Regulation of tumor cell plasticity by the androgen receptor in prostate cancer

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Abstract

Prostate cancer (PCa) has become the most common form of cancer in men in the developed world, and it ranks second in cancer-related deaths. Men that succumb to PCa have a disease that is resistant to hormonal therapies that suppress androgen receptor (AR) signaling, which plays a central role in tumor development and progression. Although AR continues to be a clinically relevant therapeutic target in PCa, selection pressures imposed by androgen-deprivation therapies promote the emergence of heterogeneous cell populations within tumors that dictate the severity of disease. This cellular plasticity, which is induced by androgen deprivation, is the focus of this review. More specifically, we address the emergence of cancer stem-like cells, epithelial–mesenchymal or myeloid plasticity, and neuroendocrine transdifferentiation as well as evidence that demonstrates how each is regulated by the AR. Importantly, because all of these cell phenotypes are associated with aggressive PCa, we examine novel therapeutic approaches for targeting therapy-induced cellular plasticity as a way of preventing PCa progression.

Key Words
- prostate
- androgen receptor
- endocrine therapy resistance

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Introduction

Prostate cancer (PCa) has become the most common form of cancer in men in the developed world, and it ranks second in cancer-related deaths, with the vast majority of these fatalities resulting from metastatic disease (Siegel *et al.* 2014). Advanced PCa is initially treated with androgen deprivation therapy (ADT), a key therapeutic approach that is based on the central role that androgens play in tumor development and growth (Heidenreich *et al.* 2014). Although it is widely used and was initially highly effective, ADT uniformly leads to the development of castration-resistant PCa (CRPC), an aggressive and usually fatal cancer state that continues to progress despite treatment. The recent development of therapeutics that block androgen receptor (AR) activity, such as abiraterone and enzalutamide, has greatly enhanced clinical management and extended the survival of CRPC patients in both the pre- and the post-chemotherapy setting (de Bono *et al.* 2011, Ryan *et al.* 2013, Beer & Tombal 2014). Nonetheless, advanced PCa remains incurable because resistance rapidly emerges via the reactivation of the AR and/or alternative adaptive mechanisms (Joseph *et al.* 2013). Tumor cell plasticity induced by androgen deprivation may play a principal role in treatment resistance and disease progression, and it potentially provides a new opportunity for therapeutic intervention.

Although the precise mechanism that governs the development of CRPC has yet to be fully realized, CRPC

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arises when cancer cells maintain AR signaling in the absence of normal levels of ligand or when they shed their dependence on the AR entirely by hijacking alternative growth and survival pathways. Several mechanisms for explaining CRPC progression have been proposed, including: altered functionality of the AR because of genetic alteration, which results in either hypersensitive (Visakorpi *et al*. 1995, Waltering *et al*. 2009), promiscuous (Fujimoto *et al*. 2007), or constitutively activated (Dehm *et al*. 2008) states; the intratumoral synthesis of androgens (Locke *et al*. 2008); and altered growth factor and/or microenvironment signaling (Lai *et al*. 2009, Sun *et al*. 2012, Lubik *et al*. 2013, Yang *et al*. 2014). Despite concerted efforts to develop pharmacological agents that are capable of suppressing AR signaling, progression is inevitable. Selection pressures imposed by therapy promote genomic rearrangements, alter inflammatory and immune responses, and change the structure of chromatin, and they thereby allow PCa cells to adapt to a plastic phenotype/genotype (Yu *et al*. 2010, Urbanucci *et al*. 2012, Sharma *et al*. 2013). In this review, we focus on therapy-induced cellular plasticity, specifically the emergence of cancer stem-like cells (CSCs), epithelial–mesenchymal or myeloid plasticity, and neuroendocrine transdifferentiation, which may contribute to disease progression. A clear understanding of these processes will help guide novel therapeutic strategies that could enhance the efficacy of clinically utilized anti-androgen therapy to cure, or at least delay, PCa.

Prostate tumor plasticity: CSCs

Cancer stem cell theory proposes that cancer cell populations have a hierarchical developmental structure, and only a fraction of tumor cells – the CSCs – can drive tumor growth and disease progression, perhaps through therapy resistance and metastasis. This framework has been based on genetic tracing studies that showed that cancers are composed of a heterogeneous population of cells that not only possess the capacity for self-renewal but also have extremely aggressive metastatic ability and heightened resistance to conventional radio- and chemotherapies (Chen *et al*. 2012, Driessens *et al*. 2012, Schepers *et al*. 2012). Accumulating evidence suggests that PCa contains a rare and distinct population of CSCs that are responsible for tumor formation and are similar to those CSCs found in other cancers (Bonnet & Dick 1997, Al-Hajj *et al*. 2003, Collins *et al*. 2005, Visvader & Lindeman 2008). To illustrate this point, PCa patients who harbor an embryonic stem cell (ESC) gene expression signature have poor survival outcomes and highly metastatic tumors (Markert *et al*. 2011). Prospective prostate CSCs have been isolated from cell lines and dissociated primary tumors based on the expression of cell surface markers, which usually include *CD44* in combination with a variety of other markers, such as *SCA1*, *CD133*, *ALDH*, and/or *α2β1 integrin*. For example, $CD44^+$ cells fractionated from PCa cell lines and patient-derived xenografts have been shown to be enriched in tumorigenic and metastatic progenitor cells as compared to isogenic $CD44^-$ cells (Patrawala *et al*. 2006). Moreover, as few as 100 $CD44^+/CD24^-$ cells derived from the LNCaP cell line demonstrated tumor-forming abilities when they were transplanted into NOD/SCID mice (Hurt *et al*. 2008). Finally, prospective CSCs have been isolated from primary human PCa cell lines based on the expression of $CD44^+/CD133^+/\alpha2\beta1^{hi}$, and these cells were able to self-renew and regenerate phenotypically mixed populations *in vitro* (Collins *et al*. 2005, Wei *et al*. 2007).

As is evident from these previous studies, a lack of consensus exists with respect to the marker expression and phenotype of the prostate CSC subpopulation. This has been complicated by an incomplete and contradictory understanding of the cellular hierarchy within the normal prostate. Studies by van Leenders *et al*. have begun to dissect the different prostate cell populations based on their unique cytokeratin expression patterns (Schalken & van Leenders 2003). However, it remains difficult to determine whether a cancer cell of origin is a stem cell, a multipotent progenitor, or of a more differentiated progeny because of the lack of *in situ* markers and our inability to isolate pure cell populations. The discordance between stem cell markers in cell lines and clinical specimens has further hampered our ability to quantify CSCs in human specimens (Hoogland *et al*. 2014). Despite these technical challenges, there is evidence to suggest that the expansion of intermediate epithelial stem cells causes PCa (van Leenders *et al*. 2000). For example, the activation of oncogenic signaling pathways (e.g., AKT) in $SCA1^+$ murine stem/progenitor cells were shown to give rise to high-grade prostatic intraepithelial neoplasia (PIN) lesions following an 8-week incubation (Xin *et al*. 2005). Early studies demonstrated that basal stem cells are capable of tumor induction in renal grafting models (Goldstein *et al*. 2010, Taylor *et al*. 2012). More recently, however, elegant lineage-marking experiments in multiple mouse models have directly implicated Nkx3.1-expressing luminal stem cells as the favored cells of origin (Karthaus *et al*. 2014, Wang *et al*. 2014).

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All of these studies fit the simple assumption that normal prostate stem cells acquire genetic and/or epigenetic alterations to transform into CSCs, which drive tumor progression. The picture became more complicated when the current doctrine regarding unidirectional normal and neoplastic stem cell hierarchies was scrutinized. Three large independent studies revealed that tumor cells display considerable plasticity; that is, differentiated, post-mitotic cells are able to ascend the cellular hierarchy and re-enter the CSC state. The paradigm-shifting work of Gupta *et al*. demonstrated that breast cancer cell populations can interconvert between phenotypic states. These initial findings indicated that CSCs can arise *de novo* from non-stem-like cells and that this process of de-differentiation can occur continually during the development of a tumor (Gupta *et al*. 2011). Additional work by the Weinberg group identified that contextual signals from the microenvironment, specifically *TGFβ* coupled to the activation of *ZEB1*, regulate the conversions from non-CSC to CSC states (Chaffer *et al*. 2011, 2013). Finally, a study by Flavahan *et al*. (2013) revealed that glioblastoma cells can de-differentiate into CSCs under the pressure of certain stressors, such as glucose deprivation. A similar phenomenon has been described in PCa, wherein cellular stress caused by anti-androgen therapy induced LNCaP and PC3 cells to convert to a CSC state (Tang *et al*. 2009). Together, these studies represent an important landmark. It is now clear that transformed cells retain some degree of phenotypic plasticity, and in response to appropriate stimuli, they can reactivate stem cell-associated self-renewal programs to drive advanced disease.

AR as a regulator of CSCs

There is accumulating evidence that ADT yields an expansion of CSCs, which suggests that the loss of canonical AR activity may be a potential inducer of the CSC state and of non-CSC-to-CSC plasticity. In both human xenograft and transgenic TRAMP models, the expression of stem cell markers increased dramatically post-ADT (Tang *et al*. 2009, Seiler *et al*. 2013), whereas PCa patients who received ADT were found to harbor an expanded $CD133^+$ CSC population (Lee *et al*. 2013*a*). Keeping with these findings, several recent studies found that some prostate CSC subpopulations express low levels of AR and are resistant to castration. Cells isolated on the expression of $CD44^+/CD133^+/\alpha2\beta1^{hi}$ were found to be AR^- (Collins *et al*. 2005), and $CD44^+/AR^-$ cells from patient-derived xenografts co-expressed stem-cell associated genes (Gu *et al*. 2007). Additionally, in the BM18 xenograft model, preexisting CSCs, which are AR^{lo} and co-express *ALDH1A1* and/or *NANOG*, were selected by castration and could reinitiate CRPC tumor growth (Germann *et al*. 2012). Most notably, Qin *et al*. (2012) discovered a cell population with low expression of prostate specific antigen (*PSA*), a direct target gene of AR, within high-grade prostate tumors that exhibited a heightened self-renewal capacity. These cells also expressed CSC-associated markers, such as *CD44*, *integrin α2*, and *ALDH1A1*, exhibited high clonogenic potential, and possessed tumor-propagating capacity. The $ALDH^+/CD44^+/\alpha2\beta1^+$ CSC subpopulation could be prospectively purified in $PSA^{-/lo}$ cells, which indicates that low AR expression and/or activity may increase the CSC population (Qin *et al*. 2012).

Although these clinical data are supportive of the implication that AR suppression is a modulator of CSC plasticity, they are not entirely sufficient to verify this suggestion. Fortuitously, studies of ESCs have provided more extensive evidence for an involvement of AR in regulating a stem-like state. It has been demonstrated that AR signaling suppresses ESC self-renewal capacity (Chang *et al*. 2006), and during ESC differentiation, AR levels continue to increase in order to 'tip the balance' from self-renewal to differentiation (Sauter *et al*. 2005). Similarly, in PCa, AR-negative cell lines have an increased ability to form non-adherent spheroids, which are a surrogate measure of self-renewal capacity (Li *et al*. 2008). More recent work using siRNA or the anti-androgen bicalutamide to suppress AR in PCa cell lines has resulted in enhanced spheroid formation (Lee *et al*. 2013*a*), which provides further support for the suggestion that AR inhibits self-renewal capacity. In a reciprocal set of experiments, overexpression of AR within the $CD133^+$ CSC population isolated from LNCaP and C4-2 cell lines dramatically reduced spheroid formation (Lee *et al*. 2013*a*). Together, these studies establish AR as a major regulator of the CSC phenotype in PCa.

Although we believe that a model in which aberrant AR signaling can enhance cellular plasticity is sound, the mechanism remains poorly understood. One possible mechanistic explanation for the association between the CSC phenotype and AR is its ability to directly regulate stem cell transcription factors, including *SOX2*, *NANOG*, and *OCT4*, which function in maintaining stem cell survival, self-renewal, and pluripotency. For example, *SOX2* is transcriptionally repressed by AR, and, as expected, treating multiple PCa cell lines with the anti-androgen enzalutamide has been shown to increase *SOX2*

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expression and to lead to CRPC tumor formation (Kregel *et al*. 2013). AR has also been reported to directly bind to the *NANOG* promoter (Kregel *et al*. 2014) to impart castration resistance in LNCaP cells driven by the expansion of $CD133^+$ and $ALDH1^+$ CSCs (Jeter *et al*. 2011). Apart from direct transcriptional regulation, AR can also indirectly modulate the Wnt (Bisson & Prowse 2009), PI3K/AKT (Dubrovska *et al*. 2009), and hedgehog (Gowda *et al*. 2013) signaling cascades, which play an important role in regulating PCa CSC self-renewal. Together, these findings beg the question of how AR is reprogrammed to facilitate CSC plasticity and CRPC progression. A pioneering study by Xu *et al*. (2012) demonstrated that in CRPC, AR is recruited to distinct genomic sites, where it executes a distinct transcriptional program to drive tumor cell proliferation and survival. This shift in the AR binding landscape requires the co-factor *EZH2*, an epigenetic regulator with a well-documented role in regulating cell identity (Margueron & Reinberg 2011). Although the resultant chromatin architectural and mechanistic consequences are not fully understood, CSC plasticity is probably mediated at least in part by the cooperation of AR and EZH2.

The impressive recent crescendo of experimental observations that implicate cytokines and growth factors in enhancing the CSC phenotype underscore the role of the tumor microenvironment in the phenotypic plasticity of tumor cells and CSCs. We recently learned from a study by Wang *et al*. (2013) that endothelial cells within the prostate are increased following ADT. These cells secrete interleukin 6 (*IL6*), which activates PI3K/AKT signal transduction to suppress AR transactivation (Yang *et al*. 2003). Intriguingly, elevated PI3K/AKT activity coincides with stem cell activation and maintenance (Segrelles *et al*. 2014), and treating mice with soluble IL6 receptor fusion protein or silencing PI3K in tumor cells has been shown to significantly suppress prostate tumor growth via a reduction in the CSC population (Dubrovska *et al*. 2009, Schroeder *et al*. 2014). *IL6* has also been reported to activate NF-κB to maintain and expand the CSC population in breast cancer (Korkaya *et al*. 2012) and PCa (Rajasekhar *et al*. 2011). Notably, the overexpression of NF-κB in LNCaP cells has been shown to confer resistance to enzalutamide (Nadiminty *et al*. 2013), possibly through the expansion of the CSC population. Hence, targeting the androgen axis modulates CSC plasticity not only through direct transcriptional regulation, such as *SOX2* and *NANOG*, but also by affecting multiple cell types that comprise the tumor microenvironment.

Prostate tumor plasticity: epithelial–mesenchymal transition

In addition to expanding CSCs, androgen deprivation is also known to direct the plasticity of transformed epithelial prostate cells toward a mesenchymal cell state, or vice versa. Epithelial–mesenchymal transition (EMT) is a developmental process wherein epithelial cells begin expressing mesenchymal markers in response to tumor microenvironmental stimuli; they also lose cell adhesions, change shape, and become more migratory and invasive. Ultimately, this process allows tumor cell dissemination and the formation of distant metastases (Kalluri & Weinberg 2009, Lim & Thiery 2012, Sun *et al*. 2012). In normal development and in PCa, this transition is controlled by the expression of the epithelial marker *E-cadherin*, which when down-regulated allows factors that drive EMT, such as *ZEB1*, *TWIST*, *SLUG* and *SNAIL*, β-catenin, and *ETS1*, to drive the expression of mesenchymal markers, including *N-cadherin*, *vimentin*, *fibronectin*, *cadherin 11*, *collagen 1*, *α2(b)β3 integrin*, and *syndecan 1* (Anose *et al*. 2008, Jennbacken *et al*. 2010, Shiota *et al*. 2010, Zhu & Kyprianou 2010, Matuszak & Kyprianou 2011, Yates 2011, Clyne 2012, Wu *et al*. 2012). Recently, the use of the term 'phenotypic plasticity' has also included reversible mesenchymal–epithelial transition (MET) that occurs in cancer cells when mesenchymal cells revert to an epithelial-like phenotype once they have established metastases in new organs (Nieto 2013). Moreover, there is close association between the CSCs and EMT in PCa, although the relationship between these two dynamic states is unclear. However, what is known is that this tumor cell epithelial plasticity is implicated in PCa metastasis and therapeutic resistance, and, like CSCs, it is controlled by the AR.

AR as a regulator of EMT

Multiple studies have shown that mesenchymal markers, including *N-cadherin*, *vimentin*, *ZEB1*, *TWIST*, and *SNAI2*, are highly expressed in androgen-deprived patient tumors, cell lines, and xenografts and mouse models, and a number of AR-dependent mechanisms of EMT control have been proposed (Liu *et al*. 2008, Zhu *et al*. 2010, Sun *et al*. 2012, Izumi *et al*. 2013, Lin *et al*. 2013*a*,*b*). In patient-derived tissue slice grafts, 6–10 weeks of flutamide or lupron treatment was shown to induce the expression of the mesenchymal marker *vimentin* and to cause the mislocalization of *E-cadherin* (Zhao *et al*. 2013). Similarly, androgen deprivation caused EMT, as was shown by the

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overexpression of *N-cadherin*, *ZEB1*, *TWIST1*, and *SLUG* in normal mouse prostate tissue, in LuCaP35 xenografts, and in PCa patients tissue xenografts as well as in LNCaP cells cultured in charcoal-stripped serum (Sun *et al*. 2012). In addition, the mesenchymal marker *cadherin 11*, which is overexpressed especially in bone metastasis of PCa, is reduced by androgens in PCa cell lines, which may be regulated by the AR at the transcriptional level (Lee *et al*. 2010). Indeed, multiple studies have shown an association between the AR and EMT transcription factors. Under ADT conditions, EMT has been shown to be mediated via a feedback loop of AR and the EMT transcription factor *ZEB1*, as was evidenced by the mutual exclusive expression of AR and *ZEB1* in castration-sensitive (LNCaP) and castration-resistant (CWR22Rv1, PC-3, and DU145) PCa cell lines. This was further supported by the up-regulation of *ZEB1* in AR-silenced, TSA, and 5-Aza treated cells and the up-regulation of AR in shZeb1 cells treated with TSA and 5-Aza (Sun *et al*. 2012). *ZEB2* has also been recently identified as being overexpressed in PCa as compared to benign prostatic hyperplasia and was described as being AR-regulated; AR positively regulates *ZEB2* in androgen-dependent cells, but it is a negative regulator of *ZEB2* in androgen-independent PCa cells (Jacob *et al*. 2014). The authors of this last study demonstrated that *ZEB2* expression is up-regulated in response to androgen stimulation and down-regulated after the silencing of AR in androgen-dependent LNCaP cells. However, androgen-independent PC-3 and DU145 cells expressed higher levels of *ZEB2* than LNCaP cells did, and forced AR expression in these cells reduced *ZEB2* expression, invasiveness, and migration and increased the levels of the *ZEB2* transcriptional target E-cadherin (Jacob *et al*. 2014). These results support the idea that AR-regulated EMT is a cell context-dependent phenomenon in PCa. Importantly, however, the detailed mechanism that describes how AR and *ZEB1/2* interact to inhibit each other has not been described.

In addition to *ZEB1/2*, the Snail family zinc-finger transcription factor *SLUG* was recently identified as being androgen-regulated and as being a coordinator of AR that promotes the development of CRPC (Wu *et al*. 2012). Wu *et al*. found that the presence of constitutively active AR induced *SLUG* expression. That study also showed that *SLUG* formed a complex with AR and acted as a co-activator by enhancing AR transcriptional activity even in the absence of androgen and thus providing a growth advantage in androgen-deprived conditions in CRPC (Wu *et al*. 2012). Furthermore, the authors indicated that PCa cells that overexpress the AR splice variants ARV7, AR3, and Arv567es show induced *SLUG* expression. Constitutively active AR splice variants are important regulators of aberrant androgen signaling, because these variants are able to maintain androgen signaling in the absence of androgens as a result of the lack of ligand binding domain. Thus, this study suggests that cooperation between *SLUG* and AR variants might drive an aggressive EMT phenotype in CRPC (Wu *et al*. 2012).

Many other pathways that converge with the AR regulate EMT in CRPC, including the growth factor receptor tyrosine kinase (RTK), PTEN, notch, hedgehog, Wnt, and TMPRSS2:ERG pathways (Leshem *et al*. 2011, Kim *et al*. 2014) and the STAT3 pathway (Karhadkar *et al*. 2004, Acevedo *et al*. 2007, Bisson & Prowse 2009, Mulholland *et al*. 2012). For example, in a study by Izumi *et al*., the silencing of AR in LNCaP cells and PCa mouse xenograft models was shown to promote cell migration by up-regulating the *CCL2*-dependent STAT3 and EMT pathways. The authors suggest that co-targeting *CCL2/CCR2–STAT3* signaling may provide a novel therapeutic approach for targeting PCa progression and metastasis at the castration-resistant stage (Izumi *et al*. 2013). In another study, ADT induced cell invasion that was reversed by targeting pSTAT3–CCL2 signaling in PCa cells and in mouse models (Lin *et al*. 2013*a*). In addition to *CCL2*, STAT3 signaling and EMT have also been shown to be regulated by *HSP27*, which is increased after ADT in CRPC (Rocchi *et al*. 2005, Shiota *et al*. 2013, Cordonnier *et al*. 2015). Silencing *HSP27* reversed an EMT phenotype by reducing *STAT3* phosphorylation and subsequent binding to the *TWIST* promoter and also by reducing EGF-mediated EMT via the modulation of the β-catenin/SLUG signaling pathway (Shiota *et al*. 2013, Cordonnier *et al*. 2015). Because β-catenin is also up-regulated in CPRC, co-localizes with AR in the nucleus, and can act as an AR co-activator, targeting β-catenin–AR interactions may represent a potential novel therapeutic strategy for preventing transcriptional activation of AR in CRPC and AR-dependent EMT (Robinson *et al*. 2008, Schweizer *et al*. 2008, Wang *et al*. 2008). Moreover, these results suggest that targeting *HSP27* and/or *STAT3* may reverse EMT in advanced PCa. Taken together, several EMT-associated pathways and EMT markers have been shown to be regulated by ADT, and aberrant AR signaling in particular has been proposed to induce EMT and to regulate epithelial cell plasticity in CRPC. However, we have yet to define the detailed molecular mechanisms that underlie how AR regulates cell plasticity and to identify potential common drivers for these phenotypes.

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Tumor cell plasticity: alternative epithelial–myeloid transition

The many observations of the emergence of CSCs and cells that have undergone EMT in therapy-resistant PCa highlight the importance of AR-regulated tumor cell plasticity as a driver of advanced disease. However, the diversity of tumor cell types that may arise from CSCs or are included in this 'plasticity' are now broader than was originally thought. For example, an alternative epithelial–myeloid version of EMT (EMyT) has been proposed for tumor cells. The EMyT theory is based on the overlap in the expression of cluster of differentiation (CD) markers, pattern recognition receptors (PRRs), cytokine and growth factor receptors, and matrix metalloproteases in cancer cells that typically define multiple myeloid cell lineages of the immune system (Schramm 2014). Although the therapeutic implications of this alternative immune EMyT for most tumor types has yet to be established, there is mounting evidence in PCa that certain myeloid marker expression correlates with aggressive tumor cell behavior. Moreover, because CSCs and EMT phenotypes are dictated by AR activity in PCa, this myeloid plasticity may likewise be androgen-regulated.

AR regulation of epithelial–myeloid transition

Depending on the AR expression in PCa cell lines, there is differential expression of toll-like receptors (TLRs), which are innate PRRs that normally function to alert immune cells and epithelial cells to infection by bacteria, viruses, or parasites. Although the mechanisms of immune evasion by cancer cells that express TLRs are outside the scope of this review, the presence of TLRs in PCa does have clinical relevance. The increased expression of TLR3 and TLR9 in patients with primary PCa has been shown to predict biochemical recurrence of CRPC (Gonzalez-Reyes *et al.* 2011), and in African American men, polymorphisms in TLR2 were shown to be a significant predictor of PCa risk (Rogers *et al.* 2013). Interestingly, whereas androgen-dependent LNCaP cells stimulated with the TLR3 agonist poly:IC (double-stranded RNA) undergo apoptosis, AR-negative PC3 cells activate an interferon response pathway that leads to the up-regulation of inflammatory mediators, which may promote tumor progression (Palchetti *et al.* 2015). LNCaP cells and PC3 cells also respond differently to stimulation with the TLR2 agonist lipoteichoic acid (LTA), which increases the invasiveness of LNCaP cells but decreases that of PC3 cells (Rezania *et al.* 2014). Although these studies have not directly addressed the androgen regulation of TLR expression or function, they suggest that the association of TLR expression with an increased risk of PCa development or recurrence could reflect differences in degrees of AR activity, and they speak to the effects that mainstay anti-androgen treatments could have on driving the emergence of an EMyT phenotype in PCa tumors.

In addition to TLRs, we have also recently reported the up-regulated expression of the T cell checkpoint molecule programmed death ligand 1 (*PDL1*) on enzalutamide-resistant CRPC cells (Bishop *et al.* 2015). The interaction between *PDL1* or *PDL2* and their receptor, *PD1*, which is located on T cells, inhibits antitumor immune responses and makes this pathway a key target of checkpoint blockade immunotherapies, especially in cancer types that up-regulate *PDL1* on the surface for immune evasion (Sharma & Allison 2015). Whereas *PDL1* on CRPC patient tumors has been difficult to detect or non-existent (Brahmer *et al.* 2010, Topalian *et al.* 2012, Taube *et al.* 2014), the expression of other inhibitory molecules in the B7 family, such as B7H3, correlate with poor prognosis in PCa (Zang *et al.* 2007). Furthermore, we have shown that the up-regulation of many B7 family members, including *PDL1*, may be unique and specific to anti-androgen-resistant CRPC (Bishop *et al.* 2015). In addition, *PDL1* was not the only immune marker expressed by these enzalutamide-resistant cells. Transcriptome profiling of these unique cell lines has indicated that they harbor a genetic signature that is associated with the differentiation and maintenance of myeloid cells from the hematopoietic compartment. For example, enzalutamide-resistant cells showed a marked up-regulation of genes that encode for the antigen presentation HLA complexes, which are cell surface markers and cytokines that are characteristic of myeloid cells, such as *FLT3/L*, *BDCA3*, *CD11B*, *IL12*, and *IL15*, as compared to CRPC controls (Fig. 1A). Moreover, these cells were characterized as overexpressing genes that encode transcription factors associated with the development of dendritic cells (DCs) and/or monocytes from common myeloid precursors, including interferon response elements (IRFs), inhibitor of differentiation factors (*ID1–ID3*), Ikaros (*IKZF1*), *BATF3*, and *RELB* (Fig. 1B). Importantly, the *PDL1* expression and myeloid markers presented in that study were only up-regulated in enzalutamide-resistant CRPC cells that showed inactivation of the classical AR pathway (Bishop *et al.* 2015), which suggests that as with TLR expression, the androgen axis may control checkpoint molecule expression in PCa.

The full extent of how myeloplastic cells affect PCa progression has yet to be defined; however, mounting

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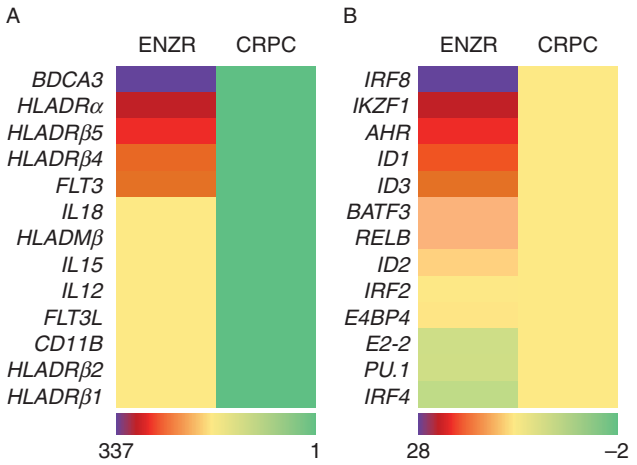

Figure 1
Myeloid differentiation signature in enzalutamide-resistant (ENZR) CRPC. Total RNA was isolated from ENZR or CRPC cells shown to have low AR activity (Bishop *et al*. 2015), and transcriptomic profiling using microarray was performed. Heat maps show that fold change in the gene expression of myeloid cell surface marker or cytokines (A) and the transcription factors required for myeloid cell differentiation (B) are up-regulated in ENZR cells as compared to CRPC (= 1).

evidence suggests that the expression of myeloid markers alters the way tumors respond to and manipulate their microenvironments. For example, beyond inhibiting T cell responses, tumor-intrinsic *PDL1*, which is up-regulated after chemotherapy or targeted therapy, can 'reverse signal' to prevent apoptosis (Azuma *et al*. 2008), thereby enhancing tumor survival. Reciprocally, *MYD88* expression in the spontaneous PCa TRAMP tumor model dictates the immune infiltrate into tumors by recruiting myeloid-derived suppressor cells and inhibiting NK cell populations, which promotes PIN and tumor progression (Peek *et al*. 2015). Manipulation of NK cell function has also been demonstrated in other studies, which indicate that the expression of the NK cell activating ligand *NKG2D* by PCa tumor-derived exosomes selectively down-regulates *NKG2D* expression on circulating NK and cytotoxic T cells; this prevents their antitumor activity *in vitro* (Lundholm *et al*. 2014). Our previous results also suggested a correlation between tumor-intrinsic myeloid marker expression and manipulation of the immune compartment, seeing as we found that only enzalutamide-resistant CRPC tumor cells that express *PDL1* were able to prevent DC infiltration into tumors and increase the frequency of circulating DC expression of *PDL1* and *PDL2* in vivo (Bishop *et al*. 2015). Again, this cross-talk between tumor and immune cell checkpoint expression may be dictated to some degree by AR activity, seeing as only enzalutamide-resistant tumors with low AR activity had these effects on circulating or tumor-infiltrating DCs (Bishop *et al*. 2015). We also found that patients who progressed on enzalutamide had significantly increased *PDL1/2*$^+$ DCs in their blood as compared to those who were naïve or responded to treatment, and in progressing patients, more *PDL1/2*$^+$ DCs were associated with a poorer initial response to enzalutamide and longer treatment duration (Bishop *et al*. 2015). Together, these studies suggest that further experiments in both patients and *in vivo* PCa tumor models should be conducted to evaluate how EMyT contributes to disease progression in the androgen-dependent, CRPC, and anti-androgen phases of PCa.

Tumor cell plasticity: neuroendocrine transdifferentiation

The correlation between altered AR expression and/or activity and a change in PCa tumor cell phenotype is best exemplified by the progression of PCa adenocarcinoma to neuroendocrine PCa (NEPC). Although the healthy prostate contains neuroendocrine cells, there is little scientific evidence to support the idea that these cells undergo transformation to give rise to NEPC (Terry & Beltran 2014). Instead, multiple studies have suggested that under the selective pressure of potent AR inhibition in late-stage CRPC, PCa adenocarcinoma 'transdifferentiates' to NEPC (Lin *et al*. 2014), which does not at all rely on AR for survival or proliferation. This transdifferentiation process is defined by a number of pathological and clinical features (Epstein *et al*. 2014) as well as molecular alterations that indicate the adenocarcinoma origin of NEPC, such as TMPRESS2–ERG rearrangements (Lapuk *et al*. 2012, Logothetis *et al*. 2013), the loss of AR and/or AR-regulated target genes, the loss of RB1, the amplification of *NMYC* and *AURKA*, and the induction of neural differentiation programs (Beltran *et al*. 2011, Logothetis *et al*. 2013, Park *et al*. 2014). Full reviews on NEPC can be found elsewhere (Tagawa 2014, Terry & Beltran 2014, Vlachostergios & Papandreou 2015); however, in the following sections, we outline key studies that show the relationship between the NEPC transdifferentiation process and AR.

AR regulation of NEPC

The rise in incidences of non-AR-driven CRPC, which may include up to 25% of patients with late-stage PCa and does include men with NEPC (Aparicio *et al*. 2011), underscores how AR activity dictates tumor cell plasticity at every stage

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of PCa progression. Indeed, multiple potential 'drivers' of NEPC have been identified, almost all of which precede, accompany, or are controlled by a loss of AR expression or activity. For example, although the AR is a major regulator of PCa cell proliferation, in NEPC models without AR expression, mitotic deregulation that leads to hyperproliferation occurs upon the loss of RB1 and cyclin D1, which suggests that the loss of this key tumor suppressor pathway may actually precede the AR loss that is observed in NEPC (Tzelepi *et al*. 2012). Indeed, up to 90% of NEPC tumors lack RB1 (Tan *et al*. 2014), which highlights the importance of cell cycle regulators in this disease. In addition, the mitotic phase kinase *AURKA* controls NEPC, which in turn is regulated by *NMYC* (Beltran *et al*. 2011) as well as REST (Svensson *et al*. 2014), both of which are inversely correlated with AR in PCa (Lapuk *et al*. 2012). Moreover, pathways that are known to also feedback on the AR, such as IL6 and cAMP signaling, can drive an NE phenotype in LnCaP cells (Cox *et al*. 2000, Spiotto & Chung 2000). Indeed, prolonged exposure to *IL6* can reduce AR expression (Debes *et al*. 2005), which further suggests that there is an important link between this cytokine and an AR-negative NEPC phenotype. In addition, a number of genes are up-regulated after ADT, and they are thus presumed to be androgen-suppressed and are associated with a progression to NEPC or non-AR-driven 'anaplastic' CRPC, including ARG2 (Kani *et al*. 2013), hASH-1 (Rapa *et al*. 2013) and protocadherins (Terry *et al*. 2013). Importantly however, although many studies have shown an inverse correlation between NEPC and AR expression or activity, no reports have indicated a direct mechanism by which a loss of AR actually drives this phenotype.

Tumor cell plasticity: overlap in aggressive cell phenotypes

Because the AR clearly plays a role in directing the CSC, EMT, EMyT, and NEPC phenotype and/or functions of PCa cells, it is not surprising that ADT induces the up-regulation of CSC, NEPC, and EMT markers simultaneously. Indeed, many reports in PCa as well as other cancers have shown a correlation between the expression of EMT and CSC markers within the same cells. For example, after androgen deprivation, both EMT and CSC populations have been shown to increase in mouse prostates and PCa cells (Scheel & Weinberg 2012). Likewise, PCa cells that were induced to an EMT phenotype, or CSCs that were isolated from PCa cell lines, have been shown to strongly up-regulate transcription factors expressed by CSCs and markers of EMT respectively, and they have been found to be highly tumorigenic in mice (Kong *et al*. 2010, Salvatori *et al*. 2012). In the aforementioned studies wherein ADT induced EMT, the authors also observed increases in CSC as well as NEPC characteristics (Sun *et al*. 2012). Reciprocally, LNCaP C-33 cells, which have an NE phenotype, express higher levels of the EMT transcription factor *SNAIL*, and the overexpression of *SNAIL* in LNCaP cells increases terminal markers of NE differentiation (McKeithen *et al*. 2010). Other studies have shown that AR splice variants simultaneously induce EMT and stem cell markers in PCa. In ADT culture conditions (CSS), AR and AR3 (ARV7) as well as the EMT/CSC markers *LIN28B*, *NANOG*, and *SOX2* and EMT markers, such as *ZEB1*, *TWIST*, *N-cadherin*, and *vimentin*, are overexpressed (Kong *et al*. 2015). The authors also indicated that AR3 expression was positively correlated with *LIN28* in PCa patient tumors and suggested that the AR3 inhibitor BR-DIM acts as a novel agent to inhibit AR, AR variants, and cancer stem cell markers in PCa (Kong *et al*. 2015). The overlap of CSC and EMT markers also occurs in mouse models of PCa: ADT has been shown to promote EMT and the expression of the CSC marker *Cd44* in castrated TRAMP mouse tumors. Seeing as a defining feature of stem cells is their pluripotent potential to give rise to other cell types, it is not surprising that the authors suggested that a switch from $CD44^{+}$ cells to EMT cells is the driver of metastasis in PCa; they showed that this switch occurs through a TGFβ1–CD44 signaling pathway (Shang *et al*. 2015). Future studies should further this work to determine whether CSCs are truly precursors or are required for the induction of EMT, EMyT, or NEPC phenotypes in PCa cells, especially under ADT conditions.

Like the close association between cells that undergo traditional EMT and CSCs, myeloplastic cancer cells may also share CSC and/or mesenchymal phenotypes. Although this concept has been less explored in PCa, examples have been found in other solid tumor types that may be relevant to PCa studies. For example, basal breast cancers, which are highly enriched for CSCs (Foulkes *et al*. 2010), have the highest expression of *PDL1* as compared to other breast cancer subtypes (Soliman *et al*. 2014), and 20% of triple negative breast cancer (TNBC) cases, of which about 80% are basal, express *PDL1* (Mittendorf *et al*. 2014). This suggests that enzalutamide-resistant tumor cells, in which *PDL1* is expressed, may have either CSC or EMT traits, and this would be in accordance with their low AR activity. Beyond the overlap between CSC and immune markers, EMyT in tumor cells may actually support mesenchymal or stem cell properties. For example,

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NKG2D expression in breast cancer has been shown to drive EMT and to support CSC maintenance via the transcription factor *SOX9* (Cai *et al*. 2014). Because *NKG2D* exists on PCa exosomes, an intriguing hypothesis may be that in addition to driving immune suppression, exosomal *NKG2D* supports tumor cell EMT and CSCs via a paracrine mechanism. Functionally, this strong overlap between CSC and myeloplastic phenotypes might dictate tumor progression by affecting how these tumors interact with the immune response. Murine metastatic lung cancer that was selectively passaged *in vivo* because of its ability to evade immune responses in vaccinated mice expressed high levels of CSC markers (Noh *et al*. 2012), and the pluripotency transcription factor *NANOG* is required to protect tumor cells from cytotoxic T cell death both *in vitro* and *in vivo* (Mao *et al*. 2014). Intriguingly, in one study, up to 50% of small-cell lung cancer patients mounted antigen-specific T cell responses to another stem cell transcription factor, *SOX2*, and these responses were associated with tumor regression after immunotherapy against *PD1* (Dhodapkar *et al*. 2013). In PCa, *SOX2* has also been identified as a tumor-associated antigen (TAA) (Shih *et al*. 2014). These studies seem to bring full circle the association between strong *PDL1* expression by solid tumor types and CSC properties, and they suggest that immune correlates of response to or progression with therapies may also benefit from investigating the CSC phenotype of tumors.

Targeting androgen response-driven cellular plasticity to improve patient outcomes

Suppressing AR signaling remains the focus of therapeutic strategies for advanced PCa, which is justified given the success of second-generation anti-androgens such as enzalutamide. Despite these advancements, many patients are impervious to further targeting of AR signaling, and none have been cured. This probably reflects the heterogeneity and plasticity of lethal prostate tumors, which are comprised of a mixed population of cells with varying degrees of AR expression. Notably, prostate CSCs probably have reduced AR signaling (Qin *et al*. 2012) and are believed to be intricately linked to EMT, EMyT, and metastasis (Mani *et al*. 2008). These observations support the process of co-targeting cellular plasticity as a rational therapeutic strategy for suppressing metastasis and CRPC. A list of potential agents is provided in Table 1.

Given the significant overlap between the plastic phenotypes of PCa tumor cells and the potential for CSCs to be the source of these phenotypes, targeting key molecules and signaling pathways that sustain prostate CSCs could lead to the development of new therapies that improve clinical disease management. One exciting preclinical study found that the inhibition of the hedgehog signaling pathway using the smoothened inhibitors cyclopamine and GDC-0449 depleted the CSC population and reduced CRPC xenograft growth (Domingo-Domenech *et al*. 2012). More recent work uncovered the therapeutic potential of inhibiting *MYC*, a transcription factor with a central function in the maintenance of CSCs and NEPC. Using a novel *in vivo* delivery system, Civenni *et al*. (2013) demonstrated that the systemic delivery of *Myc*-targeted siRNA to mice bearing PC-3 CRPC xenografts reduced the CSC population and suppressed tumor growth and metastasis. Although *MYC* inhibitor design has been difficult because of the absence of a clear ligand binding domain, BET inhibitors have been shown to reduce *MYC* expression in PCa models (Wyce *et al*. 2013) and have demonstrated astounding therapeutic efficacy in blocking CRPC tumor growth (Asangani *et al*. 2014). Alternatively, targeting *EZH2* has gained traction within the sphere of CSC-directed therapy. In LNCaP and PC-3 cells, *EZH2* was found to be up-regulated specifically within the $CD44^{+}/CD133^{+}$ CSC population (Sun *et al*. 2013), and DZNep, which induces the degradation of *EZH2*, eradicated the CSCs and attenuated DU145 CRPC tumor growth (Crea *et al*. 2011). GlaxoSmithKline is currently testing a highly specific *EZH2* inhibitor, GSK2816126, in a phase I clinical trial for relapsed/refractory lymphoma. Interestingly, their preclinical studies showed that glioblastoma stem cells responded well to this inhibitor (Kim *et al*. 2013), which suggests that it could be repositioned to eradicate prostate CSCs. Finally, *STAT3* inhibitors have also been shown to reduce CSC populations; galiellalactone was able to reduce *ALDH*-positive PCa cells (Baritaki *et al*. 2009, Hellsten *et al*. 2011), and another *STAT3* inhibitor, LLL12, has been shown to reduce the CSC phenotype in patient-derived castrate-resistant tumors (Kroon *et al*. 2013).

In addition to these small-molecule inhibitors of CSCs, a major avenue of exploration in other cancers that could translate to CRPC treatments is the use of immunotherapies to target CSC populations. For example, in glioblastoma, *CD133* has been shown to be a TAA (Ji *et al*. 2014), as has *SOX2* in small-cell (Gure *et al*. 2000, Vural *et al*. 2005, Shih *et al*. 2014) and non-small-cell (Dhodapkar *et al*. 2013) lung cancer. Antigen-specific T cells in both cases were able to induce CSC killing, which makes them strong potential vaccine candidates. In head and neck, breast, and pancreatic cancer, the transfer

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Table 1 Opportunities for therapeutic targeting of cellular plasticity

Plasticity factor	Drug	Mode of action	Clinical trials
Hedgehog	Cyclopamine	Directly binds to and inhibits the smoothened receptor to antagonize hedgehog signaling	Preclinical
	GDC-0449		Phase I/II combination study with hormone therapy in locally advanced PCa (NCT01163084) Phase I pharmacokinetic study in mCRPC (NCT02115828)
MYC	JQ1	Targets BET bromodomain proteins that are required for the transcriptional activation of *MYC*	Preclinical
	I-BET762 (GSK525762)		Phase I dose escalation study in acute leukemia (NCT01943851)
	TEN-010		Phase I dose escalation study in advanced solid tumors (NCT01987362)
EZH2	DZNep	Degrades PRC2 complex	Preclinical
	EI1	Inhibits histone methyltransferase activity	Preclinical
	EPZ-6438 (E7438)		Phase I/II combination study with standard chemotherapy in advanced solid tumors and B cell lymphoma (NCT01897571)
	GSK2816126 (GSK126 analogue)		Phase I dose escalation study in relapsed/refractory lymphoma malignancies (NCT02082977)
PKC/TWIST	Ro31-8220	Inhibits PKC	Preclinical
N-cadherin	ADH-1	Inhibits *N-cadherin*	Phase I study of Exherin (ADH-1) in advanced solid tumors. (NCT00265057)
STAT3	Galiellalactone or LLL12	Inhibits *STAT3* transcriptional activity	Preclinical
HSP27	OGX-427	Inhibits *HSP27*	Phase II study of OGX-427 in CRPC (NCT01120470)
	Silibinin	Induces MET	Preclinical
NF-κB/SNAIL/RKIP	NPI-0052	Inhibits the proteasome	Phase I study in advanced solid tumor malignancies or refractory lymphoma (NCT00396864)
Androgen receptor variant AR3	BR-DIM	Reduces the expression of AR3 and EMT markers	Phase I dose-escalation study of oral BioResponse 3,3′-Diindolylmethane (BR-DIM) in nmCRPC
ARV7	EPI-001	Blocks AR NTD transcriptional activity	Preclinical
Cadherin-11	mAbs 2C7 and 1A5	Monoclonal antibody that targets *cadherin-11*	Preclinical
	RSC (yeast)-Twist vaccine	Induces *TWIST*-specific cytoxic T cells	Preclinical
Brachyury	MVA-brachyury-TRICOM vaccine	Induces Brachyury-specific cytotoxic T cells	Phase I study in advanced tumors (prostate included) (NCT02179515)
PD1	CT-001	Monoclonal antibody that blocks *PD1* ligation	Phase II study of CT-001, Provenge, and cyclophosphamide in CRPC (NCT01420965)
PDL1	MSB0010718C	Monoclonal antibody that blocks *PDL1* ligation	Phase I study in CRPC (NCT01772004)
AURKA	MLN8237	Inhibits *AURKA*	Phase II study in mCRPC and NEPC (NCT01799278) Phase I study of Docetaxel in CRPC (NCT01094288)

of autologous CD8T cells specific for recognizing the stem cell enzyme *ALDHA1A* have also been shown to have antitumor efficacy. Seeing as *SOX2* has also been identified as a TAA in PCa (Shih *et al*. 2014), and seeing as PCa CSCs express both *CD44* and *ALDHA1A*, it may be that such immunotherapies could work against PCa CSCs as well.

A major caveat of these therapeutic targets is that they are not 'pure' CSC factors; rather, they are shared with

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normal stem cells. Therefore, a more optimal strategy might be to exploit the fact that prostate CSCs express unique cell surface markers, such as *CD44* and *CD133*, by targeting drugs specifically to these cells. *CD44*-targeted nanoparticles carrying *MDR1* siRNA have demonstrated efficacy in sensitizing ovarian cancer cells, as well as the CSCs, to paclitaxel *in vivo* (Yang *et al*. 2015). Nanoparticles are also capable of delivering anticancer therapeutics to CSCs; for example, *CD133*-coated nanoparticles carrying paclitaxel significantly reduced the CSC burden and lowered the rate of tumor relapse in a breast cancer xenograft model (Swaminathan *et al*. 2013). Although this technology is still in its infancy, it shows great promise for tumor cell-specific targeting.

Another approach to mitigating the adverse side effects of CSC targeting is to inhibit other aspects of CRPC tumor cell plasticity, such as EMT, NEPC, or immune evasion, more directly. Targeting EMT markers such as β-catenin, fibronectin, cadherin-11, or vimentin has been proposed as a potential strategy for reducing CRPC cell viability. For example, the therapeutic targeting of *N-cadherin* with a MAB has been shown to successfully delay PCa progression by reducing PC-3 and castrate-resistant LAPC4 tumor xenograft growth and invasion (Tanaka *et al*. 2010). In addition, targeting the mesenchymal marker cadherin-11 with an antibody was recently reported to reduce bone metastases in a PC3-mm2 xenograft model (Lee *et al*. 2013*b*). In addition, proteins and signaling pathways that control EMT have also been suggested to be potential drug targets in PCa cells that have EMT phenotype. For example, the proteasome inhibitor NPI-0052 is able to inhibit the NF-κB/SNAIL/RKIP pathway in metastatic PCa (Baritaki *et al*. 2009). In addition, the Hsp27 inhibitor OGX-427, which is

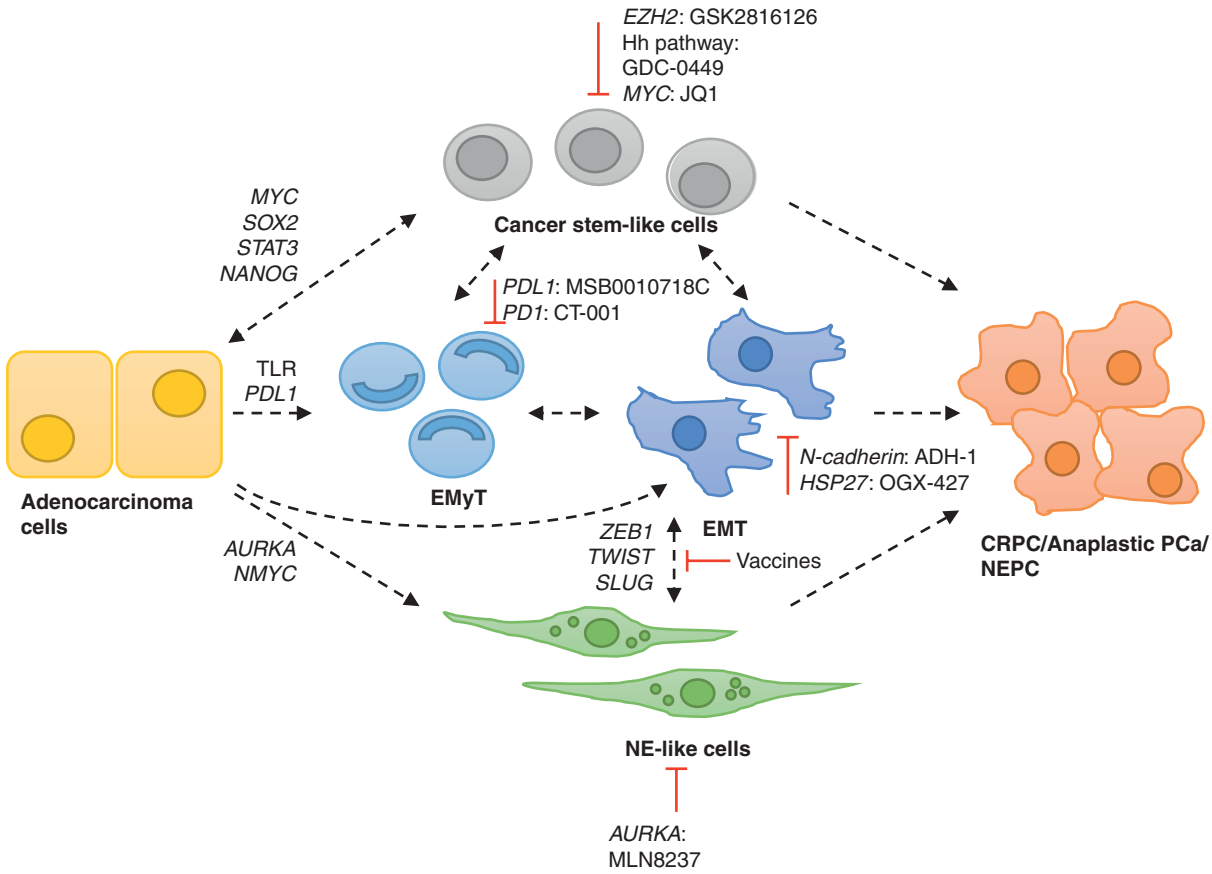

Figure 2

Targeting tumor cell plasticity in prostate cancer. Under the selective pressure of androgen deprivation, adenocarinoma of the prostate may involve heterogeneous populations of tumor cells, including cancer stem-like cells, cells undergoing epithelial-to-mesenchymal (EMT) or myeloid transition (EMyT), and neuroendocrine-like (NE) like cells. These cell types are interrelated, and the phenotypes may be dynamic, thereby underlying the phenotypic plasticity of tumor cells that have become therapy resistant. Strong evidence suggests that each cell type or plasticity between cell types contributes to prostate cancer progression to CRPC or anaplastic/NEPC. Therapies that target the mechanistic drivers of cellular plasticity and are currently under preclinical and clinical development are highlighted.

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currently in clinical trials, may be a potential means of targeting the β-catenin/SLUG and STAT3/TWIST signaling pathways in PCa, and it could thus inhibit EMT (Shiota *et al*. 2013, Cordonnier *et al*. 2015). *TWIST* can also be targeted using the *PKC* inhibitor Ro31-8220, which reduces *TWIST* signaling and the viability of LNCaP and castration-resistant C4-2 and 22Rv1 cells in combination with anti-androgen enzalutamide (Shiota *et al*. 2014). Interestingly, *TWIST* may also be a viable immunotherapy target; recently *TWIST* was shown to be a TAA in the TRAMP-C2 subcutaneous model of murine PCa, and combined treatment of TRAMP-C2 xenografts *in vivo* with enzalutamide and a *TWIST*-specific vaccine significantly reduced tumor burden (Ardiani *et al*. 2013). Similarly, the T-box transcription factor Brachyury, which drives EMT in many cancers (Fernando *et al*. 2010), has been identified as another PCa TAA, and yeast-based Brachyury vaccines are currently in development for the treatment of CRPC (Hamilton *et al*. 2013). In addition, targeted therapies are in development to prevent identified drivers of NEPC and checkpoint molecule expression by tumors. These include aurora kinase and somatostatin inhibitors as well as *PDL1* and *PD1* monoclonal antibodies. Table 1 presents a list of agents that target cellular plasticity in PCa and other tumor types.

Although we have divided these agents by their ability to modulate a particular plasticity state, like CSCs, cells undergoing EMT, or NEPC, it is clear from the ubiquitous signaling pathways they affect that these inhibitors most likely have the benefit of targeting multiple cell populations. This is underscored by the significant overlap between plastic CRPC cell phenotypes and the intimate relationships between the AR-controlled plasticity pathways. For example, the importance of ARV7 in driving therapy resistance has recently been reported (Antonarakis *et al*. 2014); thus, targeting variants with novel therapies such as Epi-001 (Myung *et al*. 2013, Martin *et al*. 2014) and BR-DIM have gained considerable momentum. Although the goal of these therapies would be to prevent ligand-independent AR activity, which drives the growth and proliferation of AR variant-expressing tumors, the ability of AR variants to drive EMT may suggest that these inhibitors can prevent metastases or reduce CSCs. Indeed, BR-DIM has been shown to inhibit both AR variants and stem cell and EMT markers in PCa (Kong *et al*. 2015). In addition, although the use of bromodomain inhibitors, such as JQ1, clearly have shown efficacy in reducing AR activity (Asangani *et al*. 2014), the close association between BRD4 and the genes that control stem cell properties and NEPC, such as *MYC* (Di Micco *et al*. 2014, Rodriguez *et al*. 2014), may mean that bromodomain inhibitors could simultaneously prevent AR signaling and the emergence of cell types that are associated with a loss of AR activity. Importantly however, we are unaware of any potential adverse side effects that targeting multiple tumor cell populations may have, such as putting strong selective pressure on various signaling pathways, which could lead to emergent mechanisms of resistance.

Conclusion

Overall, heterogeneous cell populations in tumors dictate the severity of disease by playing different roles in response to anticancer therapies, regeneration and proliferation, metastasis, and immune modulation. In PCa, AR expression and activity regulates CSCs, EMT, EmyT, and transdifferentiation to NEPC, and thus novel AR targeting agents may inhibit AR functioning as a central driver of tumor cell phenotypic plasticity. Furthermore, combined approaches that target the AR as well as individual CSC, NEPC, EMT, or immune evasion modulators will most certainly play a role in the treatment landscape of CRPC in the future. With the mounting evidence about the molecular mechanisms that underlie how different cell types emerge during CRPC and therapeutic resistance, and with the significant number of novel agents directed against these mechanisms that are currently in preclinical and clinical phases of development, we may be able to eliminate unique and aggressive PCa tumor cells to prolong survival in CRPC patients (Fig. 2).

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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