REVIEW

The epigenetic and transcriptional landscape of neuroendocrine prostate cancer

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Abstract

Tumours adapt to increasingly potent targeted therapies by transitioning to alternative lineage states. In prostate cancer, the widespread clinical application of androgen receptor (AR) pathway inhibitors has led to the insurgence of tumours relapsing with a neuroendocrine phenotype, termed neuroendocrine prostate cancer (NEPC). Recent evidence suggests that this lineage reprogramming is driven largely by dysregulation of the epigenome and transcriptional networks. Indeed, aberrant DNA methylation patterning and altered expression of epigenetic modifiers, such as EZH2, transcription factors, and RNA-modifying factors, are hallmarks of NEPC tumours. In this review, we explore the nature of the epigenetic and transcriptional landscape as prostate cancer cells lose their AR-imposed identity and transition to the neuroendocrine lineage. Beyond addressing the mechanisms underlying epithelial-to-neuroendocrine lineage reprogramming, we discuss how oncogenic signaling and metabolic shifts fuel epigenetic/transcriptional changes as well as the current state of epigenetic therapies for NEPC.

Key Words
- prostate cancer
- lineage plasticity
- androgen receptor
- epigenetics
- DNA methylation
- transcription factor

Introduction

The androgen receptor (AR) is the principal driver of prostate cancer development and resistance; accordingly, AR pathway inhibitors (ARPIs) are used to treat men at all stages of the disease. Drug resistance ultimately ensues, which in most cases occurs through reactivation of the AR signaling axis. However, a subset of prostate tumours lose dependence on the AR pathway and co-opt alternative lineage programs to bypass therapeutic pressure and sustain tumour growth (Davies et al. 2018, Ku et al. 2019). Clinically, this lineage reprogramming has been associated with loss of luminal epithelial identity and the ensuing transition from a typical prostate adenocarcinoma to an aggressive neuroendocrine prostate cancer (NEPC) (Beltran et al. 2016, Aggarwal et al. 2018). NEPC tumours are notable for attenuated AR signaling, pure or mixed small cell histology, and expression of neuroendocrine lineage markers (e.g. chromogranin A, neuron-specific enolase, and synaptophysin) (Ather et al. 2008, Aparicio et al. 2013). Recent studies have highlighted a rising incidence of NEPC in up to 15–20% of advanced prostate cancer patients due, in part, to the widespread clinical integration of potent ARPIs, such as abiraterone acetate and enzalutamide (Aggarwal et al. 2018, Abida et al. 2019). The outlook for NEPC patients remains poor as there are no established therapeutic approaches for these tumours.
To begin to dissect the molecular drivers of NEPC, genome-wide sequencing studies were undertaken between serial tumour samples from prostate cancer patients during the course of disease progression (Beltran et al. 2011, 2016). Apart from genomic loss of the tumour suppressors RB1 and TP53 (50–75% in NEPC vs 5–15% in adenocarcinoma), the overall spectrum of somatic mutations between adenocarcinoma and NEPC was found to be surprisingly similar (Beltran et al. 2016, Mu et al. 2017). Notably, the functional loss of RB1 and TP53 has been reported to facilitate the activation of pluripotency networks mediated, in part, through de-repression of the pluripotency transcription factor SOX2 as well as the epigenetic modifier, enhancer of zeste-homolog 2 (EZH2) (Choi et al. 2011, Kareta et al. 2015, Ku et al. 2017). Data from genetically engineered mouse models (GEMMs) support the notion that loss of RB1 and/or TP53 fuels the development of neuroendocrine tumours (Ku et al. 2017, Zou et al. 2017); however, inactivation of RB1 is not tightly correlated with overt neuroendocrine differentiation in neoadjuvant hormone therapy-treated primary patient tumours (Sowalsky et al. 2018). This suggests that other mechanisms are required to co-operate with genomic alterations to re-direct cell fate toward the neuroendocrine lineage.

The paucity of unique genomic aberrations between prostate adenocarcinoma and NEPC strongly imply that the evolution to NEPC is driven by coordinated epigenetic and transcriptional reprogramming. In contrast to the genetic code, epigenetic programming is dynamically regulated by a series of enzymatic writers, erasers, and readers that impart a unique pattern of chemical marks superimposed on the genetic landscape. These epigenetic marks include DNA methylation and histone post-translational modifications that alter chromatin structure and, in turn, gene expression programs to establish and maintain cell identity (Chen and Dent 2014). In NEPC, perturbed DNA methylation patterning and expression of epigenetic remodeling factors such as EZH2 and REST have been widely reported in pre-clinical models and patient tumours (Beltran et al. 2016, Ku et al. 2017, Li et al. 2017, Flores-Morales et al. 2019). Interplay between the epigenetic machinery and neuronal lineage-guiding transcription factors such as BRN2 (Bishop et al. 2017), ONECUT2 (Rotinen et al. 2018, Guo et al. 2019) and MYCN (encoding N-Myc) (Dardenne et al. 2016) may facilitate the emergent NEPC phenotype.

In this review, we introduce key concepts to understand how epigenetic and transcriptional dysregulation is a plausible driving mechanism in the reprogramming of prostate cancer cells as they lose their AR-imposed identity and transition toward the neuroendocrine lineage. Moreover, we discuss how oncogenic signaling and metabolic changes fuel epigenetic changes as well as the current state of epigenetic drugs for prostate cancer.

The epigenetic basis of NEPC

Currently available data indicate that NEPC can evolve from a prostate adenocarcinoma precursor following potent AR pathway inhibition (Lin et al. 2014, Beltran et al. 2016). Closer examination of patient tumours has revealed a spectrum of cellular differentiation states with the AR-positive luminal state and AR-indifferent neuroendocrine state at opposing ends of this cellular differentiation continuum. The existence of intermediate phenotypes, including hybrid/amphicrine and ‘double-negative’ AR and neuroendocrine marker-expressing tumours, reinforces the notion that prostate cells can undergo phenotypic conversion (Papadimitriou et al. 1994, Aggarwal et al. 2016, Bluemm et al. 2017). However, whether this occurs via direct transdifferentiation or reversion to a metastable stem-like state followed by re-differentiation toward the neuroendocrine lineage remains unclear. Recent studies have reported heightened activity of stem cell-associated transcriptional programs as tumours progress to a terminal NEPC state (Smith et al. 2015, 2018, Lee et al. 2018), suggesting that these tumours co-opt stem cell programs to enhance plasticity and lineage conversion.

Epigenetic mechanisms, particularly DNA methylation and histone modifications, have a well-established role in regulating cell plasticity. Indeed, during normal development the epigenetic machinery is central to establishing unique chromatin patterns that guide lineage commitment. Disruption in these processes can yield impaired differentiation states and epigenetic reprogramming characteristic of malignant tumours (Nieto 2013). In particular, NEPC cell lines possess a unique chromatin accessibility profile, distinct from adenocarcinoma, which supports the activation of neuronal-associated gene networks (Park et al. 2018). This chromatin flux is regulated by RB1 and TP53, which act as gatekeepers to prevent neuroendocrine differentiation; concomitant loss in partially transformed prostate epithelial cells yields marked changes in chromatin accessibility resembling NEPC (Park et al. 2018).

Although the precise mechanisms are not well understood, inactivation of RB1 and/or TP53 leads to
the upregulation of the DNA methyltransferase (DNMT) family member DNMT1 (McCabe et al. 2005, Lin et al. 2010). DNA methylation is a vital epigenetic modification that affects numerous biological processes, including transcription, cell fate decisions, and development (Greenberg & Bourc'his 2019). Notably, relative to adenocarcinoma, DNMT1 is overexpressed in NEPC, and these tumours exhibit distinct DNA methylation patterns (Beltran et al. 2016, Smith et al. 2018). This is particularly evident at gene loci of known regulators of cell fate decisions (e.g. ASCL1 and HES6), epithelial-mesenchymal plasticity, and neuronal development. Functionally, DNA methylation systems are linked to the activity of Polycomb group (PcG) proteins, namely EZH2, which serve as a recruitment platform for DNMTs (Vire et al. 2006). The expression of both EZH2 and DNMT1 is tightly coupled to the re-activation of stem cell transcriptional programs in NEPC tumours (Clermont et al. 2015, Ku et al. 2017, Smith et al. 2018), suggesting that epigenetic changes generate a chromatin landscape that supports enhanced cell pliability and, in turn, neuroendocrine differentiation.

In addition to loss-of-function of RB1 and/or TP53, neuroendocrine lineage reprogramming is frequently associated with overexpression and/or amplification of MYCN, which encodes N-Myc (Beltran et al. 2011). N-Myc has a well-established role in maintaining human embryonic stem cells as well as neural stem/progenitor cells (Varlakhanova et al. 2010). In the context of prostate cancer, forced expression of N-Myc in human prostate epithelial cells (along with AKT activation) yields aggressive tumours that histologically resemble NEPC and in which neuroendocrine markers are upregulated (Dardenne et al. 2016, Lee et al. 2016). Functionally, N-Myc is dependent on the bromodomain and extra-terminal domain (BET) family of epigenetic readers, in particular BRD4, to facilitate target gene expression (Henssen et al. 2016). BRD4 recognizes and binds acetylated lysine residues on histone tails leading to recruitment of the positive transcription elongation factor b (P-TEFb) which, in turn, phosphorylates RNA polymerase II to activate gene transcription. In this way, BRD4 maintains transcription of core stem cell genes such as OCT4 and NANOG as well as neuronal lineage gene sets, at least in part through interaction with Myc proteins (Puissant et al. 2013, Di Micco et al. 2014, Gonzales-Cope et al. 2016). In small cell lung cancer, BRD4 upregulates the expression of the neuronal lineage-guiding transcriptional factor ASCL1, rendering these tumours exquisitely sensitive to BET inhibition (Lenhart et al. 2015); whether this correlation holds true in NEPC is yet to be explored. In addition to BRD4, N-Myc has recently been reported to cooperate with EZH2 to influence chromatin architecture and gene expression in MYCN-driven NEPC (Berger et al. 2019). The interplay and coordinated regulation of epigenetic factors highlight the complexity in the dynamics of the epigenetic landscape in NEPC.

**Interplay between the epigenetic landscape and metabolism**

Modulation of the epigenome is tightly coupled to metabolic reprogramming in cancer cells; many of the chemical modifications on DNA and histones derive from intermediates of cellular metabolic pathways (Schvartzman et al. 2018). For example, pyruvate generated from glycolysis is the main substrate for acetyl-CoA, a central metabolite coordinating the activity of the histone acetyltransferase (HAT) enzymes. Treatment-induced NEPC tumours exhibit elevated expression of the histone lysine demethylase KDM8, which functions to reprogrammetabolism toward aerobic glycolysis (Wang et al. 2019). Moreover, MYCN, which is implicated in neuroendocrine lineage reprogramming, increases mitochondrial export of acetyl groups and upregulates the HAT GCN5 leading to elevated histone acetylation and DNA accessibility (Knoepfler et al. 2006). This may explain why NEPC tumours are highly glycolytic, and inhibition of glucose metabolism leads to reduced proliferation and reversion of resistance phenotypes (Li et al. 2016, Choi et al. 2018).

In addition to the heightened rate of glycolysis in NEPC, increased glutamine uptake can further increase the generation of pyruvate and, in turn, acetyl-CoA. Interestingly, epigenetic alterations in prostatic cancer-associated fibroblasts (CAFs) have been reported to promote glutamine secretion into the tumour microenvironment (Mishra et al. 2018). This process was modulated by oncogenic Ras activity resulting from epigenetic silencing of the Ras inhibitor RASAL3 in CAFs; treatment with the demethylating agent 5-aza-2′-deoxycytidine (decitabine) rescued RASAL3 expression. In 3D co-cultures of CAF-CWR22Rv1 prostate adenocarcinoma cells, increased glutamine secretion by CAFs following ARPI treatment rescues RASAL3 expression. In 3D co-cultures of CAF-CWR22Rv1 prostate adenocarcinoma cells, increased glutamine secretion by CAFs following ARPI treatment rescues RASAL3 expression. In 3D co-cultures of CAF-CWR22Rv1 prostate adenocarcinoma cells, increased glutamine secretion by CAFs following ARPI treatment rescues RASAL3 expression.
Most recently, profiling of adenocarcinoma and NEPC tumours identified NEPC-specific upregulation of the metabolic enzyme phosphoglycerate dehydrogenase (PHGDH) (Reina-Campos et al. 2019), which is the first and limiting step in the serine, glycine, one-carbon pathway (SGOCP). SGOCP activity is required for the biosynthesis of S-adenosyl methionine (SAM), the source of methyl groups that are used for DNA and histone methylation (Yang & Vousden 2016). Mechanistically, the loss of atypical protein kinase C (PKC) λ/ι in prostate adenocarcinoma fuels increased SGOCP activity, leading to aberrant DNA methylation patterns that mimic those observed in NEPC patient tumours (Reina-Campos et al. 2019). Together, these studies suggest that NEPC differentiation is dependent on metabolic reprogramming to feed epigenetic changes that favor this lineage conversion (as depicted in Fig. 1).

**EZH2 as a master regulator of NEPC reprogramming**

The epigenetic modulator EZH2 is frequently overexpressed in patients who have progressed to NEPC (Beltran et al. 2011, 2016). EZH2 is the catalytic subunit of the Polycomb repressive complex 2 (PRC2), which mediates the deposition of restrictive histone marks, namely trimethylation of histone H3 at lysine 27 (H3K27me3), to repress lineage-specifying factors thereby promoting a more stem-like state (Lee et al. 2006). Early work in prostate cancer correlated EZH2 with poorer outcomes (Varambally et al. 2002), and more recently heightened EZH2 expression and activity (e.g. H3K27me3 expression) has been reported in the majority of NEPC patients (87% vs 46% adenocarcinoma) (Clermont et al. 2015, Beltran et al. 2016, Puca et al. 2018).

In support of EZH2 as a central regulator of epigenetic rewiring in NEPC, inhibition of EZH2 in prostate adenocarcinoma cell lines precludes ARPI-mediated neuroendocrine differentiation (Zhang et al. 2018, Shan et al. 2019). Moreover, GEMM models driven by overexpression of MYCN (Dardenne et al. 2016) or deletion of R1 (Ku et al. 2017) exhibit activation of neuroendocrine and stem cell transcriptional programs along with increased expression of EZH2. Notably, these tumours were sensitive to EZH2 inhibitors, and inhibition of EZH2 re-activated the canonical AR signaling axis, thereby sensitizing tumours to ARPIs (Dardenne et al. 2016, Ku et al. 2017). A similar dependency on EZH2 has been described in NEPC cell lines (Crea et al. 2011, Zhang et al. 2018, Shan et al. 2019), patient-derived xenografts (Kleb et al. 2016), and organoids (Puca et al. 2018);

**Figure 1**

EZH2 is a master regulator of neuroendocrine lineage plasticity in prostate cancer. EZH2 functions as the catalytic subunit within the Polycomb repressive complex 2 (PRC2) to tri-methylate histone H3 at lysine 27 resulting in a condensed chromatin structure and repression of gene networks. This state is reinforced by EZH2 recruitment of DNA methyltransferases (DNMTs). Metabolic reprogramming in NEPC underlies the epigenetic re-wiring; increased glucose shuttling through the serine, glycine, one-carbon (SGOCP) pathway increases intracellular S-adenosyl methionine (SAM) – the fuel for EZH2 and DNMT enzymes. The resulting change in the chromatin architecture facilitates the conversion of an adenocarcinoma to the neuroendocrine lineage. N-Myc and AR guide EZH2 to silence genes governing the epithelial lineage identity. In addition, EZH2-mediated repression of T-cell attractant chemokines (e.g. CXCL9 and CXCL10) and the antigen presentation machinery potentiates a ‘cold’ immunosuppressive tumour microenvironment. Aberrant oncogenic signaling can yield EZH2 phosphorylation, which switches the enzyme from a Polycomb repressor to a transcriptional co-activator, functioning with the AR to support androgen-independent phenotypes.

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however, complete phenotypic reversion to an AR-driven adenocarcinoma following EZH2 inhibition appears to be context dependent. More specifically, pre-clinical NEPC models retaining AR expression are particularly plastic and sensitive to combination therapy with EZH2 inhibitors and ARPs, irrespective of genetic background (e.g. RB1 loss or N-Myc overexpression) (Dardenne et al. 2016, Ku et al. 2017). Conversely, AR-negative NEPC organoids could not be reprogrammed back to an adenocarcinoma state following EZH2 inhibition, and no additive or synergistic effects were observed with enzalutamide (Puca et al. 2018). This suggests that epigenetic inhibitors may exhibit the greatest efficacy in a therapeutic window before tumours lose AR expression.

Functionally, EZH2 co-operates with lineage-guiding transcription factors to epigenetically regulate gene expression and coordinate lineage specification. In NEPC, EZH2 directly complexes with N-Myc to transcriptionally repress genes that enforce an AR-driven adenocarcinoma state (Dardenne et al. 2016). Accordingly, conditional expression of N-Myc (along with activated AKT) in prostate epithelial cells is sufficient to induce neuroendocrine differentiation (Dardenne et al. 2016, Lee et al. 2016). MYCN-amplified neuroblastomas are absolutely dependent on EZH2 for growth and survival (Chen et al. 2018), suggesting that the interplay between N-Myc and EZH2 drives the activation of neuronal programs. Further studies in prostate cancer identified N-Myc at neuronal lineage-associated gene promoters harboring both repressive H3K27me3 and active H3K4me3 histone marks (termed bivalent chromatin). EZH2 was required to maintain bivalency at N-Myc-bound genes, and its knockdown led to de-enrichment of neuronal-associated pathways in NEPC organoids (Berger et al. 2019).

EZH2 has also been reported to synergize with other epigenetic modification systems to promote chromatin remodeling that is necessary to enforce a state of lineage plasticity. EZH2 has been linked to DNMT activity and may pre-mark genes for de novo DNA methylation (Vire et al. 2006, Schlesinger et al. 2007), potentially via a scaffolding mechanism mediated by the long ncRNA HOTAIR (Xiang et al. 2018). The EZH2:DNMT complex is not well studied in prostate cancer; however, in breast cancer, EZH2 targets DNMT1 to the promoter of genes involved in Hippo signaling to enhance epithelial-mesenchymal plasticity and stem-like phenotypes (Liu et al. 2018). Similarly, a tight association and functional link exists between EZH2 and the H3K36me2 methyltransferase nuclear receptor binding SET domain protein 2 (NSD2). NSD2 is highly expressed in NEPC (Aytes et al. 2018) and functions to reprogram the epigenome by re-directing EZH2 binding to demarcate focal H3K27me3 domains (Zheng et al. 2012, Popovic et al. 2014). The activity of NSD2 is regulated by EZH2 and, in turn, is required for EZH2-mediated oncogenic programming in prostate cancer models (Asangani et al. 2013).

Likewise, EZH2 functions with lysine-specific demethylase 1 (LSD1), a dual histone H3K4 and H3K9 demethylase. The long ncRNA HOTAIR acts as a scaffold to tether EZH2 and LSD1 (Tsai et al. 2010), which act coordinately at bivalent chromatin domains to enforce a state of heightened cell plasticity via concerted repression of developmental genes (Adamo et al. 2011). LSD1 can also demethylate and stabilize non-histone substrates, such as DNMT1 (Wang et al. 2009a), as well as having functions entirely independent of its enzymatic activity; indeed, in prostate cancer, LSD1 collaborates with ZNF217 to activate gene networks involved in the regulation of stem cell phenotype in a demethylase-independent manner (Sehrawat et al. 2018). Notably, mimicking EZH2 inhibition, silencing LSD1 re-activates the AR-driven epithelial differentiation program in androgen-independent cell lines (Sehrawat et al. 2018). In small cell lung cancer, LSD1 inhibitors reduced expression of neuroendocrine lineage genes and caused complete tumour regression in a patient-derived mouse model (Augert et al. 2019); whether NEPC is similarly addicted to LSD1 is yet to be explored.

Emerging studies are beginning to shed light on how EZH2-mediated epigenetic reprogramming facilitates neuroendocrine differentiation. In particular, EZH2 can be activated by transcription factor 4 (TCF4), a key transcription factor in Wnt/β-catenin signaling, leading to the deposition of repressive H3K27me3 histone marks along the microRNA miR-708 promoter (Shan et al. 2019). Silencing miR-708 relieves its inhibition of neuronatin (Ryu et al. 2013), a mediator of neuronal differentiation, as well as the stem cell-like factor CD44 (Saini et al. 2012). Elevated Wnt signaling is a feature of NEPC tumours (Yang et al. 2005), and inhibiting TCF4 prevented adenocarcinoma-to-NEPC conversion following androgen deprivation (Shan et al. 2019). Moreover, EZH2 activity has been reported to be coupled to cAMP-response element binding protein (CREB) activation in prostate cancer models; CREB-induced H3K27me3, and neuroendocrine differentiation could be blocked by EZH2 inhibition (Zhang et al. 2018). Mechanistically, activation of the CREB/EZH2 axis facilitates epigenetic repression of the anti-angiogenic factor thrombospondin-1 (TSP1) leading to overt angiogenesis and neuroendocrine features in
prostate tumour xenografts (Zhang et al. 2018). Finally, the MEK/ERK pathway governs EZH2 transcriptional upregulation and recruitment to the E-cadherin promoter, thereby facilitating epithelial-mesenchymal plasticity and expansion of prostate cancer stem/progenitor cells (Cai et al. 2012, Nolan et al. 2015). These studies nominate EZH2 as a central hub integrating oncogenic signaling cascades to reprogram the epigenetic landscape in support of lineage plasticity and neuroendocrine differentiation.

Although EZH2 is highly expressed in NEPC, these tumours often exhibit a more relaxed chromatin structure (Park et al. 2018). To resolve this paradox, recent evidence suggests that the function of EZH2 extends beyond PRC2 and histone methylation. In androgen-independent prostate cancer (AIPC), EZH2 has been reported to interact with the AR to transcriptionally activate target gene expression (Xu et al. 2012, Kim et al. 2018). In these tumours, the AR does not function canonically but is ‘reprogrammed’ to execute a distinct transcriptional program (Wang et al. 2009b); for example, the AR is selectively recruited to the promoter of the gene encoding UBE2C, a cell cycle checkpoint inhibitor that is highly upregulated in both AIPC and NEPC tumours (Tzelepi et al. 2012). Re-directing AR transcriptional output may be regulated by the local chromatin environment mediated, in part, by AR interaction with EZH2 (Kim et al. 2018). EZH2 has also been reported to interact with and regulate the transcriptional activity of STAT3 (Kim et al. 2013), the nuclear factor κB (NF-κB) pathway (Lee et al. 2011), and the SWI/SNF chromatin remodeling complexes (Kim et al. 2015). Further mechanistic studies are required to address the duality between the canonical, PRC2-bound and non-canonical functions of EZH2 during emergent NEPC, which are summarized in Fig. 1.

**Epigenetic reprogramming of AR function in NEPC**

While the canonical AR signaling axis is attenuated in NEPC, recent clinical data from the West Coast SU2C-PCF Dream Team has highlighted persistent AR expression in a subset of patients with histologically small cell/neuroendocrine prostate tumours (Aggarwal et al. 2018). This is suggestive of a differentiation continuum and that an alternative AR transcriptional program may exist during emergent NEPC.

The AR cistrome is fluid and influenced by the local chromatin structure. In particular, the AR preferentially binds nucleosome-deprived regions (Jia et al. 2008), suggesting that preceding chromatin remodeling exposes otherwise transcriptionally restricted AR binding sites. In support of this notion, expression of the pioneer transcription factors FOXA1 and HOXB13 – which are capable of binding highly compacted chromatin regions to promote a permissive chromatin structure – in prostate epithelial cells yields reprogramming of the AR cistrome to resemble that of a prostate tumour (Pomerantz et al. 2015). However, the mechanisms underlying FOXA1-mediated AR reprogramming remain unclear. FOXA1 signaling can be dynamically regulated by co-factors; for example, the pluripotency reprogramming factor NANOG re-directs the FOXA1:AR complex to activate stem cell-associated networks (Jeter et al. 2016). A candidate AR reprogramming factor in NEPC is another Forkhead Box A pioneer factor, FOXA2, which is expressed almost exclusively in NEPC tumours (75% NEPC vs 4% adenocarcinoma) (Park et al. 2017). FOXA2 is not only enriched in NEPC but also co-expressed with the rare synaptophysin-positive cells in the adult prostate (Mirosevich et al. 2006). Earlier work by Matusik and colleagues revealed that FOXA2 expression is restricted primarily to basal cells during early prostate development, whereas FOXA1 is broadly expressed throughout the epithelium from early development to maturity (Gao et al. 2005). This spatially and temporally distinct expression pattern is unlike other endodermal organs, such as lung and liver, in which these factors tend to be co-expressed. Collectively, these findings are indicative of distinct functionalities of FOXA1 and FOXA2 in prostate cancer and support a role for the latter in NEPC; however, whether changes to the FOXA1:FOXA2 ratio can elicit AR cistrome reprogramming to support a NEPC phenotype is an open question.

The AR can directly recruit histone modifiers to influence chromatin architecture and gene expression. LSD1 is an important regulator of AR transcriptional activity, facilitating the suppression of canonical AR target genes via H3K4 demethylation (Cai et al. 2011). Interestingly, LSD1/AR binding and transactivation are reprogrammed by RB1 loss, which may have important implications for NEPC (Liang et al. 2017). Similarly, the BET family, in particular BRD4, interact with the AR to promote chromatin de-compaction and nucleosome eviction in aggressive prostate cancer (Urbanucci et al. 2017). A recent study showed that BET inhibition disrupts the recruitment of BRD4 to AR target gene loci (Faire et al. 2017) and can re-sensitize enzalutamide-resistant prostate cancer to ARPs (Asangani et al. 2016).

AR activity can be directly regulated by epigenetic modifiers. A variety of histone deacetylases (HDACs),
such as SIRT1, are capable of deacetylating the AR and thereby preventing association with the transcriptional co-activator p300 (Fu et al. 2006). Notably, SIRT1 is upregulated in NEPC, and its forced expression in prostate adenocarcinoma cell lines promotes neuroendocrine differentiation (Ruan et al. 2018). Further studies are warranted to delineate how reciprocity between epigenetic modifiers and the AR facilitates a chromatin architecture to support the NEPC phenotype.

**Interplay between transcription factors and the epigenome in NEPC**

FOXA1 is a transcription factor that is required for the development and maintenance of epithelial cells in the prostate (DeGraff et al. 2014), a function related to its role as a pioneer factor for AR (Jozwik and Carroll 2012). In concordance with its role in maintaining the epithelial phenotype, FOXA1 is down-regulated in NEPC and its loss in cell line models of prostate cancer drives neuroendocrine differentiation (Kim et al. 2017). Mechanistically, this occurs, at least in part, via de-repression of the FOXA1 target gene IL8, leading to activation of the MAPK/ERK pathway and neuron-specific enolase (ENO2) expression (Kim et al. 2017). Notably, FOXA1 mutations are common in prostate cancer. While the majority of these mutations enhance AR activity and promote an exaggerated pro-luminal differentiation program (Adams et al. 2019, Parolia et al. 2019), mutation of R219 modifies FOXA1 binding activity and hence pioneering function, resulting in altered chromatin accessibility throughout the genome that ultimately activates a transcriptional program associated with mesenchymal and neuroendocrine phenotypes (Adams et al. 2019). Thus, whereas wild-type FOXA1 suppresses lineage plasticity in the prostate, mutant forms of this key pioneer factor can have opposing functions.

Aberrant expression and/or activity of neuronal lineage-guiding transcription factors occurs concomitantly with loss of AR and FOXA1 in NEPC. BRN2 (also known as POU3F2) is a member of the Pit-Oct-Unc (POU)-domain family with a well-described role in neural differentiation that was recently implicated in NEPC (Bishop et al. 2017). It is overexpressed in this disease subtype relative to adenocarcinoma and directly drives neuroendocrine differentiation by enhancing the expression of SOX2. Moreover, BRN2 and SOX2 physically interact and act coordinately on chromatin to activate the expression of neural genes (Bishop et al. 2017). As mentioned, SOX2 is more highly expressed in metastatic CRPC tumours that exhibit loss of TP53 and RB1 (Mu et al. 2017), but whether this phenomenon is mediated by BRN2 is currently unknown. Notably, SOX2 is part of the ‘reprogramming cocktail’ of four pioneer transcription factors that together are capable of converting differentiated fibroblasts into induced pluripotent stem cells (Takahashi & Yamanaka 2016). More recently, another POU-domain transcription factor, BRN4, was found to interact with BRN2 and enhance its regulation of SOX2 (Bhagirath et al. 2019). Importantly, BRN2 is directly repressed by AR, an example of the extensive, direct interplay that occurs between the key factors controlling cell identity in prostate cancer (Fig. 2).

Ascl1 (also referred to as MASH-1) is another neural-specific transcription factor that was first implicated in NEPC by immunohistochemical analysis of tumour samples, which showed upregulation in tumours with neuroendocrine differentiation (Rapa et al. 2008). Subsequent work demonstrated that Ascl1 was sufficient to promote neuroendocrine differentiation in LNCaP cells (Rapa et al. 2013). Although the precise mechanisms by which it facilitates neuronal lineage reprogramming remain to be elucidated, ASCL1 was identified as a transdifferentiation factor in the context of induced fibroblast-to-neuron conversion (Vierbuchen et al. 2010). Like FOXA1/2, ASCL1 acts as a pioneer factor to bind closed chromatin; via this function, it can recruit BRN2 to chromatin to regulate a reprogramming-associated transcriptional signature (Vierbuchen et al. 2010). Of relevance to NEPC, ASCL1 is also activated in small cell lung cancer where it promotes Wnt signaling and CDK5-catalyzed phosphorylation (and hence inactivation) of RB1 (Meder et al. 2016).

Two distinct methodologies for identification of NEPC drivers using clinical transcriptomic data discovered ONECUT2, which appears to have a complex, multifaceted role in neuroendocrine differentiation (Rotinen et al. 2018, Guo et al. 2019). ONECUT2 suppresses the AR signaling pathway by both down-regulating AR gene expression and antagonizing chromatin-bound AR activity at the promoters of a subset of androgen-regulated genes (e.g. KLK3) (Rotinen et al. 2018). It also directly represses FOXA1 gene expression (Rotinen et al. 2018), further highlighting the complex interplay between transcription factors in prostate cancer (Fig. 2). In addition to the repression of genes that enforce the epithelial lineage, ONECUT2 directly activates neuroendocrine lineage markers such as PEG10 (Rotinen et al. 2018). Finally, ONECUT2 regulates the genome-wide association of HIF1α with chromatin
via direct regulation of SMAD3, leading to a synergistic effect with hypoxia on neuroendocrine plasticity (Guo et al. 2019). Interestingly, another NEPC-associated transcription factor, FOXA2, also cooperates with HIF1α to regulate pro-plasticity factors such as SOX9 and the histone demethylase JMJD1A (KDM3A) (Qi et al. 2010). Indeed, more recent studies have revealed that JMJD1A is a key integrator of hypoxia-associated signals, leading to altered histone methylation patterns that provoke a neuroendocrine transcriptional program (Maina et al. 2016, 2017).

Given the similarities between neuroendocrine lineage plasticity and epithelial-mesenchymal transition (EMT; (Davies et al. 2018)), it is not surprising that EMT-associated transcription factors have been implicated in NEPC. Indeed, ZEB1 (Viswanathan et al. 2017), Snail (McKeithen et al. 2010), Slug (Esposito et al. 2015), and FOXC2 (Paranjape et al. 2016) can all promote the emergence of neuroendocrine features, highlighting the convergent transcriptional networks involved in this process and EMT. An obvious implication of this convergence is that new therapies being developed to target EMT in cancer (Du & Shim 2016) could be harnessed for NEPC; as examples, targeting Notch (e.g. rovalpituzumab tesirine, tarextumab) and stem-ness (i.e. disulfiram, an inhibitor of ALDH, which is highly expressed by cancer stem cells) are promising strategies for both. Translation of these approaches and the eventual impact on cancer patients will undoubtedly be facilitated by a more comprehensive understanding of the similarities and differences – epigenetic, transcriptional, and other – between these two different manifestations of lineage plasticity.

In summary, transcription factors play a critical role in prostate cancer cell lineage plasticity. In theory, transcription factors that can engage their target sites on nucleosomal DNA and thereby act as pioneer factors to initiate transcriptional regulation at sites of closed chromatin (e.g. FOXA1, SOX2, and ASCL1) would act as the master controllers of such plasticity. However, the extensive interplay between these factors, summarised in Fig. 2, suggests that this concept is simplistic. Unraveling this interplay and deciphering the relationships between transcription factor function, epigenetic alterations, and microenvironmental cues will be challenging but is crucial to identify the most effective therapeutic strategies for NEPC.

**Post-transcriptional regulation of neuroendocrine lineage reprogramming**

While transcription factors are likely to be the most influential regulators of gene expression, post-transcriptional mechanisms also play a crucial role in controlling the ultimate output of a functional protein product. Indeed, post-transcriptional mechanisms such as mRNA splicing appear to significantly influence the transcriptional networks that arise during the transition from adenocarcinoma to NEPC. SRRM4 is a splicing factor.
specifically required for neural cell differentiation that was found to be elevated in NEPC (Zhang et al. 2015) and, by careful analysis of whole-transcriptome sequencing data, implicated in the majority (>66%) of splicing alterations seen in this disease state (Li et al. 2017). Key targets of SRRM4 include REST, a negative regulator of neurogenesis (Li et al. 2017), as well Bif-1 (Gan et al. 2018), MEAF6 (Lee et al. 2017), and the histone demethylase BHC80 (Li et al. 2019). The identities and roles of other splicing factors beyond SRRM4 that contribute to neuroendocrine lineage plasticity remain to be determined.

Post-transcriptional gene regulation by miRNAs is another ‘epigenetic’ mechanism that influences transcriptional networks during progression to NEPC. By targeting PP2R3A, a regulatory subunit of the tumour suppressor PP2A, overexpression of miR-652 promotes neuroendocrine differentiation, EMT, and metastasis in prostate cancer cell lines (Nam et al. 2018). Similarly, an EMT-promoting metastamiR, miR-194 (Das et al. 2017), can elicit neuronal phenotypes in prostate cancer; importantly, targeting miR-194 blocks neuroendocrine differentiation and the growth of NEPC-like patient-derived organoids (Fernandes RC, Toubia J, Townley S, Hanson AR, Dredge BK, Pillman KA, Bert AG, Iggo R, Das R, Obinata D, et al., unpublished observations; https://doi.org/10.1101/752709). Collectively, these findings reveal another layer of complexity in the epigenetic and transcriptional regulation of prostate cancer lineage plasticity and nominate miRNAs as another class of therapeutic targets in NEPC.

Post-translational regulation of epigenetic modifiers

Post-translational modification of epigenetic modifiers represents another layer of regulatory control of prostate cancer lineage plasticity and neuroendocrine differentiation. For example, EZH2 has been reported to act as both a transcriptional repressor and an activator in aggressive prostate cancer (Xu et al. 2012, Kim et al. 2018). This paradox may be attributed to post-translational modification of EZH2; AKT-mediated phosphorylation of EZH2 at serine-21 shifts EZH2 from a transcriptional repressor to a co-activator of the AR in androgen-independent prostate cancer (Xuet al. 2012). Enhanced AKT signaling is characteristic of NEPC (Dardenne et al. 2016). Similarly, EZH2 phosphorylation at threonine-311 by AMP-activated protein kinase (AMPK), a metabolic sensor activated by an energy deprivation state, attenuates EZH2-mediated epigenetic silencing by disrupting the Polycomb complex (Wan et al. 2018). Activation of AMPK is essential for hypoxia-induced neuroendocrine differentiation in prostate cancer cell lines (Lin et al. 2016b), thus linking EZH2 activity to metabolic reprogramming and emergent NEPC.

DNMTs are also regulated by post-translational modifications. Cross-talk between adjacent residues on DNMT1, lysine-142 methylation by SET7, and serine-143 phosphorylation by AKT regulates DNMT1 stability, with the phosphorylated form being more stable (Esteve et al. 2011). DNMT1 activity is also linked to functional RB1 loss; inactivation of RB1 allows ATM to directly bind DNMT1, leading to decreased DNMT1 stability via sequential acetylation and ubiquitylation (Shamma et al. 2013). Altered DNMT1 stability in turn results in abnormal DNA methylation patterning, including at genes implicated in neuroendocrine reprogramming such as FOXA1 and LHX2 (Shamma et al. 2013).

PKCα-mediated phosphorylation of LSD1 at serine-112 is required for its chromatin binding and demethylase activity. Of note, phosphorylated LSD1 targets H3K4 at the CDH1 (E-cadherin) promoter to repress transcription, thereby facilitating epithelial-mesenchymal plasticity and a metastatic phenotype (Feng et al. 2016). In short, post-translational modification of epigenetic modifiers appears to play an influential role in NEPC, and further work in this area will likely reveal novel therapeutic targets.

Targeting epigenetic and transcriptional regulators in NEPC

As our understanding of the NEPC epigenome advances, the prospect of targeting epigenetic mechanisms to reverse or delay neuroendocrine lineage transformation is beginning to show promise. The correlation between specific methylation patterns and high EZH2 levels in NEPC, and the association between these tumours and heightened cell plasticity, provide a rationale for development of epigenetic targeting strategies. Recent pre-clinical and clinical studies support this notion, as summarized in Table 1.

The transition from adenocarcinoma to NEPC is accompanied by a shift in the DNA methylation landscape (Beltran et al. 2016). As described, this is in part due to metabolic reprogramming leading to aberrant SAM production, the fuel for DNMT enzymes (Reina-Campos et al. 2019). In pre-clinical models, DNMT inhibition re-sensitized hormone-refractory, neuroendocrine-like
prostate cancer cell lines, and xenografts to ARPIs (Gravina et al. 2008, 2010, Reina-Campos et al. 2019). These results suggest that inhibition of DNMTs may be an attractive therapeutic strategy for patients with NEPC. Notably, the DNMT inhibitors decitabine and azacytidine are already approved by the FDA for treatment of myelodysplastic syndromes and could therefore be rapidly re-positioned for NEPC. However, in phase II clinical trials in CRPC, DNMT inhibitors did not elicit a strong, single agent anti-tumour response, although these trials were small and without molecular biomarker selection (Thibault et al. 1998, Sonpavde et al. 2011). Future clinical trials will be needed to assess the efficacy of DNMT-directed therapies in NEPC patients, possibly using loss of PKCζ/i as a biomarker of DNMT dependency.

One of the most well-documented dysregulated epigenetic factors in NEPC is EZH2 (Clermont et al. 2015, Beltran et al. 2016). In pre-clinical NEPC models, treatment with EZH2 inhibitors reversed the neuroendocrine phenotype established by drivers of lineage plasticity, namely N-Myc (Dardenne et al. 2016, Berger et al. 2019) or RB1/TPS3 loss (Ku et al. 2017), and re-sensitized tumours to the ARPI enzalutamide. These studies are currently being translated through clinical trials testing EZH2 inhibitors. In particular, the EZH2 inhibitor PF-06821497 is currently being tested in a phase I dose-escalation study in patients with advanced/metastatic CRPC (NCT03460977). Likewise, the phase Ib/II ProSTAR trial is assessing the utility of combining the EZH2 inhibitor CPI-1205 with enzalutamide or abiraterone/prednisone in patients with metastatic CRPC (NCT03480646). Clinical activity has been reported in both the enzalutamide and abiraterone arms with ≥80% PSA reductions in 20–30% of patients, and 40–75% of patients achieved stable disease control. Aurora A inhibitors such as alisertib (MLN8237), which result in destabilization of N-Myc, have also shown some promise in clinical trials. A phase II clinical trial of alisertib in NEPC patients reported a modest clinical benefit, with a 2.3 month improvement in progression-free survival (NCT01799278). A second phase I/II clinical trial of alisertib in combination with abiraterone in CRPC with neuroendocrine differentiation was terminated early due to toxicity and lack of clinical benefit; however, a decrease in neuroendocrine marker expression was reported in a subset of patients (NCT01848067) (Lin et al. 2016a).

Table 1 Clinical trials for epigenetic therapies in prostate cancer.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Agent</th>
<th>Clinical status</th>
<th>Clinical Trial ID</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT inhibition</td>
<td>Azacitidine</td>
<td>II</td>
<td>NCT00384839</td>
<td>PSA doubling time ≥3 months in 19/34 hormone-refractory CRPC patients</td>
</tr>
<tr>
<td>DNMT inhibition</td>
<td>Azacitidine + chemotherapy (docetaxel/prednisone)</td>
<td>II</td>
<td>NCT00503984</td>
<td>PSA response in 10/19 hormone-refractory mCRPC patients; significant demethylation of GADD45A</td>
</tr>
<tr>
<td>DNMT inhibition</td>
<td>Decitabine + ARPI (enzalutamide) (pembrolizumab)</td>
<td>I/II</td>
<td>NCT03709550</td>
<td>Recruiting mCRPC; no results reported</td>
</tr>
<tr>
<td>DNMT inhibition</td>
<td>Tazemetostat</td>
<td>II</td>
<td>NCT02875548</td>
<td>Recruiting relapsed/refractory CRPC; no results reported</td>
</tr>
<tr>
<td>EZH2 inhibition</td>
<td>PF-06821497</td>
<td>I</td>
<td>NCT03460977</td>
<td>Retraining mCRPC; no results reported</td>
</tr>
<tr>
<td>EZH2 inhibition</td>
<td>CPI-1205 + ARPI (enzalutamide or abiraterone/prednisone)</td>
<td>I/II</td>
<td>NCT03480646</td>
<td>Recruiting mCRPC; no results reported</td>
</tr>
<tr>
<td>N-Myc inhibition</td>
<td>CPI-1205 + immunotherapy (ipilimumab)</td>
<td>I/II</td>
<td>NCT03525798</td>
<td>Recruiting advanced solid tumours; no results reported</td>
</tr>
<tr>
<td>BET inhibition</td>
<td>MLN8237</td>
<td>II</td>
<td>NCT01799278</td>
<td>Progression-free survival of 2.3 months in NEPC arm</td>
</tr>
<tr>
<td>BET inhibition</td>
<td>ZEN003694 + ARPI (enzalutamide)</td>
<td>I/II</td>
<td>NCT02711956</td>
<td>Ongoing in mCRPC; no results reported</td>
</tr>
<tr>
<td>BET inhibition</td>
<td>GS-5829 + ARPI (enzalutamide)</td>
<td>I/II</td>
<td>NCT02607228</td>
<td>Ongoing in mCRPC; no results reported</td>
</tr>
<tr>
<td>BET inhibition</td>
<td>OTX015</td>
<td>I</td>
<td>NCT02698176</td>
<td>Terminated; limited efficacy</td>
</tr>
<tr>
<td>LSD1 inhibition</td>
<td>INCB059872</td>
<td>I/II</td>
<td>NCT02712905</td>
<td>Recruiting, including a NE arm; no results reported</td>
</tr>
<tr>
<td>LSD1 inhibition</td>
<td>Phel Pazine</td>
<td>II</td>
<td>NCT02217709</td>
<td>Recruiting in localized prostate cancer; no results reported</td>
</tr>
<tr>
<td>LSD1 inhibition</td>
<td>Phel Pazine + chemotherapy (docetaxel)</td>
<td>II</td>
<td>NCT01253642</td>
<td>Terminated; low enrollment</td>
</tr>
</tbody>
</table>

ARPI, androgen receptor pathway inhibitor; BET, bromodomain and extra-terminal motif; CRPC, castration-resistant prostate cancer; DNMT, DNA methyltransferase; mCRPC, metastatic castration-resistant prostate cancer; NE, neuroendocrine; NEPC, neuroendocrine prostate cancer.
Immunotherapy with checkpoint inhibitors, such as PD-1 and PD-L1, has been largely unsuccessful in prostate cancer (Isaacs on Velho & Antonarakis 2018). Interestingly, in melanoma and ovarian cancer models, EZH2 is negatively associated with tumour-infiltrating CD8+ T-cells; EZH2-mediated silencing of T-cell attractant chemokines as well as genes essential for antigen processing and presentation represses tumour immunogenicity and T-cell infiltration (Peng et al. 2015, Nagarsheth et al. 2016, Zingg et al. 2017). Accordingly, EZH2 inhibition can synergize with immune checkpoint inhibitors to augment the infiltration of CD8+ T-cells into the tumour microenvironment and improve tumour control (Zingg et al. 2017). This has important implications for prostate cancer as EZH2 inhibition may have utility in reversing the immunologically ‘cold’ nature of these tumours. Clinical trials of EZH2 inhibitors in combination with Ipilimumab (a monoclonal antibody that activates the immune system by targeting CTLA-4) in patients with advanced solid tumours are ongoing (NCT03525795).

Epigenetic drugs targeting the BET family of chromatin readers and transcriptional regulators, such as BRD4, are also in clinical development. Pre-clinical studies have shown that drugs targeting BRD4 disrupt its recruitment to chromatin sites bound by AR, suppress signaling, and, alone or in combination with ARPIs, demonstrate anti-tumour activity (Asangani et al. 2014, 2016, Welti et al. 2018). This is due, in part, to the requirement of BRD4 activity to mediate chromatin accessibility and AR transcriptional reprogramming during prostate cancer progression (Urbanucci et al. 2017). A phase I/II trial is ongoing to examine the effects of the BET inhibitor ZEN003694 in combination with enzalutamide in CRPC (NCT02711956). Likewise, LSD1-targeting drugs may also have utility in NEPC since LSD1 is highly expressed in the androgen-independent disease state, modulates FOXA1-dependent AR cistrome reprogramming, and activates stem cell-associated gene networks (Cai et al. 2014, Sehrawat et al. 2018). Together, these studies suggest that concerted blockade of the AR and epigenetic reprogramming factors might be an effective approach to prevent or delay NEPC; further biomarker-driven clinical trials in this space are warranted.

Summary

It is becoming increasingly clear that epigenetic and transcriptional dysregulation is central to the emergence and maintenance of highly aggressive and lethal NEPC tumours. Aberrant function of master epigenetic regulators, such as DNMT1 and EZH2, as well as pioneer transcription factors facilitate chromatin remodeling to support the activation of alternative lineage programs that, under potent androgen receptor antagonism, largely default to neuroendocrine differentiation. These epigenetic changes are fueled, in part, through metabolic reprogramming. The exquisite dependency of NEPC tumours on the epigenetic and transcriptional machinery provides an Achilles’ heel that has already begun to be exploited therapeutically. These epigenetic therapies have shown promise clinically; however, numerous challenges remain with respect to patient stratification, timing, and combination with ARPIs and/or immunotherapy to refine the development of more precise, biomarker-driven treatment strategies for NEPC.

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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Endocrine-Related Cancer

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Epigenetics of prostate cancer plasticity

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