THEMATICAL REVIEW

The heterogeneity of prostate cancers lacking AR activity will require diverse treatment approaches

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Abstract

The use of androgen deprivation therapy and second-line anti-androgens in prostate cancer has led to the emergence of tumors employing multiple androgen receptor (AR)-dependent and AR-independent mechanisms to resist AR-targeted therapies in castration-resistant prostate cancer (CRPC). While the AR signaling axis remains the cornerstone for therapeutic development in CRPC, a clearer understanding of the heterogeneous biology of CRPC tumors is needed for innovative treatment strategies. In this review, we discuss the characteristics of CRPC tumors that lack AR activity and the temporal and spatial considerations for the conversion of an AR-dependent to an AR-independent tumor type. We describe the more prevalent treatment-emergent phenotypes arising in the CRPC disease continuum, including amphicrine, AR-low, double-negative, neuroendocrine and small cell phenotypes. We discuss the association between the loss of AR activity and tumor plasticity with a focus on the roles of transcription factors like SOX2, DNA methylation, alternative splicing, and the activity of epigenetic modifiers like EZH2, BRD4, LSD1, and the nBAF complex in conversion to a neuroendocrine or small cell phenotype in CRPC. We hypothesize that only a subset of CRPC tumors have the propensity for tumor plasticity and conversion to the neuroendocrine phenotype and outline how we might target these plastic and emergent phenotypes in CRPC. In conclusion, we assess the current and future avenues for treatment and determine that the heterogeneity of CRPCs lacking AR activity will require diverse treatment approaches.

Key Words
- castration-resistant prostate cancer
- androgen receptor
- adenocarcinoma
- amphicrine
- double-negative prostate cancer
- AR-low
- neuroendocrine

Castration-resistant prostate cancer

Prostate cancer (PC) is notable for the dependence of tumor cells on the androgen receptor (AR) for activation of a luminal differentiation program, proliferation and survival. Because of this, androgen deprivation therapy (ADT), achieved through surgical or pharmacological approaches, is the principal treatment for men with metastatic PC. Tumors from the vast majority of men (>80%) respond initially to ADT. However, ADT is not curative and
Emerging phenotypes in CRPC

With the advent of more effective therapies targeting the AR pathway, we and others have observed the emergence of tumors employing multiple AR-dependent mechanisms that contribute to resistance to AR-targeted therapies. These AR-dependent resistance mechanisms maintain a luminal differentiation program with sustained AR signaling without evidence of switching to an alternate differentiation program, most notably neuroendocrine (NE) PC. We have termed these tumors AR active without NE differentiation (AR+/NE−). However, an expanding literature highlights the increasing incidence of additional CRPC phenotypes that were previously rare prior to more widespread use of second-generation AR inhibitors (Bluemn et al. 2017). Indeed, our group characterized four additional CRPC subtypes based on the expression of established AR or NE genes. These subtypes are: (i) amphicrine; (ii) AR-low; (iii) double-negative; and (iv) small cell or neuroendocrine CRPC phenotypes (Labrecque et al. 2019). Amphicrine cells were first described by Walter and Ratzenhofer (Walter & Ratzenhofer 1979) as endocrine cells with both endocrine and exocrine functions. Amphicrine CRPC retains AR activity and luminal differentiation programs but expresses some of the classical markers of NE disease (e.g. chromogranin A, synaptophysin, and/or CD56). For the purposes of this review, we describe the amphicrine phenotype as tumors composed of cells co-expressing AR and NE genes (AR+/NE+). The AR-low phenotype displays decreased nuclear AR and decreased AR regulated genes while in many cases maintaining expression of KLK3 (i.e. prostate-specific antigen; PSA), an AR-regulated gene (ARlow/NE−) (Labrecque et al. 2019). Double-negative prostate cancer (DNPC) tumors are denoted by undetectable AR protein expression and absent AR or NE gene expression (AR−/NE−). DNPC phenotypes include rare CRPC tumors with squamous differentiation (Parwani et al. 2004, Labrecque et al. 2019, Cackowski et al. 2020). Lastly, small cell or NE prostate cancer (SCNPC) tumors are AR-null and have either classical small cell pathology or upregulated NE gene expression profiles without AR activity (AR−/NE+) (Tsai et al. 2017, Beltran et al. 2019a, Labrecque et al. 2019) (Table 1). Importantly, further refinement of CRPC classes and discovery of novel CRPC phenotypes are likely with the widespread availability of DNA and RNA sequencing datasets and continued collection and analysis of CRPC tissues (Abida et al. 2019, Labrecque et al. 2019). Thus, the shifting landscape of CRPC phenotypes in response to therapy requires the development of effective therapeutic strategies that target the diverse array of emerging aggressive tumor phenotypes that are either AR-dependent or the emerging subtypes that no longer rely on AR activity.

What is androgen receptor inactive prostate cancer?

Classically, the AR-inactive CRPC phenotype is defined by the complete loss of AR expression in PC tumor cells (Beltran et al. 2016). However, the majority of patients who die of CRPC maintain some degree of AR expression
and activity as evidenced by rising levels of serum PSA at the time of disease progression or death. Notably, the presence of AR and PSA expression does not necessarily equate with responsiveness to ADT. For example, AR mutations (Newmark et al. 1992, Gaddipati et al. 1994), AR amplification and rearrangements (Kolivisto et al. 1997, Li et al. 2020b), amplification of AR enhancer elements (Okuda et al. 2018), the expression of AR splice variants that lack the C-terminal ligand-binding domain (Zhang et al. 2011, Li et al. 2012, Sharp et al. 2019), enhancer RNAs (Hsieh et al. 2014), and altered AR co-regulators (Reebye et al. 2012) promote resistance to ADT directed therapies.

We, however, contend that not all CRPC tumors expressing AR and PSA are the same. Specifically, these cells harbor all of the clinical markers to suggest an AR-dependent phenotype, but do not require AR activity for regulating tumor cell proliferation and/or survival. This is exemplified in patients treated with ADT, where a small number of AR- and PSA-positive tumors demonstrate a loss of AR signaling and are refractory to therapy (Kumar et al. 2016). Furthermore, recent work demonstrated that AR expression in tumor cells was not a reliable predictor of response to the second-generation AR inhibitor enzalutamide; importantly, that study demonstrated that tumors from non-responders had significantly lower AR transcriptional activity despite similar protein expression as responders (Alumkal et al. 2020). Figure 1A is a RNA sequencing (RNA-Seq) heatmap of 270 CRPC biopsies from the SU2C series. Biospecimens are stratified according to the log2FPKM values of AR and further stratified based on an AR-regulated gene set GSEA enrichment score; ARG10 (Bluemn et al. 2017). Of note, a subset of adenocarcinoma with upregulated AR transcript expression display AR activity scores as low as AR-null tumors (Fig. 1A).

The AR transcript is present at high levels, but AR activity is decreased relative to other AR-expressing adenocarcinoma. When 20 AR-expressing adenocarcinoma specimens with the highest and lowest ARG10 scores were compared to each other in a gene set enrichment analysis (GSEA), the hallmark pathways androgen-response, protein-secretion and oxidative-phosphorylation were the highest pathways present in the ARG10-high group (Fig. 1B). Conversely, epithelial-mesenchymal-transition was the highest pathway in the ARG10-low group (Fig. 1B). Importantly, many of these same gene sets were activated in AR-positive, but not in AR activity-low metastatic CRPC tumors that were most resistant to enzalutamide in our recent work (Alumkal et al. 2020). We propose that the ARG10-low group – even though they display high expression of AR, KLK3, and NKK3-1 transcripts – may no longer be dependent on the AR for survival and proliferation.

In parallel, molecular profiles of CRPC biospecimens from the rapid autopsy program at the University of Washington recapitulate the SU2C observations, with a subset of AR-expressing adenocarcinomas displaying reduced AR activity scores by RNA-Seq, interestingly showing a reduction in nuclear AR, but maintaining PSA protein expression by IHC (Fig. 1C). Our group and others have also observed an AR-low phenotype where there is heterogeneous or decreased AR expression (Aggarwal et al. 2019, Labrecque et al. 2019, Alumkal et al. 2020). These tumors are losing AR expression and AR activity and appear to be transitioning to an AR-null phenotype (Fig. 1C). Importantly, PSA expression (the marker most heavily relied on to assess AR activity in CRPC patients) only displays a gradual decline with the loss of AR activity in these tumors. Furthermore, NKK3-1 expression – that is considered a surrogate for AR activation in PC – displays

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<td>Amphicrine prostate cancer</td>
<td>CRPC cells that co-express AR and NE genes (AR+/NE+)</td>
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<td>CRPC cells with attenuated or heterogeneous AR protein expression and concomitant reduction in AR transcript and some AR regulated genes (ARlow/NE−)</td>
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<td>ARPC</td>
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<td>SCPC</td>
<td>Small cell prostate cancer</td>
<td>CRPC that morphologically resembles small cell carcinoma of the lung, including small, round tumor cells, minimal cytoplasm, ‘salt and pepper’ chromatin and high mitotic index.</td>
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Table 1 Terminology and features used to describe CRPC subtypes.
an even more gradual decline in AR-low tumors (Fig. 1C) (Abate-Shen et al. 2008, Gan et al. 2019). How AR expression is suppressed and whether AR-low tumors represent an intermediate state between AR-high and AR-null CRPC or represent a stable CRPC phenotype, themselves, remain unanswered at this time.

We propose that AR inactivity is a continuum ranging from tumors that completely lack AR expression and AR activity, ‘AR-null’ on one extreme, to tumors that still express the AR and some markers of AR function, like PSA and NKX3-1, but have lost dependence on the AR for tumor cell survival and/or proliferation. An important question is whether tumors that express the AR, but lack evidence of AR target gene activity, are truly AR-inactive? Would methods that ablate AR expression in these tumors have any effect on growth or survival? Would exposing these tumors to AR ligands that activate AR signaling lead to proliferation or terminal differentiation? Ongoing work in the field seeks to better define AR indifference in appropriate clinical contexts.

Characteristics of CRPCs lacking AR activity

The complexity surrounding the transition from an AR-dependent to an AR-inactive phenotype has made it difficult to define histological or molecular features that consistently associate with the emerging CRPC phenotypes. Currently, no morphological characteristics have been described in clinical specimens to delineate an AR-active from an AR-inactive phenotype in AR-expressing CRPC. Furthermore, it is not yet clear that morphological features associate with the full spectrum of molecular phenotypes of NE CRPC (Epstein et al. 2014, Aggarwal et al. 2019, Labrecque et al. 2019). Additionally, genomic analyses of patient metastases have not clarified genomic features that reliably distinguish these phenotypes or that can be used to predict the risk of conversion to AR-null or NE-positive states. For example, bi-allelic loss of the tumor suppressor genes RB1 and TP53 are enriched in the NE phenotype. However, not all CRPC tumors that exhibit bi-allelic loss of RB1 in conjunction with TP53...
alterations are NE tumors (Ku et al. 2017, Aggarwal et al. 2019, Nyquist et al. 2020). In addition, loss of the tumor suppressor PTEN alone or in concert with TP53 alterations in CRPC mouse models have demonstrated concomitant decreases in AR expression (Zou et al. 2017, Liu et al. 2019b). Moreover, combinatorial PTEN/TP53 alterations accentuate NE lineage switching from luminal CRPC cells in response to AR pathway inhibition (Zou et al. 2017). On the other hand, and similar to the Rbl1/TP53 relationship, PTEN/TP53 alterations are not universal features of tumors with AR-loss or NE conversion in patient biospecimens (Conteduca et al. 2019). This suggests that these genomic alterations may be neither necessary nor sufficient for NE differentiation, despite their role in promoting AR-loss. Recent literature strongly suggests that epigenetic events are also important contributing factors for the development of phenotypes that are functionally independent of AR signaling (Clermont et al. 2015, Kleb et al. 2016, Lee et al. 2018a).

The temporal and spatial considerations for the clinician during the conversion of an AR-dependent to an AR-independent neuroendocrine tumor type

Currently, there are significant challenges in determining whether a specific patient’s tumor is converting to an AR-inactive state. The issue primarily rests on the interpretation of limited clinical markers that can denote an AR-inactive state. In extreme cases, such as SCNPC conversion, serum PSA levels decline with a concomitant increase in tumor volume or clinical deterioration. This may be accompanied by an increase in serum chromogranin A or other NE markers. However, to complicate matters, amphicrine tumor cells co-express AR, AR target genes and classic NE biomarkers (i.e. chromogranin A or synaptophysin). The NE aspect of the amphicrine phenotype may cause the physician to interpret an increase in serum chromogranin A as the beginning of a transition to an AR-null SCNPC phenotype. However, there is nothing to suggest that amphicrine tumors are less responsive to AR-directed therapy than AR-high adenocarcinomas (i.e. AR+/NE−). For example, the VCaP CRPC cell line displays an amphicrine molecular profile, but is responsive to enzalutamide treatment (Tran et al. 2009, Labrecque et al. 2019).

Sampling CRPC tumors and obtaining a molecular profile is currently the most effective means of determining the intermediate transitory state from an AR-high adenocarcinoma to a poorly differentiated NE phenotype. However, metastatic biopsies has not been considered standard of care, and it is even rarer to perform sequential tumor biopsies, thus limiting our understanding of the patient’s tumor phenotype. In addition, a biopsy only samples one lesion, even though a patient may have some metastases that are AR-high and responsive to ADT, while other metastases have converted to an AR-low, DNPC or SCNPC phenotype (Labrecque et al. 2019). Inter- and intra-tumoral heterogeneity within a patient is not uncommon in heavily treated patients with some tumors displaying conversion to SCNPC and other tumors containing a mix of AR+ and AR-null tumor cells within the same site. This work is highlighted in biopsies from patients with CRPC that demonstrate spatial and temporal intra-patient heterogeneity (Aggarwal et al. 2019), and in rapid autopsy specimens from patients with CRPC, where multiple sites are available for analysis from the same patient providing information that cannot be ascertained from a single biopsy (Zhang et al. 2015, Labrecque et al. 2019). The molecular characterization of circulating tumor cells (CTC) could be used in some cases to overcome sampling bias and tumor heterogeneity (Scher et al. 2017). However, challenges remain, with limited CTC available for analysis in patients with low tumor burden. In contrast to phenotypic changes, evaluating a single metastasis for genomic alterations provides a reasonable assessment of the major oncogenic driver events that are present in disseminated tumors within an individual, though DNA-sequencing-only based approaches clearly do not provide a complete assessment of a tumor’s transcriptional program (Kumar et al. 2016).

What exactly is the definition of the neuroendocrine phenotype in CRPC?

SCNPC is the most extreme AR-inactive subset vs AR-high adenocarcinoma. NE cells in the prostate are defined in current practice by immunohistochemical positivity for either synaptophysin, chromogranin A, or CD56 (Epstein et al. 2014). For some time neuroendocrine PC (NEPC) was considered a rare variant in localized hormone naïve PC. In fact, the field was somewhat dismissive of NE tumors in PC as rare and therefore not a significant focus for funding. NE tumors had been described in and appeared to be more prevalent in CRPC following ADT (Hirano et al. 2004, Beltran et al. 2011, Zhang et al. 2015). However, the implementation of second-generation AR signaling inhibitors that mediate total androgen blockade have
resulted in increased incidences of treatment-emergent NE tumors (Bluemn et al. 2017, Aggarwal et al. 2019, Beltran et al. 2019a, Labrecque et al. 2019). On this point, we need to take a step back. An NE tumor is defined in current practice by morphological characteristics and supported by immunohistochemical positivity for either synaptophysin, chromogranin A, or CD56 (Epstein et al. 2014). However, there have been a plethora of articles describing NE CRPC that do not use these specific criteria and that rely on morphologic features alone. While morphology may be an acceptable surrogate for small cell in de novo tumors that have not undergone ADT, it is possible that ADT or second-generation AR inhibitors induce morphologic changes that mirror NEPC, but without NE gene expression. Additionally, since the conversion of adenocarcinoma to an AR-null NE phenotype is most likely a continuum of disease, many models and tumor specimens have some NE molecular characteristics, but have not completely transitioned to a SCNPC phenotype (e.g. amphiocrine tumors). Due to the presence of diverse transitory and differentiated NE phenotypes, we would suggest from a molecular standpoint that the SCNPC phenotypes as a group could be identified by either synaptophysin, chromogranin A, or CD56 positivity and the lack of AR expression.

AR indifference is associated with the loss of AR and gain of tumor plasticity

Characterization of the AR gene and promoter region, and of factors inducing AR expression have been explored over the last few decades. The AR promoter region lacks a typical TATA box and AR expression across human tissues has been reported to be mediated, in part, by SP1 activity (Tilley et al. 1990, Faber et al. 1993). Additionally, cyclic-AMP induces AR expression and cyclic-AMP response elements (CRE) alongside CREB transcription factor binding in the AR promoter have been verified (Lindzey et al. 1993, Mizokami et al. 1994). In the context of CRPC, AR transcript and protein expression are directly induced through E2F1 activity in CRPC cells lacking RB expression (Sharma et al. 2010). Additionally, EZH2 and EED activity induce AR expression, directly associate with AR protein and are coactivators of AR transcription factor functions independent of canonical polycomb repressive complex 2 (PRC2) functions in PC (Xu et al. 2012, Kim et al. 2018, Liu et al. 2019a). However, the relationship between AR and polycomb group proteins is a complicated one as there is evidence to suggest that epigenetic regulation through EZH2 contributes to AR-loss (Kleb et al. 2016). This is supported by work showing that EZH2 inhibitors reactivate AR expression in AR-null NE models (Beltran et al. 2016, Kleb et al. 2016). Another mechanism that may suppress AR expression is DNA methylation. Kinoshita et al. identified consensus sites of DNA methylation in the AR promoter that are markers for gene silencing and surmised that AR DNA methylation may represent a phenotype important in the development of hormone independence in a subset of advanced PC that has lost AR expression (Kinoshita et al. 2000). Together, these data suggest that tumor cells that lose AR expression through epigenetic alterations are inherently more plastic than tumor cells that continue to express AR and have AR activity throughout ADT. This notion has led to the proposed use of epigenetic modifier inhibitors to reverse AR-loss and re-sensitize AR-inactive tumors to AR pathway inhibitors.

Loss of AR, the expression of SOX2 and the activation of LSD1 promotes NE differentiation in CRPC

If the conversion of an AR-active adenocarcinoma to an AR-null NE phenotype is an epigenetic phenomenon, then key players in the physiologic transition to a neuronal phenotype could drive the transdifferentiation process in CRPC. Alongside the silencing of AR expression in CRPC, SOX2 is expressed in many AR-null NE CRPC tumors and model systems (Yu et al. 2014, Kanan et al. 2019). SOX2 is an AR-repressed transcription factor that promotes lineage plasticity through epigenetic reprogramming (Kregel et al. 2013, Mu et al. 2017). In addition, SOX2 is considered a neural stem cell marker (Ellis et al. 2004) and has roles in neuronal development through maintaining neural progenitor identity and inhibiting neuronal differentiation (Uwanogho et al. 1995, Graham et al. 2003). Recent studies overexpressing SOX2 in LNCaP cells revealed an increase in NE gene expression, however, a complete transition to an AR-null or NE phenotype was not observed (Li et al. 2020a, Metz et al. 2020). It has been posited that SOX2 expression is permissive, but not sufficient for NE conversion in CRPC (Kwon et al. 2020a,b). Of note, SOX2 is active in neural progenitor cells, but not in differentiated postmitotic neurons (Miyagi et al. 2004). Thus, the continued expression of SOX2 in NE tumors potentially contributes to a tumor phenotype that is more plastic in nature than AR expressing tumors (Mu et al. 2017, McAuley et al. 2019). With the loss of AR expression, SOX2 activity promotes further epigenetic alterations, including upregulation
of the histone demethylase LSD1, suppression of adenocarcinoma-associated genes, and upregulation of NE programs, all of which may contribute to NE conversion (Li et al. 2020a). An alternatively spliced form of LSD1, known as LSD1+8a that is normally only expressed in neuronal tissues, was recently found to be expressed in NEPC tumor cells, and LSD1 splicing was mediated by SRRM4 (Coleman et al. 2020). In neuronal cells, LSD1+8a appears to have substrate-specificity for the repressive mark H3K9, as opposed to LSD1’s canonical histone demethylation substrate H3K4, and demethylation of H3K9 by LSD1+8a enhances neuronal gene expression in neuronal cells (Laurent et al. 2015, Jotatsu et al. 2017, Coleman et al. 2020). More work is needed to determine if this LSD1+8a function is conserved in NE CRPC tumors.

**SOX2 and the nBAF complex promote a proliferative NE phenotype**

Another epigenetic modifying complex critical for neuronal differentiation is the neuronal BAF (nBAF) complex. The BAF (mammalian SWI/SNF) complex, is a chromatin remodeling complex that regulates gene expression and cell differentiation states (Alfert et al. 2019). Brg1 is a subunit of the SWI/SNF complex involved in neurogenesis, neural crest induction, and differentiation (Eroglu et al. 2006). Seo et al. demonstrated in Xenopus that the loss of Brg1 function did not affect neural induction or neural cell fate determination. However, Brg1-loss induced the expansion of a proliferative Sox2-positive neural progenitor cell population and diminished the expression of a terminally differentiated neuronal marker, suggesting that Brg1 is required for neuronal differentiation (Seo et al. 2005). Recent work by Cyrtta et al. in prostate tumor cells recapitulate these findings; they found that BRG1 expression in CRPC was positively correlated with the expression of synaptophysin, but also showed a tendency toward positive correlation with SOX2 (Cyrtta et al. 2020). Likewise, Lessard et al. determined that BAF45A and BAF53A subunits are necessary and sufficient for neural progenitor proliferation and are replaced by BAF45B, BAF53B, and BAF45C to form the nBAF complex as neural progenitors exit the cell cycle (Lessard et al. 2007). These data demonstrate that SOX2 is associated with an expanding neural progenitor phenotype and that a switch from BAF to nBAF complexes through the insertion of BAF45B, BAF53B, and BAF45C is associated with the transition of a neural progenitor cell to a terminally differentiated neural phenotype. It is important to note that NE CRPC cells are not terminally differentiated, but are in fact highly proliferative. This suggests that the expression of SOX2, and the formation and activity of the nBAF complex provides for a proliferative stem-like NE phenotype in PC (Cyrtta et al. 2020). Additionally, it is important to note that this behavior occurs in the absence of the AR. As mentioned previously, it is possible to revert AR-null NE cells back to an AR-positive phenotype in some NE CRPC models (Beltran et al. 2016, Kleb et al. 2016). This implies that the continued expression of SOX2 and the nBAF complex could represent central components of the plastic and proliferative aspect of the NE phenotype in CRPC.

**Changes in components of the BAF complex can promote the NE phenotype in AR-null PC tumors**

Under AR pathway suppression with pharmacological agents, there is an increase in the prevalence of SCNPC and amphicrine tumors in metastatic CRPC (Labrecque et al. 2019). These two CRPC phenotypes can arise in part due to the loss of RE1-silencing transcription factor (REST) activity. REST is a master regulator of differentiation that transcriptionally represses neuronal programs in non-neuronal cells. Thus, loss of REST repressor activity in both AR-active and AR-null tumors induces NE gene expression, such as SYP, and results in the amphicrine and SCNPC phenotypes respectively (Labrecque et al. 2019). RNA splicing factors SRRM3/SRRM4 diminish REST repressor activity through a splicing-in event, producing a REST transcript that encodes a truncated REST protein variant (REST4) in prostate and other cell types (Zhang et al. 2015, Li et al. 2017, Nakano et al. 2019). Diminished canonical REST activity through REST4 alternative splicing is a hallmark of neuronal cells that allows for the expression of REST-repressed genes, many of which are NE factors or proteins involved in the vesicular secretion of NE factors. However, the loss of REST activity also induces the expression of BAF53B in PC (Cyrtta et al. 2020). As stated earlier, BAF53B is part of the nBAF complex, a neuronal chromatin remodeling complex that regulates gene expression and differentiation (Alfert et al. 2019). Post-mitotic neurons express BAF53B (Vogel-Ciernia et al. 2013) promoting a fate-determining chromatin switch to a differentiated neuronal phenotype (Olive et al. 2002, Tang et al. 2013). Yoo et al. determined that in the vertebrate nervous system, a switch in chromatin-remodeling appears to coincide with the final mitotic division of neurons. This switch involves
the exchange of the BAF53A and BAF45A subunits within the BAF complex for the homologous BAF53B and BAF45B subunits within neuron-specific BAF (nBAF) complexes in post-mitotic neurons (Yoo et al. 2009). There is a definitive increase in BAF53B at the transcript and protein level in NE CRPC and in the majority of (but not all) amphicrine CRPC metastases (Labrecque et al. 2019). BAF45B transcript is elevated in AR-null NE CRPC, but it is not elevated in amphicrine CRPC metastases, indicating that the complex may be active specifically in AR-null NE CRPC tumors (Labrecque et al. 2019, Cyrtta et al. 2020). This suggests that BAF45B is not a REST repressed message and in the absence of BAF45B, BAF53B may not be sufficient to form the nBAF complex in AR-positive amphicrine tumors. Therefore, if the AR is present, the expression of the REST-repressed chromatin modifier BAF53B alone has no impact on gene expression and can result in the amphicrine phenotype in CRPC, where only REST-repressed transcripts and not downstream master regulators of SCNPC are expressed (Labrecque et al. 2019).

How does the loss of AR, the expression of SOX2 and the loss of RB1 in neuroendocrine CRPC fit into this picture?

SOX2 is an AR-repressed transcription factor (Kregel et al. 2013). PC stem cell-like intermediates have reduced RB1 and TP53 protein expression and overexpress SOX2 (Nouri et al. 2020). In PC, it has been argued that lineage plasticity is enabled by the loss of TP53 and RB1 function, which is mediated by increased expression of the reprogramming transcription factor SOX2, and can be reversed by restoring TP53 and RB1 function or by inhibiting SOX2 expression (Mu et al. 2017). Sutter et al. have shown that inactivation of the tumor suppressor genes RB1 and TP53 in neural stem cells induced deregulated proliferation and resistance to apoptosis in vitro. Moreover, injection of these cells into mice formed medulloblastomas and that medulloblastomas originating from the neural stem cells preferentially expressed stem cell markers including SOX2 (Sutter et al. 2010). The expression of SOX2 is also high in the pediatric tumor retinoblastoma in which RB1-loss is ubiquitous, highlighting the discordance between the physiological process where neural precursors convert to terminally differentiated neurons and the maintenance of SOX2 expression in conjunction with RB1-loss in NE disease (Tong et al. 2015, Orellana et al. 2016). This suggests that there is a link between the loss of AR, the upregulation of SOX2, the loss of RB1 and the emergence of the NE CRPC phenotype. However, it must be noted that RB1-loss is not only associated with NE CRPC, it also occurs in AR-positive CRPC (Nyquist et al. 2020). Nyquist et al. determined that CRISPR-Cas9-mediated double knockout of RB1 and TP53 alone does not induce SOX2 expression or promote a plastic or NE phenotype in AR-positive LNCaP cells. Furthermore, the AR-null LNCaP derivative APiPC does not convert to the NE phenotype (Bluemn et al. 2017). These data suggest that there are various pathways to the NE phenotype involving SOX2 and RB1-loss. Identifying these pathways will be critical to predict the patients who are at a greatest risk of NE conversion and to develop therapies that block this process.

Only a subset of CRPC tumors may have the propensity for tumor plasticity and conversion to the NE phenotype

Although the incidences of AR-low, DNPC and NE tumors are rising in end-stage CRPC, the majority of CRPC metastases retain robust AR expression (Bluemn et al. 2017, Aggarwala et al. 2018). This suggests that only a subset of CRPC metastases have the propensity to lose AR expression after ADT and newer AR pathway inhibitors. The proportion of patients that fall into AR-inactive categories may represent a larger cohort over time with the introduction of more effective AR blockade, but recent work suggests that some AR-expressing tumors – particularly those that respond poorly to enzalutamide – have lower canonical AR activity (Alumkal et al. 2020).

There are several possible explanations for how the loss of AR expression and NE transdifferentiation may occur in a PC tumor cell. One possible explanation is that activation of the PRC2 complex leads to loss of AR expression and induction of AR-repressed SOX2. Similar to neural precursor cells, SOX2 could then poise the epigenetic landscape for activation of NE differentiation programs by neuronal transcription factors (Amador-Arjona et al. 2015). At the same time, sustained AR blockade can upregulate SRRM4, a splicing factor that promotes neural differentiation through REST alternative splicing and inactivation in PC (Raj et al. 2011, Zhang et al. 2015, Li et al. 2017, 2019). REST inactivation induces the expression of BAF53B in PC (Cyrtta et al. 2020). BAF53B and BAF45B are present in NE CRPC cells and are principal components of the nBAF complex that promotes neuronal gene expression in CRPC (Cyrtta et al. 2020) (Fig. 2). If these changes do not occur, a DNPC phenotype without NE differentiation may predominate. The propensity to escape AR blockade...
through the loss of AR expression, SOX2 expression and the NE phenotype could also depend on the tumor cell of origin. This population of tumors could arise from tumor progenitors originating in the prostate where SOX2 expression has been shown to be enriched in the epithelial cells of the proximal prostate adjacent to the urethra and is present in castration-resistant progenitor cells in the adult murine prostate (McAuley et al. 2019, Kwon et al. 2020a, b).

As described, the linear transition from an AR-expressing tumor cell to a plastic NE phenotype is only a hypothesis. There is currently no understanding if the amphicrine phenotype is an intermediary state and could eventually lose AR expression and convert to a classic NE phenotype. Similarly, in some cases, AR-low and AR-null DNPC may never convert to the classic NE phenotype. For example, we have observed a subset of DNPC tumors with squamous differentiation (Kibbee-Cram 1988, Parwani et al. 2004, Labrecque et al. 2019). While rare, this phenotype may represent an alternative differentiation pathway for plastic AR-low or AR-null DNPC tumors. This suggests that the DNPC phenotype could represent a transitory point where a prostate epithelial cell in a stem-like state could remain as a DNPC tumor, or convert to a squamous, NE, or other differentiated phenotype. The complexity of tumor cell evolution is evidenced by the key roles the cell of origin, the accumulated genomic hits, and microenvironment may all play in transition to different phenotypes. This is important to consider when interpreting the experimental manipulation of adenocarcinoma cell lines and organoids in vitro to produce a ‘transdifferentiated’ NE tumor cell. If the AR-inactive, AR-low and AR-null phenotypes arise based on a specific cell of origin with specific genomic alterations, and specific epigenetic alterations, then the manipulation of cell lines from an adenocarcinoma phenotype to the NE phenotype, while forced, may not be biologically relevant. There could be many pathways to the NE phenotype, but not all necessarily arise in patient tumors in response to AR blockade.

### Defining ‘phenotype’ in CRPC and the impact on clinical care in the future

We currently rely on classic biomarkers and tissue morphology to define CRPC tumor phenotypes in the clinical setting. However, this approach will change with the expansion of ‘omic’ technologies and other approaches to phenotype tumors. In this commentary, we only briefly discussed the influence of genomic alterations on AR-inactive phenotypes. Rather, we have highlighted the phenotypes that lose AR activity and acquire a plastic state or differentiate to another classic tumor phenotype via epigenetic means. This does not negate the role of genomic alterations promoting lineage plasticity or differential response to therapy of a subset of tumors in a defined phenotype due to the inherited genomic alterations acquired before a change in the tumor phenotype occurs. This demands, as we have known for some time, that a
multi-layered approach involving genomics, transcriptomics, epigenetics, and proteomics are required to define each of the treatment-emergent CRPC tumor phenotypes. More stringent definitions of these phenotypes are critically needed so that patients whose tumors exhibit a similar phenotype may be stratified to clinical trials testing agents that are predicted to work well in specific subtypes.

**Targeting plasticity and emergent phenotypes in castration-resistant prostate cancer**

In 1994, Bang *et al.* provided direct evidence of plasticity in the lineage commitment of adenocarcinoma of the prostate. They demonstrated that cAMP can drive the plasticity of prostate carcinoma cells sufficient to trigger a series of events associated with the emergence of the NE phenotype (*Bang et al.* 1994). A recent NCI Workshop focused on lineage plasticity and AR-independent PC described lineage plasticity as ‘a biologic process that occurs during normal development and later as a mechanism that promotes cell survival when adapting to their environment, evading stress, or repairing tissues’ (*Beltran et al.* 2019a). An example of this is the transdifferentiation of ADT-treated CRPC cells to an alternative NE cell lineage. In this case, the acquisition of an alternative cell lineage is an effective drug resistance mechanism. Understanding the disease continuum from adenocarcinoma to a classic small cell NE phenotype in CRPC could provide potential targets for treatment that were not present previously. Currently, treatment for confirmed or suspected SCNPC is a platinum-based regimen (*Amato et al.* 1992, *Suzuki et al.* 2020). However, the duration of response is short (*Aparicio et al.* 2013).

Cell surface markers present an opportunity for targeted drug delivery to SCNPC tumors. Carcinoembryonic antigen (CEA or CEACAM5) is enriched in SCNPC and has been suggested as a useful marker for selecting therapy (*Lee et al.* 2018b). Abnormal CEA levels predict poor overall survival suggesting a benefit from chemotherapy in patients with CRPC with an anaplastic feature (*Aparicio et al.* 2013). Further, it has been proposed as the target for an antibody-drug conjugate with efficacy in CEACAM5 expressing SCNPC patient-derived xenograft (PDX) models (*DeLucia et al.* 2021). Another cell surface marker, notch ligand delta-like protein 3 (DLL3) (*Dunwoodie et al.* 1997) is expressed in the majority of SCNPC tumors, and similar to CEACAM5 an antibody-drug conjugate that targets DLL3 has been used successfully in PDX models and in an early phase clinical trial (*Puca et al.* 2019). These two antibody-drug conjugates hold promise that cell surface markers associated with embryonic development could capture both a cell population transitioning to the NE phenotype and the NE phenotype itself.

Additional targets for treatment include the transcription factor MYCN and the mitotic serine/threonine kinase AURKA. The overexpression and gene amplification of MYCN and AURKA in a significant number of NEPC tumors, and evidence that they cooperate to induce the NE phenotype in prostate cells marks these factors as exciting targets in NEPC (*Beltran et al.* 2011). MYCN has been established as a driver of NEPC. Using genetically engineered mouse models it has been shown that N-Myc overexpression leads to the development of poorly differentiated, invasive PC that is molecularly similar to human NEPC (*Dardenne et al.* 2016). Destabilization of MYCN through AURKA inhibition has been shown to significantly impact tumor growth in MYCN driven tumors (*Lee et al.* 2016). In a clinical trial of alisertib (a drug that inhibits the interaction between MYCN and AURKA) in men with metastatic PC, patients with tumors with molecular features supporting AURKA and MYCN activation achieved significant clinical benefit from single-agent alisertib (*Beltran et al.* 2019b). Another AR repressed master regulator, BRN2/POU3F2 has been shown to regulate SOX2 expression in PC, and promote NE differentiation (*Bishop et al.* 2017). These findings nominate BRN2 as another potential target in BRN2-expressing NEPC. Fibroblast growth factors (FGFs) and their receptors have a significant role in prostate development and PC (*Kwabi-Addo et al.* 2004). Our group demonstrated that a subset of AR-null PC tumors may respond to FGFR inhibition (*Bluemn et al.* 2017). Whether the responsiveness to FGFR inhibition relates to tumors transiting from an AR expressing to an AR-null phenotype, the DNPC phenotype or the rare squamous phenotype remains to be seen. Since the AR-inactive phenotype most likely derives from treatment-induced epigenetic events (*Clermont et al.* 2015, *Beltran et al.* 2016, *Kleb et al.* 2016, *Lee et al.* 2018a), a number of investigators are assessing epigenetic modifier inhibitors as a strategy to block or reverse the emergence of new cell lineages associated with drug treatment in CRPC.

Increased EZH2 expression has been associated with the NE phenotype in PC (*Clermont et al.* 2015). Furthermore, small cell lines (including NCI-H660) have silencing histone modifications (H3K27me3 and H3K9me2) in the AR promoter and the use of an EZH2 inhibitor resulted in AR expression and growth inhibition prompting the
authors to suggest that the AR-null phenotype can be reversed with epigenetic drugs (Kleb et al. 2016). Similar results have been observed in mouse models where RB1-loss facilitates lineage plasticity and metastasis of prostate adenocarcinoma initiated by PTEN mutation (Ku et al. 2017). Additionally, MYCN also promotes an EZH2-mediated transcriptional program driving NEPC, and EZH2 inhibition can reverse MYCN-induced suppression of epithelial lineage genes (Dardenne et al. 2016, Berger et al. 2019). Since silencing EZH2 is suggested to inhibit NE differentiation in PC, this makes EZH2 a prime target for therapy in NEPC.

The bromodomain and extra-terminal (BET) protein, bromodomain protein 4 (BRD4) recruits transcriptional regulatory complexes to acetylated chromatin (Dey et al. 2003) and subsequently activates RNA polymerase II-driven transcriptional elongation. As a histone acetylation reader, BRD4 is an important component of the P-TEFb complex, where Cyclin-Dependent Kinase 9 (CDK9) heterodimerizes with a cyclin partner (cyclin T or K) and stimulates transcriptional elongation by phosphorylating RNA polymerase II (Petelin & Price 2006). It is thought that BRD4 couples the P-TEFb complex to chromatin structures via binding of its bromodomains to acetylated lysines in the histone H3 and H4 tail sequences (Vollmuth et al. 2009). CDK9 phosphorylates AR, and has been shown to mediate the reactivation of AR signaling in PC cells in vitro (Lee et al. 2001, Pawar et al. 2018).

BET inhibition has been studied in both PC pre-clinical models and a recent clinical trial. BET inhibition using the tool compound JQ1 disrupts AR recruitment to target gene loci (Asangani et al. 2014). ARV-771, a small-molecule pan-BET degrader suppresses both AR signaling and AR levels (Raina et al. 2016). This suggests that BET inhibitors could enhance AR antagonism in combination with AR antagonists (Asangani et al. 2016). Other BET inhibitors include Y08060, ABBV-075, ZEN-3694, ODM-207 and I-BET151 (Dawson et al. 2011, Fairev et al. 2017, Xiang et al. 2018, Aggarwal et al. 2020, Ameratunga et al. 2020). BET and CDK9 inhibitors can impact survival and proliferation programs through blocking MYC expression and activity (Fairev et al. 2017, Xiang et al. 2018, Coleman et al. 2019b). BET inhibitors suppress the growth of CRPC, including AR-null and NEPC models (Welti et al. 2018, Coleman et al. 2019a); however, the maintenance of MYC expression promotes de novo resistance to BET bromodomain inhibition in CRPC, demonstrating MYC’s importance for the anti-tumor activity of BET inhibition (Coleman et al. 2019b). Significantly, due to the interactions of CDK9 and BRD4, combined CDK9+BET inhibition has been suggested as a novel therapeutic approach in cancer (Moreno et al. 2017, Pawar et al. 2018). Based on the evidence above, the activity of BET inhibitors does not appear to be limited to AR-expressing CRPC. Indeed, in neuroblastoma models, BET inhibition of MYCN impaired growth, induced apoptosis, and limited tumor growth in vivo (Puissant et al. 2013, Shahbazi et al. 2016). In clinical trials, ZEN-3694 plus enzalutamide demonstrated potential efficacy in patients with androgen-signaling inhibitor-resistant CRPC. Importantly tumors with low baseline AR activity appeared to derive greater clinical benefit from treatment than those with high baseline AR activity (Aggarwal et al. 2020).

Another epigenetic modifier of interest in NEPC is LSD1 (Sehrawat et al. 2018, Etani et al. 2019). As discussed previously, Li et al. have proposed that SOX2 may promote epigenetic alterations through LSD1 and conversion to the NE phenotype (Li et al. 2020a). We also identified the splice variant LSD1+8a in NEPC, but more work is needed to determine LSD1+8a’s functional role in NEPC. Interestingly, our prior work in CRPC demonstrates that LSD1 promotes AR-independent PC cell survival and regulates gene expression independently of its demethylase function (Sehrawat et al. 2018). Thus, determining mechanisms by which LSD1 functions in NEPC will be necessary to develop the most effective class of LSD1 inhibitors.

Conclusions

The targets described above are only a few of the possible novel targets identified in the plastic AR-inactive emerging tumor phenotypes in CRPC. However, because of the complexity of these tumors, which often continue to express the AR, it will be critical to develop combinatorial therapeutic strategies targeting factors important in specific tumor subsets along with the AR. Furthermore, because of inter- and intra-patient tumor heterogeneity, it will be critical to gain a better understanding of an individual patient’s phenotype. Doing so will enable a more precise selection of patients whose tumors have similar vulnerabilities for clinical trials testing agents that target these vulnerabilities.

Declaration of interest

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