentially recapitulated the staining pattern obtained with the ALDH1-specific antibody, strongly indicating that $ALDH1^{pos}$ cells are expressing the ALDH1A1 isoform (Supporting Information Fig. S3B--S3E). Therefore, $ALDH1^{pos}$ BM18 cells will be referred hereafter as ALDH1A1 positive ($ALDH1A1^{pos}$).

Double ALDH1/CHGA immunofluorescence showed that $ALDH1A1^{pos}$ cells in xenografts from both intact and castrated hosts were consistently NE negative (NE^{neg}) (Fig. 4C, 4D). Immunohistochemical analysis of consecutive sections of xenografts in castrated hosts indicated that $ALDH1A1^{pos}$ cells were all $NKX3\text{-}1^{low}$ and AR^{low} but PSA negative (PSA^{neg}) (Supporting Information Fig. S4A). To further corroborate the SC-like nature of CR BM18 cells, we analyzed the protein expression of NANOG, OCT4, SOX2, EGR1, and CD44.

In BM18 xenografts in intact host, rare cancer cells showed cytoplasmic but no nuclear expression of NANOG. In contrast, in castrated hosts, the frequency of NANOG expressing BM18 cells increased and NANOG localization was distinctly nuclear in approximately half of them, while the other half showed both nuclear and cytoplasmic staining (Fig. 4E, 4F; Tables 1, 2). Colocalization of the luminal markers NKX3-1, PSA, and AR in sequential sections of BM18 tumors in castrated hosts showed that NANOG-positive ($NANOG^{pos}$) CR cancer cells are $PSA^{high}/NKX3\text{-}1^{low}/AR^{low}$ (Supporting Information Fig. S4B). Consistently with the evidence that the two SC-like markers ALDH1A1 and NANOG mark two different CR cancer cell populations, $PSA^{neg}/NKX3\text{-}1^{low}/AR^{low}$ and $PSA^{high}/NKX3\text{-}1^{low}/AR^{low}$, respectively, colocalization of NANOG and ALDH1A1 confirmed that these two SC-like markers are mutually exclusive (Supporting Information Fig. S5A, S5B).

BM18-cancer cells expressing CD44 ($CD44^{pos}$) were extremely rare in xenografts in intact hosts. Their frequency slightly increased in xenografts in castrated hosts (Tables 1, 2), where they tended to concentrate in a minority of the residual cell clusters (Fig. 4G, 4H). $CD44^{pos}$ cells colocalized randomly with either ALDH1A1- or NANOG-expressing cells (Supporting Information Fig. S5A--S5C). Consistently with the negligible level of α2-integrin mRNA expression, no protein expression was detectable in BM18 xenografts either in intact or castrated hosts (Table 2). Despite the upregulation of *OCT4*, *SOX2*, and *EGR1* mRNA expression following castration, their corresponding proteins could not be detected in CR BM18 cells (Table 2).

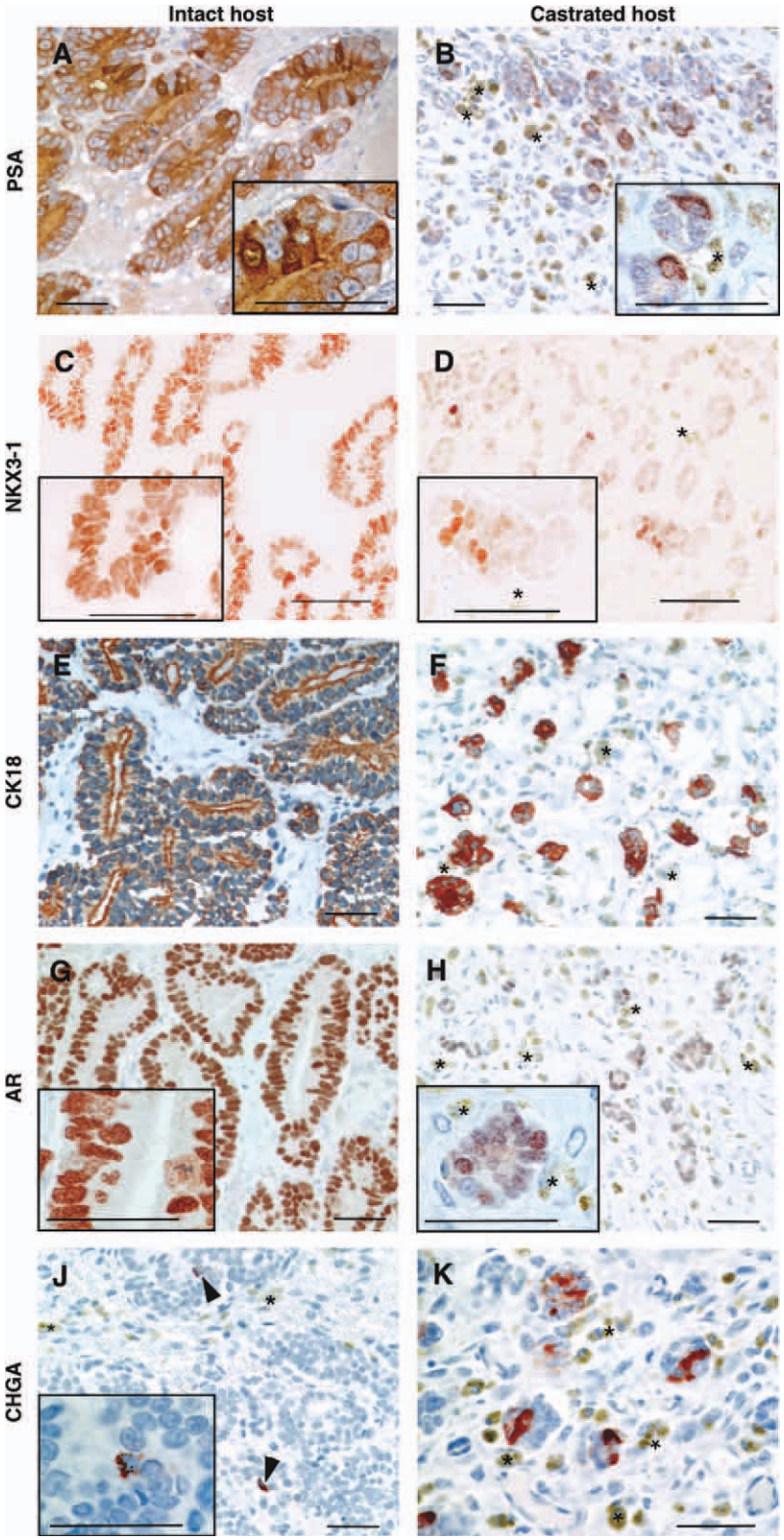

Figure 3. Castration-resistant BM18 cancer cells express luminal and neuroendocrine (NE) differentiation markers. Histological sections of BM18 xenografts in (**A**, **C**, **E**, **G,** and **J**) intact and in (**B**, **D**, **F**, **H,** and **K**) castrated hosts 23 days after surgery were derived either from (A–D and G–K) paraffin-embedded BM18 tumors or from (E and F) frozen BM18 tumors. Immunohistochemical detection of the luminal markers (A, B) PSA, (C, D) NKX3.1, (E, F) CK18, and (G, H) AR. (J, K): Immunohistochemical detection of the NE marker CHGA. (J): Arrowheads indicate CHGA-positive cells. Yellowish, granular cells (*) are infiltrating macrophages as proven by F4/80 staining (not shown). Insets represent a higher magnification of selected areas. Scale bar = 50 μm. Abbreviations: AR, androgen receptor; CHGA, chromogranin A; CK18, cytokeratin 18; NKX3-1, NK3 homeobox 1; PSA, prostate-specific antigen.

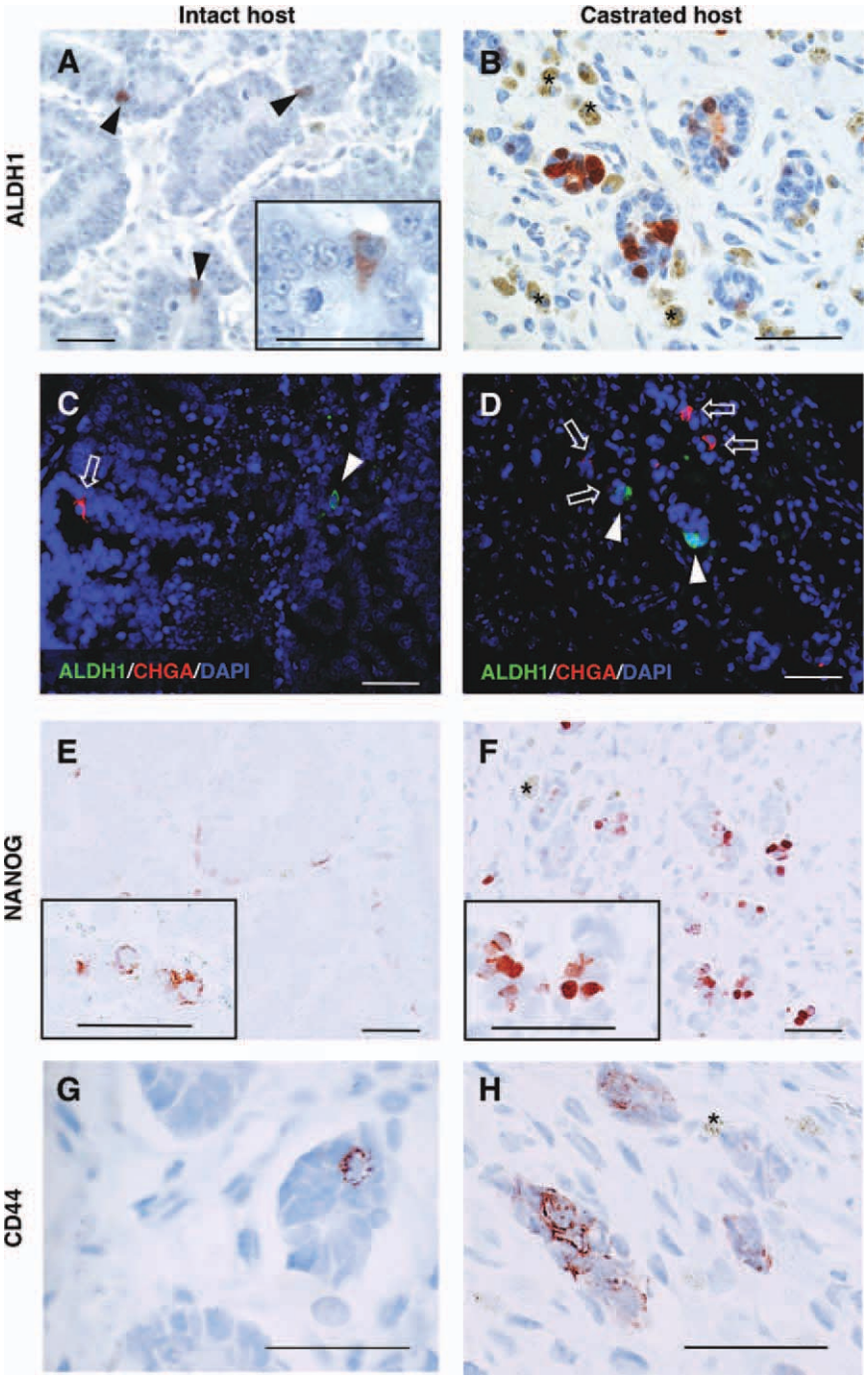

Figure 4. Castration-resistant BM18 cancer cells express stem cell (SC) markers. Histological sections of BM18 xenografts in (**A**, **C**, **E**, and **G**) intact and in (**B**, **D**, **F**, and **H**) castrated hosts 23 days after surgery were derived from paraffin-embedded BM18 tumors. (A, B and E–H): Immunohistochemical detection of the SC markers (A, B) ALDH1, (E, F) NANOG, and (G, H) CD44. Arrowheads in (A) indicate ALDH1-positive cells. (C, D): Immunofluorescent colocalization of CHGA (red, open arrows) and ALDH1 (green, arrowheads). Blue: DAPI nuclear counterstaining. Insets represent a higher magnification of selected areas. * mark macrophages. Scale bar = 50 μm. Abbreviations: ALDH1, aldehyde dehydrogenase 1; CHGA, chromogranin A; DAPI, DAPI, 4',6-diamidino-2-phenylindole.

CR Cells Result from Selection of Pre-Existing Cells and Not from Transdifferentiation

The frequency of ALDH1A1^{pos}, NANOG^{pos}, CD44^{pos}, and NE^{pos} cells in BM18 xenografts in castrated versus intact hosts attained to an apparent increase of 166-, 10-, 5-, and 38-fold, respectively. However, when this frequency was corrected by the total cancer cell loss, it resulted in a nonsignificant twofold increase in ALDH1A1^{pos} cells and even in a threefold decrease in NE^{pos} and CD44^{pos} cells after castration and 1.5-fold decrease in NANOG^{pos} cells (Table 1).

CR, SC-Like PC Cells can Reinitiate Proliferation and Luminal Differentiation Upon Androgen Replacement

BM18 xenografts do not escape androgen deprivation and, thus, cannot reinitiate growth spontaneously. Therefore, to

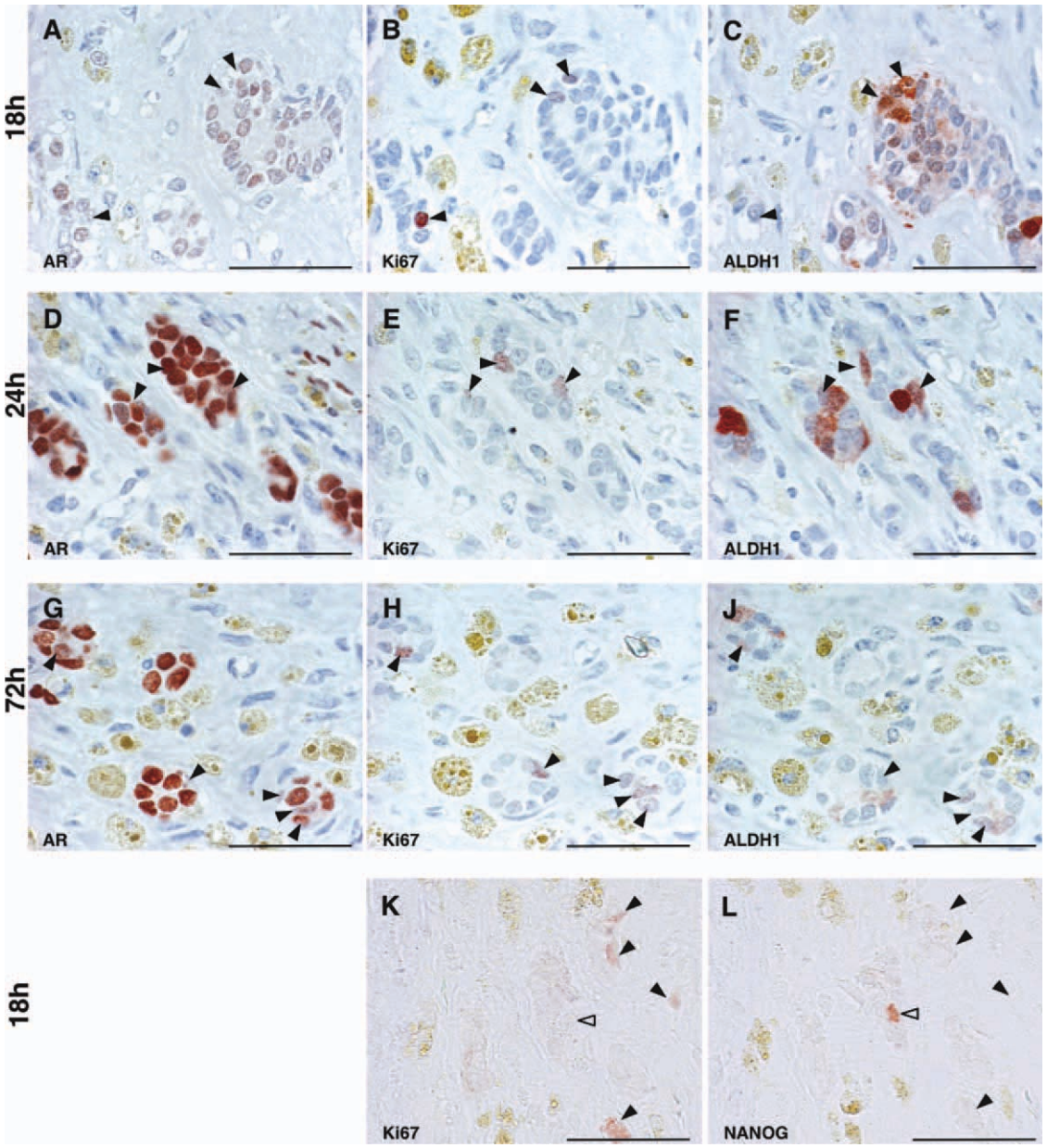

Figure 5. Castration-resistant BM18 cancer cells can reinitiate proliferation and luminal differentiation after androgen replacement. Immunohistochemical detection of (**A**, **D**, **G**) AR, (**B**, **E**, **H**, **K**) Ki67, (**C**, **F**, **J**) ALDH1, and (**L**) NANOG in consecutive sections of BM18 xenografts in castrated hosts at (A--C, K, **L**) 18 hours, (D--F) 24 hours, or (G--J) 72 hours after androgen replacement. Arrowheads indicate Ki67-positive cells and open arrowheads NANOG-positive cells. Scale bar = 50 μm. Abbreviations: ALDH1, aldehyde dehydrogenase 1; AR, androgen receptor.

identify the cell-of-origin of tumor growth reinitiation, castrated animals bearing BM18 xenografts were supplied with androgen. Within 72 hours after androgen replacement, there was a significant increase in *NKX3-1* and *PSA* mRNA expression. *AR* mRNA expression was also increased, although not significantly. This was mirrored by a significant decrease in *ALDH1A1* and *LGR6* mRNA expression. The expression of mRNA for the reprogramming factors *OCT4*, *SOX2*, *NANOG*, and *KLF4*, and for *EGR1* and *NSE* was also decreased but not significantly (Supporting Information Fig. S6). The rapid induction of AR (Fig. 5A, 5D, 5G) and PSA (not shown) after androgen replacement could be confirmed at the protein level already after 24 hours.

More importantly, androgen replacement rapidly induced Ki67 expression ($Ki67^{pos}$, Fig. 5B, 5E, 5H) in a very restricted subpopulation of CR BM18-cancer cells. The percentage of $Ki67^{pos}$ cells increased gradually within 72 hours after androgen replacement.

At the earliest time point (18 hours), approximately 60% of $Ki67^{pos}$ cells was also $ALDH1A1^{pos}/AR^{low}$, while 40% was $ALDH1A1^{neg}/AR^{low}$. Thereafter, the expression of $Ki67^{pos}$ cells occurred mostly in the $ALDH1A1^{neg}/AR^{high}$ cell population (Fig. 5A--5J; Supporting Information Fig. S7A). Interestingly, the rare $ALDH1A1^{pos}$ BM18-cancer cell population in intact hosts was mostly $Ki67^{neg}$, and $ALDH1A1^{pos}/Ki67^{pos}$ cells could be found only at an extremely low frequency (Supporting Information Fig. S7B).

Androgen replacement dramatically reduced NANOG protein expression already within the first 18 hours. The extremely rare $NANOG^{pos}$ cells were invariably $Ki67^{neg}$ (Fig.

5K, 5L). NE^{pos} cells were consistently $Ki67^{neg}$ within 72 hours following androgen replacement (not shown).

Discussion

The cell origin and mechanism(s) responsible for the irreversible progression of PC to castration-resistance are still debated [12]. Either adaptation of originally androgen-dependent cancer cells to an androgen-depleted environment or selection of pre-existing, CR cancer cells have been postulated and documented [37].

Here, we demonstrate that in the highly androgen-dependent BM-18 human PC xenograft pre-existing, luminal progenitors with SC-like characteristics and NE cells survive castrate levels of androgens as dormant. Thereby, selection, and not transdifferentiation/adaptation, is the mechanism responsible for their increased frequency. The luminal, SC-like progenitors, but not the NE cells, can reinitiate tumor growth upon androgen replacement. This is the first evidence for the existence of a CR, SC-like luminal progenitor in human PC, and it may have important implications for understanding the mechanisms of progression to CRPC.

The BM18 xenograft model closely mimics the regenerative capacity of the normal prostate epithelium, which depends on a population of self-renewing SCs able to enter dormancy upon androgen-deprivation [11]. This strongly suggests that the residual, dormant cancer cell population may be composed almost exclusively of tumor-reinitiating cells, which are CR for survival but castration-sensitive for growth.

Based on a previous investigation in the LAPC9 xenograft model of human PC, it has been suggested that CRPC progression occurs in a two-step process: the first step (stage I) consists in the enrichment, due to apoptosis of castration-sensitive cells, of pre-existing cells that are CR for survival but not for growth. The second step (stage II) is the clonal outgrowth of cells, which are CR not only for survival but also for growth [2]. According to this model of PC progression to CRPC, the BM18 xenograft is representative of only stage I and, therefore, is not a model of CRPC, where aberrant activation of the AR signaling already occurred.

Previous evidence for an enhanced expression of SC markers in androgen-depleted, human PC cells in vitro and in vivo is still scarce. A trend toward upregulation of the putative SC markers CK5 and ATP-binding cassette subfamily G member 2 has been reported in androgen-depleted DuCaP cells in vitro [27]. A transient increase of the SC-like markers CD44, c-Kit, and ALDH at relatively early time points after castration has been shown in the LNCaP xenograft model [30]. Here, we extend these findings by showing that castration induces an increased expression of four essential transcription factors required for reprogramming self-renewal and pluripotency in differentiated somatic cells, namely *OCT4*, *SOX2*, *KLF4*, and *NANOG* [38, 39] and of the putative PC SC-like cell markers *ALDH1A1* [18] and *CD44* [14, 21]. This is paralleled by upregulation of the Polycomb group transcriptional repressor *BMI1*, a crucial regulator of self-renewal of SCs from various tissues, including prostate [40, 41], and of *EGR1*, a transcriptional regulator promoting hematopoietic SCs quiescence [42] that has been associated to PC progression [43]. Additionally, the finding of an increased expression of *LGR5* and *LGR6*, markers of adult, epithelial SCs in the intestinal tract and in hair follicles of mice [44], proposes for the first time these G-protein-coupled receptors as candidate SC-like markers in PC.

ALDH1A1 and NANOG colocalization with luminal markers revealed two putative SC-like populations, namely an $ALDH1A1^{pos}/NANOG^{neg}/PSA^{neg}/NKX3\text{-}1^{low}/AR^{low}$ and a $NANOG^{pos}/ALDH1A1^{neg}/PSA^{high}/NKX3\text{-}1^{low}/AR^{low}$. This heterogeneity may reflect either clonal variation or the fact that tumor growth reinitiation and maintenance may be driven by more than one luminal SC-like population, as it has been postulated for mammary cancer [45]. Coexpression of other reprogramming factors and SC-like markers could have further refined the SC-like populations within the CR cells. However, OCT4, SOX2, and EGR1 immunoreactivity could not be shown in these cells, most likely due to the very low level of protein expression. LGR5 immunolocalization was not attempted since it is known that even in intestinal crypts, expressing high *LGR5* mRNA levels, its protein expression is too low to be detectable [44].

The prevailing view favors transdifferentiation of PC cells as the mechanism responsible for the increase in NE cell frequency following androgen-deprivation in vitro and/or in vivo [46–48]. Either dedifferentiation/reversion of luminal cancer cells to a SC-like phenotype or enrichment of pre-existing SC-like cancer cells may explain the emergence of SC-like PC cells after castration [28–30]. The analysis of the frequency of cancer cells with either SC-like or NE phenotype in BM18 xenografts in intact and castrated hosts, together with the evidence that CR BM18 cancer cells are nonproliferating, indicates an alteration in the relative abundance of the different cell populations. Therefore, our results contradict the mechanism of transdifferentiation/dedifferentiation and support, instead, a mechanism of selection of pre-existing NE and SC-like PC cells, as already postulated by others [2, 3, 10]. This seems also to rule out the possibility that increased expression of ALDH1A1, involved in the intracellular metabolism of retinoic acid, could be induced by altered testosterone levels. Evidence that testosterone modulates ALDH1A3, but not ALDH1A1, further supports this consideration [49].

In the BM18, androgen replacement induced not only proliferation but also a concomitant rise in the expression of AR target genes in AR^{low} BM18 tumor-reinitiating cells. This, together with the lack of AR expression in stromal cells, indicates that androgen-induced tumor reinitiation acts directly on cancer cells. It also excludes a paracrine role of tumor stroma, as already demonstrated in other PC xenograft models [50] and in clinical PC specimens [51]. Furthermore, the low level of nuclear AR in CR cancer cells suggests that adrenal androgens in castrated mice may provide a minimal level of AR signaling sufficient to maintain survival of BM18 tumor-reinitiating cells but still insufficient to allow tumor growth progression.

Immediately after tumor growth, reinduction by androgen-replacement proliferation occurs both in $ALDH1A1^{pos}$ and $ALDH1A1^{neg}$ cancer cells, suggesting that both fractions may possess tumor-reinitiation potential. $ALDH1A1^{pos}$ cells are quiescent in intact hosts and, therefore, they seem to contribute only to tumor reinitiation induced by androgen replacement but not to steady state tumor growth. This suggests $ALDH1A1^{pos}$ cells as self-renewing and slow-cycling cells. In addition, it proposes ALDH1A1 as the ALDH isoform identifying tumor-reinitiating cells in PC, further expanding previous studies on ALDH activity in PC cells [17, 18] and on the ALDH isoforms in others solid cancers [34–36].

It is unclear whether $ALDH1A1^{neg}$ cells are also SC-like or represent a non-self-renewing progeny of $ALDH1A1^{pos}$ cells surviving castration. However, the possibility that tumor-reinitiation potential is also possessed by the $NANOG^{pos}/ALDH1A1^{neg}$ population could not be verified due to the almost complete loss of NANOG expression already at the earliest time point (18 hours) after androgen replacement. The requirement for the repression of reprogramming factors like NANOG to initiate differentiation may underlie this effect [52].

NE^{pos} cells in BM18 tumors were consistently nonproliferative before and after androgen replacement, which is in

agreement with previous reports showing that NE cells in normal prostate and PC are quiescent and AR^{neg} [53, 54]. This strongly suggests that, at least in this model, NE cells are not responsible for tumor reinitiation, as suggested by others [55]. Nevertheless, residual NE cells may constitute a "SC-niche" promoting survival of quiescent SC-like PC cells in an androgen-deprived environment [56].

The impossibility to obtain viable, single cells dissociated from BM18 tumors has prevented us from isolating cells by flow cytometry and performing clonogenic or sphere-forming assays. Therefore, we could not determine the reinitiation and self-renewing potential in vitro and in vivo of the CR cell populations displaying different expression of SC-like markers, especially ALDH1A1.

It is still debated whether PC is initiated by a transforming event in a stem or in a progenitor cell of the normal prostate epithelium. Likewise, it is not clear whether the target cell belongs to the basal or to the luminal compartment [12, 13]. First evidence for the existence of a CR SC located in the luminal compartment has been provided in rodents [57, 58]. More recently it has been demonstrated in mice that CR cells, named CARNs, express the luminal markers Nkx3-1, CK18, and AR, but not basal markers, have SC properties, and can regenerate prostatic tissue after androgen replacement. Importantly, deletion of the tumor suppressor *Pten* in CARNs induces carcinoma after androgen-mediated regeneration, suggesting this SC population as a potential target for transformation [59]. Since PC patients are castrated only at an advanced stage of disease, it cannot be established whether an equivalent CARN population pre-exists in human PC and could be the cell-of-origin of progression to CRPC.

Conclussion

Here, we provide novel evidence demonstrating that a CARN-like population(s), expressing SC markers together with the luminal markers NKX3-1, CK18, and AR, but not basal markers, pre-exist in the BM18 xenograft model of human PC and can reinitiate tumor growth upon androgen replacement. This finding has major biological, prognostic, and therapeutic implications. It seems to indicate that, at least in early-stage PC, the SC-like cell driving tumor growth does not originate from a primitive SC within the basal compartment, as suggested by others [25]. Furthermore, identification of CR SC-/CARN-like cells in PC patients may have prognostic value in defining patients at risk for progression to CRPC. Finally, the fact that SC-/CARN-like cells are AR^{low} further supports the rationale for ADT, but suggests that they should be targeted early, before developing into CRPC cells.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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