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Graphical Abstract



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In Brief

Akamatsu et al. describe involvement of the placental gene PEG10 in driving the proliferative and invasive phenotype of lethal neuroendocrine prostate cancer (NEPC), suggesting PEG10 as a therapeutic target.

Highlights

- Placental gene PEG10 is highly expressed in neuroendocrine prostate cancer (NEPC)
- PEG10 is dynamically regulated by AR and E2F/RB during NEPC development
- Distinct isoforms of PEG10 promote proliferation and invasion of NEPC cells
- PEG10 represents a specific therapeutic target for NEPC

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The Placental Gene *PEG10* Promotes Progression of Neuroendocrine Prostate Cancer

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SUMMARY

More potent targeting of the androgen receptor (AR) in advanced prostate cancer is driving an increased incidence of neuroendocrine prostate cancer (NEPC), an aggressive and treatment-resistant AR-negative variant. Its molecular pathogenesis remains poorly understood but appears to require TP53 and RB1 aberration. We modeled the development of NEPC from conventional prostatic adenocarcinoma using a patient-derived xenograft and found that the placental gene PEG10 is de-repressed during the adaptive response to AR interference and subsequently highly upregulated in clinical NEPC. We found that the AR and the E2F/RB pathway dynamically regulate distinct post-transcriptional and post-translational isoforms of PEG10 at distinct stages of NEPC development. In vitro, PEG10 promoted cell-cycle progression from G0/G1 in the context of TP53 loss and regulated Snail expression via TGF- β signaling to promote invasion. Taken together, these findings show the mechanistic relevance of RB1 and TP53 loss in NEPC and suggest PEG10 as a NEPC-specific target.

INTRODUCTION

Prostate cancer (PCa) at diagnosis is an androgen-driven disease, dependent on ligand signaling via the androgen receptor (AR) (Attard et al., 2009). Accordingly, therapeutic strategies for advanced PCa target the AR axis, depleting ligand availability or antagonizing the AR itself. Although tumors initially respond, treatment is palliative, and, given sufficient time, patients will relapse with castration-resistant PCa (CRPC). Over recent years, the implementation of increasingly potent AR-axis interference has enjoyed success, prolonging patient survival (de Bono et al., 2011; Scher et al., 2012) but has altered the archetypal disease course. It is now relatively common to observe advanced PCa progressing in the absence of rising PSA (the chief biomarker of AR-dependent disease) and with atypical visceral metastatic sites (Aparicio et al., 2013; Beltran et al., 2012; Pezaro et al., 2014). Efforts are underway to clinically and biologically subtype AR-negative and low-AR-expressing CRPC, which can present with diverse morphology and biomarker expression, and to determine how best to recognize and manage the disease (Beltran et al., 2014; Epstein et al., 2014; Terry and Beltran, 2014).

The most recognized AR-negative CRPC variant is neuroendocrine (NE) PCa (NEPC), also termed anaplastic or small-cell carcinoma of the prostate due to the morphological resemblance to small-cell lung cancer and the gross differences to prostatic adenocarcinoma (which makes up >95% of initial diagnoses) (Aparicio et al., 2013; Beltran et al., 2011; Cindolo et al., 2007; Nelson et al., 2007). NEPC is highly aggressive, has poor prognosis, and is characterized by the expression of NE markers such as CHGA and SYP. Recent reports of NEPC tumors in up to 25% of PCa autopsies suggest that the incidence of this variant is rising (Aparicio et al., 2013; Beltran et al., 2012; Pezaro et al., 2014), and there is now little doubt that this is driven, in part, by AR-axis interference. Furthermore, the concurrent presence of identical genomic rearrangements in adenocarcinoma and NEPC foci from the same patients indicates that, at some point during progression, prostatic adenocarcinoma cells undergo "NE transdifferentiation" and become NEPC cells (Guo et al., 2011; Lotan et al., 2011; Williamson et al., 2011).

Accumulating evidence suggests that there are two conceptual stages of progression to NEPC: (1) an adaptive response to treatment through the acquisition of a NE-like phenotype



from an AR-positive adenocarcinoma and (2) the initiation of cell proliferation. Small foci of malignant cells with an NE-like phenotype can be observed in almost all adenocarcinoma tumors (Cindolo et al., 2007; Nelson et al., 2007), suggesting that most tumors are capable of achieving the initial stage. However, the second stage appears to require distinct genomic aberration, allowing malignant adaptation to a neuronal niche. RB1 loss, TP53 loss, and MYCN amplification are enriched in NEPC tumors (Beltran et al., 2011; Chen et al., 2012; Tan et al., 2014), although it is not clear whether this aberration arises in the original adenocarcinoma cells or in the treatment-induced NE-like cells. The reality is likely to be heterogeneous, with some tumors undergoing clonal selection of NE-like cells that have acquired new mutations, while other fortuitously adapted adenocarcinoma foci transform into NEPC en masse. Despite recent breakthroughs, the precise mechanisms of NE transdifferentiation remain unknown, partly due to the difficulty in modeling the transformation and capturing appropriate clinical specimens for study. Furthermore, the broader NEPC phenotype is characterized by upregulation of a wide program of non-malignant neuronal-like genes, which confound identification of cancer-specific drivers or therapeutic targets (Beltran et al., 2011; Lin et al., 2014). Consequently, there are few therapeutic options for NEPC (the disease responds briefly to platinum-based chemotherapy, and there is an AURKA inhibitor [MLN8237] in phase 2 clinical trials).

Recently, we developed a unique patient-derived xenograft model of NEPC transdifferentiation: a typical hormone-naive AR/PSA-positive adenocarcinoma (LTL331) that, upon host castration, initially regresses but rapidly relapses as terminally differentiated NEPC (LTL331R) (Lin et al., 2014). The lack of evidence for NEPC cells prior to AR blockade and the conservation of genome characteristics pre- and post-NEPC development strongly suggested an adaptive response of the adenocarcinoma cells that remained after tumor regression. We hypothesized that examination of regressing tumors during transdifferentiation, prior to the emergence of the dominant neuronal expression program that accompanies proliferative NEPC, would unmask true malignant drivers of disease progression. Therefore, in this study, we carried out longitudinal expression profiling of xenograft tumors during the transformation from adenocarcinoma to NEPC. We identified marked upregulation of the retrotransposon-derived gene PEG10 (Paternally Expressed 10), a gene normally expressed during placental development. The onset of PEG10 expression began weeks before emergence of the neuronal expression signature, persisted through to terminal NEPC, and is widely expressed in NEPC clinical tissue. We show that PEG10 is dynamically regulated by AR and the E2F/RB pathway during NEPC development and demonstrate that distinct PEG10 isoforms promote proliferation and invasion of NEPC cells.

RESULTS

Broad Programs of Gene Expression Are Altered during NE Transdifferentiation

To profile NE transdifferentiation in the LTL331 model, we used immunohistochemistry, whole-transcriptome sequencing, and microarray analysis to capture gene expression and copy-number changes in 12 tumor samples collected at different time points (adenocarcinoma pre-castration; post-castration days 1-3; weeks 1-3, 8, and 12; and post-NEPC development) during the transformation from adenocarcinoma (LTL331) to NEPC (LTL331R) (Figures 1A and 1B). Tumors exhibited a marked response to host castration, with tumor volume falling by >50% and serum PSA dropping to baseline by 12 weeks. AR protein expression was lost from the nucleus within 1 week post-castration, and PSA was undetectable in tumor foci by 3 weeks, although cells remained characteristic of adenocarcinoma rather than NEPC until the final time point (Figure 1C). CHGA and other typical NEPC markers were not overtly expressed at either the mRNA level or protein level until the final time points, suggesting that the development of a neuronal gene expression signature is a relatively late event during transdifferentiation (Figures 1C and 1D). Although genome copy-number profiles remained invariant (Figure S1A), global gene expression profiles demonstrated gross changes across the transdifferentiation series (Figure 1E). In the absence of neuronal gene expression, the differential in gene expression during transdifferentiation was not explainable by loss of the AR-responsive transcriptional program alone and suggested a phase transition period.

We searched for genes showing significant linear or non-linear increasing expression trends across the microarray series. We defined two broad classes of genes: those exhibiting transient expression, peaking at 12 weeks (i.e., low expression in both NEPC and adenocarcinoma) and genes whose expression increased throughout the series to peak in terminal NEPC (Tables S1 and S2). Genes with expression trends peaking at 12 weeks (n = 384) included several anti-apoptotic genes (e.g., BIRC3, OLFM4, and REG4) and genes involved in Wnt/ β -catenin signaling (e.g., LEF1, FZD7, and WNT2B) (Figure 1F). Components of the Wnt pathway are frequently deregulated through genomic aberration in CRPC, but not treatment-naive tumors (Grasso et al., 2012; Kumar et al., 2011), and stromal Wnt signaling contributes to therapy resistance in tumor cells (Li et al., 2008; Sun et al., 2012). It is probable that several of the genes upregulated by 12 weeks are critical for cell survival in androgen-deprived conditions. Conversely, genes that showed a significant trend of downregulation by 12 weeks (n = 245) or NEPC (n = 434) were heavily enriched with androgen-responsive genes (e.g., KLK3, BMPR1B, SLC45A3, NKX3.1, and MSMB), and the top predicted "upstream regulator" was synthetic androgen (12 weeks, $p = 3.3E^{-17}$; NEPC, $p = 2.08E^{-14}$). Genes associated with increasing expression to a peak in terminal NEPC included oncogenes MET and FYN, potentially involved in re-establishing growth, and several transcription factors, including TLX3 and BHLHE22, linked to cell fate specification.

The Placental Gene *PEG10* Is Highly Upregulated during Transdifferentiation and in Clinical NEPC

Given the absence of typical NEPC gene expression at 12 weeks post-castration in the transdifferentiation series, we sought to identify genes expressed at this time point that are also expressed in clinical NEPC, potentially representing biomarkers or mechanistic drivers of NEPC emergence. First, to identify robust signatures of clinical NEPC, we compared transcriptome-sequencing-derived gene expression profiles of



Figure 1. Modeling NE Transdifferentiation in Patient-Derived Xenograft LTL331

(A) Overview of the model.

Day3(ii)

-Day1(ii)

(B) Schematic depicting time points at which tumors were collected along progression to NEPC.

NEUROD1 ASCL1 SRRM4

(C) Immunohistochemistry demonstrating changes in marker expression.

adenocarcinoma to NEPC from two independent centers (Weill Cornell Medical College and Vancouver Prostate Centre). The most upregulated NEPC genes in both cohorts were associated with typical neuronal-like biology, such as synapse function and neurite extension (Figure 2A). To unmask malignant drivers among this neuronal program, we overlaid all genes upregulated over 8-fold by week 12 in the transdifferentiation series (Figure 2B). Few typical neuronal genes remained, but PEG10 stood out as markedly overexpressed during transdifferentiation and in clinical NEPC (Figures 2B and 2C). PEG10 mRNA expression was also significantly higher in patient tumors treated with neoadjuvant androgen deprivation therapy (ADT) than in treatment-naive tumors (Figure 2D) (Wyatt et al., 2014). At the protein level, PEG10 expression increased rapidly post-castration in the transdifferentiation series and increased again to high intensity in terminal NEPC, consistent with the mRNA data (Figure 2E). Clinical specimens showed the same trend, with PEG10 demonstrating significantly higher staining intensity in tumors with a NEPC component (p = 0.01; Wilcoxon rank-sum test) and colocalizing with CHGA expression (Figures 2F and S1B). In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model (which expresses the PB-Tag Line 8247 transgene and is considered a model of sorts for NEPC), PEG10 also correlated strongly with CHGA, indicative of PEG10 expression in NEPC tumor foci (Figure S1C) (Haram et al., 2008). Furthermore, high PEG10 mRNA expression in small-cell lung cancer (Kastner et al., 2012; Takeuchi et al., 2006) suggests that PEG10 may be globally expressed in NE tumors (Figure S1D).

The patient whose treatment-naive radical prostatectomy specimen (Gleason score 4 + 5 = 9) led to the establishment of LTL331 did not reach PSA nadir post-prostatectomy and received an initial 18 months of ADT. Unfortunately, he recurred with metastatic disease 5 years post-prostatectomy. He had rising serum PSA, indicative of AR-driven adenocarcinoma, and was treated with a gonadotropin-releasing hormone superagonist and an AR antagonist, to which he experienced a PSA response (Figure 2G). One year later, he progressed with painful lytic bone metastases, and a biopsy revealed AR-negative, CHGA-positive NEPC that was also strongly positive for PEG10.

PEG10 is a retrotransposon-derived gene that integrated into the therian mammalian genome after the split with prototherians. Although PEG10 no longer retains reverse transcriptase activity, it is remarkably unusual among mammalian genes, harboring an active –1 ribosomal frameshift element allowing translation of two overlapping reading frames from the same transcript (RF1 and RF1/2) (Clark et al., 2007; Lux et al., 2010). *PEG10* also possesses two translation initiation sites ("a" and "b," where "b" is non-ATG) and an active protease domain capable of generating a self-cleavage product (termed cleaved N-terminal fragment: CNF), suggesting highly complex biology (Figure S2A). PEG10 has limited expression in adult tissues but is required for placental development, with heterozygous knockout Peg10^{+/-} mice demonstrating embryonic lethality by embryonic day (E) 10.5 (Ono et al., 2001). Although *PEG10* is reported to be overexpressed in some cancers, including hepatocellular carcinoma (Wang et al., 2008), the function of PEG10 is unclear, and previous work has focused exclusively on the RF1a isoform, which is less abundantly expressed than other isoforms. Indeed, we have confirmed that RF1b and RF1b/2 are the predominant isoforms expressed in PCa cell lines (Figure S2A). In addition, using a protease-inactive mutant of PEG10, we have identified a second product of asparytyl protease cleavage around 22 kDa, which we named CNF2 (Figure S2B). We confirmed both CNF and CNF2 to be cleaved N-terminal fragments of PEG10 by mass spectrometry (Figure S2C). Consequently, this study focused on expression and function of the RF1b, RF1b/2, CNF, and CNF2 isoforms, which are all expressed in PCa cell lines and LTL331R.

PEG10 Protein Isoforms Are Dynamically Regulated by AR and E2F/RB at Distinct Stages of Transdifferentiation

We explored whether the upregulation of PEG10 observed during NEPC transdifferentiation could be recapitulated in vitro using PCa cell lines. Consistent with expression data from clinical samples, native PEG10 mRNA expression was low in the androgen-dependent cell line LNCaP, moderate in an androgen-independent cell line DU145, and high in the cell line PC3, which is considered similar to NEPC (Figure S2A). Since the AR controls a broad transcriptional program in PCa, we examined the possibility that PEG10 is AR regulated by inhibiting the AR pathway in LNCaP cells. Growing cells in charcoalstripped media (ligand depletion) or treatment with enzalutamide (AR antagonist) resulted in a marked increase in PEG10 expression that was reversed by treatment with the synthetic androgen R1881 (Figure 3A). This suggests that PEG10 expression is repressed by the AR. Indeed, computational analysis predicted AR-binding sites within the PEG10 minimum promoter region (Figure S3A), and PEG10 promoter activity was increased by enzalutamide and decreased by treatment with R1881 (Figure 3B), supporting repression at the transcriptional level. Furthermore, published chromatin immunoprecipitation sequencing (ChIPseq) data suggested increased AR occupancy at the PEG10 promoter upon R1881 treatment of the AR-positive cell line VCaP (Yu et al., 2010) (Figure S3B), a finding we confirmed in LNCaP cells. Conversely, AR occupancy at the PEG10 promoter was decreased upon treatment with enzalutamide (Figure 3C). Taken together, these data demonstrate that PEG10 is directly transcriptionally repressed by AR binding to the PEG10 promoter region.

Although AR inhibition explains the initial onset of *PEG10* expression, it is not sufficient to explain the second increase that occurs at the emergence of proliferative NEPC. However, *PEG10* is also reportedly directly regulated by E2F transcription factors (Wang et al., 2008), a finding we confirmed in PC3 and

⁽D) Plot showing divergence of gene expression profiles during transdifferentiation.

⁽E) Gene expression clustering demonstrating that weeks 2–12 post-castration are markedly different to earlier in the series.

⁽F) Selected genes exhibiting a significant trend of increasing mRNA expression during transdifferentiation. Circle size represents log₂ fold changes in gene expression normalized to pre-castration levels. Adeno., adenocarcinoma. See also Tables S1 and S2.



DU145, where E2F1 knockdown resulted in decreased PEG10 (Figure S3C). Additionally, we show that, in PCa cell lines, PEG10 promoter activity is significantly increased by transient overexpression of E2F1 (Figure S3D), with concomitant increase in PEG10 expression both at the mRNA level and protein level in LNCaP (Figure S3E). Furthermore, E2F1 was significantly upregulated in NEPC, compared to adenocarcinoma in both the LTL331 model and in clinical cohorts (Figures 3D, S3F, and S3G). The pivotal regulator of E2F1 activity, RB1 is frequently lost in NEPC (Tan et al., 2014), and genomic analyses demonstrated that the LTL331 system had a single-copy loss of RB1 and a frame-preserving amino acid insertion in the pocket domain of the remaining allele (Figure S3H). Application of the "RB1 loss signature" (Ertel et al., 2010) to mRNA expression data from the LTL331 system and a panel of other PDX tumors, including multiple adenocarcinoma and NEPC tumors, further suggested significant RB pathway aberration in LTL331R (Figure 3E). Overall, these observations suggest that the second increase in PEG10 expression that occurs upon NEPC emergence in the LTL331 system is associated with aberration in the E2F/RB pathway; aberration that may also partly explain the clear predisposition of LTL331 to NE transdifferentiation.

At the protein level, we observed dynamic post-translational regulation of PEG10 isoforms upon AR inhibition. Concordant with the transcript level, DU145 and PC3 expressed high levels of RF1b and RF1b/2 compared to LNCaP, where RF1b especially was almost undetectable (Figure 3F, left panel: short exposure). However, upon AR inhibition of LNCaP with enzalutamide, there was a marked increase in CNF abundance and decrease in RF1b/2, suggesting accelerated proteolytic self-cleavage (Figure 3F, right panel: long exposure). This effect was reversed with R1881 treatment, together with an additional switch in proteolytic cleavage pattern from CNF to CNF2. The protein isoform expression pattern of LNCaP under AR inhibition mirrored the adenocarcinoma of LTL331 post-castration (rapid increase in CNF abundance), whereas the high expression of RF1/2 and RF1b in PC3 and DU145 was very similar to the terminal NEPC tumor in LTL331R (Figure 3G). The inability of LNCaP cells to achieve comparable levels of RF1/2 and RF1b may be related to its intact RB1 gene and further highlights the context dependency of PEG10 isoform expression.

TP53 loss has also been associated with NEPC (Chen et al., 2012), and analysis of microarray data from a previous study

demonstrated decreased *PEG10* expression upon introduction of wild-type *TP53* into PC3 cells (Spurgers et al., 2006). LTL331 harbored a single-copy loss of *TP53* and a functional C277G mutation in the remaining allele (Figure S3G), which resulted in accumulation of stabilized non-functional TP53 (Figure S3I).

Overall, these data demonstrate that PEG10 is dynamically regulated at distinctly different stages of NEPC development, and that PEG10 protein isoforms are associated with hallmarks of the malignant NEPC phenotype.

PEG10 Drives Cell Cycle Progression from G0/G1

To test the potential of PEG10 to be a therapeutic target in NEPC, we evaluated the effect of PEG10 knockdown on PCa cell growth. Both PEG10 transient knockdown using two independent small interfering RNAs (siRNAs) and stable knockdown using small hairpin RNA (shRNA) resulted in significant growth suppression in PC3 and DU145 cells (Figures 4A and S4A). We performed a bromodeoxyuridine (BrdU) incorporation assay after cell-cycle synchronization by double-thymidine block using PC3 cells with an intact RB1 pathway to evaluate the effect of PEG10 knockdown on cell-cycle progression (Figures 4B and S4B). We observed a significantly higher population of control cells entering S phase early after cell-cycle release, compared to PEG10 knocked-down cells, and most control cells had progressed to G2/M after 10 hr. Conversely, a significantly higher population of PEG10 knocked-down cells remained in G0/G1 or exhibited cell-cycle delay compared to controls. Concurrent protein expression analysis in the control cells showed that phospho-RB1 (pRB1) peaked early (5 hr) after cell-cycle release, and CCNE1, whose expression generally peaks at G0/G1 to S phase, decreased smoothly as cells progressed to G2/M. However, in PEG10 knocked-down cells, the pRB1 level was lower or peaked later than controls, and a significantly higher level of CCNE1 was expressed even 10 hr after cell-cycle release, reflecting slower cell-cycle progression and delay in exit from G0/G1 (Figure 4C). In parallel, we also observed a delay in exit from G0/G1 after PEG10 knockdown, when cells were treated with paclitaxel, which blocks cells from exiting G2/M to re-enter G0/G1 (Figure S4C). Interestingly, we noted that transient PEG10 knockdown induced significantly higher expression of key cell-cycle-dependent kinase inhibitors CDKN1A (p21) and CDKN1B (p27) than observed in controls. CDK2 expression was

Figure 2. PEG10 Is Upregulated during NE Transdifferentiation and Is Highly Expressed in Clinical NEPC

⁽A) Significantly upregulated genes in clinical NEPC compared to adenocarcinoma. The most significant genes are annotated with their predominant function, demonstrating the neuronal-like phenotype. VPC, Vancouver Prostate Centre.

⁽B) Intersection of NE transdifferentiation and clinical NEPC. This plot is the same as in (A), but genes are only plotted if upregulated by 12 weeks post-castration in the LTL331 model. Circle size is proportional to gene expression upregulation. *PEG10* is the most upregulated significant gene, and the plot is notable for the absence of expression of the majority of genes shown in (A).

⁽C) mRNA expression of PEG10 during NE transdifferentiation in the LTL331 system, highlighting the biphasic pattern.

⁽D) RNA-sequencing (RNA-seq)-derived expression of *PEG10* in primary prostate tumors, showing increased expression in NEPC tumors and in adenocarcinomas treated with neo-adjuvant hormone therapy (NHT).

⁽E) PEG10 protein expression in the LTL331 system. Cx, castration.

⁽F) PEG10 and CHGA back-to-back protein expression in clinical specimens showing high intensity of PEG10 expression in NEPC foci.

⁽G) Clinical progression of the LTL331 index patient to NEPC. The left panel shows serum PSA over time from the patient whose prostatectomy tissue led to the establishment of the LTL331 system. The right images show recent development of NEPC in this patient, who initially recurred with AR-driven disease but progressed to NEPC upon ADT, as predicted by the LTL331 model. See also Figure S1.



inversely correlated with CDKN1A and CDKN1B, instead mirroring the changes observed in pRB1 (Figure 4C). CDK4/6 expression remained unchanged (data not shown). This suggests that *PEG10* knockdown affects cell-cycle progression through upregulation of CDKN1A and CDKN1B.

Next, we evaluated the effects of forced PEG10 overexpression on cell growth. Given the potential for the different protein isoforms of PEG10 to possess distinct biological functions, we created three different constructs expressing: (1) RF1b only; (2) RF1b/2 with an active protease domain (capable of generating both CNFs); and (3) RF1b/2 with an inactive protease domain (note that constructs 2 and 3 cannot express RF1b because of modification of the frameshift sequence and skipping of the first STOP codon; see Figure S2). We transiently overexpressed each of these different PEG10 isoform constructs in DU145 cells. Since DU145 cells harbor mutations in TP53 and RB1, PEG10 overexpression in this cell line closely mimics the molecular landscape in clinical NEPC. In this context, we found that the protease mutant form of RF1b/2 significantly promotes growth. Growth was also promoted, but to a lesser extent, when protease active RF1b/2 (which expresses less RF1b/2 compared to protease mutant because of active selfcleavage) was expressed, indicating that growth is promoted primarily by the RF1b/2 isoform in a dose-dependent manner (Figure 4D). Overexpression of protease mutant RF1b/2 promoted transition from G0/G1 to S phase of the cell cycle, as evidenced by more cells entering S phase at an early time point after release from synchronization (Figure S4D). This construct also led to the downregulation of CDKN1B and upregulation of CCND1, further reflecting accelerated cell-cycle (Figure 4E). Growth promotion by RF1b/2 was also observed in 293T cells, which have inactivated TP53 and RB1 (Figure S4E). The isoform-specific effect of RF1b/2 on cell growth was also confirmed by a rescue experiment using siRNA-resistant overexpression plasmids, where different PEG10 isoforms were re-expressed in DU145 cells after PEG10 transient knockdown (Figure S4F). These observations are compatible with the fact that PEG10 inhibition also decreases the growth of LNCaP cells, which express RF1b/2 but not RF1b (Figure S4H). However, interestingly, when the RF1b/2 isoform was transiently overexpressed in LNCaP, which has wild-type TP53, there was no growth promotion; instead, TP53 expression was induced (Figures 4F and S4G), indicating that the effect of excess RF1b/2 is counterbalanced by wild-type TP53. Consistent with these data, when LNCaP cells were stably transfected with wild-type (full-length) PEG10, overexpression of PEG10 functioned as an oncogenic signal, inducing tumor suppressors. The cell-cycle-promoting effect of PEG10 that was characterized by increased pRB1 and CCND1 and decreased CDKN1B was matched by marked induction of CDKN2A (p14 and p16), TP53, and CDKN1A, resulting in decreased cell growth overall (Figure S4I). Furthermore, when PEG10 was stably overexpressed in LNCaP cells with TP53 knockdown, cell growth was increased (Figure S4J). Taken together, these data indicate that the PEG10 RF1b/2 isoform in particular, which can promptly regulate its expression by active self-cleavage, contributes to cell-cycle progression through downregulation of CDKN1B and that this, in turn, acts as a potent oncogenic signal that cannot be compensated by the induction of tumor suppressor expression in TP53- and RB1defective NEPC.

Finally, we examined whether knockdown of *PEG10* affects in vivo tumor growth. PC3 cells with stable knockdown of *PEG10* showed significantly reduced growth, compared to controls, when subcutaneously implanted in nude mice (Figure 4G). Consistent with PEG10's role in cell-cycle progression, there was a statistically significant reduction in Ki-67 score in *PEG10* knocked-down tumors (Figure 4H). Interestingly, although all mice in the control group harbored tumors (n = 10), only 50% (5/10) of those in the *PEG10* knocked-down group developed tumors, indicating the potential involvement of PEG10 in tumor cell invasion and establishment.

PEG10 Regulates Snail Expression via TGF- β Signaling and Promotes PCa Invasion

Clinical NEPC is characterized by high invasiveness and metastatic potential (Beltran et al., 2014). In four PCa datasets (Chandran et al., 2007; Grasso et al., 2012; Tomlins et al., 2007; Varambally et al., 2005), we found a significant association of *PEG10* mRNA upregulation with metastatic tumors compared to benign and primary tissue (Figure S5A). Given the role of *PEG10* in placental development (Ono et al., 2006), where invasion of maternal tissue is a fundamental step, we evaluated whether *PEG10* knockdown affects the invasive capability of PCa cells. Using a Matrigel chamber, we observed a significant

Figure 3. Dynamic Regulation of PEG10 Isoform Expression

(A) *PEG10* mRNA expression in LNCaP cells after androgen pathway inhibition or treatment with R1881, as measured by real-time qPCR (CSS, charcoal-stripped serum; Enza, enzalutamide). Values are normalized by β -actin (ACTB) and expressed as means \pm SD.

(D) Expression of E2F1 in NEPC compared to adenocarcinoma (Adeno).

(G) Expression of different PEG10 protein isoforms and E2F1 in the LTL331 model. Cx, castration.

See also Figures S2 and S3.

⁽B) *PEG10* promoter activity after AR pathway inhibition or treatment (treat) with R1881 as measured by luciferase assay (mean \pm SD). IgG, immunoglobulin G. (C) ChIP assay demonstrating AR occupancy at the *PEG10* promoter in LNCaP cells upon treatment with R1881 and reversal with enzalutamide treatment. The PSA promoter region (harbors a known AR-binding site) was used as a positive control. AR occupancy is expressed as percentage of input \pm SD. *p < 0.05. **p < 0.01.

⁽E) Heatmap showing the expression pattern of genes that constitute the *RB1* loss signature in the LTL331 model and other PCa specimens, showing upregulation of the signature only in NEPC tissue.

⁽F) Schematic of *PEG10* mRNA and protein domains (left) and immunoblots (right) demonstrating the expression of different PEG10 protein isoforms in PCa cell lines. 60 µg of protein was loaded for LNCaP, and 20 µg was loaded for DU145 and PC3 cells. Expression of different PEG10 isoforms upon treatment of LNCaP with enzalutamide or R1881 is also shown. Although RF1b/2 is expressed in LNCaP as shown in the right panel, the expression is significantly lower compared to DU145 and PC3 and, therefore, hardly visible on the left panel. Conversely, RF1b is not detectable in LNCaP, even with a very long exposure.



Figure 4. PEG10 Promotes Cell Proliferation in PCa Cell Lines

(A) Reduced cell growth after *PEG10* knockdown by siRNA in PC3 (left panel) and DU145 (right panel) cells, as measured by WST-8 assay. Values are normalized by absorbance on day 0 and expressed as mean ± SD. *p < 0.01. **p < 0.001. Inset immunoblots confirm target knockdown. siCtrl, control.
(B) Cell-cycle analysis using BrdU and 7AAD after *PEG10* knockdown, showing delay in cell-cycle progression. Cell cycle was initially synchronized (syn) at G0/G1 with double-thymidine block and then released and analyzed at the indicated time points after release. The bar represents the distribution of cell population in each phase of cell cycle.

decrease in cell invasion after *PEG10* knockdown in both PC3 and DU145 cells (Figures 5A and S5B). This was particularly interesting, since PEG10 has been reported to directly interact with multiple receptors of the transforming growth factor β (TGF- β) family (Lux et al., 2010), a family known to play an important role in PCa invasion and metastasis. Consequently, we used a growth-factor-reduced Matrigel chamber to demonstrate that PC3 cell invasion was significantly increased with TGF- β treatment but showed no change after *PEG10* knockdown (Figure 5B). We observed a similar trend in cell migration after TGF- β treatment, as measured by a scratch assay (Figure S5C).

These data suggested a potential for PEG10 to play a role in canonical TGF- β pathway activation. Indeed, after TGF- β treatment, we observed significantly more activation of the canonical TGF-β pathway in control cells compared to PEG10 knockdown, as evidenced by increased SMAD2 and SMAD3 phosphorylation and, in a separate assay, increased SBE-4 luciferase reporter activity (Figures S5D and S5E). Although TFG-β-mediated phosphorylation of SMAD2 and SMAD3 can be associated with decreased cell proliferation (Wilding et al., 1989), the net effect of overexpressing all PEG10 isoforms is increased cell proliferation, suggesting that any growth inhibitory effect is countered (Figure S5F). Of more relevance to the invasive NEPC phenotype, phosphorylation of SMAD2 and SMAD3 are also reported to induce expression of sentinel genes associated with invasion and motility, including members of the Snail, Zeb, and Twist families (Brandl et al., 2010; Lamouille et al., 2014; Smith et al., 2009). Interestingly, these families are typically associated with epithelial mesenchymal transition (EMT) (Peinado et al., 2007), and although the phenotype of NEPC is distinct from that of mesenchymal cells, our data raised the possibility of an overlap in terms of invasion mechanisms. In partial support of this hypothesis, we observed clear upregulation of SNAI1 and ZEB1 in LTL331R versus LTL331 and in clinical NEPC compared to adenocarcinoma; note that other typical EMT markers, including E-cadherin (CDH1) and vimentin (VIM), were unchanged (Figures 5C, S5G, and S5H). Furthermore, TGF- β treatment of PC3 cells induced significant mRNA and protein expression of SNAI1 and ZEB1 under control conditions but not after PEG10 knockdown (Figures 5D, 5E, and S5I). This differential between control and PEG10 knockdown after TGF-β treatment was also apparent after short-term treatment (<24 hr) using a higher dose of TGF- β (Figure S5J), but only at the protein level, suggesting that there exist multiple levels of association between PEG10, TGF- β , and SNAI1/ZEB1. We performed a rescue experiment using siRNA-resistant PEG10 isoforms to determine which isoform was responsible for promoting invasion. On the background of *PEG10* knockdown, only re-introduction of PEG10 RF1b isoform was sufficient to restore SNAI1 expression (Figure 5F) and TGF- β -induced invasion (Figures 5G and S5K). The association of PEG10 with SNAI1 and ZEB1 expression was also observed in the tumor tissue samples of mouse xenografts implanted with either control or *PEG10* knocked-down PC3 cells (Figure S5L).

DISCUSSION

This study identified the placental gene *PEG10* as a driver and potential therapeutic target for NEPC, a poorly understood and lethal disease. Importantly, PEG10 promotes the invasive and proliferative phenotype of NEPC cells, and its function is intimately linked to *RB1* and *TP53* loss, the genomic hallmarks of NEPC.

The development of novel therapeutic strategies for NEPC is complicated by the similarity of NEPC expression signatures to those of neuronal lineages as well as the logistical difficulties of studying clinical metastatic NEPC tissue. Previous studies focused on the terminal NEPC phenotype, where neuronal gene expression can overwhelm attempts to identify malignant drivers and inform little about earlier development. Our strategy leveraged a unique patient-derived xenograft model to investigate the development of NEPC, identifying gene expression programs associated with cell survival and dedifferentiation. Although "transient" programs of expression are likely to have mechanistic relevance, we focused on the "persistent" genes (those that were also highly expressed in terminal NEPC), for the sake of diagnosis and targeting but also for ease of modeling. PEG10 was the most upregulated gene during transdifferentiation, and typical NEPC markers were massively underrepresented in the persistent gene set, conferring implications for future work on biomarker development and early diagnosis of NEPC. Remarkable validation for our xenograft model can be drawn from the subsequent development of PEG10-positive NEPC in the original patient, whose initial adenocarcinoma prostatectomy specimen led to the model's establishment.

Among its human counterparts, *PEG10* is highly unusual, possessing two start codons and two reading frames controlled by a -1 ribosomal frameshift signal permitting "skipping" of a stop codon (Clark et al., 2007). This complexity is inherited from a retrotransposon ancestor, and although transposon ability was lost in the past 120 million years, PEG10 retains the ability

(H) PEG10 and Ki-67 staining of representative PC3 xenograft tumors. Values are expressed as mean \pm SEM. **p < 0.01.

See also Figure S4.

⁽C) Immunoblots showing changes in G0/G1-related genes after cell-cycle release in *PEG10* knocked-down cells. Cell lysates were collected at the indicated time points after cell-cycle release from G0/G1 synchronization.

⁽D) Immunoblots demonstrating transient overexpression of different PEG10 isoforms in DU145 cells (left) and the effect of each isoform on the growth of DU145 cells measured by WST-8 assay (right). Values are normalized by absorbance on day 0. Values are expressed as mean \pm SD. *p < 0.01. **p < 0.001. (E) Expression of cell-cycle-related genes in DU145. Robust expression of TP53 is due to mutated non-functional protein.

⁽F) Effect of transient overexpression of different PEG10 isoforms in LNCaP cells (left), showing that RF1b/2 has a negative effect on cell growth in this *RB1* and *TP53* wild-type cell line, as measured by WST-8 assay. Values are normalized by absorbance on day 0. *p < 0.05. **p < 0.01. The right panel shows that p53 expression is induced after transfection of RF1b/2 protease mutant, and other cell-cycle-related genes are unchanged. Values are expressed as mean \pm SD.

⁽G) Growth curves of subcutaneous xenograft tumors from PC3 subclones infected with control or PEG10 shRNAs. The numbers in parentheses indicate the number of animals developing tumors/total number of animals injected in each group. The values are expressed as mean ± SEM.



Figure 5. PEG10 Promotes PCa Cell Invasion

(A) Representative images (left) from an invasion assay after siRNA *PEG10* knockdown. The number of invading cells from four different microscopic fields is expressed as mean ± SD in the right panel. *p < 0.05 (compared to siCtrl [control]).

to self-cleave, in an apparently homologous manner, to HIV. PEG10 is required for placental development, and there is currently intense interest in the reactivation of placental and developmental genes in cancer, given the inherently "oncogenic" tissue invasion and immune evasion properties of the placenta (Rousseaux et al., 2013). Cancer cells under treatment stress must reprogram their transcriptome to express new biological properties and adapt to a different microenvrinoment, and the reactivation of placental genes offers an attractive solution. Interestingly, PEG10 is also maternally imprinted (paternally expressed). Imprinting is tightly associated with placental and embryonic genes and is only found in therian mammals; in fact, there are <50 known imprinted genes in humans, several of which are retrotransposon derived (Hamed et al., 2012; Morison et al., 2005). Paternally expressed genes (e.g., PEG10) tend to be associated with growth promotion, while maternally expressed genes tend to limit growth (Moore and Haig, 1991). As such, maternally imprinted placental genes have clear oncogenic characteristics. Indeed, among the 13,497 cancer samples with copy-number data on the cBioPortal for Cancer Genomics, there are only seven cases with PEG10 deletion.

PEG10 showed a distinct biphasic expression pattern during NEPC development, regulated by two distinct mechanisms and closely associated with two conceptual steps of NEPC phenotype acquisition: (1) adaptive response (to survive) and (2) cell proliferation. We report that PEG10 is AR repressed, but under the stress of AR inhibition, cancer cells must express survival genes in a reversible manner. Therefore, as PEG10 expression rapidly rises during AR inhibition, the pro-proliferative PEG10 RF1b/2 isoform is self-cleaved, presumably to slow cellcycle and metabolic requirement. PEG10 has an established anti-apoptotic role in hepatocellular carcinoma, and together, these data suggest that the cleavage products (CNF and the CNF2 reported here) may also play an active part in cell survival. Interestingly, the long terminal repeat (LTR) sequences of retrotransposons are reportedly stress responsive, and although the LTRs of PEG10 are not conserved, it is possible that some functional elements remain. Cancer cells surviving under treatment stress must inherit or gain beneficial genetic or epigenetic aberration to allow the resumption of proliferation. Recent evidence suggests RB1 and TP53 loss as important steps in this process, but their functional relevance to transdifferentiation was unclear. Our data suggest that these tumor suppressor pathways overlap during NEPC development, as PEG10 is directly regulated by E2F1 (which is overexpressed in NEPC) and can amplify the effect of E2F1 on cell cycle in tumors with RB1 and TP53 loss. Deregulation of PEG10 RF1b/2 induces TP53 (another sign of an oncogene), but on the background of *TP53* loss, it leads to downregulation of major cell-ycle-dependent kinase inhibitors CDKN1A and CDKN1B. Furthermore, aside from their well-documented function through pRB1, CDKN1A and CDKN1B can affect cell cycle directly by inhibiting E2F1 even in the context of *RB1* loss (Dimri et al., 1996).

PEG10 has been reported to interact directly with members of the TGF-β receptor family. In particular, co-expression of ALK1 and PEG10 (RF1a) in COS-1 and CHO-K1 cells resulted in cell spreading that resembled neuronal cell morphology (Lux et al., 2010). However, in PCa cell lines, we did not observe any difference in the activation of ALK1 signaling mediated by SMAD1/5/8 upon PEG10 knockdown (data not shown), nor did we observe typical neuronal-like cell morphology after PEG10 overexpression. Nevertheless, PEG10 expression in PCa cell lines affected canonical TGF-B signaling mediated by ALK5, SMAD2, and SMAD3 and resulted in differential expression of SNAI1, one of the direct downstream mediators of the canonical TGF- β signaling pathway. Though TGF- β is linked with EMT in PCa and other cancers (Akhurst and Hata, 2012; Lamouille et al., 2014; Shiota et al., 2012; Wu et al., 2014; Zavadil and Böttinger, 2005), typical EMT markers such as CDH1 and VIM were not affected by PEG10 knockdown. Concordantly, in xenograft tumors and human NEPC, ZEB1 and SNAI1 were upregulated in the absence of typical EMT markers. NEPC and EMT are fundamentally different in terms of cell morphology, but these data suggest a partial overlap in the genes mediating invasion and migration between the two phenotypes. Interestingly, we also demonstrate that high-dose (5 ng/ml) TGF- β treatment can induce SNAI1 expression at the protein level within 24 hr without changes in mRNA, suggesting that SNAI1 expression is also regulated by non-canonical TGF- β signaling through Rac, Rho, or other pathways, and that PEG10 has influence over these pathways as well.

PEG10 is a potent growth promoter controlled by imprinting in the placenta/embryo, silencing in adult tissue, and cleavage in treatment-stressed cancer cells. However, when left unchecked by deregulation to TP53 and RB1 pathways, PEG10 supports the progression of lethal NEPC. The unique genomic features of *PEG10*, together with its lack of expression in most adult tissues, its oncogenic characteristics, and its intimate relationship with aberrant cancer cells, make it a highly specific therapeutic target for NEPC.

EXPERIMENTAL PROCEDURES

For additional details, see the Supplemental Experimental Procedures.

See also Figure S5.

⁽B) Invasion assay showing effect of *PEG10* knockdown in PC3 cells upon treatment with TGF-β. Cells were pre-treated with or without 0.1 ng/ml TGF-β for 24 hr and then seeded in Growth Factor Reduced Matrigel invasion chambers.

⁽C) Microarray-derived expression of select master regulators of invasion during NEPC transdifferentiation in the LTL331 model.

⁽D) Expression of SNAI1, as measured by real-time qPCR, after treatment of control or *PEG10*-knocked-down PC3 cells with 0.1 ng/ml TGF- β for the indicated time. Values are normalized by *ACTB* and expressed as mean \pm SD. *p < 0.05.

⁽E) Protein expression of select master regulators of invasion associated with the TGF- β pathway.

⁽F) Immunoblots showing rescue of ZEB1 and SNAIL expression when *PEG10* knocked-down PC3 cells were treated with siPEG10-resistant PEG10-isoform-expressing plasmids. Cells were sequentially treated with siRNA- and *PEG10*-expressing plasmids.

⁽G) Invasion assay, as described in (B), conducted after the rescue experiment shown in (F). Number of invaded cells from four different microscopic fields are expressed as mean \pm SD. *p < 0.05.

Patient-Derived Xenografts and Clinical Datasets

Twelve LTL331 patient-derived xenografts were raised in non-obese diabetic (NOD) severe combined immunodeficiency (SCID) mice as previously described (Lin et al., 2014). After host castration, tissue was harvested, measured, fixed for histopathology, and processed for DNA/RNA analysis. Copy number and gene expression microarray profiling was performed using the Agilent SurePrint G3 Human CGH 8x60K platform and GE 8x60K Microarray respectively, as previously described (Lin et al., 2014). We used RNA-sequencing data from two clinical cohorts: Weill Medical College of Cornell University (Beltran et al., 2011) and the Vancouver Prostate Centre. For the latter, specimens were obtained following a protocol approved by the Clinical Research Ethics Board of the University of British Columbia and the BC Cancer Agency (all patients signed a consent form approved by the ethics board).

Cell Culture and Transfection

PCa cell lines (LNCaP, DU145, and PC3) were obtained from the American Type Culture Collection and maintained in RPMI 1640 (Thermo Scientific) supplied with 10% FBS. All cell lines were tested and found to be free of mycoplasma contamination. For transient loss-of-function studies, Silencer Select siRNAs (Table S3) were transfected using Lipofectamine RNAiMAX Reagent (Life Technologies) at a final concentration of 10 nM. Transient transfections of cells with *PEG10*-expressing plasmids were performed using Lipofectin (LNCaP) (Life Technologies) or DU145 Cell Avalanche (DU145, PC3) (EZ Biosystems) reagents according to the manufacturer's protocol. Transfection efficiency was monitored using the hrGFP II expression vector. *PEG10* stable knocked-down cells were established by transfecting cells with *PEG10* shRNA Lentiviral Particles (Santa Cruz Biotechnology, sc-152158-V) according to the manufacturer's protocol and were selected and maintained in 2 µg/ml puromy-cin-containing media. LNCaP cells with stable *TPS3* knockdown were provided by Dr. Xuesen Dong (Vancouver, BC, Canada).

Generation of PEG10 Expression Plasmids

Full-length PEG10 and PEG10 RF1b were amplified from PC3 cell line cDNA using primers listed in Table S3 and cloned into BAMHI and Xbal restriction sites of pcDNA3.1/hisA vector (Life Technologies). The PEG10 RF1b/2 vector, which does not express RF1b, was created by mutating the -1 frameshift sequence GGGAAAC to TGGCAAT, followed by the insertion of one base (C). These changes result in disruption to the frameshift mechanism and fixation of the reading frame to express only RF1/2, without changes in amino acid sequence (Figure S2). The PEG10 RF1b/2 protease mutant vector was constructed by site-directed mutagenesis using the QuikChange Lightening Kit (Agilent Technologies). For rescue experiments, PEG10 siRNA#1resistant PEG10 expression vectors were constructed for each of the aforementioned vectors by introducing silent mutations to the PEG10 siRNA#1 target sequence. Transfection of siRNA-resistant plasmids was performed 24 hr after treatment by the indicated siRNAs. For stable overexpression. full-length PEG10 was cloned into pLenti4/V5-DEST Gateway Vector (Life Technologies). Lentiviral stocks were produced in 293T cells. Virus-containing medium was collected at 48 hr post-transfection and filtered through a 0.45-mm filter. Lentiviral infection into LNCaP cells was performed by replacing the medium with medium containing the virus and 8 mg/ml Polybrene (Millipore), followed by centrifugation at 1,000 × g for 30 min. Stably infected cells were selected with Zeocin (200 mg/ml; Life Technologies). All plasmid constructs were verified by Sanger sequencing.

Antibodies and Immunoblot

Antibodies used for immunoblots are listed in Table S4. Protein-transferred polyvinylidene difluoride membranes were incubated with primary antibodies overnight at 4°C, and bands were detected either by chemiluminescence using ECL Western Blotting Detection (GE Healthcare) or by fluorescence using the Odyssey Imaging System (LI-COR Biosciences). In cases of low protein abundance, Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific) was used.

Growth Assay and Cell Cycle Analysis

Cell growth was assessed by WST-8 assay using Cell Counting Kit 8 (Dojindo Molecular Technologies) according to the manufacturer's protocol. For loss-

of-function studies using siRNA, cells were first transfected with siRNA in six-well plates, and then seeded into a 96-well plate at 1,000 cells per well 24 hr after transfection. Absorbance at 450 nm was measured at the indicated time points after seeding, and values were normalized by baseline (4 hr after seeding). For transient overexpression experiments, cells were seeded in 48-well plates, transfected, and measured for absorbance without re-seeding. All experiments were performed in at least hexaplicates and repeated more than three times. Cell-cycle distribution was analyzed by double staining with BrdU and 7AAD using the FITC BrdU Flow Kit (BD Biosciences) according to the manufacturer's protocol. For the double-thymidine block, 24 hr after transfection in six-well plates, cells were first treated with 2 mM thymidine for 12 hr. After washing three times with PBS, cells were released from the thymidine block by growth in normal media supplemented with 10% fetal bovine serum (FBS) for 14 hr and then blocked again with 2 mM thymidine for another 12 hr. Cells were finally released by adding back normal media after PBS washes, and 1 mM BrdU was added 30 min prior to harvest at each time point.

Cell Invasion and Migration Assays

Cell invasion was assessed using BD Biocoat Matrigel invasion chambers (BD Biosciences). In brief, 5 × 10⁴ cells were seeded in the upper chamber in a serum-deprived condition, and the lower chamber was supplemented with 20% FBS. 16–24 hr after seeding, the upper chamber was scrubbed with a cotton swab, fixed in methanol, and then stained with crystal violet. Invading cells were counted visually at four different microscopic fields and averaged. To examine the effect of TGF- β on cell invasion, cells were treated with or without 0.1 ng/ml TGF- β for 24 hr and then seeded in Growth Factor Reduced Bd Matrigel invasion chambers with or without TGF- β . For scratch assays, cells were seeded to sub-confluence and then similarly pre-treated with 0.1 ng/ml TGF- β for 24 hr prior to scratch. Next, cells were grown in a serum-deprived condition with or without TGF- β for 24–48 hr, and the degree of wound healing was assessed. 0.3 μ g/ml mitomycin C was also added after the scratch to suppress cell growth.

ACCESSION NUMBERS

Microarray data have been deposited to the NCBI GEO and are available under accession number GEO: GSE59986. Sequencing reads have been deposited to the European Nucleotide Archive and are available under accession number ENA: PRJEB9660.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.07.012.

AUTHOR CONTRIBUTIONS

S.A., A.W.W., Y.W., M.E.G., and C.C.C. conceived and designed the study. D.L., H.X., X.D., and Y.W. performed xenograft studies. A.W.W., A.H., and S.B. were involved in microarray and sequence data acquisition. A.W.W., K.W., F.M., R.B., H.T., and T.L.L. performed microarray and sequence data analysis. L.F., M.K., J.M.M., M.A.R., and H.B. performed immunohistochemistry work and provided patient samples. H.A. carried out proteomic analysis. S.A., S.L., F.Z., S.K., C.T., and Y.K. carried out in vitro experiments. A.Z. advised on experiments. S.A. and A.W.W. interpreted data and wrote the manuscript. All authors approved the final manuscript.

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REFERENCES

Akhurst, R.J., and Hata, A. (2012). Targeting the TGF β signalling pathway in disease. Nat. Rev. Drug Discov. *11*, 790–811.

Aparicio, A.M., Harzstark, A.L., Corn, P.G., Wen, S., Araujo, J.C., Tu, S.M., Pagliaro, L.C., Kim, J., Millikan, R.E., Ryan, C., et al. (2013). Platinum-based chemotherapy for variant castrate-resistant prostate cancer. Clin. Cancer Res. *19*, 3621–3630.

Attard, G., Cooper, C.S., and de Bono, J.S. (2009). Steroid hormone receptors in prostate cancer: a hard habit to break? Cancer Cell *16*, 458–462.

Beltran, H., Rickman, D.S., Park, K., Chae, S.S., Sboner, A., MacDonald, T.Y., Wang, Y., Sheikh, K.L., Terry, S., Tagawa, S.T., et al. (2011). Molecular characterization of neuroendocrine prostate cancer and identification of new drug targets. Cancer Discov. *1*, 487–495.

Beltran, H., Tagawa, S.T., Park, K., MacDonald, T., Milowsky, M.I., Mosquera, J.M., Rubin, M.A., and Nanus, D.M. (2012). Challenges in recognizing treatment-related neuroendocrine prostate cancer. J. Clin. Oncol. *30*, e386–e389.

Beltran, H., Tomlins, S., Aparicio, A., Arora, V., Rickman, D., Ayala, G., Huang, J., True, L., Gleave, M.E., Soule, H., et al. (2014). Aggressive variants of castration-resistant prostate cancer. Clin. Cancer Res. *20*, 2846–2850.

Brandl, M., Seidler, B., Haller, F., Adamski, J., Schmid, R.M., Saur, D., and Schneider, G. (2010). IKK(α) controls canonical TGF(β)-SMAD signaling to regulate genes expressing SNAIL and SLUG during EMT in panc1 cells. J. Cell Sci. *123*, 4231–4239.

Chandran, U.R., Ma, C., Dhir, R., Bisceglia, M., Lyons-Weiler, M., Liang, W., Michalopoulos, G., Becich, M., and Monzon, F.A. (2007). Gene expression profiles of prostate cancer reveal involvement of multiple molecular pathways in the metastatic process. BMC Cancer 7, 64.

Chen, H., Sun, Y., Wu, C., Magyar, C.E., Li, X., Cheng, L., Yao, J.L., Shen, S., Osunkoya, A.O., Liang, C., and Huang, J. (2012). Pathogenesis of prostatic small cell carcinoma involves the inactivation of the P53 pathway. Endocr. Relat. Cancer *19*, 321–331.

Cindolo, L., Cantile, M., Vacherot, F., Terry, S., and de la Taille, A. (2007). Neuroendocrine differentiation in prostate cancer: from lab to bedside. Urol. Int. 79, 287–296.

Clark, M.B., Jänicke, M., Gottesbühren, U., Kleffmann, T., Legge, M., Poole, E.S., and Tate, W.P. (2007). Mammalian gene PEG10 expresses two reading frames by high efficiency -1 frameshifting in embryonic-associated tissues. J. Biol. Chem. *282*, 37359–37369.

de Bono, J.S., Logothetis, C.J., Molina, A., Fizazi, K., North, S., Chu, L., Chi, K.N., Jones, R.J., Goodman, O.B., Jr., Saad, F., et al.; COU-AA-301 Investigators (2011). Abiraterone and increased survival in metastatic prostate cancer. N. Engl. J. Med. *364*, 1995–2005.

Dimri, G.P., Nakanishi, M., Desprez, P.Y., Smith, J.R., and Campisi, J. (1996). Inhibition of E2F activity by the cyclin-dependent protein kinase inhibitor p21 in cells expressing or lacking a functional retinoblastoma protein. Mol. Cell. Biol. *16*, 2987–2997.

Epstein, J.I., Amin, M.B., Beltran, H., Lotan, T.L., Mosquera, J.M., Reuter, V.E., Robinson, B.D., Troncoso, P., and Rubin, M.A. (2014). Proposed morphologic classification of prostate cancer with neuroendocrine differentiation. Am. J. Surg. Pathol. *38*, 756–767.

Ertel, A., Dean, J.L., Rui, H., Liu, C., Witkiewicz, A.K., Knudsen, K.E., and Knudsen, E.S. (2010). RB-pathway disruption in breast cancer: differential association with disease subtypes, disease-specific prognosis and therapeutic response. Cell Cycle *9*, 4153–4163.

Grasso, C.S., Wu, Y.M., Robinson, D.R., Cao, X., Dhanasekaran, S.M., Khan, A.P., Quist, M.J., Jing, X., Lonigro, R.J., Brenner, J.C., et al. (2012). The mutational landscape of lethal castration-resistant prostate cancer. Nature *487*, 239–243.

Guo, C.C., Dancer, J.Y., Wang, Y., Aparicio, A., Navone, N.M., Troncoso, P., and Czerniak, B.A. (2011). TMPRSS2-ERG gene fusion in small cell carcinoma of the prostate. Hum. Pathol. *42*, 11–17.

Hamed, M., Ismael, S., Paulsen, M., and Helms, V. (2012). Cellular functions of genetically imprinted genes in human and mouse as annotated in the gene ontology. PLoS ONE 7, e50285.

Haram, K.M., Peltier, H.J., Lu, B., Bhasin, M., Otu, H.H., Choy, B., Regan, M., Libermann, T.A., Latham, G.J., Sanda, M.G., and Arredouani, M.S. (2008). Gene expression profile of mouse prostate tumors reveals dysregulations in major biological processes and identifies potential murine targets for preclinical development of human prostate cancer therapy. Prostate *68*, 1517–1530.

Kastner, S., Voss, T., Keuerleber, S., Glöckel, C., Freissmuth, M., and Sommergruber, W. (2012). Expression of G protein-coupled receptor 19 in human lung cancer cells is triggered by entry into S-phase and supports G(2)-M cellcycle progression. Mol. Cancer Res. *10*, 1343–1358.

Kumar, A., White, T.A., MacKenzie, A.P., Clegg, N., Lee, C., Dumpit, R.F., Coleman, I., Ng, S.B., Salipante, S.J., Rieder, M.J., et al. (2011). Exome sequencing identifies a spectrum of mutation frequencies in advanced and lethal prostate cancers. Proc. Natl. Acad. Sci. USA *108*, 17087–17092.

Lamouille, S., Xu, J., and Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal transition. Nat. Rev. Mol. Cell Biol. *15*, 178–196.

Li, X., Placencio, V., Iturregui, J.M., Uwamariya, C., Sharif-Afshar, A.R., Koyama, T., Hayward, S.W., and Bhowmick, N.A. (2008). Prostate tumor progression is mediated by a paracrine TGF-beta/Wnt3a signaling axis. Oncogene *27*, 7118–7130.

Lin, D., Wyatt, A.W., Xue, H., Wang, Y., Dong, X., Haegert, A., Wu, R., Brahmbhatt, S., Mo, F., Jong, L., et al. (2014). High fidelity patient-derived xenografts for accelerating prostate cancer discovery and drug development. Cancer Res. 74, 1272–1283.

Lotan, T.L., Gupta, N.S., Wang, W., Toubaji, A., Haffner, M.C., Chaux, A., Hicks, J.L., Meeker, A.K., Bieberich, C.J., De Marzo, A.M., et al. (2011). ERG gene rearrangements are common in prostatic small cell carcinomas. Mod. Pathol. *24*, 820–828.

Lux, H., Flammann, H., Hafner, M., and Lux, A. (2010). Genetic and molecular analyses of PEG10 reveal new aspects of genomic organization, transcription and translation. PLoS ONE 5, e8686.

Moore, T., and Haig, D. (1991). Genomic imprinting in mammalian development: a parental tug-of-war. Trends Genet. 7, 45–49.

Morison, I.M., Ramsay, J.P., and Spencer, H.G. (2005). A census of mammalian imprinting. Trends Genet. *21*, 457–465.

Nelson, E.C., Cambio, A.J., Yang, J.C., Ok, J.H., Lara, P.N., Jr., and Evans, C.P. (2007). Clinical implications of neuroendocrine differentiation in prostate cancer. Prostate Cancer Prostatic Dis. *10*, 6–14.

Ono, R., Kobayashi, S., Wagatsuma, H., Aisaka, K., Kohda, T., Kaneko-Ishino, T., and Ishino, F. (2001). A retrotransposon-derived gene, PEG10, is a novel imprinted gene located on human chromosome 7q21. Genomics 73, 232–237.

Ono, R., Nakamura, K., Inoue, K., Naruse, M., Usami, T., Wakisaka-Saito, N., Hino, T., Suzuki-Migishima, R., Ogonuki, N., Miki, H., et al. (2006). Deletion of Peg10, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality. Nat. Genet. *38*, 101–106.

Peinado, H., Olmeda, D., and Cano, A. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat. Rev. Cancer 7, 415–428.

Pezaro, C.J., Omlin, A., Lorente, D., Nava Rodrigues, D., Ferraldeschi, R., Bianchini, D., Mukherji, D., Riisnaes, R., Altavilla, A., Crespo, M., et al. (2014). Visceral disease in castration-resistant prostate cancer. Eur. Urol. *65*, 270–273.

Rousseaux, S., Debernardi, A., Jacquiau, B., Vitte, A.L., Vesin, A., Nagy-Mignotte, H., Moro-Sibilot, D., Brichon, P.Y., Lantuejoul, S., Hainaut, P., et al. (2013). Ectopic activation of germline and placental genes identifies aggressive metastasis-prone lung cancers. Sci. Transl. Med. *5*, 186ra66.

Scher, H.I., Fizazi, K., Saad, F., Taplin, M.E., Sternberg, C.N., Miller, K., de Wit, R., Mulders, P., Chi, K.N., Shore, N.D., et al.; AFFIRM Investigators (2012). Increased survival with enzalutamide in prostate cancer after chemotherapy. N. Engl. J. Med. *367*, 1187–1197.

Shiota, M., Zardan, A., Takeuchi, A., Kumano, M., Beraldi, E., Naito, S., Zoubeidi, A., and Gleave, M.E. (2012). Clusterin mediates $TGF-\beta$ -induced epithelial-mesenchymal transition and metastasis via Twist1 in prostate cancer cells. Cancer Res. 72, 5261–5272.

Smith, A.P., Verrecchia, A., Fagà, G., Doni, M., Perna, D., Martinato, F., Guccione, E., and Amati, B. (2009). A positive role for Myc in TGFbeta-induced Snail transcription and epithelial-to-mesenchymal transition. Oncogene *28*, 422–430.

Spurgers, K.B., Gold, D.L., Coombes, K.R., Bohnenstiehl, N.L., Mullins, B., Meyn, R.E., Logothetis, C.J., and McDonnell, T.J. (2006). Identification of cell cycle regulatory genes as principal targets of p53-mediated transcriptional repression. J. Biol. Chem. *281*, 25134–25142.

Sun, Y., Campisi, J., Higano, C., Beer, T.M., Porter, P., Coleman, I., True, L., and Nelson, P.S. (2012). Treatment-induced damage to the tumor microenvironment promotes prostate cancer therapy resistance through WNT16B. Nat. Med. *18*, 1359–1368.

Takeuchi, T., Tomida, S., Yatabe, Y., Kosaka, T., Osada, H., Yanagisawa, K., Mitsudomi, T., and Takahashi, T. (2006). Expression profile-defined classification of lung adenocarcinoma shows close relationship with underlying major genetic changes and clinicopathologic behaviors. J. Clin. Oncol. *24*, 1679– 1688.

Tan, H.L., Sood, A., Rahimi, H.A., Wang, W., Gupta, N., Hicks, J., Mosier, S., Gocke, C.D., Epstein, J.I., Netto, G.J., et al. (2014). Rb loss is characteristic of prostatic small cell neuroendocrine carcinoma. Clin. Cancer Res. *20*, 890–903.

Terry, S., and Beltran, H. (2014). The many faces of neuroendocrine differentiation in prostate cancer progression. Front. Oncol. *4*, 60. Tomlins, S.A., Mehra, R., Rhodes, D.R., Cao, X., Wang, L., Dhanasekaran, S.M., Kalyana-Sundaram, S., Wei, J.T., Rubin, M.A., Pienta, K.J., et al. (2007). Integrative molecular concept modeling of prostate cancer progression. Nat. Genet. *39*, 41–51.

Varambally, S., Yu, J., Laxman, B., Rhodes, D.R., Mehra, R., Tomlins, S.A., Shah, R.B., Chandran, U., Monzon, F.A., Becich, M.J., et al. (2005). Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. Cancer Cell 8, 393–406.

Wang, C., Xiao, Y., Hu, Z., Chen, Y., Liu, N., and Hu, G. (2008). PEG10 directly regulated by E2Fs might have a role in the development of hepatocellular carcinoma. FEBS Lett. *582*, 2793–2798.

Wilding, G., Zugmeier, G., Knabbe, C., Flanders, K., and Gelmann, E. (1989). Differential effects of transforming growth factor beta on human prostate cancer cells in vitro. Mol. Cell. Endocrinol. *62*, 79–87.

Williamson, S.R., Zhang, S., Yao, J.L., Huang, J., Lopez-Beltran, A., Shen, S., Osunkoya, A.O., MacLennan, G.T., Montironi, R., and Cheng, L. (2011). ERG-TMPRSS2 rearrangement is shared by concurrent prostatic adenocarcinoma and prostatic small cell carcinoma and absent in small cell carcinoma of the urinary bladder: evidence supporting monoclonal origin. Mod. Pathol. *24*, 1120–1127.

Wu, Y., Fu, Y., Zheng, L., Lin, G., Ma, J., Lou, J., Zhu, H., He, Q., and Yang, B. (2014). Nutlin-3 inhibits epithelial-mesenchymal transition by interfering with canonical transforming growth factor- β 1-Smad-Snail/Slug axis. Cancer Lett. *342*, 82–91.

Wyatt, A.W., Mo, F., Wang, K., McConeghy, B., Brahmbhatt, S., Jong, L., Mitchell, D.M., Johnston, R.L., Haegert, A., Li, E., et al. (2014). Heterogeneity in the inter-tumor transcriptome of high risk prostate cancer. Genome Biol. *15*, 426.

Yu, J., Yu, J., Mani, R.S., Cao, Q., Brenner, C.J., Cao, X., Wang, X., Wu, L., Li, J., Hu, M., et al. (2010). An integrated network of androgen receptor, polycomb, and TMPRSS2-ERG gene fusions in prostate cancer progression. Cancer Cell *17*, 443–454.

Zavadil, J., and Böttinger, E.P. (2005). TGF-beta and epithelial-to-mesenchymal transitions. Oncogene 24, 5764–5774.