A rare castration-resistant progenitor cell population is highly enriched in Pten-null prostate tumours

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Abstract

Castration-resistant prostate cancer is a lethal disease. The cell type(s) that survive androgen deprivation remain poorly described, despite global efforts to understand the various mechanisms of therapy resistance. We recently identified in wild-type (WT) mouse prostates a rare population of luminal progenitor cells that we called LSCmed according to their FACS profile (Lin−/Sca-1+/CD49fmed). Here, we investigated the prevalence and castration resistance of LSCmed in various mouse models of prostate tumourigenesis (Pb-PRL, Ptenpc−/−, and Hi-Myc mice). LSCmed prevalence is low (~8%), similar to WT) in Hi-Myc mice, where prostatic androgen receptor signalling is unaltered, but is significantly higher in the two other models, where androgen receptor signalling is decreased, rising up to more than 80% in Ptenpc−/− prostates. LSCmed tolerate androgen deprivation and persist or are enriched 2–3 weeks after castration. The tumour-initiating properties of LSCmed from Ptenpc−/− mice were demonstrated by regeneration of tumours in vivo. Transcriptomic analysis revealed that LSCmed represent a unique cell entity as their gene expression profile is different from luminal and basal/stem cells, but shares markers of each. Their intrinsic androgen signalling is markedly decreased, explaining why LSCmed tolerate androgen deprivation. This also illuminates why Ptenpc−/− tumours are castration-resistant since LSCmed represent the most prevalent cell type in this model. We validated CK4 as a specific marker for LSCmed on sorted cells and prostate tissues by immunostaining, allowing for the detection of LSCmed in various mouse prostate specimens. In castrated Ptenpc−/− prostates, there was significant proliferation of CK4+ cells, further demonstrating their key role in castration-resistant prostate cancer progression. Taken together, this study identifies LSCmed as a probable source of prostate cancer relapse after androgen deprivation and as a new therapeutic target for the prevention of castrate-resistant prostate cancer.

Keywords: prostate cancer; castration resistance; epithelial cells; progenitor cell; androgen signalling; CK4

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Introduction

Prostate cancer remains a major cause of cancer death, due to resistance to androgen deprivation therapy in advanced stages of the disease [1]. Despite global efforts to understand the mechanisms of androgen-deprivation resistance, the underlying aetiology remains incompletely understood. Prostate tumours are composed of heterogeneous populations of cells, and identifying the cell type that is androgen-independent and castration-resistant remains challenging, although it is the relevant cell type to be targeted.

In postnatal development of the prostate [2], and more rarely in the adult [3,4], basal/stem cells differentiate to luminal progenitors to maintain the luminal lineage. Following castration of adult mice, there is a
significant decline in luminal cell number, with little or no decline in basal cells [5]. The latter population is therefore considered to be castration-tolerant and to repopulate the epithelium when androgen signalling is restored [5]. In addition, recent studies have shown that the adult mouse prostate contains rare luminal-like cells with stem cell characteristics termed CARN (castration-resistant Nkx3.1-expressing cells) [6] and CARB (castration-resistant Bmi1-expressing cells) [7] that also survive castration. Finally, using human patient-derived xenografts (PDXs) of treatment-naïve prostatic tumours, we showed that a luminal [cytokeratin (CK) 8/18+ ] population of castrate-tolerant cells with stem-like properties survives androgen withdrawal. These cells remain quiescent upon androgen deprivation and regenerate tumours upon restoration of androgens [8]. Both in mouse and in human prostates, castrate-tolerant luminal cells exhibiting stem-like properties remain poorly characterized, as specific markers are yet to be identified.

We recently identified in wild-type (WT) mouse prostates a rare prostate epithelial cell population called LSCmed (Lin−/Sca-1+/CD49fmed) that displays luminal progenitor properties [9,10]. This luminal-like (CK8+, CK5−), stem cell antigen-1 (Sca-1)-positive, Nkx3.1-negative cell population generated 2D colonies and 3D spheres in androgen-free conditions. In addition, in agreement with their condition of luminal progenitors, LSCmed lost Sca-1 expression upon androgen stimulation in vitro, suggesting differentiation to mature luminal cells [9]. LSCmed from WT prostates can also generate organoids in vitro and prostate-like structures in vivo and survive androgen deprivation [11]. Of particular interest, we have also reported a marked amplification of LSCmed in the premalignant prostates of Pb-PRL mice [9]. These transgenic mice display Stat5 overactivation in prostate luminal cells [12], mimicking what is observed in human prostate cancer [13].

The androgen-independent and progenitor characteristics of LSCmed suggest their potential role in prostate tumourigenesis and in the progression to castrate-resistant prostate cancer (CRPC). However, very little is known about this recently discovered cell population, including their presence in prostate cancer. Here, we show that LSCmed (i) are present and tolerate androgen deprivation in various mouse models of prostate tumourigenesis; (ii) represent, before and after castration, more than 80% of prostate epithelia in Ptenpc−/− mice (a relevant model of CRPC), massively proliferate after castration, and have tumour-initiating capacity in vivo; (iii) exhibit a unique gene expression signature that distinguishes them from prostate cell populations designated as luminal and basal/stem cells [14]; (iv) display intrinsically low activation of androgen receptor (AR) signalling consistent with their resistance to androgen deprivation; and (v) can be identified on prostate tissue sections by CK4 immunostaining. In all, this study identifies LSCmed as a probable source of prostate cancer relapse after androgen deprivation and as a new therapeutic target for the prevention of CRPC.

Materials and methods

Animals

Pb-PRL mice (prolactin transgene driven by the short probasin promoter) were generated in the Paris laboratory on a C57BL/6J background (> 20 backcrosses), as previously described [12]. Ptenpc−/− mice (PtenloxPflloxP mice crossed with P-Cre4 transgenic males) were generated in the Vienna laboratory as described previously [15] and maintained on a C57BL/6 and Sv129 mixed genetic background. Hi-Myc (ARR2/Pb-MYC) mice maintained on a pure FVB/N background were bred at Monash University. Experiments were performed using 6- to 8-month-old mice, i.e. when premalignant (Pb-PRL) and aggressive malignant (Ptenpc−/− and Hi-Myc) phenotypes were well established. For all genotypes, non-transgenic littersmates were used as controls and are referred to as WT animals. Colonies were housed in controlled conditions, on a 12/12-h light/dark cycle with normal food and water provided ad libitum. Where indicated, mice were surgically castrated and analysed 2 (WT and Pb-PRL) or 3 weeks (Ptenpc−/−) later. Prostate samples were obtained by microdissection immediately after sacrifice by cervical dislocation. Animal experiments using Pb-PRL mice were approved by the local ethics committee for animal experimentation (authorization CEEA34.VG.095.12). Animal experiments using Ptenpc−/− mice were reviewed and approved by the Austrian ministry authorities and conducted according to relevant regulatory standards (BMWF-66.009/0281-I/3b/2012). Animal experiments using Hi-Myc mice were reviewed and approved by the Monash Animal Research Platform Animal Ethics Committee (approval No MARP/2011/161).

Prostate subpopulation sorting by FACS

The procedures for cell sorting were performed as described previously by FACS using the antigeic profile Lin/Sca-1/CD49f [9,16]. Cell sorting was performed on a BD FACSAria III (BD Biosciences, San José, CA, USA). A brief methodological summary is provided in the supplementary material, Supplementary materials and methods, and sequential gates and staining controls are shown in Figure S1 of the supplementary material. Sorted cells were collected in DMEM medium, supplemented with 50% FBS, glutamine, and penicillin–streptomycin, or in RA1 Lysis Buffer (Macherey-Nagel, Düren, Germany) to perform RNA extraction as described in the supplementary material, Supplementary materials and methods.

Microarray analysis

Gene expression analysis was performed using GeneChip® Mouse Transcriptome Arrays 1.0 (Affymetrix, Santa Clara, CA, USA), interrogating...
more than 6.0 million probes covering coding transcripts (70% of probes) and non-coding transcripts (30% of probes). Prior to hybridization, cDNA was fragmented and biotin-labelled using the Encore Biotin Module (NuGEN) as recommended by the manufacturer. Biotinylated DNA fragments were hybridized onto the array chips using the Hybridization Wash Stain kit (Affymetrix). The chips were washed, stained, and scanned using the Affymetrix Model 450 Fluidics Station, the Affymetrix Model 3000 scanner, and the Command Console software for piloting the GeneChip systems. For data analysis, raw data CEL files were imported in R/Bioconductor using the Oligo package (http://www.r-project.org/). Expression levels were normalized using the RMA algorithm from the affy package and background noise was computed using a custom algorithm within R as follows. Assuming that a maximum of 80% of genes are expressed on any given microarray, we tagged the 20% of probes with the lowest intensity as background for each microarray as background. A threshold was fixed at two standard deviations over the mean of the background. All probes for which normalized intensities were lower than the computed threshold were designated as background for each array. When comparing gene expression levels between two groups, a probe was included in the analysis if its intensity exceeded the background in at least 80% of the samples from at least one group. Group comparisons were done using Student’s t-test and lists were filtered at p < 0.05 and fold change >1.5. Cluster analysis was performed by hierarchical clustering using the Spearman correlation similarity measure and average linkage algorithm. Heatmaps were generated using Java Treeview [17] and a custom R script. Functional analyses were carried out using Ingenuity Pathway Analysis (IPA; Qiagen, Hilden, Germany). Microarray data have been deposited on the ArrayExpress site (accession number E-MTAB-4991) (http://www.ebi.ac.uk/arrayexpress). Data from transcriptomic studies available online on GEO were analysed using a strategy similar to that described above for our own transcriptomic data.

Reverse transcription–quantitative PCR (RT-qPCR)

For RT-qPCR, iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA, USA) was used, and reactions were run on a Vii7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) or a qTower 2.0 Real-Time Thermal Cycler (Analytik Jena, Jena, Germany). Primers are listed in the supplementary material, Table S1. Results were normalized to Eef1a1 expression.

Cytospins

Sorted cells were loaded onto the cytospin slide chambers of a Shandon Cytospin 2 (Thermo Scientific, Rockford, IL, USA) and centrifuged at 500 rpm at room temperature for 10 min.

In vivo regeneration assay

The in vivo prostate regeneration assay was performed as described previously [18]. In brief, 8 × 10^7 FACS-sorted LSCmed cells (or basal/stem cells used as a control) harvested from three pooled Ptenpc−/− prostates were combined with 1.5 × 10^6 urogenital sinus mesenchymal cells, mixed (1:1, v/v) with growth factor-reduced Matrigel (Corning, Corning, NY, USA) in a final volume of 150 μl, and injected subcutaneously into immunodeficient SCID male mice (Janvier, Saint Berthevin, France). Grafts were harvested 10 weeks later.

Immunohistochemistry/immunocytochemistry (IHC/ICC) and immunofluorescence (IF)

All samples were fixed in 4% PFA, paraffin wax-embedded, and sections underwent heat-induced antigen retrieval in citrate buffer at pH6. IHC/ICC and IF were performed as described previously [9] using the antibodies listed in the Supplementary materials and methods (supplementary material), a Vector Elite ABC HRP kit with DAB substrate (Vector Laboratories, Burlingame, CA, USA), and haematoxylin as a counterstain. Slides were scanned with a Nanozoomer 2.0 (Hamamatsu, Massy, France) and analysed using NDP.view 2.5.14 software (Hamamatsu). For IF, nuclei were stained with Hoechst dye, and samples were analysed with a 10× or 20× objective under an Axios Observer.Z1 inverted microscope (Carl Zeiss Microscopy, Göttingen, Germany).

Statistics

Two-way analysis of variance (ANOVA) with Šidák’s multiple comparison test was used to compare population percentages among intact and castrated mouse groups. Repeated measures one-way ANOVA tests were used to evaluate gene expression differences among the cell types for each genotype. Post-hoc multiple comparisons were performed using Tukey’s test. Statistical analysis of transcriptome data is described in the corresponding methods section. A value of p < 0.05 was used as the significance cut-off for all tests. All analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

LSCmed are the most abundant epithelial cell type in intact and castrated Ptenpc−/− prostate tumours

In humans, PTEN deficiency is observed in 69% of prostate cancer cases and up to 86% of CRPCs [19,20]. Accordingly, prostate-specific Pten-deficient mice (Ptenpc−/−) develop invasive prostate adenocarcinomas [21,22]. This model was therefore chosen to test the prevalence and androgen independence of LSCmed in a cancer context. Strikingly, 82% of the whole Ptenpc−/− epithelia of intact prostates was LSCmed (Figure 1A).
Figure 1. LSC\textsuperscript{med} are the most abundant epithelial cell type in intact and castrated \( Pten^{pc-/} \) prostate tumours. Representative FACS profiles of \( Pten^{pc-/} \) (A), Pb-PRL (B), and WT (C) mouse prostates. Graphs depict epithelial cells only (gated as Lin\textsuperscript{−}CD49f\textsuperscript{+}), with large gates for percentage analysis. Complete Lin\textsuperscript{−} populations, including stromal cells, are shown in the supplementary material, Figure S1. Intact and castrated conditions are shown in the left and right panels, respectively. Each FACS profile shows gated epithelial populations: basal/stem, LSC\textsuperscript{med} (green squares), and luminal cells. Percentages are noted under each gate name. Bar graphs show mean (± SD) percentages of basal/stem, LSC\textsuperscript{med}, and luminal cells for each genotype in \( n=2\text{–}5 \) independent experiments consisting of one to ten pooled prostates each. Stars denote significant differences (\( p<0.001 \)) in a repeated-measures two-way ANOVA with Sidak’s multiple comparisons. NS = not significant.

Since \( Pten^{pc-/} \) prostate tumours are castration-resistant [21], we hypothesized that LSC\textsuperscript{med} may tolerate castration, reflecting a role of these cells in the progression to castration resistance. Indeed, there was no change in LSC\textsuperscript{med} prevalence in the castrated \( Pten^{pc-/} \) epithelium (Figure 1A). We also castrated Pb-PRL mice and observed that LSC\textsuperscript{med} increased from 36\% (Figure 1B and ref 9) to more than 70\% of the epithelia before and after castration, respectively (Figure 1B). Wild-type mice served as controls, showing the expected low
Castration-resistant progenitors are enriched in Pten\textsuperscript{−\textasciicircum} prostates

prevalence (< 10\%) in intact prostates and a significant increase (> 20\%) after castration (Figure 1C and refs 9 and 11).

Together, these results demonstrate that LSC\textsuperscript{med} are the most abundant epithelial cell population in malignant Pten\textsuperscript{pc−}\ prostate, and they can survive androgen deprivation in all the models tested (normal, premalignant, and malignant prostate).

LSC\textsuperscript{med} are a unique prostate epithelial cell entity

To establish the cell identity of LSC\textsuperscript{med}, we performed a microarray analysis of FACS-sorted LSC\textsuperscript{med}, basal/stem, and luminal cell populations. This analysis was performed on WT prostates to circumvent any bias due to prostate pathology. Our results show that the gene expression profiles of the three epithelial cell subpopulations are different (Figure 2A). Gene expression in LSC\textsuperscript{med} shares some similarity to basal/stem and to luminal cells (Figure 2A, gene clusters identified as b and c). A group of genes was specifically up-regulated in LSC\textsuperscript{med} compared with basal/stem and luminal cells (Figure 2A, gene cluster identified as a). Gene ontology analysis of genes differentially expressed between LSC\textsuperscript{med} and basal/stem or LSC\textsuperscript{med} and luminal cells highlighted functions related to tumorigenesis (cancer, cellular movement, inflammatory response), proliferation/survival/regeneration (cell death and survival, cell growth and proliferation, organismal injury and abnormalities), and urological diseases (Figure 2B).

Unsupervised cluster analysis of the microarray results segregated LSC\textsuperscript{med} as a separate prostate cell population, different from luminal and basal/stem cells but closer to the former (Figure 2C). Further analysis of established markers proved that LSC\textsuperscript{med} share some similarities with basal/stem and some with luminal cells (Figure 2D; RT-qPCR validations in Figure 2E). Certain luminal markers, e.g. Krt18, Krt14, and Krt19, were present in both luminal and LSC\textsuperscript{med}, consistent with LSC\textsuperscript{med} being similar to luminal cells (Figure 2C). Nevertheless, markers of mature (secretory) luminal cells (Pbsn, Msmb) were not expressed in LSC\textsuperscript{med}. Several stem cell markers were expressed in LSC\textsuperscript{med}, e.g. Tacstd2 (Trop2) and Cldn4, implying some similarity to basal/stem cells, but traditional basal markers (Krt5, Krt14, and Trp63) were not expressed in LSC\textsuperscript{med}. Importantly, LSC\textsuperscript{med} did not express Nkx3.1 (Figure 2E), implying that LSC\textsuperscript{med} and CARN cells [6] are distinct epithelial cell populations.

In summary, LSC\textsuperscript{med} express genes typical of both luminal and basal/stem cells but represent a distinct, newly identified epithelial cell entity.

The LSC\textsuperscript{med}-specific gene expression signature is conserved across mouse models

To establish whether the identity of LSC\textsuperscript{med} is maintained in pathological conditions, we generated an LSC\textsuperscript{med}-specific gene expression signature. In WT LSC\textsuperscript{med}, 111 genes were differentially expressed (> 2-fold, \(p < 0.05\)) compared with basal/stem and luminal cells. Most of these genes were overexpressed in LSC\textsuperscript{med}, while a minor portion was down-regulated (Figure 3A). Certain genes are implicated in prostate cancer or other cancers (e.g. Psca, Edn1, and Reg3b) and others are associated with activation of the innate immune response (e.g. Cxcl17, Cxcl15, Pglyrp1, LTF, Gsdmc, C3, and Reg3g).

Given that LSC\textsuperscript{med} represent the vast majority of epithelial cells in Pten\textsuperscript{pc−}\ mouse prostates (Figure 1A), we reasoned that if the LSC\textsuperscript{med} identity is conserved in a cancer context, the gene expression profile of unsorted Pten\textsuperscript{pc−}\ mouse prostates should reflect an enrichment of LSC\textsuperscript{med}-specific genes. We tested this hypothesis by using the LSC\textsuperscript{med} signature generated above to query two independent transcriptomic datasets of Pten\textsuperscript{pc−}\ mouse prostate tissue relative to WT (GSE46799 and GSE46473) [23,24]. Both analyses revealed a strong overlap of LSC\textsuperscript{med}-specific genes with those differentially expressed in Pten\textsuperscript{pc−}\ mice versus WT. Of a total of 9293 LSC\textsuperscript{med}-specific genes present in these two datasets, 84/85 were concordantly up- or down-regulated in both unsorted Pten\textsuperscript{pc−}\ prostates and sorted WT LSC\textsuperscript{med} (Figure 3B–D and supplementary material, Figure S2A–C). This strongly supports the notion that the LSC\textsuperscript{med} signature is intrinsic to these cells and independent of the prostate pathological status.

To confirm this hypothesis, we selected 13 genes from the LSC\textsuperscript{med} signature and compared their actual level of expression in sorted cell populations from WT, premalignant Pb-PRL, and malignant Pten\textsuperscript{pc−}\ prostates by RT-qPCR (Figure 3E and supplementary material, Figure S2D). In the three models, significant up-regulation of these genes was observed in LSC\textsuperscript{med} compared with basal/stem and luminal cell populations. Importantly, castration did not affect this population-specific expression pattern, even when the absolute expression levels varied post-castration (e.g. Psca). For some genes (e.g. Reg3b, Reg3g), a slight up-regulation was observed in the basal/stem compartment which nevertheless remained lower than in LSC\textsuperscript{med}.

Altogether, this analysis identified a unique gene expression signature of LSC\textsuperscript{med} that is conserved irrespective of the pathophysiological and androgen signalling status of the prostate.

Cytokeratin 4 is a protein marker for LSC\textsuperscript{med} before and after castration

Cytokeratins are conventionally used to distinguish basal/stem from luminal prostate cells. Since the LSC\textsuperscript{med} signature included CK4 (Krt4, Figure 3A, E), we aimed to use CK4 as a protein marker to identify LSC\textsuperscript{med} on tissue slides. To confirm that CK4 protein expression was specific for LSC\textsuperscript{med}, we immunostained all three sorted cell populations. Only LSC\textsuperscript{med} were positive for CK4 (Figure 4A), which was confirmed using another anti-CK4 antibody (not shown). LSC\textsuperscript{med} also
Figure 2. LSCmed are a unique prostate epithelial cell entity. (A) Heatmap of selected gene expression results for sorted WT basal/stem, LSCmed, and luminal cells (n = 3 replicates each). Note that the gates used for sorting the different cell populations (see supplementary material, Figure S1) were more restricted in size than those used for analysis (shown in Figure 1) to avoid contaminations. Genes up-regulated specifically in LSCmed (a), and genes shared by LSCmed and luminal (b) or basal/stem populations (c) are marked in boxes (p < 0.05; fold change > 1.5). (B) The top 15 functions associated with differentially expressed genes between LSCmed and basal/stem cells (blue) or between LSCmed and luminal cells (orange). The green line marks the threshold of statistical significance (p < 0.05) for association by Ingenuity. (C) Hierarchical clustering of WT cell populations analysed by transcriptomics showing that LSCmed are more similar to luminal than to basal/stem cells. (D) Heatmap of the gene expression levels for known markers of prostate basal, stem, and luminal cells. (E) Validation of the gene expression of known cell-type markers by RT-qPCR on sorted WT populations (n = 3–5 samples of each sorted population were isolated from three to ten pooled mice in independent experiments). Different letters denote significant differences (p < 0.05) in a repeated-measures one-way ANOVA with Tukey’s post-hoc test. Basal/S or B = basal/stem; Lm = LSCmed; L = luminal cells.
Castration-resistant progenitors are enriched in Pten<sup>−/−</sup> prostates

Figure 3. Legend on next page.

coeff-expressed CK8, but not CK5, proteins (Figure 4A), consistent with gene expression studies (Figure 2D, E).

In tissue sections of intact prostates, we detected CK4<sup>+</sup> cells that increased in frequency from WT to Pb-PRL and to Pten<sup>−/−</sup> tissue (Figure 4B), consistent with FACS analyses (Figure 1). In WT prostates, CK4<sup>+</sup> cells were restricted to the proximal regions of the gland (supplementary material, Figure S3). In contrast, in Pb-PRL mice, the CK4<sup>+</sup> cells were distributed throughout the proximal and distal ductal regions (supplementary material, Figure S3), where p63<sup>+</sup> basal/stem cells reside in this model [12]. Pten<sup>−/−</sup>
mouse tissues showed large clusters of CK4+ cells located adjacent to negative regions, suggesting clonal expansion (Figure 4B, blue and red arrows, respectively). Again consistent with FACS profiles (Figure 1), castration increased the frequency of CK4+ cells in WT and Pb-PRL prostates, while their prevalence remained high in LSCmed−/− mice (Figure 4B). Together, these analyses validated CK4 as a specific marker of LSCmed in tissue sections, irrespective of the pathophysiological and androgen signalling status of the prostate.

LSCmed of Ptenpc−/− prostates highly proliferate and are tumour-initiating cells

To address the role of LSCmed in cancer progression, we first analysed their proliferation in intact and castrated Ptenpc−/− tumours using Ki-67 and CK4 co-immunostaining (Figure 5A). In intact mice, 18.3 ± 1.5% LSCmed were Ki-67+ (> 20 000 CK4+ cells counted in randomly chosen fields from three animals). The mean proliferation index of CK4+ cells remained high 3 weeks after castration (14.6 ± 2.4%), although Ki-67 staining was more heterogeneous (Figure 5A, c versus d). This suggests that some LSCmed actively participate in cancer relapse. Very few proliferating LSCmed could be detected in WT prostates before (< 0.7%) and even less after (< 0.15%) castration.

We next addressed the tumorigenic potency of Ptenpc−/− LSCmed using the in vivo prostate regeneration assay that was used to assess the tumour-initiating properties of Pten-deficient basal/stem cells [25]. All cell grafts involving LSCmed (n = 3) or basal/stem cells used as a control (n = 2) generated palpable tumours (Figure 5B). The histomorphology of prostatic structures generated from LSCmed (Figure 5C, a–h) was comparable to carcinoma in situ with focal micro-invasion (red arrows in Figure 5C, c–f), prostate intraepithelial neoplasia (PIN), and extended atrophic, cystic glands. The histomorphology of grafts generated from basal/stem cells (Figure 5C, i–k) was comparable to high-grade PIN lesions, with some foci of carcinoma in situ but no sign of micro-invasion. Immunofluorescence experiments showed the presence of basal/stem (CK5+), luminal (CK8+), and LSCmed (CK4+CK8+) cells in tumours generated from LSCmed (Figure 5C, h) and basal/stem cells (Figure 5C, k). The micro-invasion phenotype observed in some non-atrophic irregular glands of LSCmed grafts was associated with the absence of a basal cell layer (Figure 5C, b, c, e, f), while the latter was mostly present in non-invasive glands of LSCmed (Figure 5C, h) and basal/stem (Figure 5C, k) cell grafts.

Together, these experiments demonstrate that LSCmed exhibit tumour-initiating properties and not only survive castration but also massively proliferate under these conditions of androgen depletion, supporting their key role in the progression to CRPC.

LSCmed display low androgen signalling but are sensitive to androgen manipulation

We hypothesized that the castration-tolerant properties of LSCmed could reflect intrinsically low AR signalling. This was investigated using a published list of 148 androgen-responsive genes [22]. As expected in the WT prostate, luminal cells displayed an up-regulation of AR-activated genes and a down-regulation of AR-repressed genes (Figure 6A). In contrast, androgen signalling was markedly lower in LSCmed, with AR-activated genes significantly down-regulated and AR-repressed genes up-regulated or unchanged relative to luminal cells (Figure 6A). In fact, based on androgen signalling, the hierarchical clustering places LSCmed closer to basal/stem cells (Figure 6B), which, as expected, display the lowest levels of AR signalling activation (Figure 6A and supplementary material, Figure S4). The intrinsically low androgen signalling of LSCmed likely explains why they tolerate androgen deprivation. This phenotype was not due to the lack of AR expression as LSCmed of all the genotypes studied exhibited AR mRNA levels similar to those found in WT luminal cells (Figure 6C). However, the cytoplasmic AR staining observed in LSCmed (identified by CK4+ staining on serial sections and circled with dashed lines in the right panels of Figure 6D) contrasted with the exclusively nuclear AR staining observed in mature luminal cells (black arrows). This reduced nuclear AR translocation in LSCmed (especially marked in Pb-PRL prostates)

Figure 3. The LSCmed-specific gene-expression signature is conserved across mouse models. (A) Heatmap of gene expression in WT sorted populations for 111 genes that were specifically up-regulated or down-regulated in LSCmed compared with basal/stem and luminal cells (p < 0.05; fold change > 2). (B–D) Enrichment of the LSCmed signature in an independent transcriptomics study of Ptenpc−/− tumours [GEO: GSE46799]. (B) Heatmap of gene expression showing marked enrichment of the LSCmed signature in Ptenpc−/− prostates (data were available for 92 genes of the LSCmed signature; red asterisks denote genes that are significantly different between groups and consistent with LSCmed enrichment). (C) Number of genes of the LSCmed signature that are up- and down-regulated in LSCmed (versus basal/stem and luminal cells) and in Ptenpc−/− mice (versus WT). An overlap of 85 out of 92 genes was found, out of which 73 (72 up + 1 down) were significantly different between Ptenpc−/− and WT prostates (p < 0.05 in a two-tailed independent samples t-test). (D) Hierarchical clustering of Ptenpc−/− and WT gene expression profiles [GEO: GSE46799] based on the LSCmed signature (92 genes) and showing a clear separation of both genotypes. Similar results were obtained with a different set of data (GEO: GSE46473), as shown in the supplementary material, Figure S2A–C. (E) Validation of the expression of LSCmed-specific genes by RT-qPCR on sorted cell populations from WT, intact, and castrated Pb-PRL and intact and castrated Ptenpc−/− mouse prostates (per group, n = 2–5 samples of each population isolated from one to ten pooled mice in independent experiments). Within mouse groups, different letters denote significant differences (p < 0.05) in a repeated-measures one-way ANOVA with Tukey’s post-hoc test. Basal/S or B = basal/stem; Lm = LSCmed; L = luminal cells. See also supplementary material, Figure S2.
Castration-resistant progenitors are enriched in Pten\(^{-/-}\) prostates

Figure 4. CK4 is a protein marker of LSC\(^{med}\). (A) Representative images of sorted epithelial cell subpopulations of Pb-PRL prostates stained for CK4, CK5, and CK8. (B) Representative IHC staining for CK4 in prostates of intact and castrated WT, Pb-PRL, and Pten\(^{-/-}\) mice. Black arrows indicate CK4\(^+\) cells in the left and middle panels. A strongly positive region (blue arrow) and a mainly negative region (red arrow) are shown for intact Pten\(^{-/-}\) tissue (right panels). Insets show higher-magnification images.

Figure 4

confirms the intrinsically low AR signalling activation in this cell population. Castration further down-regulated AR-activated genes that were expressed at detectable levels before castration in LSC\(^{med}\) (Mme, Fkbp5, and Msmb, taken as examples) without down-regulating AR levels (Figure 6E).

Together with our earlier findings that dihydrotestosterone stimulation of LSC\(^{med}\) led to the loss of Sca-1 expression [9], these results indicate that LSC\(^{med}\) are sensitive to androgen manipulation.

LSC\(^{med}\) are enriched in low androgen signalling contexts

Our previous findings showed that dihydrotestosterone induced the loss of Sca-1 expression in LSC\(^{med}\), suggesting that androgens stimulate their differentiation into mature luminal cells [9]. Hence, we hypothesized that the enrichment of LSC\(^{med}\) and decrease in luminal cells in intact Pten\(^{-/-}\) and Pb-PRL prostates compared with WT prostates (Figures 1 and 4) could reflect a
Figure 5. LSCmed of Ptenpc⁻/⁻ prostates highly proliferate and are tumour-initiating cells. (A) CK4 (green), Ki-67 (red) and DAPI (blue) co-immunostaining of intact and castrated prostates from WT and Ptenpc⁻/⁻ mice as indicated. Representative images of low (a, d) and high (b, c) proliferating zones are shown for both genotypes (original magnification, 60×). (B) Tumours obtained 10 weeks after engrafting sorted Ptenpc⁻/⁻ basal/stem cells (n = 2) or LSCmed (n = 3) into host mice. (C) Histological and immunostaining analysis of regenerated tumours. Representative images of H&E stains [a, d, g, i, j] and co-immunostaining [b, c, e, f, h, k] of CK4 (white), CK5 (green), and CK8 (red) are shown. Scale bars: 250 μm (a, i); 100 μm (d, g, j).

block in LSCmed differentiation into luminal cells due to decreased AR signalling in these models. Consistent with this hypothesis, several groups previously showed reduced AR output in intact Ptenpc⁻/⁻ prostates due to the down-regulation of AR expression and/or AR transcriptional activity [21,22,26]. This is in agreement with the AR cytoplasmic staining observed in Ptenpc⁻/⁻ LSCmed, which represent the majority of cells in Ptenpc⁻/⁻ prostate tumours (Figure 6D). In addition, our results show that AR signalling is also significantly
Castration-resistant progenitors are enriched in Pten−/− prostates

Figure 6. AR signalling is decreased in LSCmed. (A) Heatmap of the gene expression for genes activated (top) and repressed (bottom) by the AR in WT basal/stem, LSCmed, and luminal cells. Red asterisks denote genes whose expression was significantly down-regulated (top) or up-regulated (bottom) in LSCmed compared with luminal cells (p < 0.05 in a two-tailed, independent samples t-test). (B) Hierarchical clustering for the analysed samples based on the expression levels of AR-regulated genes. (C) Expression level of AR in sorted LSCmed of the indicated genotypes relative to sorted WT luminal cells as determined by RT-qPCR (n = 2–4 independent sorting experiments each involving one to ten mice depending on genotype). (D) Representative images of CK4 (left panels) and AR (right panels at two different magnifications) immunostaining of serial sections of prostates from intact mice of the three genotypes (as indicated) are shown (scale bars: 50 μm). In the right panels, dotted lines encircle CK4+ areas and arrows point to some luminal cells in WT and Pb-PRL prostates. (E) Effect of castration on the expression level of AR and three selected AR-activated genes in sorted LSCmed of the indicated genotypes relative to WT luminal cells sorted from intact prostates (n = 2–4 independent sorting experiments each involving one to ten mice depending on genotype, except for castrated WT mice for which one sample obtained from five animals was tested in duplicate). B = basal/stem cells; Lm = luminal progenitors (LSCmed); L = luminal cells.
reduced in Pb-PRL prostatic tissue; (i) low/absent AR nuclear staining in mature luminal cells (CK4+/− of Pb-PRL) compared with WT prostates (supplementary material, Figure S5A); and (ii) altered expression of several androgen-regulated genes denoting significantly lower AR signalling in sorted luminal cells from Pb-PRL compared with WT prostates (supplementary material, Figure S5B).

To further challenge the involvement of decreased prostatic AR-signalling activity in LSCmed enrichment, we investigated another established model of prostate cancer named Hi-Myc. These mice harbour the c-myc oncogene driven by the ARRR2/probasin promoter [27]. We used an independently generated set of transcriptomic data (GSE53202) to establish that, as opposed to Pten-null and similar to WT prostates, AR signalling was mainly unaffected in Hi-Myc mouse prostates (supplementary material, Figure S6A, B). Consistent with our hypothesis, the FACs profiles of Hi-Myc prostates bearing adenocarcinomas showed no LSCmed enrichment (supplementary material, Figure S6C, D). Furthermore, the expression of LSCmed signature genes in prostates from Hi-Myc mice was similar to that in WT mice, and markedly lower than that in Pten-null mice (supplementary material, Figure S6E, F), confirming a lack of LSCmed accumulation in the Hi-Myc model.

Together, these results show that LSCmed enrichment prior to castration is specific to mouse models with decreased AR signalling (Ptenpc−/−, Pb-PRL).

Discussion

CRPC remains a lethal disease, and the development of efficient therapeutic strategies requires a better understanding of the cellular and molecular mechanisms driving cancer recurrence. Here, we identify LSCmed cells as an important therapeutic target because they have progenitor properties; they can regenerate tumours in host mice; they increase proportionally upon disrupted AR signalling; they withstand androgen blockade in vivo; and they massively proliferate after castration in a relevant model of CRPC.

This study challenges the conventional paradigm of searching for castrate-resistant cells based on ‘basal/stem’ versus ‘luminal’ markers [28] because the transcriptional profile of LSCmed defines them as a third, distinct prostatic cell entity sharing markers of each. This is consistent with an intermediate position between a basal/stem and a mature luminal cell in the prostate lineage hierarchy and with their proposed role as luminal progenitors capable of forming organoids and spheres in vitro and tubule-like structures in vivo [9,11]. The intermediate nature of LSCmed is implied also by their proximal ductal location in WT prostate glands, where stem/progenitor cells are known to reside [29].

The fact that they have overlapping markers of luminal and basal/stem cells adds further complexity to the interpretation of previous studies, where LSCmed are likely to have been analysed together with basal/stem or with luminal cells. For example, sorting strategies using Sca-1, Trop2 or CD44 would result in their inclusion in the basal/stem population [25,30–32], whereas immunostaining or lineage tracing using CK8/18 would capture them as luminal cells [3,33]. Also, in models with low AR signalling such as the widely used Ptenpc−/− mouse, where they represent the bulk of the epithelia, LSCmed will influence the isolation of pure basal/stem or luminal cell types depending on the markers used for selection.

As evidenced from our analysis involving four different mouse models, LSCmed enrichment is linked with the intrinsic level of AR signalling activation in the prostatic tissue more than with malignancy per se, as illustrated by the lack of LSCmed enrichment in Hi-Myc prostate adenocarcinomas. This finding is particularly relevant since the standard of care of prostate cancer is androgen deprivation, which should lead to LSCmed accumulation. The molecular mediators that contribute to LSCmed enrichment in low AR-signalling contexts remain to be established. Interestingly, a recent study suggested a role for prostatic inflammation in the amplification of progenitor-like luminal cells in the human prostate [34]. Both Pb-PRL [35] and Pten-null [21] prostates exhibit significant inflammation, which was further amplified by castration. The presence of various immune genes in the LSCmed signature suggests their responsiveness to inflammatory signals. Further studies will elucidate the role of inflammation in regulating LSCmed homeostasis.

In the in vivo regeneration assay, Ptenpc−/− LSCmed generated micro-invasive lesions in glands devoid of a basal cell layer, reminiscent of what is observed in human prostate cancer. The fact that glands with a well-formed basal cell layer co-existed in the same graft suggests that LSCmed may comprise cells displaying distinct stem-like properties. Of note, WT LSCmed were shown to generate different types of organoids in vitro [11]. Although Ptenpc−/− prostate tumours are referred to as castration-resistant, significant cell death and reduction of prostate volume are observed within the first days post-castration [21]. The unchanged prevalence of the different epithelial cell populations before and after castration in this model suggests that some LSCmed subsets may be more tolerant to castration than others, leading to clonal expansion. Accordingly, we identified large clusters of highly proliferative CK4+ cells in castrated Ptenpc−/− prostates. This most probably reflects the mitogenic effect of cell-autonomous Akt signalling [22] and may be observed with other oncogenic pathways that emerge in androgen deprivation resistance [1]. Ultimately, as seen in WT, Pb-PRL, and Ptenpc−/− mouse models, LSCmed will evade androgen deprivation therapy regardless of their genetic makeup, highlighting the clinical implications of their castration resistance.

Although there is increasing evidence that epithelial cells exhibiting both luminal and stem/progenitor cell characteristics play a major role in prostate tumour progression [6–8,34], the study of these cells has been hampered by the lack of specific phenotypic markers.
Here, we validated the use of CK4 as a specific marker of LSCmed on mouse prostate sections. In addition, the genes of the LSCmed signature that we validated by RT-qPCR were all specifically overexpressed in LSCmed, irrespective of the genetic background and stage of prostate pathology. Given the large inter-patient and intra-patient tumour heterogeneity observed in prostate cancer, markers of therapy-resistant cells that are independent of genetic mutations driving disease progression represent optimal targets for successful treatment.

To translate these findings to the clinic requires robust detection and targeting of LSCmed in human specimens without using Sca-1, for which a human homologue is unknown. Based on our results, CK4 may be a promising candidate for detection. Of interest, a recent study reported CK4 expression in some prostate cancer specimens [36]. Also, luminal progenitor-like cells exhibiting tumourigenic capacity were recently identified in normal and pathological human prostate as low CD38-expressing cells [34]. Although expression of CK5 and Nkx3.1 distinguishes these cells from LSCmed, typical LSCmed markers including CK4 deserve to be investigated in low CD38-expressing cells.

As there is a worldwide effort to treat lethal CRPC, this article addresses a major need in prostate cancer treatment. Our focus on cells that survive castration identified LSCmed as new cellular targets. The LSCmed signature provides an important resource and reference for future studies on cell population targeting for the prevention of therapy-resistant prostate cancer.

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Author contributions statement

LSS, J-EG, and VG conceived and designed the study. LSS, FB, GM, AGP, ML, JC, NL, AC, NC, and CBF collected and assembled the data. LSS, GPR, J-EG, and VG analysed and interpreted the data. JP, RAT, GPR, and LK provided mice and prostate material, and intellectual input. LSS, JP, RM, GPR, RAT, LK, J-EG, and VG wrote the manuscript. LSS, J-EG, and VG approved the final version of the manuscript.

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