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Cellular rewiring in lethal prostate cancer: the architect of drug resistance

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Abstract

Over the past 5 years, the advent of combination therapeutic strategies has substantially reshaped the clinical management of patients with advanced prostate cancer. However, most of these combination regimens were developed empirically and, despite offering survival benefits, are not enough to halt disease progression. Thus, the development of effective therapeutic strategies that target the mechanisms involved in the acquisition of drug resistance and improve clinical trial design are an unmet clinical need. In this context, we hypothesize that the tumour engineers a dynamic response through the process of cellular rewiring, in which it adapts to the therapy used and develops mechanisms of drug resistance via downstream signalling of key regulatory cascades such as the androgen receptor, PI3K–AKT or GATA2-dependent pathways, as well as initiation of biological processes to revert tumour cells to undifferentiated aggressive states via phenotype switching towards a neuroendocrine phenotype or acquisition of stem-like properties. These dynamic responses are specific for each patient and could be responsible for treatment failure despite multi-target approaches. Understanding the common stages of these cellular rewiring mechanisms to gain a new perspective on the molecular underpinnings of drug resistance might help formulate novel combination therapeutic regimens.

Targeted therapies such as immunotherapy or inhibition of multiple signalling pathways will remain an important component of cancer treatment. Following decades of research, our perspective on cancer cell biology has evolved from a generalist approach based on the histological appearance of the tumour towards increasingly complex genomic and molecular profiles that dictate the management of patients with cancer. Unfortunately, patients who

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Competing interests

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initially benefit from these therapies commonly progress to refractory states owing to the phenomenon of cellular rewiring — regulation of signalling cascades involved in cell growth, proliferation and survival, and switching towards an undifferentiated phenotype — which ultimately enables cancer cells to evade death and survive despite drug exposure.

This phenomenon is particularly seen in prostate cancer, partly owing to specific tumour characteristics but also as a result of current therapy regimens. In this context, advanced prostate cancer frequently evolves into castration-resistant and chemotherapy-resistant stages. At present, therapeutic modalities for prostate cancer initially include androgen deprivation therapy (ADT) that consists of either chemical castration using gonadotropin-releasing hormones or surgical castration via bilateral orchiectomy. Once prostate cancer progresses to become castration-resistant prostate cancer (CRPC), treatments are mainly focused on androgen receptor (AR) inhibition and/or taxane chemotherapy, among other therapeutic approaches including radium-223-mediated α-emission[1] or active cellular immunotherapy using sipuleucel-T[2] (FIG. 1). Anti-androgen therapy is based on the use of second-generation anti-androgens, such as enzalutamide[3,4], apalutamide[5] and darolutamide[6], and inhibitors of androgen biosynthesis such as abiraterone[7–10]. First-generation and second-generation antimitotic taxane agents such as docetaxel[11,12] and cabazitaxel[13], which block cell cycle progression from metaphase to anaphase by binding and stabilizing β-tubulin, have been shown to improve survival in patients with prostate cancer and have become mainstays of treatment[11,12]. However, despite these therapeutic efforts, prostate cancer invariably progresses towards a castration-resistant and taxane-resistant phenotype, meaning that novel therapeutics are still required. Although the acquisition of resistance to a targeted therapy such as anti-androgens mainly arises via cellular rewiring, resistance can also be explained by the clonal evolution model, in which cells harbour specific molecular alterations and the fittest cells are selected by therapy.

Combination therapies enabling targeting of multiple different molecular pathways are a widely used approach to overcoming drug resistance but in many cases fail to do so because of cellular rewiring mechanisms. Historical studies from the 1960s and early 1970s demonstrated that cancers with a poor prognosis such as acute lymphocytic leukaemia and Hodgkin lymphoma became curable when combination therapy with three or more agents was administered[14]. However, despite our increased understanding of the mechanisms of drug resistance, the majority of current combination regimens have been developed empirically, without taking into account the particular mechanisms of cellular rewiring that are involved in drug resistance.

The advantages of combination therapy in prostate cancer are well known (FIG. 1), and the combination of taxanes or anti-androgen agents with ADT in the context of hormone-sensitive disease at the time of initial metastasis have revolutionized the management of patients with prostate cancer. Indeed, large randomized studies testing the addition of docetaxel (the CHAARTED and STAMPEDE trials), abiraterone plus prednisone (the LATITUDE and STAMPEDE trials) or enzalutamide (the ARCHES trial) to standard ADT in patients with metastatic, hormone-sensitive prostate cancer have shown striking overall survival (OS) benefits[15–19]. Taken together, these studies have demonstrated that combined, rather than sequential, therapy is more beneficial in patients with prostate cancer and that

tumours that are treated early — when they are less aggressive and heterogeneous — respond better and for longer to these combined therapeutic strategies than those that are treated after they have evolved into a castration-resistant stage.

This new evidence, albeit empirical, indicates the need to develop studies that take into account the process of cellular rewiring, for example, through the description of distinct tumour profiles that comprise the prostate cancer landscape, in order to identify the combination therapy that best fits the needs of each patient. In this context, clinical and biomarker studies are warranted to establish personalized therapeutic approaches for a patient's first-line treatment for prostate cancer and to understand how the initial therapeutic approach affects the clinical benefit from second-line therapies. Moreover, targeting the mechanisms involved in the development of acquired resistance might abrogate disease progression and ensure prolonged survival. To this end, understanding the molecular mechanisms that drive drug resistance is crucial to the better design of combined targeted therapies.

General mechanisms of drug resistance

Mechanisms of general drug resistance are unspecific and are often mediated by the impaired delivery of the drug to the target cancer cell. Membrane-bound efflux proteins, especially ATP-binding cassette (ABC) transporters such as MDR1 (also known as ABCB1 or P-glycoprotein), are able to pump therapeutic agents out of the cell[20]. This mechanism is especially relevant in the setting of prostate cancer, as docetaxel has high affinity for the transporter P-glycoprotein, accounting for both a primary and an acquired mechanism of resistance, which results in a decrease in the intracellular concentration of this agent in cancer cells[21–23]. In accordance with these findings, the docetaxel-resistant prostate cancer cell lines DU-145-R and 22RV1-R express higher levels of P-glycoprotein than their docetaxel-sensitive counterparts, and P-glycoprotein inhibition can be sufficient to overcome their resistant status[24–26]. Another mechanism of impaired drug delivery could be an increased interstitial fluid pressure that results from vasculature leakages or alterations in the lymphatic drainage system[27]. Increased interstitial fluid pressure acts as a barrier against transcapillary transport and, therefore, underlies impaired uptake of therapeutic agents, an effect seen in many solid tumours including prostate cancer[27]. Related to this process, heterogeneity in the tumour microenvironment can lead to regions of hypoxia and acidity, which influence the response of the tumour cells to therapy[28,29]. A notable example of this response is hypoxia-inducible factor 1α (HIF1α), which under normoxic conditions is degraded via its interaction with the von Hippel-Lindau protein (pVHL). However, under hypoxic conditions, HIF1α is induced and overexpressed[30]. Overexpression of HIF1α also occurs in human cancers including CRPC, where it regulates the abilities of cell proliferation and invasion[30]. HIF1α induces sphingosine kinase 1 (SK1), which is implicated in chemoresistance. Everolimus, an mTOR inhibitor, decreases SK1 and HIF1α mRNA and resensitizes cancer cells to docetaxel[31]. Another study has shown that inhibition of TR4 nuclear receptor using an antagonist, bexarotene, also resensitizes CRPC cells to docetaxel via inhibition of HIF1α-mediated signalling[32]. Finally, combined targeting of the AR using enzalutamide and HIF1α using chemotin, a disruptor of HIF1α–p300 interactions, in prostate cancer cell lines LNCaP and 22Rv1 resulted in synergistic inhibition of CRPC[33].

In summary, induction of HIF1α decreases the efficacy of chemotherapy and ADT, whereas its inhibition resensitizes cells to conventional therapy, providing another valuable target for the treatment of prostate cancer.

Cellular rewiring in drug resistance

Cellular rewiring is an additional mechanism that can be exploited by prostate cancer cells to acquire drug resistance by enabling alternative bypass signalling pathways after therapy exposure and, therefore, allowing continued tumour proliferation and survival. Whole-exome and whole-transcriptome sequencing have been used to analyse the mutational landscape of CRPC, and have shown that the vast majority of patients with metastatic CRPC (mCRPC) show molecular aberrations in key genes such as *AR*, *ETS*, *TP53* and *PTEN*. Indeed, *AR* amplification, ETS gene family fusions and *PTEN* loss are well-characterized molecular alterations in prostate cancer[34]. A plethora of downstream signalling pathways have been described that might drive and sustain drug resistance based on the alteration of these key regulators. The cellular rewiring that takes place in this setting is likely to be an adaptive mechanism, regulated by the activation of master regulator transcription factors that act as oncogenic drivers. Many of these signaling pathways are clinically relevant and offer potential therapeutic targets that could be used to counter the effects of cellular rewiring (FIG. 2).

AR signalling cascades

The AR is one of the most well-characterized drivers of prostate cancer (FIG. 2). Despite being initially bound to the cytoplasm by heat-shock proteins such as HSP90 (REF.[35]), binding with dihydrotestosterone (DHT) induces a conformational change that enables the AR to dimerize and translocate to the nucleus, where it binds with androgen response elements (AREs)[35]. The signalling cascade triggered by the AR has been extensively studied, and its persistent activation even after anti-androgen therapy is a prime example of adaptive drug resistance.

AR signalling is sustained in CRPC by several mechanisms that include AR overexpression[36], which can arise via gene amplification[37] or owing to reduced turnover and increased stability of the AR[38]. Furthermore, structural changes in the AR induced by mutations can reduce the native sensitivity of the AR to DHT and enable its activation by other binding elements. For example, a single point mutation changing the sense of codon 868 (Thr to Ala) in the ligand-binding domain of the AR allows binding of androgens, progestagens, oestrogens and anti-androgens and, therefore, activates AR-dependent gene expression[39]. Apart from this, mutations in the AR can also alter interactions with its co-regulators. For example, the T877A variant, which is found at a high frequency in prostate cancer, is less responsive to repression by co-repressor NCoR1 and responds better to potentiation by co-activator steroid receptor co-activator 1 (SRC1)[40].

AR splice variants.—The creation of AR splice variants after genomic rearrangements and/or aberrant alternative mRNA splicing is another well-characterized mechanism of drug resistance[41] (FIG. 2). These variants lack the ligand-binding domain found in the full-length AR (AR-FL) and are, therefore, constitutively active in a ligand-independent environment[42].

Moreover, splicing variants have different taxane-binding domains from AR-FL and, therefore, interact in a unique way with the microtubule network that is responsible for the nuclear translocation of the AR, which can potentially impair taxane efficacy[41]. An increase in androgen receptor variant 7 (AR-V7), the most well-studied variant, has been associated with disease progression and is frequently observed after the use of second-generation anti-androgens such as enzalutamide and abiraterone[43–45]. In a cohort of patients with mCRPC who were treated with enzalutamide ($n = 31$) or abiraterone ($n = 31$), detectable AR-V7 in circulating tumour cells was found in 39% and 19% of the patients, respectively. The presence of AR-V7 is rare in primary disease and its absence correlates with improved prognosis and response to anti-androgen therapy[46].

The importance of AR splice variants in conferring drug resistance has prompted interest in inhibiting their activity or expression. These novel targeting opportunities are under investigation in several clinical trials and include interfering with AR variant co-activators or targeting the N-terminal domain (NTD) or DNA-binding domain of the AR, which are retained by most AR variants[47]. In 2018, EPI-506, an AR NTD inhibitor, was tested in a phase I study (NCT02606123)[48] in men with CRPC that had progressed on enzalutamide or abiraterone. However, the trial was discontinued after showing minor PSA declines due to high pill burden. Most therapeutic efforts have focused on the suppression of AR-V7, for example through the development of agents that degrade this splice variant, such as niclosamide, an anti-helminthic drug that acts as a potent AR-V7 inhibitor in prostate cancer cells by causing protein degradation via a proteasome-dependent pathway[49,50]. Niclosamide is being studied in several clinical trials, which include a phase II study of abiraterone plus niclosamide (NCT02807805)[51] and a phase I trial of niclosamide plus enzalutamide (NCT03123978)[52] in recurrent CRPC or mCRPC.

AR-V7 is created by aberrant pre-mRNA splicing, which is also important in the generation of other oncogenic protein products such as truncated BRAF-V600E splice variants that confer vemurafenib resistance in melanoma[53]. Thus, another strategy to reduce resistance could be direct targeting of this mechanism using spliceosome inhibitors such as thailanstatins — anti-AR-V7 molecules that inhibit AR splicing by interfering in the interaction between U2AF65 and SAP155 (REF.[54]) — or using small-molecule inhibitors that target the transactive domain of AR-Vs, such as the small molecule peptidomimetic D2 (REFS[55,56]).

Glucocorticoid receptor upregulation.—AR signalling can also be sustained via upregulation of the glucocorticoid receptor (GR) (FIG. 2). After treatment with enzalutamide, GR upregulation has been linked to the activation of a subset of AR target genes and a poor response to this drug[57,58]. The transcriptomes of AR and GR overlap — >50% of their target genes can be regulated by both receptors[57,59]. In prostate cancer progression, ligand-activated AR binds to negative AREs present at the GR promoter, directly repressing GR expression[59]. Thus, in the context of androgen signalling suppression after enzalutamide treatment, the AR is functionally replaced by the GR and is, therefore, overexpressed in AR-deficient preclinical models[60,61]. This mechanism of adaptive resistance offers interesting targeting opportunities. For example, an ongoing clinical trial

(NCT02012296)[62] is investigating the effects of therapy with RU-486, a competitive GR antagonist, in combination with enzalutamide.

GR expression can also be inhibited by the bromodomain and extra-terminal motif protein (BET) inhibitor JQ1, which restored sensitivity to enzalutamide in a subset of patients with CRPC with acquired resistance to enzalutamide. BET family proteins bind to acetylated lysine motifs at enhancers and help drive the expression of key tissue-specific genes, including the GR. JQ1 was first identified as a BET inhibitor as it disrupted the binding between bromodomain-containing 4 (BRD4), which belongs to the BET family class of chromatin readers, and the NTD of the AR[63]. BRD4 binding had already been the focus of studies that demonstrated that the use of BET inhibitors such as JQ1 could abrogate downstream AR signalling and reduce tumour size in mouse xenograft models of prostate cancer[64]. Thus, novel BET degraders, which target degraded BET proteins via proteasomal action, could become useful in the treatment of lethal mCRPC[65]. However, no clinical studies have yet confirmed the benefits of BET inhibitors in the treatment of patients with CRPC. Moreover, if BET inhibition (which inhibits GR expression) is potentially beneficial in prostate cancer, these data[66] would suggest that corticosteroids could, in fact, be detrimental in patients with prostate cancer and could contribute to tumour progression[57]. This effect seems counterintuitive, as corticosteroids are used extensively in the treatment of prostate cancer[60], owing to their dual action of adrenocorticotrophic hormone (ACTH) suppression, which diminishes the expression of adrenal androgens, and immunosuppressive effects when administered concomitantly with chemotherapy[60]. The use of corticosteroids is controversial, as a 2012 phase III clinical trial that demonstrated an improvement in OS with enzalutamide therapy also showed that patients treated with corticosteroids had a worse survival[4]. Thus, further studies are warranted to elucidate their purpose in the treatment of advanced CRPC.

Rewiring the AR.—AR signalling is sustained beyond castration by multiple mechanisms. These mechanisms can be AR dependent, such as AR overexpression and the synthesis of aberrant AR variants, or AR independent, such as GR overexpression[67]. These findings support the hypothesis that AR-mediated drug resistance mechanisms emerge when prostate cancer cells rewire their signalling to adapt to androgen depletion, transitioning from androgen dependence to an AR-independent state. Moreover, many of these mechanisms are likely to coexist, given the heterogeneous pattern of AR-V7 and AR-FL expression, which indicate that AR-V7$^+$ and AR-FL$^+$ cells simultaneously persist with AR$^-$ cells in small-cell prostate cancer[68,69].

Data showing that AR-V7 antagonizes the AR transcriptional programme[70] support the hypothesis that AR splice variants can contribute to phenotype switching towards a more undifferentiated state. This hypothesis has been confirmed by the observation that AR splice variants induce epithelial-mesenchymal transition (EMT) and the expression of stemness markers[71], and by data showing a correlation between AR degradation using the steroidal anti-androgen galeterone and decreased EMT and stem cell markers in cell line models[72–74]. Despite these promising results, galeterone use failed to improve OS compared with enzalutamide use in patients with AR-V7$^+$ prostate cancer (NCT02438007)[75]. The established link between these drug resistance mechanisms and phenotype switching

indicates that cell plasticity and adaptive drug resistance mechanisms are likely to act in concert in the rewiring of the prostate cancer cell.

PI3K–AKT–MAPK signalling cascades

Alterations in the PI3K–AKT signalling pathways (FIG. 2) are very frequent; they can be found in 40% of primary prostate tumours and in up to 70% of metastatic prostate cancers, and are frequently the result of *PTEN* loss[76–78]. Indeed, *PTEN* loss has been linked to resistance to castration[79]; however, loss of *PTEN* alone seems to be insufficient to cause castration resistance, as the LNCaP and LAPC9 cell lines, which do not express *PTEN*, are sensitive to ADT[80,81]. These findings suggest that inhibition of PI3K signalling in PTEN-deficient prostate cancer cells could be of clinical interest. Unfortunately, studies using mTOR inhibitors in a *PTEN*-depleted setting and for the treatment of CRPC have been negative (NCT00629525)[82,83]. This lack of effect might be owing to the fact that mTOR inhibition with rapamycin leads to a negative-feedback loop that upregulates AKT and MAPK[84,85]. AKT phosphorylation is increased during resistance to castration, as indicated by studies in LNCaP cells and in Nkx3 *Pten*-mutant mice as well as in clinical post-prostatectomy specimens[86–88]. Similarly, MAPK signalling pathways are activated in combination with AKT in advanced prostate cancer and jointly contribute to tumour growth and drug resistance[89]. Related to this, a 2019 study discovered that the atypical chemokine receptor CXCR7 is one of the most upregulated genes in enzalutamide-resistant prostate cancer cells and that it acts by stimulating MAPK-ERK activation via interaction with β-arrestin 2 (ARRB2)[90]. CXCR7 is a membrane protein that is internalized when activated and then forms a complex with ARRB2, which acts as a scaffold protein for MAPK protein assembly and activation. Targeting this axis could prove beneficial, but combined targeting of these pathways might be necessary for an effective antitumoural response, as has already been demonstrated with dual inhibition of PI3K–AKT using perifosine and MEK–ERK pathways using trametinib in diffuse intrinsic pontine glioma cells[91].

The association between *PTEN* loss and resistance to castration indicates a possible cross-resistance mechanism between the PI3K and AR signalling cascades[79]. Interestingly, pharmacological inhibition of PI3K or AKT increases AR protein expression by activating a subset of AR-related genes through a mechanism dependent on HER3 (REF.[86]). Clinical interest in the functional interplay between these signalling pathways has led to the development of several ongoing trials to explore the combination of AR and PI3K inhibition. A phase II study[92] combining treatment with the small-molecule AKT inhibitor ipatasertib and abiraterone has shown a dose-dependent improvement in OS in patients with docetaxel-resistant mCRPC, especially in those with PTEN-deficient tumours, without reaching statistically significant levels ($P = 0.22$ versus placebo in patients treated with 400 mg ipatasertib). These results led to an ongoing phase III study (NCT03072238)[93], which is currently active and is expected to be completed by 2023.

Interestingly, in a 2019 phase II study (NCT01331083)[94] in unselected patients with mCRPC with and without *PTEN* loss who had progressive disease despite treatment with abiraterone plus prednisone, addition of a pan-isoform inhibitor of PI3K (PX-866) did not lead to any antitumoural activity[95]. These results indicate that, despite the potential benefits

of PI3K and AR combined inhibition, selecting patients with PTEN-deficient tumours and starting PI3K inhibition in early stages of disease could prove more beneficial than using PI3K inhibitors in unselected patients or in advanced stages of disease. Indeed, a separate phase II study (NCT00814788) demonstrated a significant PSA decrease in men with bicalutamide-naive CRPC who were treated with bicalutamide plus the mTOR inhibitor everolimus[96]. These results are in direct contrast to those of a previous phase II trial, which found that adding everolimus to bicalutamide in men with bicalutamide-resistant CRPC was ineffective[97], which could mean that inhibiting the PI3K–MAPK pathway in patients with androgen-dependent tumours could be more useful than in patients with androgen-resistant tumours. Additional combination therapies are currently under study, such as the combination of a novel AR-signalling inhibitor with everolimus (NCT02106507)[98] and the combination of enzalutamide with a novel mTOR kinase inhibitor (CC-115) (NCT02833883)[99].

In summary, the molecular interplay between the PI3K and AR signalling pathways justifies combined inhibition of PI3K and AR to potentially overcome androgen resistance in CRPC, but the timing and selection of patients remain important topics to address in future studies.

The GATA2-dependent signalling network

The transcription factor GATA2 is a master regulator that mediates aggressiveness throughout all stages of prostate cancer (FIG. 2). In AR-expressing prostate cancer, GATA2 acts as a pioneer transcription factor that drives androgen-responsive gene expression through a three-tiered role: first, by binding to AREs in response to androgens; second, by physically enabling chromatin activation in the AR enhancer elements; and finally, by recruiting the MED1 complex, which is necessary for building and maintaining chromatin regulatory loops between *AR* distal enhancers and *AR* promoters[100,101]. Moreover, GATA2 further contributes to AR function by enabling its binding to *PSA*, *TMPRSS2* and *PDE9A* enhancers[102]. Owing to this multifunctionality, GATA2 has been suggested as a critical factor in the theoretical hierarchical regulation network that drives androgen-dependent *AR* expression[102]. Altered AR signalling is the most prominent mechanism of growth and progression of prostate cancer cells throughout the castration-resistant stage[103], and GATA2 directly contributes to CRPC progression by maintaining AR signalling and transcriptional activity[104]. Consequently, GATA2 silencing correlates with a decrease in AR gene and protein expression[105], and knockdown of *GATA2* expression resensitizes chemotherapy-resistant cells to docetaxel and cabazitaxel[106].

As a master regulator, GATA2 overexpression has been linked to resistance to chemotherapy and progression to lethal stages of disease through regulation of a set of critical cancer-related genes, including *IGF2* and *POM121* (REF.[107]). GATA2 regulates *IGF2* expression through direct binding with its promoters, and *IGF2* expression is increased in line with prostate cancer progression, particularly in androgen-independent tumours[106]. Insulin-like growth factor 2 (IGF2) exhibits structural homology with insulin and can activate both the insulin-like growth factor 1 receptor (IGFR1) and insulin receptor (INSR), which, in turn, activate PI3K and MAPK4 signalling, as well as other downstream effectors such as AKT, JNK, ERK1 and ERK2, and p38 (REF.[108]). Inhibition of IGFR1 in combination with

castration and docetaxel has been proposed as an effective combined therapy in in vivo studies using LuCaP35V tumours in surgically castrated mice[109], but inhibition of the IGFR1 axis in clinical trials has yet to be proven as an effective strategy. Neither the combination of ADT and cixutumumab (a human monoclonal antibody that targets IGFR1) in a phase II study in men with metastatic hormone-sensitive prostate cancer (NCT00313781) nor the combination of figitumumab (which also targets IGFR1) and docetaxel in a phase II study in men with CRPC (NCT01120236)[110,111] has yielded statistically significant results, which can be partially attributed to compensatory signalling through the INSR[112]. In this context, dual inhibition of IGFR1 and INSR is an attractive option and the use of OSI-906, a selective and orally bioavailable dual IGFR1 and INSR kinase inhibitor, was shown to increase response to taxane therapy and OS in xenograft mouse models using patient-derived lethal prostate cancer and 22Rv1-DR cells[106].

POM121, another key downstream element of GATA2, is a nucleoporin with important roles for the structural conformation of the nuclear pore complex[113]. Aberrant nucleocytoplasmic transport has long been linked to cancer[114], and several nucleoporins have been identified as kinase drivers or regulators of gene and chromatin expression[115,116]. Indeed, POM121 has been identified as a key regulator of the nucleocytoplasmic transport of the oncogenic factors MYC and E2F1 and of the prostate-specific growth transcription factors AR and GATA2 in aggressive prostate cancer via its interaction with importin-β[117]. Importantly, combination therapy of docetaxel and mitoxantrone plus importazole, a pharmacological inhibitor of the POM121-importin-β axis[118] resensitizes tumour cells to chemotherapy in mice bearing 22Rv1-DR xenografts and patient-derived lethal prostate cancer cells. Thus, GATA2 inhibition could be a valuable therapeutic approach for prostate cancer at both the CRPC and taxane-resistant stage.

Other signalling cascades

Many other downstream molecular pathways have been suggested to influence resistance to taxanes and hormonal therapies. For example, signal transducer and activator of transcription 1 (STAT1), a transcription factor that translocates from the cytoplasm to the nucleus of the cell and mediates several crucial cell processes[119], is overexpressed in a DU145 docetaxel-resistant cell line[120]. Elevated STAT1 levels correlate with an increase in clusterin, which is an anti-apoptotic protein that defends the cell from the pro-apoptotic triggers induced by docetaxel[121], which suggests that a STAT1–clusterin-dependent mechanism might mediate docetaxel resistance. Congruently, antisense knockdown of this protein with custirsen (OGX-011) correlates with chemoresistance reversal in prostate cancer cells[122]. However, a 2017 phase III clinical trial (NCT01188187) failed to demonstrate positive results derived from this inhibition[123]. The results of this clinical trial were unexpected, as custirsen had demonstrated positive results in a previous phase II study and could be explained by poor selection and timing of the patients included in the study.

Moreover, docetaxel is able to activate STAT phosphorylation, which in turn activates PIM1 and improves survival of docetaxel-treated prostate cancer cells in a mice model mainly via NF-κB activation[124]. This mechanism is yet another example of how the tumour cells

dynamically adapt to therapy exposure, in this case activating the STAT3–PIM1–NF-κB stress pathway in response to the use of docetaxel.

Other studies have also confirmed a central role of NF-κB and have shown that its inhibition could be beneficial in overcoming docetaxel resistance. In particular, IL-6, an NF-κB target, has been shown to be increased in tumours that were resistant to docetaxel and inhibition of NF-κB by PS-1145 (an IKK2 inhibitor) decreased IL-6 production and resensitized prostate cancer cells to docetaxel[125,126]. Other examples of potential therapeutic targets are the transcription factors Twist1 and Y-box binding protein (YB1), which have been associated with increased taxane resistance in CRPC cells[127,128] and are also associated with increased AR expression in castration-resistant LNCaP cells[129].

A number of other pathways also seem to be involved in signalling in prostate cancer. For example, the retinoic acid receptor-related orphan receptor-γ (RORγ) is overexpressed and amplified in metastatic CRPC tumours, and drives AR expression[130]. Treatment with RORγ antagonists, in particular compound XY018, which was developed by combining the structural features of RORγ-specific antagonists SR221 and GSK805, suppressed tumour growth in AR-expressing prostate cancer cell lines such as 22Rv1 and showed increased efficacy when combined with enzalutamide[130]. HER2 (also known as ERBB2; a member of the epidermal growth factor receptor (EGFR) family) is a classic regulator of cell growth and proliferation, in particular in the progression of prostate cancer in patients with low androgen levels[131]; HER2 activates several downstream signalling pathways, including MEK, ERK and PI3K–AKT[131]. Indeed, it has been found that both HER2 and EGFR1 (also known as HER1) levels increase as prostate cancer progresses and are associated with poor prognosis and AR androgen-independent activation[132–134]. Thus, lapatinib, a dual inhibitor of EGFR and HER2 used to treat $HER2^+$ metastatic breast cancer, has been proposed as a suitable candidate for prostate cancer therapy. However, phase II studies using this inhibitor have failed to demonstrate a decrease in PSA levels in patients with hormone-sensitive prostate cancer[135] and in unselected patients with CRPC[136], despite the fact that combined EGFR and HER2 dual inhibition using lapatinib plus ADT yielded positive results in androgen-dependent LNCaP cells[137].

These findings indicate that multiple pathways might be mediators of cross-resistance to both hormonal and taxane therapy; thus, their inhibition using combined therapies could be useful for prostate cancer therapy at various disease stages.

Tumour microenvironment

The tumour microenvironment is crucial in the genesis of drug resistance in cancer[138,139] — it has been documented that cultured cells that are sensitive to a particular therapeutic agent can develop resistance to that agent when grown in complex, 3D models that recapitulate their natural environment[140,141]. A reason for this could be the paracrine production of growth factors such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) and interleukins such as IL-6 that can be found in the tumoural stroma or are involved in the adhesion of cells to the extracellular matrix (ECM)[142,143]. In 2009, a heterogeneous population of immune cells with immunosuppressing activity, termed

myeloid-derived suppressor cells, were shown to infiltrate the CRPC microenvironment and support tumorigenesis and AR independence by secreting IL-23, which activates AR signalling[144]. The ECM can also induce chemoresistance via AKT-mediated inhibition of apoptosis[145], a pathway that is dependent on transforming growth factor-β-induced (TFBI) protein and that can be reversed upon loss of this protein[146]. Finally, and particularly in the case of prostate cancer, taxanes have been noted to disrupt focal adhesion dynamics via an effect on microtubules[147]; alterations of microtubule-mediated adhesion dynamics are observed in prostate cancer cell lines DU145-Rx and PC3-Rx that are resistant to docetaxel[148]. Inhibition of protein tyrosine kinase 2 (PTK2), a cytoskeleton-related protein that is involved in focal adhesion processes, using defactinib, overcomes resistance to docetaxel in docetaxel-resistant CRPC cells and mice with PC3 xenografts, in particular when coadministered with docetaxel[149]. Likewise, reactive changes occur in the tumour stroma, creating a suitable microenvironment for cell growth and proliferation. These changes include an abundance of fibroblasts and myofibroblasts and a loss of immune and endothelial cells[150]. As in other tumours, T lymphocyte infiltration is observed in prostate cancer; however, the overall immune response is dysfunctional owing to the presence of TGFβ, IL-10 and other immunosuppressive agents. Thus, the majority of tumour-infiltrating T lymphocytes are non-functional and their presence has been linked to conflicting evidence in terms of prognosis[151].

In other tumours, such as pancreatic cancer, the presence of activated fibroblasts (cancer-associated fibroblasts (CAFs)) has been associated with an immune-suppressed state and overproduction of ECM and has led to targeted immunotherapies[152]. For example, blocking the activity of CXCL12 cytokine secreted by CAFs acts synergistically with anti-PD-L1 immunotherapy in pancreatic cancer[153]. However, combined treatments that include targeting of CAFs in the setting of prostate cancer have yet to be designed. Other types of immunotherapy are currently in development for prostate cancer in combination with chemotherapy, but no favourable results have been published so far in phase III trials[151].

Thus, tumour cells can dynamically adapt in response to their microenvironment, and the result of such an interaction can result in cellular rewiring processes that eventually lead to drug resistance and is an area of growing interest that merits further investigation, which could result in development of innovative therapeutic strategies to be implemented in stand-alone or combination regimens.

Lineage plasticity and phenotype switching

The acquisition of drug resistance requires that tumour cells recognize the harmful events triggered by therapeutic assaults and, in return, adapt to them by rewiring their signalling cascades and phenotype. Some mechanisms of adaptive drug resistance consist of the activation of molecular signalling cascades driven by transcription factors, eventually leading to the enhancement of several pro-survival properties of the cell[154]. Another adaptive mechanism by which the prostate cancer cell can shift towards a drug-resistant status involves the acquisition of a phenotype that does not directly depend upon the drug target to survive. This phenomenon is described in the literature as ‘cell plasticity’ or ‘lineage plasticity’, and in the setting of androgen resistance has also been termed

'treatment-induced lineage crisis'[155,156]. All these terms ultimately refer to an elemental principle: the cell rewires itself towards a more undifferentiated, stem-like phenotype, the plasticity of which enables it to revert back and forth between differentiated and undifferentiated states as needed and to continue to thrive despite therapeutic interventions (FIG. 3).

Transdifferentiation to a neuroendocrine phenotype

A classic example of lineage plasticity is seen in metastatic prostate cancer treated with anti-androgen therapy that relapses with morphological features of neuroendocrine carcinoma[154,157]. These tumours, classified as neuroendocrine prostate cancer (NEPC), are characterized by small-cell morphology and positive staining for neuroendocrine markers, low-to-absent expression of AR, and secretion of neuropeptides and other growth factors that enable their survival and maintain a poor response to therapy[158,159]. Development of NEPC occurs in ~25% of patients with mCRPC treated with anti-androgen therapy and is infrequent in patients with primary tumours (~1%)[156]. As low-to-absent AR expression is a hallmark of aggressive prostate cancer[160,161], NEPC might arise through a process of divergent evolution in which an AR-poor precursor is selected through therapy to eventually progress to a neuroendocrine phenotype[155,161]. This mechanism has been demonstrated both in vitro and in vivo, in which AR suppression was shown to drive the neuroendocrine transdifferentiation of adenocarcinoma-type cells to NEPC[162–165]. In in vitro studies, LNCaP cells cultured for long periods in hormone-deprived conditions acquired neuroendocrine features. In in vivo studies, mice bearing patient-derived xenografts underwent castration, which gave rise to a neuroendocrine phenotype including markers such as synaptophysin, chromogranin or CD56.

Comparison of the molecular profiles of NEPC and AR-driven CRPC using whole-exome sequencing has shown significant genomic overlap despite the marked clinical and pathological differences of these types of tumour, which indicates a divergent clonal selection mechanism of lineage conversion[160]. For example, *RB1* loss was found in 70% of NEPC and 32% of CRPC adenocarcinomas ($P = 0.003$, proportion test), *TP53* loss was found in 66.7% of NEPC and 31.4% of CRPC adenocarcinomas ($P < 0.0004$; proportion test), and concurrent *RB1* and *TP53* loss was found in 53.3% of NEPC and 13.7% of CRPC adenocarcinomas ($P < 0.0004$, proportion test). Given the genomic similarity and epigenetic disparity observed in this study and revealed by genome-wide DNA methylation, epigenetic modulation is likely to have a role in neuroendocrine transdifferentiation, rather than the linear progression of somatic mutations that are archetypical of a Darwinian model of evolution[166].

This elegant mechanism of drug resistance exists across several tumour types. In lung tumours, EGFR-mutant adenocarcinomas are known to switch phenotypes and relapse as small-cell carcinomas after anti-EGFR therapy[167]. In melanoma, phenotype switching is also thought to occur after BRAF inhibition, which confers growth and invasiveness properties on the tumour[168].

Several genomic aberrations in tumour suppressors, such as loss of *PTEN*, *TP53* and *RB1*, are integral to the development of CRPC and are probably drivers of the evolution towards

this state[160]. Given the shared features of CRPC and NEPC, accelerated progression towards the acquisition of neuroendocrine features owing to combined inactivation of *PTEN* and *TP53* is not surprising[169]. Consistent with these findings, a 2017 study demonstrated that loss of *PTEN* and *TP53* as well as overexpression of transcription factor *SOX2* were responsible for the phenotype shift from AR-dependent epithelial cells to enzalutamide-resistant AR-independent neuroendocrine cells[170].

SOX2 is a master regulator of pluripotent embryonic stem cells and multipotent neural progenitor cells[171] and acts as a critical factor in the reprogramming of fibroblasts to induced pluripotent cells[172]. SOX2 also acts as a key regulator of self-renewal and maintenance of cancer stem cells (CSCs) in a variety of tumours, including prostate cancer[173]. In prostate cancer, SOX2 acts as a marker of neuroendocrine differentiation[174] and EMT induction[175], which supports its role as a driver of lineage plasticity. Interestingly, AR directly represses SOX2 expression in prostate cancer, and a loss of AR signalling is, therefore, paralleled by increased SOX2 expression, which in turn supports progression of the tumour[176].

According to this wealth of data, SOX2 inhibition can lock prostate cancer cells in their epithelial state and block changes in their phenotype, preventing EMT. Even though targeting of SOX2 is not currently available, a feasible strategy would be to prevent its transcriptional upregulation by targeting *TP53* and *RB1*, as *TP53* directly inhibits SOX2 (REF.[177]) and RB1 directly represses SOX2 recruitment to E2F binding sites in fibroblasts[178]. Some studies have shown SOX2 inhibition using short hairpin RNA (shRNA) or zinc-finger-based artificial transcription factors (ZF-ATFs)[179,180]. The downside of these approaches is that they require infection with a viral vector to work, which can increase the risk of infection-related diseases or immune-related responses to the introduced viral vector. Other techniques of SOX2 targeting, such as the use of peptide aptamers or aptamer-small interfering RNA (siRNA) complexes, are under investigation[173].

Unlike *PTEN*, *TP53* and *RB1* loss are rarely seen in primary prostate cancer, despite being frequent in metastatic and prostate cancer that recurs after ADT (53.3% and 21%, respectively)[181,182], and have been linked to resistance to ADT[183]. *RB1* loss is sufficient to drive AR signalling in CRPC[184] and a distinct E2F1 cistrome expansion is observed depending on whether *RB1* is lost or functionally inactivated[185]. Genomic aberrations in *TP53* and *RB1*, among others, are also commonly found in almost all NEPC tumours[186], and another study has shown that simultaneous *RB1* and *TP53* loss occurs more frequently in NEPC than in CRPC adenocarcinomas (53.3% of NEPC and 13.7% of CRPC; $P < 0.0004$, proportion test)[187]. In accordance with these findings, a 2017 study linked combined *RB1* and *TP53* loss to increased expression of *SOX2* and *EZH2*, which created a stem-like environment that was permissive for lineage plasticity[188]. In this same study, $Rb1^{-/-}$ mice developed metastatic prostate cancer tumours genetically[188]. Moreover, a transcriptional programme mediated by N-MYC induced a neuroendocrine phenotype as well as androgen-resistance features (NE-CRPC) through the activation of enhancer of zeste homologue 2 (EZH2)[189,190]. Thus, EZH2 and SOX2 are likely to work together to drive lineage plasticity in CRPC and the phenotype switch towards NEPC (FIG. 2). Selective EZH2 inhibition has been achieved using the small molecule CPI-1205, which is currently under investigation in

mCRPC as a potential strategy to prevent enzalutamide resistance in a phase Ib/II study that combines oral administration of CPI-1205 with either enzalutamide or abiraterone/prednisone (NCT03480646)[191].

The importance of transcription factors in driving not only the progression of prostate cancer but also the transition towards a neuroendocrine phenotype was emphasized in a 2018 study, which used a bioinformatics model to identify the transcription factor one cut homeobox 2 (ONECUT2) as highly active in mCRPC[192]. According to this study, the atypical homeobox protein ONECUT2 activates an AR-independent transcriptional programme in CRPC that contributes to the neuroendocrine differentiation of the prostate cancer cells mainly through negative regulation of FOXA1 (REF[192]). FOXA1 is a transcription factor with paramount importance in the development and differentiation of epithelial cells, and FOXA1 loss has been demonstrated to enable prostate cancer progression to NEPC by leading to AR reprogramming and EMT[193]. RE-1 silencing transcription factor (REST) acts in a similar way to FOXA1 in inhibiting neuroendocrine differentiation and acts via ONECUT2 repression[192,194,195]. Downregulation of REST is commonly seen in NEPC via activation of the RNA splicing factor SRRM4 (REF.[196]). Taking these data together, we believe it is plausible that loss of REST probably releases ONECUT2, which in turn downregulates FOXA1 and initiates a transcriptional programme independent of AR signalling, which eventually leads to the acquisition of a neuroendocrine phenotype (FIG. 2). Of note, inhibition of ONECUT2 by CSRM617, a novel small molecule identified through a structure-based drug design screen, suppresses metastasis growth in mice bearing the mCRPC cell line 22Rv1 (REF[192]). Thus, the clinical development of this small molecule might lead to novel therapeutic opportunities in the treatment of NEPC.

Several other transcription factors are under investigation as drivers of neuroendocrine differentiation in prostate cancer. Like ONECUT2, BRN2 is a POU-domain neural transcription factor that is upregulated in enzalutamide-resistant prostate cancer cell lines[197]. BRN2 is also expressed in small-cell lung carcinoma, in which it has a role in tumour progression by acting as an upstream regulator[198], and in melanoma, in which it mediates invasiveness and migration[199,200]. In prostate cancer, BRN2 interacts with SOX2 and together they act as drivers of neuroendocrine differentiation in enzalutamide-resistant prostate cancer cell lines[197].

The role of transcription factors as key regulators of phenotype switching is just beginning to be uncovered. As these signalling cascades by which the cell rewires itself are elucidated, they could be exploited as a source of novel therapeutic targets.

EMT and acquisition of stem-like properties

A primary mechanism of drug resistance is found in the existence of tumour heterogeneity, so that while some tumour cells are effectively targeted by therapy, others remain unaffected and continue with their progression and growth under therapeutic selective pressure[201]. According to the clonal evolution hypothesis, genomic instability is a major force in the generation of intratumour heterogeneity and accounts for the existence of several molecularly distinct subpopulations of cancer cells within the tumour[201]. Another mechanism of tumour heterogeneity is described by the CSC model[202]. Indeed, that some

tumours follow a hierarchical structure that begins with CSCs is well known[202]. CSCs are cancer cells with inherent resistance to chemotherapy that have tumour-initiating capabilities so that they not only survive the therapy used against them but continue proliferating despite all therapeutic efforts[203,204].

The seminal studies of CSC were performed in acute myeloid leukaemia[205]; these studies revealed a group of cells that exhibit a differential cell surface antigen expression profile from the rest of the tumour population[205]. This hierarchical structure that presents the tumour as a set of cells deriving from an initial CSC population has also been demonstrated in the setting of prostate cancer, wherein CSCs bear a $CD44^{+}\alpha2\beta1^{high}CD133^{+}$ phenotype. Moreover, most cells in the $CD44^{+}$ population are AR^{-} (REFS[206,207]). Interestingly, prostate cancer cells that exhibited a PSA^{-} or PSA^{low} profile survived castration-level androgen concentration in the hormone-dependent setting[208], and a group of undifferentiated prostate cancer cells with resistance to docetaxel chemotherapy also exhibited a PSA^{-} or PSA^{low} and a HLA class I^{-} or HLA class I^{low} profile[209]. This latter group also demonstrated increased activity of the Notch and Hedgehog signalling pathways, which could be inhibited using shRNAs to knockdown critical genes such as *NOTCH2*, *GLI1* and *GLI2*, in combination with radiotherapy or chemotherapy in mice bearing DU145 and 22Rv1 xenografts, enabling elimination of these drug-resistant cells[209]. Both Hedgehog and Notch signalling have been linked to self-renewal properties in various tumour models including prostate cancer and provide novel targeting opportunities. Thus, the distinct molecular and functional profiles of CSCs offer vulnerabilities that might be exploitable with the use of combination therapy.

Although CSCs arise from pre-existing stem and/or progenitor cells, their origin can feasibly be traced back to the de-differentiation of terminally differentiated states[204]. Interestingly, a number of oncogenic transcription factors, including SOX2, OCT3 and OCT4, have been defined as required elements for pluripotency reprogramming in prostate cancer[210].

Another mechanism of lineage plasticity involves the process of EMT by the prostate cancer cell in order to gain stem-like properties and aggressiveness[211]. EMT is a bidirectional and reversible process by which adherent epithelial cells can de-differentiate and gain migratory and invasive properties as well as stem-like status, and is directly involved in embryogenesis[211–213]. EMT is believed to underlie the pathogenesis of both CSCs and circulating tumour cells, so might be responsible for the metastatic as well as the drug-resistant status of the cancer cell[214–216]. Notably, EMT is a transitional process[217]; thus, intermediate phenotypes are likely to be the most associated with stem-like features[217], whereas progression towards a definitive mesenchymal phenotype is detrimental for the tumour-initiating capacity of the cells as well as their invasion and proliferation properties[218,219].

Importantly, EMT occurs not only under physiological conditions such as embryonic development or protection of the epithelial phenotype but can also be induced in the pathological context when the tumour encounters adverse conditions such as chemotherapy, which stimulate the induction of EMT in cancer cells through increased signalling via molecular pathways such as Wnt, Notch and Hedgehog, which drive CSC renewal and maintenance[220,221]. Moreover, maintenance of AR signalling is necessary for EMT

regulation, and androgen deprivation induces EMT, further reducing the ability of the cells to respond to ADT[222–224]. Treatment-induced reversal of EMT has also been demonstrated in the context of combination therapy with enzalutamide and cabazitaxel[225]. In particular, this study found that cabazitaxel contributed to AR inhibition and that addition of enzalutamide overcame cabazitaxel resistance in androgen-responsive tumours in human CRPC xenografts, apart from reversing EMT to mesenchymal–epithelial transition.

Activation of the PI3K–AKT–mTOR pathway has been shown to induce EMT and enhance the CSC phenotype in radioresistant prostate cancer cell lines[226]. The PTEN–PI3K–AKT signalling pathway is linked to CSC expansion and maintenance in the prostate and offer interesting therapeutic opportunities[227].

Other findings related to EMT and advanced prostate cancer are the overexpression of the cell adhesion molecule N-cadherin, which correlates with metastatic potential and castration resistance[228–230], aberrant activation of the Wnt–β-catenin pathway, which correlates with EMT features and positively affects proliferation and invasiveness in prostate cancer[231], and an increased role of the growth factor TGFβ, which is also found in advanced prostate cancer[232]. RAS–MAPK activation paired with *PTEN* loss has also been shown to accompany EMT and macrometastasis in mice[233]. Novel crosstalk between the AR and the EGF–SRC signalling pathways has also been linked to EMT induction in TMPRSS2–ERG tumours[234]. This mechanism occurs via miR-30b modulation, a tumour suppressor that when silenced leads to ERG induction and EMT despite the absence of androgen[234].

Many of the molecular pathways that are dysregulated in advanced prostate cancer have a clear link to the induction of EMT. For example, a study using whole-transcriptome and whole-genome sequencing identified the Wnt–β-catenin pathway as the most highly enriched pathway among enzalutamide-resistant patients and also found that having two DNA alterations in *RB1* was associated with reduced OS in a cohort of men with mCRPC[235]. Thus, the acquisition of stem-like properties is likely to be another example of phenotype switching that can be exploited by drug-resistant prostate cancer cells. This effect was elegantly illustrated in a study demonstrating the differential expression of EMT and stem-like cell markers between prostate cancer cells that were treated with neoadjuvant docetaxel and ADT and cells that were not treated. Transcriptional levels of a subset of 93 genes from a docetaxel-resistant prostate cancer cell line microarray study were analysed using low-density arrays in tumours from patients with advanced prostate cancer. These data support EMT as a marker of resistance to therapy and cancer progression[236,237].

Linking neuroendocrine and stem-like phenotypes

The neuroendocrine phenotype has been widely linked to the induction of stemness properties and plasticity in prostate cancer. A study that used a gene signature specific for human prostate basal cells showed that metastatic NEPC is molecularly more basal and stem-like than the adenocarcinoma phenotypes[238]. SOX2, the transcription factor that acts as a driver of transdifferentiation in prostate cancer[197], is also involved in self-renewal properties of stem cells[170], providing a clear link between NEPC and stem-like properties. A 2019 study found that — following castration and before recurrence — a group of $BMI1^{+}SOX2^{+}$ prostate cancer cells underwent a transient phenotype switch towards a more

undifferentiated state, which was characterized by the expression of basal markers $CK14^+$ and $p63^+$ and overexpression of SOX2 (REF.[239]). Interestingly, overexpression of SOX2 occurred mainly within a group of progenitor cells that expressed BMI1, which had been previously described as potential cells of origin of prostate cancer[240].

Furthermore, the signalling cascades that drive neuroendocrine differentiation and/or plasticity overlap considerably. For example, the expression of TROP2 and CD49 markers that define basal cell plasticity is also high in NEPC[241]. Additionally, forced expression of the SNAIL transcription factor, an inducer of EMT, has been shown to promote a neuroendocrine phenotype in LNCaP prostate cancer cells[242].

This confluence between cell rewiring and cell plasticity is illustrated by the role of STAT3, which integrates different signalling pathways involved in the differentiation of NEPC, induction of EMT and maintenance of CSC populations[243]. Indeed, STAT3 upregulation by IL-6 in an androgen-depleted context has been linked to the acquisition of stem-like properties[244] as well as neuroendocrine differentiation of prostate cancer cells[245]. Similarly, the Wnt–β-catenin pathway, which is upregulated in CRPC[246], contributes to both neuroendocrine differentiation and the acquisition of stem-like properties[247,248], both of which can be reversed using anti-androgen therapy[249].

In summary, the acquisition of undifferentiated features by the prostate cancer cell is another plausible mechanism of cell rewiring that further extends the aggressiveness and drug resistance of the tumour. These examples of phenotype switching can occur through transdifferentiation to a neuroendocrine phenotype, selection of undifferentiated CSCs or the employment of the EMT process to acquire a basal and stem-like phenotype. However, based on the evidence, we believe that these processes can probably be integrated into a continuum of cell plasticity, in which intermediate phenotypes exist, which explains why the neuroendocrine and stem-like phenotypes are linked at a molecular level. Finally, many of these processes seem to be driven by transcription factors, many of which also drive resistance to anti-androgen and taxane therapy. Notably, this dynamic response transcends the genome and might also involve important epigenetic regulations[187], and examples of such are EZH2 and SOX2, which are involved in epigenetic reprogramming.

Considerations for clinical trial design

A plethora of cell rewiring mechanisms are employed by the prostate cancer cell in order to acquire drug resistance. The study and characterization of these mechanisms is likely to translate into specific molecular profiles for each individual tumour, which has considerable implications in the era of personalized medicine. Thus, future combination therapies could be designed to target cellular rewiring mechanisms and restrain acquired resistance in prostate cancer, but several clinical considerations should be taken into account for clinical trials of such agents.

Early application of combined therapies

First, combined therapies in prostate cancer are most likely to succeed when administered in early stages of the disease. Genetic mutations are one of the main operators of adaptive drug

resistance and, according to the Goldie–Coldman hypothesis, the probability of the appearance of a resistant phenotype increases with the mutation rate[250]. Genomic studies addressing the mutational landscape of early-stage and late-stage prostate cancer[78,182,183,251,252] indicate that the mutational rate is highest in advanced tumours, which would favour the rapid acquisition of resistance in late stages of the disease. Indeed, supporting this hypothesis, clinical survival data from the CHAARTED (docetaxel plus ADT)[15], STAMPEDE (docetaxel or abiraterone plus ADT)[17], LATITUDE (abiraterone and prednisone plus ADT)[16] and ARCHES (enzalutamide plus ADT)[19] trials showed substantially better clinical outcomes when these agents were administered in patients with early-metastatic, therapy-naive prostate cancer than when similar combination therapies were administered in patients with advanced, castration-resistant, metastatic prostate cancer. Thus, these trials support the development and use of combination therapies in the context of early-stage disease, before the mutational burden induced by therapy has increased and the fittest and more resistant cells are selected.

However, use of combined therapy in early disease stages can also lead to an overestimation of the clinical benefit of treatment, similar to the lead time bias in early disease screening[253]. If a tumour is detected and treated with combined therapy early in its course, the OS of the patient will seem to be longer, even if early combined therapy has no real effect on the overall length of survival when compared with treatments administered sequentially later in the disease course[253]. In order to address this possibility, clinical trials should ideally be designed to measure survival time after the use of sequential treatments in early-stage disease, in order to demonstrate that combined therapy is actually improving survival.

Use of appropriate biomarkers.—Moreover, several important considerations will need to be taken into account when assessing the efficacy of combined treatments. In addition to biochemical and radiographic measures of tumour burden, assessing for specific biomarkers is key to selecting patients for a precise combined treatment and to monitoring the effectiveness of these treatments during the course of treatment. One such biomarker is the expression of AR-V7. Detection of AR-V7 in circulating tumour cells[43,254,255], whole blood[46,256] and extracellular vesicles[257] can select patients who will not respond to anti-androgen therapies and who might benefit from other therapy options, taking into account the process of cell rewiring when considering which therapeutic option to use. Other biomarkers that relate to cell rewiring, and that can be used to select patients, are likely to arise in the future and can include, for example, the *TMPRSS2–ERG* gene fusion[258]. In the future, specific biomarkers that predict the response to a particular therapy would ideally be measured dynamically using non-invasive liquid biopsy methods to track and ensure the success of combined therapies[259–261].

Monitoring toxic effects.—Finally, consideration of toxic effects is of particular importance when developing combined therapies. In vivo models including genetically engineered mice and xenografts generated from patients with advanced prostate cancer transplanted into mice should be used to address potential adverse effects of newly developed therapeutic agents. Indeed, a study reviewed here examined the toxicity impact of EZH2 inhibitors in combination with anti-androgen therapy in mouse models for the first

time, demonstrating minimal toxicity[188]. Notably, the toxicity and efficacy of combining EZH2 inhibition with either enzalutamide or abiraterone plus prednisone are currently being tested in a phase Ib/II clinical trial (NCT03480646)[191]. Thus, although adverse effects need to be tested in appropriately designed clinical trials, in vivo models might provide valuable information to anticipate toxicities derived from combined therapies.

Toxic effects of treatment might be particularly important in the context of prostate cancer, as many patients are elderly and have comorbidities. Thus, in addition to prioritizing the use of the most efficacious combination therapy, special attention must be paid to identifying those patients most susceptible to the development of severe adverse effects. Additionally, sequential administration of drugs could be considered as an alternative option to combining agents. Indeed, swift sequential administration of drugs might decrease the probability of developing cross-resistance mechanisms between agents by decreasing exposure time to the drug and could, therefore, improve clinical outcomes, while minimizing the toxic effects induced by combined drugs[155]. The protocol for switching sequential treatments will need to take into account the individual toxicity profiles of each of the drugs, as well as the patient's specific comorbidities in order to administer drugs in the optimal sequence to maximize clinical benefit.

Overall, the implementation of combined therapies to minimize cellular rewiring and suppress acquired resistance to standard therapy will necessitate several careful considerations, including careful design of clinical trials, use of biomarkers for appropriate selection of the patients most likely to benefit from combination strategies and the anticipation of toxic effects that are emphasized with combined agents.

Conclusions

Combined therapy is becoming a critical component in the therapeutic landscape of prostate cancer. However, most of the combined therapeutic strategies that are currently in clinical use have been developed empirically, meaning that prostate cancer cells have been able to develop a number of mechanisms to develop and maintain resistance to therapies. These mechanisms of drug resistance are part of a dynamic process so that, when tumour cells are confronted with the disadvantageous environment of therapy, they maintain survival and growth by initiating a myriad of molecular events that transcend genome modifications and regulate distinct transcriptional states and the acquisition of phenotypes in the process of cellular rewiring. The molecular elucidation of these distinguishable pathways offers a tremendous number of potential targets for cancer therapy that can be targeted with combined therapy to prevent the development of cell rewiring in cancer cells. The results of clinical trials using combination therapies have demonstrated that combined therapy, rather than traditional sequential therapy, can prolong survival in prostate cancer. However, a 'best' target is unlikely to exist. Instead, there are only 'better' targets and therapeutic regimens depending on the biological nature of each tumour and the dynamic responses of the cell to enable it to become resistant to therapy. Thus, research should be focused on studying these dynamic responses, to distinguish their common stages, and to better understand how the tumour shapes its response against therapy through the use of cell rewiring mechanisms. Importantly, the detailed dissection of these mechanisms has uncovered novel actionable

targets that can be modulated to inhibit the acquisition of resistance. Thus, the development of drugs to target or prevent cellular rewiring could help establish effective combination therapies to improve the survival of patients with prostate cancer.

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Key points

- Targeting mechanisms involved in the acquisition of drug resistance could result in more effective therapeutic strategies for patients with prostate cancer.
- Cellular rewiring can be exploited by prostate cancer cells to acquire drug resistance by implementing alternative bypass signalling pathways after therapy exposure, thus enabling continued tumour proliferation and survival.
- Tumour cell crosstalk with the microenvironment can also result in cellular rewiring processes that eventually lead to drug resistance.
- Cellular rewiring mechanisms can induce phenotype switching towards a neuroendocrine phenotype and acquisition of stem-like properties.
- Clinical trials are investigating the combination of standard therapies, such as anti-androgens, with agents targeting cellular rewiring mechanisms.
- However, introducing these combinations that target cellular rewiring pathways into the prostate cancer armamentarium will require the development of predictive assays to anticipate toxicities and identify the most effective combinations.

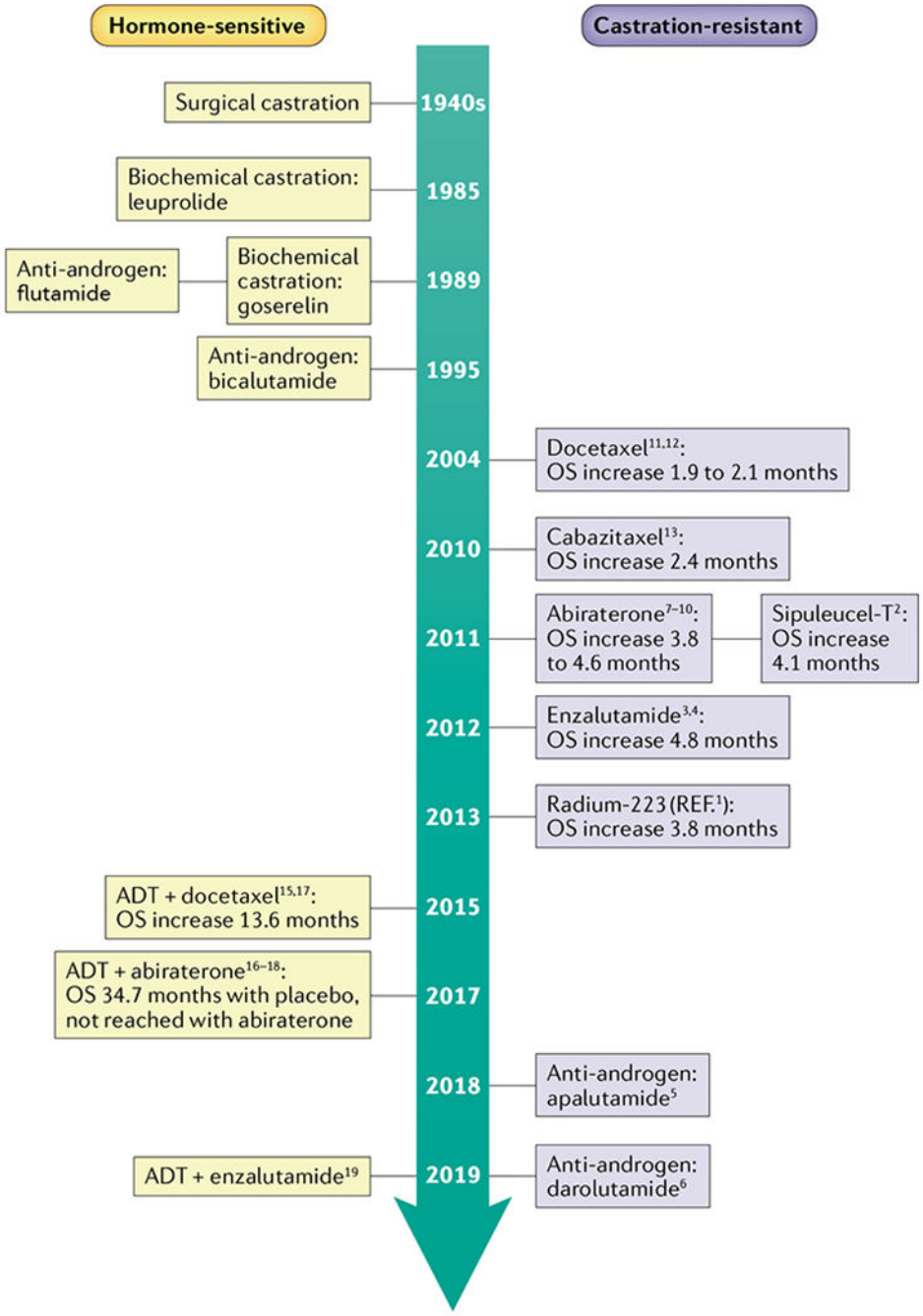

Fig. 1 |. Timeline of treatments for advanced prostate cancer.

Timeline of milestone treatments used for the current management of prostate cancer, in both androgen-responsive and castration-resistant stages. Note how novel therapies include combinatory options such as androgen deprivation therapy (ADT) plus docetaxel (CHAARTED trial, 2015 and STAMPEDE, 2016), ADT plus abiraterone (LATITUDE trial, 2017) or ADT plus enzalutamide (ARCHES trial, 2019). OS, overall survival.

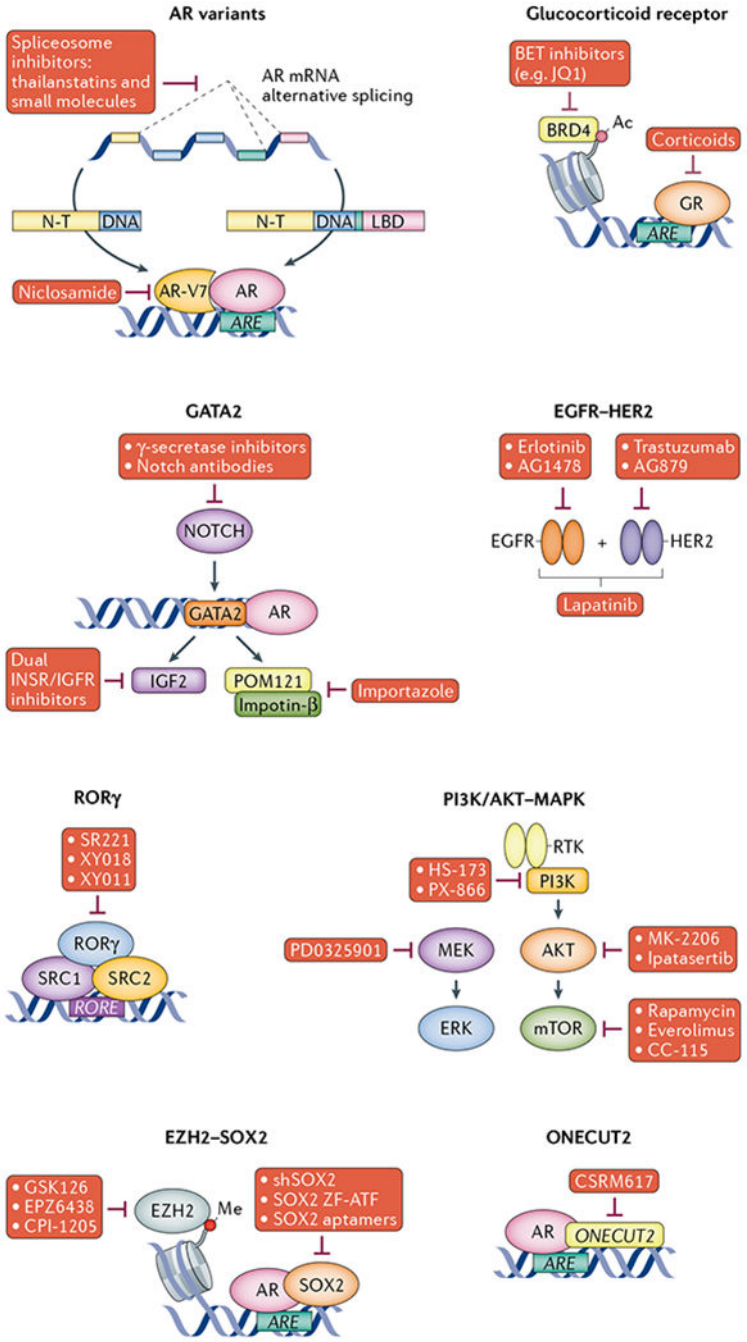

Fig. 2 |. Therapeutic targeting of cell rewiring mechanisms contributing to acquired drug resistance.

Various cellular rewiring pathways contribute to acquired drug resistance; some of these pathways offer potential treatment targets. Key regulatory cascades include the AR, PI3K–AKT, GATA2 and GR pathways. Other pathways that are currently under investigation are mediated by master regulator transcription factors such as SOX2 and ONECUT2. A number of agents (highlighted in red) have been used experimentally to inhibit cellular rewiring mechanisms and have been shown to resensitize drug-resistant cells. AR, androgen receptor; AR-V7, androgen receptor variant 7; BET, bromodomain and extra-terminal domain; BRD4,

bromodomain containing protein 4; ECFR, epidermal growth factor receptor; EZH2, enhancer of zeste homologue 2; GATA2, GATA binding protein 2; GR, glucocorticoid receptor; HER2, human epidermal growth factor receptor 2; IGF2, insulin-like growth factor 2; IGFR, insulin-like growth factor receptor; INSR, insulin receptor; LBD, ligand-binding domain; N-T, N-terminal; ONECUT2, one cut homeobox 2; POM121, POM121 transmembrane nucleoporin; RORγ, retinoic acid receptor-related orphan receptor-γ; RTK, receptor tyrosine kinase; SOX2, SRY (sex determining region Y)-box2; SRC1, steroid receptor co-activator 1; SRC2, steroid receptor co-activator 2.

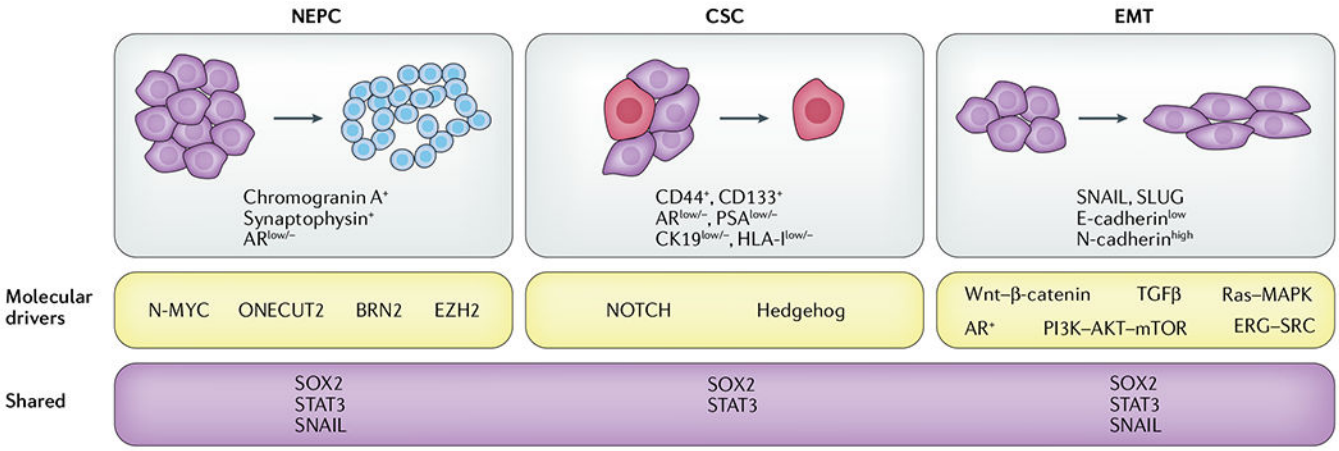

Fig. 3 |. Cellular plasticity and switching phenotypes associated with acquired drug resistance.

A number of distinct phenotypes and molecular pathways are associated with the acquisition of drug resistance. NEPC is characterized by the presence of markers such as chromogranin A and synaptophysin and an $AR^{low/-}$ status, and is driven by transcription and epigenetic factors such as ONECUT2 and EZH2, among others. CSCs are characterized by a $CD44^{+}CD133^{+}AR^{low/-}PSA^{low/-}$ $CK19^{low/-}HLA^{low/-}$ phenotype. EMT is promoted by transcription factors such as SNAIL and SLUG and driven by pathways such as ERG–SRC and PI3K–AKT–mTOR, resulting in a switch in cell expression from E-cadherin to N-cadherin. AR, androgen receptor; CSC, cancer stem cell; EMT, epithelial mesenchymal transition; ERG, ETS transcription factor; EZH2, enhancer of zeste homologue 2; MAPK, mitogen-activated protein kinase; NEPC, neuroendocrine prostate cancer; N-MYC, MYCN proto-oncogene; ONECUT2, one cut homeobox 2; SOX2, SRY (sex determining region Y) box 2; SNAIL, Snail family transcriptional repressor 1; SLUG, Snail family transcription repressor 2; SRC, steroid receptor co-activator; STAT3, signal transducer and activator of transcription 3; TGFβ, transforming growth factor-β.