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PEG10 is associated with treatment-induced neuroendocrine prostate cancer

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Abstract

Neuroendocrine (NE) differentiation of advanced prostate adenocarcinoma following androgen receptor (AR) axis-directed therapy is becoming increasingly recognized. Several models of this transdifferentiation provide insight into its molecular pathogenesis and have highlighted the placental gene *PEG10* for further study. Using our unique model of enzalutamide resistance (ENZR) and NE differentiation, we studied PEG10/AR interplay in enzalutamide treatment-resistant cell lines 42D^{ENZR} and 42F^{ENZR} compared to LNCaP and castration-resistant 16D^{CRPC} cells. ENZR cell lines with positive terminal NE marker status also displayed higher baseline expression of PEG10 compared to LNCaP and 16D^{CRPC}. Antagonism of AR activity increased PEG10 expression followed by an increase in terminal NE markers. Conversely, stimulating AR activity via androgen supplementation reversed PEG10 and NE marker expression in a time and dose-dependent manner. These results were supported by human data showing that PEG10 expression is highest in NEPC and that AR-dependent gene, PSA, is negatively correlated with PEG10 in adenocarcinoma. Further, ChIP assay confirmed binding of activated AR to the *PEG10* enhancer, decreasing PEG10 expression. While PEG10 did not drive NEPC, its knockdown reduced NE markers in our cell lines. Moreover, PEG10 knockdown *in vitro*- and *in vivo*-attenuated tumor growth. Overall, these observations indicate that *PEG10* is an AR-repressed gene which modulates NE markers in ENZR cells and targeting PEG10 in advanced prostate cancer with NE features is a rational and viable option.

Key Words

- ▶ prostate cancer
- ▶ neuroendocrine
- ▶ treatment resistance
- ▶ PEG10
- ▶ androgen receptor

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Introduction

Prostate cancer (PCa) is the most commonly diagnosed and the third leading cause of cancer mortality among Canadian men (CCSACoC 2017). Initially, most cases of prostate adenocarcinoma are hormone driven and respond to androgen deprivation therapy (ADT); however, treatment resistance is inevitable and tumors progress to castration-resistant PCa (CRPC) (Davies *et al.* 2018). Persistent androgen receptor (AR) activity continues to be a driving force behind disease progression in CRPC and

consequently, second-generation AR antagonists such as enzalutamide (ENZ) have demonstrated an overall survival benefit in CRPC, highlighting the ongoing ‘targetable’ nature of the AR even in advanced disease (Scher *et al.* 2012). Unfortunately, with the wider use of this agent, enzalutamide resistance (ENZR) is an increasingly common clinical entity. In some cases it may be mediated by AR mutations creating an agonist relationship between enzalutamide and the AR ligand-binding domain (LBD)

(Balbas *et al.* 2013, Joseph *et al.* 2013, Korpai *et al.* 2013, Li *et al.* 2013). In other cases, after multi-agent AR-axis inhibition, aggressive CRPC cancers demonstrate a non-AR-driven phenotype with neuroendocrine (NE) features absent in typical PCa and CRPC (Wright *et al.* 2003, Akamatsu *et al.* 2015, Bishop *et al.* 2017). This has given rise to the term treatment-induced NE PCa (tNEPC) (Terry & Beltran 2014, Aggarwal *et al.* 2018). The cellular origin of tNEPC is actively debated but increasing evidence suggests a 'transdifferentiation' from an AR-positive PCa to an AR-negative NE state characterized by minimal AR-dependent gene activity (e.g. PSA) and an increase in NE marker expression such as synaptophysin (SYP), chromogranin A (CHGA), neural cell adhesion molecule 1 (NCAM1) and neuron-specific enolase (NSE, gene symbol: ENO2) (Beltran *et al.* 2014, Bishop *et al.* 2017).

A number of molecular mechanisms were proposed to facilitate the progression of CRPC to NEPC (e.g. amplification of *MYCN* and *AURKA*; Beltran *et al.* 2011, loss of *RB* or *PTEN*; Tzelepi *et al.* 2012, Tan *et al.* 2014 and overexpression of epigenetic modulator *EZH2*; Dardenne *et al.* 2016). More importantly, the AR still plays a crucial, yet to be completely deciphered, role in NEPC (Bishop *et al.* 2015). Indeed, loss of AR activity and acquisition of a NE phenotype under the pressure of ENZ has raised the question if AR can act as a repressing agent on NE-like phenotype, a potential theory which is tested by mechanistically studying the interplay between AR- and NE-driving forces such as neural differentiation regulator *BRN2* (Bishop *et al.* 2017) or growth-promoting paternally expressed gene 10 (PEG10) (Akamatsu *et al.* 2015).

Utilizing a unique *in vivo*-derived model of ENZ resistance (Kuruma *et al.* 2013, Bishop *et al.* 2017), our lab further explored the role of PEG10 in tNEPC as well as the interplay between PEG10 and AR in non-AR driven ENZR cells (42D^{ENZ}, 42F^{ENZ}) that display NE features compared to original LNCaP and 16D^{CRPC} cells. Here, we elucidate the AR/PEG10 interplay by demonstrating that the AR binds to the *PEG10* downstream enhancer and suppresses PEG10 expression. Moreover, we characterize the changes in PEG10 and NE marker expression in LNCaP, 16D^{CRPC}, 42D^{ENZ} and 42F^{ENZ} following androgen depletion and supplementation. We also silenced *PEG10* both *in vitro* and *in vivo* to further delineate its association with NE marker expression and tumor growth, respectively. *PEG10* silencing affects cell proliferation, confirming its pro-growth properties (Akamatsu *et al.* 2015). Together, these findings endorse a mechanism whereby AR acts to repress PEG10 expression and also reveal further evidence for PEG10 involvement in NEPC progression. This study also

highlights the utility of our novel ENZR cell model, which by mimicking the heterogeneity of ENZ resistance, permits evaluation of mechanistic features of NEPC progression and provides a future platform for studying NEPC drug targets, including PEG10.

Materials and methods

Generation of ENZR xenografts and cell lines

The detailed procedure for generation of CRPC and ENZR cell lines can be found in our previously published report (Bishop *et al.* 2017).

Cell culture and transfection

LNCaP cells were kindly provided by Dr Leland W.K. Chung (Emory University) and authenticated in January 2013 using short tandem repeat (STR) profile. LNCaP and castration-resistant LNCaP-derived V16D cell lines were grown in RPMI 1640 (Thermo Scientific) with 10% FBS (Invitrogen), and enzalutamide-resistant 42F and 42D cell lines were grown in RPMI 10% FBS along with 10 µM Enzalutamide (Selleck Chemicals). All cell lines were grown at 37°C in a humidified air atmosphere with 5% CO₂ and were kept at passages less than 25. For induction of AR activity experiments, R1881 (0–5 nM) was added to the cell lines after culturing the cells in RPMI with 10% CSS (Invitrogen) for 6 days prior. For transient loss of function experiments, Silencer Select siRNA *PEG10* (si*PEG10*) (#s23005, Thermofisher) and custom-designed control siRNA (siCTRL) 5'-AUCAAACUGUCAGCGCUG-3' (Dharmacon) were transfected twice to improve silencing using Oligofectamine (Invitrogen) at the final concentration of 20 nM according to the manufacturer's protocol. The duration for the first transfection was 16 hours, and the duration of the second transfection 4 h. Stable *PEG10*-knockdown cells were generated by transfecting *PEG10* shRNA (Lentiviral Particles sc-152158-V (Santa Cruz)) and control shRNA (Lentiviral Particles-A sc-108080) into cell lines according to the manufacturer's protocol. After transfection, the cells were isolated via selection with 2.3 µg/mL puromycin (Gibco) containing media. Stably *PEG10*-knockdown cells were challenged with enzalutamide for 6 days prior to experiments to ensure knockdown of *PEG10*. *PEG10* overexpression experiments were carried in LNCaP cells for 7 days using 1 µg of full-length PEG10 plasmid (kindly provided by Dr Akamatsu) and TransIT®-2020 (Mirus Bio.).

Quantitative RT-PCR

Total RNA was extracted using TRIzol (Invitrogen) and 2 µg was reversed transcribed using MMLV reverse transcriptase and random hexamers (Invitrogen). Levels of *PEG10*, *AR*, *PSA*, *NCAM1*, *NSE*, *SYP*, *CHGA* and *GAPDH* transcripts were quantified using FastStart Universal SYBR Green Master (Roche) with custom primers (listed in Supplementary Table 2, see section on [supplementary data](#) given at the end of this article). The average change in threshold cycle (Δ CT) relative to endogenous GAPDH levels was determined for each sample and compared to control. All experiments were performed in triplicate and mean standard error was determined. Student *T* tests were performed to test for differences between treatments.

Western blot analysis

Total protein was extracted using RIPA lysis buffer and the concentration was determined by using Pierce BCA Protein Assay Kit (Thermo Scientific). Forty microgram protein were separated by SDS-PAGE and blotted to a nitrocellulose membrane. The membranes were probed with primary antibodies against PEG10 (Novus Biologicals: rabbit NBP2-13749 or mouse NBP1-28875) at 1:1000 and 1:500 dilution respectively, AR N-20 sc-816 (Santa Cruz) at 1:5000 dilution, and PSA C-19 sc-7638 (Santa Cruz) at 1:500 dilution. All antibodies were dissolved in 2.5% bovine serum albumin + TBS-T solution and the membranes were incubated overnight at 4°C. Vinculin MA5-1690 (Thermo Scientific) at 1:10,000 was blotted as a loading control. Proteins were visualized using the Odyssey system (Li-Cor Biosciences). Alternately, HRP detected the bands with Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Proliferation assay

Three thousand cells from each condition of the cell lines were plated onto 96-well plates in RPMI + 10% FBS + 2.3 µg/mL puromycin, where ENZ-resistant cell line media was supplemented with enzalutamide 10 µM. Over the span of 8 days, the cells were fixed with 1% glutaraldehyde and stained with crystal violet. Sorenson's buffer was used to solubilize the stain for absorbance measurement at 540 nm on the Epoch reader (BioTek). Mean optical density values of PEG10 shRNA cells were compared to those of control to derive relative cell proliferation percentages.

AR chromatin immunoprecipitation (ChIP)

All cell lines were cultured in RPMI + 10% CSS on 15 cm plates with or without 1 nM R1881 for 4 days. The sequence for the AR-binding site is summarized in Supplementary Table 3. The ChIP assay was performed using EZChIP kit (EMD Millipore) according to the manufacturer's protocol. Briefly, once the cells grew to 90% confluency, they were cross-linked with 1% formaldehyde and the cross-linking was quenched with 0.125 M glycine. Upon cell collection, the nuclei were extracted and sonicated into 200 bp fragments. The DNA-protein complex was immunoprecipitated with either 5 µg AR N-20 sc-816 (Santa Cruz) or rabbit IgG overnight in 4°C. The complex was washed with low-salt buffer (0.1% SDS, 1% Triton X, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl), LiCl buffer (250 mM LiCl, 1% NP40, 1% sodium deoxycolate, 1 mM EDTA, 10 mM Tris-HCl) and finally with TE buffer. The complex was reverse cross-linked and purified. The extracted DNA fragments were subjected to qRT-PCR using PEG10 primers that were designed to cover the predicted AR-binding site on the *PEG10* enhancer region CACTTGCTGTTCTCGCCCTA (forward), AGGGGAAGCAAAGATGAGCC (reverse). The *KLK3* enhancer primers (5'-TCTGCCTTTGTCCCCTAGAT-3' (forward), 5'-AACCTTCATTCCCCAGGACT-3' (reverse)) were used as a positive control for AR activity.

In vivo tumor formation

All experimental protocols used in this study were approved by the Canadian Council on Animal Care as well as the Animal Care Committee of University of British Columbia (certificate number A016-0246) and were carried out in accordance with institutional guidelines. Two million 42F cells stably transfected with either control or *PEG10* shRNA were injected subcutaneously into the flank region of 4- to 6-week-old male athymic nude mice post castration. Mice were purchased from Jackson Labs and were kept in the animal facility of Jack Bell Research Centre where veterinarians and staff monitored the mice multiple times a week. Each experimental group consisted of ten mice. Tumor volume was measured once a week (length × width × depth × 0.5432) until the tumor volume was ≥10% of body weight. Tumors were harvested for evaluation of gene expression and protein expression by qRT-PCR and Western blot respectively.

Statistical analysis and data representation

Unpaired, two-tailed, Student's *T* tests were performed to analyze statistical significance between groups using Graph Pad Prism (Graph Pad Software). Significance is indicated as follows: **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. Unless otherwise indicated, graphs show representative data from one of at least three independent experiments ± S.E.M.

Results

PEG10 is highly expressed in NE-like ENZ resistance and in human NEPC

To investigate the clinical relevance of PEG10 in human NEPC, we assessed the level in prostatic hormone-naïve adenocarcinoma, CRPC and NEPC patients in a recently published cohort (Beltran *et al.* 2016) and found that PEG10 level was significantly increased in NEPC compared with CRPC or adenocarcinoma (Fig. 1A). PEG10 mRNA expression was also tested across our established ENZR cell lines as well as the NEPC tumor-derived NCIH660 cell line. Reflecting the clinical data, compared to adenocarcinoma LNCaP and castrate-resistant V16D^{CRPC} cells, PEG10 expression was highest in human NEPC cell line, NCIH660 cells, followed by cell lines with neuroendocrine-like features, 42D^{ENZ}R and 42F^{ENZ}R (Bishop *et al.* 2017) (Fig. 1B). Interestingly, the expression levels of NE markers (ENO2, NCAM1, CHGA and SYP) correlate with PEG10 expression in these cell lines (Fig. 1C). Similar data were observed in human specimens from The Cancer Genome Atlas (TCGA) prostate cancer database (publicly available on <https://genome-cancer.ucsc.edu/>) where NE representative markers ENO2, NCAM1, CHGA and CHGB were elevated

in tumor samples with higher PEG10 expression (shown as red on heatmap) (Fig. 1D, left). Interestingly, analysis of the same samples showed that AR canonical targets PSA, FKBP5 and NKX3-1 are lower in tumors with high PEG10 expression (Fig. 1D, right). These findings were supported by more public data mining for the relationship between PEG10 and serum PSA from CRPC samples generated by Grasso *et al.* (2012) which further confirmed PSA/PEG10 inverse correlation suggesting that AR activity may be involved in PEG10 regulation (Fig. 1E). Furthermore, data from two other cohorts (Rajan *et al.* 2014, Beltran *et al.* 2017) showed that PEG10 is increased upon ADT (Supplementary Table 1).

AR activity negatively regulates PEG10 in enzalutamide-resistant cell lines

Following the observation from our ENZR model and inverse correlation between PEG10 and PSA from Grasso analysis (Fig. 1E), we tested the hypothesis if AR is a negative regulator of PEG10. We confirmed that in LNCaP cells, AR inhibition using 10 μM ENZ leads to decreased PSA expression, while PEG10 expression is increased in a time-dependent manner at both the mRNA and protein levels (Fig. 2A). This is in harmony with previous data showing PEG10 is repressed by AR in LNCaP cells (Akamatsu *et al.* 2015). The effect of ENZ is accentuated in charcoal-stripped serum (CSS) media, with highest PEG10 expression occurring after 7 days of exposure to CSS+ENZ, which represents maximal androgen blockade condition. Similar results were reproduced in CRPC cells 16D^{CRPC} (Supplementary Fig. 1A). It is important to note that PEG10 has several isoforms due to alternative translation initiation sites, alternative splicing, posttranslational

Figure 1

PEG10 is highly expressed in non-AR driven human NEPC, and ENZR cells feature increased levels of PEG10 and a NE differentiation signature. (A) PEG10 expression in Adeno (*n* = 37), CRPC (*n* = 34) and NEPC (*n* = 15) patients obtained from Beltran cohort (Beltran *et al.* 2016). (B) Relative mRNA expression of PEG10 and (C) relative mRNA expression of NE markers in V16D^{CRPC}, 42D^{ENZ}R, 42F^{ENZ}R and NCIH660 cells compared to LNCaP (=1). (D) Heat map of PEG10 and neuroendocrine-associated genes as well as canonical AR targets PSA, FKBP5 and NKX3-1 in RNA-sequencing data obtained from The Cancer Genome Atlas (TCGA) prostate cancer samples (*n* = 499). (E) PEG10 expression in CRPC tumors versus patient serum PSA (Grasso *et al.* 2012). Statistical significance is shown as **P* < 0.05; *****P* < 0.0001.

Figure 2

PEG10 and PEG10-dependent neuroendocrine marker expression are induced by ENZ and suppressed by reintroduction of AR activity. (A) Protein and relative mRNA expression of PEG10 and PSA in LNCaP cells treated $\pm 10 \mu\text{mol/L}$ ENZ for 2, 4, 7 days compared with day 0 (=1) (Relative PSA expression after treatment is <0.2). (B) Relative mRNA expression of NE markers expression in LNCaP cells treated $10 \mu\text{mol/L}$ ENZ for 2, 4, 7 days compared with day 0 (=1) in FBS or CSS. (C and D) Relative mRNA expression of PEG10, PSA and NE markers expression in LNCaP, V16D^{CRPC}, 42D^{ENZR} and 42F^{ENZR} with reintroduction of androgen after 7 days of $10 \mu\text{mol/L}$ ENZ treatment (=1). Graphs show representative data from three independent experiments. (E) Chromatin immunoprecipitation showing AR binding to the enhancer region of PEG10 in 42D^{ENZR} and 42F^{ENZR} treated with 10 nmol/L R1881 for 6 h non-treated control (=1); Corresponding PSA binding used as positive control. Graphs show pooled data from three independent experiments.

proteolytic cleavage and –1 ribosomal frameshift translation. We were interested in looking at CNF band as it was previously shown to augment upon ENZ treatment (Akamatsu *et al.* 2015). Parallel to PEG10 expression, NE markers were increased in both FBS+ENZ and CSS+ENZ (Fig. 2B).

Inversely, we assessed the effects of AR stimulation with synthetic androgen (R1881) on PEG10 expression after 7 days of androgen deprivation. R1881 significantly reduced PEG10 mRNA levels across multiple cell lines (Fig. 2C) and was accompanied by a reduction in terminal NE markers (Fig. 2D). Interestingly, the PSA expression was recovered in all cell lines including 42D^{ENZ} and 42F^{ENZ} in a time- and dose-dependent fashion (Fig. 2C and Supplementary Fig. 1B). Importantly, publicly available ChIP-seq data for AR shows a strong AR-binding peak in the enhancer region of the *PEG10* gene (www.cistrome.org) (Supplementary Fig. 2). Indeed, using chromatin immunoprecipitation (ChIP) on our cell lines, 42D^{ENZ} and 42F^{ENZ}, we observed drastic increase in AR occupancy at this ARE after R1881 stimulation (Fig. 2E). PSA enhancer served as an AR-dependent positive control (Fig. 2E). Together, these results indicate that PEG10 expression is negatively regulated by AR activity in CRPC and ENZR cells. Also, the expression of NE markers is positively correlated to PEG10 in these cells.

PEG10 is required for NE marker expression across multiple cell lines

Using gain- and loss-of-function approaches, we investigated whether PEG10 is a driver of NEPC. We observed no significant upregulation of NE markers following PEG10 overexpression indicating that PEG10 is not a driver of NEPC (Fig. 3A and Supplementary Fig. 3). However, our previous data suggested that PEG10 is supporting a NE phenotype across cell lines. To confirm this, we generated transient and stable *PEG10*-knockdown conditions via small-interference (siRNA) and short-hairpin RNA (shRNA) respectively (Supplementary Fig. 4). Transient *PEG10* knockdown (si*PEG10*) lead to a marked reduction in mRNA levels of CHGA, NCAM1, NSE and SYP in ENZ-treated LNCaP and V16D^{CRPC} and also in 42D^{ENZ} and 42F^{ENZ} (Fig. 3B). Similar results were observed when sh*PEG10* prevented the ENZ-induced upregulation of NE markers (only the most effective clones are demonstrated) (Fig. 3C). Overall, these results confirm that although PEG10 is not a driver of NEPC, it is indeed important for maintaining the NE marker expression.

PEG10 plays a role in cell proliferation *in vitro* and *in vivo*

In addition to the expression of terminal NE markers, we questioned whether PEG10 is involved in regulating proliferation of cells. Previous studies have shown that PEG10 plays a role in breast and pancreatic cancer cell proliferation (Li *et al.* 2016, Peng *et al.* 2017). More importantly, Akamatsu *et al.* showed a supporting role for PEG10 in LNCaP and PC3 prostate cancer cells (Akamatsu *et al.* 2015) but not in NE-like prostate cells. Therefore, we investigated the effects of *PEG10* knockdown on cell proliferation ENZ-treated LNCaP and V16D^{CRPC} as well as 42D^{ENZ} and 42F^{ENZ} cell lines. *PEG10* knockdown significantly reduced proliferation across all cell lines *in vitro* (Fig. 4A). Similar data were observed *in vivo* where sh*PEG10* 42F^{ENZ} murine xenograft was established with significant attenuation of tumor growth compared to control (Fig. 5A, left). Waterfall plot of tumor sizes between groups highlights four mice from the sh*PEG10* group which failed to grow any tumor beyond 10 weeks (Fig. 5A, right). Tumor tissue analysis confirms PEG10 knockdown and significantly reduced expression of CHGA, a representative NE marker (Fig. 5B). These *in vitro* and *in vivo* results suggest that PEG10 may be an ideal target to reduce tumor proliferation in ENZR.

Discussion

Although *de novo* NEPC is rare and includes less than 1% of all diagnosed PCa cases, there is strong clinical evidence for the emergence of a more aggressive, treatment-induced neuroendocrine phenotype after treatment with strong APIs such as enzalutamide (17%) (Beltran *et al.* 2016, Aggarwal *et al.* 2018). While the mechanisms of such neuroendocrine transdifferentiation remain unclear, numerous putative targets have been identified as related to this process, including AURKA, MYCN, REST, BRN2 and PEG10 (Beltran *et al.* 2011, Akamatsu *et al.* 2015, Zhang *et al.* 2015, Bishop *et al.* 2017). How each of these proteins confers a change from an AR-positive adenocarcinoma to an AR-negative neuroendocrine phenotype, is only beginning to be elucidated. Our study builds up on previous findings that *PEG10* is an AR-repressed gene and a potential therapeutic target in NEPC. Mining human data, we demonstrate that in NEPC tumors expression of NE markers are positively and expression of PSA is negatively correlated to PEG10. More importantly, utilizing an *in vivo*-derived model of ENZ resistance, we show for the first time that AR activity negatively regulates PEG10

Figure 3

PEG10 is required to maintain NE marker expression across multiple cell lines. (A) Relative mRNA expression of PEG10 in LNCaP after overexpression compared with control (Ctr = 1). (B) (Top) Relative mRNA expression of PEG10 and NE markers in LNCaP and V16D^{CRPC} transfected with PEG10 siRNA (siPEG10) or scrambled siRNA (siCtrl) and treated with 10 μ mol/L ENZ for 48 h compared to basal levels (basal = 1). Statistics show the decrease from siPEG10 to siCtrl, (Bottom) Relative mRNA expression of PEG10 and NE markers and protein expression of PEG10 in 42D^{ENZR} and 42F^{ENZR} transfected with PEG10 siRNA (siPEG10) or scrambled siRNA. Western blots validate protein levels of PEG10 after knockdown. (C) (Left panel) PEG10 protein expression in stable PEG10 knockdown (shPEG10) compared with control-transfected cells (shCtrl) in LNCaP, V16D^{CRPC}, 42D^{ENZR} and 42F^{ENZR} cells maintained in 10 μ mol/L ENZ for 7 days; (Right panel) Corresponding relative mRNA expression of PEG10 and NE markers compared with control (shCtrl = 1). Statistical analyses were performed on pooled data from at least three independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001, **** P < 0.0001.

Figure 4

PEG10 promotes the proliferation of ENZR cell lines *in vitro*. Proliferation rate of shCtrl and shPEG10 in LNCaP, V16D^{CRPC}, 42D^{ENZR} and 42F^{ENZR} cells maintained in 10 μ mol/L ENZ over the course of 8 days. Statistics were performed on pooled data from at least three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

expression in the context of ENZ resistance (42D^{ENZR} and 42F^{ENZR} cells) as well as ENZ-treated adenocarcinoma cells (LNCaP and 16D^{CRPC}). Finally, we show that PEG10 is required for maintaining NE phenotype in ENZR cells and targeting PEG10 reduces proliferation rate and expression of NE markers both *in vitro* and *in vivo*.

Figure 5

PEG10 knockdown via shPEG10 attenuates tumor growth *in vivo*. (A) (Left) Tumor volume of 42F^{ENZR} shCtrl and shPEG10 xenografts grown *in vivo* ($n = 10$), with (Right) representation in waterfall plot. Graph represents pooled data from five shPEG10 and five shCtrl tumors. (B) Relative mRNA expression of PEG10 and representative NE marker chromogranin A (CHGA) in 42F^{ENZR} shPEG10 and shCtrl xenografts ($n = 1$), harvested at 12 weeks after inoculation. Values are expressed as mean $\pm$ S.E.M.

PEG10 is an evolutionary conserved retrotransposon-derived gene located within an imprinted domain on chromosome 7q21 and is highly expressed during embryogenesis while mostly silenced in adult tissues. It is required for placental development and murine PEG10 knockout results in early embryonic lethality due to placental defects (Ono *et al.* 2006). There is an increasing interest in genes with restricted expression during placental development as this phenomenon is observed in natural models of invasion as well as oncologic states (Rousseaux *et al.* 2013). During embryogenesis, such genes may serve pivotal roles in driving coordinated proliferation and invasion to allow proper embryonal development, while in adult cancers, reactivation of these genes especially in the context of inappropriate coordination may drive advanced disease phenotypes. PEG10 is suggested to be an inhibitor of apoptosis and mediator of proliferation and was shown to be overexpressed in several malignancies including acute and chronic lymphocytic leukemia, hepatocarcinoma, pancreatic cancer (Ribarska *et al.* 2012) and small cell bladder cancer (Robertson *et al.* 2017). In prostate cancer, our collaborators identified PEG10 as one of the upregulated genes in a human xenograft model that recapitulated transdifferentiation of adenocarcinoma to NEPC. Analysis of two independent cohorts revealed that PEG10 expression is elevated upon castration, and further significantly upregulated in clinical NEPC. *In vitro* mechanistic experiments demonstrated PEG10's role in promoting a proliferative, invasive phenotype as a result of direct transcriptional upregulation by E2F1 (major transcription factor in the RB signaling pathway) in the context of TP53 loss and enhanced TGF-beta signaling (Akamatsu *et al.* 2015). This is in harmony with results of a recent study which showed PEG10 is regulated by E2F1 and its overexpression promotes cell proliferation, migration and invasion in pancreatic cancer (Peng *et al.* 2017). Akamatsu *et al.* also reported PEG10 promoter occupancy by AR in LNCaP cells. However, the relation between PEG10 and AR was not delineated further as the majority of their experiments were carried out in PC3 and DU145 cells.

Despite the fact that tNEPC exhibits an AR-independent characteristic with low PSA production, there is little doubt that AR still plays a significant role in the emergence of NEPC. PC3 and DU145 cells have mutant TP53, express higher endogenous PEG10 compared to LNCaP and provide a good model for studying TGF-beta signaling; however, they are unable to accurately mimic NEPC as they do not express AR. AR is a regulator of cell plasticity and was suggested to be a

repressor of several NEPC-promoting genes (Bishop *et al.* 2015, Akamatsu *et al.* 2018). Therefore, to understand the true role of PEG10 in tNEPC, it is imperative to study it in an AR-positive model under the pressure of an AR inhibitor. Indeed, we confirmed that AR not only binds to the enhancer region of PEG10 in LNCaP, V16D^{CRPC}, 42D^{ENZR} and 42F^{ENZR} cells but that treatment with synthetic androgen R1881 increases AR occupancy. We also searched TCGA human data to provide clinical evidence for the role of PEG10 in NEPC. More importantly, analysis of prostate adenocarcinoma data available from Grasso *et al.* demonstrated that PEG10 expression is negatively correlated to PSA, AR's classic target gene. Also, a recent study on metastatic CRPC showed PEG10 is upregulated in cases treated with neoadjuvant docetaxel and androgen deprivation before going through radical prostatectomy (Beltran *et al.* 2017). Using systematic experiments, we elucidate this interaction in more detail by showing androgen deprivation increases PEG10 expression, while AR stimulation decreases PEG10. These findings highlight the adaptive nature of the AR axis present in this disease state and once again demonstrate how maximum AR inhibition not only reduces AR transcription activity but also removes AR-dependent transcriptional repression and consequently enhances other signaling pathways including NE differentiation.

Insight into tNEPC, specifically its development from a conventional adenocarcinoma, is an increasingly recognized clinical scenario that needs further investigation. Early studies were pivotal in identifying that the expression of NE markers in PCa was inversely related to AR activity (Bang *et al.* 1994, Burchardt *et al.* 1999). We examined this in further detail via our cell models which recapitulate the cellular phenotype of ENZR and neuroendocrine PCa. Our modeling of ENZR provides a unique research platform as 42D^{ENZR} and 42F^{ENZR} cells proliferate under the pressure of ENZ, remain AR positive but PSA negative and also demonstrate markers of terminal NE differentiation (Bishop *et al.* 2017). We show that maintaining NE marker status in these cell lines is dependent on PEG10 expression, meaning knockdown of PEG10 resulted in the attenuation of NE marker status while PEG10 overexpression alone was not sufficient to induce NE differentiation in LNCaP cells. These findings suggest that PEG10 is not a driver of NEPC but is crucial in maintaining this phenotype. Moreover, PEG10 inhibition reduced proliferation *in vitro* and attenuates tumor growth *in vivo* making it an attractive therapeutic target in advanced prostate cancers where NE differentiation

or growth may be PEG10 dependent. Our results were supported by a recent study that shows inhibition of PEG10 may be a novel treatment approach in a subset of bladder cancer (Kawai *et al.* 2017).

In summary, this study adds to our understanding of the mechanisms involved in tNEPC and the molecular underpinnings driving neuroendocrine differentiation. We highlight the non-canonical role that AR plays in mediating neuroendocrine transdifferentiation, namely its effect to repress the NE phenotype in prostate cancer by suppressing PEG10 expression. Utilizing *in vitro*, *in vivo* study design and corroborating our findings with human cancer databases, we have shown that PEG10 is one of the few genes that have been identified in multiple datasets as being upregulated during and following transdifferentiation (Beltran *et al.* 2011, 2016, Akamatsu *et al.* 2015) and a potential therapeutic target in NEPC.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-18-0226>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S K: Experimental design, data acquisition, statistical analysis; D T and S V: manuscript preparation, data acquisition, data analysis; S B: manuscript preparation; S A, P T and C C: manuscript editing; J L B: experimental design, data analysis, manuscript preparation; A Z: experimental design, study overview, data analysis, manuscript preparation.

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