The $\beta_2$-Adrenergic Receptor Is a Molecular Switch for Neuroendocrine Transdifferentiation of Prostate Cancer Cells

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Abstract

The incidence of treatment-related neuroendocrine prostate cancer (t-NEPC) is rising as more potent drugs targeting the androgen signaling axis are clinically implemented. Neuroendocrine transdifferentiation (NEtD), an apparent initial step in t-NEPC development, is induced by androgen-deprivation therapy (ADT) or anti-androgens, and by activation of the $\beta_2$-adrenergic receptor (ADRB2) in prostate cancer cell lines. Thus, understanding whether ADRB2 is involved in ADT-initiated NEtD may assist in developing treatment strategies that can prevent or reverse t-NEPC emergence, thereby prolonging therapeutic responses. Here we found that in primary, treatment-naive prostate cancers, ADRB2 mRNA was positively correlated with expression of luminal differentiation markers, and ADRB2 protein levels were inversely correlated with Gleason grade. ADRB2 mRNA was upregulated in metastatic prostate cancer, and progressively downregulated during ADT and t-NEPC emergence. In androgen-deprived medium, high ADRB2 was required for LNCaP cells to undergo NEtD, measured as increased neurite outgrowth and expression of neuron differentiation and neuroendocrine genes. ADRB2 overexpression induced a neuroendocrine-like morphology in both androgen receptor (AR)-positive and -negative prostate cancer cell lines. ADRB2 downregulation in LNCaP cells increased canonical Wnt signaling, and GSK3β inhibition reduced the expression of neuron differentiation and neuroendocrine genes. In LNCaP xenografts, more pronounced castration-induced NEtD was observed in tumors derived from high than low ADRB2 cells. In conclusion, high ADRB2 expression is required for ADT-induced NEtD, characterized by ADRB2 downregulation and t-NEPC emergence.

Implications: This data suggest a potential application of β-blockers to prevent cancer cells committed to a neuroendocrine lineage from evolving into t-NEPC.

Introduction

Androgen-deprivation therapy (ADT), involving surgical or chemical castration with agonists or antagonists of luteinizing hormone-releasing hormone, remains the pillar in treatment of metastatic hormone-sensitive prostate cancer. The majority of patients initially respond due to innate tumoral androgen dependence, but treatment resistance predictably develops as castration-resistant prostate cancer (CRPC; ref. 1). Despite the introduction of second-line androgen receptor pathway inhibitors (ARPI) such as abiraterone, enzalutamide, and apalutamide, which have offered a survival benefit (2), CRPC remains a lethal disease.

Recent studies indicate that increasing use of existing and novel ARPIs is introducing new challenges in the management of advanced prostate cancer. More potent AR pathway inhibition is believed to impose a therapeutic pressure on prostate cancer cells, which can promote adaptation and selection for traits that alleviate tumoral androgen dependence (3, 4). In recent years, novel CRPC subtypes displaying AR negativity and/or low reliance on AR signaling have gained attention because of their increased incidence in the post-abiraterone/
enazalutamide era (3, 5, 6). These subtypes are believed to develop through lineage plasticity, whereby prostate cancer cells escape androgen dependence and sensitivity through relying on alternative pathways for sustained survival (7, 8), and may display stem cell/basal-like features and/or neuroendocrine differentiation (9).

A significant proportion of androgen-independent CRPCs display neuroendocrine differentiation, and are commonly termed treatment-related neuroendocrine prostate cancer (t-NEPC). As many as 13–27% of patients with CRPC have tumors which display NEPC (3, 10, 11), and almost 80% of metastatic CRPCs show at least focal neuroendocrine features (12). t-NEPC is more frequently observed in tumors challenged with ARPIs, but a significant proportion of abiraterone/enzalutamide-naive, ADT-treated tumors also develop t-NEPC (11). Molecularly, NEPCs typically feature positive IHC staining with neuroendocrine markers, low or negative AR expression, and disproportionally low or negative PSA expression. Clinically, NEPCs commonly metastasize to visceral tissue, form osteolytic bone metastases, are very aggressive, and not responsive to androgen signaling–targeted therapies (5, 13). These molecular and clinical features resemble the highly aggressive de novo small-cell carcinoma of the prostate, which accounts for less than 1% of all primary prostate cancer diagnoses.

A model where neuroendocrine cells present in advanced prostate cancer are clonally selected during ADT has been suggested at least in part to explain the emergence of NEPC (14). However, compelling evidence from both preclinical models and genetic studies on autopsy-derived metastases (4, 15) suggested that these cells divergently transdifferentiate from adenocarcinoma cells during androgen-targeting therapy modalities.

Adrenergic stimulation, like androgen withdrawal, has been shown to induce neuroendocrine-like features in prostate cancer cell lines (18, 19). In this study, we have investigated the role of β-adrenergic receptor (ADRB2) in prostate cancer progression to NEPC by analyses of clinical specimens and functional assays on preclinical model systems with modulated ADRB2 levels. We find that ADRB2 acts as a molecular switch for NEtD in prostate cancer, and is progressively lost during tumor dedifferentiation and emergence of therapy resistance.

Materials and Methods

Tissue microarray and IHC

The tissue microarray (TMA) includes radical prostatectomy tissue from 122 patients treated at Skåne University Hospital between 1999 and 2002 (20). The TMA was IHC stained as described previously (21) with an anti-ADRB2 primary antibody (MC2656; MBL Int.) in a 1:4,000 dilution. Time of biochemical recurrence was defined as the first date of two consecutive rises in PSA above nadir (>0.2 ng/mL).

Manual scoring of the ADRB2 antibody staining on a scale from 1 to 3 was performed by two experienced uropathologists (W. Wang and A. Svindland). The most profound Gleason grade in each spot was also reported. In cases with more than one cancerous spot from an individual patient, the staining intensity for the spot(s) with the highest Gleason grade was chosen for further analysis. All patients included on the TMA provided informed consent. The study was approved by the Regional Committees for Medical and Health Research Ethics (434-04153, S-04153c, 2009/373, and 09/00450-2/bso 2009/1028). Oslo University Research Support Unit (2011/3286), and by the Regional Ethical Review Board at Lund University (Sweden, approval number DN. 494-2005).

Whole sections of fresh-frozen paraffin-embedded xeno grafts were stained with an anti-ADRB2 antibody (1:4,000) as described above. Staining procedures for tubulin β 3 class III (TUBB3; 1:800; 801201, Nordic BioSite) and anti-CD31 (1:50; ab28364, Abcam) are detailed in Supplementary Materials and Methods. Whole-tumor section brightfield 10 × scans were acquired using an AxioScan Z1 (Carl Zeiss), and were imported to ImageJ. Staining positive areas were measured by altering threshold and related to the total tumor area, excluding adjacent mouse stroma.

Cell lines and reagents

Prostate cancer cell lines LNCaP, VCaP, and PC-3 were purchased from ATCC. All cells were maintained in RPMI1640 (#R0883; Sigma) with 10% FCS (androgen-proficient medium; #F7524; Sigma), 100 units/mL penicillin and 50 mg/mL streptomycin (#P4458; Sigma), and 1% alanyl-glutamine (#G8541; Sigma) at 37°C with an atmosphere of 5% CO2 and humidified air. In experiments involving androgen depletion, RPMI1640 was replaced with phenol red-free RPMI1640 (#32404014; Thermo Fisher Scientific), and FCS was replaced with 2% charcoal-stripped serum (CSS; #A3382101; Thermo Fisher Scientific). Stable knockdown of ADRB2 in LNCaP cells was performed as described previously (21). In the current study, we used an additional stable ADRB2 knockdown cell line (LNCaP shADRB2-3; insert sequence gaagctatcctcaata). Hence, we utilized three shADRB2 cell lines (LNCaP shADRB2-1, shADRB2-2 and shADRB2-3) and one LNCaP control cell line stably transfected with control shRNA (shCtrl). Stably transfected cells were kept in culture medium supplied with 200 µg/mL G418 sulphate (#10131-027; Thermo Fisher Scientific). Cell IDs of VCaP, PC-3, parental LNCaP, LNCaP shADRB2, and shCtrl cell lines were verified using the STR PowerPlex16 System (Promega), and the cells were routinely checked for Mycoplasma infections. The GSK3β/β inhibitor, CHIR990012, was ordered from Selleckchem (#S2924). MSAB (#SML1726), [±] isoproterenol (#15627), propranolol (#P0884-1G), and forskolin (F6886) were purchased from Sigma.

Semi-quantitative real-time RT-PCR

Total RNA was extracted using the TRizol Reagent (#15596018; Thermo Fisher Scientific) following the manufacturer’s protocol (Invitrogen). A total of 100 ng of total RNA was used with the qScript One-Step qRT-PCR Kit (Quantabio) as described previously (21). The primer sequences are listed in Supplementary Materials and Methods. ALAS1 or POLR2A mRNA expression were used for reference. To display relative gene expression the ΔΔC method was used.

DNA microarray

The integrity of extracted RNA was assessed using the 2100 Bioanalyzer (Agilent). A total of 500 ng RNA was amplified and labeled according to the TotalPrepTM RNA Amplification Kit protocol (Illumina), and concentrations were measured using the NanoDrop Spectrophotometer (Thermo Fisher Scientific), and quality and size distributions were assessed using the 2100 Bioanalyzer. A total of 750 ng of biotin-labeled cRNA was hybridized to Illumina’s HumanHT-12 v4 Expression BeadChip
Protein extraction and Western blot analysis

The cells were harvested, lysed in whole-cell buffer (21), and centrifuged at 16,000 × g for 20 minutes. The NE-PER Nuclear and Cytoplasmic Extraction Reagents (#78833; Thermo Fisher Scientific) were used for fractionation of cells. Western blots were prepared and visualized as reported previously (21). Antibodies and their dilutions are listed in Supplementary Materials and Methods. Where predicted molecular weights were similar for two or more antibodies, the same set of protein extracts were probed on separate gels.

ADRB2 radioligand binding assay

Cell membrane fractions from shCtrl and shADRB2 cells were prepared as described in (23). A radioligand binding assay was used to measure the ADRB2 protein binding activity as described previously (23, 24).

Neurite outgrowth measurements

For manual neurite length measurements, at least two representative regions of the images containing cells with similar aggregation and confluence were analyzed from ≥3 independent experiments. Measurement of the length of all neurites present in experiments. Measurement of the length of all neurites present in the captured, representative fields was performed using the NeuronGrowth plug-in in ImageJ (25) and was related to the manually counted number of cells in each field. Phase contrast light microscopy images were acquired with the IncuCyte live-cell analysis system equipped with a 10 × objective (ESSEN Bioscience) or an IX81 Microscope (Olympus). Automatic neurite length and branch points per cell (mm²) quantifications were performed using the NeuroTrack Software module in six technical replicates of representative fields acquired on the IncuCyte S3 imaging system equipped with a 10 × objective from ≥3 independent experiments.

ADRB2 overexpression experiments

The pcDNA3 Flag beta-2-adrenergic-receptor (pADRB2) containing the full-length ADRB2 gene and a Flag-tag was a kind gift from Finn Olav Levy (University of Oslo, Oslo, Norway) and Robert LeKowitz (Duke University Medical Center, Durham, NC) (#14697; Addgene; ref. 26). A nonexpressing/empty pcDNA3.1/Zeo vector (pEmpt; #V860-20; Invitrogen) was used as transfection control. LNCaP parental, shCtrl and shADRB2, PC-3, and VCaP cells were transfected for 48 hours using XtremaGene HP DNA Transfection Reagent (#06366236001; Sigma) under androgen-proficient conditions.

Luciferase assay

In brief, cells were transfected using the DharmaFECT Duo Reagent (#T-2010-0; Dharmacon). The plasmid pGL4.49 luc2P/TCF-LEF RE/Hygro (#E4611; Promega), containing eight copies of the TCF-LEF response elements, was used as control. The pEZX-PG04 vector expressing secreted alkaline phosphatase (SEAP; GeneCopoeia) was used to normalize for differences in transfection efficiency. The SEAP activity in medium samples was measured 24 or 48 hours after transfection using the Secretre-Pair Dual Luminescence Assay kit (#SPDA-D100; GeneCopoeia) on a Victor Wallac Spectrophotometer (PerkinElmer). To determine intracellular luciferase activity, cells were lysed in 1× Reporter Lysis Buffer (#E397A; Promega), the supernatant mixed with Luciferase Assay Reagent (#E152A; Promega), and the activity measured on a TD-20/20 Luminometer (Turner Designs).

Animal studies

Fifty-eight in-house bred, 4 weeks old male NOD-SCID gamma/mmu null mice weighing 28.2 ± 4 g were administered 0.03 mg/mL testosterone (#NA-151 Sigma) in drinking water 1 week prior to subcutaneous injection into the hind flank with 2 × 10⁶ cells of shCtrl (n = 30), shADRB2-2 (n = 13), or shADRB2-2 (n = 15). Tumor volumes were assessed as described in (21). Time to take was defined as the time to the first measurement of the first two consecutive measurements of a palpable tumor. The mice were surgically castrated, and following termination, tumors were harvested, fresh-frozen, and paraffin-embedded for IHC analyses. The experiment was approved by the National Animal Research Authority (FOTS ref. 7132) and was performed according to the guidelines and safety standards set by the Federation of European Laboratory Animal Science Association.

In silico analyses on published datasets

Median RNA-seq RNA expression values and clinopathologic data from Neuroendocrine Prostate Cancer (Trento/Cornell/ Broad 2016, hereby called NEPC WC; ref. 4) were downloaded from cbioPortal (27, 28). Median expression values were scale normalized in R v3.4.3. Gene expression values and clinopathologic data from the Fred Hutchinson CRC (FHCRC; expression microarray; ref. 29) and the Stand Up To Cancer/PCF Dream Team (SU2C/PCF; RNA-seq) 2015 (RPM; ref. 30) and 2019 (FPKM; ref. 31) cohorts were downloaded from cbioPortal using the CDSR package in R. Hierarchical clustering (Ward method) and heatmaps were generated using the pheatmap package in R. Where more than one sample was present for a patient, the samples were analyzed individually. Scale-normalized gene expression levels were downloaded from GSE35998 (GPL6848 and GPL6480; ref. 32) and GSE41193 (16) using the GEOquery package in R from GEO. Quantile-normalized ADRB2 expression levels in cell lines were acquired from data deposited under GSE25183 (33). Gene set enrichment analyses were performed using DAVID v6.7 Functional Annotation (34).

Statistical analysis

For cell line studies, two-sided Student t test was applied to test for statistical significance, and error bars depict SEM. The Shapiro–Wilks test was used to test normality. For animal experiment data, statistical significance was calculated using Wilcoxon rank sum tests or t tests where indicated. Spearman correlation (p) was applied to evaluate gene expression correlations. Wilcoxon rank sum tests were used to evaluate differences in ADRB2 gene expression in clinical datasets, assuming nonnormal distributions determined by Shapiro–Wilks tests. For our microarray analysis, differential gene expression was assessed by significance analysis
of microarrays comparing mRNA levels between shCtrl and shADRB2-2 and -3 LNCaP cells. A FDR of less than 0.05 was considered statistically significant.

Cox proportional hazards modeling was used for multivariable survival analyses. Kendall tau-b correlation analysis was performed to evaluate correlations between Gleason grade and ADRB2 and AR staining intensities. Statistical analyses were performed using STATA version 15 and R v3.4.3. A two-sided $P < 0.05$ was considered statistically significant for all analyses.

**Results**

ADRB2 is associated with luminal differentiation

IHC staining of a TMA consisting of specimens from 76 radical prostatectomies with an anti-ADRB2 antibody showed high ADRB2 levels in differentiated and low levels of ADRB2 in poorly differentiated prostate cancers (Fig. 1A). ADRB2 was positively correlated with AR staining intensity (Kendall Tau-b correlation 0.30; $P = 0.011$) and inversely correlated with Gleason Grade in the spot (Kendall tau-b correlation $-0.49$; $P < 0.0001$; Fig. 1B). Baseline patient characteristics are shown in Supplementary Table S1. Clinical follow-up data from this cohort showed that patients with low ADRB2 staining intensities had a higher risk of biochemical recurrence than patients with high ADRB2 [HR, 0.51; 95% confidence interval (CI), 0.25–1.04; $P = 0.064$]. This was fully explained by Gleason grade in the spots (HR for ADRB2 adjusted for Gleason grade, 1.81; 95% CI, 0.61–5.39; $P = 0.29$).

**Corroborative of an association with luminal differentiation,** ADRB2 mRNA was also highly correlated with NKX3.1, AR, KLK3, KLK4, and HOXB13 mRNAs in hormone-naïve tumors in the Memorial Sloan Kettering Cancer Center (MSKCC) dataset (ref. 35; Fig. 1C). Similarly, we also observed a positive correlation between ADRB2 and NKX3.1 in radical prostatectomy specimens from the Prostate Biobank at the Oslo University Hospital ($n = 20$; Spearman $r = 0.64$; $P < 0.01$; Supplementary Fig. S1).

ADRB2 is functionally involved in NEtD of prostate cancer cell lines

Epigenetic silencing of ADRB2 is associated with progressive differentiation of embryonic stem cells toward terminally differentiated neural cells (36), and ADRB2 was recently reported to be among the top downregulated genes in a meta-analysis–developed NEPC gene signature (37). However, the direct involvement of ADRB2 in NEPC development has not been investigated. To test the implication of ADRB2 downregulation in a hormone-sensitive adenocarcinoma model system, we employed LNCaP cell line derivatives stably transfected with three different shRNAs targeting ADRB2 mRNA (shADRB2-1, shADRB2-2, and shADRB2-3) and a negative control shRNA (shCtrl; ref. 21). shADRB2-1, -2, and -3 presented about 50%, 85%, and 80% lowered $b$-adrenergic receptor ligand binding activity, respectively, compared with shCtrl, which was also apparent by IHC staining of formalin-fixed cells (Supplementary Fig. S2A and S2B).
ADRB2 is functionally involved in androgen depletion- and β-adrenergic signaling pathway–mediated NETD. A, Venn diagram of genes differentially expressed in shADRB2-2 and shADRB2-3, both compared with shCtrl (FDR < 0.05). The top five enriched pathways identified by DAVID v6.7 are shown, with differentially expressed genes involved in neuron differentiation indicated on the right. B, ENO2 and TUBB3 mRNA levels in shADRB2 and shCtrl cells incubated in 2% CSS for 5 days were measured using sqRT-PCR. Bars represent mean ADRB2 mRNA levels relative to shCtrl. C, Representative Western blots visualizing ENO2 (NSE) and TUBB3 protein levels in whole-cell protein extracts from shCtrl and shADRB2 cells incubated in 2% CSS for 5 days. α-Tub, α-Tubulin. D, Relative changes in ENO2 and TUBB3 mRNA levels in shCtrl and shADRB2 cells (n = 2 for shADRB2-3) after withdrawal of androgens. Expression levels are shown as CSS (5 days) relative to FCS-treated cells. The dotted line indicates 1 (no change in expression). E, Representative fields from phase contrast light microscopy images of LNCaP shCtrl and shADRB2 cultured in 2% CSS for 5 days. F, Neurite lengths were automatically quantified using the IncuCyte S3 Imaging System in six representative fields captured from shCtrl and shADRB2 cells challenged with 2% CSS for 72 hours. Bars represent mean field-wise neurite lengths per cell number in each field relative to shCtrl, with >2 technical replicates per independent experiment. G, In independent biological experiments, neurite lengths were quantified in shCtrl and shADRB2 cells incubated in 2% CSS for 5 days by manual neurite tracing, and bars represent mean field-wise neurite lengths per cell number in each field relative to shCtrl, with >2 technical replicates per independent experiment. H, The number of branch points in CSS-challenged shCtrl and shADRB2 cells were measured per field and related to the total area. I, Mean neurite lengths per cell were manually quantified in fields acquired from cells incubated in androgen-proficient conditions (FCS) with vehicle, or 10 μM/L of either isoproterenol (ISO), isoproterenol + propranolol (ISO + PRO), or forskolin (FSK) for 90 minutes. All experiments were performed in ≥3 biological replicates. Statistical significance was tested using two-sided t tests, and categorized significance levels are indicated by asterisks. * P < 0.05; ** P < 0.01; *** P < 0.001. Error bars indicate SEM.
Microarray expression analysis of shADRB2-2 and shADRB2-3 against shCtrl-treated cells revealed 51 commonly differentially expressed genes (FDR < 0.05). Pathway enrichment analysis of these genes showed "neuron differentiation" and "neuron projection development" as among the top enriched pathways (Fig. 2A). Using semi-quantitative real-time RT-PCR (sqRT-PCR), we validated the decreased expression of four of five genes implicated in neuron differentiation upon knockdown of ADRB2: Signal transducer CD24 (CD24), SLIT, and NTRK-Like family member 3 (SLITRK3), jagged canonical Notch ligand 1 (JAG1) and kelch-like family member 1 (KLHL1, Supplementary Fig. S2C). Downregulation of JAG1 and NTRK-Like family member 3 (SLITRK3), jagged canonical Notch ligand 1 (JAG1) and kelch-like family member 1 (KLHL1; Supplementary Fig. S2C). Downregulation of JAG1 and NTRK-Like family member 3 (SLITRK3), jagged canonical Notch ligand 1 (JAG1) and kelch-like family member 1.

Previous studies on the β-adrenergic signaling pathway (18, 19), and our gene expression analysis on shADRB2 cells suggested a role of ADRB2 in NeD. Therefore, we hypothesized that lowered expression of neuron differentiation genes in shADRB2 cells would result in impaired NeD. To this end, we incubated shCtrl and shADRB2 androgen-sensitive LNCaP cells in androgen-depleted medium, thus mimicking ADT. After 5 days, both transcript and protein levels of the neuronal markers enolase 2 (ENO2) and tubulin β 3 class III (TUBB3) were lower in shADRB2 than shCtrl cells (Fig. 2B and C). ENO2 and TUBB3 mRNA levels were induced in response to androgen deprivation, as expected, but the effect was less prominent in shADRB2 cells than shCtrl cells (Fig. 2D). In the presence of higher testosterone levels (10% FCS), ENO2 mRNA and protein levels, but not TUBB3 mRNA, were lower in shADRB2 cells than shCtrl cells (Supplementary Fig. S2D and S2E), suggesting that ENO2 is under control of ADRB2 independently of the androgen levels to which the cells are exposed.

In agreement with previous reports on the LNCaP cell line model (38, 39), LNCaP shCtrl cells progressively developed complex cell protrusion networks reminiscent of neurite outgrowths and smaller and more compact soma following androgen depletion (Fig. 2E). After 72 hours of androgen depletion, the length of neurites per cell was significantly (t test all P < 0.001) lower in shADRB2 cells compared with shCtrl cells (Fig. 2F). These findings were corroborated by quantification of neurite lengths in independent experiments (n = 3) where the cells were incubated in androgen-depleted medium for 5 days (Fig. 2G). Interestingly, the moderately ADRB2-depleted shADRB2-1 cells only showed a trend toward shorter neurite lengths after 5 days of androgen withdrawal (P > 0.05), suggesting ADRB2 dosage dependency. Similar results were obtained after 48 hours of androgen depletion (Supplementary Fig. S2F). Moreover, the average number of branch points per field was also lowered in shADRB2 cells (Fig. 2H).

In line with our previous findings (21), LNCaP shADRB2 cells were less sensitive to antiandrogen treatment than shCtrl cells (Supplementary Fig. S3). These data indicated that, upon challenge with androgen depletion, LNCaP shADRB2 cells had reduced capabilities toward undergoing NeD. Moreover, these cells were more resistant to treatments targeting the androgen-signaling axis than high ADRB2-expressing cells, supporting that shADRB2 cells represent an adeno-CRPC model.

The well-known NeD-promoting effect of β-adrenergic receptor stimulation made us hypothesize that ADRB2 knockdown would abate agonist-induced NeD under androgen-proficient conditions. After short exposure to the β-adrenergic receptor agonist isoproterenol in androgen-proficient medium, shCtrl and shADRB2-1 developed long and complex neurite outgrowths, whereas the effect was modest in shADRB2-2 and shADRB2-3 (Supplementary Fig. S2C), in-line with the latter two cell lines having the lowest ADRB2 protein levels. The effects of isoproterenol were abrogated by co-supplementation with the β-adrenergic antagonist propranolol, suggesting the effects were specific. Interestingly, in vehicle-treated cells, shADRB2-2 (P = 0.1) and shADRB2-3 (P < 0.05) tended to have less neurite outgrowth than shCtrl and shADRB2-1. Interestingly, forskolin, activating adenylyl cyclase downstream of ADRB2, rescued neurite outgrowth in the shADRB2 cells, yielding similar neurite lengths per cell as stimulated shCtrl cells (Fig. 2H; Supplementary Fig. S2G).

The observation that shADRB2 cells were capable of transdifferentiating upon cAMP elevation (bypassing ADRB2) suggested that ADRB2 overexpression in shADRB2 cells would mimic this effect and induce NeD. Indeed, the three shADRB2 cell lines transiently overexpressing ADRB2 developed extensive neurite outgrowth in androgen-proficient medium (Fig. 3A; Supplementary Fig. S4A). We quantified neurite outgrowth and found that it was elevated in all the ADRB2-overexpressing cells compared with pEmpty-transfected cells (Fig. 3B). Total neurite length per cell were similar in ADRB2-overexpressing shCtrl and shADRB2 cells (t test all P > 0.05), and neurite lengths per cell were not different from those developed upon short-term forskolin treatment (t test all P > 0.10). We could confirm expression and plasma membrane localization of ADRB2 using an anti-Flag tag antibody (Supplementary Fig. S4B), and overexpression by radioligand binding assay (data not shown). Surprisingly, transient ADRB2 overexpression did not increase immunofluorescence intensities of TUBB3, although it was present in neurites (Supplementary Fig. S4C). Moreover, both mRNA and protein levels of TUBB3 and other neuroendocrine markers such as synaptophysin (SYP) and chromogranin A (CHGA) were unchanged following ADRB2 overexpression (data not shown).

To further substantiate the finding that ectopic ADRB2 overexpression induced neuroendocrine-like morphologic characteristics in LNCaP cells, we overexpressed ADRB2 in VCaP and PC-3 cells, both expressing lower ADRB2 protein (Fig. 3C) and mRNA (Fig. 3D) levels than parental LNCaP cells. Consistent with our results in LNCaP cells, ADRB2 overexpression increased neurite outgrowth also in a subset of VCaP and PC-3 cells (Supplementary Fig. S4D). In both VCaP and PC-3 cells, ADRB2 overexpression led to a higher proportion of cells with neurite outgrowths, particularly in VCaP cells (Fig. 3E). The increase in neurite length was particularly clear and significant in PC-3 cells (Fig. 3F). The NeD-like morphologic changes in PC-3 and VCaP cells upon overexpression of ADRB2 were accompanied by unchanged levels of the neuroendocrine markers TUBB3 (Supplementary Fig. S4C), CHGA, and SYP, in-line with what was observed in LNCaP cells.

Collectively, these data show that ADRB2 promotes neurite outgrowth but not induction of neuroendocrine markers, whereas ADRB2 depletion abrogates both neurite outgrowth and neuroendocrine marker elevation.

Depletion of ADRB2 increases β-catenin activity, and inhibition of GSK3β reduces neuroendocrine characteristics

The canonical Wnt pathway is believed to be a central factor in the development of CRPC through interaction with AR (40).
Moreover, canonical Wnt signaling has been shown to be elevated in neuroendocrine-transdifferentiated LNCaP cells and in neuroendocrine tumors from TRAMP mice (41). We sought to investigate whether stable ADRB2 knockdown could impact this pathway and thereby reduce NEtD in these cells. Western blotting of nuclear cell extracts from shADRB2 LNCaP cells revealed elevated levels of active $\beta$-catenin (unphosphorylated at S37 and T41; Fig. 4A). Using a luciferase reporter system, we found that, as expected, androgen depletion led to an elevated TCF/LEF (transcription factors activated by nuclear $\beta$-catenin) transcriptional activity, which was significantly higher in shADRB2-treated cells, particularly in androgen-depleted medium (Fig. 4B). Reintroduction of ADRB2 in the shADRB2-treated cells tended to decrease the TCF/LEF activity in shADRB2-2 (paired $t$ test $P = 0.18$; Fig. 4C). VCaP cells which express lower ADRB2 than LNCaP, showed a higher ratio of active $\beta$-catenin to total $\beta$-catenin and had higher TCF/LEF reporter activity than LNCaP cells (Supplementary Fig. S5A and S5B).

We tested whether siRNA-mediated knockdown of $\beta$-catenin or direct $\beta$-catenin inhibition by supplementation with MSAB would rescue NEtD in shADRB2 cells, and found no effect on NEtD morphology or neuroendocrine marker expression (not shown). Activation of $\beta$-catenin by GSK3$\alpha$/3$\beta$ inhibition with CHIR99021, however, reduced androgen depletion–induced NEtD in shCtrl (Fig. 4D and E). CHIR99021 increased nuclear localization of $\beta$-catenin, TCF/LEF reporter activity, and total levels of active $\beta$-catenin in shCtrl cells (Supplementary Fig. S5C–S5E). Interestingly, CHIR99021 reduced expression of the neuron differentiation–related genes that were downregulated in shADRB2 cells, as well as ENO2 and TUBB3.

**Figure 3.** Ectopic ADRB2 overexpression induces neurite outgrowth in cell line models with different ADRB2 levels. A, Representative fields from phase contrast light microscopy images captured from LNCaP shCtrl, shADRB2, and parental LNCaP cells following 48 hours transfection with pEmpty (pcDNA3.1/Zeo) or pADRB2 (pcDNA3 Flag pADRB2) under androgen-proficient conditions (scale bars, 50 $\mu$m). B, Neurite lengths per cell in LNCaP shCtrl and shADRB2 cells transfected for 48 hours with pEmpty or pADRB2 in androgen-proficient medium. C, $^{35}$S-CYP specific binding to membrane protein fractions from parental LNCaP, VCaP, and PC-3 cells was determined. Membrane bound ADRB2 levels are shown as mean fmol/mg protein in the membrane fractions. D, Quantile normalized ADRB2 mRNA levels in LNCaP, VCaP, and PC-3 cells accessible from GSE25183. E, Fraction of cells displaying neurite outgrowth in VCaP and PC-3 cells transfected with pEmpty or pADRB2 for 48 hours in androgen-proficient medium. F, Neurite length per cell in pADRB2-transfected PC-3 cells relative to pEmpty-transfected cells. All experiments were performed in ≥3 biological replicates. Statistical significance was tested using two-sided $t$ tests, and categorized significance levels are indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars indicate SEM.
ADRB2 is suppressed in NEPC and associated with neuroendocrine characteristics

We have previously shown that ADRB2 mRNA is upregulated in primary prostate cancer and ADT-naïve metastases, and downregulated following neoadjuvant ADT in primary prostate cancer (24), suggesting an association with disease progression due to the near inevitable emergence of CRPC among these patients. Using multiple publicly available prostate cancer datasets, we confirmed our previous findings that ADRB2 levels were consistently upregulated in primary prostate cancer and elevated in metastases from ADT-naïve patients compared with benign tissue samples (Fig. 5A; Supplementary Fig. S6A). Moreover, ADRB2 mRNA levels were downregulated in CRPC samples, primarily characterized by reactivation of AR and AR overexpression, compared with treatment-naïve metastases.

Figure 4.
ADRB2 knockdown increases β-catenin activity, and GSK3β-inhibition reduces neuroendocrine characteristics. A, Representative Western blot of active β-catenin in nuclear extracts from shCtrl and shADRB2 cells. Histone H3 was used as loading control. B, SEAP-normalized TCF/LEF-driven luciferase activities of shCtrl and shADRB2 cells transfected with after incubation in 10% FCS or 2% CSS for 48 hours. C, SEAP-normalized TCF/LEF activities of shADRB2-2 cells transfected with pEmpty or pADRB2 in androgen-proficient medium. D, Representative fields from phase contrast light microscopy captured images of LNCaP parental cells incubated for 48 hours in 2% CSS supplemented with vehicle or 10 μmol/L CHIR99021 (CHIR). E, Neurite lengths per cell quantified in each cell treated as in D. F, Normalized mRNA levels of neuron differentiation genes differentially expressed in shADRB2 cells (JAG1, SLITRK3, CD24, and KLHL3) and neuroendocrine (NE) markers ENO2 and TUBB3 in LNCaP parental cells incubated in androgen-proficient medium supplemented with either vehicle or 10 μmol/L CHIR99021 for 24 or 48 hours. mRNA levels were measured using qRT-PCR, and bars represent expression levels relative to vehicle-treated cells. All experiments were performed in ≥3 biological replicates. Statistical significance was tested using two-sided t-tests, and categorized significance levels are indicated by asterisks. *P < 0.05; **P < 0.01; ***P < 0.001. Error bars indicate SEM.
and primary prostate cancers (Fig. 5A; Supplementary Fig. S6A and S6C).

It has been shown that at least a subset of NEPCs are clonally derived from adeno-CRPCs, and NEPC tumors express low levels of prostate luminal genes (4). Accordingly, in metastatic CRPC samples with NEPC histology, ADRB2 levels were further significantly downregulated compared with adeno-CRPC samples (Fig. 5B). This finding led us to perform a correlative analysis between ADRB2 expression levels with a panel of genes previously shown to be altered in NEPC (Fig. 5C). The gene panel consisted of previously published NEPC/small-cell phenotype-associated genes (4, 37) and genes coding for neuroendocrine markers used in pathologic assessment of NEtD/NEPC. Notably, the NEPC WCM cohort showed that the majority (13/16) of metastases with low ADRB2 expression (lower tertile) had histologically confirmed NEPC. Low ADRB2 expression was associated with
elevated expression of neuroendocrine markers and inversely associated with prostatic luminal markers. Similar associations were found in the FHCRC and SU2C/PCF 2019 cohorts by hierarchical clustering (Supplementary Fig. S6D). Collectively these data corroborate the functional studies presented in this work, namely that loss or lowered ADRB2 expression is a characteristic of, and may be involved in, NEPC development through enabling ADT-induced NiEtD.

To understand the molecular downstream effect of ADRB2 activity, we selected the top 400 genes showing positive or negative correlation with ADRB2 expression (800 genes in total) in the NEPC WCM, SU2C/PCF 2015, and FHCRC mCRPC data sets. We then performed an explorative gene ontology analyses on the 400 top genes negatively correlated with ADRB2 in each dataset individually, and 13 enriched pathways were found to overlap between the datasets (Supplementary Fig. S6E). Interestingly, these pathways included generation of neurons (GO:0048699), nervous system development (GO:0007399), and cell differentiation (GO:0030154).

A total of 23 ADRB2-correlated genes were found to overlap between all the datasets. Within these 23 genes we identified 15 genes (Supplementary Table S2) that showed expression levels associated with NEPC histology in the NEPC WCM dataset. Hierarchical clustering revealed that genes negatively correlated with ADRB2 clustered with neuroendocrine-up genes, while genes positively correlated with ADRB2 expression clustered with high expression of luminal genes (Fig. 5D). Several of the identified genes correlating with ADRB2 have previously been included in ADRB2-correlated genes and ADRB2 as a t-NEPC gene expression signature of NEPCs but not adeno-CRPCs in the NEPC WCM dataset (Supplementary Fig. S7A). Collectively, these results suggest a utility of the ADRB2 signature as a transcriptomic signature for t-NEPC.

To elucidate mechanisms responsible for the observed downregulation of ADRB2 following therapy resistance, we performed a reanalysis of the NEPC WCM cohort for ADRB2 copy-number variation, but found no differences in frequencies of amplifications (20% vs. 22 %) or shallow deletions (3% vs. 7 %) in adeno-CRPCs and NEPCs ($\chi^2$ test, both $P > 0.05$; Supplementary Fig. S7B). These data suggested the ADRB2 is epigenetically or transcriptionally silenced, or destabilized at the mRNA level in NEPC. ADRB2 has been identified as an Zeste homolog 2 (EZH2) target gene (43) and we observed that EZH2 was inversely correlated with ADRB2 mRNA levels in t-NEPCs but not adeno-CRPCs in the NEPC WCM dataset (Supplementary Fig. S7C). Accordingly, a reanalysis of the GSE107782 dataset (44) showed that both EZH2 inhibition and transient EZH2 knockdown led to modest increases in ADRB2 mRNA levels (Supplementary Fig. S7D). Furthermore, we have previously shown that ADRB2 is highly downregulated in LNCaP cells following androgen depletion (24), and as in LNCaP, a reanalysis of transcriptome data of the LuCaP35 PDX (GSE33316; ref. 45) showed ADRB2 downregulation following castration (Supplementary Fig. S7E). Likewise, enzalutamide-challenged CWR-R1, LAPC, and LNCaP cells (GSE78201; ref. 46) showed reduced ADRB2 mRNA levels, with further downregulation of ADRB2 upon acquisition of enzalutamide resistance (Supplementary Fig. S7F). Although these data collectively suggested that transcription of ADRB2 could be androgen dependent, we have previously shown in androgen-depleted LNCaP cells that ADRB2 protein levels are not elevated upon restimulation with varying concentrations of R1881 (24). This is supported by gene expression profiling of androgen depletion–challenged LNCaP cells reintroduced to androgen, showing mRNA upregulation of several androgen responsive genes, but not ADRB2 (Supplementary Fig. S7G).

ADRB2 promotes emergence of neuroendocrine features during ADT in prostate cancer xenografts

As the transcriptomic data presented showed that ADRB2 expression was consistently and progressively downregulated in CRPC and NEPC, we performed a reanalysis of our LNCaP xenograft model, which underwent castration treatment (Fig. 6A; ref. 21). We found that xenografts of shADRB2 LNCaP cells had significantly increased tumor latencies following injection compared with LNCaP shCtrl xenografts (Fig. 6B and C). To corroborate this finding, we performed colony formation assays with shCtrl and shADRB2 LNCaP cells and VCaP cells, which showed consistently lower colony-formation capabilities of shADRB2 and VCaP cells in soft-agar compared with shCtrl (Supplementary Fig. S8A and S8B). Knockdown of ADRB2 had no or a minor negative effect on proliferation (Supplementary Fig. S8C and S8D), and was thus likely not responsible for the lowered amount of colonies formed in these cells. GSK3β inhibition using CHIR99021, which reduced NiEtD, also reduced colony formation in shCtrl, shADRB2, and VCaP cells (Supplementary Fig. S6E), suggesting that GSK3β could be responsible for loss of both anchorage-independent growth and NiEtD.

Importantly, IHC analyses of tumors that had progressed following castration revealed that tumors that expressed high ADRB2 levels at time of injection expressed more TUBB3 than their low ADRB2 counterparts ($t$ test $P = 0.038$; Fig. 6D and E). As expected, loss of ADRB2 expression was observed in all tumors, regardless of precastration ADRB2 levels (Fig. 6D). This suggested that downregulation of ADRB2 mRNA in tumors recurring on ADT and/or ARPI results in lowered protein expression. As tissue vascularization was recently reported to increase with NiEtD in prostate cancer (47), we also investigated CD31 by IHC. CD31 expression was consistently and progressively downregulated of androgen responsive genes, but not ADRB2 (Supplementary Table S2). Guided by the hierarchical clustering, we applied the 15 ADRB2-correlated genes and ADRB2 as a t-NEPC gene expression signature (ADRB2 signature). We found that the ADRB2 signature genes were capable to cluster LITL PDX models with an NEPC histology (Fig. 5E; ref. 16). ADRB2 signature scores were higher among PDXs with an NEPC histology (median z-score sums of 0.295 and 17.8 for NEPCs and adenocarcinomas, respectively; Wilcoxon rank sum test $P < 0.0001$). By in silico reanalysis of longitudinal samples from the LTL331 xenograft, which develops NEtD, also reduced colony formation in shCtrl, shADRB2, and VCaP cells (Supplementary Fig. S6E), suggesting that GSK3β could be responsible for loss of both anchorage-independent growth and NiEtD.
Figure 6.
Castration induces NEtD and ADRB2 loss, but a high pretreatment tumoral ADRB2 level promotes NEtD, in LNCaP xenografts. A, Schematic overview of the used xenograft model. B, Tumor growth was monitored weekly by caliper measurements following injection of LNCaP shCtrl (n = 30), shADRB2-1 (n = 13), and shADRB2-2 (n = 15) in NSG mice, and is shown as mean tumor size (mm²) ± SEM. Statistical significance was tested for tumor volumes at each timepoint for each shADRB2 xenograft model relative to shCtrl (Wilcoxon rank sum test). C, Boxplot showing the distribution of time from subcutaneous injection to first detection of a palpable tumor for each xenograft model individually. Two mice injected with shADRB2-1 cells did not develop palpable tumors. Statistical significance of differences in time to take relative to shCtrl was calculated by Wilcoxon rank sum test. D, Representative light microscopy images of sectioned tumors from two representative mice (#40: shCtrl and #34: shADRB2-2) at termination (post-castration) stained with anti-ADRB2 or anti-TUBB3 antibodies. All tumors lacked expression of ADRB2. The TUBB3-stained sections illustrate areas with the most pronounced staining intensity for the two mice individually. Scale bars, 100 μm. E, Whole-tumor sections from shCtrl (n = 12) and shADRB2-2 (n = 10) stained with anti-TUBB3 and anti-CD31 antibodies were analyzed for percentage of positive areas per whole tumor, and are shown as box-and-beeswarm plots. Statistical significance between distributions were calculated using two-sided t tests. F, Proposed model for the divergence in clonal evolution of advanced prostate cancer depending on treatment-naive ADRB2 levels. Statistical significance was tested by Wilcoxon rank sum tests and is indicated by asterisks (*, P < 0.05; **, P < 0.01; *** P < 0.001).
castrate androgen levels and thereby promotes adeno-CRPC development.

**Discussion**

Progression to NEPC is an increasing challenge as more drugs targeting the androgen signaling axis become part of the toolbox for treating patients with prostate cancer. In this study, we have shown that ADRB2 is a luminal marker associated with a well-differentiated growth pattern in hormone-sensitive tumors. ADRB2 expression was progressively downregulated upon treatment-related resistance development, and the level of ADRB2 in hormone-naïve prostate cancer determines whether or not the cells are able to undergo NEtD upon androgen deprivation both in cell lines and a mouse xenograft model. High ADRB2 cells had longer neurite outgrowth and higher expression of neuron differentiation and neuroendocrine genes than low ADRB2 cells. Overexpression of ADRB2 in low ADRB2 cells induced growth of neurite protrusions, and ADRB2 expression was associated with low canonical WNT signaling activity.

The clinical appearance of both adeno-CRPC and t-NEPC is associated with loss of differentiation of luminal cell lineages and gain of lineage plasticity, and these characteristics are most pronounced in NEPC. Recently it was reported that men with high grade disease and disproportionally low serum PSA not only have poor outcomes, but also displayed a neuroendocrine/small-cell gene signature (48). The finding that ADRB2 expression in advanced prostate cancer correlated with markers of luminal differentiation and inversely correlated with genes typically upregulated in t-NEPC indicates that suppression of ADRB2 is a feature of AR-low/null prostate cancer variants. In fact, a recent transcriptomic meta-analysis-driven search for markers associated with NEPC included ADRB2 in its Meta-12 gene signature (37).

Certain primary tumor characteristics are likely to predispose for t-NEPC development (42), and neuronal genes are the most frequently enriched genes in t-NEPC (37). Androgen withdrawal predictably induces neuroendocrine features in prostate cancer cell lines (38, 39) and patient tumors (49). The extent of neuroendocrine differentiation has been shown to increase with duration of ADT (50) and the majority of metastatic CRPCs display at least focal neuroendocrine differentiation (12). This corroborates the putative successive relationship between NEtD and t-NEPC (51).

Ample evidence that stimulation of ADRB2 and its’ downstream signaling factors confers NEtD in androgen-dependent and -independent prostate cancer cells exists (18, 19, 47), and the sympathetic nervous system and the catecholamine signaling axis are increasingly acknowledged as major factors in prostate cancer oncogenesis and progression (52–54). Our findings illustrate that downregulation of ADRB2 not only repressed ARβonization-driven NEtD, but also impaired transdifferentiation upon androgen withdrawal. Although these findings seemingly contradict the clinical data presented, t-NEPC development may be considered a multistep clonal evolution process involving initial growth inhibition and transdifferentiation (NEtD) followed by a ARPI challenge-induced proliferative switch involving, for example, loss of tumor suppressor function (55, 56).

We propose that high ADRB2 prostate cancer cells are inherently more prone to undergo NEtD upon challenge with ADT, which may represent an initial, essential step in t-NEPC development appended by transcriptional repression of ADRB2. In support of this hypothesis, we have shown that a low ADRB2 protein level is associated with rapid adeno-CRPC progression in patients, and that low ADRB2 LNCaP cells better retain bioavailable androgens (21) and are less sensitive toward antiandrogens. This theoretically renders them more likely to recur as conventional adeno-CRPCs by reactivating the androgen signaling axis, and not as t-NEPCs. In support of this, our xenograft model showed that although tumoral ADRB2 protein expression was predictably lost in castrated mice, the level of ADRB2 at time of castration was decisive for neuroendocrine-like lineage commitment during treatment. Moreover, in absence of treatment challenge, both hormone-sensitive and -insensitive ADRB2-overexpressing prostate cancer cells developed a morphology reminiscent of neurons. This was surprisingly not accompanied by elevated neuroendocrine marker expression. Although this finding may solely represent an in vitro phenomenon, ADRB2’s role in NEtD may primarily related to the neural component of neuroendocrine cells, while treatment-related pressure is necessitous to further drive emergence of a true neuroendocrine phenotype. A paired longitudinal patient biopsy study on tumor tissue sampled prior and after ARPI treatment was published during revision of this article (57), and it will be interesting to retrospectively see whether treatment-naïve tumors with high ADRB2 are more likely to recur as t-NEPC.

The mechanism(s) responsible for downregulation of ADRB2 upon treatment challenge, NEtD and t-NEPC development remain elusive. Nouri and colleagues showed in multiple prostate cancer cell lines that NEtD is likely preceded by a transient neural/neural crest stem-like reprogramming wherein ADRB2 mRNA was consistently downregulated in four prostate cancer cell lines upon acquisition of this state (58). Likewise, ADRB2 mRNA was downregulated in Enz-challenged and -resistant prostate cancer cell lines. These findings suggest that transcriptional repression of ADRB2 is prerequisite for repressing luminal characteristics and allowing reprogramming to lineages with less reliance on AR signaling.

Expanding on this notion, we have found that androgen depletion-mediated ADRB2 downregulation, which is recapitulated in tumors from patients who have undergone ADT, is not rescued by androgen resupplementation. Moreover, expression levels of ADRB2 and AR were correlated in primary, treatment-naïve prostate cancers, whereas CRPCs were characterized by high AR and low ADRB2 mRNA level. This suggests that ADT-induced transcriptional repression of ADRB2 is not primarily androgen dependent, but may involve other epigenetic and/or transcriptional alterations. In advanced prostate cancer, ADRB2 was anticorrelated with enhancer of EZH2, which is enriched in t-NEPCs (4). EZH2 has been shown to silence ADRB2 in a polycomb repressive complex 2–dependent manner (43), and we found that ADRB2 and EZH2 mRNA levels to be inversely correlated in t-NEPCs, but not adeno-CRPCs in the NEPC WCM dataset. Moreover, during revision of this work, androgen depletion was shown to drive NEtD in prostate cancer cells through induction of β-adrenergic signaling and cAMP response element-binding protein (CREB), which in turn enhanced EZH2 activity (47). In another study, however, knockdown of EZH2 and small-molecule EZH2 inhibitors in LNCaP and LNCaP C4-2B cells only led to modest increases in ADRB2 expression. More studies are needed to unravel how ADRB2 expression is regulated during prostate cancer progression and therapy resistance development.
β-blockers (β-adrenergic receptor antagonists) were recently shown to induce expression of EZH2 target genes, including ADRB2, and were shown to represent a compelling therapeutic option for NEPCs (47). Our group has previously shown an association between β-blocker use and reduced cancer-specific mortality (59), potentially more so in patients with prostate cancer undergoing ADT (60). If the prosurvival effect of β-blockers was accompanied by a modest increase in canonical Wnt/β-catenin signaling, our finding that tumors with high Wnt/β-catenin activity were resistant to undergoing NEtD is conflicted by studies indicating a NEtD-promoting effect of Wnt/β-catenin activation (41, 61). Regardless, the ramifications of this signaling pathway are likely context dependent (62). For instance, β-catenin has been shown to promote transcription of AR target genes in prostate cancer cells (62). Low ADRB2 LNCaP cells are more androgen responsive (21), and the observed elevation of β-catenin activity in these cells may thus limit androgen depletion–induced NEtD through maintaining an AR transcriptional program that promotes luminal differentiation. We propose that β-blockers may have therapeutic potential for patients with metastatic prostate cancer through potentiating AR-targeted therapy regimens by sustaining a therapy-sensitive luminal lineage and thereby preventing t-NEPC emergence. Conversely, β-blockers should theoretically lower CAMP-mediated EZH2 activation. As EZH2 inhibition has been shown to resectinse prostate cancer tumors in mice harboring functional loss of TP53 and RB1 to androgen withdrawal (56), studies evaluating the efficacy of combining β-blockers with EZH2 inhibitors are prompted.

In conclusion, we have shown that ADRB2 expression is required for androgen depletion–induced NEtD, a putative precursory stage of t-NEPC development. Moreover, ADRB2 overexpression was associated with acquisition of neuroendocrine-like morphologic characteristics, and high ADRB2 xenograft tumors developed NEtD to a higher degree than low ADRB2 tumors, while castration conferred loss of ADRB2 protein expression. Accordingly, development of adeno-CRPC, and in particular t-NEPC, are associated with downregulation of ADRB2. We suggest that AR-positive prostate adenocarcinoma cells expressing high levels of ADRB2 are prone to develop t-NEPC, whereas low ADRB2 cells associate with reactivation of the AR signaling axis and are biased toward adeno-CRPC development.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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