BRN4 Is a Novel Driver of Neuroendocrine Differentiation in Castration-Resistant Prostate Cancer and Is Selectively Released in Extracellular Vesicles with BRN2

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Abstract

Purpose: Neuroendocrine prostate cancer (NEPC), an aggressive variant of castration-resistant prostate cancer (CRPC), often emerges after androgen receptor–targeted therapies such as enzalutamide or de novo, via trans-differentiation process of neuroendocrine differentiation. The mechanistic basis of neuroendocrine differentiation is poorly understood, contributing to lack of effective predictive biomarkers and late disease recognition. The purpose of this study was to examine the role of novel proneural Pit-Oct-Unc-domain transcription factors (TF) in NEPC and examine their potential as noninvasive predictive biomarkers.

Experimental Design: Prostate cancer patient-derived xenograft models, clinical samples, and cellular neuroendocrine differentiation models were employed to determine the expression of TFs BRN1 and BRN4. BRN4 levels were modulated in prostate cancer cell lines followed by functional assays. Furthermore, extracellular vesicles (EV) were isolated from patient samples and cell culture models, characterized by nanoparticle tracking analyses, Western blotting, and real-time PCR.

Results: We identify for the first time that: (i) BRN4 is amplified and overexpressed in NEPC clinical samples and that BRN4 overexpression drives neuroendocrine differentiation via its interplay with BRN2, a TF that was previously implicated in NEPC. (ii) BRN4 and BRN2 mRNA are actively released in prostate cancer EVs upon neuroendocrine differentiation induction; and (iii) enzalutamide treatment augments release of BRN4 and BRN2 in prostate cancer EVs, promoting neuroendocrine differentiation induction.

Conclusions: Our study identiﬁes a novel TF that drives NEPC and suggests that as adaptive mechanism to enzalutamide treatment, prostate cancer cells express and secrete BRN4 and BRN2 in EVs that drive oncogenic reprogramming of prostate cancer cells to NEPC. Importantly, EV-associated BRN4 and BRN2 are potential novel noninvasive biomarkers to predict neuroendocrine differentiation in CRPC.

Introduction

Prostate cancer, a leading cause of male cancer-related mortality in the United States (1), is dependent on androgen receptor (AR) signaling. Therefore, ablation of AR signaling by androgen deprivation is the goal of first-line therapy (2) that initially results in cancer regression. However, 2–3 years after androgen deprivation in 25%–40% of cases, the disease develops into castration-resistant prostate cancer (CRPC) that has limited therapeutic options (3). Patients with CRPC are treated with AR pathway inhibitors (API) such as enzalutamide (MDV3100/ENZ) and abiraterone as a second-line of therapy that improves survival initially (2, 4). However, patients with CRPC develop drug resistance over a certain period owing to heterogeneous molecular mechanisms such as AR bypass signaling or complete AR independence (5, 6). A subset of API-resistant tumors undergo a reversible trans-differentiation process known as neuroendocrine differentiation, that is associated with altered expression of lineage markers such as decreased expression of AR and increased expression of neuroendocrine (NE) lineage markers including enolase 2 (ENO2), chromogranin A (CHGA), and synaptophysin (SYP; refs. 7, 8). Because of lack of AR signaling, these prostate cancer variants, referred to as neuroendocrine prostate cancer (NEPC), are impervious to antiandrogen therapy and constitute an aggressive variant of advanced CRPC with shorter survival times and limited therapeutic options (7). Although NEPC is thought to arise late in the disease course subsequent to treatment with enzalutamide and abiraterone, this variant can also arise de novo in metastatic CRPC (mCRPC) after primary docetaxel therapy or early on after API treatment (7–9). Furthermore, it is not clear whether therapy-induced neuroendocrine differentiation is the same disease as de novo small-cell prostate cancer that emerges from rare neuroendocrine cell populations in the prostate (10). NEPC variants are associated with the presence of visceral metastasis to liver, lung, and central nervous system, in addition to lytic bone metastases and low serum PSA levels relative to disease burden (7). The molecular mechanistic basis of neuroendocrine...
POU-Domain/BRN Factors in Neuroendocrine Prostate Cancer

Translational Relevance

The emergence of neuroendocrine differentiation in castration-resistant prostate cancer (CRPC) poses significant clinical challenge as the survival rates are extremely poor. The molecular basis of this trans-differentiation process is poorly understood, contributing to a lack of robust molecular biomarkers for its diagnosis and prediction. This study identifies for the first time, BRN4 as a novel transcription factor (TF) that drives neuroendocrine differentiation in prostate cancer and interplays with a reported master neural TF, BRN2. Importantly, this study demonstrates that BRN4 and BRN2 mRNA are actively released in prostate cancer extracellular vesicles (EV) upon neuroendocrine differentiation induction and that EV-associated BRN4 and BRN2 are potential novel noninvasive biomarkers to predict neuroendocrine differentiation in patients with CRPC.

differentiation is poorly understood that contributes to lack of effective predictive biomarkers and late recognition of the disease. The genetic and epigenetic alterations underlying neuroendocrine differentiation has been investigated (11–15) recently that shows these states are derived via clonal evolution from adenocarcinomas (11). The key genetic events driving this transition include loss of the tumor suppressor retinoblastoma (RB1), tumor protein 53 (TP53), phosphatase and tensin homolog (PTEN; refs. 11–15), frequent TMPRSS2-ERG gene rearrangements (14), EZH2 overexpression, and amplifications of NMYC and Aurora Kinase A (AURKA; refs. 11–13, 15). AURKA is a cell-cycle kinase that stabilizes N-Myc oncoprotein and prevents N-Myc degradation (12, 13, 16). Disruption of the molecular interaction between AURKA and N-Myc via a therapeutic agent alisertib is being examined as a therapeutic modality for NEPC (17) with promising results. Furthermore, the upregulation of Delta-like protein 3 (DLL3) has been reported in NEPC cases as compared with CRPC adenocarcinomas. In fact, DLL3 expression can be exploited for therapeutic targeting of NEPC by employing a humanized DLL3 antibody (18–20). Although these studies have characterized the key alterations driving neuroendocrine differentiation, we are still far from understanding the genetic alterations driving this transition.

POU (Pit-Oct-Uncl)-domain/Oct proteins are a set of programming transcription factors (TF) that are critical regulators of gene expression programs determining cellular identities and play important roles in neurogenesis (21–23). Out of six classes, class III POU genes (POU3F1/OCT6, POU3F2/BRN2, POU3F3/BRN1, and POU3F4/BRN4) are considered to be crucial for neurogenesis (24). POU3F2/BRN2 was recently reported as an AR-repressed, master neural TF that drives prostate cancer neuroendocrine differentiation by controlling SOX2 expression (25). BRN3a has also been reported to be upregulated with NEPC (26). However, the roles of other class III pro-neural TFs have not been unequivocally studied in prostate cancer. Here we report for the first time, that BRN4 is a novel driver of neuroendocrine differentiation states in CRPC. BRN4 (Brain4) is located on X chromosome and is involved in the patterning of the neural tube, paraventricular, and supraoptic nuclei of the hypothalamus in the developing embryo (27). BRN4 mutations have been linked to X-linked nonsyndromic deafness (28). However, its involvement in prostate cancer has never been studied.

Recently, extracellular vesicles (EV)/exosomes have emerged as key regulators of cancer progression and metastasis. Exosomes are small membranous EVs, typically between 30 and 150 nm in size, (29) that are gaining significant interest as alternate disease biomarkers. EVs can be detected noninvasively in biological fluids such as serum (30) and can be used as a liquid biopsy for prostate cancer (31–33). EVs mediate intercellular communication by transferring their cargo such as mRNA and proteins to recipient cells to modulate target cell functions (34, 35). We hypothesized that in addition to cell intrinsic genetic determinants of neuroendocrine differentiation, tumor exosomes/EVs (referred to as EVs subsequently) are important determinants that facilitate this trans-differentiation by mediating intercellular communication between cancer cells via horizontal transfer of functional neuronal factors. In this study, we validated our hypothesis and demonstrate for the first time that EV-mediated signaling is important for neuroendocrine differentiation induction via the release of BRN4 and BRN2 mRNA in prostate cancer EVs.

Materials and Methods

Cell lines and cell culture

Nonmalignant prostate epithelial cell line RWPE-1 and prostate cancer cell lines (LNCaP, Du145, PC3, C42B, and NCI-H660; ref. 36) were obtained from the ATCC and cultured under recommended conditions. All cell lines were maintained in an incubator with a humidified atmosphere of 95% air and 5% CO2 at 37°C. The experiments with cell lines were performed within 6 months of their procurement/resuscitation. Prostate cell lines were authenticated by DNA short-tandem repeat analysis. Cell lines were checked at periodic intervals for Mycoplasma contamination by DAPI staining.

Clinical samples

The study was conducted in accordance with ethical guidelines of U.S. Common Rule and was approved by the University of California San Francisco Committee on Human Research. Written informed consent was obtained from patients. Serum samples (0.5–1 mL) from patients with prostate cancer and unidentified clinical information were obtained from Prostate Cancer Biorepository Network and stored at −80°C till processed. Cohort included metastatic human CRPC clinical samples with adenocarcinoma features (CRPC-adeno) versus those with neuroendocrine features (CRPC-NE; Supplementary Table S1). CRPC-adeno included patients with no evidence of neuroendocrine differentiation, while CRPC-NE included AR patients with therapy-induced neuroendocrine differentiation with features of small-cell/large-cell neuroendocrine carcinoma. Follow-up information included prior therapies for all the clinical samples.

Isolation of EVs

Serum-derived EVs were isolated from 250–500 μL of serum using the Total Exosome Isolation Reagent (Life Technologies; catalog no. 478360) as per the manufacturer’s instructions and as described in (37). For isolation of EVs from cell culture media, cells were grown in recommended media with exosome-depleted FBS for 48 hours. Conditioned media were collected and EVs were isolated with Exosome Isolation Reagent (Life
We hypothesized that multiple POU-domain TFs act in concert to promote prostate cancer neuroendocrine differentiation. To test our hypothesis, we examined the copy-number alterations (CNA) of these factors in patients with CRPC with adenocarcinomas. Importantly, BRN4 amplifications were present in approximately 20%, 9%, and 16% of CRPC-NE cases in these two cohorts, suggestive of a potential role for these factors in patients with CRPC with adenocarcinomas. In view of our data showing induction of BRN1 and BRN4 mRNA in neuroendocrine cellular models, we examined the clinical relevance of these alterations using patient-derived xenograft (PDX) models (Fig. 2A). We assessed their levels in PDX models with CRPC-adenoc characteristics (LuCaP 70, 78, 81, and 92) versus those with CRPC-NE alterations (LuCaP 49, 145.1, and 145.2; ref. 42) by RT-PCR analyses. While the expression of BRN1 was not significantly different between CRPC-adeno versus CRPC-NE PDXs (Fig. 2A), BRN4 expression was significantly upregulated in CRPC-NE xenografts LuCaP 49, 145.1, and 145.2 (Fig. 2B). These data suggest that BRN4 upregulation is a clinically relevant alteration associated with transition of adenocarcinomas to neuroendocrine states. In view of these data, we focused on BRN4. To validate the association of BRN4 with NEPC, we examined BRN4 mRNA alterations in Beltran and colleagues’ (11) cohort using cBioPortal (39, 40). We found that BRN1 and BRN4 mRNAs were upregulated in LuCaP-AR-ENZR cells, suggesting the potential association of these factors with enzalutamide resistance in CRPC (Fig. 1E, left). BRN4 protein was found to be upregulated in LuCaP-AR-ENZR cells as compared with LuCaP-AR/LNCaP cells along with induction of BRN2, CHGA, and ENO2 (Fig. 1E, right).

BRN4 expression is selectively upregulated in CRPC-NE patient-derived xenograft models, clinical samples, and cellular neuroendocrine differentiation models

In view of our data showing induction of BRN1 and BRN4 mRNA in neuroendocrine cellular models, we examined the clinical relevance of these alterations using patient-derived xenograft (PDX) models (Fig. 2A). We assessed their levels in PDX models with CRPC-adenoc characteristics (LuCaP 70, 78, 81, and 92) versus those with CRPC-NE alterations (LuCaP 49, 145.1, and 145.2; ref. 42) by RT-PCR analyses. While the expression of BRN1 was not significantly different between CRPC-adeno versus CRPC-NE PDXs (Fig. 2A), BRN4 expression was significantly upregulated in CRPC-NE xenografts LuCaP 49, 145.1, and 145.2 (Fig. 2B). These data suggest that BRN4 upregulation is a clinically relevant alteration associated with transition of adenocarcinomas to neuroendocrine states.
TP53 and RB dual knockdown in LNCaP/AR cells led to BRN4 mRNA induction (Fig. 2E). BRN4 induction with BRN2 and other neuroendocrine markers by androgen withdrawal was confirmed in additional prostate cancer cell lines, C42B and 22Rv1 (Fig. 2F), consolidating the association of BRN4 expression with induction of prostate cancer neuroendocrine differentiation.

BRN4 interplays with BRN2 and regulates SOX2 expression
To understand the mechanistic role of BRN4 in NEPC, we overexpressed control/BRN4 construct in LNCaP-AR and C42B cell lines (Fig. 3A) followed by expression analyses of neuroendocrine markers. Because BRN2 was recently implicated as a principal driver of NEPC (25), we included BRN2 overexpression as a positive control. Interestingly, we found that BRN4 overexpression led to SOX2 overexpression in both cell lines compared with control and BRN2 overexpression (Fig. 3B) concomitant with induction of ENO2, a neuroendocrine marker. Because SOX2 is a critical TF that has been implicated in NEPC (45) and BRN2 has been reported to an upstream regulator of SOX2 in driving NEPC (25), we sought to determine whether there is potential interaction between BRN4 and BRN2. We performed coimmunoprecipitation (co-IP) with BRN4 in LNCaP-AR and C42B cell lines followed by Western blotting for BRN2 (Fig. 3C). In a converse approach, we pulled down BRN2 with BRN2 antibody and probed for BRN4 (Fig. 3C). Our data shows that these two TFs interact directly suggesting that they may act in concert in driving NEPC. To further understand the interplay between these factors, we examined BRN4 levels upon BRN2 overexpression in LNCaP-AR and C42B cell lines (Fig. 3D). Interestingly, we found that BRN2 overexpression led to BRN4 upregulation in both cell lines. In a converse approach, we knocked down BRN2 expression in neuroendocrine cell line...
NCI-H660 and C42B cells and observed low BRN4 expression concomitant with BRN2 knockdown (Fig. 3E). These data suggest that BRN4 interplays with BRN2. We further examined the correlation of BRN4 with BRN2 expression in Prostate Cancer Transcriptome Analyses (PCTA) dataset (46), a large cohort of patients with mCRPC (n = 260) and observed a significant positive correlation between BRN4 and BRN2 in mCRPC (P < 0.001; Fig. 3F). These data validate BRN4 as a crucial TF that interacts with master neural TF BRN2 in prostate cancer (25). We also examined the correlation of BRN4 with AR in the PCTA cohort and observed a negative correlation (Fig. 3G) suggesting that BRN4 is expressed upon AR downregulation. We further examined the effects of BRN4 overexpression on LNCaP-AR cells grown in regular media/androgen-depleted media + enzalutamide (Fig. 3H). We observed that BRN4 overexpression confers a growth advantage to cells grown
BRN4 regulates SOX2 expression and controls expression of neuroendocrine genes. LNCaP-AR and C42B cells were stably transfected with control/BRN2/BRN4 overexpression constructs followed by Western blot analyses of BRN2 and BRN4 protein levels (A) and SOX2 and ENO2 protein levels (B). GAPDH was used as a loading control. C, LNCaP-AR and C42B cells were used to perform co-IP with control IgG, BRN4 antibody, and BRN2 antibody followed by Western blot analyses for BRN2 and BRN4. D, LNCaP-AR and C42B cells were stably transfected with control/BRN2 overexpression constructs followed by RT-PCR analyses of BRN4 mRNA. GAPDH was used as an endogenous control. E, NCI-H660 and C42B cells were stably transfected with control/BRN2 shRNA constructs followed by RT-PCR analyses of BRN2 and BRN4 mRNA. GAPDH was used as an endogenous control. Correlation between BRN2 and BRN4 expression (G) in PCTA dataset of patients with mCRPC (n = 260). H, Cellular viabilities of LNCaP-AR cells transfected with control/BRN4 grown in regular media/androgen-depleted media + enzalutamide. I, Transwell in vitro migration and invasion assays upon control/BRN4 expression in LNCaP-AR and C42B cell lines. J, Schematic representation showing the proposed role of BRN4 in NEPC.

Figure 3.

Alterations in EV secretion pathways upon induction of neuroendocrine differentiation states in prostate cancer

Recent results from our laboratory suggest that EVs play a key role in mediating neuroendocrine differentiation states in advanced prostate cancer. We propose that enzalutamide resistance is mediated via EVs, whereby these vesicles act as...
vehicles for exchange of neuronal TFs between heterogeneous populations of tumor cells, promoting neuroendocrine differentiation and engendering a transmitted API resistance. Toward this, we assayed EVs released from clinical samples (CRPC-adeno, \( n = 42 \) vs. CRPC-NE, \( n = 6 \); Supplementary Table S1). Isolated exosomal preparations were comprehensively characterized by electron microscopy, nanoparticle tracking analyses (NTA; Fig. 4A), and immunoblot analyses for presence of multiple exosomal markers (CD9, CD63, and TSG101) and absence of contaminating proteins such as GRP94 (Fig. 4B). NTA analyses showed that the average particle size (Fig. 4A, lower left panel) and numbers (Fig. 4A, lower right panel) were not significantly different between CRPC-adeno and CRPC-NE, although CRPC-NE samples trended toward higher particle number and size.

Figure 4.
Alterations in EV secretion pathways upon induction of neuroendocrine differentiation states in prostate cancer and release of BRN2 and BRN4 mRNA in prostate cancer EVs upon enzalutamide treatment. A, EVs were isolated from sera of patients with prostate cancer with CRPC-adeno, \( n = 42 \) and CRPC-NE, \( n = 6 \). NTA of representative CRPC-adeno (top, left) and CRPC-NE (top, right) cases showing size and concentration of isolated particles. Average particle size (bottom, left) and particle concentration in CRPC-adeno versus CRPC-NE cases (bottom, right) as determined by NTA analyses. B, Western blot analyses for EV markers CD9, CD63, TSG101, and negative marker GRP94 to confirm the integrity of EVs isolated from sera of CRPC-adeno (\( n = 7 \)) and CRPC-NE (\( n = 4 \)) cases. C, Genomic alteration frequencies (left) and relative mRNA expression (right) for CD9 and CD63 in CRPC-adeno and CRPC-NE cases in Beltran and colleagues’ (11) cohort. mRNA data are represented as mean ± SEM. D, EVs were extracted from conditioned media of LNCaP, LNCaP-AR, and ENZ-R cell line followed by RNA isolation and RT-PCR–based expression profiling for EV-associated BRN2 and BRN4 mRNA. Data were normalized to GAPDH control and represented as mean ± SEM. E, LNCaP-AR ENZ-R cell line was treated with exosome inhibitor GW4869 (20 \( \mu \)mol/L) for 48 hours followed by clonogenicity assay. Representative images from control/GW4869-treated cells are shown above. F, Expression of indicated genes in cellular (top) and EV (bottom) fractions from RWPE-1, LNCaP, and NCI-H660 cells. Data were normalized to GAPDH control and represented as mean ± SEM.
While we confirmed EV markers by Western blotting (Fig. 4B), we found that CD9 expression is variable, decreasing predominantly in CRPC-NE cases. To gain further insights, we examined CD9 alterations in Beltran and colleagues’ (11) cohort using cBioPortal (39, 40). We found that CD9 is amplified in 4% cases of CRPC-NE versus approximately 13% in other prostate cancer cases, while CD63 amplification frequency in NEPC is not significantly different (Fig. 4C, left). In concordance with amplifications, average CD9 mRNA expression was found to be approximately 2.5-fold lower in CRPC-NE versus CRPC-adeno, while CD63 is not altered significantly (Fig. 4C, right). These data suggest that prostate cancer neuroendocrine differentiation is associated with alterations in EV secretion pathways.

**BRN2 and BRN4 mRNA are released in prostate cancer EVs upon enzalutamide treatment.**

Importantly, we identified that BRN4 and BRN2 mRNA are specifically released into prostate cancer EVs. We extracted EVs from conditioned media of LNCaP, LNCaP-AR, and LNCaP-AR-ENZR cell line followed by expression profiling (Fig. 4D) and found that BRN4 and BRN2 mRNA (denoted as EV-BRN4 and EV-BRN2, respectively) were significantly increased in prostate cancer EVs isolated from LNCaP-AR-ENZR cell line. These data point to an association of enzalutamide resistance to an increased secretion of these TRs in EVs. We hypothesize that secretion of these factors in prostate cancer EVs in CRPC underlie enzalutamide resistance and may be an adaptive mechanism for prostate cancer cells to survive under the selective pressure of APs. Furthermore, treatment of LNCaP-AR-ENZR cell line with exosome calcium chelator GW4869 (Fig. 4E) could partially restore the sensitivity of this cell line to enzalutamide as monitored by clonogenicity assay. This supports a key role of exosome-mediated intercommunication in enzalutamide resistance. We also examined the expression of BRN4 and BRN2 in cells (Fig. 4F, top) and EVs (Fig. 4F, bottom) from RWPE-1 cells versus prostate cancer cell lines and found that their levels are specifically upregulated in EVs from neuroendocrine cell line NCI-H660, while the increases in LNCaP EVs were statistically insignificant. To confirm the specific release of BRN4 and BRN2 in EVs in NEPC, we examined the expression of additional genes, CUX1 and ATP2A3, in cells and corresponding EVs from prostate cancer cell lines (Fig. 4F). CUX1 encodes Cut-like homeobox 1 TF, while ATP2A3 encodes a calcium-translocating P-type ATPase that has been shown to be regulated in prostate cancer (47). While CUX1 and ATP2A3 were found to be expressed in LNCaP and NCI-H660 (Fig. 4F, top), their expression in EVs were undetected in all analyzed cell lines (Fig. 4F, bottom). These observations validated our findings on EV-BRN2 and -BRN4.

**EV-associated BRN4 and BRN2 are upregulated in sera from patients with NEPC and can predict neuroendocrine differentiation induction noninvasively.**

In view of our data showing presence of BRN4 and BRN2 mRNA in EVs and increase in their levels upon enzalutamide treatment and elevated levels in neuroendocrine cell line, we asked whether these factors could be used as noninvasive markers to predict prostate cancer neuroendocrine differentiation (Fig. 5). EVs were extracted from the sera of CRPC-adeno and CRPC-NE cases (cohort 1; Supplementary Table S1). Following extensive characterization of EVs by NTA analyses and Western blotting for positive and negative EV markers (4A-B), vesicle-associated RNAs were extracted and profiled by RT-PCR. EV-BRN4 was significantly upregulated (~7-fold) in CRPC-NE compared with CRPC-adeno cases (Fig. 5A). To assess the potential of EV-BRN4 to be a diagnostic biomarker for assessing neuroendocrine differentiation, we performed ROC curve analyses based on AUC, values in CRPC-adeno and CRPC-NE (Fig. 5B) cases. Our analyses showed that EV-BRN4 expression is an excellent marker to diagnose neuroendocrine differentiation in CRPC cases with an AUC of 1 [P < 0.0001; 95% confidence interval (CI), 0.832–1.000], 100% specificity and 100% sensitivity. Furthermore, in view of our data with cellular models showing release of BRN2 mRNA in EVs, we also evaluated its levels in sera of CRPC-adeno and CRPC-NE patients (Fig. 5C). Similar to BRN4, EV-BRN2 was found to be significantly higher (~4-fold) in CRPC-NE as compared with CRPC-adeno. ROC curve analyses for EV-BRN2 (Fig. 5D) showed that it can diagnose neuroendocrine differentiation with an AUC of 0.944 (P < 0.0001; 95% CI 0.782–0.998), 94.4% specificity and 100% sensitivity. These data demonstrate the promising potential of EV-associated BRN4 and BRN2 to predict neuroendocrine differentiation in patients with CRPC noninvasively.

**Enzalutamide treatment increases EV-associated BRN4 and BRN2 levels in patients with CRPC.**

In view of our preceding results, we analyzed the expression of EV-BRN4 and EV-BRN2 in additional patients with CRPC (cohort 2, n = 23; Fig. 5E and F; Supplementary Table S1). While this cohort did not include patients with proven neuroendocrine differentiation, it included CRPC-adeno patients with/without enzalutamide treatment. The levels of BRN4 (Fig. 5E) and BRN2 (Fig. 5F) were found to range from low to high. We further examined whether EV-associated BRN4 and BRN2 levels in CRPC-adeno patients (cohort 1+2) are correlated with clinico-pathologic parameters (Supplementary Fig. S2). On the basis of median expression of EV-BRN4 (4.04) and EV-BRN2 (5) in CRPC-adeno cases, these patients were stratified into two groups (<median and >median expression). While no correlations were observed with Gleason score of primary tumor, age at diagnosis, or final serum PSA, EV-BRN4 and EV-BRN2 were higher in CRPC-adeno patients treated with enzalutamide (67% and 83%, respectively) versus those that were non-enzalutamide treated (48% and 43%, respectively; Supplementary Fig. S2), although it failed to reach statistical significance. Furthermore, the median expression levels of EV-BRN4 and EV-BRN2 were found to be higher in enzalutamide-treated cases versus non-enzalutamide CRPC (adeno + neuroendocrine) cases (Fig. 5G), with the levels of EV-BRN2 significantly higher (~2-fold higher; P = 0.029 * (Asterisk refers to significant P value.) in enzalutamide-treated cases (Fig. 5G, right). These data suggest that these factors are increasingly released in EVs upon enzalutamide treatment. We propose that their release in EVs promote enzalutamide-induced neuroendocrine differentiation in patients with CRPC.

**BRN4 and BRN2 protein are selectively released in prostate cancer EVs, and their release increases upon neuroendocrine differentiation induction.**

In addition to BRN4 and BRN2 mRNA, we found that EVs contain BRN4 and BRN2 protein (Fig. 6A). EVs were isolated from control/enzalutamide-treated LNCaP and NCI-H660 cells and subjected to Western blot analyses. We found that BRN4 (Fig. 6A, left) and BRN2 (ref. 25; Fig. 6A, right) are selectively released in prostate cancer EVs with their release increasing upon...
Figure 5.
EV-associated BRN4 and BRN2 are upregulated in sera from patients with neuroendocrine prostate cancer and can predict neuroendocrine differentiation induction in patients with prostate cancer noninvasively. A, Relative EV-BRN4 levels in CRPC-adeno (n = 14) and CRPC-NE samples (n = 6) as assessed by RT-PCR. Data were normalized to GAPDH control and represented as mean ± SEM (left). Average EV-BRN4 expression in CRPC-adeno versus CRPC-NE cases (right). B, ROC curve analyses for EV-BRN4 as a parameter to discriminate between non-neuroendocrine and neuroendocrine cases based on ΔCt values in CRPC-adeno versus CRPC-NE cases. C, Relative EV-BRN2 levels in CRPC-adeno versus CRPC-NE cases (left). Average EV-BRN2 expression in CRPC-adeno versus CRPC-NE cases (right). Data were normalized to GAPDH control and represented as mean ± SEM. D, ROC curve analyses for EV-BRN2 based on ΔCt values in CRPC-adeno (n = 19) and CRPC-NE (n = 6). Relative EV-BRN4 levels (E) and EV-BRN2 levels (F) in sera of cohort 2 of CRPC-adeno patients (n = 23) as assessed by RT-PCR. Data were normalized to GAPDH control and represented as mean ± SEM. G, Median EV-BRN4 (left) and EV-BRN2 expression (right) in CRPC-adeno cases treated with/without enzalutamide. P values are based on Mann-Whitney U test.
enzalutamide treatment and enrichment in EVs derived from the neuroendocrine cell line NCI-H660. To validate our EV preparations, we performed Western blot analyses for EV markers CD9 and CD63 (Fig. 6A). While we confirmed the purity of our preparations as they stained positive for these markers, we found that CD9-containing vesicles decrease significantly upon neuroendocrine differentiation induction as compared with CD63-positive vesicles, further supporting our data suggesting that alterations in EV secretion pathways occur with neuroendocrine differentiation induction (Fig. 4B and C). To validate the release of BRN2 and BRN4 into prostate cancer EVs, we inhibited EV release in our cellular LNCaP neuroendocrine differentiation model (Supplementary Fig. S1) by treatment with inhibitor GW4869 followed by BRN2/4 expression analyses in cellular and EV fractions (Fig. 6B). EV secretion inhibition increased cellular BRN2 levels and decreased EV-associated BRN2 upon neuroendocrine differentiation induction by enzalutamide treatment and/or androgen depletion. Similarly, increased BRN4 was observed in EVs upon androgen depletion and/or enzalutamide treatment and these increases were attenuated by GW4869 treatment. Interestingly, probing for BRN4 in EVs yield a higher band in addition to expected size, which may reflect glycosylated protein. Furthermore, GW4869 treatment led to increased cellular BRN4 in control and C/D FBS-treated cells, while expected increase was not observed in combined treatment with enzalutamide suggesting a different regulatory control of BRN4 and BRN2 secretion and expression. We also assayed the release of BRN4 and BRN2 in EVs derived from RWPE-1/BPH1 and prostate cancer cell lines (Fig. 6C) and found that these genes are specifically expressed in EVs derived from prostate cancer cell lines LNCaP, PC3, and Du145 while EVs from RWPE-1 and BPH1 cells had undetectable levels. Treatment of prostate cancer cell lines PC3 and LNCaP with EV inhibitor GW4869 led to decreased EV-associated BRN2 and BRN4 mRNA concomitant with increased cellular levels (Fig. 6D).

**EV-associated BRN4 and BRN2 mediates neuroendocrine differentiation states in prostate cancer**

In view of our results showing the release of BRN factors in EVs upon enzalutamide treatment, we hypothesized that as an adaptive mechanism to androgen deprivation conditions/enzalutamide treatment, prostate cancer cells express and secrete these factors in EVs that act in a paracrine manner on neighboring cancer cells to drive oncogenic reprogramming to neuroendocrine-like states (Fig. 6I). To test our hypothesis, we performed “uptake experiments” (Fig. 6E–H). EVs were isolated from control/enzalutamide-treated LNCaP cells, labeled with SYTO RNASelect Green Fluorescent Stain (Thermo Fisher Scientific; ref. 48), and followed by incubation with parental LNCaP cells. As a negative control, parental LNCaP cells were incubated with EV-free media. After 48 hours, parental LNCaP cells were harvested and analyzed. Cellular uptake of labeled EVs was confirmed by fluorescence microscopy (Fig. 6E, left). We performed BRN2 immunofluorescence (IF) staining on parental LNCaP cells (Fig. 6E, right), which showed augmented cellular BRN2 staining upon treatment with enzalutamide EVs, suggesting that BRN2 protein is increasingly released in prostate cancer EVs upon enzalutamide treatment and horizontally transferred to neighboring cancer cells. Interestingly, BRN2 protein showed as tiny speckles inside the cells, colocalized with EVs (green label) validating the transfer of BRN2 protein in EVs. RT-PCR analyses of parental LNCaP cells after “uptake experiment” showed an induction of BRN2 and BRN4 along with neuroendocrine genes ENO2 and SYF by RT-PCR (Fig. 6F). Western blot analyses after “uptake experiment” (Fig. 6G) showed that treatment of parental LNCaP cells with enzalutamide EVs led to a significant increase in the expression of BRN2, BRN4, CHGA, and SYF with a concomitant decrease in AR expression. We examined the effects of enzalutamide EVs on AR target genes (NKX3.1 and KLK3) and neuroendocrine/stem cell marker CD44 in LNCaP-AR cells (Supplementary Fig. S3A) and found a significant repression of NKX3.1 and KLK3, while CD44 expression was upregulated. To further consolidate the transfer of BRN4 in EVs, we labeled nascent RNA in donor cells (control/BRN4-overexpressing LNCaP-AR cells) with 5-ethynyl uridine (5EU) followed by tracking of EU-labeled RNA release and uptake in recipient parental LNCaP-AR cells (non-EU labeled), to determine whether labeled BRN4 mRNA transferred from donor cells could be detected in recipient cells (Fig. 6H, left). Interestingly, we found that parental cells treated with EU-labeled EVs from BRN4-expressing cells showed higher expression as compared with corresponding control EV-treated cells (Fig. 6H, right). This data validates our hypothesis of transfer of BRN4 via EVs.

Because BRN2 was reported as a key neuronal factor driving prostate cancer neuroendocrine differentiation (25), we further tested the role of EV-associated BRN2 in prostate cancer neuroendocrine differentiation (Supplementary Fig. S3B). We performed shRNA-mediated stable BRN2 knockout in NCI-H660 cells (Supplementary Fig. S3B, left). EVs were harvested from conditioned media of control shRNA (shCON) versus shBRN2-transfected cells (Supplementary Fig. S3B, right) and used in an “uptake experiment” with parental LNCaP cells incubated with shCON or shBRN2 EVs (Supplementary Fig. S3C). Our data shows that treatment of parental LNCaP cells with shBRN2 EVs led to decreased expression of BRN2 and CD44 concomitant with AR upregulation. Collectively, these data support a role of EV-associated BRN2/4 in inducing neuroendocrine states (Fig. 6I).

**Discussion**

While the widespread use of enzalutamide and other second-generation APIS has led to transformative impact in the management of patients with mCRPC (2, 4), API resistance (5, 6) is near-universal leading to significantly increased incidences of therapy-induced neuroendocrine differentiation (9) with aggressive clinical course. Therapy-induced neuroendocrine differentiation emerges in patients with mCRPC either late upon treatment with enzalutamide/abiraterone, but is also believed to occur early in disease course upon treatment with these APIs or even during de novo post-docetaxel therapy (9, 10). The genetic and epigenetic changes underlying this trans-differentiation process has been investigated and has been reported to involve key events including loss of the tumor suppressors retinoblastoma (RB1), tumor protein 53 (TP53), and MYC overexpression among others (11–15). Here we show that BRN4, encoding a proneural TF, is a crucial factor that is upregulated in NEPC. While both BRN1 and BRN4 were induced upon enzalutamide treatment of prostate cancer cell lines along with BRN2, BRN4 alterations were found to be associated more specifically with NEPC. In this regard, we identified that (i) in addition to BRN2, BRN4 is upregulated upon enzalutamide treatment/androgen withdrawal of prostate

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controls for EV, while tubulin/GAPDH was used as controls for cellular fractions. Cell fractions were extracted after various treatments followed by Western blot analyses for BRN2 and BRN4 in the two fractions. CD9 and CD63 were used as

Figure 6.
BRN4 and BRN2 are selectively released in prostate cancer EVs upon neuroendocrine differentiation induction that mediate neuroendocrine differentiation states in prostate cancer. A, EVs were isolated from control (DMSO)/1µmol/L enzalutamide-treated LNCaP cells and NCI-H660 cells and subjected to Western blot analyses for indicated proteins. B, LNCaP cells were cultured under regular conditions (control) or under androgen-depleted conditions (C/D FBS) or treated with 20 µmol/L enzalutamide in C/D FBS media. These treatments were followed by treatment with exosome inhibitor GW4869 for 48 hours. Cellular and EV fractions were extracted after various treatments followed by Western blot analyses for BRN2 and BRN4 in the two fractions. CD9 and CD63 were used as controls for EV, while tubulin/GAPDH was used as controls for cellular fractions. C, BRN2 and BRN4 protein levels in EVs derived from normal immortalized (RWPE-1)/benign prostate epithelial (BPH1) cells and prostate cancer cell lines (PC3, LNCaP, and Du145). (Continued on the following page.)
cancer cell lines and in enzalutamide-resistant prostate cancer cell line; (ii) BRN4 is selectively upregulated in CPC- neuroendocrine PDX models as compared with CRPC-adeno PDXs (42); (iii) BRN4 is upregulated in inducible cellular neuroendocrine differentiation models including MYCN overexpression and dual TP53 and RB1 knockdown (11, 12, 16, 44); and (iv) analyses in Beltran and colleagues’ (11) cohort showed that BRN4 mRNA is upregulated in NEPC. In view of these lines of evidence, we propose that BRN4 upregulation is a clinically relevant alteration associated with transition of CRPC-adeno to CRPC-NE and that BRN4 may play a critical role in reprogramming prostate cancer cells to neuroendocrine states. In agreement with this hypothesis, BRN4 overexpression led to upregulation of neuronal markers including SOX2, a critical TF that drives NEPC (44) and ENO2, a canonical neuroendocrine marker. BRN2 was previously reported to be an AR-repressed and an upstream regulator of SOX2 in driving NEPC (25). In view of our results showing an interplay between BRN2 and BRN4, we speculate that BRN4 and BRN2 act synergistically to control SOX2 expression in regulating NEPC (Fig. 3I).

Our co-IP data showing that BRN4 directly interacts with BRN2 support our hypothesis. Overexpression/knockdown of BRN2 led to corresponding changes in BRN4 levels. We speculate that in response to APIs such as enzalutamide treatment, BRN4 and BRN2 are upregulated that work together to initiate a proneural program that drives prostate cancer neuroendocrine differentiation (Fig. 3I). Future mechanistic studies mapping the binding sites and regulation of BRN4 are warranted and are the subject of ongoing investigations in our laboratory. Interestingly, our preliminary analyses suggest that BRN4 promoter possess multiple SOX2 and AR-binding sites suggesting a potential regulatory interplay between these TFs in driving NEPC.

It has been suggested and supported that NEPC transformation is a potentially reversible epigenetic phenomenon (49). We hypothesized that EVs mediate intercellular signaling in NEPC and plays a role in oncogenic reprogramming of CRPC-adeno to CRPC-NE states via the transfer of functional TFs. Importantly, treatment of enzalutamide-resistant cell line with EV inhibitor could restore the sensitivity of these cells to enzalutamide, supporting a key role of EVs in imparting enzalutamide resistance and suggesting EV inhibition as a potential strategy to reverse enzalutamide resistance. Our data lend support to our hypothesis that EVs are crucial to neuroendocrine differentiation induction and that key oncogenic factors including BRN2 and BRN4 are released in prostate cancer EVs. We found that enzalutamide causes alterations in vesicular sorting pathways such as increased release of BRN2 and BRN4 mRNA in EVs. Our data suggests that EV-associated BRN4 and BRN2 are horizontally transferred to neighboring cancer cells to propagate neuroendocrine differentiation states. We propose that as an adaptive mechanism to APIs, prostate cancer cells express BRN2 and BRN4, which in turn, drives oncogenic reprogramming of prostate cancer cells. Furthermore, these reprogramming TFs are selectively sorted into prostate cancer EVs to mediate intercellular communication between prostate cancer cells, leading to induction of neuronal genes, thereby promoting perpetuation of neuroendocrine differentiation states (Fig. 6I). Because amplification of N-Myc and overexpression of EZH2 have been identified as key oncogenic factors in NEPC (11–13), we also assayed these factors in EVs (Supplementary Fig. S4). We found that EZH2 and N-Myc are released in EVs by prostate cancer cells undergoing neuroendocrine differentiation (Supplementary Fig. S4). Lending support to our hypothesis that neuroendocrine differentiation induction is associated with release of oncogenic TFs that perpetuate these states in advanced prostate cancer.

Importantly, we identified that: there is increased expression of EV-BRN4 and EV-BRN2 in CRPC-NE cases as compared with CRPC-adeno and that EV-BRN4 and EV-BRN2 have promising potential as noninvasive diagnostic/predictive biomarkers for NEPC/neuroendocrine differentiation. Rigorous validation of the proposed EV-associated BRN4 and BRN2 as novel, noninvasive markers for detection and prediction of NEPC in larger cohorts are warranted. If validated, these markers can provide a significant advancement over existing methods of assessing neuroendocrine differentiation based on histopathologic criteria that are often flawed owing to the heterogeneity of neuroendocrine differentiation (7, 8). Furthermore, we found that CD9-containing vesicles decrease significantly upon enzalutamide treatment and that NEPC is associated with lower CD9 amplification, lower mRNA expression, and low CD9-positive vesicles suggesting that the amount of CD9-positive vesicles may act as an indicator of neuroendocrine differentiation induction in CRPC. Previous studies have associated CD9-positive vesicles with advanced metastatic prostate cancer (50). A limitation of our study was limited number of CRPC-NE samples. Our current findings need to be validated in larger cohorts.

(Continued.) CD9 was used as an exosomal control. D, Relative BRN2 mRNA (top) and BRN4 mRNA (bottom) expression in cellular and EV fractions of PC3 and LNCaP cell lines with/without exosome inhibitor GW4869 treatment as assessed by RT-PCR. Data were normalized to vinculin control and represented as mean ± SEM. E–G, “Uptake experiment” with labeled EVs in parental LNCaP cells. EVs were isolated from control (DMSO)/1 μmol/L enzalutamide-treated LNCaP cells, labeled with SYTO RNA Select green fluorescent stain followed by incubation of labeled EVs (40 μg/mL) with parental LNCaP cells. As a negative control, parental LNCaP cells were incubated with media with no EVs. E, Fluorescent microscopy analyses to confirm uptake of labeled EVs (green, left), DAPI staining (blue, middle), and BRN4 IF staining (red, right) after EV treatment. F, Western blot analyses for indicated proteins after “uptake experiment” with labeled EVs in parental LNCaP cells, labeled with SYTO RNA Select green fluorescent stain followed by incubation of labeled EVs (40 μg/mL) with parental LNCaP cells, labeled with SYTO RNA Select green fluorescent stain followed by incubation of labeled EVs (40 μg/mL) with parental LNCaP cells. As a negative control, parental LNCaP cells were incubated with media with no EVs. F, Western blot analyses for indicated proteins after “uptake assay.” G, Uptake experiment with labeled EVs in parental LNCaP cells. EVs were isolated from control (DMSO)/1 μmol/L enzalutamide-treated LNCaP cells, labeled with SYTO RNA Select green fluorescent stain followed by incubation of labeled EVs (40 μg/mL) with parental LNCaP cells. As a negative control, parental LNCaP cells were incubated with media with no EVs. E, Fluorescent microscopy analyses to confirm uptake of labeled EVs (green, left), DAPI staining (blue, middle), and BRN4 IF staining (red, right) after EV treatment. F, Western blot analyses for indicated proteins after “uptake experiment” with labeled EVs in parental LNCaP cells, labeled with SYTO RNA Select green fluorescent stain followed by incubation of labeled EVs (40 μg/mL) with parental LNCaP cells, labeled with SYTO RNA Select green fluorescent stain followed by incubation of labeled EVs (40 μg/mL) with parental LNCaP cells. As a negative control, parental LNCaP cells were incubated with media with no EVs. F, Western blot analyses for indicated proteins after “uptake assay.”
In conclusion, our study has important clinical implications and transformative potential as it identifies BRN4 as an important player in NEPC/therapy-induced neuroendocrine differentiation. We propose that selective modulation of BRN4 can be exploited to prevent neuroendocrine differentiation induction. Importantly, we identified novel, noninvasive, EV biomarkers for detection and neuroendocrine differentiation prediction that can potentially improve clinical management of CRPC.

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

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BRN4 Is a Novel Driver of Neuroendocrine Differentiation in Castration-Resistant Prostate Cancer and Is Selectively Released in Extracellular Vesicles with BRN2

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