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Clonal Evolution and Epithelial Plasticity in the Emergence of AR-independent Prostate Carcinoma

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Abstract

In spite of an initial clinical response to androgen deprivation therapy (ADT), the majority of prostate cancer patients eventually develop castration-resistant prostate cancer (CRPC). Recent studies have highlighted the role of epithelial plasticity, including transdifferentiation and epithelial-to-mesenchymal transition (EMT), in the development of AR pathway-negative CRPC, a form of the disease that has increased in incidence after the introduction of potent AR inhibitors. In this review, we will discuss the switches between different cell fates that occur in response to AR blockade or acquisition of specific oncogenic mutations, such as those in *TP53* and *RB1*, during the evolution to CRPC. We highlight the urgent need to dissect the mechanistic underpinnings of these transitions and identify novel vulnerabilities that can be targeted therapeutically.

Keywords

CRPC; plasticity; EMT; cancer stem cells

Resistance to AR-Directed Therapy in Prostate Cancer

Prostate carcinoma (PCa) is a common solid tumor and a leading cause of cancer-related deaths. While low- and intermediate-risk PCa patients (PSA<10, Gleason score<8) are effectively treated with surgery or radiation, high-risk patients (Gleason score >8, PSA>20)

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will likely progress to metastatic disease [1, 2]. Given the dependence of prostate adenocarcinoma on Androgen Receptor (AR) signaling, **androgen deprivation therapy** (ADT) is currently the backbone for the systemic treatment of locally advanced or metastatic PCa patients [3, 4]. Although PCa patients initially respond to ADT, they eventually develop androgen independence [5]. In many cases, resistance to hormone therapy appears to be driven by mechanisms that restore or enhance AR signaling, including genomic AR amplifications and expression of AR splicing variants that are constitutively active, such as V7. In addition, resistance can be driven by mutations that render the AR insensitive to antagonists, but sensitive to promiscuous ligands such as adrenal androgens, estrogen, and progesterone. Some mutations even convert AR antagonists into agonists [5].

The prevalence of AR alterations in castration-resistant prostate cancer (CRPC) and the demonstration that AR signaling drives progression to CRPC in preclinical models has provided a compelling rationale for the development of second-generation AR-targeted therapies, including abiraterone acetate (targeting *de novo* steroidogenesis) and enzalutamide (potent AR antagonist)[6–8]. These agents cause tumor regression in a large fraction of chemotherapy-naïve and chemotherapy-refractory CRPC patients [9–13]. Moreover, as the recent LATITUDE, STAMPEDE and ARCHES trials have indicated, next-generation inhibitors significantly improve radiographic progression-free survival and afford a significant overall survival benefit in *de novo* metastatic patients when they are used in combination with ADT [12, 13]⁽¹⁾. In spite of initial efficacy, intrinsic or acquired resistance to enzalutamide and abiraterone develops rapidly, highlighting the urgent need to understand the mechanisms of resistance to these agents.

Recent studies have pointed to two major categories of mechanisms of resistance: AR bypass and AR indifference. The glucocorticoid receptor (GR) can bypass the AR by upregulating the expression of a large fraction of AR target genes and is associated with acquired resistance to enzalutamide in preclinical studies and in patients [5, 14]. AR indifference or independence is associated with low or absent AR pathway activity based on tumor gene expression or low PSA in the blood, but its origin and consequences are not well known. AR-indifferent cancers comprise a group of histologically heterogeneous but clinically aggressive diseases collectively referred to as **aggressive variant prostate cancer** (AVPC) (Figure 1) [15, 16]. Here we discuss the role of epithelial plasticity, encompassing reversible **epithelial-to-mesenchymal transition** (EMT), **dedifferentiation**, and **transdifferentiation**, in resistance to AR-directed therapy and development of AVPC.

The clinical problem

Aggressive variant prostate cancers were initially identified as tumors that shared the clinical and therapy response profiles of small cell prostate cancer (SCPC)/neuroendocrine prostate cancer (NEPC). Indeed, AVPCs appear histologically as classic adenocarcinomas or as poorly differentiated carcinomas with or without neuroendocrine (NE) features [15–17]. The AVPCs encompass the NEPCs but include also tumors that don't necessarily display NEPC morphology. More recent studies have shown that AVPCs exhibit basal-like and stem-like

¹<https://clinicaltrials.gov/ct2/show/NCT02677896>

features, epigenetic aberrations, and combined defects in three tumor suppressors - *PTEN*, *TP53*, and *RBI* [17, 18].

Genomic studies have suggested that NEPCs arise from luminal prostate adenocarcinomas by divergent clonal evolution under the selective pressure of AR inhibitors, but alternative explanations also exist [19–21]. Intriguingly, recent studies revealed that inactivation of *RBI* and *TP53* can reprogram prostate adenocarcinoma to NEPC, especially in the context of pre-existing mutations in *PTEN* or overexpression of *MYCN*, suggesting a role for epithelial plasticity in the development of NEPC [22–24]. In principle, NEPC may originate from adenocarcinoma through a process referred to as **transdifferentiation** [25]. In contrast, the origin of a newly recognized form of AVPC devoid of neuroendocrine traits and referred to as double-negative prostate cancer (DNPC) is not known [26]. Intriguingly, the incidence of DNPC has increased substantially after the clinical advent of second-generation AR-inhibitors, pointing to a role for AR blockade in their emergence. Despite significant efforts, the biological mechanisms leading to the development and maintenance of the two major forms of AVPC, NEPC and DNPC, are not well understood.

Towards a Definition of Cell-of-Origin

In order to understand the role of **epithelial plasticity** in prostate tumor progression, it is important to define the prostate progenitors that are sensitive to oncogenic transformation. The epithelia of human and mouse prostate tissues consist of basal, luminal, and rarer neuroendocrine cells. Luminal progenitors were suggested as PCa-initiating cells, given the predominant expansion of luminal-like progenitors and luminal-like cells as well as the lack of a mature basal epithelium in human prostate adenocarcinoma. Consistently, genetic lineage-tracing demonstrated that rare luminal cells expressing the transcription factor Nkx3.1 and low levels of AR undergo self-renewal in the absence of testicular androgens. They finally differentiate into luminal and basal cells in response to physiological signals *in vivo*. Targeted deletion of *PTEN* leads to conversion of luminal bipotential progenitors that are Nkx3.1-positive into neoplastic cells, suggesting that luminal stem cells might be cells of origin [27]. However, subsequent studies showed that overexpression of AR, AKT and ERG transforms human basal cells into luminal-like adenocarcinoma cells [28], and additional findings supported the notion of basal epithelial stem cells as cells of origin [29]. Resolving this apparent contradiction, several groups showed that basal and luminal progenitors independently sustain the basal and luminal lineages in the adult mouse prostate and prostate cancer can be initiated by neoplastic conversion of either progenitor [30]. However, mouse tumors derived from luminal progenitors resemble more closely human PCa tumors [31]. In addition, prostate luminal cells are more sensitive to mitogenic signaling, while basal cells are more resistant to transformation [30].

In contrast to studies in adult mice, recent quantitative lineage reconstruction studies in adult human prostates identified multipotent basal stem cells that are located at the junction between proximal ducts and the urethra. These stem cells generate bipotent basal progenitors that give rise to luminal cells in the most proximal ducts. Additionally, a rarer and distally confined lineage-restricted luminal progenitor exists but contributes minimally to epithelial homeostasis [32]. Strikingly, the reconstitution of human prostate glands in organoid culture

argues for the existence of a bipotential luminal progenitor [33], suggesting a translatability of the findings obtained in mouse models to the human system. Microenvironmental cues may play a role in the initiation of prostate cancer from a basal cell of origin, as it has been shown that prostate basal cells acquire functional plasticity and differentiate into luminal cells in an inflammatory milieu [34].

Recent studies suggested that luminal cells expressing low levels of CD38 are enriched for progenitor activity, survive under low androgen conditions, and contribute to the initiation of CRPC [35]. Genetic labeling of prostate epithelial basal cells using tamoxifen (TAM)-inducible Cre-recombinase (Cre) driven by the CK5 promoter supports the model of bipotentiality of prostate basal cells able to give rise to both luminal and basal cell progeny *in situ* during adult tissue regeneration and homeostasis [36]. By using *PTEN/TP53*-null GEMM as well as organoid cultures, several studies identified two classes of luminal progenitors with different stem- cell abilities: a) multipotent luminal-like progenitors [27, 33], and b) luminal-committed progenitors [37]. Combined loss of *PTEN* and *TP53* leads to the development of adenocarcinomas containing a subpopulation of luminal progenitors, which are absent in *PTEN* null lesions [37]. This finding evokes an intriguing role for TP53 in repressing lineage plasticity, serving as a “guardian” against cellular dedifferentiation. Indeed, it has been shown that loss of TP53 induces dedifferentiation of mature hepatocytes into progenitor-like cells through overexpression of *Nestin*, a stem and progenitor associated protein that contributes to the development of hepatocellular carcinomas (HCCs) or cholangiocarcinomas (CCs) [38]. Overall, there is still no consensus regarding the cell of origin for prostate adenocarcinoma or mCRPC. The aforementioned findings are not mutually incongruent as no single experimental approach and condition may faithfully mimic human tumor progression. Moreover, as implied by these models, different forms of PCa may arise from different cells of origin, resulting in tumors of different subtypes and sensitivities to therapy [39].

Although embryonal and prepubertal development of the prostatic epithelium is largely driven by a single bipotential stem cell, which produces progenitors committed to either the basal or the luminal lineage, independent progenitors maintain the two compartments in adult life [30]. One of the major effects of neoplastic transformation is a differentiative block. In AR-driven PCa adenocarcinoma, the differentiation block appears to occur late, as demonstrated by the expression and nuclear accumulation of luminal differentiation transcription factors, such as NKX3.1 and AR, in the majority of tumor cells [40]. Yet, even tumors that appear well differentiated at the histological and molecular level contain a subpopulation of undifferentiated or poorly differentiated cells, consistent with the presence of a stem cell compartment [41]. It is possible, and indeed likely, that different oncogenic mutations transform different cells of origin within the differentiation hierarchy of the normal epithelium or within the differentiation trajectory of the tumor if they occur late in tumor development. Therefore, as tumor-propagating cells within the same tumor acquire additional oncogenic mutations, their stem/progenitor and aberrantly differentiated compartments may change in nature [42]. For example, ERG and ETV1 cause a basal-to-luminal switch in differentiation in *PTEN*-mutant PCa cells [43]. The presence of multiple, genetically distinct tumor clones in individual cancers potentially complicates matters, although there is evidence suggesting that clones containing combinations of mutations that

sustain metastatic fitness ultimately prevail [44]. A deeper understanding of prostate tumor initiation and differentiation in response to oncogenic transformations will allow us to distinguish indolent from aggressive tumors based on the cell type of origin, which is crucial for patient stratification and personalized treatment.

Lineage Switching is a Mechanism of Resistance to Therapy Resistance in NEPC

Epithelial plasticity has emerged as a major mechanism for therapy resistance in cancer [45, 46]. Different forms of epithelial plasticity, including epithelial-to-mesenchymal transition (EMT), dedifferentiation from a lineage-committed to a stem cell-like state, and transdifferentiation from the luminal to another cell fate may play a role in PCa [23, 47]. Recent studies showed that lineage switching and transdifferentiation are key processes in the acquisition of resistant neuroendocrine phenotypes [25, 48] (Figure 2). NEPC is defined by loss of expression of AR and AR-driven gene expression (PSA-negative), acquisition of NE differentiation traits by a substantial proportion of tumor cells (chromogranin A- and synaptophysin-positive), and small cell morphology [22]. Although NEPC can arise *de novo*, it most commonly develops as a form of primary or adaptive resistance to ADT [49]. Under the former mechanism, it is presumed that targeting the predominant AR-driven luminal differentiated tumor generates a strong selective pressure that enables the expansion of a pre-existing minor subpopulation of AR-negative or AR-low tumor progenitors. A large fraction of the constituent tumor cells then aberrantly differentiates to acquire NE traits. Under this latter scenario, AR-directed therapy promotes switching from the luminal to the NE cell fate through an epigenetic reprogramming. In principle, this process may occur through transdifferentiation or through dedifferentiation to a progenitor state followed by aberrant NE differentiation. Although the relative roles of clonal selection and reprogramming in the development of NEPC remain to be established, it is apparent that both clonal divergence and transdifferentiation from adenocarcinoma play a role [19, 50].

Recent exome and whole-genome sequencing of human metastatic prostate cancer tissues have revealed an accumulation of genetic alterations associated with metastatic disease progression (see Text Box 1)(Figure 4). Genomic studies have indeed shown that AR-pathway negative tumors (AVPC) exhibit prototypical genetic alterations, such as *TMPRSS2-ERG* gene fusions, which are commonly found in prostate adenocarcinomas, but also additional specific mutations, suggesting that these tumors evolve from typical adenocarcinomas [19]. The conversion of prostate adenocarcinoma to NEPC is often associated with the increased activation of *MYCN* and Aurora kinase A (*AURKA*) in *in vitro* models. Consistently, overexpression of *Mycn* promotes the development of aggressive NEPC-like tumors characterized by low AR activity and stem cell traits in *Pten* mutant mice [20]. In addition, direct transformation assays have demonstrated that co-expression of *MYCN* and *AKT* in human prostate epithelial cells can drive the development of both prostate adenocarcinoma and NEPC, both arising from a common epithelial clone; however, castrate conditions lead to NEPC enrichment over prostate adenocarcinoma [51]. Treatment with *AURKA* inhibitors destabilizes N-MYC and induces tumor regression in pre-clinical models, suggesting that N-MYC is necessary for tumor maintenance in these systems [51].

In addition, although the AURKA inhibitor Alisertib failed to meet the primary endpoint of a Phase II trial in patients with AVPC, exceptional responders were identified, suggesting that a subset of AVPC patients may be sensitive to AURKA inhibition [52]. Different subtypes of prostate cancer are likely to respond differently to different therapies. Thus, a classification based on their molecular and biological features is critical toward the design of successful and informative clinical trials.

Extensive genomic analyses have revealed that loss of *RB1* and mutation or deletion of *TP53* occur more frequently in AVPC than in prostate adenocarcinoma [19, 53]. Pointing to a causative role, earlier observations had indicated that transgenic expression of the SV40 large and small T antigens, which inactivate Rb1 and Tp53, leads to the development of prostate adenocarcinomas with NE features in mice [50]. These tumors exhibit decreased expression of the androgen-responsive transcription factor NKX3.1, whose loss is associated with dysregulation of prostate cell differentiation [54]. *In vitro* studies have revealed that combined inactivation of *TP53* and *RB1* is sufficient to rapidly reprogram the *PTEN*-mutant prostate adenocarcinoma LNCaP-AR cells to an AR-independent, basal-like, and neuroendocrine state, which is insensitive to enzalutamide [23]. In GEMMs, the inactivation of *Pten* and *Rb1* is sufficient to initiate the conversion to NEPC, but the additional inactivation of *Tp53* is required to induce expression of *Sox2* and *Ezh2*, thus completing this process [47]. Lineage tracing studies in mice carrying a combined inactivation of *Tp53* and *Pten* in their prostatic epithelium have suggested that NE tumors can also arise through anti-androgen-induced transdifferentiation of luminal tumor cells. Treatment with abiraterone accelerated tumor growth and metastasis in double-knockout mice, suggesting that dual loss of function of *PTEN* and *TP53* contributes to resistance to therapy with abiraterone [48]. Additional findings have corroborated the role of *TP53* and *RB1* aberrations in the development of NEPC and identified *PEG10* as a novel mediator of proliferation and invasion negatively regulated by AR and RB during transdifferentiation to NEPC [55]. Although these studies provide strong evidence that inactivation of *RB1* and *TP53* drives progression to lethal NEPC, especially in the context of an earlier loss of *PTEN*, the epigenetic changes and transcriptional networks that coordinate the conversion to NEPC remain to be fully elucidated.

Intriguingly, recent findings have provided evidence that NE tumors of the lung and prostate arise through similar oncogenic mechanisms and share not only histological, but also epigenetic and transcriptional features. Direct transformation assays have indicated that a combination of oncogenic factors, such as inactivation of *TP53* and *RB1* and activation of *AKT* and *MYC*, is able to reprogram normal human prostate and bronchial epithelial cells to small cell prostate cancer or NEPC and small cell lung cancer (SCLC), respectively [24]. Moreover, mutations of *RB1* and *TP53* are frequent in NEPC and occur in nearly all cases of SCLC [56–58], indicating that these mutations may serve as initiating events in both tumor types. These observations support the notion that these aggressive and lethal cancers originate through a shared evolutionary trajectory dominated by common epigenetic changes. Reprogramming to a common, lethal, small cell phenotype may require a specific cellular context, whereby specific mutations alter cell fate and shape the tumor phenotype.

EMT and stemness in the context of drug resistance

Several studies have indicated that the EMT plays a key role in metastatic dissemination and therapeutic resistance [59]. Cancer cells undergoing an EMT transit through a discrete series of metastable states [46, 60]. Intriguingly, whereas the conversion to a mesenchymal state confers invasive abilities upon tumor cells but inhibits their stemness, the acquisition of hybrid epithelial and mesenchymal (EM) traits is associated with increased self-renewal and tumor initiation capacity [61, 62]. Consistently, studies of breast, lung, and squamous cell carcinoma development have indicated that the EMT facilitates dissemination, but a reversion of this process, the Mesenchymal-to-Epithelial Transition (MET), is required for metastatic colonization [63–67]. Although the link between the hybrid EM state and stemness has not been decisively explored in PCa, it is plausible that cancer stem cells (CSCs) with such hybrid traits exist in prostate adenocarcinomas and drive recurrence under the selective pressure imposed by AR blockade (Figure 3).

In support of this hypothesis, early studies have shown that LNCaP luminal adenocarcinoma cells contain a small subpopulation of PSA^{-lo} cells harboring intrinsic cancer stem cell properties. These cells express very low levels of AR and are refractory to ADT, suggesting that a similar subpopulation may serve as a source of castration-resistant stem cells in PCa [68]. Consistently, transcriptional profiling has shown that human PCa stem cells resemble their normal counterparts in their expression of basal cell-specific markers, lack of expression of luminal differentiation markers, diminished expression of AR and dependency on AR signaling [69]. In addition, prospective identification studies have indicated that the prostatic adenocarcinomas arising in PB-*TAg* mice are characterized by a basal compartment consisting of integrin $\beta 4$ (ITGB4)-positive stem cells able to undergo self-renewal as well as rapid proliferation upon activation of ErbB2 and MET signaling. Mechanistic studies indicated that deletion of the ITGB4 signaling domain physically uncouples the integrin from ErbB2 and MET, preventing it from buttressing joint signaling [70]. Intriguingly, recent studies have shown that ITGB4 marks cancer stem cells possessing a hybrid EM phenotype in triple negative breast cancer (TNBC) [71]. Consistent with their castration resistance and metastatic capacity, ITGB4-positive cells are detected at increased frequency in CRPC metastases [70].

It is also possible that, rather than pre-existing in prostate adenocarcinomas, PCa stem cells with mesenchymal traits are produced through therapy-induced or partial EMT. The existence of a negative feedback loop between AR and the EMT-inducing transcription factor ZEB1 [72] and the ability of AR blockade to induce EMT via STAT3 activation in cell models [73, 74] indicate a potential link between inhibition of androgen signaling and EMT. Of note, AR activity has been shown to be inversely correlated with expression of the EMT transcription factor Twist1, which is transcriptionally repressed by the androgen-regulated transcription factor NKX3.1 [75]. These observations suggest that inhibition of AR signaling may induce implementation of the EMT program, thus driving the development of treatment-resistant CRPC. Acquisition of mesenchymal traits may even drive metastasis in these tumors, as antibodies blocking the function of N-cadherin can block metastasis in preclinical xenograft models of CRPC [76, 77].

GEMMs have been instrumental in studying the connection between EMT, stemness, and therapy resistance in PCa. An analysis of *Pb-Cre4; Pten^{flox/flox}* mice has indicated that the percentage of cancer cells with tumor-initiating capacity increases with disease progression [78]. In the same GEMM, castration induces a large increase in the number of Lin⁻ Sca1⁺ CD49^{high} (LSC^{high}) tumor cells endowed with tumor-propagating capacity [79]. However, inactivation of *Pten* is insufficient to produce stem/progenitor subpopulations with mesenchymal characteristics; this process requires concurrent activation of *Ras*. Notably, tumor cells isolated from *Pten Kras^{G12D}* compound mutant mice not only show mesenchymal traits but also possess high metastatic potential upon orthotopic transplantation *in vivo* [80]. Subsequent studies using a vimentin-GFP reporter showed that the compound mice contain two types of mesenchymal-like tumor-propagating cells, EpCAM⁻ Vim⁺ and EpCAM⁺ Vim⁺. Whereas the former was not able to produce macroscopic metastases upon transplantation, the latter was able to do so, consistent with the notion that hybrid EM traits are conducive to metastasis [81]. These studies are consistent with the demonstrated ability of Ras signaling to induce EMT [82]. Although *RAS* is rarely mutated in PCa, recent studies have indicated that DNPC is driven, at least in part, by activation of the RAS-ERK signaling cascade downstream of the FGF-R [26]. It remains to be examined if these tumors possess subpopulations of mesenchymal stem-like cells.

Despite intense investigation, the origin and characterization of prostate cancer stem cells (CSCs) remains controversial and highly debated. Studies in other cancer types have shown that CSCs can arise *de novo* from differentiated non-stem-like cells [83, 84], but whether they exist at the time of diagnosis or arise as a result of therapy-induced reprogramming is yet to be determined. In fact, although it is possible that CSCs may derive from normal adult stem cells, it is also plausible that they are generated from non-stem-like tumor cells as a consequence of treatment-induced lineage plasticity in a reprogramming-permissive environment induced by alterations of tumor suppressors, such as *PTEN*, *RB1*, and *TP53* among others. The strength of this hypothesis resides in emerging evidence that links the ability of cancer cells in other tumor types to dedifferentiate upon lineage plasticity to a less mature progenitor state in order to replenish a pre-existing CSC pool, thus enabling tumor maintenance [85, 86].

Targeting lineage plasticity: state of the art and future challenges

The hypothesis of tumor cells dedifferentiating or otherwise reprogramming to a CSC-like state partially explains the short-term sensitization of bulk tumors to standard therapies. In fact, M-CRPC is often characterized by admixed AR-positive and AR-negative cells, the latter potentially comprising stem-like cells able to resist to AR targeting. The identification of cell-specific markers or pathways associated with CSCs is crucial for the development of new therapeutic approaches for the treatment of PCa. Currently, a promising line of research focuses on dissecting the signaling pathways used by CSCs to retain their self-renewal capacity and survive cancer treatments. Among those, Wnt and Notch are the major pathways sustaining the self-renewal, proliferation, and aberrant differentiation of CSCs [87].

The canonical Wnt signaling pathway has been shown to be involved in stem cell maintenance and self-renewal [88]. Loss of the tumor suppressor DAB2IP, which restrains Ras signaling and the EMT [89], increased stemness and chemoresistance of non-tumorigenic normal prostate epithelial cell lines with activated WNT- β -catenin signaling [90]. Another CSC marker and direct target of Wnt signaling called ALDH1 was found to be upregulated in radioresistant PCa cells [91]. Inhibition of Wnt signaling by using the tankyrase inhibitor XAV939 restored PCa cell sensitivity to irradiation and reduced ALDH1-positive CSC population [91]. Motivated by these findings, two Wnt inhibitors are being currently tested in clinical trials: vantictumab (OMP-18R5), a monoclonal antibody targeting the frizzled receptors on cancer cells, is being tested in solid tumors including PCa ⁽ⁱⁱ⁾; and Foxy-5 is being used in two clinical trials for treatment of metastatic PCa patients ⁽ⁱⁱⁱ⁾ and ^(iv). However, given the pleiotropic effects of the Wnt signaling pathway and its essential role in tissue homeostasis in multiple organs, it may difficult to target this pathway without unacceptable toxicity. In a similar vein, other studies showed that pro-oncogenic Notch signaling is often enriched in prostate CSCs as well as in chemoresistant PCa [87]. Analysis of mouse models indicated that activation of Notch is not required for tumor initiation in a *Pten*-null mouse model, but it promotes cancer cell proliferation and metastasis by regulating FOXC2 and hence EMT [92]. Interestingly, a γ -secretase inhibitor (GSI) of Notch triggered cellular senescence and constrained tumor progression in *Pten*-null and *Pten-p53*-null mutant mice [93]. Additionally, TMPRSS2-ERG-positive tumors showed a cis-regulatory dependence on Notch signaling, as ERG activated core regulatory regions surrounding Notch pathway genes, such as *HES1*, *JAG1*, and *DLL1* [94]. Inhibition of ERG in TMPRSS2-ERG-positive cells decreased Notch signaling pathway gene expression and enhanced sensitivity to ADT [95]. A number of agents targeting Notch are currently being evaluated; among them, GSI is being tested in combination with the antiandrogen bicalutamide for patients with recurrent PCa after surgery or radiation ^(v).

Tumors are complex mixtures containing a wide array of stromal, immune, and vascular cells. The complex crosstalk between CSCs and their tumor microenvironment (TME) regulates their ability to undergo self-renewal and to proliferate [96], as well as to undergo an EMT that facilitates metastatic spread [97]. Several studies reported the existence of chemoresistance niches dependent on paracrine factors secreted by the TME in response to genotoxic stress. Among them, *WNT16B* secreted by fibroblasts upon *NFKB* activation enhances cell survival and acquisition of invasive mesenchymal tracts [98]. In a *Pten*-null mouse model, additional loss of Smad4 increased Yap-1-mediated Cxcl5 expression, which in turn promoted PCa progression by recruiting polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) [99]. Interestingly, a *Pten/Zbtb7a* double deletion led to an increase in Sox9-mediated activation of Cxcl5, a chemokine known to attract granulocytic cells, thus reinforcing PMN-MDSC-induced tumor progression [100]. Therefore, considering the profound influence of the TME on cancer cell behavior, it is plausible that treatment-resistant CSCs are generated by a plastic reprogramming based on specific

II. <https://clinicaltrials.gov/ct2/show/NCT01608867>

III. <https://clinicaltrials.gov/ct2/show/NCT02655952>

IV. <https://clinicaltrials.gov/ct2/show/NCT02020291>

V. <https://clinicaltrials.gov/ct2/show/NCT01200810>

microenvironmental conditions. Cancer-associated fibroblasts (CAFs) isolated from CRPC tumors strongly supported the ability of CSCs to form organoids *in vitro* and glandular structures *in vivo* as compared to CAFs isolated from androgen-dependent PCa [101]. Recent findings have highlighted the intrinsic ability of cancer stem cells to evade recognition by the immune system. This effect is, at least in part, mediated by specific downregulation of the antigen presentation machinery in these cells [102]. Overall, these studies support the urgent need to understand the dynamic interplay between the TME and CSC plasticity in PCa. These investigations will necessitate the establishment of GEMMs able to recapitulate the aggressive forms of prostate cancer.

In order to improve the clinical care of men with advanced PCa, it will be important to fully elucidate the dependencies of prostate cancer stem cells. The compounds described above are still under clinical development and have not been tested in biomarker-driven trials. An important challenge to take into account is that PCa stem cells can rely on more than one of the previously mentioned pathways. Thus, their efficacy may be limited by a functional redundancy of their targets. One appealing possibility would be to pharmacologically induce the differentiation of PCa stem-like cells and then target the aberrantly differentiated cells emerging from this treatment. Under such a scenario, it would be important to rationally sequence combination therapies incorporating CSC inhibitors and AR inhibitors or oncogene-targeted therapies. Inhibition of PI3K-mTOR pathways are currently being investigated in clinical trials in combination with second generation AR inhibitor therapy in CRPC patients [103]. Additionally, poly(ADP-ribose) polymerase (PARP) inhibitors (e.g., olaparib, rucaparib, veliparib, talozaparib) are being tested in patients carrying mutations in *BRCA1* and *BRCA2* genes who had previously received enzalutamide or abiraterone [104, 105]. Recent studies demonstrated that AR-positive CRPC cell lines and xenograft mouse models are sensitive to epigenetic therapies targeting bromodomain-containing proteins BRD2/3/4 and BRDT [106]. Accordingly, enzalutamide-resistant LNCaP-AR and VCAP cells displayed growth inhibition upon exposure to BET inhibitors, and a higher efficacy was observed when these agents were combined with antiandrogens *in vivo* [107]. Another study proposed a BROMO-10 signature to identify patients likely to respond to BET inhibitors, possibly in combination with other agents to avoid resistance [108]. Currently, the BET bromodomain inhibitor ZEN-3694 is being tested in combination with enzalutamide in mCRPC patients ^(vi).

CSC maintenance and PCa progression depend on epigenetic mechanisms. For instance, EZH2, a subunit of PRC2, functioning as a coactivator for transcription factors strongly expressed in CRPC patients (such as AR) [109, 110], has been associated with the acquisition of a stem-like phenotype in advanced PCa. In fact, EZH2 inhibitors can revert the CSC phenotype and resensitize PCa cells to ADT and androgen receptor blockade [47, 111]. An EZH2 inhibitor (CPI-1205) is currently under investigation in a phase 1b/2 study in combination with enzalutamide or abiraterone/prednisone in patients with mCRPC ^(vii).

VI. <https://clinicaltrials.gov/ct2/show/NCT02711956>

VII. <https://clinicaltrials.gov/ct2/show/NCT03480646>

Finally, although ineffective as single agents in CRPC, immune checkpoint inhibitors have shown strong potential when combined with conventional therapies [112]. Pembrolizumab, a PD-1 inhibitor, is currently in a phase 2 study for patients with metastatic CRPC after ADT therapy (^{viii}). More importantly, double checkpoint immunotherapy has demonstrated to produce durable complete pathological responses in about 18% of M-CRPC patients (^{ix}). A greater understanding of the biological traits of CSCs will be the key to defining new effective combinational trials for the treatment of metastatic CRPC.

Concluding remarks

Although the genomic alterations of late stage AR-independent M-CRPC are known, fundamental questions remain regarding its origin, evolution, and metastatic spread (see outstanding questions). Firstly, it will be important to define both intratumoral and interpatient heterogeneity of the disease so that biomarker-driven clinical trials can be designed on a rational basis. Genomic and functional studies have already revealed that certain genomic aberrations, such as loss of *PTEN*, *TP53* and *RBI*, lead to the development of the androgen-indifferent AVPC. Unfortunately, it is very difficult to reactivate the function of multiple tumor suppressor. However, genetic screening for synthetic lethal interactions in AVPC may reveal novel targets that could be pursued in preclinical models. In this context, the lack of spontaneous GEMM that faithfully mimic the development of M-CRPC is still an important challenge to contend with. Indeed, certain combinations of critical genomic alterations potentially involved in the development of the androgen-independent M-CRPC phenotype have not yet been fully modeled. In particular, there is currently no PCa GEMM that reproducibly develops macroscopic bone metastases. One important caveat of the traditional GEMMs is that transgene expression is driven by AR-dependent promoters, and this complicates the assessment of their hormone dependence and response to ADT. Improvements in GEMMs resembling the advanced stages of human PCa will help us model response and resistance to AR-targeted therapy and identify novel targets in AR-independent disease.

The mechanistic relationship between androgen deprivation therapy and cellular plasticity is still unclear. Whether they are consequently linked, whereby ADT induces plasticity, or cellular plasticity primes the tumor to acquire drug resistance needs to be determined. In this context, it will be critical to assess whether distinct CSC populations do pre-exist, or if they arise as a consequence of spontaneous EMT, drug-induced plasticity, and/or upon oncogenic insults on non-CSCs in the normal prostate tissue. The aggressiveness of each subpopulation would help to distinguish between indolent and aggressive disease. Additionally, *in vivo* lineage-tracing experiments using stem-cell specific promoters (e.g., Sox9, Lgr5, p63) in combination with oncogenic mutations will help to understand the role of PCa stem cells in response to therapy in mouse models, and to determine which niches impact the trajectory from stem to differentiated states and *vice versa*. The use of cutting-edge technologies such as single-cell RNA sequencing of mouse and human biopsy specimens may help to determine key targets restricted to the CSC subpopulations. Finally, the impact of non-cell

VIII-<https://clinicaltrials.gov/ct2/show/NCT02312557>

IX-<https://clinicaltrials.gov/ct2/show/NCT02985957>

autonomous mechanisms on drug-induced plasticity is another issue that deserves urgent investigation. The delineation of the crosstalk between the tumor microenvironment and the progression to CRPC will clarify whether and how stromal components promote the survival and therapy resistance of PCa cells. Taken together, these studies should enable the identification of novel and promising therapeutic targets for AR-independent M-CRPC.

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Glossary

Androgen deprivation therapy (ADT)

type of hormone therapy used to block the production of androgens and the effects of AR signaling in PCa patients

Aggressive Variant Prostate Cancer (AVPC)

subset of prostate cancers defined clinically by the presence of at least 1 of 7 criteria (AVPC-C), including: 1) Small cell prostate carcinoma; 2) Visceral metastases only; 3) Lytic bone metastases; 4) Bulky nodes or prostate mass; 5) Low PSA relative to volume; 6) Neuroendocrine markers; 7) Primary castration-resistance

Dedifferentiation

Biological phenomenon whereby differentiated cells regress to a less differentiated state. In prostate, AR⁺ luminal epithelial cells revert to AR^{low/-} progenitor-like cells

Transdifferentiation

process whereby a fully committed somatic cell converts into another mature cell

Epithelial-to-mesenchymal transition

physiological process in which epithelial cells lose their polarity and acquire mesenchymal, migratory and invasive features

Plasticity

ability of cells to reversibly switch between different phenotypic states

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Text Box1.**Oncogenic Drivers in Prostate Cancer**

Significant effort has been devoted to identifying genetic drivers in both hormone-naïve primary PCa and mCRPC [19, 113, 115–119]. More recently, classification criteria based on AR and NE gene expression scores allowed transcriptional segregation of CRPC tumors into three tumor subtype categories: ARPC, NEPC, DNPC [26]. As displayed in Figure 4, with the exceptions of *AR* (mainly altered in the ARPC subtype) and *RB1* (prevalently inactivated in NEPC), both NEPC and DNPC show enrichment of *TP53*, *PTEN*, *RB1*, *ERG* fusions and *MYC* aberrations [114, 119]. Mutations of genes involved in the DNA damage response are less frequent.

Many of these mutations and combinations thereof have been demonstrated to be oncogenic in genetically engineered mouse models (GEMMs) of PCa [47, 113]. *Pten* knockout in GEMMs develop high-grade prostate intraepithelial neoplasia (PIN) and progress from adenocarcinoma [120–123] to androgen-independent CRPC [123–126], with minimal metastasis [122, 124]. Loss of *PTEN* promotes cellular senescence [127], which is bypassed by castration or by additional genetic alterations such as loss of *TP53* [127], gain of function of *MYC* [128], or activation of TGFβ signaling via loss of *SMAD4* [129]. Inactivation of *TP53* drives prostate tumor progression only when combined with loss of function of additional tumor suppressors (*Pten*, *Rb1*, *Brca2*, *Nkx3.1*) [47, 130]. Interestingly, combined inactivation of *Pten* and *TP53* in mice induces prostate tumors that are resistant to ADT, whereas inactivation of *Pten* alone only delays the response to ADT. This difference is due in part to the capacity of *TP53* loss to counteract the inhibition of proliferation and induction of apoptosis caused by downregulation of the X-linked inhibitor of apoptosis protein-associated factor-1 (*Xaf1*) [131].

Aberrations of the *RB1* tumor suppressor gene are probably acquired late in the progression of PCa. *RB1* mutations were indeed found to be enriched in patients previously exposed to taxanes and anti-androgens, favoring the hypothesis that these mutations are sub-clonal events arising post-treatment [132]. Previous studies have established an *RB/E2F1/AR* axis in CRPC, wherein the *RB1/E2F1* complex binds to the AR promoter and represses its expression. Consequently, it was argued that *RB1*-deficient tumors are able to bypass hormonal therapy leading to a castration-resistant state [133]. In GEMMs, inactivation of *Rb1* alone does not confer an aggressive phenotype [134], but combined inactivation of *Rb1*, *Pten*, and *TP53* promotes the development of AR-negative invasive adenocarcinomas that display NE traits and metastasize to the lung, liver, and in rare cases bone [47]. These observations suggest that inactivation of *RB1* may have distinct consequences in AR signaling-driven and AR-indifferent mCRPC.

A *MYC* family, namely *MYCN*, has been showed to be involved in aggressive CRPC and amplified in 40% of NEPCs [22]. Combined loss of *Pten* and overexpression of *N-Myc* in GEMMs promotes the development of invasive tumors comprising foci of poorly differentiated tumor cells, which exhibit NE traits, attenuated AR signaling, and

activation of Ezh2, a component of the polycomb repressive complex 2 (*PRC2*) [20]. Strikingly, *EZH2* inhibitors can sensitize the NEPC tumors arising in *Rb1/Tp53* mutant mice to enzalutamide treatment by partially inhibiting lineage conversion and thus restoring AR expression and signaling [47].

Outstanding Questions

- Are CSCs pre-existing in the bulk tumor or do they arise upon ADT therapy in the metastatic target organ and drive outgrowth?
- What is the influence of the tumor microenvironment on tumor cell plasticity?
- Can we identify specific markers and cellular programs that sustain the survival and resistance to therapy of CSCs?
- Is metastatic CRPC a pre-determined disease event or is it a result of genetic mutations sequentially acquired as a consequence of therapy?
- What is the exact roadmap of the reprogramming process?

Highlights

- Patients with prostate cancer on Androgen Deprivation Therapy (ADT) will progress to androgen-independent disease.
- Tumor cells can acquire a plastic phenotype and give rise to AR-indifferent variants of prostate cancer (AVPC/NEPC)
- Plasticity may drive acquisition of EMT traits and cause the acquisition of metastatic traits as well as therapeutic resistance.
- Cancer stem cells (CSCs) can arise *de novo* from differentiated non-stem-like cells; it is unclear if they are present at time of diagnosis or arise as a consequence of ADT-induced reprogramming.

Figure 1. Oncogenic drivers and therapeutic resistance to second-generation AR inhibitors. The rapid clinical progression to CRPC is indicated by increasing PSA levels after the failure of androgen-deprivation therapy (ADT). The use of second-generation androgen receptor (AR) inhibitors significantly improves the survival of men with CRPC and M-CRPC; however, resistance to these agents eventually emerges, ultimately leading to death. The resistant tumors will be driven by either reactivation of AR signaling (ARPC) or rely on alternative AR- indifferent mechanisms and will be characterized by low PSA levels and more aggressive clinical features (aggressive variant prostate cancer-AVPC). Combination therapy will most likely benefit M-CRPC patients. Abbreviations: ADT, androgen deprivation therapy; ARPC, androgen receptor pathway active prostate cancer; AVPC, aggressive variant prostate cancer.

Figure 2. NEPC-induced by androgen targeted therapy.

The clonal selection model indicates that existing neuroendocrine (NE) clones will gain growth advantage in an androgen-deprived condition. Recent studies showed that treatment-resistant NEPC arise from adenocarcinoma *via* transdifferentiation. Amplifications of *NMYC* and *AURKA* have been linked to this aggressive subtype. PCa tumors with loss *RB1* and *TP53* undergo lineage plasticity and transdifferentiate to a basal/NE state that is no longer dependent on AR signaling. A dynamic epigenetic reprogramming enables the transdifferentiation process; indeed, increased expression of *SOX9*, *SOX9*, *SOX11* and *EZH2* drives the insurgence of NEPC phenotype. Alternatively, a broader *spectrum* of de-differentiation process may exist. Therapy-induced CSCs could arise as a consequence of a dedifferentiation from a mature luminal (AR-positive) to progenitor/stem-like state (AR-low or negative). Whether these CSCs re-differentiate into NE-like cells or they form mesenchymal-like tumors before converting in NE-like phenotype is still unclear. Abbreviations: NE, neuroendocrine; NEPC, neuroendocrine prostate cancer; CSCs, cancer stem cells.

Figure 3. Origin of therapy-resistant Cancer Stem Cells.

Once exposed to androgen blockade, pre-existing CSCs can survive, self-renew and give rise to metastatic relapse. The *de novo* generation of CSCs model suggests that mature luminal cells could instead dedifferentiate and acquire CSCs traits *via* signaling pathways aberrantly activated. In both cases, treatment-resistant CSCs will be characterized by low PSA and AR expression, EMT traits, activation of STAT3 signaling, CD44⁺, CD133⁺, ALDH1⁺ cells. In both proposed models, CSCs will ultimately drive tumor recurrence. Abbreviations: EMT, epithelial-to- mesenchymal transition; CSCs, cancer stem cells

Figure 4. Frequency of genomic aberrations in CRPC subtypes.

Columns represent individual patients and rows specific genes. Percentage of gene altered in each subtype is shown. The patients are grouped based on AR activity score and NE signature score as previously described [26]. SU2C and FHCRC genomic/clinical data were acquired via cBioPortal [113, 114] and queried specifically for the gene alterations listed.