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Understanding and targeting prostate cancer cell heterogeneity and plasticity

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Abstract

Prostate cancer (PCa) is a prevalent malignancy that occurs primarily in old males. Prostate tumors in different patients manifest significant inter-patient heterogeneity with respect to histo-morphological presentations and molecular architecture. An individual patient tumor also harbors genetically distinct clones in which PCa cells display intra-tumor heterogeneity in molecular features and phenotypic marker expression. This inherent PCa cell heterogeneity, e.g., in the expression of androgen receptor (AR), constitutes a barrier to the long-term therapeutic efficacy of AR-targeting therapies. Furthermore, tumor progression as well as therapeutic treatments induce PCa cell plasticity such that AR-positive PCa cells may turn into AR-negative cells and prostate tumors may switch lineage identity from adenocarcinomas to neuroendocrine-like tumors. This induced PCa cell plasticity similarly confers resistance to AR-targeting and other therapies. In this review, I first discuss PCa from the perspective of an abnormal organ development and deregulated cellular differentiation, and discuss the luminal progenitor cells as the likely cells of origin for PCa. I then focus on intrinsic PCa cell heterogeneity in treatment-naïve tumors with the presence of prostate cancer stem cells (PCSCs). I further elaborate on PCa cell plasticity induced by genetic alterations and therapeutic interventions, and present potential strategies to therapeutically tackle PCa cell heterogeneity and plasticity. My discussions will make it clear that, to achieve enduring clinical efficacy, both intrinsic PCa cell heterogeneity and induced PCa cell plasticity need to be targeted with novel combinatorial approaches.

Keywords

Cancer cell heterogeneity; Prostate cancer; Androgen receptor; Cancer stem cells; Cancer cell plasticity

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Declaration of Competing Interest

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1. Introduction

Human prostate cancer (PCa) is a prevalent malignancy with >1.4 million new cases and >375,000 deaths worldwide in 2020. In the U.S, more than 250,000 new PCa cases are diagnosed each year with ~30,000 PCa-associated deaths. Why is the adult prostate so susceptible to cancer development? The normal human prostate (NHP) is a pseudostratified two-layer epithelial glandular organ that consists of basal and luminal epithelial cells together with rare neuroendocrine (NE) cells [1] (Fig. 1A–B). Luminal cells, characterized by the expression of cytokeratin (CK) 8 (CK8), CK18, androgen receptor (AR), prostate-specific antigen (PSA), are 'functional' cell types that secrete prostatic fluid into the lumen, which constitutes a major part of the ejaculate. Basal cells express CK5, CK14 and the p53 family member, p63, and interface the luminal compartment with the underlying stroma through basal lamina (Fig. 1A–B).

Primary PCa is pathologically graded in the clinic by a combined Gleason score (GS) system – the higher the GS the more malignant (less differentiated) the tumor is (Fig. 1C). Most prostate tumors are diagnosed as adenocarcinomas, meaning that tumors contain glandular structures that resemble the normal prostatic glands (see Fig. 2; below). Although the majority of low-grade tumors (GS6–7) can be 'cured' by the surgical procedure called radical prostatectomy, many patients with high-grade tumors (GS9–10) have extra-prostatic involvement and lymph node (LN) metastasis and are treated with drugs called AR signaling inhibitors or ARSIs (Fig. 1C). There are two broad classes of ARSIs: those that block the production of AR ligands (mainly testosterone, T) in the testis (e.g., Lupron) or adrenal gland (e.g., abiraterone acetate) and those that block AR nuclear translocation and AR functions through direct interactions with AR (e.g., enzalutamide, Enza). In most clinics, PCa patients with advanced tumors and ineligible for surgery are first treated with androgen synthesis inhibitors, a protocol called androgen-deprivation therapy (ADT), which generally is effective for ~6–23 months (Fig. 1C). The ADT-resistant PCa are then treated, as the second-line therapy, with Enza or Enza together with chemotherapeutic drugs such as docetaxel. Tumors that have failed ADT or ADT/Enza are often 'generically' called castration-resistant PCa or CRPC (Fig. 1C) although sometimes tumors that have failed the first-line ADT are called primary CRPC whereas tumors that have become resistant to both ADT and second-line antiandrogens such as Enza are called secondary CRPC. Advanced primary PCa and, in particular, CRPC patients often have metastases in many organs including bone, LN, lung, liver, adrenal gland and brain (Fig. 1C). PCa patients who have only a few metastases in a limited number of organs are frequently said to have oligometastatic disease. Regardless, metastatic CRPC (mCRPC) patients have a poor prognosis and very limited treatment options, which generally include chemotherapeutics such as cisplatin and docetaxel.

ARSIs represent among the most remarkable and most effective targeted anti-cancer therapeutics [2], with the majority of PCa patients responding impressively with clear clinical evidence of tumor regression (Fig. 1C–D). The response of PCa patients to ARSIs follows the 3 'pro-totypical' phases, i.e., the response (or shocking), adaptation and recurrence (or relapse) phases (Fig. 1D). The dramatic initial response to ARSIs can be explained by the fact that the majority of prostate tumors are adenocarcinomas with most

tumor cells being AR^+ and dependent on AR signaling for their survival. It is unclear what types of PCa cells survive ADT/Enza and enter the adaptation phase (Fig. 1D); however, as this phase is characterized by dormancy, it's possible that a population of dormant or slow-cycling cells [3] such as PCa stem cells (PCSCs; see below) preferentially survive ADT/Enza and mediate subsequent tumor relapse (Fig. 1D). Interestingly, recent studies suggest that chemotherapy-treated tumors during 'drug holiday' enter a dormant stage resembling the developmental program called diapause, during which subsets of cancer cells downregulate mTOR signaling but upregulate autophagy to survive [4–7].

2. Cellular heterogeneity of the normal prostate and luminal progenitor cells as potential cells of origin of human prostate cancer

The adult prostate, a relatively dormant organ during homeostasis, nevertheless, consists of heterogeneous subpopulations of cells in both basal and luminal cell compartments (Fig. 1A) that may undergo dynamic changes during aging and inflammatory responses. Thus, the basal cell layer consists of differentiated $CK5^+/CK14^+/p63^+$ basal cells as well as the basal stem/progenitor cells (i.e., cells with certain stem cell properties such as bi-potential or multi-lineage differentiation) expressing high levels of CD44, CD49b (integrin α2), CD49f (integrin α6) CD133, and BCL-2 (Fig. 1A). Likewise, the luminal cell layer contains not only differentiated $CK8^+/CK18^+/AR^+/PSA^+/CD26^+$ luminal cells but also the luminal progenitor (LP) cells (Fig. 1A) that are frequently double-positive for both CK5 and CK8 (i.e., $CK5^+CK8^+$), express high levels of CK19 ($CK19^{+/hi}$), and retain significant proliferative capacity [8–23]. The LPs are also endowed with an enhanced ability to form organoids *ex vivo* [18,21,23] and express high levels of prostate stem cell antigen (PSCA) and low levels of CD38 (i.e., $CD38^{lo}$) [22–25]) (Fig. 1A). Recent single-cell RNA-seq (scRNA-seq) studies in human and mouse prostate have also revealed heterogeneous cell populations in the epithelial and stromal compartments and, particularly, have uncovered plastic LP cell populations that might be involved in mediating castration resistance [26–37].

Studying the NHP epithelial cell heterogeneity and hierarchy has an important bearing on understanding the potential cell(s) of origin of human PCa.—Like other glandular organs such as the breast and pancreas, development of the prostate follows the basic guiding principles of branching morphogenesis [38]. 'Lineage tracing' studies in the adult human prostate using mitochondrial DNA mutations (identified by sequencing) combined with cytochrome c oxidase/succinate dehydrogenase enzyme histochemistry (to identify areas of respiratory chain deficiency) have suggested that both basal and luminal epithelial (as well as the NE) cells are derived from a common stem/progenitor cell [39,40]. Further mapping studies reveal that primitive prostate stem cells are localized in the basal cell layer proximal to the urethra, which then direct stem cell differentiation along the prostate ductal tree with diminishing numbers of progenitors towards the distal acini [41]. scRNA-seq studies in the NHP have also implicated less mature prostate epithelial cells close to the urethra [26]. Recent whole-genome sequencing of targeted microdissections of normal/benign prostate from fetuses to aged adults suggests that the fetal organ development starts with 24–30 ductal subunits with each duct deriving from 5 to 10 embryonic progenitor cells [42]. During puberty, hormones and growth factors act on local stem cells in ductal units to promote branching morphogenesis and increase branching complexity [42].

Interestingly, somatic mutations generally accumulate slowly in the adult prostate at ~16/year/cell [42], consistent with the human adult prostate being a rather dormant organ with slow cellular turnover.

Exactly which cells, basal or luminal, differentiated or stem/progenitor cells, become tumorigenically transformed to develop into PCa remains an important outstanding question.—RNA expression profiling [17,43], coupled with prospective tumor transformation studies [e.g., 18], suggests that some cells in both basal and luminal cell compartments can function as the cells of origin for human PCa, although basal-originated PCa appears to be associated with the minor but more aggressive subtypes of PCa (such as neuroendocrine PCa, NEPC) whereas the luminal-originated PCa tend to be adenocarcinomas [17,43], the major type of PCa. Evidence is accumulating that LPs in the luminal cell compartment (Fig. 1A) may represent the preferred cellular targets of tumorigenic transformation [18,20–25]. The NHP LPs represent <2% of the $CD26^{+}CD49^{lo}$ (or $TROP2^{+}CD49^{lo}$) luminal cell population, can readily establish organoids containing both luminal and basal cells (i.e., bipotentiality; [18,21,23]), and have been phenotyped as $PSCA^{hi}CD38^{lo}$ [15]. Increasing numbers of LP cells have been observed to accumulate in the aging prostate [24]. Significantly, the LPs freshly purified from the $CD26^{+}$ luminal cell population and propagated in 3D organoid conditions can be transformed to PCa by lentiviruses expressing c-MYC together with constitutively active AKT [21]. We have similarly shown that organoid-forming LPs in $TROP2^{+}CD49f^{lo}$ isolates from the NHP can be transformed by AR, AKT and ERG to form typical $AR^{+}PSA^{+}$ adenocarcinomas [18]. Notably, we have developed a novel 2D culture system that could extend the lifespan of the LPs beyond 100 population doublings (PDs) [18]. The 2D-propagated LPs possess cardinal features of stem/progenitor cell properties (e.g., forming more and larger holoclones, spheres and organoids; regenerating prostatic glands in vivo containing both $CK5^{+}P63^{+}$ basal cells and $CK8^{+}AR^{+}PSA^{+}$ luminal cells), and are phenotyped as $CK5^{+}CK18^{+}CK19^{hi}$ with increased mRNA (but not protein) levels of *AR* and *PSA* and increased AR activity (compared to basal cells). These LP cells express a unique stem/progenitor cell-enriched gene expression profile linked to PCa aggressiveness, castration resistance, metastatic propensity and poor patient survival, and, significantly, can be oncogenically transformed by AR/AKT/ERG overexpression as well as by c-MYC together with PTEN-KO [18].

How do the LP cells (or other prostate epithelial cells) become trans- formed into tumor cells?—As PCa manifests a distinct association with age and LPs accumulate and expand in the aging prostate [24], it is reasonable to speculate that PCa etiology may be linked to immune senescence and impaired immune surveillance in the aging organ [44, 45]. Mounting evidence starts to link chronic inflammation to development of PCa precursor lesions and to prostate tumorigenesis [46,47]. There are two putative precursor lesions to PCa [48,49]: prostatic intraepithelial neoplasia (PIN) and prostate inflammatory atrophy (PIA). PIN is characterized by a hyperplasia phenotype such that proliferative luminal cells, likely LPs, would often fill up the lumen. Genomics studies using micro-dissected tissues to track clonal mutations [50–52] suggest that high-grade PIN (HGPIN) represents a genomically distant precursor to invasive adenocarcinomas and that the clinically common GS7 PCa are derived from branched evolution of clonal lesions with Gleason 3 and 4

patterns. On the other hand, PIA displays an atrophic phenotype with a single layer of smaller luminal cells lining the prostatic ducts and acini, which are frequently associated with prominent inflammatory infiltration [48,49]. Although the PIA glands are atrophic, luminal cells, which have the cardinal features of LPs being $CK5^{+}CK8^{+}$ (i.e., intermediate cells), are nevertheless highly proliferative in association with, among others, reduced levels of $p27^{Kip1}$ [48,49, 53]. There is evidence that at least some HGPIN and early adenocarcinomas may arise from the PIA, likely driven by inflammatory cytokines and inflammation-derived oxidants, electrophiles, and genotoxins [46–49,53]. A recent study from the same group provided striking evidence to directly link bacterial prostatitis to PIA and TMPRSS2-ERG fusion, the most prevalent (up to 50 %) genomic alterations in PCa [54]. The authors identified ERG^{+} PIA transitioning to early invasive cancer and also identified pathogen-derived genotoxin colibactin as a potential source of DNA damage [54]. This remarkable new work [54] suggests that infection-induced ERG fusions represent an early alteration during prostate tumorigenesis and that PIA indeed may serve as a direct precursor to PCa.

One hypothetical scenario is that inflammatory insults induce apoptotic death of differentiated luminal cells pushing LP cells to the cell cycle and leading to expansion of the LP cell population and transitioning from PIA to PIN. Uncontrolled proliferation of LPs in HGPIN, driven by the chronic inflammation and activated oncogenes such as c-MYC and AKT (due to *PTEN* loss or mutations), would lead to telomere attrition and subsequent genetic, genomic and chromosomal instability, which, together with additional genetic and epigenetic alterations, would facilitate transition of HGPIN to adenocarcinomas and promote PCa progression. In support of this hypothesis, ~25% of the HGPIN are DNA aneuploid [55] and telomere shortening, with or without concomitant telomerase activation, represents an early and the most frequently observed and consistent somatic genomic alteration in HGPIN and early-stage adenocarcinoma [56–68]. In fact, as much as ~96 % of precursor lesions to multiple epithelial cancers (including those in prostate, bladder, breast, pancreas, esophagus, large intestine, oral cavity and uterine cervix) manifest shortened telomeres and abnormalities in telomere length [59]. Notably, telomere erosion occurs exclusively in luminal (but not basal) cells in HGPIN [56], different HGPIN lesions often exhibit different levels of telomere shortening [57], telomere loss in HGPIN is correlated with abnormalities on chromosome 8 and also with the levels of oxidative stress as measured by malondialdehyde [62], HGPIN and PCa often display prominent telomere fusions and anaphase bridges [63], and prostatic glands with shortened telomeres (e.g., due to reduced TRF2 expression) developed age-and p53-dependent hyperplasia whereas prostate tumors with *TRF2* loss were mostly high-grade AR-negative adenocarcinomas [67].

Recent whole-genome sequencing studies [69–72] have identified many somatic clonal mutations in apparently normal tissues, especially fast-renewing tissues such as the skin, esophagus and colon, some of which accumulate with age and become driver clones of cancer. It is not clear whether apparently normal prostate or PCa precursor lesions also harbor such driver mutations although cells in PCa precursor lesions that have sustained TMPRSS2-ERG fusion appear to be clonal. It also remains unknown whether prostate tumorigenesis involves the phenomenon of (stem) cell competition and clonal fitness [73–77].

3. PCa cell heterogeneity

3.1. Genomic, transcriptomic, histological and cellular heterogeneity of PCa

—Intra-tumor heterogeneity (ITH) makes significant contributions to therapy resistance [78,79]. PCa manifests ITH at every interrogated level [80,81]. *At the genomic level*, multiple genomic sequencing studies [82–96] in various type of PCa, including localized indolent, localized non-indolent, early-onset, metastatic castration-sensitive (mCSPC), and metastatic castration-resistant tumors (mCRPC) have made it clear that primary prostate tumors are multi-focal containing genomically distinct clones and subclones whereas PCa metastases are clonal, meaning that each metastasis in different organs was derived from the outgrowth of a single 'metastatic stem cell'. *At the epigenome level*, the epigenetic landscape of PCa can be correlated with germline genomic features [97]. Whole-genome bisulfite sequencing in 100 mCRPC revealed that ~20 % of the metastases exhibited a novel epigenomic subtype associated with hypermethylation and somatic mutations in *TET2, DNMT3B* and *IDH1* [98]. Notably, differential methylation during CRPC progression preferentially occurs at somatic mutational hotspots and putative regulatory regions [98]. Furthermore, DNA methylation-mediated 3D genome architecture acts, synergistically, with some risk SNPs (single nucleotide polymorphism) to confer predispositions to PCa [99]. *At the transcriptional level*, prostate tumors are found to harbor abnormal RNA transcripts such as circular RNAs (circRNAs) [100] as well as a myriad of unspliced mRNAs [101], and, to display tremendous heterogeneity in the global transcriptomes even in tumors pathologically classified as the same Gleason Grade [102]. Of clinical significance, molecular subtyping of PCa based on transcriptional profiling has identified a luminal subtype of adenocarcinomas that have an aggressive progression trajectory and are associated with poor patient outcomes [103–108]. It is likely that this aggressive subtype of luminal PCa is driven by LPs [20].

Accompanying the genomic, epigenomic and transcriptomic heterogeneity, PCa also exhibit wide variations in histological presentations. As illustrated in Fig. 2, the 3 human PCa (HPCa57, 58, 70) that are all diagnosed pathologically as GS7 look nothing alike histologically. Finally, prostate tumors are characterized with pervasive cellular heterogeneity, manifested as variegated morphologies, nuclear-to-cytoplasm ratio, metabolic activity, and antigenic marker expression (see discussions below). Recent scRNA-seq studies [109–115] have also revealed heterogeneous cell subpopulations in LNCaP cultures [109], carcinoma-associated fibroblasts (CAFs) [110] and cancer-associated macrophages [115] in patient tumors, and in primary prostate tumors [111–113] as well as mCRPC [114]. In fact, even circulating tumor cells (CTCs) in PCa patients can be epithelial, mesenchymal or a hybrid phenotype and similarly exhibit heterogeneity in their transcriptomes at the single-cell levels [116].

3.2. PCa as an abnormally differentiated organ and PCa cell heterogeneity in AR and PSA—

Since 2002, our lab has been studying PCa from the perspective of deregulated organ differentiation by systematically and meticulously dissecting phenotypic and functional properties of heterogeneous PCa cell populations and studying molecular regulators in HPCa samples, PDX, xenografts and cell cultures [2,3,16–18,20,117–169] (Figs. 2–13). Nearly 20 years of focused studies in our lab on PCa cell heterogeneity and plasticity, and their impact on therapy response, have uncovered several fundamental

principles relevant to our understanding of tumor progression and development of therapy resistance in patients (Fig. 14; see discussions below). PCa development and progression are accompanied by a decrease and loss of normal differentiation program at the organ and cellular levels. Hence, in low-grade (GS6–7) tumors, both differentiated (characterized by the presence of glandular structures) and undifferentiated (lack of glandular structures) tumor regions can be observed (Fig. 2C, F, I; zoom-in images). The 'differentiated' areas in these tumors are populated by significantly increased numbers of glands, as if these prostatic glands had undergone fission (Fig. 2C, F, I; zoom-in images). In contrast, most high-grade (GS9–10) tumors present an undifferentiated or a poorly differentiated phenotype that lacks significant glandular structures (Fig. 2L) [131,144,149]. The concept of prostate tumors as an organ of increasingly deregulated differentiation is also supported by the gradual loss of normal prostatic basal cells expressing CK5, CK14 and p63.

At the cellular level, significant heterogeneity in the expression of AR and PSA and prominent discordance between AR and PSA expression have been observed (Figs. 2; 3) [131,144,147,163]. For example, in GS7 and GS9 tumors, both AR^{+} and $AR^{-/lo}$ regions and PCa cells can be readily observed in whole-mount (WM) sections (Fig. 2B, E, H, K). Similarly, both PSA^{+} and $PSA^{-/lo}$ PCa regions and PCa cells can be observed in GS7 tumors (Fig. 2A, D, G), and, in GS9 tumors, most PCa cells appear to be $PSA^{-/lo}$(Fig. 2J). Heterogeneity in AR and PSA expression in human PCa has also been observed at the mRNA levels (Fig. 3). In fact, heterogeneous expression in AR and PSA has been reported for decades in the literature [2,147,170–214]. AR and PSA heterogeneity, and, $AR^{-/lo}$ and $PSA^{-/lo}$ PCa cells have been observed across the entire spectrum of PCa development, progression, therapy resistance and metastasis from treatment-naïve primary tumors to CRPC to mCRPC (Figs. 2; 3B; 4) [2,131,132,144,147,164]. Importantly, discordant AR and PSA expression has been observed at both mRNA and protein levels and at the tumor as well as the cellular levels (Figs. 3B; 4A). Indeed, at the tumor level, discordance in *AR* and *PSA* mRNA expression is apparent across GS6–9 tumors (Fig. 3B) [131,144]. Similarly, discordance in AR and PSA protein expression also exists in treatment-naïve primary HPCa such that although the majority cells in these adenocarcinomas, as expected, have the $AR^{+}PSA^{+}$ (i.e., double-positive) pheno-type, $AR^{+}PSA^{-/lo}$, $AR^{-/lo}PSA^{+}$ and $AR^{-/lo}PSA^{-/lo}$ PCa cells are also observed at different abundance (Fig. 4A–B) [144]. Of note, PCa cells of the above 4 AR/PSA phenotypes have also been observed in xenograft models and shown to manifest distinct responses to ARSIs and other therapeutics (Fig. 5) [144] (see discussions below).

3.3. Tumor cell PSA is a PCa differentiation marker and tumor PSA levels correlate with better patients' survival—

Do phenotypically distinct PCa cells display different biological and functional properties? We started addressing this question by focusing on PSA^{+} and $PSA^{-/lo}$ PCa cells. Serum PSA levels have been routinely utilized in the clinic to help diagnose PCa and, upon ADT/Enza treatment, rising (rebound) serum PSA levels represent a biomarker for tumor relapse. However, PSA, normally regulated by AR, a master pro-differentiation transcription factor (TF), represents probably one of the most lineage-specific differentiation markers in the human epithelia, as it is only expressed in fully differentiated human prostatic luminal cells. PSA is not expressed in rodents. As

a prostate-specific differentiation marker, tumor cell *PSA* (*KLK3*) mRNA levels gradually decrease with increasing tumor malignancy, i.e., Gleason grade (Fig. 3A–E) [131,144]. This inverse correlation between the *PSA* mRNA levels and tumor grade can be best appreciated by analyzing the TCGA PRAD data set (Fig. 3F). As expected, early-stage (GS6) tumors showed significantly increased PSA mRNA levels than the normal prostate tissues (Fig. 3F), likely due to increased AR activity early during prostate tumorigenesis initiated and driven by the transformed LPs [20]. However, as tumors progress to higher Gleason grades, the tumor *PSA* mRNA levels gradually declined (Fig. 3F). This tumor grade-associated decrease in *PSA* mRNA levels may, again, be related to AR signaling since during PCa progression, AR signaling intensity becomes attenuated [215,216] due to increasing numbers of genetic and epigenetic mutations and alterations such as MYC and AKT overexpression and loss of tumor suppressors. Importantly, reduced tumor (cell) *PSA* mRNA levels were also observed in PCa metastases and recurrence (Fig. 3G–J), and prostate tumor *PSA* mRNA levels positively correlate with patients' overall and BCR (Biochemical Recurrence) free survival (Fig. 3K–L) [131,144].

Consistent with the changes in *KLK3* mRNA, intra-tumor PSA protein levels also decrease accompanying PCa progression (i.e., increasing Gleason grade) and become largely lost in CRPC (e.g., Fig. 4D) [131,144, 147]. Similar to PSA, another prostatic luminal differentiation marker, CD26 (also called DPP4; Fig. 1A), is also downregulated and lost during PCa progression and its loss causally promotes CRPC development [217]. It should be noted that our discussions herein linking tumor PSA (mRNA and protein) levels to overall tumor (cell) differentiation and to better PCa patient survival should NOT be confused with increased serum PSA levels as a PCa diagnostic and prognostic biomarker. PSA, as a secreted protein, is normally restricted to prostatic ducts and constitutes part of the seminal fluid, and should not be detected in the blood at significant levels. Increasing blood PSA levels only signal that the organ and vascular integrity of the prostate has been compromised allowing the access of PSA to the circulation.

3.4. The undifferentiated *(PSA$^{-/lo}$)* compartment harbors self-renewing and therapy-resistant PCSCs—

Associated with the heterogeneity in PSA expression are (differentiated) PSA^{+} and (undifferentiated) PSA$^{-/lo}$ PCa cells that are readily observed in treatment-naïve human prostate tumors (e.g., Fig. 2A,D,G,J; Fig. 4A–B) as well as in PCa cell cultures (Fig. 5D) and xenografts (Fig. 5A,B,E) [131,144,149]. The PSA$^{-/lo}$ PCa cells are significantly increased in high-grade (GS9/10) tumors compared to low-grade (GS6/7) tumors (e.g., compare Fig. 2J and A,D,G) and become the predominant cell population in treatment-failed PCa and CRPC (Fig. 4C–E). Similarly, all castrated xenografts such as LNCaP-AI (Fig. 5A–B) and LAPC4-AI (Fig. 5E), as well as LNCaP cells subjected to chronic castration in vitro (Fig. 5C; also see Fig. 11, below), are enriched in PSA$^{-/lo}$ cells [131,144,149,152].

Do PSA^{+} and PSA$^{-/lo}$ PCa cells have distinct biological behavior, functional properties and therapeutic responses? To directly address this question, we developed a series of lentiviral-based lineage reporters in which a compound *PSA* promoter (*PSAP*) and enhancer was used to drive GFP (or other fluorescent reporter) expression. The *PSAP*-GFP lentiviral reporter was found to faithfully report and separate PCa cells that express abundant endogenous PSA

(GFP$^+$) vs. those that express little/no PSA (GFP$^{-/lo}$) (Fig. 6) [131,144,152]. As expected, most PSA$^+$ PCa cells express high levels of nuclear AR whereas most PSA$^{-/lo}$ cells are AR$^{-/lo}$ (Fig. 6A). Comprehensive biological studies and functional interrogations reveal that the PSA$^{-/lo}$ PCa cell population harbors authentic PCSCs that can long-term self-renew and propagate tumors in vivo, and are inherently therapy-resistant [131,144,149,152]. *First of all*, the PSA$^{-/lo}$ PCa cells are enriched in stem cell gene expression profiles, much like the normal (AR$^{-/lo}$PSA-) basal/stem cells [17,43]. *Secondly*, PSA$^{-/lo}$ PCa cells also possess some of the epigenetic makeups of normal stem cells such as poised bivalent chromatin characterized by simultaneous association of genes with the H3K4me3 and H3K27me3 histone marks [144]. *Thirdly,* 5–18 % of the cells in PSA$^{-/lo}$ PCa cell population can undergo Notch-regulated asymmetric cell division (ACD; Fig. 6B) [131,144,149], a cardinal feature of epithelial stem cells. *Fourthly*, similar to many normal adult stem cells, PSA$^{-/lo}$ PCa cells are much more quiescent and have longer cell-cycle transit times than PSA$^+$ cells (Fig. 6C). The dormant nature of PSA$^{-/lo}$ cells can also be appreciated from the single-cell tracing: while the single GFP$^+$ LNCaP cell developed into a clone of 6 cells within 66 h, the single GFP- LNCaP cell only generated a 3-cell progeny in 127 h (Fig. 6B). *Fifthly*, the PSA$^{-/lo}$ PCa cells possess much greater serial tumor-propagating capacity than corresponding PSA$^+$ cells in that the PSA$^{-/lo}$ PCa cells can initiate xenograft tumors that can be propagated indefinitely whereas PSA$^+$ PCa cell-initiated xenografts can only be propagated for a limited number of generations (Fig. 6D) [131,144,149]. *Sixthly*, PSA$^{-/lo}$ PCa cells are much more resistant to apoptosis induction and become greatly enriched by castration, prooxidants and chemotherapeutic drugs (Fig. 6E–F) [131, 144,152]. *Lastly*, purified PSA$^{-/lo}$ PCa cells could readily initiate and propagate CRPC in castrated mice at low cell input [131,144,149]. Together, these observations indicate that the undifferentiated PSA$^{-/lo}$ PCa cell population harbors long-term self-renewing CSCs (i.e., PCSCs), which can indefinitely propagate xenograft tumors, are inherently insensitive to castration and other therapeutics, and may function as cell (s) of origin for CRPC.

3.5. Heterogeneity in PCSC phenotypes and challenges of studying PCSCs in treatment-naïve primary prostate tumors—

Our studies on PCa cell subpopulations and their molecular regulation [117–169] in multiple xenograft and PDX models as well as >200 patient tumors and tumor-derived cells and organoids (e.g., Figs. 2, 4, 7, 8) suggest that human PCSCs that meet at least some of the CSC definitions [3,120,129,140,145,160] are phenotypically heterogeneous. For example, ABCG2$^+$ [171], CD44$^{+/hi}$ [118], CD44$^+\alpha2\beta1^+$ [119], holoclone-forming [121], and NANOG-expressing [122,125] PCa cells all possess some of the classically defined CSC properties and identify overlapping and non-identical subpopulations of tumor-initiating and tumor-propagating cells depending on specific cell types and genetic backgrounds of the model and patient tumors [144]. Interestingly, these phenotypically diverse subsets of PCSCs all seem to be encompassed in the PSA$^{-/lo}$ PCSC pool [144] (see Fig. 14A, below). Importantly, despite the phenotypic diversity, the functionally defined PCSCs share one common property, i.e., they are intrinsically resistant to various therapeutics including castration, antiandrogens, radiation, chemotherapy drugs, and prooxidants. I should point out that the cell(s) of origin for cancer and CSCs are two different concepts that should not be mixed up and used inter-changeably [145].

Along with our efforts in dissecting the functional properties of PCa cell subpopulations and PCSCs, numerous groups worldwide have reported on PCSCs [218–324]. A Pubmed search with 'Prostate Cancer Stem Cells' as the Keyword would turn up >3000 references. These reported PCSCs manifest a wide diversity of phenotypes including, among others, $ABCG2^{+}$ [218], $CD133^{+}$ [219,220,236], $CD133^{+}\alpha2\beta1^{+}$ [219], $CD44^{+}$ [219,232,299,311], $ALDH^{+/hi}$ [224], $MHC^{-/lo}$ (and $PSA^{-/lo}$) [226], LP-like [227,238] or basal-like [231], $Trop2^{+/hi}$ [230], TOP2A-[243], $hTERT^{hi}$ [250], $Bmi1^{+}Sox2^{+}$ [260], $CD24^{+/hi}$ [261], $CXCR2^{+}$ [272], $Zeb1^{+}$ [280], holoclone-forming cells [291], and $CD117(KIT)^{+}$ [310]. Many of these PCSC phenotypes overlap with the PCSCs reported by our lab. Of clinical relevance, expression of some PCSC phenotypic markers has been linked to PCa aggressiveness and poor patient prognosis and survival [220,240,294].

Studying PCSCs or tumor-initiating PCa cells in treatment-naïve patient primary tumors are generally challenging, based on our working experience on 232 primary HPCa at the M.D Anderson Cancer Center (from Feb. of 2005 to April of 2016; Figs. 7, 8) and 48 primary HPCa at the Roswell Park Comprehensive Cancer Center (May of 2016 – present). Unlike CSCs freshly purified out from some other aggressive human cancers (e.g., colorectal and lung cancers and GBM) that can readily initiate serially transplantable xenografts, putative PCSCs acutely purified from patient primary tumors, especially the low-grade tumors, frequently do not manifest significant tumor-regenerating capabilities, even in the presence of 'helper' cells such as CAFs and mesenchymal stem cells or MSCs [123,132,144]. The difficulty in experimentally reconstituting the tumorigenicity in immunodeficient mice of freshly purified HPCa cells and/or PCSCs may be related to the well-known indolent nature of most low-grade tumors (i.e., GS6–7 tumors, representing >70 % of the HPCa; Fig. 7A) and the complex tumor microenvironment (TME) of PCa [155]. In support of these points, primary prostate tumor pieces, when xeno-transplanted into various types of immune-deficient mice (regular or irradiated (IR) NOD/SCID, NSG, Rag2, etc) at different sites including subcutaneum (s.c), anterior prostate (AP) or subrenal capsule in Matrigel or together with rUGM (rat urogenital sinus mesenchyme), frequently initiated PDX that could often be serially passaged (Figs. 7D–E; 8). As expected, high-grade tumors (≥GS8) generally exhibit higher tumor take rates (Fig. 7D) although we have succeeded in generating multiple PDX lines from primary prostate tumors of GS7-GS9 (Fig. 8), and many PDX lines, e.g., HPCa101 from a GS9 (4 + 5) patient tumor (Fig. 7E) and HPCa70 from a GS7 patient tumor (Fig. 8E), have been passaged for many generations (G) in immune-deficient mice. Occasionally, we could even reconstitute the high tumorigenicity in PCSCs freshly isolated from primary tumors. For instance, the $CD44^{+/hi}$ PCa cells acutely purified from the GS8 HPCa52 patient tumor demonstrated significantly higher tumor-initiating frequency (TIF) than the corresponding $CD44^{-}$HPCa52 cells (Fig. 7C). Significantly, we have employed HPCa cells freshly purified from many high-volume primary tumors (i.e., with ≥80 % tumor involvement) in our dissection of PCa cell heterogeneity and studies on PCSCs [17,18, 118–120,122,123,125,126,130–132,139,144,149,151,152,154,160, 163–165]. As illustrated in Fig. 7F, most of the $CD44^{+}$ HPCa cell populations freshly purified from a dozen of GS7-GS9 HPCa were found to express much lower levels *AR* and *PSA* mRNA than the corresponding $CD44^{-}$ HPCa cells [144]. These observations in patient primary tumors are consistent with our data showing that the $CD44^{+/hi}$ PCa cell population

is enriched in PCSCs [118,119,126,130,131,144] (Fig. 7C) and with the concept that PCSCs are less differentiated expressing lower levels of AR and PSA [131,144,149,152].

3.6. Therapy resistance and metastatic propensity in PCSCs and molecular regulators of PCSCs—Studying the genetic, transcriptomic and cellular heterogeneity in PCa [2,80–116,325] and in-depth elucidation of PCSCs [117–169, 218–324] have direct relevance to understanding the mechanisms of therapy resistance [326,327]. Recent studies in PCa patients undergoing neoadjuvant ADT trials have directly linked the clonal heterogeneity, i. e., ITH, to distinct clinical responses [328–330]. For example, primary tumor clones with alterations in multiple oncogenic drivers are preferentially selected for by neoadjuvant-intensive ADT [328]. In a case of PCa with two primary tumor foci, a large GS9 tumor and an adjacent GS7 nodule, DNA sequencing showed that the GS9 tumor had suffered single-copy loss of both *PTEN* and *TP53* [329]. Strikingly, neoadjuvant intensive ADT completely eliminated the GS7 nodule whereas subclones from the GS9 tumor that had sustained mutations to the remaining copies of *PTEN* and *TP53* emerged to mediate ADT resistance [329]. These studies [328–330] highlight the importance of nascent PCa cell genetic heterogeneity in dictating tumor response to clinical treatments.

PCSCs, in spite of phenotypic diversity, are commonly endowed with high clonogenic and tumor-initiating/tumor-propagating activities. Importantly, PCSCs have been reported to be intrinsically therapy-resistant, including resistance to castration, chemotherapy, radiotherapy and, likely, immunotherapy [2,3,125,131,138,218,226,227, 251–253,265,278,299,300,310,313,315]. For example, the $PSA^{-/lo}$ PCa cells are resistant to castration, antiandrogens, prooxidants and chemodrugs [131,144] and $ABCG2^{+}$ PCSCs are resistant to ARSIs due to increased efflux of androgens [218]. Similarly, the $PSA^{-/lo}/MHC^{-/lo}$ PCSCs are resistant to docetaxel as a result of enhanced Notch and Hedgehog signaling [226], and the $CD4^{4}$+ [299], LP-like [227,252] and hypoxia-induced [265] PCSCs are all inherently castration- and chemo-resistant. Finally, sphere-derived PCSCs are resistant toγ?d T cell-mediated cytotoxicity [300] and PCSCs, like many other CSCs, are generally ‘immuno-deficient’ lacking the expression of many immunogenic molecules [160].

PCSCs are also invasive and possess high metastatic potential [3,118, 126,128,155,159,228,309,313]. For example, our early studies indicate that the $CD44^{+}$ PCSCs are highly metastatic [118] and their metastatic capacity can be suppressed by miR-34a [126] and miR-141 [154]. Of interest, PCSCs metastasized to the bone are rather dormant [3], and this dormancy is maintained, at least partly, by BMP7 [225]. The high invasive and metastatic capabilities in PCSCs may be intrinsic to PCSCs [3,155,159] and/or are conferred by TME-derived inflammatory and cytokine molecules that induce epithelial-mesenchymal transition or EMT [3,273–275]. For example, TGF-β in the TME has been shown to promote PCa stemness [287,311] whereas signaling from IL-30/IL-27p28 (an IL-6 family member) shapes PCSC behavior and regulates their metastasis [254]. Similarly, CXCL12γ promotes the development of metastatic CRPC [255], CCL5 generated by tumor-associated macrophages (TAM) promotes PCSCs and their metastasis via β-catenin/STAT3 signaling [286], and MSC-derived IL-28 drives selection of apoptosis-resistant bone-homing PCSCs [313]. It’s noteworthy that PCSCs, slow-cycling (dormant) PCa cells, and PCa cells

undergoing EMT share many biological properties (e.g., dormancy) and frequently identify overlapping but non-identical cell populations [reviewed in 3].

Studies on PCSCs have identified many signaling pathways that positively regulate the PCSC properties, which include PTEN/PI3K/AKT [222,228], NF-kB [224], Notch [137,158,226], Hedgehog [226], Wnt/β-catenin [230,250,257,259,286,314], JAK/STAT [235], β4 integrin [239], hypoxia through HIF1α [246], IL-30/IL-27p28 [254, 266], BRCA1/EZH2 [271], and FGF/FGFR [308]. Also reported are positive molecular regulators of PCSCs including, among others, MYC [234],STAT3[235,237,241,245,286,296],ERG[236,297],BMI-1[139, 249], Skp2 [251], YAP1 [253], CXCL12γ [255], LIN28B [248,295], ASPM [259], NANOG [122,125,135,137,142,143,150,156,262,263], SOX2 [270,276], OCT4 [270], CXCR2 [272], SOX9 [277], HOTAIR [296], BMP5 [298], and MUC-1 [279,306,318]. Finally, our studies have uncovered several tumor-suppressive microRNAs (miRNAs) as the negative regulators of PCSCs [127], which include miR-34a [126,167], let-7 [130,248], miR-128 [139], miR-199–3p [151], and miR-141 [154].

3.7. PCa cell heterogeneity in AR expression: Relationship to PCSCs and direct impact on tumor response to ARSIs—AR signaling has been the clinical therapeutic target, and different forms of ARSIs have been used to treat PCa patients for nearly 80 years. In principle, in order for ARSIs to exhibit significant and long-lasting therapeutic efficacy, most, if not all, PCa cells in patient tumors should express AR (i.e., AR^{+}) and rely on AR activity for their survival and wellbeing. In reality, however, AR expression and AR activity are highly heterogeneous in PCa. *FIRST*, shortly after the *AR* gene was cloned and antibodies against AR were developed, numerous studies have reported the heterogeneity in AR expression and existence of $AR^{-/lo}$ PCa cells across the entire spectrum of PCa, from treatment-naïve primary tumors to tumors treated with neoadjuvant ADT to mCRPC [2, 147,175–190,192–200,202–209]. *SECOND*, transcriptomic profiling also defined a *de novo* low AR-activity subclass in treatment-naïve PCa [204]. *THIRD*, studies from PCa metastases, especially in specimens obtained from warm autopsy patients, have revealed many subtypes of mCRPC with respect to AR expression including the $AR^{-}NE^{-}$ metastases called double-negative PCa or DNPC [201,203,207,325,331]. *FOURTH*, in support, our recent study [163] showed that ~27 % of 195 CRPC TMA cores were classified as $AR^{-/lo}$ (Fig. 9A). AR IHC analysis in 6 WM sections also revealed the $AR^{-/lo}$ PCa cells as the prominent (Fig. 9B–C) or even predominant (Fig. 9D) cell population in castration-resistant tumors in the prostate [163]. *FIFTH*, recent dual-modality PET/CT imaging studies using ^{18}F-DHT (to image AR expression) and ^{18}F-deoox-yglucose (to image overall tumor glycolytic activity) similarly revealed both AR^{+} and $AR^{-/lo}$ metastatic foci in PCa patients with mCRPC [213, 214] (Fig. 9E). *FINALLY*, transcriptional profiling in treatment-failed tumors identified an AR activity-low stemness program that was linked to Enza resistance [288]. This last study [288] implies that ARSIs, while blocking AR activity (as expected), also upregulate the stemness in patient PCa cells, consistent with many other studies (see below).

What are these $AR^{-/lo}$ PCa cells and how are they molecularly regulated?— There is substantial evidence that the $AR^{-/lo}$ cells in untreated PCa models and patient tumors represent 'undifferentiated' cells and possess many PCSC properties, and therapies including chemotherapy and radiotherapy frequently enrich the $AR^{-/lo}$ PCSCs [reviewed in 147]. For example, we have reported that the $CD44^{+}$ [118] and $PSA^{-/lo}$ [131,144, 149] PCSCs in untreated PCa models and patient tumors are mostly $AR^{-/lo}$ (e.g., Fig. 7F). Similarly, many PCSC populations reported by others in untreated PCa models or treatment-naïve primary tumors are also $AR^{-/lo}$, including $ABCG2^{+}$ [218], $CD133^{+}$ [219,220], $CD133^{+}\alpha2\beta1^{+}$ [219], $CD44^{+}$ [219,224,232], $(PSA^{-/lo})MHC^{-/lo}$ [226], LP-like [227, 238], basal-like [231], $hTERT^{hi}$ [250], $CD24^{+/hi}$ [261], and $CXCR2^{+}$ [272] cells. In fact, repression of AR expression/activity has been shown to reprogram bulk PCa cells to generate $AR^{-/lo}$ PCSCs [e.g., 244,245,264, 289,292] (also see Fig. 11, below). For example, loss of AR expression was shown to promote PCSCs via STAT3 signaling [245], and repression of AR signaling by cytokines secreted from the infiltrated BM MSCs led to significant expansion of $AR^{-/lo}$ PCSCs [244]. Notably, preferential expression of the oncogene MDM2 in $AR^{-/lo}$ PCa cells led to AR degradation, which helped maintain their PCSC properties [264]. Finally, androgen deprivation has been shown to reprogram bulk PCa cells, both functionally and transcriptionally, into PCSCs [289,292], consistent with our studies [152,163] (also see below) and with recent transcriptional profiling of treated patient tumors that identified a low-AR activity stemness program to be associated with Enza resistance [288].

How would the PCa cell heterogeneity, particularly, the $AR^{-/lo}$ PCa cells, impact tumor response to ARSIs and other clinical treatments?—To directly answer this question, we have established several paired AD/AI xenograft models that mimic the primary (1 °; ADT resistant) and secondary (2 °; ADT/Enza-resistant) CRPC in treated PCa patients (see Introduction; Fig. 10A) [163]. Interestingly, the 4 primary CRPC models displayed unique changes in AR (and PSA) during their evolution to becoming fully castration-resistant (Fig. 10B–D). Specifically, LNCaP 1 ° CRPC showed upregulated nuclear AR, LAPC9 1 ° CRPC showed loss of AR, and LAPC4 and VCaP 1° CRPC displayed mostly cytoplasmic AR (Fig. 10B). Therapeutic studies in mice bearing these 1° CRPC revealed that the 3 AR^{+} CRPC models (LNCaP, LAPC4 and VCaP) responded to Enza treatment although Enza-resistant tumors started to emerge after ~6 (for LNCaP and LAPC4) and ~13 (for VCaP) weeks of Enza treatment (Fig. 10E) [163]. In contrast, the $AR^{-/lo}$ LAPC9 1° CRPC showed *de novo* resistance to Enza (Fig. 10E) [163]. The insensitivity of $AR^{-/lo}$ PCa/CRPC to Enza was also demonstrated using isogenic AR^{+} and AR-KO LNCaP clones [163]. These results suggest that persistent castration (ADT) would eventually lead to two subtypes of CRPC, the $AR^{+/hi}$ LNCaP-like CRPC with most cells expressing high nuclear AR and the $AR^{-/lo}$ LAPC9-like CRPC with most cells losing AR expression [163] (Fig. 14C). Notably, our xenograft-based CRPC modeling studies have pinpointed BCL-2 as a vitally important therapeutic target for both $AR^{+/hi}$ and $AR^{-/lo}$ CRPC [163] (Fig. 10C–D; also see Figs. 12, 13, below).

How are $AR^{-/lo}$ PCa cells/clones maintained and what unique signaling mechanisms drive their non-responsiveness to ARSIs and other

therapeutics?—The inherent PCSC properties in these cells may partly answer these questions although we have just begun to understand these cells at the detailed molecular and cellular levels. Our early studies found that the $PSA^{-/lo}(AR^{-/lo})$ PCSCs actually require the corresponding $PSA^{+}(AR^{+})$ PCa cells for their survival and for maintenance of their properties such as the relative dormancy [131,144]. Others have reported that both AR^{+} and $AR^{-/lo}$ PCa cells cooperate to establish and maintain the metastatic ecosystem (niche) in the bone marrow [200]. These observations are consistent with the co-existence of both $AR^{-/lo}$ and AR^{+} PCa cells in treatment-naïve tumors as well as in castration-resistant primary (prostate) tumors and metastases. These observations also suggest that the two subpopulations of PCa cells may constantly interact with each other to form a tumor-sustaining cellular network, which will be disrupted by, and also dynamically evolves in response to, therapeutic interventions. Recent studies begin to unravel signaling pathways and molecules that may play uniquely important roles in supporting the $AR^{-/lo}$ PCa cells, clones and metastases, including, e.g., β-catenin [197], FGF/FGFR [201], the polycomb repressor complex-1 [203], T-type calcium channels [206], and stem cell regulators [163].

4. PCa cell plasticity and PCa lineage plasticity

PCa cell heterogeneity in expression of AR, the therapeutic target, and the presence of $AR^{-/lo}$ PCa cells and PCSCs in primary tumors are not the only problems in PCa treatment. Like most other cancer cells, PCa cells are also phenotypically and functionally plastic due to their fluid epigenome and genetic alterations. The PCa cell plasticity can be best appreciated from our studies in LNCaP cells [e.g., 150,152] (Fig. 11), one of the most frequently used PCa cell line model that has manifested heterogeneity in every phenotypic marker (including AR) we have analyzed (e.g., Fig. 5D), that has been used to generate dozens of sublines including LNCaP-abl (Figs. 5C; 11C–D), and that has been shown, by scRNA-seq analysis, to be very heterogeneous containing a subpopulation with attenuated androgen response [109]. Chronic exposure of LNCaP cells to 3 regimens of castration, i.e., CDSS, CDSS plus bicalutamide and Enza (also called MDV3100), in 2–3 months (Fig. 11A) turned the bulk AR^{+} (and PSA^{+}) LNCaP cultures into AR-PSA-cells [152] (Fig. 11B). Importantly, the reprogrammed $AR^{-}PSA^{-}$ LNCaP cells acquired many PCSC phenotypic markers including CD44, integrin α2β1 and ABCG2 [152] (Fig. 11B, table below). These $AR^{-}PSA^{-}$LNCaP cells were resistant to not only antiandrogens (Enza and bicalutamide) but also docetaxel (Fig. 11C) but, interestingly, displayed sensitivities to the BCL-2 inhibitor (ABT-199) and several kinase inhibitors [152] (Fig. 11D). This study [152] vividly illustrates PCa cell plasticity caused by a clinically relevant treatment regimen, i.e., castration (Fig. 14B). Numerous studies by others have also shown that repression of AR expression/activity can reprogram bulk PCa cells to generate $AR^{-/lo}$ PCSCs [e.g., 244,245,264,289,292].

PCa cell plasticity can also be induced by stemness factor reactivation or overexpression. Our studies have implicated NANOG in PCSCs (and other CSCs) and in regulating cancer aggressiveness and castration resistance [122,125,135,137,142,143,150,156]. Interestingly, castration of LNCaP and LAPC9 models, in which most cells are differentiated ($AR^{+}PSA^{+}$), led to upregulation of NANOG that subsequently contributed to CRPC development [125] (Fig. 11E). Strikingly, induction of NANOG expression in bulk LNCaP cells through a

transgene, in a time-dependent manner (~3–4 weeks), gradually reprogrammed these cells into androgen-insensitive CRPC cells [150] (Fig. 11F). Time-resolved RNA-seq coupled with NANOG ChIP-seq, together with other molecular studies reveal that NANOG-induced reprogramming of LNCaP cells to the castration-resistant state involved 4 sequential steps: inhibition of the AR-mediated differentiation program due to competitive NANOG binding to many canonical pro-differentiation AR/FOXA1 sites (step 1), increased expression of other stemness genes including ABCG2 and WNT5A as a result of NANOG cooperation with AR/FOXA1 (step 2), AR/FOXA1-independent NANOG binding to the genome leading to increased transcription of genes associated with migration/invasion/metastasis (step 3), and, finally, transactivation of genes associated with cell-cycle progression and proliferation [150] (Fig. 11F). This reprogramming process, resembling the reprogramming of fibroblasts to induced pluripotent cells, leads, eventually, to the generation of highly proliferative, aggressive and castration-resistant LNCaP cells [150]. This study [150] provides a prime example of PCa cell plasticity induced by a single stemness factor (Fig. 14B).

PCa cell plasticity can also be induced by genetic perturbations and diffusible inflammatory cytokines in the TME (Fig. 14B). For example, oncogenic Kras can cooperate with AR signaling to induce PCSCs and tumor-propagating cells [229], and mutant Kras may induce CD24 upregulation leading to the expansion of PCSCs and increased PCa stemness [261]. C-MYC in combination with activated AKT1 can induce *de novo* plasticity in human prostate luminal epithelial cells [302]. Infiltrating BM MSCs have been reported to secrete cytokines to repress AR signaling and expand PCSCs [244]. Other TME factors such as TGF-β1 (please change to symbol of beta) [287,311], elevated ROS levels [237] and hypoxia [265] may also induce PCa cell plasticity.

The above examples of PCa cell plasticity generally refer to the ‘de-differentiation’ of AR^{+} PCa cells to $AR^{-/lo}$ cells of the same lineage, i.e., adenocarcinoma [Fig. 14B]. Frequently, however, treated and reprogrammed PCa cells may change the lineage identity to become NE-like cells in a process often dubbed as PCa lineage plasticity or transdifferentiation [129,279,280,283,295,307,314,327,332–334]. As much as 20–25 % of the treatment-failed patient CRPC may contain NE-like cells and sometimes the entire CRPC clone or tumor may be populated by NE-like cells [327,334]. Such ‘transdifferentiated’ tumors are called CRPC-NE or NEPC [327]. Obviously, development of resistance to ARSIs does not necessarily always lead to CRPC-NE [335], and most metastases in mCRPC patients have the adenocarcinoma phenotype whether AR^{+} or $AR^{-/lo}$. Indeed, in our xenograft modeling studies, both $AR^{+/hi}$ LNCaP-type of CRPC and $AR^{-/lo}$ LAPC9-type of CRPC, although exhibiting increase NE score (a cohort of neurogenesis and NE differentiation related genes), have the typical adenocarcinoma cell phenotypes without increased NE cells [163]. One of the best examples of both PCa cell plasticity and PCa lineage plasticity is provided in a genetic mouse model of PCa [332]. When only *Pten* was deleted in the mouse prostate, mice developed indolent prostate adenocarcinomas with homogeneous AR^{+} PCa cells. However, when *Pten* and *Rb1* were co-deleted, the double knockout (DKO) prostate developed more aggressive adenocarcinomas with significantly increased AR heterogeneity and increased $AR^{-/lo}$ PCa cells [332]. The DKO tumors were initially sensitive to castration, which then gradually became castration-resistant. Strikingly, the castration-resistant DKO tumors turned completely to $AR^{-/lo}$ phenotype and some tumors even became NE-like [332]. When *p53*

was also deleted together with *Pten* and *Rb1*, the triple knockout (TKO) prostate tumors were typical AR^- *de novo* NEPC [332]. This study [332] elegantly illustrates both PCa cell plasticity and PCa lineage plasticity elicited by genetic perturbations together with therapeutic interventions (castration). In a similar fashion, the NHP epithelial cells can be reprogrammed by different genetic drivers to form AR^+ adenocarcinomas [18,21,302] or AR- NE-like cancers [258].

In addition to *Rb1* and *p53*, whose mutations have been convincingly linked to PCa cell/lineage plasticity and to therapy resistance [326,327, 332–336], other molecules and signaling pathways have also been implicated in PCa lineage switch to NE, which include MUC-1 [279, 306], ASPM [259], LIN28B [248,295], c-MYC/AKT1 [302], N-MYC [314], SOX2 [270,276,307], ONECUT2 [337], etc. Interestingly, many of these lineage infidelity-inducing genes (N-MYC, SOX2, LIN28B, ASPM, ASCL1, etc) belong to the functional class of neural development/neurogenesis genes that are also preferentially expressed in AR^- PSA^- normal prostate basal/stem cells [17,43], $PSA^{-/lo}$ PCSCs [131,144], and in *de novo* NEPC, CRPC-NE and CRPC-adeno [163,327] (Fig. 14D). *De novo* NEPC, large-cell neuroendocrine PCa, and CRPC (including both CRPC-NE and CRPC-adeno), as a group, are sometimes called aggressive variant PCa or AVPC (Fig. 14D). We propose that the lineage-regulating and neurogenesis genes may present a common cohort of genes required to establish the ‘ground state’ of the aggressiveness, stemness and therapy resistance associated with the PCSCs and AVPC cells (Fig. 14D).

5. Therapeutic targeting of PCa cell heterogeneity and plasticity: focus on PCSCs and PCa stemness

Xenograft-based CRPC modeling and retrospective studies in patient CRPC specimens suggest many phenotypically divergent subtypes of endpoint CRPC [163,327,331] including the $AR^{+/hi}$ and $AR^{-/lo}$ CRPC. As the AR^+ PCa cells in treatment-naïve tumors are exquisitely sensitive to ARSIs and $AR^{-/lo}$ PCa cells possess PCSC properties and are inherently resistant to ARSIs, the focus of the therapeutic development should be on identifying key regulators in PCSCs that can become drug targets. In principle, inhibitors of positive PCSC regulators such as Wnt/β-catenin and pluripotency molecules such as NANOG could be developed and used together with ARSIs to target both PCSCs and differentiated (AR^+) PCa cells (Fig. 14A). In support, studies have shown that inhibition of pluripotency regulators such as NANOG [262,263] and SOX2/OCT4 [270] or critical signaling nodes including STAT3 [241], GSK-3β [242] and Wnt/β-catenin (please change to symbol of beta) [257] could lead to depletion of PCSCs and help overcome PCa resistance to ASRIs. As many of the TME-derived cytokines could reprogram PCSCs (discussed above), neutralizing antibodies could potentially be deployed together with ARSIs to treat PCa. PCSCs might also be targeted from different angles, e. g., by exploiting their unique metabolism [161,267] associated with the mitochondrial fission [268]. Some PCSC-specific surface markers, such as CD133 [338], could be developed into targeted imaging/therapeutic ligands for lethal CRPC. Finally, tumor-suppressive miRNAs such as miR-34a [126,167] and miR-141 [154], which are normally devoid in PCSCs, could potentially be developed into replacement therapeutics to be used in the clinic in conjunction with ARSIs (Fig. 14A).

Our recent studies [163] have nominated BCL-2 as a critical therapeutic target in both $AR^{+/hi}$ LNCaP-type (Fig. 12) and $AR^{-/lo}$ LAPC9-type (Fig. 13) of CRPCs. Indeed, in the LNCaP AD/AI models, BCL-2 was slightly induced in primary CRPC but significantly upregulated in ADT/Enza-resistant secondary CRPC (Figs. 10C–D; 12B–D). In vitro, LNCaP and freshly purified HPCa cells upregulated BCL-2 in response to Enza treatment (Fig. 12–E–F) [163]. Overexpression of BCL-2 in LNCaP cells was sufficient to increase tumor incidence and promote tumor growth [163] (Fig. 12G). Consequently, the BCL-2 inhibitor, ABT-199, although not appreciably inhibiting the primary LNCaP CRPC (Fig. 12H), significantly blocked the development of LNCaP secondary CRPC when used in combination with Enza (Fig. 12I) [163]. ABT-199 also sensitized *in vitro* reprogrammed (AR^{-}) LNCaP-CRPC cells (Fig. 11D) [152]. Similarly, BCL-2 was significantly upregulated in $AR^{-/lo}$ LAPC9 primary CRPC (Fig. 13A–C), which did not respond to Enza (Fig. 10E) [163]. ABT-199 alone inhibited the growth of both LAPC9-AI organoids (Fig. 13D) and tumors (Fig. 13E–F), and its tumor-inhibitory effect was further enhanced by the BET inhibitor JQ1 (Fig. 13E–F) [163]. These observations promoted us to initiate a phase Ib/II clinical trial of treating Enza-naïve CRPC patients with a combination of Enza and BCL-2 inhibitor venetoclax (NCT03751436; clinical PI, Dr. Gurkamal Chatta). A recent study has also implicated BCL-2 as a therapeutic target in small-cell NEPC [339].

Of interest, one PCa treatment regimen, called SPT (supraphysiological testosterone) or SPA (supraphysiological androgens), exploits cyclic treatment of PCa patients with ADT followed by giving patients high doses of testosterone [340–344]. The clinical benefit of the SPT protocol was thought to be derived, mostly, from DNA damage caused by high doses of T. It will be interesting to elucidate the detailed cellular mechanisms of the SPT regimen under the framework of our current understanding of PCa cell heterogeneity and plasticity and PCa development as deregulated prostate differentiation, and in the context of potentially very different biological effects of T on AR^{+} vs. $AR^{-/lo}$ PCa cells.

Finally, can we potentially target PCSCs and AVPC with immunological approaches? PCSCs have been evinced to be ‘immune-privileged’ lacking the expression of MHC [226] and other immune recognition molecules [160] while overexpressing immune-evading molecules such as Dickkopf-1 [211]. Therefore, PCSCs may not be very susceptible to immune-mediated cell killing [160]. For example, PCSCs have been shown to be refractory to γδ T cell-mediated cytotoxicity [300]. On the other hand, a recent phase I/II clinical trial hints that vaccination in PCa patients against RhoC could elicit long-lasting immune response [305]. Also, radioresistant PCSCs [138] could be targeted by B7-H3 CAR-T cells [315]. Furthermore, inhibition of N-cadherin, preferentially expressed in $PSA^{-/lo}$ PCSCs [131] and in $AR^{-/lo}$ CRPC [163], appears to ‘normalize’ the PCa TME that protects tumor-infiltrated lymphocytes (TILs) from immune checkpoints and Tregs [316]. A recent study also suggests that epigenetic reprogramming through EZH2 inhibition may be able to enhance PCa response to anti-PD-1 due to the activation of STING-interferon signaling axis [345].

6. Perspectives & outstanding questions

It has long been known that the differentiation level of human tumors is inversely correlated with their malignancy. An embryonic stem cell gene expression signature characterizes undifferentiated human cancers [346], human adult stem cell signature marks aggressive subtypes of epithelial cancers [256], and induction of core stem cell and pluripotency master regulators in cancers defines poor clinical outcomes and therapy resistance [269]. My discussions proposed the LP cells as potential cells of origin for human PCa. LP-like PCSCs are intrinsically castration-resistant and survive castration [227], and LPs mediate the progression of basal cell-originated tumors to aggressive adenocarcinomas [238]. Understanding PCa etiology, progression, treatment response and development of therapy resistance from the perspective of abnormal organ and cellular differentiation has a great significance in helping identify both naturally therapy-resistant and induced treatment-refractory PCa cell populations, and subsequently develop novel therapeutics and therapeutic combinations to target these cells. With this goal in mind, I identify many outstanding questions in the field and list several below:

1. What exactly is the phenotype of LP cells in the human prostate and how do their phenotypes and functions change during organ development, maturation and aging and in response to inflammatory challenges?
2. How do LPs (or other prostate epithelial cells) become tumorigenically transformed and what epigenetic mechanisms are involved in prostate tumorigenesis instigated by inflammation?
3. How can we culture and propagate differentiated $AR^{+}PSA^{+}$ human prostate epithelial and PCa cells?
4. What PCa cells survive ADT/Enza, enter the adaptation phase and mediate tumor relapse (Fig. 1D)? Are they pre-existent $AR^{-/lo}$ PCa cells or de-differentiated and reprogrammed AR^{+}PCa cells? Are they dormant slow-cycling cells and PCSCs [3]? Do prostate tumors in the adaptation phase enter a diapause-like state to survive?
5. There are notable challenges in reconstituting the tumorigenicity and stemness of PCSCs from primary prostate tumors. In contrast to CSCs freshly isolated from aggressive human tumors such as lung cancer, colorectal cancer and glioblastoma multiforme that can readily regenerate tumors in immune-deficient mice, putative PCSCs freshly purified from treatment-naïve PCa are generally deficient in initiating tumor development in mice. Why?
6. Modeling of CRPC development in xenografts has revealed distinct changes in AR expression and subcellular distribution patterns [163] (Fig. 10B). How do the AR^{+} and $AR^{-/lo}$ PCa cell populations in treatment-naïve tumors reciprocally interact with and regulate each other? How are $AR^{+/hi}$ and $AR^{-/lo}$ PCa cells generated during CRPC development? How does cytoplasmic AR function?
7. What exactly are the cellular and molecular mechanisms underlying the therapeutic efficacy of intermediate ADT using cyclic ADT and SPT regimen in addition to the proposed DNA damage response?

8. What strategies can we adopt to turn the PCa TME, generally considered as immunologically ‘cold’ with limited infiltration of $CD8^+$ cytotoxic T cells, into a ‘hot’ proinflammatory TME? What PCSC-specific tumor antigens or neoantigens can be uncovered to help design novel immunological approaches to target PCSCs and AVPC? Can the deregulated alternative splicing program and prominently increased intron retention in aggressive PCa [165] be exploited to identify tumor neoantigens and develop novel anti-PCa immunotherapies?

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Abbreviations:

ACD	asymmetric cell division
AD	androgen-dependent
ADT	androgen deprivation therapy
AI	androgen-independent
AP	anterior prostate
AR	androgen receptor
AR-KO	AR knockout
ARSIs	AR signaling inhibitors
AR-Vs	AR splice variants
AVPC	aggressive variant prostate cancer
BCR	biochemical recurrence
BM	bone marrow
BMP	bone morphogenic protein
circRNA	circular RNA
CAF	carcinoma associated fibroblasts
CDSS	charcoal and dextran-stripped serum
CK	cytokeratin

CRPC	castration-resistant prostate cancer
CSCs	cancer stem cells
CTCs	circulating tumor cells
DKO	double knockout (i.e., *Pten*$^{-/1-}$*Rb1*$^{-/-}$)
DNPC	double negative prostate cancer
DHT	5α-dihydrotestosterone
DNPC	double-negative PCa
EMT	epithelial mesenchymal transition
Enza	enzalutamide
GR	glucocorticoid receptor
GS	Gleason Score
HGPIN	high grade prostate intraepithelial neoplasia
HPCa	human PCa (specimens)
IF	immunofluorescence
IHC	immunohistochemistry
IL	interleukin
IR	irradiated
ITH	intra-tumor heterogeneity
KO	knockout
LN	lymph node
LP	luminal progenitor cells
mCSPC	metastatic castration-sensitive PCa
mCRPC	metastatic castration resistant prostate cancer
MDV3100	e nzalutamide
miRNA	microRNA
MSCs	mesenchymal stem cells
NE	neuroendocrine
NEPC	neuroendocrine prostate cancer
NHP	normal human prostate

PCa	prostate cancer
PCSCs	prostate cancer stem cells
PDX	patient (tumor) Derived Xenografts
PIA	prostate inflammatory atrophy
PIN	prostate intraepithelial neoplasia
***PSAP*-GFP**	PSA Promoter-driven GFP expressing (lentiviral reporters)
PSCA	prostate stem cell antigen
PSA	prostate specific antigen
ROS	reactive oxygen species
s.c	subcutaneously
SCCs	slow-cycling cells
scRNA-seq	single-cell RNA sequencing
SNP	single nucleotide polymorphism
SPA	supraphysiological androgens
SPT	supraphysiological testosterone
T	testosterone
TAM	tumor-associated macrophages
TILs	tumor-infiltrating lymphocytes
TF	transcription factor
TMA	tissue microarray
TME	tumor microenvironment
Tregs	regulatory T cells
WM	Whole-mount (sections)

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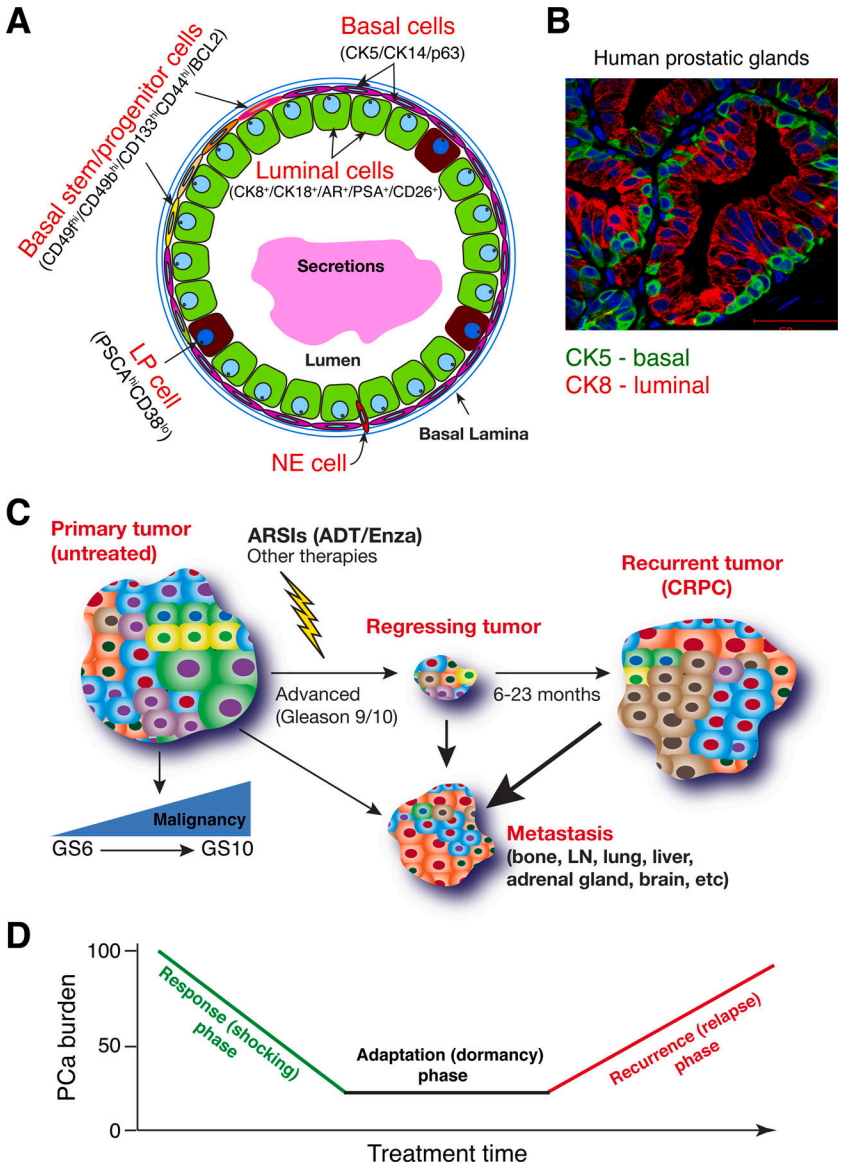


Fig. 1. Normal human prostatic glands and PCa.

A. Schematic of human prostatic glands. Both basal and luminal cell compartments harbor differentiated as well as less mature stem/progenitor cells. LP, luminal progenitor; NE, neuroendocrine.

B. Double immunofluorescence staining of CK5 and CK8 in benign human prostatic glands.

C. Human PCa at different clinical stages of development. Shown are 4 representative types of PCa, i.e., primary, regressing tumors (during therapy), recurrent tumors (i.e., CRPC in the prostate) and metastasis in different organs.

D. PCa response to clinical treatments goes through 3 typical phases, i.e., response, adaptation and recurrence. ‘PCa burden’ is measured by serum PSA levels, primary tumor sizes, and imaging analyses. see Text for details.

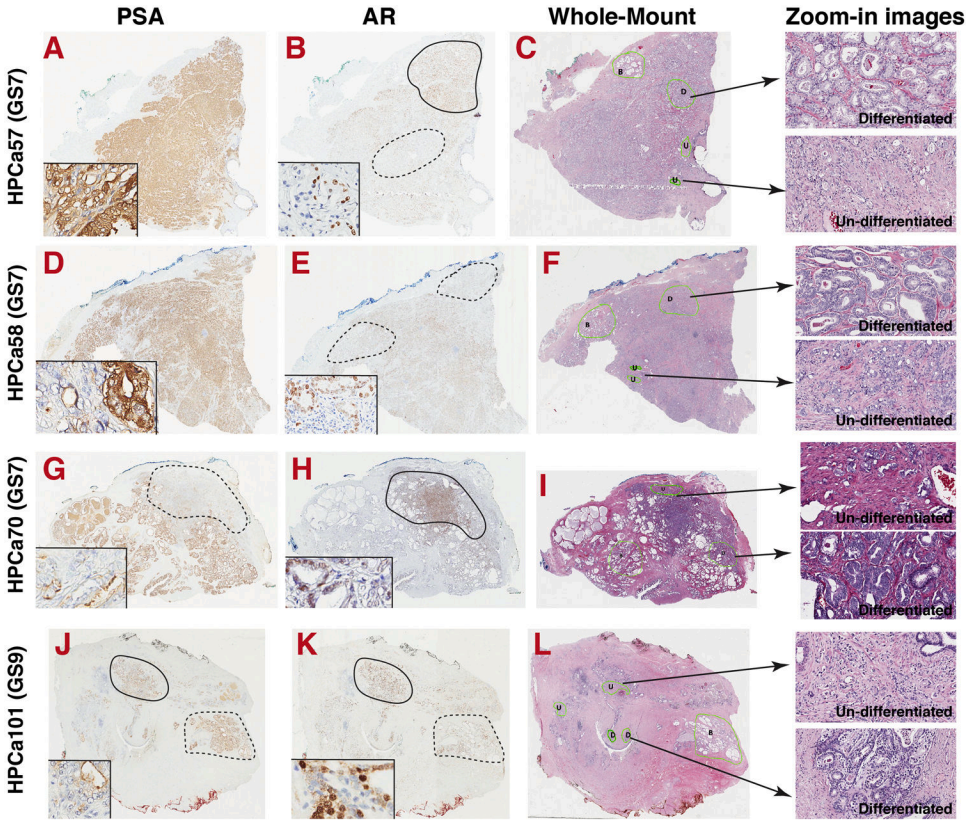


Fig. 2. Histo-structural, cellular and molecular heterogeneity in human PCa.

Shown are the Aperio ScanScope images of whole-mount sections (4x) of HPCa57 (A–C), HPCa58 (E–F), HPCa70 (G–I) and HPCa101 (J–L) stained for HE (C, F, I, L), AR (B, E, H, K), and PSA (A, D, G, J). HPCa 57, 58 and 70 are Gleason score 7 (GS7) tumors while HPCa101 is a Gleason 9 tumor. Serially transplantable PDX have been established from all 4 HPCa samples. Shown on the right are representative enlarged (zoom-in) HE images (200x) of differentiated (D) and poorly or undifferentiated (U) areas. For AR/PSA IHC images, a higher magnification (400x) inset was shown for each HPCa sample to illustrate $AR^{-/lo}$ and $PSA^{-/lo}$ PCa cells, and to illustrate the frequently discordant AR and PSA expression. For instance, in HPCa57, AR^{hi} (demarcated by a solid circle) and $AR^{-/lo}$ (dashed line) areas could be discerned. In HPCa58, two $AR^{-/lo}$ areas are indicated by dashed circles. In HPCa70 and HPCa101, areas marked by dashed lines indicate discordant expression of AR and PSA. Note that AR IHC was performed using an antibody against the N-terminus, which recognizes both full-length AR and all c-terminally truncated splice variants.

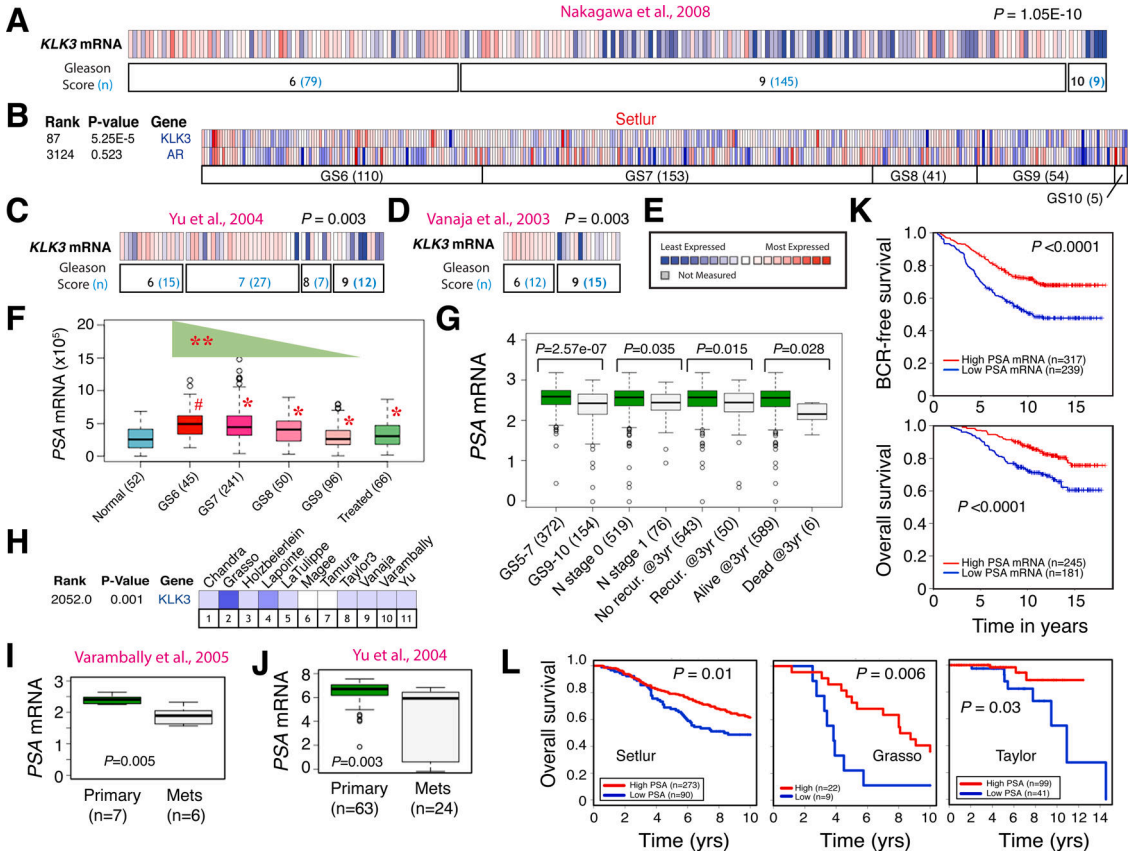


Fig. 3. PCa heterogeneity: Inverse correlation between tumor *PSA* (*KLK3*) mRNA levels and tumor grade, metastasis, and patient survival.

A-E. Tumor *KLK3* mRNA levels are inversely correlated with the tumor grade. Shown are results from 4 PCa cohorts (A–D) in Oncomine. E, the heatmap legend. Data in B also illustrates discordant *PSA* and *AR* mRNA expression in patient tumors.

F. Reducing *PSA* (*KLK3*) mRNA levels with increasing Gleason grade (GS) of prostate tumors in TCGA. Patient numbers are indicated in parentheses. #, P < 0.001 compared with normal and *P < 0.00001 compared with GS6 tumors (paired Student's *t*-test). The *PSA* mRNA levels display a statistically significant decreasing trend with increasing tumor grade (**P < 0.0001, Proportion Trend test).

G. Tumor *PSA* mRNA levels inversely correlate with tumor grade, metastasis, recurrence and survival in the Nakagawa data set. Shown are reduced *PSA* mRNA levels in high-grade (GS9–10) tumors, patients with lymph node (N) metastasis, patients that had recurrent diseases within 3 years, and in patients who were dead 3 years after diagnosis.

H-J. Tumor *PSA* mRNA levels were reduced in metastases in most Oncomine data sets (H) as illustrated in Varambally (I) and Yu (J) data sets. P values were determined by Student's *t*-test.

K-L. High tumor *PSA* mRNA levels correlate with better biochemical recurrence (BCR) free and overall survival of PCa patients in the Nakagawa data set (K) and with better patient survival in 3 other data sets (L). P values were determined by log-rank test.

Panels A, C–E, G, I–K were adapted with permission from reference [131]. Panels B, H and L were adapted with permission from reference [144].

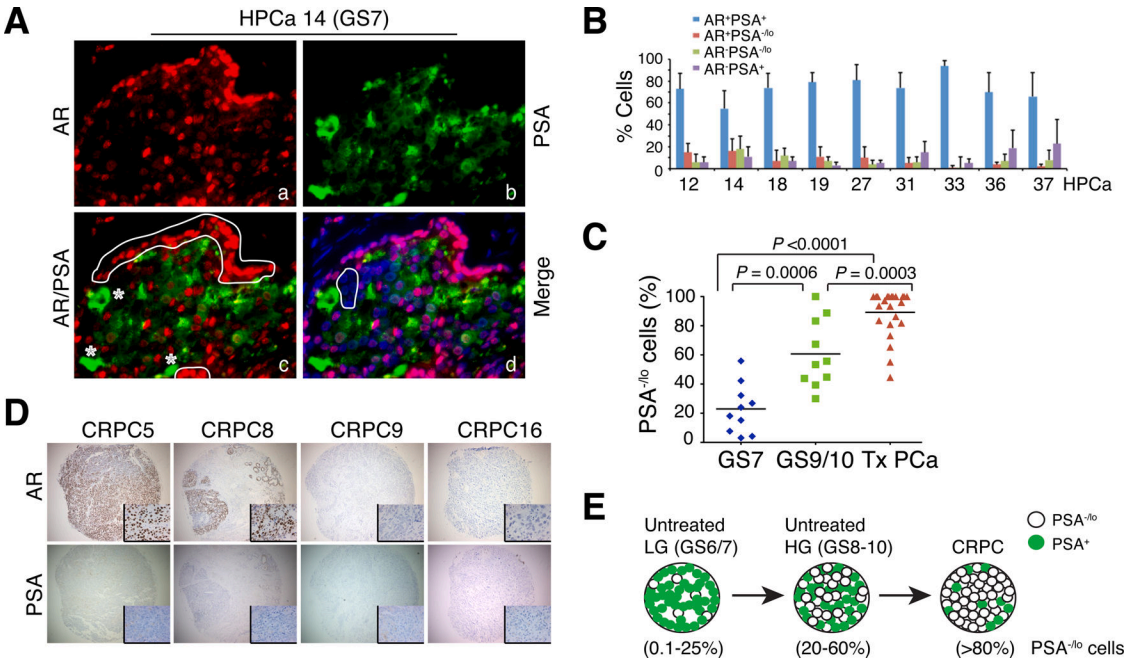


Fig. 4. PCa cell heterogeneity: Pre-existence of PSA$^{-/lo}$ PCa cells in treatment-naive HPCa and enrichment of PSA$^{-/lo}$ cells in advanced and treatment-failed PCa.

A-B. Representative immunofluorescence images (x400) illustrating discordant PSA and AR protein expression and the presence of PSA$^{-/lo}$ PCa cells in untreated HPCa 14 (GS7; A) and quantification of 4 subpopulations of PCa cells in the 9 HPCa samples (B). In A, AR^{+}PSA^{+} PCa cells are marked by red nuclei and green cytoplasm, AR^{+}PSA$^{-/lo}$ cells by red staining (panel c, white circled areas), AR-PSA^{+} cells by green staining (panel c, asterisks), and AR-PSA$^{-/lo}$ /lo cells by being negative (or low) for both red and green staining (panel d, white circle).

C. The abundance of PSA$^{-/lo}$ tumor cells is increased in high-grade (GS9/10) and in treatment-failed (Tx) PCa compared to untreated low/intermediate grade (GS7) tumors (n = 20–24 for each type of tumor samples).

D. IHC analysis of AR and PSA in the TMA samples. Shown are 4 CRPC samples illustrating homogeneous loss of PSA in all 4 samples and heterogeneous expression of AR (insets: 400x).

E. Schematic illustration of increasing PSA$^{-/lo}$ PCa cells from low-grade (LG) tumors to untreated high-grade (HG) tumors and to CRPC. Panels A, B and D were adapted with permission from reference [144].

Panel C was adapted with permission from reference [131].

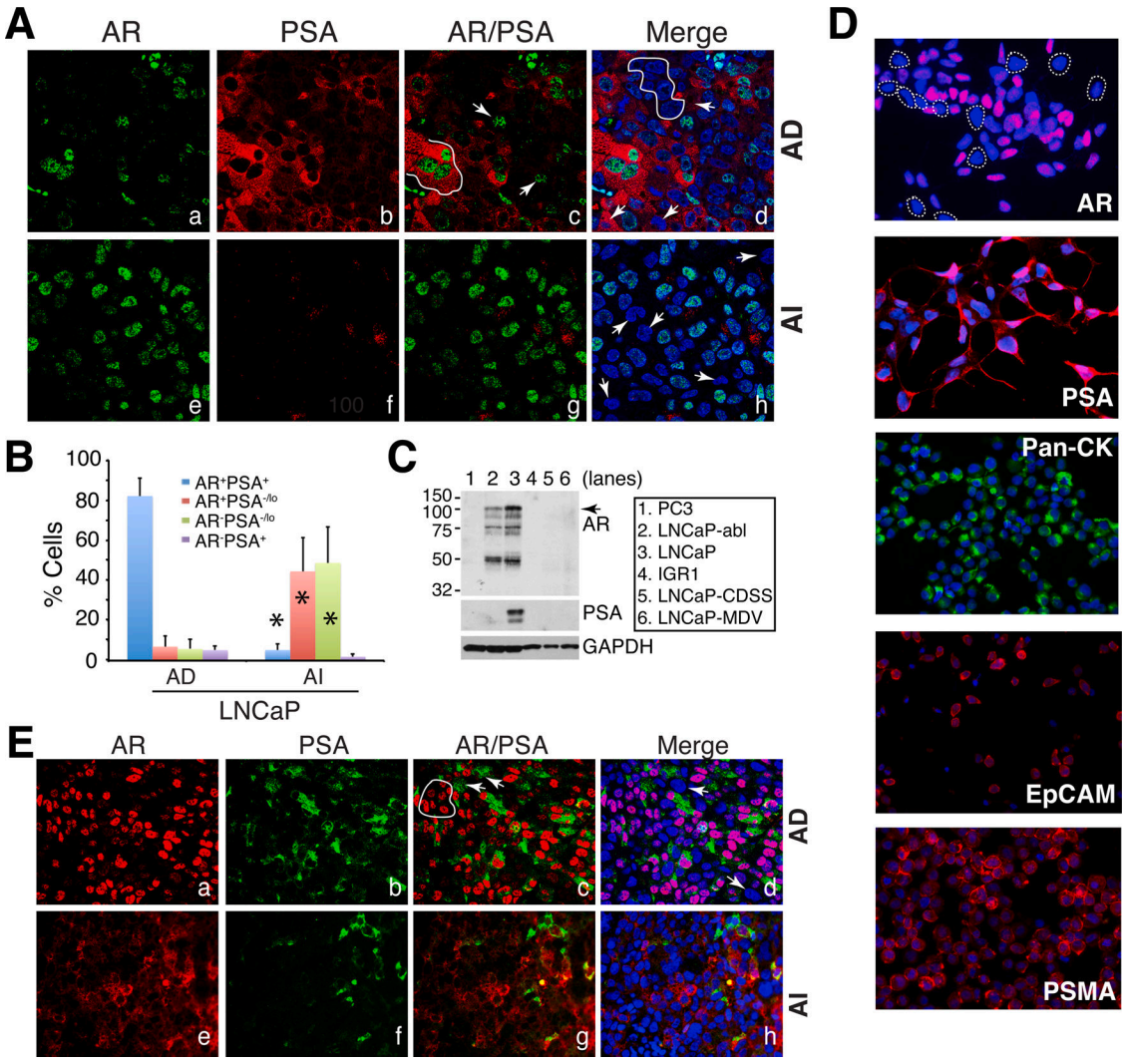


Fig. 5. Studying PCa cell heterogeneity in PCa cell and xenograft models: PSA$^{-/lo}$ PCa cells accumulate in experimental CRPC models.

A–B. Accumulation of PSA$^{-/lo}$ cells in LNCaP-AI tumors. (A) Double immunofluorescence (IF) staining of AR and PSA in AD (androgen-dependent) vs. AI (androgen-independent) LNCaP xenograft tumors. In panel c, the white line demarcates 3 AR^{+}PSA^{+} cells and the arrows point to 2 AR^{+}PSA$^{-/lo}$ cells. In panel d, the white circle demarcates several AR-PSA$^{-/lo}$ cells and the arrows point to 3 AR-PSA^{+} cells. In panel h, the arrows illustrate several AR-PSA$^{-/lo}$ cells. Shown are representative confocal images (original magnification; x400). (B) Quantification of the 4 subtypes of PCa cells in AD and AI LNCaP xenograft tumors. A total of 809 and 907 cells were counted from several AD and AI tumors, respectively. *P $<$0.001 in AI compared in AD tumors.

C. WB analysis of AR and PSA heterogeneity in cultured LNCaP cell sublines. PC3 and IGR1 cells, which are negative for both proteins, were used as controls. Note that the wild-type LNCaP cells (lane 3) were AR^{+}PSA^{+} whereas LNCaP-abl cells AR^{+}PSA^{-} (lane 2). LNCaP-CDSS and LNCaP-MDV cells were both AR^{-}PSA^{-} (lanes 5–6). The arrow

indicates the ~114 kD full-length AR and lower bands represent AR splice variants (top panel).

D. Heterogeneity in expression of phenotypic markers in cultured LNCaP cells. Shown are representative IF images of AR, PSA, pan-cytokeratin, EpCAM and PSMA.

E. Double IF staining of AR and PSA in AD vs. AI LAPC4 xenograft tumors. In panel c, the white circle indicates several $AR^{+}PSA^{-/lo}$ cells and arrows indicate AR-PSA^{+} cells. In panel d, arrows point to AR-$PSA^{-/lo}$ cells. Note significantly increased $PSA^{-/lo}$ LAPC4 cells in the AI tumor (f). Shown are representative confocal images (original magnification; x400).

All panels (except most of D) were adapted with permission from reference [144].

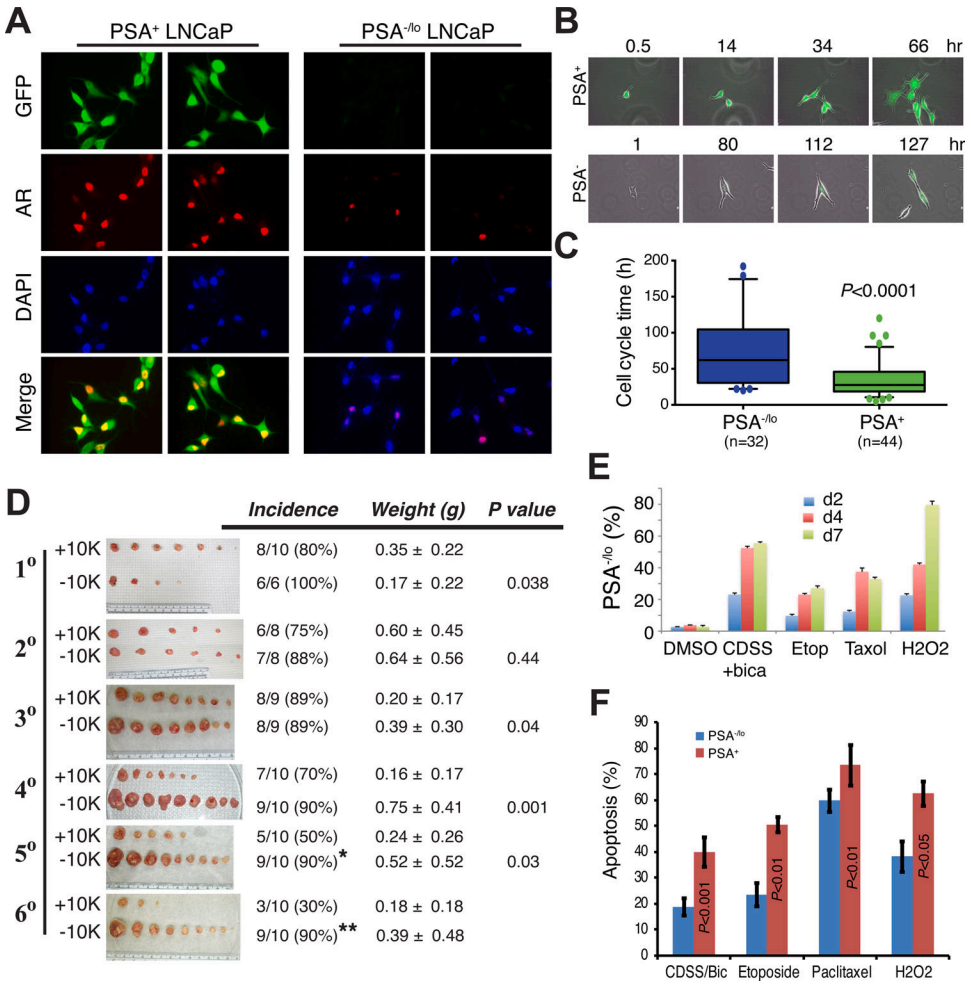


Fig. 6. The PSA$^{-/lo}$ PCa cell population harbors cells that exhibit CSC properties.

A. Most PSA$^{-/lo}$ PCa cells are also AR^{-}. Shown are IF images of LNCaP cells separated by the *PSAP*-GFP lentiviral reporter and stained for AR. Note that all GFP^{+} (PSA^{+}) LNCaP have high nuclear AR whereas the majority of PSA$^{-/lo}$ cells are also AR$^{-/lo}$.

B. PSA$^{-/lo}$ PCa cells are more dormant and can undergo ACD (bottom) whereas PSA^{+} PCa cells are more proliferative and only undergo symmetrical cell division. Shown are time-lapse images of LNCaP cells infected with the *PSAP*-GFP reporter and recorded at the indicated time points.

C. PSA$^{-/lo}$ LNCaP cells have much longer cell-cycle transit times than the corresponding PSA^{+} cells. The number of cells analyzed by time lapse videomicroscopy are indicated in parentheses.

D. The PSA$^{-/lo}$ PCa cells can initiate long-term xenografts that can be serially propagated. PSA^{+}(GFP^{+}) and PSA$^{-/lo}$ (GFP$^{-/lo}$) LAPC9 cells were purified from reporter tumors and 10,000 cells each (+10 K or −10 K) were implanted subcutaneously in intact male NOD-SCID mice. The first-generation (1 °) tumors were harvested ~2 months later. One +10 K tumor and two −10 K tumors were utilized to purify GFP^{+} and GFP-/lo LAPC9 cells, respectively, which were used in secondary (2 °) tumor transplantation experiments. One +10 K and one −10 K 2 ° tumors were utilized to purify GFP^{+} and GFP- LAPC9 cells,

respectively, for 3 ° transplantation. The 4 °, 5 ° and 6 ° transplantation experiments were carried out in a similar fashion. For each generation, tumor images (shown not to the same scale), tumor incidence (%; *P = 0.045 and **P = 0.006, when compared to the corresponding +10 K groups, χ^2 test), time (d) of harvest, tumor weights (mean ± S.D), and P values (Student's *t*-test) were indicated. For the 6 ° generation, statistics was not applicable due to the small number of tumors developed in the PSA^+ group.

E. $PSA^{-/lo}$ LNCaP cells are resistant to androgen deprivation (i.e., CDSS plus bicalutamide) as well as chemotherapeutics and hydrogen peroxide. Shown are % $PSA^{-/lo}$ cells in PSAP-GFP infected LNCaP cells treated with the conditions indicated for 2, 4, and 7 days (d). Differences between all individual treatments and DMSO are statistically significant ($P < 0.01$).

F. $PSA^{-/lo}$ PCa cells are more resistant to apoptosis as assessed by the Vybrant apoptosis assays. Bulk cultured LNCaP cells infected with the *PSAP*-GFP lentiviral reporter were plated at 120,000 cells/well in 6-well plates. Cells were treated for 4 days with either DMSO, 2% CDSS plus 20 μM Bicalutamide (CDSS/Bic), 20 nM Paclitaxel, 1 μM etoposide, or 1 μM H2O2. The % apoptosis represents the mean ± S.D (n = 3) and *P* values determined by Student's *t*-test. No difference in apoptosis was observed in the two populations in response to vehicle DMSO (not shown).

Panels A–C and F were adapted with permission from reference [144]. Panels D and E were adapted with permission from reference [131].

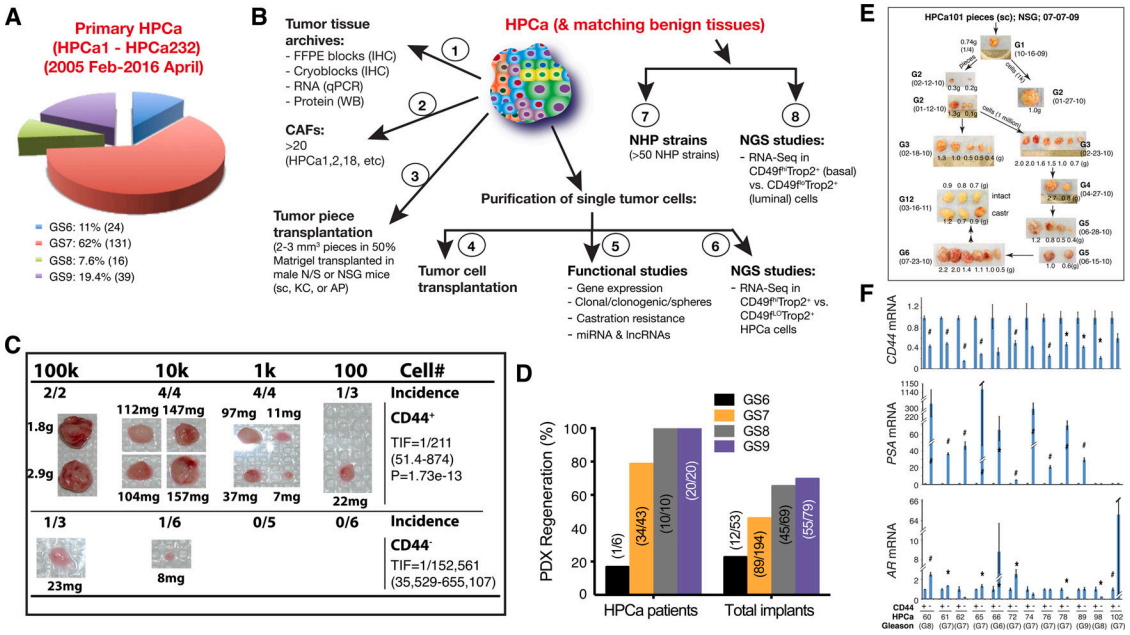


Fig. 7. Studying PCa cell heterogeneity in treatment-naïve patient primary prostate tumors.

A. Pie chart presentation of the Gleason grade distribution of 210 HPCa samples out of a total of 232 (please change the number to 232) HPCa our lab used and studied from Feb. of 2005 to April of 2016 at the M.D Anderson Cancer Center. HPCa, human prostate cancer (specimens or samples).

B. Experimental uses for the HPCa (1–6) and matching benign samples (7–8) in our lab. CAFs, carcinoma-associated fibroblasts; FFPE, formalin-fixed and paraffin embedded; NGS, next-generation sequencing (studies); NHP, normal human prostate (epithelial) cells; N/S, NOD/SCID mice; NSG, NOD/SCID-γ mice.

C. An example of tumor regeneration from purified CD44$^+$ HPCa cells. CD44$^+$ and CD44$^-$ HPCa52 cells were MACS-purified from patient tumor (GS8) and co-injected, at the indicated cell numbers, with 10k Hs5 cells in 50 % Matrigel s.c into irradiated NOD/SCID-γ mice. The 10k and 100k tumors were harvested at ~4 months whereas 100 and 1k tumors were harvested at 7 months after implantation. Shown on the right are TIF (tumor-initiating cell frequency). The difference in CD44$^+$ HPCa 52 cell TIF and the corresponding CD44- cell TIF is highly statistically significant (P = 1.73e-13).

D. Success rate (%) of PDX (patient-derived xenograft) regeneration plotted based on total # of HPCa patient specimens (left bars; e.g., for GS7 tumors, 34 of the 43 patient HPCa pieces implanted in immunodeficient mice regenerated PDX [79 %]) or total # of implants (right bars; e.g., 89 of the 194 GS7 HPCa pieces implanted in all 3 sites (i.e., sc, KC, and AP) regenerated PDX [thus 46 %]).

E. Serially transplantable HPCa101 (GS9[4 + 5]) PDX derived from tumor piece implantation. Shown are representative tumor images of HPCa101 PDX at multiple generations (G). castr, castrated; sc, subcutaneously.

F. qPCR analysis of *CD44*, *AR*, and *PSA* mRNAs in CD44$^+$ and CD44$^-$ HPCa cells freshly purified from untreated primary prostate tumors. The results are expressed as the mRNA

levels in $CD44^+$ HPCa cells relative to those in the matched $CD44^-$ HPCa cells. *P < 0.05; #P < 0.01 (Student's *t*-test).

Panels F was adapted with permission from reference [144].

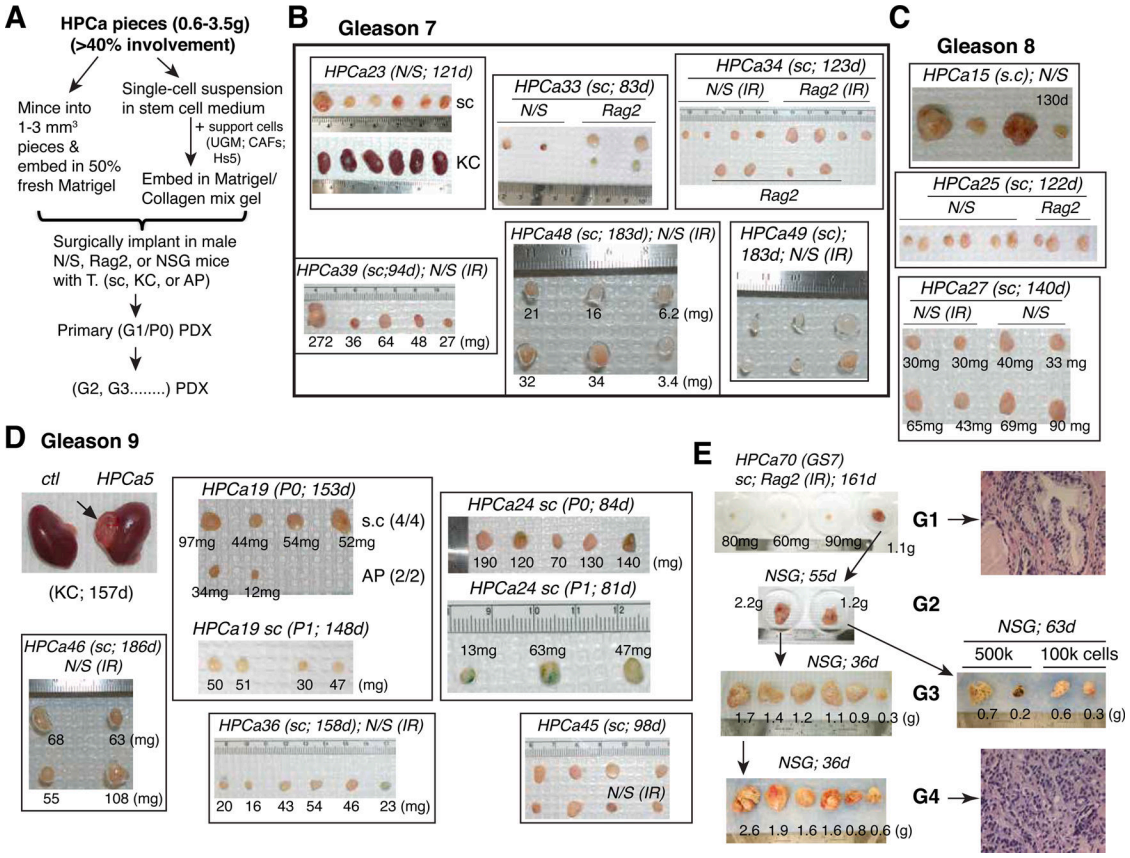


Fig. 8. Studying PCa cell heterogeneity in treatment-naïve patient primary prostate tumors.

Shown are examples of PDXs established in our lab from treatment-naïve patient prostate tumors using tumor pieces xenotransplanted in immune deficient mice.

A. Basic experimental flow. AP, anterior prostate; CAFs, carcinoma-associated fibroblasts; Hs5, immortalized human BM mesenchymal stem cells; KC, kidney capsule; sc, subcutaneously; UGM, urogenital mesenchyme.

B-D. Examples of PDXs established from Gleason 7 (B; 6 HPCa examples shown), Gleason 8 (C; 3 HPCa examples shown), and Gleason 9 (D; 6 HPCa samples shown) HPCa samples. IR, irradiated; P, passage.

E. Four generations (G) of HPCa70 PDX tumors are illustrated. A representative HE staining for G1 and G4 tumor was shown on the right.

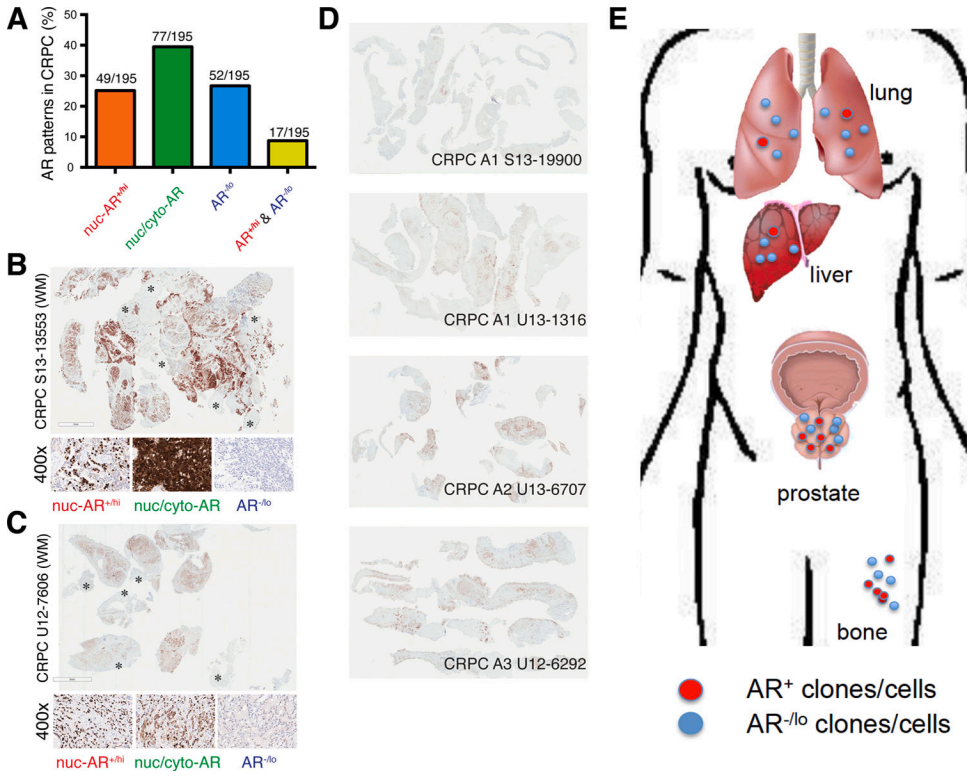


Fig. 9. AR heterogeneity in CRPC and PCa metastases.

A. Quantitative summary of the 4 AR expression patterns in CRPC based on the AR IHC analysis in 195 TMA cores.

B-C. Whole-mount (WM) sections showing AR heterogeneity in both expression levels and subcellular distribution patterns in two cases of patient CRPC. AR was stained using an antibody recognizing an N-terminal epitope that will capture full-length AR and all C-terminally truncated AR splice variants. $AR^{-/lo}$ cells/areas are indicated by asterisks (*). Shown below the WM sections are enlarged images of 3 main patterns of AR expression, i.e., $AR^{-/lo}$, nuclear AR (nuc-$AR^{+/hi}$) and mixed nuclear/cytoplasmic AR (nuc/cyto-AR).

D. AR IHC in another 4 cases of patient CRPC. AR staining in WM sections was conducted as in B–C.

E. Metastases in PCa patients also exhibit AR^{+} and $AR^{-/lo}$ clonal patterns, based on recent imaging analysis [212,213].

Panels A–D were adapted with permission from reference [163].

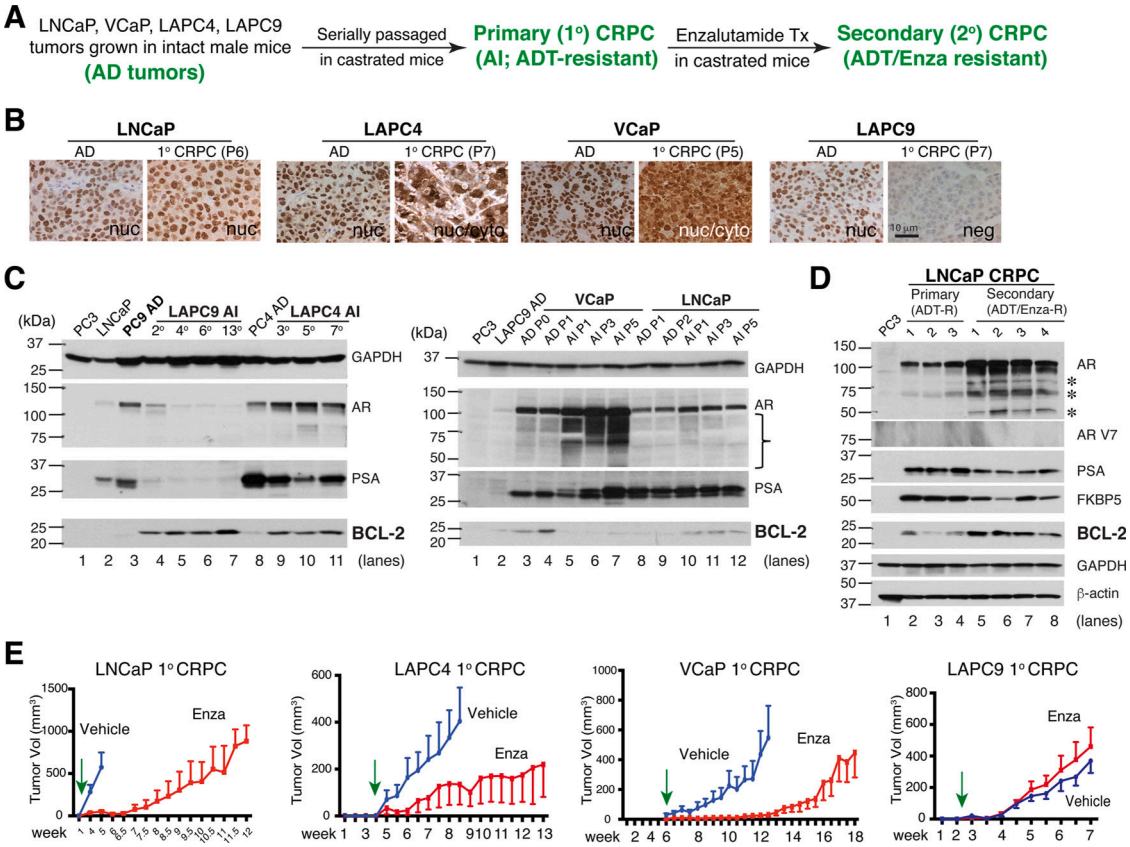


Fig. 10. AR$^{-/lo}$ CRPC is insensitive to Enza and BCL-2 is upregulated by castration.

A. Schema in generating paired xenograft AD tumors, primary CRPC (ADT-resistant) and secondary CRPC (both ADT/Enza-resistant) in LNCaP, VCaP, LAPC4 and LAPC9 models.

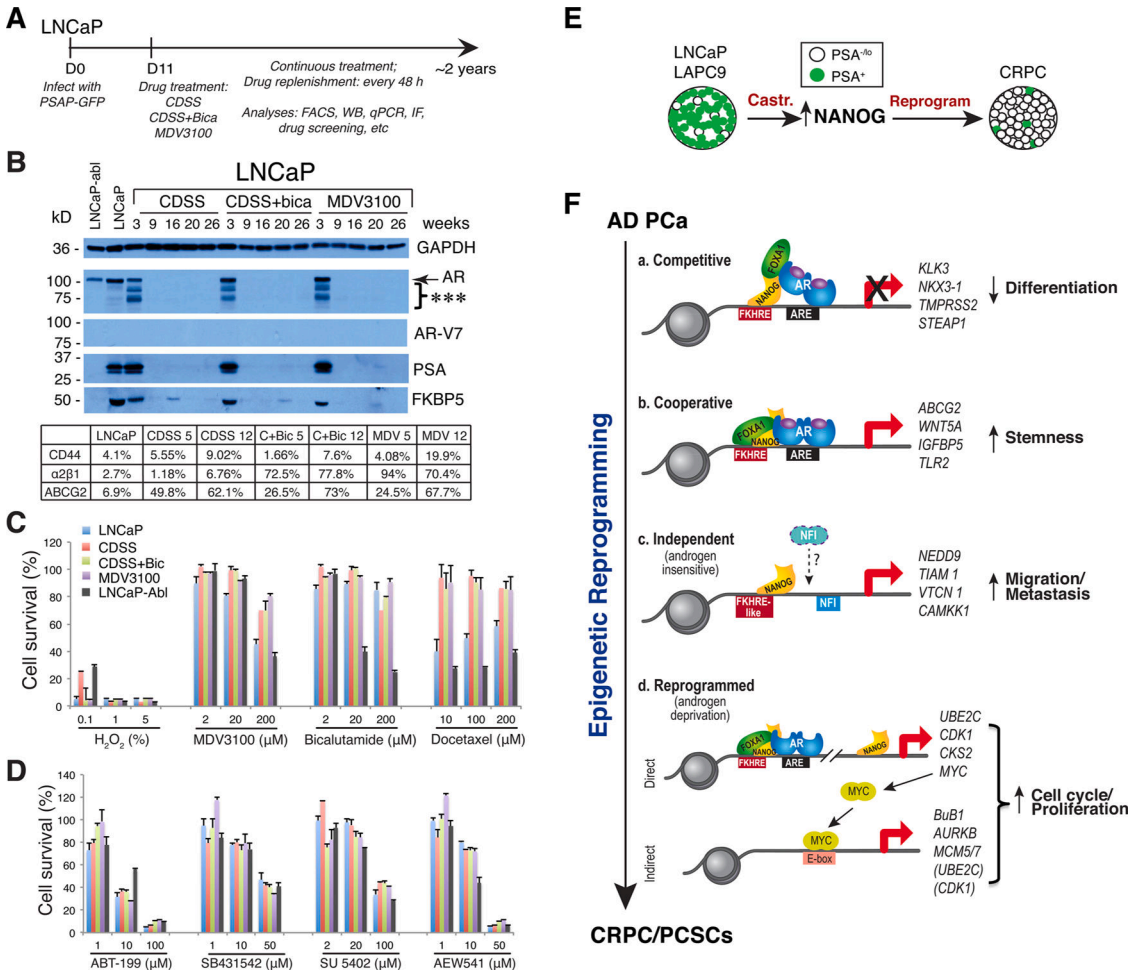
B. The above 4 xenografts develop unique AR expression patterns as they evolve towards CRPC. P, passage (of tumors in mice).

C. BCL-2 was elevated in 3 of the 4 CRPC xenograft (except VCaP) models.

D. BCL-2 was further increased in the LNCaP secondary CRPC model.

E. Distinct responses of the 4 xenograft CRPC models to Enza (see Text). Green arrows indicate the time when treatment of tumor-bearing mice was started.

All panels in the Figure were adapted with permission from reference [163].



	LNCaP	CDSS 5	CDSS 12	C+Bic 5	C+Bic 12	MDV 5	MDV 12
CD44	4.1%	5.55%	9.02%	1.66%	7.6%	4.08%	19.9%
α2β1	2.7%	1.18%	6.76%	72.5%	77.8%	94%	70.4%
ABCG2	6.9%	49.8%	62.1%	26.5%	73%	24.5%	67.7%

Fig. 11. PCa cell plasticity induced by ARSIs and stemness factor NANOG.

A–D. LNCaP cell plasticity and reprogramming by ARSIs. Shown in (A) is the experimental schema, in which bulk LNCaP cells were infected with the *PSAP*-GFP lentiviral reporter. In (B), LNCaP cells treated with 3 regimens of castration, i.e., CDSS, CDSS plus bicalutamide, and MDV3100 (Enza) for up to 26 weeks were used in Western blot analysis of AR, AR-V7 and two AR downstream targets, PSA and FKBP5. Regular LNCaP cells and LNCaP-abl cells were used as controls. Shown below is the summary of flow analysis of 3 PCSC markers in regular LNCaP and in 3 LNCaP-CRPC cells treated with CDSS, CDSS/bicalutamide (C + Bic) and MDV3100 (MDV) for 5 or 12 weeks. LNCaP-CRPC cells, in contrast to LNCaP and LNCaP-abl cells, were highly refractory to Enza, Bicalutamide and docetaxel (C) but were sensitive to BCL-2 inhibitor (ABT-199) and inhibitors of ALK (SB431542), VEGFR2/FGFR/PDGFR (SU 5402) and IGFR1 (AEW541).

E–F. Inducible NANOG expression time-dependently reprograms LNCaP-AD cells to the CRPC state. Shown in E is a schematic to illustrate that in LNCaP and LAPC9, $PSA^{-/lo}$ PCa cells normally comprise the minor population but castration upregulates NANOG that subsequently contributes to the generation of CRPC in which $PSA^{-/lo}$ cells now constitute the majority. Shown in F is a model depicting modes of operation of NANOG during reprogramming of androgen-dependent PCa cells to the CRPC state (see Text).

Panels A–D were adapted with permission from reference [152] and panels E–F from reference [150].

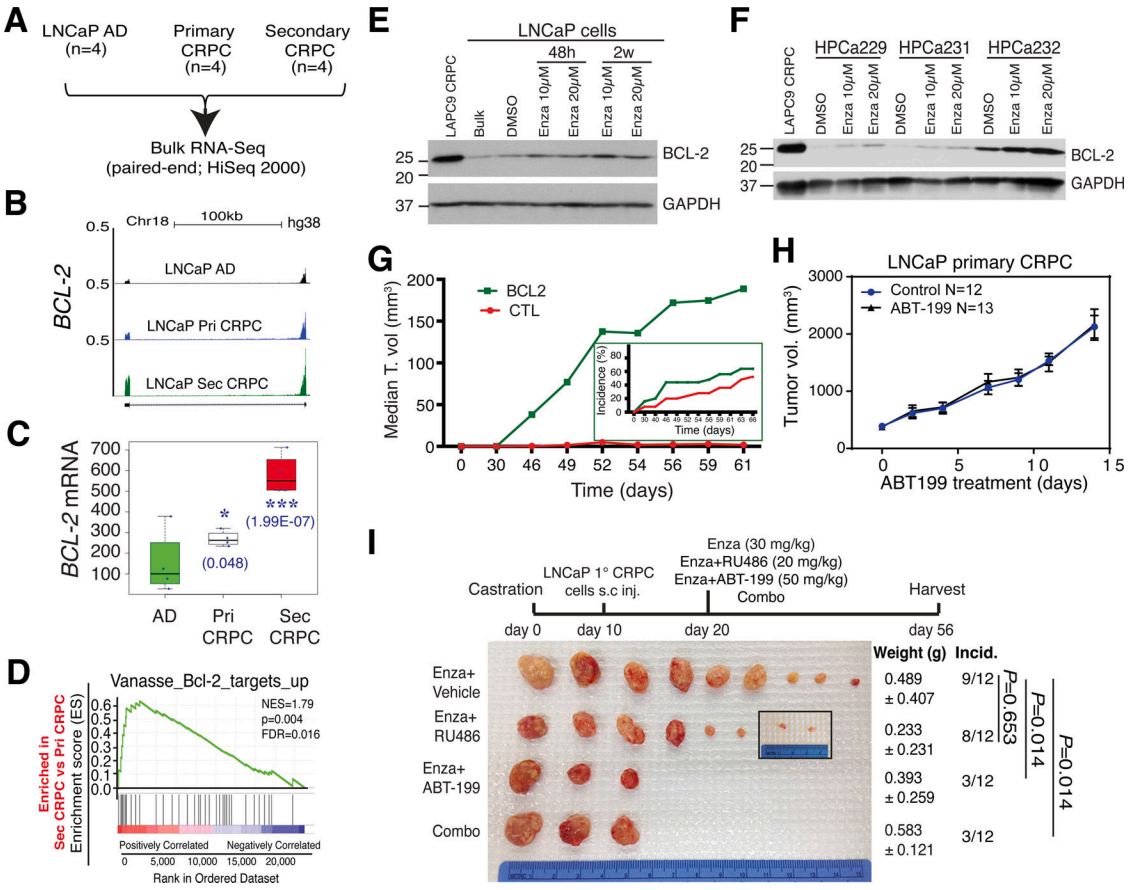


Fig. 12. BCL-2 represents a therapeutic target in AR$^{+/hi}$ subtype of CRPC.

A–D. In whole-genome RNA-seq experiments (A), *BCL-2* mRNA was increased in LNCaP primary (Pri; ADT-resistant) CRPC but dramatically upregulated in LNCaP secondary (Sec; both ADT/Enza-resistant) CRPC (B–C). Consequently, a BCL-2 target signature was enriched in LNCaP secondary CRPC compared to the LNCaP primary CRPC (D).

E–F. BCL-2 protein was upregulated by Enza in cultured LNCaP cells (E) as well as in primary tumor cells freshly purified from 3 HPCa patients.

G. BCL-2 overexpression is sufficient to promote LNCaP tumor growth (i.e., median tumor (T) volume) and also increase tumor incidence (inset). Please introduce a space between this sentence and the next sentence (in front of H).

H-I. BCL-2 inhibitor, ABT-199, did not appreciably affect the LNCaP primary CRPC growth (H) but, when used in combination with Enza, dramatically curbed development of castration/Enza-resistant secondary CRPC (I).

All panels in this Figure were adapted with permission from reference [163].

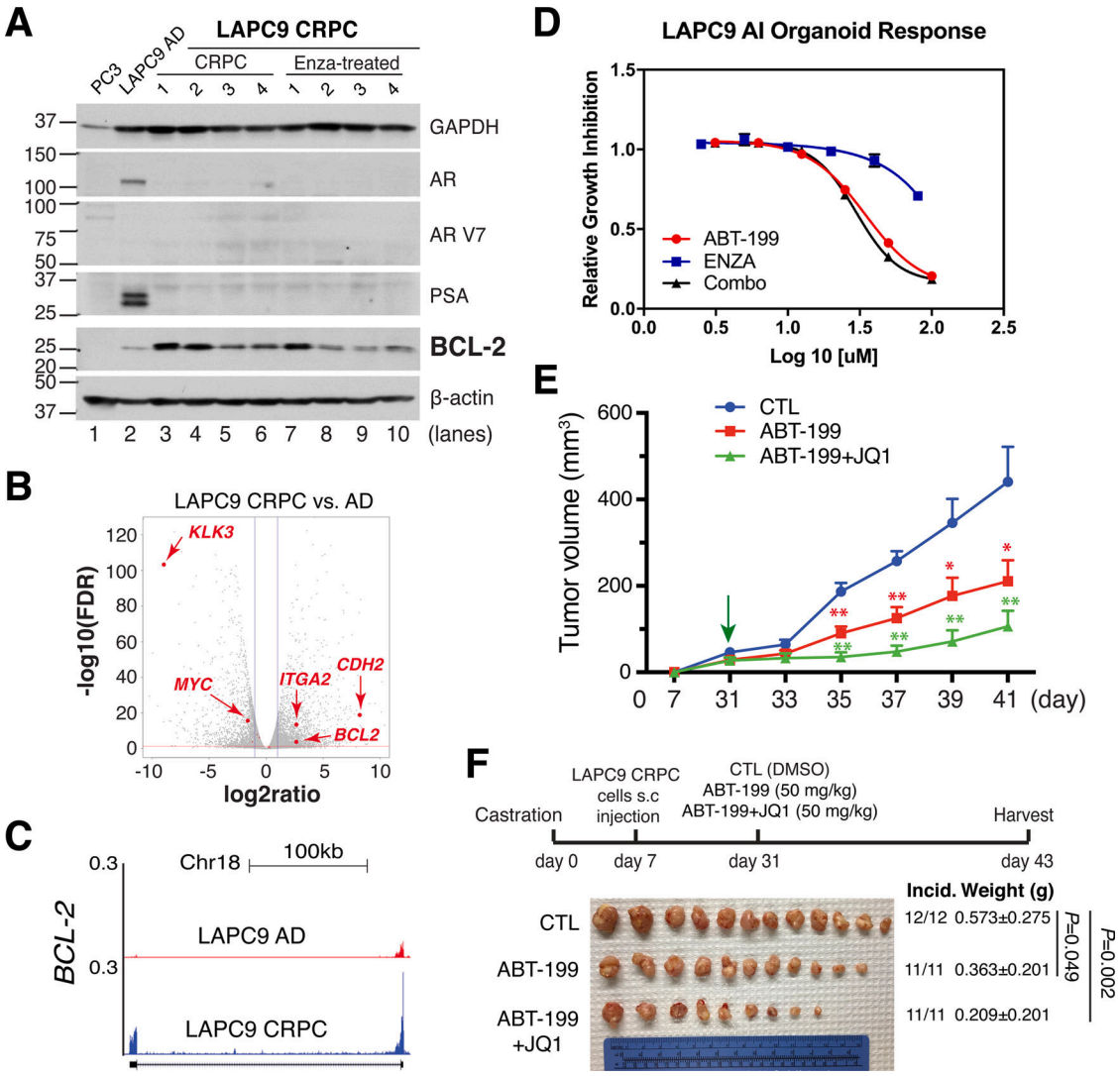


Fig. 13. BCL-2 represents a therapeutic target in AR$^{-/lo}$ subtype of CRPC.

A. BCL-2 was upregulated in LAPC9-CRPC and remained at increased levels in LAPC9-CRPC further treated with Enza.

B-C. *BCL-2* mRNA was significantly upregulated in LAPC9-CRPC compared to LAPC-AD. Also upregulated in LAPC9-CRPC were integrin α2 (ITA2) and N-cadherin (CDH2) (B).

D. BCL-2 inhibitor, ABT-199, dose-dependently inhibited the growth of LAPC9-CRPC (i.e., LAPC9-AI) organoids whereas Enza demonstrated minimal effects and combination of ABT-199 and Enza (Combo) exhibited similar inhibitory effects to ABT-199 alone.

E–F. BCL-2 antagonist, ABT-199, alone inhibited LAPC9-CRPC in vivo, which was further inhibited by the combination of ABT-199 and JQ1.

All panels in this Figure were adapted with permission from reference [163].

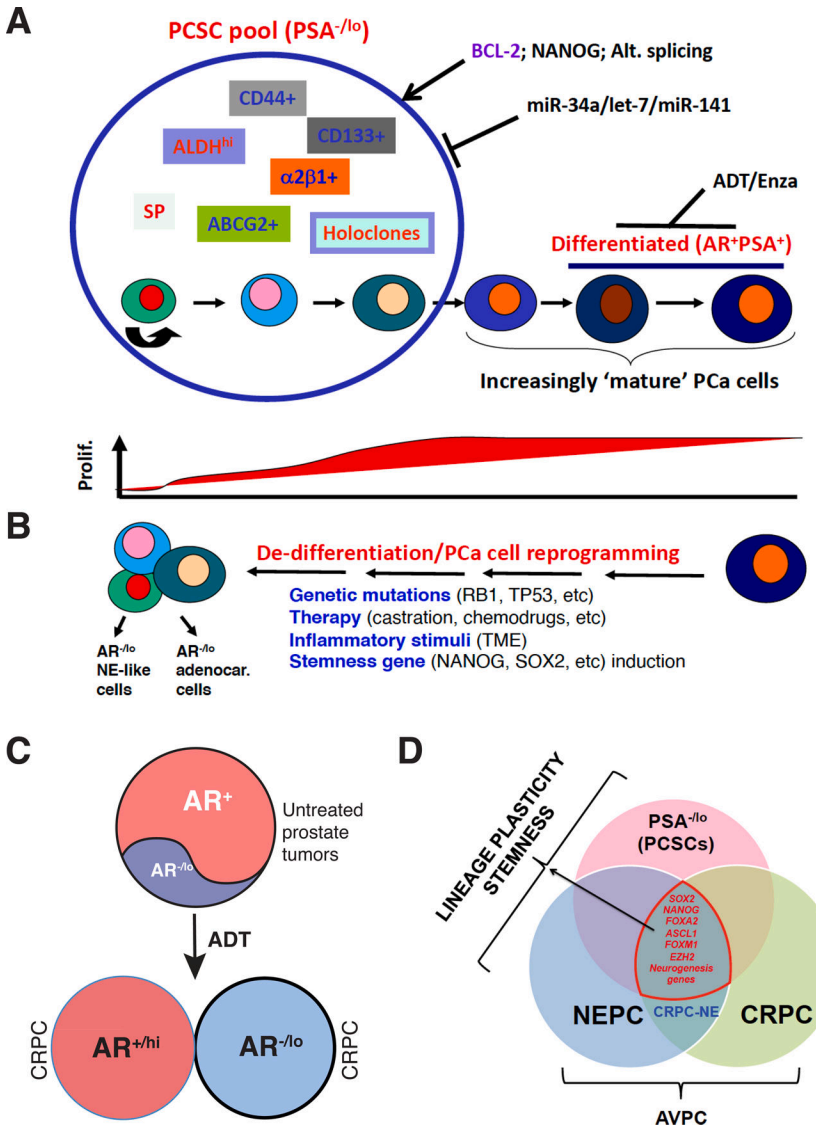


Fig. 14. Potential strategies to tackle PCa cell heterogeneity and plasticity.

A. A hypothetical model of intrinsic PCa cell heterogeneity. Primary (treatment-naïve) PCa consist of both bulk differentiated (AR^+PSA^+) cells as well as a subset of phenotypically undifferentiated ($PSA^{-/lo}$) cells. The latter cells represent the PCSC pool in which other subsets of tumorigenic cells can be further identified and purified. While AR^+PSA^+ PCa cells are highly proliferative, most cells in the $PSA^{-/lo}$ PCSC pool remain dormant (below). ARSIs including ADT and Enza primarily and preferentially target the differentiated AR^+PSA^+ PCa cells while largely ignoring the $PSA^{-/lo}$ cells. We have identified BCL-2, NANOG and deregulated alternative splicing program as positive regulators of PCSCs and several tumor-suppressive miRNAs including miR-34a, let-7 and miR-141 as negative regulators of PCSCs. In principle, these tumor-suppressive miRNAs or the inhibitors of positive regulators can be used in conjunction with ADT/Enza to tackle PCa cell heterogeneity and plasticity.

B. PCa cell plasticity (or de-differentiation) can be induced by genetic mutations, therapeutic treatments, inflammatory cytokines in the TME and by stemness gene overexpression. The reprogrammed PCa cells can be $AR^{-/lo}$ adenocarcinoma or $AR^{-/lo}$ NE-like cells (left).
C. Persistent ADT leads to two subtypes of CRPC, $AR^{+/hi}$ and $AR^{-/lo}$.
D. Many of the stemness and neurogenesis genes are commonly enriched in $PSA^{-/lo}$ PCSCs, NEPC, CRPC and CRPC-NE, which are often called AVPC.
Panel A was adapted with permission from reference [144].