Resistance to Androgen Receptor Signaling Inhibition Does Not Necessitate Development of Neuroendocrine Prostate Cancer

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Abstract

Resistance to AR signaling inhibitors (ARSi) in a subset of metastatic castration-resistant prostate cancers (mCRPC) occurs with emergence of AR-negative Neuroendocrine Prostate Cancer (NEPC), coupled with mutations/deletions in PTEN, TP53, RB1, and overexpression of DNMTs, EZH2, and/or SOX2. To resolve whether lack of AR is the driving factor for the emergence of the NE phenotype, molecular, cell, and tumor biology analyses were performed on 23 prostate cancer patient-derived xenografts recapitulating the full spectrum of genetic alterations proposed to drive NE differentiation. Additionally, phenotypic response to CRISPR-Cas9-mediated AR knockout in AR-positive CRPC cells was evaluated. These analyses document that: 1) ARSi-resistant NEPC can develop without androgen deprivation treatment; 2) AR signaling in ARSi-resistant AR+/NE+ double positive “ampicrine” mCRPCs does not suppress NE differentiation; 3) lack of AR expression does not necessitate acquiring a NE phenotype despite concomitant mutations/deletions in PTEN and TP53, and loss of RB1, but can occur via emergence of an AR-/NE- double negative prostate cancer (DNPC); 4) despite DNPC cells having homogeneous genetic driver mutations, they are phenotypically heterogeneous, expressing basal lineage markers alone or in combination with luminal lineage markers; and 5) AR loss is associated with AR promoter hypermethylation in NEPCs but not in DNPCs.
Introduction

The normal adult human prostate is composed of a simple stratified epithelium, the homeostasis of which is maintained via adult stem/progenitor cell turnover producing a steady-state, self-renewing condition (1). In the human adult male, prostate epithelial stem cells undergo asymmetric division for self-renewal while producing progenitor cells with limited proliferative ability (2). The percentage of epithelial cells proliferating per day (i.e. 0.19 +/- 0.03%) is remarkably low in the human adult benign prostate, which balances the equally low percentage of epithelial cells dying per day (3). During this steady-state maintenance condition, the turnover time (i.e. the time required to renew the epithelium) is 500 +/- 79 days (3). Neither stem nor progenitor cells express AR protein; however, they require AR-dependent paracrine factors (i.e. andromedins) from the stroma for their proliferation but not survival (4, 5). It is proposed that a rare subset (i.e. 0.59%) of adult prostate basal cells are the epithelial stem/progenitor cells, which co-express the full spectrum of prostate epithelial cell markers [i.e. keratins (KRT) 5, 6A, 8, 14, 18, and 19, the transcription factor p63, and Glutathione-S-Transferase [pi (GSTP1)], but not AR (2, 6). This is supported by the fact that while the growth fraction in basal cells based upon Ki67 expression is quite low (i.e. 1.65 +/- 0.12% positive), it is 12-fold higher than the growth fraction in luminal cells [i.e. 0.14 +/- 0.06% positive, (Figure 1A)] (7, 8). Consistent with this basal location for the proliferating epithelial stem/progenitor cells is the nuclear expression of c-MYC by AR-negative cells within this compartment in benign human glands (Figure 1B).

These basal adult progenitor cells differentiate into one of three lineage progeny (6-11). In the first lineage, progenitors at a very low frequency (i.e. <1%) differentiate into proliferation-quiescent neuroendocrine (NE) cell progeny with loss of expression of p63, GSTP1, and basal cell keratins (KRT 5, 6A, 14, and 19) without gaining expression of AR (9-13). While the majority of these NE cells lack detectable expression of the highly prostate-specific transcription factor, HOXB13 (Figure 1C), a small subset (i.e. 17%) do express this protein at a low level equal to that expressed by basal cells. In contrast, these cells acquire high expression of NE lineage markers like chromogranin A (CHGA) (Figure 1C), B (CHGB), and synaptophysin (SYP) (9-13). In the second lineage, progenitors differentiate into basal cell progeny, which mature and maintain expression of KRT 5, 6A, 14, and 19, p63 (Figure 1D), and GSTP1 (Figure 1E), while losing KRT8 and KRT18 expression without gaining expression of AR (6-13). Also, these basal cells characteristically express the transcription factors SOX2 (Figure 1F) (14), YAP-1 (15), and nerve growth factor receptor (NGFR a.k.a. p75)] (Figure 1D) (16) coupled with low to moderate HOXB13 expression (Figure 1G) (17); consistent with its expression being AR-independent (18). In the third lineage, progenitors differentiate into luminal cell progeny, which maintain expression of KRT 8 and 18 while losing expression of all other stem/progenitor markers [i.e. KRT 5, 6A, 14, 19, p63 (Figure 1D), GSTP1(Figure 1E), SOX2 (Figure 1F), YAP-1, and NFG (Figure 1D)] (6-11, 15). In addition, they acquire expression of AR (6), and prostate-specific membrane antigen (PSMA a.k.a. FOLH1) (19), along with a 6-fold increase in HOXB13 (Figure 1G-H) (17, 20). AR transcriptional activity is not required, however, for commitment to luminal cell differentiation (21) as HOXB13 is not an AR-target gene (18) and PSMA transcription is inhibited by AR (22). In contrast, AR expression and ligand occupancy is required for terminal luminal differentiation into a mature proliferation-quiescent secretory cell (21). This terminal
differentiation is characterized by gain of expression of AR-dependent prostate luminal cell lineage markers, such as NKX3.1, prostate-specific antigen (PSA a.k.a. KLK3), and hK2 (KLK2) with no expression of NE markers (6, 23) or the proliferation markers, Ki67 (Figure 1A) or c-Myc (Figure 1B).

During prostate carcinogenesis, molecular changes occur in prostate epithelial cells such that AR signaling is subverted from a growth suppressor of c-Myc expression to a cell autonomous oncogenic stimulator of c-Myc expression and thus malignant growth (24-26). Due to this acquired oncogenic AR signaling addiction, androgen deprivation therapy (ADT) is the standard-of-care for mCRPC. This is because ADT not only inhibits prostate cancer cell proliferation, but also induces apoptotic cell death (4). While initially responsive to such “castration therapy”, metastatic cancer cells inevitably progress to a castration-resistant (CR) state given enough time and selective pressure (27). In the majority of cases, these mCRPC cells continue to express AR and their lethal growth is still stimulated by AR-dependent transcription despite greater than 90% suppression of serum androgen by ADT (27). These results validate that further disrupting AR function is a rational therapeutic approach for mCRPCs progressing on ADT.

Based upon this realization, next generation AR signaling inhibitors (ARSi), such as Abiraterone Acetate (Abi), Enzalutamide (Enza), and Apalutamide, were developed and clinically documented to increase the survival of men with mCRPC progressing after first-line ADT and when given in combination with first-line ADT (27). Despite these advances, mCRPC remains a lethal disease due to the inevitable progression of these cancers to an ARSi-resistant state (28, 29). Approximately 1/3 of these ARSi-resistant cancers are AR-negative (28). The proposed mechanism for this progression involves an initially AR-positive adenocarcinoma (ARPC) “losing” its luminal cell differentiation via loss of AR activity (30-34). It has been proposed that such loss of AR-dependent transcription enables “lineage plasticity”, driving trans-differentiation of the initial ARPC to a more aggressive lethal AR-negative treatment-related neuroendocrine prostate cancer (NEPC) phenotype (30-35).

Therefore, to interrogate the relationship between ARSi-resistance and NE differentiation, three complementary approaches were taken. First, the growth characteristics and expression of basal vs. luminal vs. NE lineage markers were evaluated in a large series (n = 23) of previously characterized patient-derived xenografts (PDXs) in addition to several newly established PDXs, which collectively recapitulate the full spectrum of clinically-important genetic alterations in mCRPC. Second, hypermethylation of the AR promoter as a putative mechanism for the loss of AR expression was evaluated. Third, the in vitro and in vivo growth characteristics vs. marker expression of the ARPC LNCaP-95 (LN-95) mCRPC cells initially exhibiting AR activity were determined following CRISPR-Cas9 dependent elimination of total AR protein expression.

Results

Development of ARSi-Resistant Neuroendocrine Prostate Cancer Does Not Require Prior ADT
The NCI-H660 cell line was established from a cervical lymph node metastasis from an untreated 63 year old male diagnosed with small cell cancer of the prostate who presented with metastatic sites in the brain, liver, lymph nodes, subcutaneous tissue, bone, and bone marrow (36). The patient died 4 days post-tissue harvest (i.e. 18 days post-initial diagnosis) having never received ADT. The in vivo growth of H660 in adult male NSG hosts is ARSi-resistant as it grows equally well in intact or castrated hosts with a doubling time of 10 +/- 5 days (Figure 2A), which is not affected by daily oral treatment with a therapeutically effective dose of either Abiraterone Acetate (AA) or Enzalutamide (Enza). Histologically, this xenograft has been classified as is a small cell carcinoma (Figure 2B). It has a TP53 exon 9-11 deletion (37) and TMPRSS2-ERG fusion due to a homozygous intronic deletion (38), but expresses neither AR (Figure 2C) nor ERG (Figure 2D). It also does not express HOXB13 (Figure 2E) (39) or the basal cell markers GSTP1, KRT5, NGFR (Figure 2F), or p63 (Figure 2F). It does, however, uniformly express NE markers like SYP (Figure 2G) and CHGA (Figure 2H). Thus, H660 is a “classic” ARSi-resistant NEPC, which developed without ADT treatment.

AR Does Not Suppress NE differentiation in Amphicrine Prostate Cancer PDXs

LvCaP-2 is a newly described prostate cancer PDX derived from a liver metastasis obtained at rapid autopsy at Hopkins from an ARSi-resistant mCRPC patient (29). When adult male hosts bearing the LvCaP-2 PDX are castrated, the cancer stops growing for ~1 month before relapsing (29). Subsequent passage of a relapsing tumor in castrated hosts results in a variant, named LvCaP-2R, that grows equally well in intact and castrated hosts (doubling time of 10 +/- 3 days vs. 9 +/- 2 days, respectively). Growth of LvCaP-2R in castrated mice is resistant to daily oral treatment with Abi or Enza (29). Histologically, LvCaP-2 and LvCaP-2R are high grade adenocarcinomas, which genetically have a hemizygous loss-of-function (LOF) truncating mutation in TP53 (T211fs) and hemizygous deleterious mutation (R130Q) in PTEN with loss of PTEN protein expression (29). While they have wild type RB1, there is only limited focal expression of RB1 protein. In addition to expressing prostate-specific HOXB13 and luminal-, but not basal-specific markers (Figure 3A), they express NE markers (Figure 3B). This is despite expressing AR at a 52-fold higher mRNA level (Figure 3A) with 11-fold higher nuclear localization of AR protein compared to normal prostate luminal cells (29). Importantly, AR and NE markers like SYP are co-expressed in the same cell (Figure 3C-E). This AR is functional as documented by expression (Figure 3F) and secretion of AR-target proteins like PSA (Supplemental Figure 1A). They also express REST and YAP1 (Figure 3A), the latter of which is a basal lineage marker, and neither of which are expressed in NEPC (15, 40). These cancers thus represent “amphicrine” prostate carcinomas [AMPC, (i.e. AR+/NE+)] (41, 42). A similar co-expression of luminal and NE markers without basal marker expression occurs in the AR-positive LuCaP-77CR PDX, which is an ARSi-resistant AMPC variant of LuCaP-77 derived from a bone (femur) metastasis at rapid autopsy (43). These results document that AR signaling does not suppress NE differentiation in ARSi-resistant AR+/NE+ double positive AMPCs, and demonstrates that NE differentiation can occur in the presence of ARSi-resistant AR signaling, resulting in ARSi-resistant AR+/NE+ double positive AMPCs.
ARSi-Resistance in PDXs Lacking AR Expression Does Not Necessitate NE Differentiation

The BCaP-1 PDX is derived from a soft tissue metastasis adjacent to the right tibia obtained at rapid autopsy from a 63-year old African-American who at the time of initial presentation had bone and lymph node metastases and an initial diagnostic prostate biopsy that was positive for carcinoma with a Gleason sum score of 9. Over the next year, the patient was treated with ADT followed by palliative external beam radiation of the bone before death, but never received treatment with next generation ARS-inhibitors (Table 1, Supplemental Figure 2A). At autopsy, 3 of the 4 metastatic lesions collected were completely negative for AR, PSA, and NKX3.1, including the bone met used to establish the BCaP-1 PDX (Supplemental Table 1). In 1 of the collected metastases (i.e. Bone – L4) and the localized prostate lesion, AR staining was heterogeneous (Supplemental Table 1). Expression of AR-dependent genes (e.g. PSA, NKX3.1, etc.) were consistent with the AR expression pattern in these lesions. In contrast, all 4 of the metastatic lesions collected from the patient in addition to the localized cancer in the prostate had PTEN-loss, RB1-loss, and mutated TP53 (i.e. genetic drivers), which are consistent with the PDX. Despite the patient never being exposed to ARSi-treatment, the in vivo growth of BCaP-1 PDX is ARSi-resistant as it grows equally well in intact or castrated hosts with a doubling time of 20 +/- 5 days (Figure 4A), which is not affected by additional treatment with either Abi or Enza. This is consistent with the fact that the metastatic lesion from which this PDX is derived was AR-negative. Histologically, this PDX is a high grade carcinoma (Figure 4B). Based upon a combination of RNAseq, western blotting, immunohistochemical (IHC) staining, and targeted DNA sequencing analyses, Table 1 summarizes the most relevant characteristics of the BCaP-1 PDX. Consistent with the metastatic lesions in the patient, BCaP-1 uniformly expresses mutated TP53 (R175H) (Figures 3A, 4C) coupled with minimal expression of a mutated RB1 (P298fs) allele and loss of the other wild type RB1 allele (Figures 3A, 4D). These cells uniformly lack PTEN expression due to a homozygous deletion (Figure 3A), resulting in no detectable PTEN protein (Figure 4E). They also express a mutated CTNNB1 (S45F) (Figure 3A), which is localized in the nucleus (Figure 4F), presumably activating CTNNB1 driven gene expression. BCaP-1 has high expression of c-MYC and Ki67 (Figure 3A) with a high proportion of cells showing nuclear localization of these proteins (Figures 4G-H). This is despite minimal expression of AR or glucocorticoid receptor (GR) mRNA (Figure 3A), and no expression of AR or GR protein. Consistent with the lack of AR protein, BCaP-1 does not express AR-target genes like PSA and NKX3-1 that are characteristic of luminal cells (Table 1, Figure 3E, and Supplemental Figure 1A). However, they do uniformly express other markers characteristic of luminal cells, such as KRT8 and KRT18 (Figures 3A, 4I), while heterogeneously expressing characteristic basal cell markers like KRT5 (Figures 3A, 4J), GSTP1 (Figure 4K), NGFR (a.k.a. p75 neurotrophin receptor) (Figure 4M), and p63 (Figure 4M), coupled with uniform expression of SOX2 (Figure 4N) (14, 44, 45). Additionally, they heterogeneously express a moderate to high level of nuclear HOXB13 protein (Figure 4O). Retention of HOXB13 expression confirms its prostatic origin. This moderate to a high level HOXB13 expression in a subset of cells is significant since this is the level of nuclear expression characteristic of prostatic luminal cells (20), and is consistent with its expression being AR-independent (18). These cells also express FOXA1 (Figure 3A), which is an important co-regulator of HOXB13 via its binding to a 37-bp regulatory element that activates expression independent of
AR transcriptional activity (18). This heterogeneous moderate to high expression is consistent with why this PDX has a lower level of HOXB13 mRNA (Figure 3A) and protein detected by western blot (Supplemental Figure 1B) than ARPCs. BCaP-1 lacks expression of ERG (Figure 3A), and the majority of NE-related genes (Figure 3B), including CHGA (Figure 4P). They also express REST (Figure 3A) and the basal marker YAP1 (Figure 3A); neither of which are expressed in NEPC (15, 40). Collectively, these results document that despite BCaP-1’s lack of AR expression, even when coupled with a LOF mutations in PTEN and RB1, plus putative gain-of-function (GOF) mutation in TP53 together with overexpression of DNMT1 and EZH2 (Figure 3A), does not drive their differentiation into a NEPC. Rather, it is an example of an AR-/NE- DNPC heterogeneously composed of cells expressing both basal and luminal cell characteristics, suggestive that their cancer-initiating cell is an AR-negative progenitor cell whose malignant transformation does not require exposure to ARSi.

Three additional PDXs were established from a 65-year old European-American patient with a germline BRCA2 mutation (Y2215fs) who underwent resection for a non-invasive urothelial carcinoma of the bladder a year before having a prostate biopsy that was positive for Gleason 9 prostate cancer with perineural invasion. Over the next 3 years, the patient had a radical prostatectomy for locally advanced disease (i.e. extra-prostatic extension, seminal vesicle invasion, and lymphatic invasion), followed by ADT, external beam radiation, taxane chemotherapy, and olaparib before undergoing a rapid autopsy upon his death (Supplemental Figure 2B). All metastatic lesions collected from this patient had PTEN- and RB1-loss, in addition to mutated TP53 (Supplemental Table 1). From this autopsy, 3 independent PDXs were established from a liver metastasis (LvCaP-3), a lung metastasis (LgCaP-1), and a peri-pancreatic lymph node metastasis (PLNCaP-1). The in vivo growth of each of these PDXs is ARSi-resistant as documented by the fact that each grows equally well in intact vs. castrate hosts and is not affected by the addition of treatment with either Abi or Enza. Interestingly, though derived from the same patient with lethal mCRPC, these 3 ARSi-resistant PDXs have different in vivo growth rates (Table 1, Figure 5A), despite the fact that all 3 PDXs are histologically high grade carcinomas (Figures 5B, 6A, 7A).

Table 1 summarizes the most relevant characteristics of these 3 additional ARSi-resistant PDX models. All 3 of these additional PDXs lack expression of ERG (Figure 3A) and NE markers including CHGA, CHGB, and SYP (Figure 3B), with minimal expression of either mutated BRCA2 (Y2215fs) not sure which allele is being expressed, if at all (Figure 3A) or mutated TP53 (R282W), and no expression of PTEN due to homozygous deletion. In addition, they minimally express RB1 mRNA with no detectable nuclear expression of RB1 protein (Figure 3A, Table 1). None of the cancer cells in these 3 PDXs express AR protein; thus, explaining why they are resistant to ARSi. The tumors have a high proportion (>50%) of cells with nuclear staining for c-MYC (Table 1, Figures 5C, 7B) and Ki67 (Table 1, Figure 5D). They do not express KLK3 (PSA), FOLH1 (PSMA), or NKX3.1 (Table 1, Figures 3A, 3E, and Supplemental Figure 1A). Again, like AR-negative BCaP-1 cells, the fact that there is no expression of PSA or NKX3.1 in LvCaP-3, LgCaP-1, or PLNCap-1 cells is predictable since these are known AR-target genes. This is despite the fact that these cells express GR (Figure 3A). All 3 heterogeneously express a moderate to high level of nuclear HOXB13 protein (Figures 5E, 6B, 7C). Again, this cellular heterogeneity is consistent with these PDXs having a lower level of HOXB13 mRNA (Figure 3A).
and protein detected by western blot (Supplemental Figure 1B) than ARPCs. Retention of HOXB13 expression in all 3 of these PDXs confirms their prostatic origin. Unlike BCaP-1 cells, however, HOXB13 expression in all 3 of these latter PDXs is independent of FOXA1 since they essentially have no expression of this transcription factor (Figure 3A).

All 3 of these PDXs retain expression of luminal characteristic KRT8 (Figures 3A, 6C, 7D) and KRT18 (Figure 3A). Interestingly, while neither LvCaP-3 nor LgCaP-1 express basal characteristic KRT5 or KRT14, PLNCaP-1 co-expresses KRT5, 8, 14, 18, and 19 (Figure 3A). They also all uniformly express the basal marker GSTP1 (Figures 5F, 6D, 7E), in addition to other basal characteristic markers like NGFR focally and p63 sporadically (Figures 5G, 6E-F, 7F). Thus, despite the lack of AR and RB1 expression coupled with mutations in PTEN and TP53, and overexpression of DNMT1 and EZH2 (Figure 3A), they are not NEPCs. Again, they express REST and the basal marker YAP1 (Figure 3A), which are not expressed in NEPC (15, 40). Thus, these PDXs are again examples of AR-/NE- DN prostate carcinomas with heterogeneous basal and luminal cell characteristics.

Progression of ARPC PDX to ARSi-Resistance

These PDX models document that the lack of AR-dependent transcription in AR-negative prostate cancer cells does not necessitate differentiation into NEPC even when combined with loss-of-function/expression of PTEN, RB1 and p53. A possible explanation is that the cancer-initiating cells in these DN-PDXs are derived from transformed progenitor cells that never expressed AR and thus are unresponsive to AR-targeted therapy. Clearly, however, the majority of mCRPCs express AR. This raises the issue of whether the subset of ARPCs that lose AR expression in their progression to ARSi-resistance induces lineage trans-differentiation into NEPC.

To test this possibility, another newly derived PDX, LvCaP-1, was used as a model system. LvCaP-1 is derived from a liver metastasis obtained at rapid autopsy from a 64-year old European-American who was treated over a 17-year period starting with a radical prostatectomy (Gleason Sum 8), then with a prostate cancer vaccine (GVAX) followed by ADT, docetaxel plus strontium-89 (89Sr) and external beam radiation, Abi, etoposide, and cisplatin (Supplemental Figure 2C) (46). Like the radical prostatectomy specimen, all metastatic lesions collected from this patient at the time of autopsy were AR-positive and NKX3-1-positive, in addition to having PTEN-loss and mutated TP53 (Supplemental Table 1). The most relevant characteristics of this PDX are summarized in Table 1. Histologically, LvCaP-1 is a high grade adenocarcinoma (Figure 8A). Like the original patient-derived liver metastasis (46), this PDX has a 20-fold amplification of the AR gene locus and a 64-fold higher level of wild type AR mRNA as compared to localized prostate cancer (Figure 3A). Essentially, all LvCaP-1 cells exhibit high nuclear staining of AR-FL (Figure 8B). It has a high proportion (>70%) of cells expressing c-MYC and Ki67 (Figure 3A). LvCaP-1 expresses NKX3-1, HOXB13 (mutated G84E), FOLH1, KLK2, and KLK3 (Figures 3A, 3E, 8C), but with only a low level of PSA secretion [i.e. serum PSA of 1.4 +/- 0.4 ng/ml/g tumor, (Supplemental Figure 1A)]. This is coupled with expression of mutated TP53 (R248Q) (Figure 8D), SOX2, mutated SPOP (F133L), and mutated PTEN (V317fs), but no expression of NE
markers like CHGA, CHGB, and SYP (Figures 3A-B). LvCaP-1 also does not express basal cell markers like GSTP1 (Figure 8E), p75 (Figure 8F), or p63 (Figure 8F).

When intact mice bearing LvCaP-1 PDXs growing with a 12 +/- 2 day doubling time are castrated, the cancers regress by >90% over the next 80 days before relapsing (Figure 8G), documenting that LvCaP-1 is an androgen-responsive ARPC. A relapsing tumor was serially passaged in castrated male mice to produce the LvCaP-1R PDX which grows with a doubling time of 15 +/- 5 days in castrated hosts (Figure 8H). Oral treatment with effective daily doses of either Abi or Enza (29) had no effect upon the growth of LvCap-1R in castrated male hosts, documenting its ARSi-resistance. Histologically, the LvCaP-1R is a high grade carcinoma with focal pleomorphic giant cell features (Figure 8I). LvCaP-1R retains a 20-fold amplification of the AR gene. Despite this amplification, LvCaP-1R has minimal expression of AR mRNA (Figure 3A) and no detectable AR protein (Figure 8J). This PDX also lacks detectable expression of AR-dependent NXK3.1 and PSA, but retains a large proportion of cells expressing c-MYC and Ki67 (Figures 3A, 8K). Like the parental LvCaP-1 PDX, it also retains mutated TP53 (R248Q), mutated PTEN (V317fs), mutated SPOP (F133L), and mutated ATRX (Table 1, Figure 3A) and uniform expression of basal marker SOX2 (Figure 8L). In addition, there is no gain of NE marker expression, including CHGA, CHGB, and SYP (Table 1, Figure 3B). However, it does gain heterogeneous expression of basal markers like KRT5, KRT14, p63 (Figures 8M), and NGFR (Figure 8N-O), but not GSTP1 (Figure 8P). Importantly, it retains heterogeneous moderate to high expression of both of the AR-independent HOXB13 (Figure 8Q) and FOLH1 genes (Figures 3A, 3E, Supplemental Figure 1B) characteristic of luminal cells. These results document that lack of AR expression by ARSi-resistant LVCaP-1R results in the acquisition of an AR-/NE- DN phenotype with heterogeneous expression of both basal and luminal cell markers, but lacks NE differentiation.

**Response to Acute Loss of AR by ARSi-Resistant Prostate Cancer Cells**

To directly test whether the acute loss of AR signaling can drive NE trans-differentiation of ARSi-resistant ARPC cells, a molecular approach was taken using the ARSi-resistant LN-95 prostate cancer cell line as a model. This cell line is a variant of LNCaP produced by long-term *in vitro* growth in charcoal-stripped fetal bovine serum (CS-FBS) media containing low androgen (47). ARSi-resistance is documented by the fact that both its *in vitro* and *in vivo* growth is resistant to Enza (29). This *in vivo* ARSi-resistance is not due to intratumoral synthesis of androgens as documented by the fact that serial passaging of LN-95 in castrated NSG hosts results in equivalent levels of intra-tumoral androgen as seen in ARSi (i.e. Abi/Enza)-treated patients (48, 49). Importantly, we have documented previously that such ARSi-resistance is not associated with a NE morphology, but that LN-95 remains a poorly differentiated ARPC whose growth (i.e. high Ki67 positivity) is dependent upon AR expression and signaling even when grown in a castrated host (Figure 9A, upper panels) (29).

Based upon this validation, CRISPR-Cas9 editing was used to delete the AR in LN-95 cells. Multiple clones were obtained in which both full-length and AR-V7 are simultaneously knocked out [i.e. total AR KO, (Figures 9B-C)]. The *in vitro* growth of these total AR knockout clones is slower than the parental AR-expressing LN-95
cells in CS-FBS media (Figure 9D). Importantly, these AR knockout clones do not acquire NE (i.e. dendritic) morphology in vitro (Supplemental Figure 3), nor upregulate NE markers like SYP, CHGA, or CHGB (Figure 9E). Neither do they gain expression of basal markers like KRT5, KRT14, TP63, NGFR, or GSTP1 (Figure 9E). They do, however, downregulate expression of AR-target genes like NKX3-1, TMPRSS2, KLK2, and KLK3 (Figure 9F, Supplemental Figure 1C). Despite loss of AR, they retain a high proportion of Ki67-positive cells, while remaining a high grade carcinoma histologically in vivo (Figure 9A, lower panels). These AR knockout clones, however, grow at a rate that is significantly slower (p<0.05) than the parental AR-expressing LN-95 cells in castrated male hosts (Figure 9G). Importantly, the AR knockout clones retain the same high expression of AR-independent luminal genes (e.g. KRT8, KRT18, HOXB13, FOLH1, etc.) as the parental LN-95 cells despite the loss of AR expression (Figure 9F). These results document that AR is not suppressing expression of NE or basal markers in the parental LN95 ARSi-resistant ARPC cells.

Transcriptome-based Subtype Clustering of Prostate Cancer PDXs

RNAseq analysis was performed to allow transcriptome-based clustering of an enlarged PDX series, including the newly developed DN PDXs described in this paper as well as previously characterized prostate cancer PDXs from both Johns Hopkins and the University of Washington Prostate Cancer Group (28, 29, 43). Four of these (i.e. PC-82, CWR22, SkCaP-1, LvCaP-1) are androgen-responsive ARPCs that regress when intact NSG immune-deficient adult male mice bearing these growing PDXs are castrated (29, 50). One (i.e. LvCaP-2) is an androgen-responsive AMPC, which regresses when tumor-bearing intact adult male mice are castrated, and two (i.e. LvCaP-2R and LuCaP77CR) are castration-resistant (CR) AMPC grown in castrated hosts (29, 43). The remaining 14 PDXs are also CR models grown in castrated hosts. Four (i.e. CWR22-RH, SkCaP-1R, 78CR, and 147CR) are castration resistant ARPCs. Four (i.e. LuCaP 93, 145.1, 145.2, and 173.1) are NEPCs, and the remaining 6 (i.e. BCaP-1, LgCaP-1, LvCap-3, PLNCaP-1, LuCaP 173.2, and LvCaP-1R) are DNPCs. Multidimensional scaling (MDS) was performed based on the expression of a panel of 21 genes (10-gene “NE” and “AR” signatures plus AR) (28). This MDS documents the expected clustering of ARPC vs. NEPC PDXs (Figure 10A). The AMPC PDXs demonstrate an intermediate clustering due to co-expression of AR and NE gene signatures (Figure 10A). In contrast, DNPC PDXs are distinctly clustered from the other phenotypes (Figure 10A).

Androgen Receptor Promoter Hypermethylation Differs in DN vs. NE PDXs

DNPC and NEPC PDXs show differential clustering despite neither expressing AR. To determine whether these transcriptional differences were in part due to differences in AR transcriptional silencing, we performed genome scale and site-specific DNA methylation analyses. Infinium methylation EPIC array studies revealed a region of hypermethylation around the AR transcriptional start site that is present in the NEPC line LuCaP 93, but not in the DNPC lines LvCaP-1, BCaP-1, LgGaP-1, LvCaP-3, or PLNCaP-1 (Figure 10B-C). As a positive control, AR-negative DU145 prostate cancer cells were used since they are known to have hypermethylation around the transcriptional start site and expanding into the first exon (51, 52). As a negative control, PC3 cells were included (51, 52).
These findings were further corroborated by targeted assessment of DNA methylation of the **AR** locus using COOMPARE-MS (53). Of the 9 ARPCs evaluated, 8 showed minimal to no **AR** exon 1 DNA hypermethylation (Figure 10D), which is consistent with their high **AR** expression. Of note, in the 6 evaluated DNs lacking **AR** expression, no significant **AR** exon 1 DNA hypermethylation was detected (Figure 10B, D). This is in contrast to the 6 NEPC PDXs evaluated, where 5 out of the 6 lines showed DNA hypermethylation at this site (Figure 10B, D), consistent with the lack of **AR** expression. These results suggest that NEPC PDXs evaluated here are derived from cells that lose **AR** expression due to promoter hypermethylation while **AR** is transcriptionally silenced in DNPC PDXs via a different mechanism.

As a potential mechanism for the suppression of the NE phenotype within DN PDXs, promoter methylation analysis of the 10 NE-related genes described in Figure 3B was performed. This analysis documented that only 4 of the 10 genes (i.e. **CHRNB2**, **PCSK1**, **ASCL1**, and **NKKX2-1**) showed differential methylation consistent with suppression of the NE phenotype in the DN vs. NE PDXs (Supplemental Figure 3). Importantly, in only 1 of the genes (i.e. **CHRNB2**) is the promoter methylation pattern in the LvCaP-2/-2R amphicrine PDXs consistent with that expected based on the NE phenotype in LuCaP93 (Supplemental Figure 3).

**Discussion**

Lineage plasticity of prostate cancer-initiating cells into a NE phenotype is supported by observations that multiple treatments can induce varying degrees of morphologic and phenotypic NE differentiation of prostate ARPC cells in vitro, including cAMP, interleukin-6 (IL-6), and serum starvation (54-57). In addition, **AR** signaling in ARPC cells can repress such NE differentiation in cell culture models (58-62). Based upon these results, it has been proposed that ARSi-treatment of mCRPC patients drives ARPCs into a more aggressive and lethal AR-negative NEPC phenotype (28, 31-34). In particular, it has been suggested that such differentiation into NEPC requires the loss of **AR** signaling in combination with mutations in **PTEN**, **RB1**, and **TP53**, together with overexpression of DNMTs, EZH2, and/or SOX2 (28, 31-34, 63).

The present studies demonstrate that lack of **AR** signaling in combination with these molecular alterations does not always necessitate progression of ARPCs to an ARSi-resistant NEPC phenotype. This conclusion is supported by previous reports. For example, Frigo and McDonnell demonstrated that only incomplete NE differentiation of ARPCs in vitro is produced by inhibition of **AR** signaling with **AR** antagonists or siRNA-mediated downregulation of **AR** (62). Additionally, while HSP-90 inhibition downregulates **AR** expression, it has no effect on NE differentiation (62). In contrast, histone deacetylase (HDAC) inhibitor-induced epigenetic changes can promote such NE differentiation (62). Similarly, ADT is reported to activate the CREB/EZH2 axis resulting in epigenetic activation of NE differentiation (64). Interestingly, the PDX models used in this study express both CREB and EZH2, and yet only a subset of the PDXs that lack **AR** expression are NEPCs. Also, LNCaP cells stably transfected with MYCN phenotypically resemble NEPC with upregulation of the NE markers and EZH2 coupled with downregulation of **AR** and androgen-regulated genes compared to parental cells (30).
However, in the PDXs used in our studies, MYCN is highly expressed by BCaP-1, LVCaP-3, and LgCaP-1, and despite no AR expression, these cells do not exhibit NEPC characteristics. Hyperactive mTOR is reported to induce NE differentiation in vitro in LNCaP cells with concurrent upregulation of interferon regulatory factor 1 (IRF1) and downregulation of AR signaling associated with upregulation of CDKN1A (a.k.a. p21) and growth arrest (65). However, all of the AR-negative PDXs reported herein express a high level of p21, but only a subset undergo NE differentiation and none are growth arrested. Thus, the present results document that ARSi-resistance can occur without ARPC differentiation into a NEPC. These results suggest that in addition to the loss of AR, other molecular changes are needed for NEPC differentiation. There are several candidate changes for such NEPC drivers, including downregulation of REST and YAP, in addition to upregulation of ASCL1, a transcription factor important in neuronal development (15, 40, 66). Of note, it is significant that the DNPC PDXs do not downregulate REST and YAP1, nor upregulate ASCL1 (Figure 3A); whereas, the NEPC PDXs do undergo these changes (Figure 3A).

Collectively, these results document that ARSi-resistance occurs in both NEPC and DNPC via loss of AR expression. In NEPC, this loss is frequently associated with hypermethylation and silencing of the AR promoter, which is consistent with previous studies supporting their derivation from ARPC cells via lineage plasticity (28, 31-34). In contrast, hypermethylation of the AR promoter is not detected in the current DNPC PDX series. In addition, differential promoter hypermethylation does not provide a simple explanation for the suppression of NE-related gene expression in the different phenotypes.

Importantly, all of the DNPC PDXs described herein have a mixture of malignant cells that heterogeneously express basal markers either alone or in combination with luminal markers. This phenotypic heterogeneity is present despite all cancer cells within each PDX having identical genetic driver mutations. There are at least two potential mechanisms to explain this phenomenon and the emergence of ARSi-resistant DNPC: 1) an initially AR-positive cancer-initiating cell loses AR expression under ADT and acquires this phenotypic heterogeneity via lineage plasticity (i.e. adaptation), or 2) an AR-negative prostate progenitor cell is the cancer-initiating cell and gives rise to malignant progeny heterogeneously expressing various combinations of basal and luminal markers (i.e. selection). In this second scenario, no promoter hypermethylation-dependent silencing of AR would be necessary for selective outgrowth of these DN cells under extreme androgen deprivation. Presently, these PDX models are being utilized to resolve whether adaption vs. selection is the mechanism for the emergence of ARSi-resistant DNPC. Earlier studies demonstrated PC with a luminal phenotype could be derived from genetically-manipulated primary human benign prostate basal cells, suggesting that histology does not necessarily correlate with cell-of-origin (67).

Regardless of whether adaption or selection is responsible, approximately 1/3 of ARSi-resistant cancers are either DNPCs or NEPCs that lack AR expression, and the frequencies of such AR-negative PCs is increasing (26). Thus, there is an urgent need for the development of therapies that do not depend upon AR activity for their efficacy (28, 68, 69). Thus, the PDXs characterized in the present report provide a credentialed platform for such drug development.
Material and Methods

Detailed procedures describing PDX establishment, cell culture, proliferation assays, cytogenetic, genetic and epigenetic characterization, plasmid construction and transfection of CRISPR-Cas9 vectors, isolation of clonal cell lines by FACS, RNAseq, DNA sequencing, methylation, Western blot, IHC, animal studies, and statistical analyses are included in the Supplemental Materials and Methods document.

Author Contributions:

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for the Oncology Tissue Services, Animal Resources, Cell Imaging, Rapid Autopsy Core, Genetic Resources, and Experimental and Computational Genomics Core Facilities.

References


Table and Figure Legends

**Table 1:** Phenotypic and Growth Characteristics of a Novel Lethal mCRPC PDX Series.

**Figure 1:** Lineage marker expression in the benign prostate. a.) Immunofluorescent (IF) staining of the AR (green) and Ki67 (pink), which documents the majority of proliferation is restricted to the basal epithelial layer. Nuclei stained with DAPI (blue). b.) IF staining of c-Myc (green) and AR (red) documenting that the small subset basal cells expressing c-Myc, do not express AR. Nuclei stained with DAPI (blue). c.) IF staining of HOXB13 (green) and Chromogranin A (red). Nuclei stained with DAPI (blue). Arrowheads indicate HOXB13-high luminal cells. Arrows indicate HOXB13-low basal cells. Diamond indicates CHGA-positive neuroendocrine cell.
Asterisks indicate HOXB13-negative stromal cells. d.) dual IHC staining of NGFR (pink) and p63 (brown) identifies the basal layer; e.) IHC staining of GSTP1 (brown) in basal layer; f.) IHC staining of SOX2 in basal layer; g.) IHC staining of HOXB13. Arrowheads indicate HOXB13-high luminal cells. Arrows indicate HOXB13-low basal cells. Asterisks indicate HOXB13-negative stromal cells. h.) Box plots indicate median and interquartile range for the Mean fluorescence intensity (MFI) of HOXB13 staining normalized to nuclear area in neuroendocrine (n = 97), basal (n = 97), and luminal cells (n = 24) of the normal prostate (whiskers = min/max values).

**Figure 2: Characteristics of the NCI-H660 xenograft.** a.) Growth rate in castrated NSG male mice (n = 5). b.) H & E histology (200X), [inset (400x)]; c.-h.) IHC (200X) for c.) AR; d.) ERG, note positive staining in tumor endothelial cells; e.) HOXB13; f.) NGFR (pink) and p63 (brown) dual stain; g.) SYP; and h.) CHGA.

**Figure 3: Lethal mCRPC PDXs analyzed for RNA expression of** a.) select genes; b.) NE-associated genes; c.-e.) IHC step-section of LvCaP-2R PDX stained for c.) AR (200X); d.) SYP (200X); and e.) dual staining (400X) for AR (pink) and SYP (brown) documenting co-expression of both markers in the same cell (i.e. amphicrine), in addition to f.) RNAsseq analysis for AR-regulated genes in panel of PDXs representing different phenotypes (e.g. DN, AR-PCa, Amphicrine, and NE).

**Figure 4: Characteristics of the BCaP-1 PDX.** a.) Growth rate in intact vs. castrated NSG male mice (n = 5). b.) H & E histology (200X), [inset (400x)]; c.-p.) IHC (200X) for c.) p53; d.) Rb [note that endothelial cell nuclei are an internal positive control for staining, (black arrows)]; e.) PTEN [note that endothelial cells are an internal positive control for staining, (black arrows)]; f.) β-Catenin; g.) c-MYC; h.) Ki67; i.) CK18; j.) focal CK5; k.) GSTP1 in BCaP-1 (positive); l.) GSTP1 in SkCaP-1 (negative control); m.) dual staining for p75 (pink) and p63 (brown); n.) Sox2; o.) HOXB13; and p.) CHGA.

**Figure 5: Characteristics of the LvCaP-3 PDX.** a.) Comparative growth rate in castrated NSG male mice (n = 5) of LvCaP-3, LgCaP-1, and PLNCaP-1. b.) H & E histology (200X), [inset (400x)]; c.-f.) IHC (200X) for c.) c-MYC; d.) Ki67; e.) HOXB13; f.) GSTP1; g.) dual staining for NGFR (pink) and p63 (brown).

**Figure 6: Characteristics of the LgCaP-1 PDX.** a.) H & E histology (200X), [inset (400x)]; b.-e.) IHC (200X) for b.) HOXB13; c.) CK8; d.) GSTP1; e.) NGFR (pink) and p63 (brown); and f.) p63.

**Figure 7: Characteristics of the PLNCaP-1 PDX.** a.) H & E histology (200X), [inset (400x)]; b.-f.) IHC (200X) for b.) c-MYC; c.) HOXB13; d.) CK8; e.) GSTP1; f.) NGFR (pink) and p63 (brown).

**Figure 8: Characteristics of the LvCaP-1 PDX.** a.) H & E histology (200X), [inset (400x)]; b.-f.) IHC (200X) for b.) AR; c.) HOXB13; d.) p53; e.) GSTP1; and f.) dual staining for NGFR (pink) and p63 (brown). g.) Growth rate in intact and subsequent regression and relapse to castration in NSG male mice (n = 5 each).

**Characteristics of the LvCaP-1R PDX.** h.) Growth rate in castrated NSG male mice (n = 5 each); i.) H & E histology (200X), [inset (400x) showing a pleomorphic giant cell]; j.-q.) IHC (200X) for j.) AR; k.) Ki67; l.) SOX2; n.-o.) dual staining for NGFR (pink) and p63 (brown); p.) GSTP1; and q.) HOXB13.
Figure 9: Characterization of LN-95 parental vs total AR knockout (KO) cells. a.) Left panels are the histology (200X), [inset (400x)]; middle panels the AR protein expression (200X); and right panels the Ki67 expression (200X) of the PDXs; b.) Western blot documentation of the successful knockout of AR protein in multiple clones of LN-95 cells; c.) IHC (200X) staining of parental LN-95 cells expressing both full length AR (AR-FL) and AR variant 7 (AR-V7) vs. AR-negative PC-3 cells and the AR-KO clones using an N-terminal AR antibody and an AR-V7 specific antibody; d.) in vitro growth of the parental LN-95 cells vs. total AR-KO clones in 10% CS-FBS media with asterisks denoting significant difference at the p < 0.05; e.) RNAseq-based comparison of the expression of NE- and basal-specific genes in total AR-KO clones compared to parental LN-95 cells; f.) RNAseq-based comparison of the expression of AR-independent and –dependent luminal-specific genes in total AR-KO clones compared to parental LN-95 cells (Note: significant difference in magnitude of y-axes between panels; and g.) in vivo growth of the total AR-KO clones vs. the parental LN-95 in castrated hosts.

Figure 10: Clustering of lethal mCRPC PDXs and analysis of AR promoter methylation. a.) Clustering of PDX models based on multidimensional scaling (MDS). b.) Analysis of methylation levels at the single CpG level using Illumina EPIC arrays reveals hypermethylation of a region encompassing the transcriptional start site and first exon of AR in LuCaP 93 and DU145 cells; c.) Schematic of the AR locus showing CpG islands, the putative differentially methylated region (DMR) and the region interrogated in this COMPARE-MS study; and d.) Heatmap of methylation indices in the first exon of AR in PDX lines assessed COMPARE-MS.

Supplemental Table and Figure Legends

Supplemental Table 1: Characteristics of patient-derived samples, including metastatic lesions from which the PDXs were derived, based on immunohistochemical staining.

Supplemental Table 2: STR profile of the novel PDXs and LN-95 xenografts.

Supplemental Table 3: IHC pre-treatment and primary antibody details.

Supplemental Figure 1: Expression of prostate-specific differentiation markers. a.) in vivo serum PSA of DN-PDXs (i.e. BCap-1 , LvCaP-3, LgCaP-1, PLNCaP-1, and LvCaP-1R) vs. ARPCs (i.e. LvCap-1, CWR22, PC-82) or AMPCs (i.e. LvCaP-2 and LvCap-2R) (n = 3-28). b.) Western blot for HOXB13 documenting prostatic origin of each of the DN PDX. ARPC LvCaP-1 and the AMPC LvCaP-2 are used as positive controls. c.) in vitro PSA secretion from LNCaP cultured in standard and CS-media conditions, in addition to LN-95 and the AR-KO derivative in CS-media (n = 3-8). Box plots display the median and interquartile range (whiskers = min/max values).

Supplemental Figure 2: Treatment histories of patients from which each of the novel PDX model was derived.
**Supplemental Figure 3:** Lack of differential promoter methylation of NE-related genes across PDX phenotypes. Analysis of methylation levels at the single CpG level using Illumina EPIC arrays reveals inconsistent differential hypermethylation of a region encompassing the transcriptional start site of a.) *CHRNB2*, b.) *PCSK1*, c.) *ASCL1*, d.) *NKX2-1*, e.) *CHGA*, f.) *CHGB*, and g.) *SYP*. Yellow box highlights differential promoter methylation in *CHRNB2*, *PCSK1*, *ASCL1*, and *NKX2-1*, but not in *CHGA*, *CHGB*, and *SYP*.

**Supplemental Figure 4:** *in vitro* morphology of LNCaP, LN-95, and LN-95 total AR-KO #1 and AR-KO #2. Cells grown in media indicated in parentheses. 400x.

**Supplemental Figure 5:** Centromere-telomere PNA FISH (Cen/Tel PNA FISH) on BCaP-1 PDX tissue showing human cells surrounding rodent endothelial cells. A human- and mouse-specific FITC-labeled centromeric FISH probe (green) was combined with a Cy3-labeled telomere FISH probe (red), which highlights the longer rodent telomeres counterstained with DAPI (blue) to identify nuclei. Such a method allows unambiguous discernment between human cells (Cen-positive; Tel-dim) denoted by arrows and mouse cells (Cen-positive; Tel-bright) denoted by triangles.
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Figure 4

(a) Graph showing tumor volume (cc) over days post-inoculation for BCaP-1. The graph indicates a significant increase in tumor volume over time, with data points for castrate and intact conditions.

(b) H&E staining showing tissue structure.
(c) p53 staining highlighting cellular changes.
(d) Rb staining indicating cellular cycle regulation.
(e) PTEN staining showing protein expression.
(f) β-catenin staining for cellular signaling.
(g) c-Myc staining for cellular proliferation.
(h) Ki67 staining for cell cycle progression.
(i) CK18 staining for epithelial differentiation.
(j) CK5 staining for stem cell marker.
(k) GSTP1 staining for detoxification enzymes.
(l) GSTP1 staining for detoxification enzymes.
(m) p75 p63 staining for epithelial markers.
(n) SOX2 staining for stem cell marker.
(o) HOXB13 staining for developmental markers.
(p) ChgA staining for epithelial differentiation.
Figure 5

(a) Graph showing tumor volume (cc) over days post-inoculation for LvCaP-3, LgCaP-1, and PLNCaP-1.

(b) H&E staining of LvCaP-3 tissue.

(c) c-Myc staining of LvCaP-3 tissue.

(d) Ki67 staining of LvCaP-3 tissue.

(e) HOXB13 staining of LvCaP-3 tissue.

(f) GSTP1 staining of LvCaP-3 tissue.

(g) p75 and p63 staining of LvCaP-3 tissue.
Figure 6

LgCaP-1

a. H&E

b. HOXB13

c. CK8

d. GSTP1

e. p75 p63

f. p63
Figure 7

PLNCaP-1

a. H&E

b. c-Myc

c. HOXB13

d. CK8

e. GSTP1

f. p75
p63
Figure 9