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Original Article

Wntless expression promotes lineage plasticity and is associated with neuroendocrine prostate cancer

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Abstract: Resistance to androgen receptor (AR) targeted therapies remains as the main reason for most prostate cancer related deaths. Lineage plasticity resulting in altered, treatment insensitive prostate tumor cell phenotypes such neuroendocrine differentiated prostate cancer is a common manifestation within resistant tumors upon AR-targeted therapies. The mechanisms responsible for lineage plasticity in prostate cancer remain incompletely understood. Here we demonstrate that the enzalutamide resistant MDVR cell line possesses lineage plastic characteristics associated with overexpression of the Wnt transporter Wntless (WLS). Furthermore, we present evidence that overexpression of WLS is common in varying cell line models of lineage plastic prostate cancer, is higher in neuroendocrine patient samples, and positively correlates with the neuroendocrine marker SYP in clinical data. Targeting WLS in lineage plastic cellular models reduces viability and represses lineage plasticity associated gene expression. Our study provides insight into the importance of WLS to the development of lethal resistant prostate cancer and provides a potential target for the treatment of advanced disease.

Keywords: Prostate cancer, WLS, lineage plasticity, neuroendocrine, enzalutamide

Introduction

Prostate cancer has historically been treated with androgen deprivation therapy (ADT) since androgen receptor (AR) stimulation of oncogenic signaling is the main driver of tumor growth. However, while initial treatment is modestly effective, prolonged ADT eventually fails leading to castration-resistant prostate cancer (CRPC). The recent development and clinical implementation of next-generation anti-androgens (NGAT's) including enzalutamide, abiraterone, apalutamide, and darolutamide have enhanced our ability to target AR and improve patient outcomes, but the emergence of resistance to treatment is still a challenge to long term disease control [1]. Ongoing investigations have revealed that the mechanisms mediating resistance to AR-directed therapies are numerous and heterogeneous, but further study promises to lead to the development of novel treatments for advanced disease.

One of the mechanisms responsible for the development of treatment resistance is lineage plasticity [2]. Lineage plasticity describes a process whereby a cell may differentiate into a cell with a new identity with altered characteristics. Through this process, tumor cells can differentiate into more aggressive and potentially AR pathway independent lineages. Treatment induced neuroendocrine prostate cancer (t-NEPC) is a recognized variant of CRPC which represents one potential fate of lineage plastic prostate cancer. t-NEPC is commonly characterized by decreased AR expression and/or canonical signaling and high expression of one or more neuroendocrine (NE) markers including synaptophysin (SYP), neuron-specific enolase (NSE/ENO2), CD56 and chromogranin A (CHGA) [3]. However, to date, there is no standard signature to diagnose t-NEPC. While t-NEPC represents one CRPC variant of lineage plasticity, it does not represent all potential fates. Resistant prostate cancer lineages also include but are

not limited to a double negative phenotype which is both AR-null and NE-null, and a gastrointestinal lineage characterized by expression of the transcription factors HNF4G and HNF1A [4, 5]. Furthermore, additional, hybrid lineages with overlapping characteristics are thought to arise, suggesting there may be a spectrum or continuum of lineages capable of conferring resistance to treatment [2]. Thus, lineage plasticity represents a heterogeneous process which may give rise to multiple resistant prostate cancer subtypes. Further complicating this phenomenon is our lack of understanding of how cells gain access to lineage plasticity and how differentiation occurs. It is thought that lineage plasticity is associated with altered activation of developmental, stem, and EMT signaling programs [6]. While the mechanistic specifics are not yet understood, the development and characterization of treatment resistant models promises to aid elucidation of lineage plastic characteristics and their drivers.

We previously described and characterized an enzalutamide resistant CRPC cell line, C4-2B-MDVR (MDVR), derived from long-term exposure of C4-2B CRPC cells to enzalutamide [7]. Our past work has largely focused on AR-dependent mechanisms of resistance [8-10]. In the present study, we present evidence that MDVR cells also represent lineage plastic disease in that they appear less reliant on traditional AR-signaling and to have acquired EMT, stem, and neural signaling programs. These findings are in line with the notion that lineage plasticity may give rise to a spectrum of phenotypes with overlapping characteristics. Additionally, MDVR cells present with high expression of Wntless (WLS), a Wnt transporter thought to play an indispensable role in Wnt signaling [11]. Wnt signaling has been suggested to play a role in prostate cancer progression to a NE phenotype [12]. Here, we present evidence that WLS is both associated with and regulates lineage plastic characteristics and is highly expressed in several models of lineage plastic disease. We also show that WLS inhibition decreases viability of lineage plastic prostate cancer cells. Altogether, our study demonstrates the utility of MDVR cells in studying lineage plasticity and suggests that WLS is a mediator of the lineage plastic phenotype.

Materials and methods

Cell lines and reagents

CWR22Rv1, PC3, and NCI-H660 cells were obtained from the American Type Culture Collection (ATCC). ATCC uses short tandem repeat profiling for testing and authentication of cell lines. All cell lines are routinely tested for mycoplasma using ABM mycoplasma PCR detection kit (Cat#: G238). CWR22Rv1 and PC3 cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 IU penicillin and 0.1 mg/ml streptomycin. Enzalutamide-resistant C4-2B cells (C4-2B-MDVR) were previously described and maintained in complete RPMI 1640 supplemented with 20 μM enzalutamide [7]. NCI-H660 cells were cultured in RPMI-1640 base medium with 5% FBS, 10 nM β-estradiol, 10 nM hydrocortisone, and 1% insulin-transferrin-selenium. All cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide. Enzalutamide (Cat#: S1250) was purchased from Selleckchem. All transfections were performed using Lipofectamine RNAiMAX (Cat#: 13778150) purchased from ThermoFisher according to manufacturer's instructions using 10-50 nM siRNA. Non-targeting control siRNA (Cat#: 12935112) and Stealth Wntless (WLS) targeting siRNA were purchased from ThermoFisher. WLS-F-Oligo (Cat#: 10620318): GGUAUUGGAGGAGGAUCACCAUGAU. WLS-R-Oligo (Cat#: 106203-19): AUCAUGGUGAUCCUCCUCCAAUACC.

RNA-sequencing and GSEA analysis

RNA isolated from 1) C4-2B cells treated with vehicle (DMSO) or enzalutamide (20 μM) for 48 hours, 2) C4-2B and C4-2B-MDVR cell lines 48 hours after plating without selection agent, or 3) MDVR cells treated with non-targeting or WLS-targeting siRNA for 72 hours was subjected to next generation sequencing (NGS) as described elsewhere [13]. Vehicle or enzalutamide treated C4-2B cells and C4-2B versus MDVR samples were submitted to Novogene for RNA-sequencing and analysis according to their pipeline methodology. Alternatively, non-targeting or WLS-targeting siRNA treated MDVR samples were submitted to the UC Davis Comprehensive Cancer Center's Genomics Shared Resource (GSR) for RNA-Sequencing (RNA-Seq) and analysis according to the follow-

ing methodology; Indexed, stranded mRNA-seq libraries were prepared from total RNA (100 ng) using the KAPA Stranded mRNA-Seq kit (Roche) according to the manufacturer's standard protocol. Libraries were pooled and multiplex sequenced on an Illumina NovaSeq 6000 System (150-bp, paired-end, > 20 × 10^6 reads per sample) at the QB3 Vincent J. Coates Genomics Sequencing Lab University of California, Berkeley. RNA-Seq data was analyzed using a Salmon-tximport-DESeq2 pipeline. Raw sequence reads (FASTQ format) were mapped to the reference human transcriptome index (GRCh38/hg38, GENCODE release 31) and quantified with Salmon [14]. Gene-level counts were imported with *tximport* [15] and differential expression analysis was performed with DESeq [16].

GSEA was performed using the Java desktop software (http://software.broadinstitute.org/gsea/index.jsp) [17, 18]. The HALLMARK_ANDROGEN_RESPONSE, REACTOME_WNT_LIGAND_BIOGENESIS_AND_TRAFFICKING, HALLMARK_EMT, BOQUEST_STEM_CELL_UP, and WP_NEURAL_CREST_DIFFERENTIATION datasets were downloaded from the Molecular Signatures Database and were used in the GSEA analysis.

Quantitative PCR (qPCR)

Total RNA from 1) C4-2B cells treated with vehicle (DMSO) or enzalutamide (20 μM) for 48 hours, 2) C4-2B and C4-2B-MDVR cell lines 48 hours after plating without selection agent, or 3) MDVR cells treated with non-targeting or WLS-targeting siRNA for 72 hours was extracted using TRIzol reagent (Cat#: 15596018) purchased from ThermoFisher. cDNAs were prepared from 1 ug RNA using ImPromII reverse transcriptase (Cat#: M314C) purchased from Promega. The cDNAs were subjected to quantitative-PCR (qPCR) using SsoFast EvaGreen Supermix (Cat#: 172-5205) purchased from Bio-Rad according to the manufacturer's instructions. Triplicates of samples were run on default settings of Bio-Rad CFX-96 real-time cycler. Each reaction was normalized by co-amplification of Actin. Data was calculated using the efficiency corrected method. Primers used for qPCR are as follows.

ACTIN Forward - CCCAGCCATGTACGTTGCTA, ACTIN Reverse - AGGGCATACCCCTCGTAGATG; WLS Forward - TCATGGTATTTCAGGTGTTTCG, WLS Reverse - GCATGAGGAACTTGAACCTAAAA; PSA Forward - GCCCTGCCCGAAAGG, PSA Reverse - GATCCACTTCCGGTAATGCA; NKX3.1 Forward - CCGAGACGCTGGCAGAGACC, NKX3.1 Reverse - GCTTAGGGGTTTGGGGAAG; FKBP5 Forward - AGAACCAAACGGAAAGGAGA, FKBP5 Reverse - GCCACATCTCTGCAGTCAAA; UGT2B15 Forward - GTGTTGGGAATATTATGACTA, UGT2B-15 Reverse - GGGTATGTTAAATAGTTCAGC; NSE Forward - CTGGCTAAATACAACCAGCTCA, NSE Reverse - CACAGCACACTGGGATTACG.

Western blot

Whole cell extracts were prepared by lysing cells with SDS cell lysis buffer and proteins were quantitated by BCA assay (Cat#: 23225, Pierce, Rockford, IL). Proteins were separated by SDS-PAGE. The blots were stained overnight with primary antibodies at 4°C and detected by enhanced chemiluminescence (Cat#: WBLUR0500, Millipore, Burlington, MA) following incubation with a peroxidase-labeled secondary antibody. WLS primary antibody (MABS87) and NSE (MAB324) were purchased from Millipore Sigma. Tubulin (T5168) was purchased from Sigma-Aldrich. AR 441 (sc-7305) and SYP (sc-9116) were purchased from Santa Cruz Biotechnology.

cBioPortal

WLS expression was analyzed in neuroendocrine (NEPC) and adenocarcinoma CRPC samples in the Neuroendocrine Prostate Cancer (Multi-Institute, Nat Med 2016) database from cBioPortal [19, 20]. A two-tailed, unequal variance t-test was used to assess significance between the two groups. Additionally in the same dataset, WLS expression was correlated with that of Synaptophysin either in all samples or individually in either NEPC samples or adenocarcinoma samples using the cBioPortal co-expression tool.

Cell growth assay

Cells were seeded at 25,000-50,000 cells per well in 24-well plates in complete media. After 24 hours, cells were transfected with either non-targeting or WLS-targeting siRNA. Cell viability was assessed 72 or 96 hours post transfection using Cell Counting Kit-8 (Cat#: CK04-20) purchased from Dojindo Molecular Tech-

nologies, Inc. Data are displayed as percent of control cell growth ± SD. All conditions were performed in triplicates.

Statistical analysis

All quantitated qPCR and cell growth assay data is displayed as fold change or percent of control mean ± standard deviation. Significance was assessed using a two tailed two sample equal variance students t-test. A p-value of ≤ 0.05 was accepted as significant.

Results

Enzalutamide resistant MDVR cells display decreased canonical AR signaling and increased Wnt signaling associated characteristics of lineage plastic prostate cancer

In this study, we sought to understand whether our previously characterized enzalutamide resistant MDVR cell line possesses lineage plastic characteristics and putative drivers of plasticity. We began our study by first characterizing sensitive C4-2B cells response to enzalutamide. RNA isolated from C4-2B cells treated with enzalutamide was submitted for RNA-sequencing. Gene Set Enrichment Analysis (GSEA) of the data revealed significant downregulation of the HALLMARK_ANDROGEN_RESPONSE in line with the expected effect of enzalutamide treatment (**Figure 1A**). qPCR validation of these findings demonstrated significantly decreased transcription of canonical AR target genes PSA, NKX3.1, and FKBP5, and increased expression of the androgen repressed gene UGT2B15 (**Figure 1B**). Further GSEA exploration of enzalutamide treated C4-2B cells revealed enrichment of several pathways associated with Wnt signaling, with near significant enrichment of the REACTOME_WNT_LIGAND_BIOGENESIS gene set (**Figure 1C**). At the leading edge of this gene set, we found upregulation of several Wnt ligands, including Wnt4 and Wnt5a, and the Wnt transporter WLS. All three of these have been associated with enzalutamide resistance and/or neuroendocrine prostate cancer [21, 22].

We have previously shown that MDVR cells express higher levels of both full-length AR and AR-variants which are thought to contribute to drug resistance [7, 23]. Despite increased AR protein expression in MDVR cells, AR canonical signaling is not likewise upregulated, exemplified by GSEA analysis that indicates suppression of the HALLMARK_ANDROGEN_RESPONSE (**Figure 1D**). This is further supported by qPCR which shows decreased levels of PSA mRNA and general lack of expected effects on AR target genes (**Figure 1E**). Decreased canonical AR activity suggests the advent of lineage plasticity in our MDVR cell line. In line with our past study, MDVR cells maintain upregulation of the REACTOME_WNT_LIGAND_BIOGENESIS pathway with significantly increased expression of WLS compared to C4-2B, suggesting augmented Wnt signaling activity in enzalutamide resistant MDVR cells (**Figure 1F** and **1G**) [24]. Put together, these data indicate that MDVR cells may have acquired lineage plastic characteristics including an altered AR signaling program and increased developmental Wnt signaling.

WLS regulates features associated with lineage plastic prostate cancer

We further analyzed our RNA-seq data comparing enzalutamide resistant MDVR cells to parental C4-2B cells for additional evidence of lineage plastic characteristics. Lineage plasticity is thought to be associated with EMT, stem, and neural gene expression. GSEA shows significant enrichment of several pathways which may be associated with lineage plasticity in resistant MDVR cells, including HALLMARK_EMT, BOQUEST_STEM_CELL_UP, and WP_NEURAL_CREST_DIFFERENTIATION gene sets (**Figure 2A**). Additionally, we found significant upregulation of the neuroendocrine phenotype marker NSE in MDVR (**Figure 2B**). These data support that MDVR cells possess lineage plastic characteristics.

Whether WLS may regulate lineage plasticity is not clear, but our previous report demonstrated that inhibition of WLS significantly reduced MDVR cellular viability [24]. Interestingly, siRNA-mediated knockdown of WLS also reduced NSE expression as well as lineage plasticity associated gene sets found to be upregulated in MDVR cells (**Figure 2C**, **2D**). Altogether, these data support the hypothesis that WLS regulates features associated with lineage plasticity.

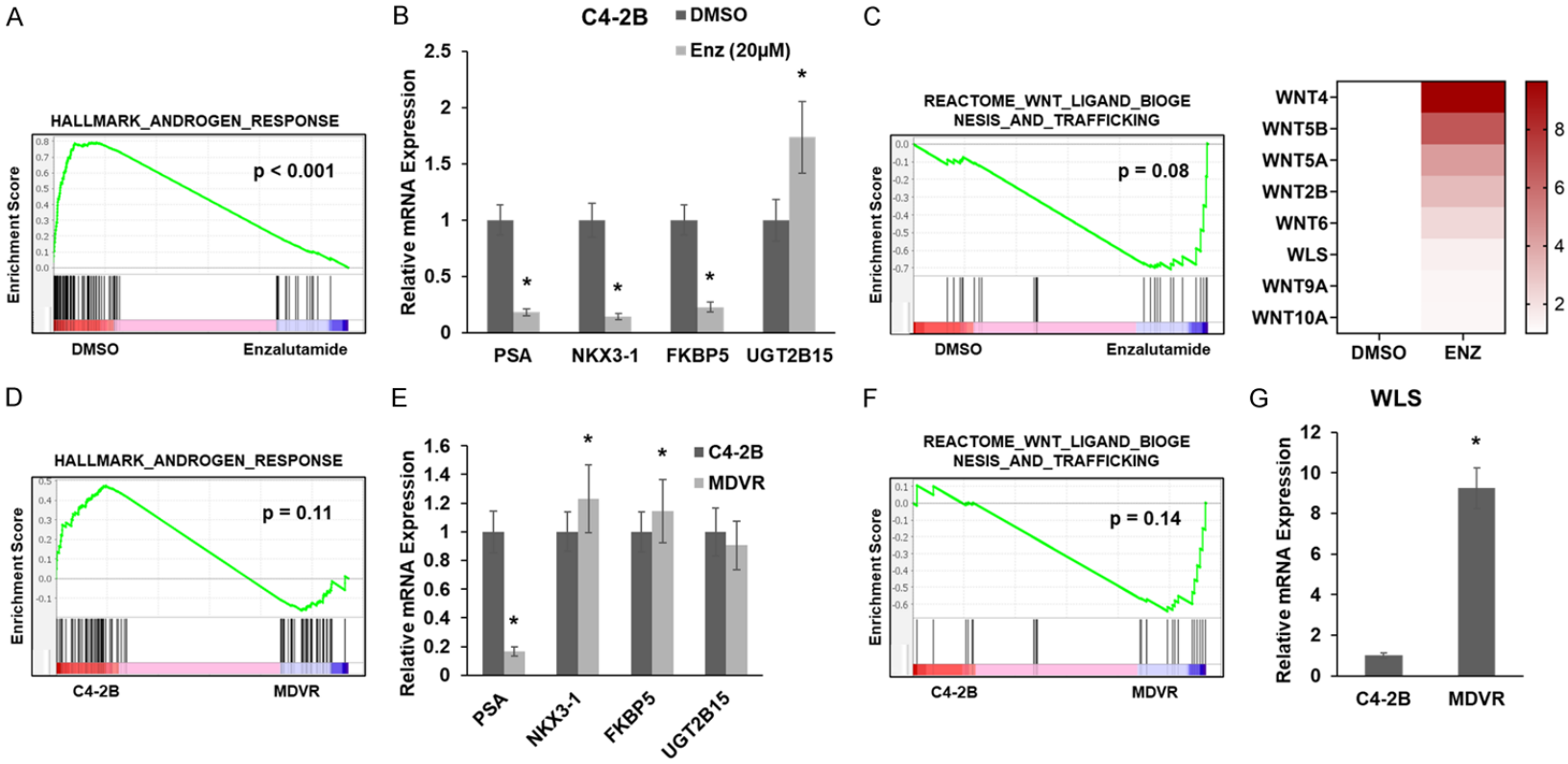

Figure 1. Enzalutamide resistant MDVR cells display lineage plastic characteristics. A. GSEA analysis of C4-2B cells treated with enzalutamide (20 μM) for 48 hours shows decrease of AR signaling in enzalutamide treated cells. B. Relative quantification of mRNA expression by qPCR of AR target genes in C4-2B cells treated with enzalutamide (20 μM) for 48 hours shows decreased AR transcriptional activity. C. GSEA analysis showing enhancement of Wnt biogenesis and trafficking in C4-2B cells treated with enzalutamide (20 μM) for 48 hours. Heatmap represents most upregulated genes in the set by fold change relative to vehicle (DMSO) treated control cells. D. GSEA comparing MDVR cells to parental C4-2B cells suggests downregulation of canonical AR signaling in MDVR cells. E. qPCR of AR target genes in C4-2B and MDVR shows lack of expected increase in AR pathway activity in MDVR cells despite AR expression. F. GSEA shows enrichment of Wnt ligand biogenesis and trafficking in MDVR cells compared to C4-2B cells. G. Relative mRNA expression analysis by qPCR of WLS between MDVR and C4-2B cells. *p-value ≤ 0.05.

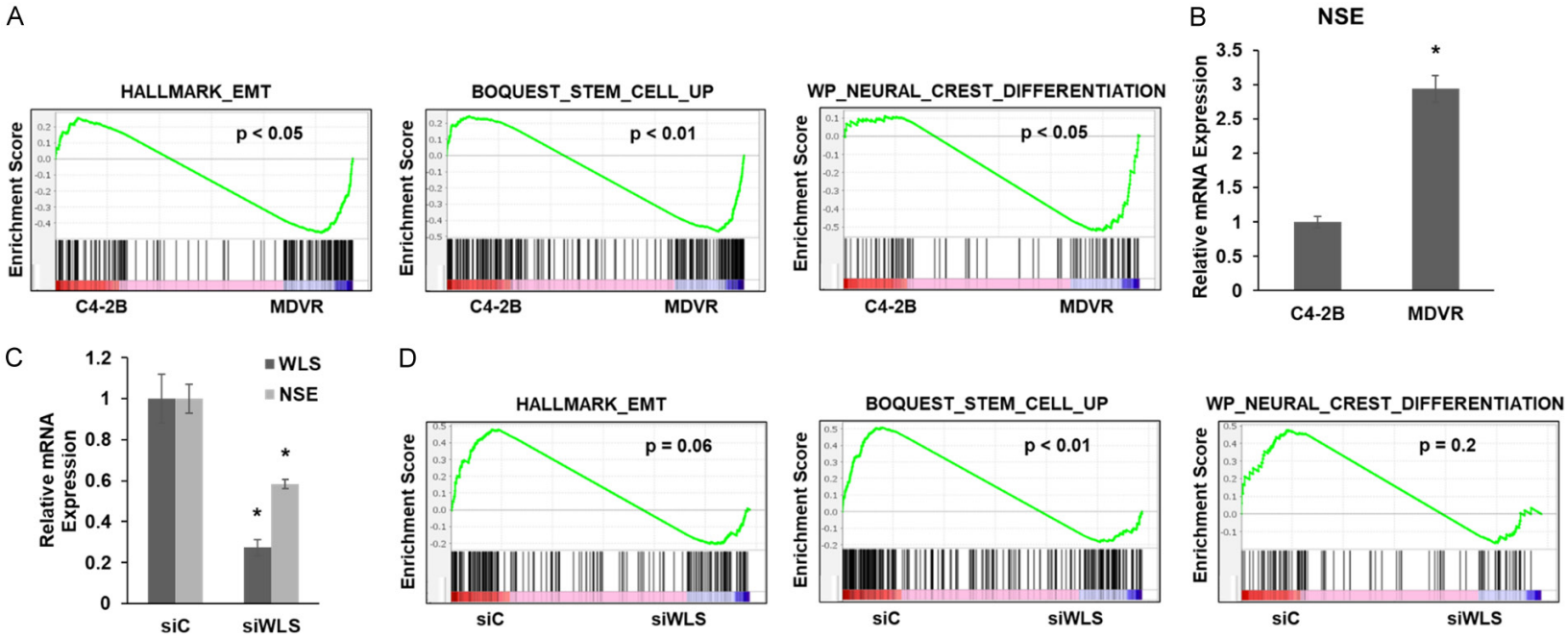

Figure 2. WLS regulates lineage plasticity features in MDVR cells. A. GSEA analysis shows significant enrichment of EMT, stem, and neural gene sets in MDVR cells. B. qPCR demonstrates increased mRNA expression of the neuroendocrine marker NSE in MDVR cells. C. qPCR shows decreased expression of WLS and NSE in MDVR cells 72 hours post treatment with WLS targeting siRNA. D. GSEA analysis shows that siRNA mediated WLS inhibition in MDVR cells decreases EMT, stem, and neural gene sets found to be enriched in these cells. siC = control non-targeting siRNA. siWLS = WLS-targeting siRNA. *p-value ≤ 0.05.

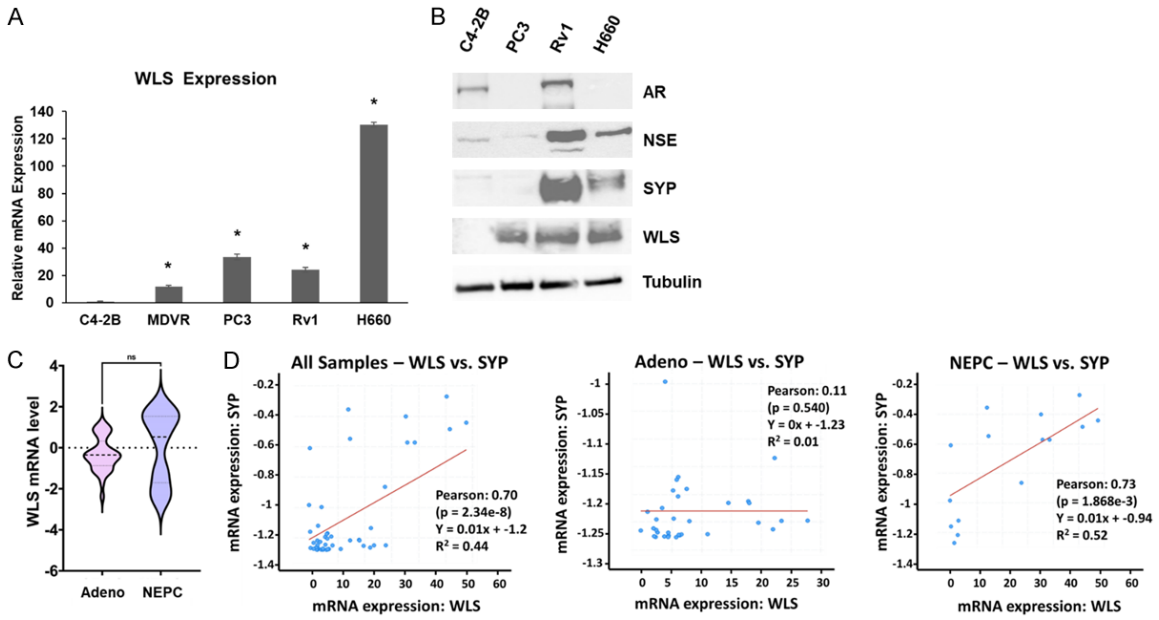

Figure 3. High expression of WLS correlates with lineage plastic and neuroendocrine features in prostate cancer. A. qPCR shows increased WLS mRNA expression in varying lineage plastic models of prostate cancer (MDVR, PC3, Rv1, H660) relative to C4-2B cells. B. Western blots show protein levels of WLS, AR, NSE, and SYP in C4-2B, PC3, Rv1, and H660 cells. Tubulin served as a loading control. C. WLS mRNA expression comparison between CRPC adenocarcinoma and NEPC patients from Beltran's 2016 cohort data available from cBioPortal depicting an upward trend in the NEPC patients' population. D. Co-expression data of WLS with SYP in Beltran's 2016 clinical data obtained from cBioPortal showing positive correlation between WLS and SYP in NEPC patients but not in adenocarcinoma patients. *p-value ≤ 0.05.

WLS is highly expressed in models of lineage plastic prostate cancer and associated with neuroendocrine disease

Having demonstrated that WLS regulates the lineage plastic features of the enzalutamide resistant MDVR cell line, we sought to investigate its expression in varying additional lineage plastic prostate cancer cell lines. PC3 cells are AR-negative and thought to possess features of neuroendocrine disease [25]. CWR22Rv1 (Rv1) cells are thought to possess an amphicrine phenotype as they are both AR-positive and display NE features [21]. NCI-H660 (H660) are a model of AR-null neuroendocrine prostate cancer [26]. Levels of WLS mRNA in MDVR, PC3, Rv1 and H660 are all increased when compared to C4-2B (**Figure 3A**). Western blots support past studies of AR expression in these models and the increased expression of WLS as well as demonstrate increased expression of the neuroendocrine makers SYP and NSE in Rv1 and H660 cells (**Figure 3B**).

To validate our result of increased WLS expression in lineage plastic prostate cancer, we investigated a clinical data set available in cBioPortal [6, 19]. Although not significant, WLS shows an upward trend in NEPC clinical specimens compared to adenocarcinomas (**Figure 3C**). Using this same database, we show that there is a positive correlation between the expression of WLS and SYP, a NE marker. Further analysis suggests this correlation is only present in neuroendocrine patients, while there is no correlation of expression in adenocarcinoma patients (**Figure 3D**), supporting our hypothesis that WLS plays a critical role in the development of lineage plasticity in prostate cancer.

Inhibition of WLS reduces lineage plastic prostate tumor cell viability and SYP expression

Having presented evidence that WLS is highly expressed in multiple models of lineage plastic disease and that WLS knockdown in MDVR cells regulates lineage plastic characteristics, we next wanted to investigate the effects of silencing WLS on viability of lineage plastic PC3, Rv1, and H660 cells. Viability of all three cell lines was significantly decreased by siRNA

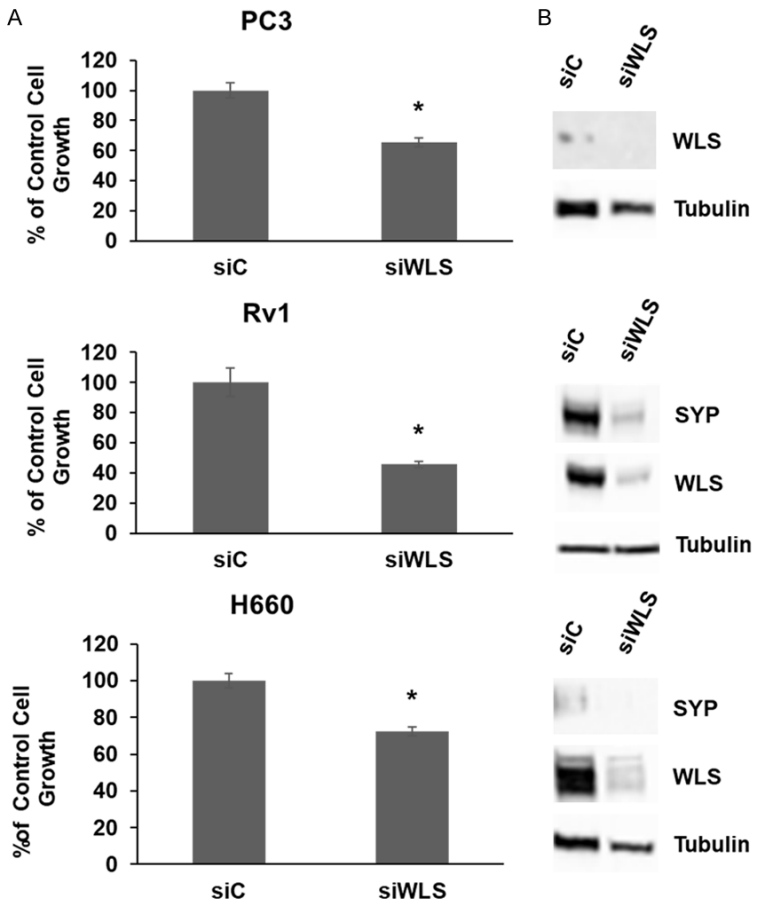

Figure 4. WLS knockdown decreases viability and associated marker SYP in lineage plastic prostate cancer cell lines. A. Cell viability assays show decreased viability in lineage plasticity models of prostate cancer (PC3, Rv1, and H660) treated with WLS-targeting siRNA versus control treatment. B. Western blots show expression of WLS and SYP in cells treated with either WLS-targeting siRNA or control non-targeting siRNA. Tubulin served as a loading control. siC = control non-targeting siRNA. siWLS = WLS-targeting siRNA. *p-value ≤ 0.05.

mediated WLS inhibition (**Figure 4A**). Knockdown was verified by western blots (**Figure 4B**). Additionally, knockdown of WLS decreased the expression of the NEPC marker SYP in both Rv1 and H660 cells, supporting our findings regarding the correlation of WLS and SYP expression in neuroendocrine prostate tumor samples. Altogether, our findings support that WLS expression is associated with lineage plasticity and may promote progression of tumors toward resistant phenotypes.

Discussion

Acquired resistance to therapy remains a significant impediment to further progress in improving patient outcomes. Several mechanisms have been proposed to give rise to anti-androgen resistance including the advent of lineage plasticity [27]. Despite this knowledge, little remains understood regarding this phenomenon. Here we characterize our previously developed enzalutamide resistant C4-2B derived cell line, MDVR, as a model of lineage plastic prostate cancer. MDVR cells are shown to have decreased canonical AR signaling and increased expression of EMT, stem, and neural gene signatures which are thought to be associated with lineage plastic disease. We also confirm increased expression of the Wnt transporter WLS, which we have previously shown to be associated with increased Wnt pathway activation [24]. WLS is shown here to be highly expressed in diverse models of lineage plastic prostate cancer and its inhibition reduces characteristics of lineage plasticity and significantly diminishes viability in several models. Our data suggest that WLS may contribute to lineage plasticity and be a potential therapeutical target.

It is thought that lineage plasticity gives rise to cancer cells with new properties which render them less or un-responsive to treatment. Prostate cancer cell lineage plasticity is often associated with decreased AR expression and/or canonical activity (ie. PSA expression) and may involve acquisition of and differentiation into a completely altered cellular lineage [2]. Development of treatment induced neuroendocrine prostate cancer (t-NEPC) is one such fate of lineage plasticity by which cells may evade AR-targeting treatments, but it is not the only fate [28]. It is thought that a spectrum of phenotypes harboring varying characteristics may exist [2]. Many questions regarding lineage plasticity remain including 1) how do cells acquire lineage plasticity and what are its drivers, 2) what cellular

lineages are possible, and 3) what can we target to treat lineage plastic disease? Characterization of our enzalutamide resistant cell line as a model of lineage plasticity provides evidence that overexpression of WLS may be linked to this process.

We show that enzalutamide diminishes AR activity and induces Wnt ligand biogenesis and expression of the master Wnt regulator, WLS. These data are in line with a previous report that WLS is an AR repressed target gene [21]. It is of note that increased Wnt signaling is thought to be involved in the development of anti-androgen resistance [22, 29]. Interestingly, we show that despite increased expression of both full-length and variant AR in MDVR cells, which we have previously shown, canonical AR activity is repressed compared to parental C4-2B cells [23]. These findings suggest that MDVR cells may have developed a degree of lineage plasticity during acquisition of resistance. We also show that MDVR cells retain enrichment of Wnt ligand biogenesis gene expression and confirm WLS is highly expressed in these cells as previously shown [21, 24]. Wnt signaling plays a pivotal role in development and may regulate several characteristics known to be associated with lineage plasticity including stemness and EMT [30-32]. Furthermore, recent reports suggest Wnt signaling may be involved in the development of neuroendocrine prostate cancer [21, 33, 34].

Past reports have implicated several phenotypic and genetic changes to be associated with lineage plastic disease including alterations of EMT programs, stem signaling, and neural gene signatures [2, 6]. Using GSEA, here we report that relative to parental C4-2B cells, MDVR cells display significant enrichment of several gene signatures including the hallmark EMT signature, a stem signature, and a neural crest differentiation signature. It is interesting that prostatic neuroendocrine cells are thought to be derived from neural crest and a past report suggested a role for an intermediary neural/neural crest stem cell phenotype en route to neuroendocrine transdifferentiation [35, 36]. We also show that MDVR cells overexpress neuron-specific enolase, a gene frequently associated with the advent of neuroendocrine disease. Interestingly, inhibition of WLS was able to decrease features of lineage plasticity to varying degrees, implicating it as a potentiator and driver of lineage plastic characteristics.

The study by Bland et al. showed that WLS supports neuroendocrine development through the ROR2/PKCδ/ERK signaling pathway [21]. The study presented here supports these findings by showing a trend toward increased WLS expression in a clinical data set comparing neuroendocrine disease to adenocarcinoma and showing a positive correlation between WLS and the well characterized neuroendocrine marker SYP in neuroendocrine patient samples. However, our current study expands on past work by supporting a role for WLS not only in neuroendocrine differentiation but more broadly in lineage plasticity. Our data support that MDVR cells represent a resistant tumor cell line with characteristics of lineage plasticity including decreased canonical AR signaling and increased EMT, stem, and neural gene expression. It is thought that lineage plasticity may give rise to a continuum of phenotypes between well differentiated adenocarcinoma and disparate lineages [2]. While our data do not support full neuroendocrine differentiation in MDVR cells, results presented here support the hypothesis that MDVR cells represent a fate on the lineage plastic spectrum and that WLS potentiates this change. In line with these data, we see WLS highly expressed in additional cell line models of lineage plasticity including PC3, Rv1, and H660. Several studies propose mechanistic models for the transdifferentiation from adenocarcinoma to lineage plastic disease including upregulation of MYCN, BRN2, and ONECUT2 [37-41]. We suggest a model whereby WLS expression supports broad spectrum lineage plasticity. Further study will be needed to better understand the contribution of WLS to prostate cancer progression.

The question of how to treat lineage plastic prostate cancer remains largely unanswered. Here we show that inhibition of WLS by siRNA significantly reduces viability in PC3, Rv1, and H660 models of lineage plastic prostate cancer, consistent with the reports shown that inhibition of WLS significantly reduce viability of MDVR cells [21, 24]. PORCN is a membrane bound O-acyl transferase upstream of WLS in the Wnt ligand biogenesis pathway and is a more druggable target to inhibit overactive Wnt

activity. Inhibition of PORCN using a small molecule inhibitor could also be used to treat CRPC xenograft tumors [21, 42]. These data support that WLS may serve as a putative therapeutic target for lineage plastic prostate cancer.

In conclusion, our data collectively support a role for WLS in the advent of lineage plasticity. Additionally, we demonstrate that the enzalutamide resistant cell line C4-2B-MDVR may be used as a model of lineage plastic disease and that inhibition of WLS may potentially be pursued as a means to treat these aggressive and difficult to manage tumors.

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Disclosure of conflict of interest

None.

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References

[1] Rice MA, Malhotra SV and Stoyanova T. Second-generation antiandrogens: from discovery to standard of care in castration resistant prostate cancer. Front Oncol 2019; 9: 801.

[2] Beltran H, Hruszkewycz A, Scher HI, Hildesheim J, Isaacs J, Yu EY, Kelly K, Lin D, Dicker A, Arnold J, Hecht T, Wicha M, Sears R, Rowley D, White R, Gulley JL, Lee J, Diaz Meco M, Small EJ, Shen M, Knudsen K, Goodrich DW, Lotan T, Zoubeidi A, Sawyers CL, Rudin CM, Loda M, Thompson T, Rubin MA, Tawab-Amiri A, Dahut W and Nelson PS. The role of lineage plasticity in prostate cancer therapy resistance. Clin Cancer Res 2019; 25: 6916-6924.

[3] Beltran H and Demichelis F. Therapy considerations in neuroendocrine prostate cancer: what next? Endocr Relat Cancer 2021; 28: T67-T78.

[4] Shukla S, Cyrta J, Murphy DA, Walczak EG, Ran L, Agrawal P, Xie Y, Chen Y, Wang S, Zhan Y, Li D, Wong EWP, Sboner A, Beltran H, Mosquera JM, Sher J, Cao Z, Wongvipat J, Koche RP, Gopalan A, Zheng D, Rubin MA, Scher HI, Chi P and Chen Y. Aberrant activation of a gastrointestinal transcriptional circuit in prostate cancer mediates castration resistance. Cancer Cell 2017; 32: 792-806, e797.

[5] Bluemn EG, Coleman IM, Lucas JM, Coleman RT, Hernandez-Lopez S, Tharakan R, Bianchi-Frias D, Dumpit RF, Kaipainen A, Corella AN, Yang YC, Nyquist MD, Mostaghel E, Hsieh AC, Zhang X, Corey E, Brown LG, Nguyen HM, Pienta K, Ittmann M, Schweizer M, True LD, Wise D, Rennie PS, Vessella RL, Morrissey C and Nelson PS. Androgen receptor pathway-independent prostate cancer is sustained through FGF signaling. Cancer Cell 2017; 32: 474-489, e476.

[6] Beltran H, Prandi D, Mosquera JM, Benelli M, Puca L, Cyrta J, Marotz C, Giannopoulou E, Chakravarthi BV, Varambally S, Tomlins SA, Nanus DM, Tagawa ST, Van Allen EM, Elemento O, Sboner A, Garraway LA, Rubin MA and Demichelis F. Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. Nat Med 2016; 22: 298-305.

[7] Liu C, Lou W, Zhu Y, Nadiminty N, Schwartz CT, Evans CP and Gao AC. Niclosamide inhibits androgen receptor variants expression and overcomes enzalutamide resistance in castration-resistant prostate cancer. Clin Cancer Res 2014; 20: 3198-3210.

[8] Liu C, Lou W, Zhu Y, Yang JC, Nadiminty N, Gaikwad NW, Evans CP and Gao AC. Intracrine androgens and AKR1C3 activation confer resistance to enzalutamide in prostate cancer. Cancer Res 2015; 75: 1413-1422.

[9] Liu C, Armstrong C, Zhu Y, Lou W and Gao AC. Niclosamide enhances abiraterone treatment via inhibition of androgen receptor variants in castration resistant prostate cancer. Oncotarget 2016; 7: 32210-32220.

[10] Gao AC, Armstrong CM, Liu C, Liu L, Yang JC, Lou W, Zhao R, Ning S, Lombard AP, Zhao J, D'Abronzo LS, Evans CP and Li PK. Steroid sulfatase stimulates intracrine androgen synthe-

sis and is a therapeutic target for advanced prostate cancer. Clin Cancer Res 2020; 26: 6064-6074.
[11] Nygaard R, Yu J, Kim J, Ross DR, Parisi G, Clarke OB, Virshup DM and Mancia F. Structural basis of WLS/Evi-mediated Wnt transport and secretion. Cell 2021; 184: 194-206, e114.
[12] Murillo-Garzon V and Kypta R. WNT signalling in prostate cancer. Nat Rev Urol 2017; 14: 683-696.
[13] Lombard AP, Liu C, Armstrong CM, D'Abronzo LS, Lou W, Evans CP and Gao AC. Wntless promotes cellular viability and resistance to enzalutamide in castration-resistant prostate cancer cells. Am J Clin Exp Urol 2019; 7: 203.
[14] Patro R, Duggal G, Love MI, Irizarry RA and Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. Nat Methods 2017; 14: 417-419.
[15] Soneson C, Love MI and Robinson MD. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. F1000Res 2015; 4: 1521.
[16] Anders S and Huber W. Differential expression analysis for sequence count data. Genome Biol 2010; 11: R106.
[17] Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES and Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005; 102: 15545-15550.
[18] Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D and Groop LC. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 2003; 34: 267-273.
[19] Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C and Schultz N. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012; 2: 401-404.
[20] Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C and Schultz N. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013; 6: pl1.
[21] Bland T, Wang J, Yin L, Pu T, Li J, Gao J, Lin TP, Gao AC and Wu BJ. WLS-Wnt signaling promotes neuroendocrine prostate cancer. iScience 2021; 24: 101970.
[22] Miyamoto DT, Zheng Y, Wittner BS, Lee RJ, Zhu H, Broderick KT, Desai R, Fox DB, Brannigan BW, Trautwein J, Arora KS, Desai N, Dahl DM, Sequist LV, Smith MR, Kapur R, Wu CL, Shioda T, Ramaswamy S, Ting DT, Toner M, Maheswaran S and Haber DA. RNA-Seq of single prostate CTCs implicates noncanonical Wnt signaling in antiandrogen resistance. Science 2015; 349: 1351-1356.
[23] Lombard AP, Liu L, Cucchiara V, Liu C, Armstrong CM, Zhao R, Yang JC, Lou W, Evans CP and Gao AC. Intra versus inter cross-resistance determines treatment sequence between taxane and AR-targeting therapies in advanced prostate cancer. Mol Cancer Ther 2018; 17: 2197-2205.
[24] Lombard AP, Liu C, Armstrong CM, D'Abronzo LS, Lou W, Evans CP and Gao AC. Wntless promotes cellular viability and resistance to enzalutamide in castration-resistant prostate cancer cells. Am J Clin Exp Urol 2019; 7: 203-214.
[25] Tai S, Sun Y, Squires JM, Zhang H, Oh WK, Liang CZ and Huang J. PC3 is a cell line characteristic of prostatic small cell carcinoma. Prostate 2011; 71: 1668-1679.
[26] Li Y, Chen R, Bowden M, Mo F, Lin YY, Gleave M, Collins C and Dong X. Establishment of a neuroendocrine prostate cancer model driven by the RNA splicing factor SRRM4. Oncotarget 2017; 8: 66878-66888.
[27] Boudadi K and Antonarakis ES. Resistance to novel antiandrogen therapies in metastatic castration-resistant prostate cancer. Clin Med Insights Oncol 2016; 10: 1-9.
[28] Aggarwal R, Huang J, Alumkal JJ, Zhang L, Feng FY, Thomas GV, Weinstein AS, Friedl V, Zhang C, Witte ON, Lloyd P, Gleave M, Evans CP, Youngren J, Beer TM, Rettig M, Wong CK, True L, Foye A, Playdle D, Ryan CJ, Lara P, Chi KN, Uzunangelov V, Sokolov A, Newton Y, Beltran H, Demichelis F, Rubin MA, Stuart JM and Small EJ. Clinical and genomic characterization of treatment-emergent small-cell neuroendocrine prostate cancer: a multi-institutional prospective study. J Clin Oncol 2018; 36: 2492-2503.
[29] Zhang Z, Cheng L, Li J, Farah E, Atallah NM, Pascuzzi PE, Gupta S and Liu X. Inhibition of the Wnt/beta-catenin pathway overcomes resistance to enzalutamide in castration-resistant prostate cancer. Cancer Res 2018; 78: 3147-3162.
[30] Nusse R. Wnt signaling and stem cell control. Cell Res 2008; 18: 523-527.
[31] Heuberger J and Birchmeier W. Interplay of cadherin-mediated cell adhesion and canoni-

cal Wnt signaling. Cold Spring Harb Perspect Biol 2010; 2: a002915.
[32] Abedini A, Sayed C, Carter LE, Boerboom D and Vanderhyden BC. Non-canonical WNT5a regulates epithelial-to-mesenchymal transition in the mouse ovarian surface epithelium. Sci Rep 2020; 10: 9695.
[33] Wen YC, Liu YN, Yeh HL, Chen WH, Jiang KC, Lin SR, Huang J, Hsiao M and Chen WY. TCF7L1 regulates cytokine response and neuroendocrine differentiation of prostate cancer. Oncogenesis 2021; 10: 81.
[34] Uysal-Onganer P, Kawano Y, Caro M, Walker MM, Diez S, Darrington RS, Waxman J and Kypta RM. Wnt-11 promotes neuroendocrine-like differentiation, survival and migration of prostate cancer cells. Mol Cancer 2010; 9: 55.
[35] Szczyrba J, Niesen A, Wagner M, Wandernoth PM, Aumuller G and Wennemuth G. Neuroendocrine cells of the prostate derive from the neural crest. J Biol Chem 2017; 292: 2021-2031.
[36] Nouri M, Caradec J, Lubik AA, Li N, Hollier BG, Takhar M, Altimirano-Dimas M, Chen M, Roshan-Moniri M, Butler M, Lehman M, Bishop J, Truong S, Huang SC, Cochrane D, Cox M, Collins C, Gleave M, Erho N, Alshalafa M, Davicioni E, Nelson C, Gregory-Evans S, Karnes RJ, Jenkins RB, Klein EA and Buttyan R. Therapy-induced developmental reprogramming of prostate cancer cells and acquired therapy resistance. Oncotarget 2017; 8: 18949-18967.
[37] Beltran H, Rickman DS, Park K, Chae SS, Sboner A, MacDonald TY, Wang Y, Sheikh KL, Terry S, Tagawa ST, Dhir R, Nelson JB, de la Taille A, Allory Y, Gerstein MB, Perner S, Pienta KJ, Chinnaiyan AM, Wang Y, Collins CC, Gleave ME, Demichelis F, Nanus DM and Rubin MA. Molecular characterization of neuroendocrine prostate cancer and identification of new drug targets. Cancer Discov 2011; 1: 487-495.
[38] Dardenne E, Beltran H, Benelli M, Gayvert K, Berger A, Puca L, Cyrta J, Sboner A, Noorzad Z, MacDonald T, Cheung C, Yuen KS, Gao D, Chen Y, Eilers M, Mosquera JM, Robinson BD, Elemento O, Rubin MA, Demichelis F and Rickman DS. N-Myc induces an EZH2-mediated transcriptional program driving neuroendocrine prostate cancer. Cancer Cell 2016; 30: 563-577.
[39] Lee JK, Phillips JW, Smith BA, Park JW, Stoyanova T, McCaffrey EF, Baertsch R, Sokolov A, Meyerowitz JG, Mathis C, Cheng D, Stuart JM, Shokat KM, Gustafson WC, Huang J and Witte ON. N-Myc drives neuroendocrine prostate cancer initiated from human prostate epithelial cells. Cancer Cell 2016; 29: 536-547.
[40] Bishop JL, Thaper D, Vahid S, Davies A, Ketola K, Kuruma H, Jama R, Nip KM, Angeles A, Johnson F, Wyatt AW, Fazli L, Gleave ME, Lin D, Rubin MA, Collins CC, Wang Y, Beltran H and Zoubeidi A. The master neural transcription factor BRN2 is an androgen receptor-suppressed driver of neuroendocrine differentiation in prostate cancer. Cancer Discov 2017; 7: 54-71.
[41] Guo H, Ci X, Ahmed M, Hua JT, Soares F, Lin D, Puca L, Vosoughi A, Xue H, Li E, Su P, Chen S, Nguyen T, Liang Y, Zhang Y, Xu X, Xu J, Sheahan AV, Ba-Alawi W, Zhang S, Mahamud O, Vellanki RN, Gleave M, Bristow RG, Haibe-Kains B, Poirier JT, Rudin CM, Tsao MS, Wouters BG, Fazli L, Feng FY, Ellis L, van der Kwast T, Berlin A, Koritzinsky M, Boutros PC, Zoubeidi A, Beltran H, Wang Y and He HH. ONECUT2 is a driver of neuroendocrine prostate cancer. Nat Commun 2019; 10: 278.
[42] Ma F, Arai S, Wang K, Calagua C, Yuan AR, Poluben L, Gu ZK, Russo JW, Einstein DJ, Ye HH, He MX, Liu Y, Allen EV, Sowalsky AG, Bhasin MK, Yuan X and Balk SP. Autocrine canonical Wnt signaling primes noncanonical signaling through ROR1 in metastatic castration-resistant prostate cancer. Cancer Res 2022; 82: 1518-1533.