Elucidation of AR Impact on the Paternally Expressed Gene 10 (PEG10) in Enzalutamide-resistant Prostate Cancer

by

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Abstract

Despite advances in therapeutics, castration resistant prostate cancer (CRPC) continues to be a major problem due to ongoing androgen receptor (AR) activity driving disease progression. This lack of control of AR inhibition in drug resistant CRPC may reflect a shift in the natural history of classically "AR-driven" disease to a "AR non-driven" phenotype, characterized by low circulating levels of prostate specific antigen (PSA) despite high metastatic burden in soft tissues. This hypothesis is supported by recent data from the Stand up to Cancer-Prostate Cancer Foundation East Coast and West Coast Dream Team (SU2C) indicating up to 27% of CRPC patients resistant to AR pathway inhibitors, including ENZ or ABI, develop an AR non-driven disease. Notably, our pre-clinical model of ENZ-R also reflects the clinical distribution of disease heterogeneity. We have shown that while 75% of ENZ-R tumors recur *in vivo* with AR re-activation and rising PSA, 25% of tumors show downregulation of canonical AR target genes and maintain an AR non-driven phenotype. Analysis of ENZ-R cells using RNAseq revealed that ENZ-R cells with downregulation of canonical pathway and NE phenotype up-regulate the paternally expressed gene 10 (PEG10). PEG10 is a retrotransposon-derived gene that was recently reported to be upregulated in neuroendocrine prostate cancer (NEPC) and regulates cell survival and metastasis in NEPC. However the underlying mechanisms by which PEG10 is regulated in ENZ-R and NEPC remain unexplored.

We found that AR negatively regulates PEG10 and that activated AR directly binds to the PEG10 promoter and suppresses its expression while ENZ inhibits AR activity which allows for the NE differentiation to commence in CRPC and ENZ-R cell lines. In the next step, we investigated whether PEG10 is a potential target of NEPC progression in CRPC and ENZ-R cell

lines. Through our experiments, we demonstrated that targeting PEG10 reduces cell proliferation *in vitro*, while knocking down PEG10 *in vivo* attenuates tumor growth. The results justify PEG10 as potential therapeutic target for NEPC.

Preface

The publications in this thesis are based on the work that I performed towards the completion of my Master's program. The manuscript present in this thesis will be submitted for publication as co-authored work.

A version of Chapter 2 of this thesis will be submitted for a publication in the *Endocrine-related Cancer* as noted below:

Chapter 2: PEG10 is an Androgen Receptor-regulated Gene Involved in Prostate Cancer Neuroendocrine Differentiation After AR-targeted Therapies

Soojin Kim, Samir Bidnur, Daksh Thaper, Jared Allman, Ka Mun Nip, Paul Toren, Shusuke Akamatsu, Jenna L. Bishop, Colin Colllins, Amina Zoubeidi. *Endocrine-related Cancer*, 2016

Dr. Zoubeidi was the principal investigator for the manuscript. All the *in vitro* and *in vivo* experiments performed in this chapter was designed, performed, and analyzed by myself and reviewed by Dr. Zoubeidi. Dr. Paul Toren provided help with the initial western blot analysis and Mr. Jared Allman provided help with *in vivo* studies. Dr. Jennifer Bishop and Mr. Daksh Thaper reviewed the data and provided helpful comments. The manuscript was edited by Dr. Samir Bidnur and reviewed by Dr. Zoubeidi.

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List of Abbreviations

ADT	Androgen deprivation therapy
AF-1/2	Activation function domain
AR	Androgen receptor
AURKA	Aurora kinase A
CHGA	Chromogranin A
CNF	Cleaved N-terminus fragment
CRPC	Castration-resistant prostate cancer
CTD	C-terminal binding domain
DBD	DNA-binding domain
DHEA	Dehydroepiandrosterone
DHT	5α-dihydrostestosterone
DRE	Digital rectal exam
EBRT	External beam radiation therapy
EMT	Epithelial-to-mesenchymal transition
ENZ	Enzalutamide
ENZ-R	Enzalutamide-resistant
FSH	Follicle-stimulating hormone
GM-CSF	Granulocyte-macrophage colony stimulating factor
GnRH	Gonadotropin-releasing hormone
LBD	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
NCAM1	Neural cell adhesion molecule 1
NE	Neuroendocrine
NEPC	Neuroendocrine prostate cancer
NES	Nuclear export signal
NLS	Nuclear localization signal
NSE	Neuron-specific enolase

NTD	NH2-terminal domain
PAP	Prostatic acid phosphatase
PCa	Prostate cancer
PCA3	Prostate cancer antigen
PEG10	Paternally expressed gene 10
PSA	Prostate-specific antigen
PSMA	Prostate membrane-specific antigen
R1881	Metribolone
RF	Reading frame
SRRM4	Serine/arginine repetitive matrix 4
SSC	Small-cell carcinoma
stAR	Steroidogenic acute regulatory protein
siRNA	Small interfering RNA
SYP	Synaptophysin
TMPRSS2	Transmembrane protease, serine 2
TNM	Tumor, nodes, metastasis
TRUS	Transrectal ultrasound
UGS	Urogenital sinus
μM	Micro molar
nM	Nano molar

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To my family, Sookyi and Elyas

CHAPTER 1: Introduction

1.1. The Prostate

1.1.1. Anatomy and Development

The male accessory glands include the prostate gland, bulbourethral glands, and the seminal vesicles [1]. Among them, the prostate gland is the largest gland and is situated below the bladder and in front of the rectum while it surrounds the urethra in the pelvic cavity. During embryonic development, the prostate gland is developed through a mesenchymal-epithelial interaction [2]. Initially, the testis differentiates with the influence of the Y chromosome [2]. At 10-13 weeks, the prostate develops from the urogenital sinus (UGS), a midline structure with an endodermally derived epithelial layer surrounded by a mesodermally derived mesenchymal layer. Circulating fetal testosterones interact with androgen receptors (AR) on mesenchymal cells and influence the epithelial cells to form the primordial prostate buds [1, 3]. The buds undergo proliferation, cell adhesion, and cell migration to coordinate the outgrowth of the prostate, prostatic urethral, and bulbourethral glands [2]. The buds then signal back to the overlying epithelium to stimulate the formation of prostate ductal progenitor [2]. At maturity, growth of the gland ceases but androgens such as testosterone continue to maintain the prostate function [4].

1.1.2. Histology

Mature prostate gland consists of 70% glandular components and 30% fibromuscular stroma [5]. The glands branch off and make up the long tubuloalveolar glands that radiate from the urethra [1]. The glands are embedded into the fibromuscular stroma which consists of smooth muscle cells, fibroblast, and endothelial cells [1, 6]. There are two main types of cell layers lining the glands: an outer basal layer of cuboidal epithelial cells and the secretory inner layer composed of tall columnar epithelium which changes to a transitional epithelium near the openings of the ducts at the urethra [4, 7]. The secretory columnar cells project into the lumen of the gland as papillary projections and contribute to the irregular shape of the alveolar branch [8]. The secretory columnar cells express androgen receptor (AR), prostatic acid phosphatase

(PAP), and prostate-specific antigen (PSA), and are dependent on the regulation of AR by ligands to maintain their secretory activity [9, 10].

The basal cuboidal epithelial cells on the other hand are independent of AR. Because they have stem cell characteristics, studies have suggested that they may be the reservoir of progenitors that differentiate into luminal cells [2, 11].

The third type of cells is the neuroendocrine (NE) cells. These cells are distributed irregularly throughout the basal and luminal layers of the prostate and secrete neuropeptides including bombesin, calcitonin, serotonin, as well as growth factors such as VEGF [12, 13, 14]These cells are believed to maintain homeostasis of the surrounding epithelial cells and confer a role in growth and differentiation of the gland [14, 15]. Unlike the columnar cells they do not express AR or PSA [16]. NE cells are more commonly present in the prostate gland than in any other genitourinary organs [17]. The NE cells' characteristics and functions are still largely unknown, and current studies are underway to decipher them.

The human prostate gland can be further divided into three concentric zones: the peripheral zone, transition zone, and the central zone [18]. The peripheral zone is located closest to the rectum and contains large glands whose ducts open into the urethra [18]. This zone is the site responsible for 70% of adenocarcinoma occurrence. The transition zone is located between the peripheral and the central zone, and comprises of submucosal glands, and gives rise to 20% of the cancers [18]. The central zone is relatively resistant to carcinoma and consists of the mucosal glands [18].

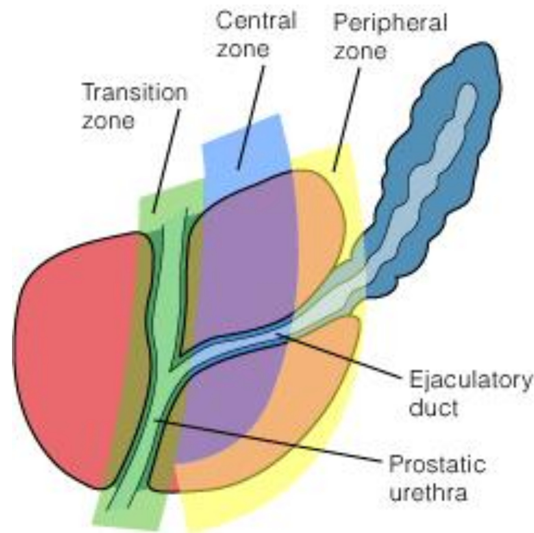


Figure 1. 1 General structure of the prostate gland
Diagram of the prostate showing the central zone (CZ), anterior fibromuscular stroma (FM), transition zone (TZ), and posterior zone (PZ). The peripheral zone is located closest to the rectum and responsible for 70% of adenocarcinoma occurrence. The transition zone is located between the peripheral and the central zone.

1.1.3. Function

The prostate plays a major role in the male sexual reproduction as both exocrine gland and endocrine gland [19]. As an exocrine gland, it secretes fluid that makes up 20-30% of the semen which facilitates the coagulation-liquefaction sequence of the fertilization process [20, 21, 22]. The seminal fluid contains coagulation factors to prevent out-flow of the sperms from the vagina and ensure protective delivery to the egg [21]. About 15-20 minutes later, the proteolytic enzymes and a-amylase from the prostatic fluid liquefies the coagulation, thereby liberating the sperms to freely fertilize the egg [21]. Furthermore, the prostatic fluid is alkaline and neutralizes the acidic environment of the vagina and protects the sperms from degradation [21]. The prostatic fluid also contains nutrients to maintain sperm life. As the female egg is surrounded by an outer layer of cumulus cells embedded in the matrix rich in hyaluronic acid, the citric acid from the prostatic fluid plays a major role in hyaluronidase activity, to penetrate the cumulus cell layer surrounding the egg [22, 23]. The zinc ion present in the prostatic fluid has an antibacterial effect, which allows the safeguarding of the sperms against bacteria [22]. Physiologically, the muscles of the prostate gland contracts to ensure that the semen is

expelled outwards during ejaculation, and at the same time controls the urine output. As an endocrine gland, the prostate converts the male androgen, testosterone (T), to a more potent form dihydrotestosterone (DHT) [21].

1.2. Androgens

1.2.1. Production, Functions, and Metabolism

Androgens are steroid hormones composed of 19 carbons that control the development and maintenance of the male reproductive function [24]. The principal androgenic steroid, testosterone, is synthesized from cholesterol through a series of enzymatic steps in the Leydig cells of the testis. Weaker androgens such as dehydroepiandrosterone (DHEA) and androstenediones are also produced by the zona reticularis of the adrenal cortex and ovaries [25].

Approximately at 8 weeks of gestation during embryonic development, fetal testis differentiates and fetal testosterone formation is initiated [25]. The fetal testosterone plays a major role in the development of the Wolffian ducts which give rise to the development of epididymis and seminal vesicles, and also plays an important role in the development of the prostate and the penis [25]. During the second trimester, the level of fetal testosterone specifies gender formation where it can be used as a predictor for the feminine or masculine behaviors of the individual after birth [26].

During puberty, androgens produced by the Leydig cells of the testes and adrenal glands play a major role in the development of secondary sexual characteristics in male, such as penis enlargement, growth of chest and facial hair, increased muscle strength and mass, deepening of voice, growth of spermatogenic tissue, and prostate development [25]. Androgens are also important in maintaining the male sexual characteristics throughout life [24].

Androgen synthesis and regulation is closely controlled by the hypothalamus-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) is released by the hypothalamus which then stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) at the anterior pituitary gland [27, 28]. These hormones circulate in the blood stream and bind to the

target Leydig cells of the testes, and increase the expression of steroidogenic acute regulatory protein (stAR) [28, 29]. This protein promotes the transfer of cholesterol to the inner mitochondrial membrane to initiate steroidogenesis where cholesterol is converted to pregnenolone [29]. The pregnenolone is then converted to DHEA through a lysation process, and with the precursors androstenediol and androstenedione, DHEA is converted to testosterone [28]. Once at the intracellular prostate gland, testosterone is converted into a more potent 5α-dihydrotestosterone (DHT) by the action of 5α-reductase, which then promotes the growth and survival of prostate cells [28, 29].

DHT is a more potent form of androgen than testosterone and binds to the androgen receptor with five-fold higher affinity [30]. The metabolite of testosterone, estradiol, inhibits further secretion of GnRH and FSH/LH through a negative feedback loop to regulate the amount [30].

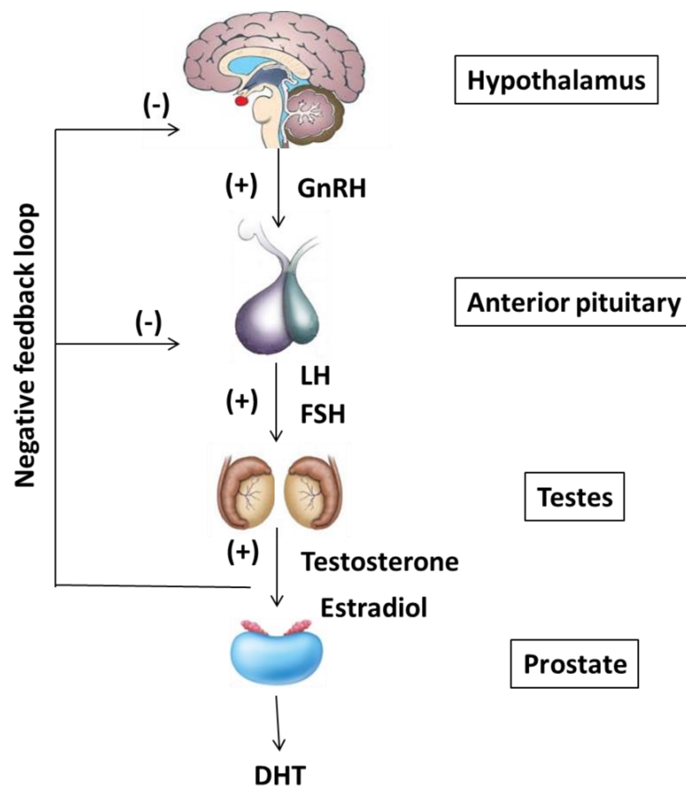


Figure 1. 2 Hypothalamus-pituitary-gonadal hormonal axis
Androgen production is regulated through the secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus as well as luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland. The amount of androgen production is also regulated by a negative feedback loop.

1.3. Androgen Receptor

1.3.1. Biology, Structure, and Function

Androgen Receptor (AR) (NR3C4, nuclear receptor subfamily 3, group C, gene 4) is a ligand-dependent transcription factor that controls the expression and maintenance of male sexual characteristics [31]. It belongs to the steroid hormone nuclear receptor family which include glucocorticoid receptor, progesterone and estrogen receptors [32].

The human AR gene has been mapped on the X-chromosome at the locus Xq11-q12 and contains eight exons spanning 2757 nucleotides [32]. The exons code for a 110kDa protein consisting of three important functional domains which include: a NH2-terminal domain (NTD) (residues 1-555) coded by exon 1, a DNA binding domain (DBD) (residues 555-623) coded by exons 2 and 3, and a hinge (residues 623-665) containing ligand binding domain (LBD) (residues 665-919) located at the C- terminal domain (CTD) coded by exons 4 to 8. Total of 919 amino acids make up the AR protein [33]. The **NTD** of AR is the main transcriptional regulatory region where it interacts with the LBD (N/C interaction) upon ligand binding [31, 33]. It has been shown that NTD of AR can also be activated by protein kinase pathways even in the absence of androgenic ligand binding [33]. Generally, NTD contains repeats of polyglutamine (CAG; poly-Q) regions that affect the folding structure of NTD which in turn impact the dependency of AR transcriptional activity on the repeat length [33, 34]. The shorter repeats impose higher transactivation activity, whereas longer repeats cause reduced activity. The deleterious mutation of the poly-Q tract causes a four-fold increase in AR activation function compared to the wild-type AR [33]. The sequence and length of the poly-Q repeats in AR are highly variable in the human population. This gives the structural plasticity of the NTD that allows interactions with many androgen responsive genes, such as Prostate specific antigen (PSA), Transmembrane protease, serine 2 (TMPRSS2), and NKX3.1 [33, 34]. Apart from the poly-Q regions, NTD also holds the AF-1 region which contains transcription activation units that are required for full activity of the AR [33].

The **DBD** is composed of two zinc fingers each consisting of four cysteine residues characteristic of steroid hormone receptors [31]. The specific recognition and binding to DNA response

element is first mediated by the proximal box (glycine-serine-valine; P-box) located on one of the zinc fingers, which recognizes the specific hexameric half-site on the AREs [35]. While the specificity is achieved by the P-box on one of the zinc fingers, the selectivity of AREs by the AR is achieved by the second zinc finger which contains the Distal box (D-box) that allows AR receptor dimerization in a "head-to-head" manner [31]. This allows the AR to bind to the half-sites of promoter region on the specific AREs.

The inactive form of AR predominantly resides in the cytoplasm, and requires active transportation through the nuclear pore [36]. Upon binding to its native ligands DHT and testosterone, the large 110kDa AR undergoes conformation change [31, 36]. The nuclear localization signal (NLS) harbored between the DBD and the hinge region is responsible for the import of the receptor; and upon ligand binding to the AR, NLS is exposed and importin-α is bound to the NLS allowing the conformational change of the AR for import [37, 38]. Recently, the hinge region was not only found to harbor the NLS, but it has been shown to play a role in DNA binding, coactivator recruitment, and N/C interaction [39].

The **LBD** plays an important role in the recognition of androgenic ligands and mediates disassociation from AR's large hetero-complex state. The hetero-complex consists of chaperone and heat-shock proteins that hold the receptor in a ligand binding competent state [40]. AR LBD consists of 11 α-helices and two small β-sheets in an antiparallel helical fold [40]. This folding forms a hydrophobic pocket where the ligand can bind. Four N-termini α-helices surround the pocket to form the activation function 2 domain (AF-2) that acts as a lid to hold the androgen upon binding [31, 40]. Apart from playing a role as a secure holder for the androgen, AF-2 also mediates N/C interaction as well as regulation of co-regulator binding site. Upon androgen binding, AR dissociates from the hetero-complex, dimerizes and moves to the nucleus [31]. Apart from mediating AR nuclear localization, LBD also plays an important role in the regulation of AR nuclear export [31]. Once the androgen is withdrawn, AR is exported to the cytoplasm. The nuclear export signal (NES) present in the vicinity of the LBD senses the withdrawal and facilitates to complete the AR shuttling [41, 31].

The AR is able to accommodate both physiological and synthetic ligands by modifying the LBD conformation. It has been shown that the potent synthetic androgen R1881 (metribolone) binds to the LBD and causes similar agonist conformation change as the DHT and testosterone [31].

AR and androgens play a critical role in expression of the male physiology, and mutation in the AR gene can hinder the normal development and function of male structures. Current discoveries of the AR gene mutations include: 1) single point mutations resulting in premature stop codons, 2) partial or complete deletion of the AR gene, 3) frameshift mutation resulting from nucleotide insertions or deletions, and 4) mutations in the introns within the AR gene that result in AR splice variant [42]. These mutations have been shown to contribute to alteration in AR functions that give rise to diseases such as prostate cancer in males [30].

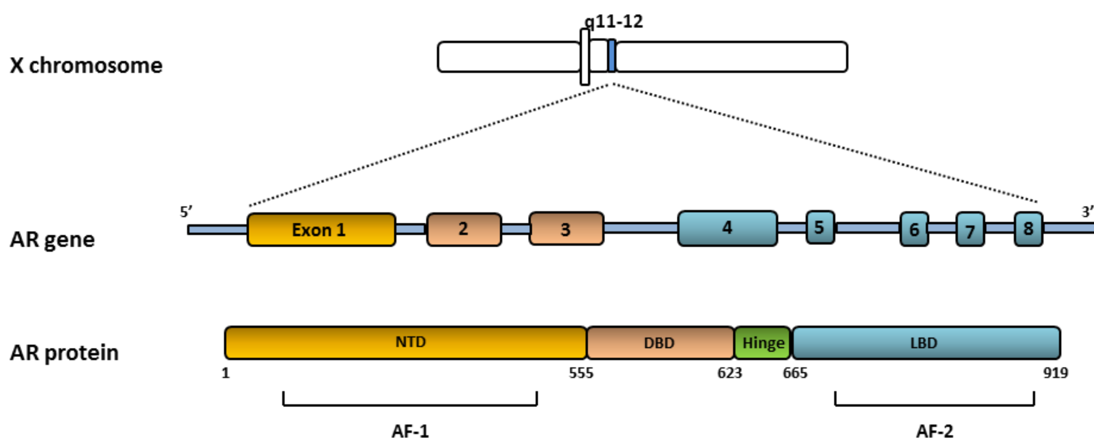


Figure 1. 3 Schematic diagram of the androgen receptor (AR) gene and protein with indication of its specific motifs and domains

The AR gene at Xq11-12 contains eight exons that code for a 110kDa protein consisting of three domains. NH2-terminal domain (NTD) (residues 1-555) is coded by exon 1 and contains AF-1, DNA binding domain (DBD) (residues 555-623) is coded by exons 2 and 3, and a hinge containing the ligand binding domain (LBD) (residues 665-919) is coded by exons 4 to 8. The LBD also contains AF-2.

1.4 Prostate Cancer

1.4.1 Epidemiology and Risk Factors

Prostate cancer (PCa) is the second leading cause of cancer-related mortality and most common non-cutaneous cancer in North-American men after lung cancer, affecting one in six men [43]. In 2012, there were an estimated 2,795,592 men living with prostate cancer in North America. In 2015 an estimate of 220,800 prostate cancers will be diagnosed, resulting in approximately 27,540 deaths among Canadian men [43]. The well-established risk factors of PCa development has been shown to be affected by: 1) age, 2) race/ethnicity, and 3) family history.

Age is the leading risk factor associated with PCa development and progression. According to the statistical studies conducted in 2012, approximately 20% of PCa-related death occurred in the age groups of 65 to 74, 37% of death in 75-84 years old group, and 33% in the 85 years and older group. Studies on PCa autopsies have also revealed that about 56% of PCa are diagnosed in men of 65-75 age, and 97% in men 50 years and older [44].

Race/ethnicity is an important factor, where the incidence rates are about 60% higher in men of African ancestry than Caucasians [45]. The PCa incidence and associated- mortality rate has been shown to be lower in most Asian men, but the rate has been reported to be increasing [45].

Genetic studies reveal that **familial predisposition** also contributes to the increased risk of developing PCa. It has been reported that men with first relative with PCa history has a 2.41 fold higher risk of PCa development than men without [46]. Men whose paternal history involved PCa have about 15% higher risk than the control population [46]. Various genes have been reported to contribute to PCa susceptibility, and include AR, KLK3, CYP18A1, BRCA2, SRD5A2 [46, 47].

Apart from these factors, studies have also suggested that a diet high in dairy product and processed meat may increase the risk of PCa [31].

1.4.2 Development of Prostate Cancer

Prostate cancer is thought to arise as a result of disrupted cellular homeostasis in the prostate gland. It is also recognized as a histologically heterogeneous disease due to its multifocal nature consisting of one or more separate tumors in a single prostate [47]. As a result of accumulation of molecular abnormalities which in turn cause genomic instabilities, abnormal proliferation of the secretory luminal cells is triggered. Increased luminal cells contribute to bulk of tumor mass, with progressive destruction of basement membrane and basal cells [47]. 95% of all prostate cancers arise from the luminal cells in the gland, termed adenocarcinomas, and occur more commonly in the peripheral zone of the prostate. Recent studies have indicated that origin of PCa can also be from the basal cuboidal cell population which have stem cell characteristics [48, 49]. Other types of cancers that arise in the prostate gland include sarcomas, small cell carcinomas, and neuroendocrine tumors [48].

1.4.3 Diagnosis

Common detection methods for PCa in North America include digital rectal exam (DRE), PSA blood test screening, and transrectal ultrasound (TRUS).

Prostate-specific antigen (PSA) is a serine protease (kallikrein 3) produced almost exclusively from the luminal secretory epithelial cells of the prostate. PSA is normally secreted into the seminal fluid but when the disruption of the basal cell layer occurs such as in the case of PCa, PSA leaks into the blood circulation. The standard level of PSA is below 4ng/mL, and when the elevated levels of PSA in the blood stream are detected above this level, further diagnosis is required to confirm the disease [50]. Although PSA screening is a supersensitive and relatively inexpensive procedure, it may provide misleading results to patients with poorly differentiated tumors, or patients whose PSA secretion is naturally less elevated.

DRE is a basic procedure to detect the PCa in the earlier stage. The main advantage of DRE is its cost-friendly and time-efficient procedure. It may also detect tumors in patients whose PSA level is normal. The limitation of DRE is that significant tumors may be located in the distant parts of the gland evasive to digital palpation [50].

TRUS and needle biopsy are highly sensitive procedures and enable early screening of PCa [50]. Any positive results from these procedures will undergo further histological examination to assess the biology of the tumor and its degree of spread depending on the accurate staging of the tumors.

The tumor, nodes, metastasis (TNM) staging method classifies the tumors according to their characteristics and the degree to which they have spread (T stage), whether there is lymph node involvement (N stage), or if metastasis occurred (M stage) [51].

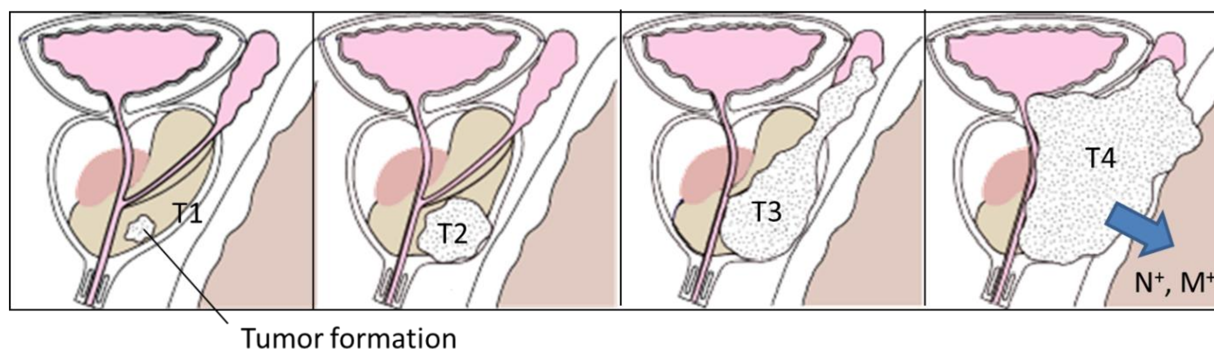


Figure 1. 4 Diagram of TNM classification of the prostate cancer
The progress of prostate cancer is classified based on the extent of the primary tumor spread (T), whether the tumor has spread to lymph nodes (N), and whether the metastasis has occurred (M).

The Gleason system is used to grade the biopsy samples from 1 to 5 based on the degree of tumor differentiation [52]. On a specimen, an area with well-differentiated tumor pattern is assigned 1, and poorly differentiated area is assigned 5. These two grades are added together to give the Gleason score of a specimen [52]. Gleason grade 1 indicates a near-normal pattern, while scores between 2 and 4 indicate low grade/well differentiated tumors. Scores between 5 and 7 indicate moderately differentiated tumors, and scores from 8 to 10 indicate high grade/poorly differentiated tumors [52]. Men between the ages of 65 and 74 years with PCa Gleason score of 2-4 have no loss of life expectancy, while those with scores between 5 and 10 have decreased life expectancy on the grade. [52, 53].

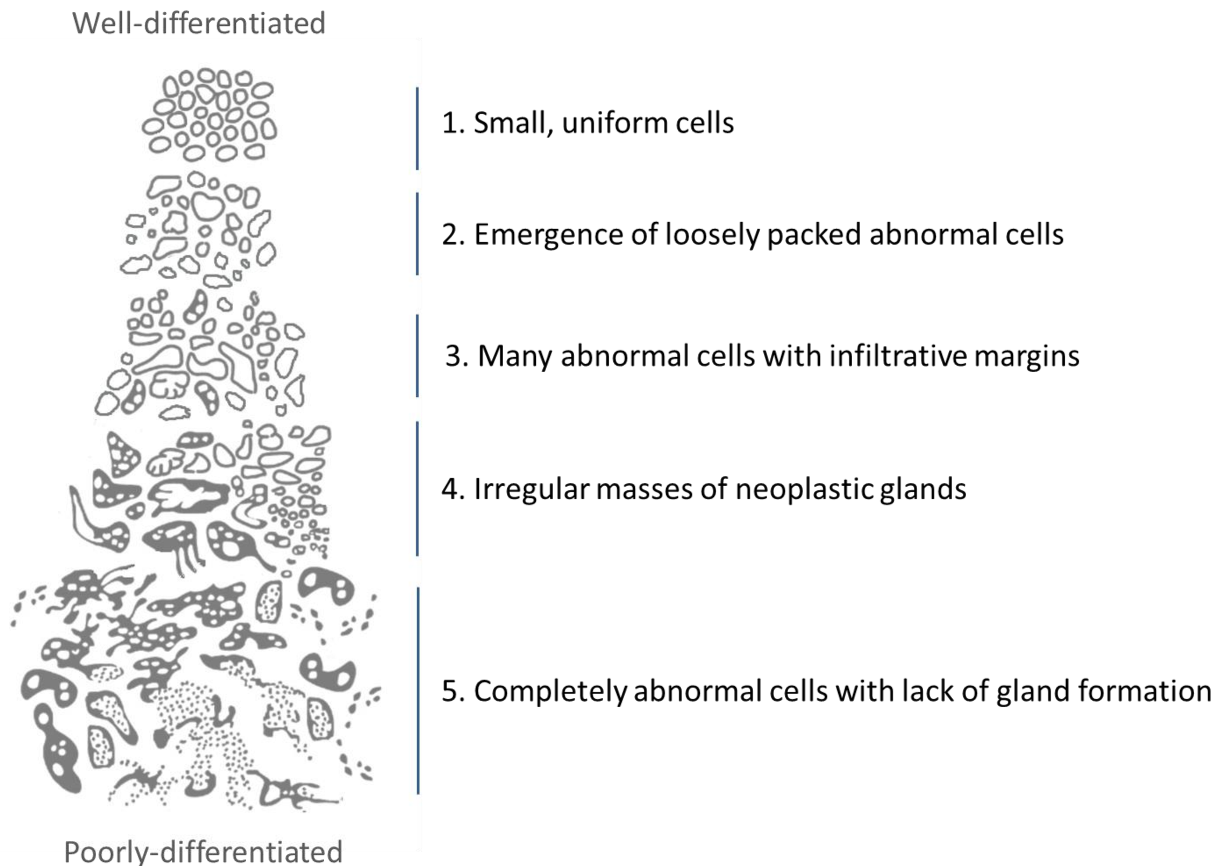


Figure 1. 5 Diagram of the Gleason grade scale
Gleason grading of the prostate cancer is based on the appearance of the biopsy sample. Grade 1 is well-differentiated appearing to be similar to the normal prostate tissue. Grade 5 is poorly differentiated. Due to the common presence of more than one pattern on a biopsy, the two most commonly seen patterns are added together to give a Gleason score.

Although these methods have helped to detect the disease at a much earlier stage, further discovery of PCa biomarkers is required to improve the diagnosis and prognosis in PCa patients. PSA as a biomarker is vastly utilized in current clinical practice, but its reduced specificity for PCa results in over-diagnosis. Biomarkers such as TMPRSS2-ERG gene fusion, prostate cancer antigen 3 (PCA3), prostate membrane-specific antigen (PSMA), prostate stem cell antigen (PSCA), and circulating tumor cells, have emerged in recent science as new potential markers [54]. These markers and further discoveries may contribute to improvement on the detection of PCa in turn improving the quality of life. Furthermore, given the

heterogeneity of PCa, a combination of markers would improve the predictive accuracy and characterization of the cancer.

1.4.4 Treatment

Upon diagnosis of PCa, treatment options for confined cancer include radiation therapy, surgery, and active surveillance depending on the stage. Patients with tumors at stage (T1-2), Gleason score of 6 or less and low PSA levels are considered to be in **stage 1** of the PCa and are recommended to be under active surveillance [52, 55]. Active surveillance is a strategy where patients are subjected to regular PSA screening, collection of biopsies, and DRE to closely monitor the progress of the PCa. The decision is made based on the progress of the tumors as some tumors regress with well-maintained life. The active surveillance may also be an option for older men with limited life expectancy, as other therapies may be harder to endure than younger and healthier patients. Patients are recommended the radiation therapy.

Radiation therapy is an option for the patients who prefer to avoid the risks of invasive surgical removal of the tumors. There are two types of radiation therapies: the external beam radiation therapy (EBRT) and the internal radiation therapy (brachytherapy) [56, 57].

Radical prostatectomy is the surgical removal of the prostate gland and the attached seminal vesicles [57]. Radical prostatectomy is one of the most effective options to maximize the treatment outcomes for patients with locally confined PCa; however, it is considered to be an invasive procedure and side effects include erectile dysfunction and decline in urinary function.
Stage 2 tumors are still confined, but are larger and have higher Gleason scores accompanied by higher PSA levels. Treatment options for stage 2 patients include radical prostatectomy which may be followed by EBRT, or single or combined radiation therapy. Patients also have an option to take part in clinical trials of newer treatments [56, 57].

Stage 3 tumors are grown outside of the prostate but have not metastasized. The treatment options at this stage may include radical prostatectomy followed by radiation therapy, or combined radiation therapy, or and androgen deprivation therapy (ADT) [57, 58].

Stage 4 indicates the condition where the cancer has already metastasized to other organs. Hormone therapy, surgery, radiation therapy, and active surveillance are utilized as treatment options. Treatment of stage 4 PCa may also include treatments to help patients to alleviate pain.

1.5 Castration-resistant Prostate Cancer (CRPC)

1.5.1 Development and Biology

As the growth of PCa cells depends on the presence of androgens, androgen deprivation therapy (ADT) remains the standard treatment for patients with advanced PCa. The principal behind ADT arises from the androgen dependent growth of the PCa cells. Several types of ADT are designed to lower the levels of testosterone, such as orchiectomy (surgical castration) and luteinizing hormone-releasing hormone (LHRH) therapy [58, 59]. While orchiectomy physically removes the source of androgen production, LHRH therapy down-regulates the LHRH receptors in pituitary gland thereby decreasing the secretion of luteinizing hormone to obtain a reduced amount of testosterone. As the initial LHRH therapy causes a brief increase of testosterone level, anti-androgens are administered together at the start of the therapy [58]. Use of antiandrogens alone is another method of ADT to abolish the action of androgens. Antiandrogens such as bicalutamide and flutamide act as potent antagonists of AR by competing with the androgens to bind to the receptor [60, 61]. Moreover, once at the promoters of AR target genes bicalutamide impairs AR transcriptional activity by promoting the incorporation of corepressors upon transcription complex assembly [60]. Bicalutamide was approved in 1995 and has been regarded as the standard of care antiandrogen for the treatment of PCa. Cyproterone acetate and megestrol acetate are antiandrogens that suppress androgen synthesis and interaction with the AR [62].

Almost all PCa patients initially respond to ADT; however, the median duration of response to ADT is approximately 18-24 months and virtually all patients experience a relapse in PCa and progress to a more aggressive stage termed castration resistant prostate cancer (CRPC) [63, 64].

CRPC cells, despite the castrate levels of androgens, reactivate AR and drive initiation and progression of CRPC [63, 64]. This is accompanied by re-expression of androgen-dependent genes such as PSA as indicated by the rising PSA level in the serum. The mechanisms of sustained AR activity in CRPC is achieved through mutation, amplification, altered coactivators and corepressors, splice variants, and acquired ability to synthesize or use androgenic precursors [65].

In the presence of castrate levels of androgens, overexpression of the AR is necessary for the growth of the cancer cells [65]. Studies have found that CRPC cells increase transcription of the AR and the receptor persists in the nuclei of the cancer cells. Alternate studies have also observed that AR genes were amplified by the ligands other than the androgens in CRPC. This phenomenon is due to the mutation in the LBD of the AR, and results in decreased specificity for the ligands [66]. Non-androgenic steroids, adrenal androgens, and even anti-androgens have been shown to bind and act as agonists for AR activation which may explain the ADT withdrawal response [65, 67]. Overexpression of co-activators, such as ARA70, TIF2, and SRC-1, observed in CRPC also enhances the sensitivity of AR to concentrations of adrenal androgens [65].

Some CRPC cells display a variation in AR protein which results in lack of LBD altogether (ARΔLBD). These variants are derived from alternative splicing which include intronic insertions downstream of the coding sequences for the AR DBD [65, 68]. Due to the premature stop codons present in the insertions, the translated AR is truncated that lack the LBD. These AR variants are constantly active despite the absence of androgens or in the presence of anti-androgens, allowing CRPC cells to survive and proliferate.

Another mechanism that activates AR in CRPC is by the cross-regulation of oncogenic pathways. Studies have shown that AR can be reactivated through a reciprocal feedback regulation between inhibited AR and other pathways by the action of growth factors and protein kinases [69].

In recent years, reports from several groups demonstrated that CRPC cells are capable of synthesizing testosterone from cholesterol despite castrated levels of androgens. The

intraprostatic androgens are synthesized from cholesterol through a de novo steroidogenesis pathway mediated by steroidogenic enzymes such as CYP17A1 and HSD3B1. Upregulation of these enzymes is another hallmark of CRPC, and their output of de novo androgens together with adrenal androgens have been shown to be sufficient to activate AR even after ADT [70, 71, 72].

The knowledge of continued dependence of the AR by the cancer cells has allowed new regimens to emerge in hopes to treat CRPC patients thereby prolonging the life.

1.5.2 Treatment for CRPC

Despite the early response to ADT, almost all patients advance to CRPC. Although hormone therapies such as use of bicalutamide are initially effective, with eminent emergence of progressive mutations in the AR, the therapy renders ineffective over time. New generation of chemotherapies, hormonal therapies, antiangiogenic and immune strategies have been developed and are available for treating CRPC.

A number of chemotherapeutic agents for the treatment of CRPC include mitoxantrone, estramustine, and docetaxel [73]. Among them, docetaxel has been shown to be most efficacious [73, 74]. This taxane drug induces polymerization of microtubules to disrupt mitosis and also promotes phosphorylation of oncoprotein bcl-2, which promotes apoptosis of cancer cells that resisted previous apoptotic inducing mechanism [74]. Based on promising results obtained from two phase III clinical trials, docetaxel was approved by FDA in 2004 and has been the main chemotherapeutic option for CRPC. However, the median increase in survival is only 4 months where majority of the patients treated with docetaxel develop resistance to this treatment agent or are not able to tolerate the side effects [73]. In 2010, cabazitaxel received FDA approval as a second-line chemotherapeutic option after docetaxel failure [73].

Several studies have recently reported that PCa may be more immunogenic than previously appreciated. Sipuleucel-T has been FDA approved in 2010 as the first immunotherapy for minimal CRPC [73, 75]. This vaccine utilizes patients' own antigen-presenting cells (APC) which are extracted and engineered to include fusion protein (PA2024) [75]. The PA2024 consists of antigen prostatic acid phosphatase (PAP), which is present in 95% of PCa cells, and an immune

signalling factor granulocyte-macrophage colony stimulating factor (GM-CSF). The activated blood product is reintroduced into the patients to elicit an immune response against cancer cells carrying the PAP antigen. The median survival for this therapy is 4.1 months, and research continues to explore immunotherapy as an option for CRPC treatment [75]. Studies to target main markers of angiogenesis are also underway as a strategy to treat CRPC. As growth factors and pathways such as RAF/MEK/ERK are crucial in controlling the cell division and proliferation of PCa, drugs are currently being developed to target these markers in CRPC cells [69, 76]. Although current clinical trials have not shown encouraging results due to heterogeneous nature of the cancer, continued understanding of the PCa biology and optimal timing of angiogenic pathways may improve the clinical activity for the CRPC patients. After docetaxel failure, more antiandrogens have been developed as a second generation treatment. One of the agents is abiraterone acetate, which was FDA approved in 2011 [73, 77]. Based on the knowledge of continued synthesis of androgens in CRPC, abiraterone acetate was designed to inhibit CYP17A1, an enzyme involved in steroidogenesis of testosterones. Upon inhibition, the conversion of pregnenolone and progesterone to their 17α-hydroxy derivatives is inhibited, thereby halting the formation of DHEA and androstenedione in the testes, adrenal glands, and even in the tumor cells [77, 78]. Despite the therapeutic effort, a "backdoor pathway" to DHT synthesis exists that bypasses the inhibition.

With the inhibition of CYP17A1, other steroidogenic enzymes such as 5-α reductase 1 and 218 have been observed to convert the accumulated progesterone into DHT. With the backdoor pathway of androgen production and adaptive changes to AR to promote AR reactivation, the resistance to abiraterone occurs and the median survival is only 3 months [73]. Studies are continuing to investigate the use of abiraterone in combination with other regimens to possibly overcome the resistance.

Another potent antiandrogen that has been developed as a second-generation therapy for CRPC is enzalutamide (ENZ). During the international phase III clinical trial AFFIRM, patients given the drug were observed to live for approximately 8.4 months longer than those taking placebo, with reduction of PSA concentration in serum [78, 79, 80]. The trial was stopped early after an interim analysis and received FDA approval in 2012.

Pharmacologically, ENZ has about 5 to 8 fold higher binding affinity for the AR than bicalutamide and only 2 to 3 fold reduced affinity than the DHT [79, 80]. ENZ not only prevents the nuclear localization of AR in metastatic PCa cells, but the drug also inhibits DNA binding and coactivator recruitment in CRPC cells that have become resistant to bicalutamide [79, 80]. With inhibition of transcriptional activity of AR, studies have observed suppressed growth and induced apoptosis of CRPC cells regardless of previous chemotherapy status [81]. On the basis of these findings, ENZ has become the most potent agent in prolonging overall and progression-free survival and improved quality of CRPC patient life.

Despite early success, resistance to ENZ inevitably occurs. The AR pathway is still activated in these cells, with full length AR and its target genes upregulated. Due to maximal targeting of AR, cancer cells also develop alternate pathways to progress the disease to a more aggressive form such as neuroendocrine prostate cancer, a lethal and completely AR-independent subtype of disease [82, 83, 84]. Therefore, there remains a need for continued research on the mechanisms that confer resistance to CRPC cells, as well as identify key biomarkers for improved therapeutic strategies.

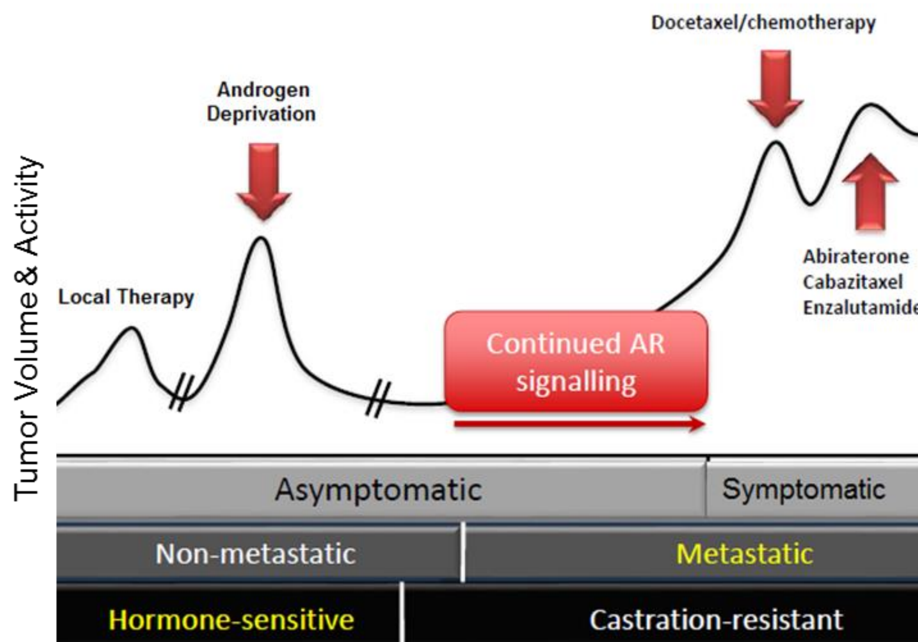


Figure 1. 6 Treatment landscape of prostate cancer tumor progression with various therapies
For localized PCa, surgery and radiation are the therapeutic options, whereas ADT is the first line of treatment for advanced and metastatic PCa. Chemically or surgically castrating the patients show good

response in tumor regression, but the disease progresses again after a median time of 18-24 months with continued PSA secretion detected in serum. This stage is known as castration resistant PCa, and it is driven by the selective pressure of treatment. Several therapies including use of docetaxel are initially effective but the disease becomes resistant. Treatment with second generation drugs such as enzalutamide and abiraterone has been the standard measure that has been shown to be most effective since its FDA approval in 2012, but it inevitably is rendered resistant and there remains a need for new therapy to treat PCa.

1.6 Neuroendocrine Prostate Cancer (NEPC)

Neuroendocrine prostate cancer (NEPC), or small cell carcinoma of the prostate, is a lethal subset of PCa that was first described in 1977. Since then, it has been reported to occur in only two percent of all diagnosed prostate cancer patients, although autopsy studies from men who have died of CRPC revealed the presence of NEPC up to 30% [85, 86]. Recent evidence suggests that the disease arises from pre-existing prostate adenocarcinoma, with higher occurrence in CRPC patients who underwent maximal androgen blockade [87, 17]. NEPC patients no longer benefit from hormonal therapies that target AR, and the prognosis of these patients remain dismal with less than one year of survival time [88, 89]. NEPC is an under-recognized disease and currently without an effective therapy. Therefore, further studies are required to help distinguish NEPC on the basis of distinct molecular biomarkers and improve the current diagnostic features that rely on morphological characterization.

1.6.1 Pathology and Clinical Features

While CRPC patients experience bone and lymph node metastases accompanied by rising serum PSA, patients who progress to NEPC experience rapid metastasis to other abdominal visceral organs with low PSA level and is unresponsive to hormonal therapy [90, 91, 92]. Unlike prostate adenocarcinoma, NEPC patients frequently present neurological symptoms such as confusion and sensory or motor deficits, due to NEPC’s greater tendency to metastasize to the brain [90, 93]. NEPC also releases ectopic neuropeptides, adrenocorticotropic hormone and gastrin-releasing peptides, and is associated with Cushing syndrome, hypercalcaemia, and syndrome of inappropriate antidiuretic hormone [90, 94, 95, 93, 96].

Histological features of NEPC are similar to that of small-cell carcinomas (SSC) of other organs such as lungs [90]. It is highly populated by NE-like cells which consist of scant cytoplasm,

irregular borders, granular chromatin, inconspicuous nucleoli, and have high mitotic count [97, 90]. These cells express neuroendocrine markers including neuron-specific enolase (NSE), synaptophysin (SYP), chromogranin A (CHGA), and express low to no level of PSA and AR [98, 99, 100]. Due to these features, the origin of NE-like cells gave rise to two theories: evolution from the normal NE cells in the prostate, and the more recently supported NE transdifferentiation from pre-existing prostatic adenocarcinoma cells.

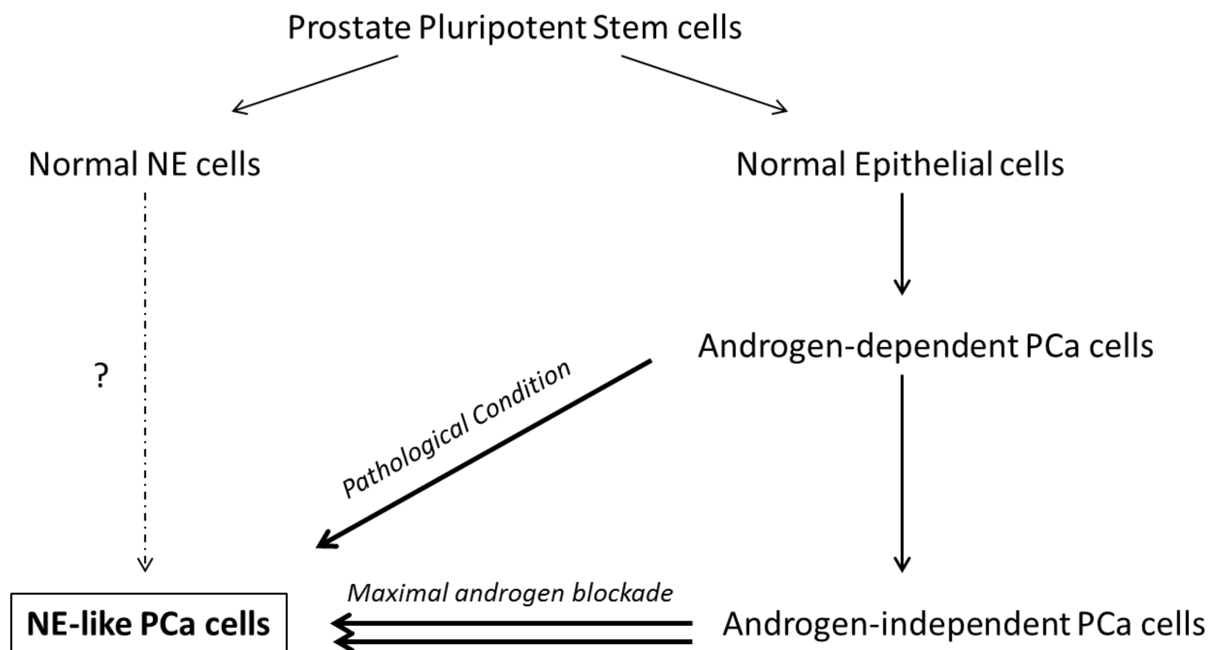


Figure 1. 7 Schematic diagram showing postulated origin of NE-like PCa cells
The origin of NE-like PCa cells has been debated. Because NE-like cells express markers of normal NE cells, it led to a postulation that ADT-resistant NE cell survived the treatment and proliferated. However, as normal NE cells are non-proliferative this theory still requires validation. Another theory postulates that NE-like cells may have transdifferentiated from existing adenocarcinoma cells. Supporting evidences include TMPRSS2-ERG gene fusion found in NE-like PCa cells, downregulation of REST, and amplification of AURKA and nMYC genes found in both adenocarcinoma and NEPC cells.

1.6.2 NE Transdifferentiation

Because NE-like cells in NEPC also express markers of normal NE cells, such as p63 and high-molecular-weight cytokeratin, it was originally postulated that the cells have been influenced by the normal NE cells in the prostate gland [101]. According to this theory, while ADT

eliminates AR-dependent PCa cells since they require androgen for survival, the NE cells that lack AR and are devious to the therapy remain and persist to proliferate [101]. However, this theory still requires further validation.

Alternatively, increasing evidence reveal that the NE-like cells are divergently evolved from pre-existing prostatic adenocarcinoma cells through NE transdifferentiation, and drive NEPC. Firstly, recurrent ERG rearrangements in the androgen-regulated 5'end partners including TMPRSS2 and SLC45A3, as well as ETS genes are reported in approximately 50% of NE-like cells [102, 103, 90, 104]. These features are prostatic adenocarcinoma-specific mutations, and the rearrangement occurs similarly in PCa cells, suggesting a common origin. Secondly, down-regulation of REST, a transcription factor involved in the repression of neuronal differentiation, is observed in both NEPC and in CRPC patients who eventually progressed to NEPC [105]. Thirdly, an identical mutation in the DNA-binding domain of TP53, a tumor-suppressor gene, is reported in both NEPC and PCa cells [106]. Additionally, amplification of Aurora kinase A (AURKA) and N-Myc (MYCN) genes are reported in advanced PCa patients and at a higher rate in patients with NEPC, suggesting that PCa and NE-like cells are related [107, 86]. This also distinguishes the cells from having arisen from normal NE cells. Finally, recent xenograft models have identified loss of AR expression as a mechanism of resistance to enzalutamide supporting the NE transdifferentiation to AR-independent state in NEPC [98, 99, 108, 100]. These findings support the hypothesis that existing PCa cells may have transdifferentiated to acquire NE phenotype at some point of progression. Unlike the increasing report of NEPC progression in CRPC patients who have become resistant to second generation therapy, the progression is uncommon in PCa patients who did not receive any prior ADT [98, 100]. A recent study by Dong *et al*. revealed that SRRM4, an RNA splicing factor, is upregulated in adenocarcinoma patients who received maximal ADT. Under the ADT condition, SRRM4 has been shown to splice negative neuronal regulators such as REST, driving the NEPC [109] Thus, the transdifferentiation model is becoming more recognized as an adaptation mechanism due to maximal ADT.

1.6.3 Aggressive Nature of NEPC

NEPC metastasizes more aggressively than CRPC and the average survival period is less than one year [85, 101]. Although NE-like cells may have evolved from CRPC, there exists diversity between the CRPC and NEPC cells. Distinguishing them would improve diagnostic methodology and earlier detection, and in turn improve the prognosis of NEPC patients.

Apart from higher expression of AURKA and MYCN gene which are involved in cell proliferation, NEPC cells also display promoter hypermethylation and reduced expression of the SPDEF, a transcriptional activator involved in suppression of tumor metastasis through inhibition of epithelial-to-mesenchymal transition (EMT) in PCa [110]. This may contribute to more aggressive and rapid metastatic behavior of NEPC compared to CRPC. EZH2, a histone methyltransferase which is associated with aggressive PCa, is two-fold more abundant in NEPC than in CRPC [102, 111, 112, 113]. Furthermore, EZH2-repressed target genes such as DKK1 and NKD1, repressors of Wnt pathway, are down-regulated in NEPC. These features may also contribute to rapid cell division and metastasis, thereby attributing to aggressive nature [113, 114].

Currently, various studies are under way to identify other molecular components unique to NEPC. Understanding the markers involved in NE transdifferentiation process will help us track disease progression. This would allow the patients to have an option of receiving NEPC-directed therapy rather than the rendered-ineffective AR therapy, or receive co-targeting therapies, thereby improving the outcomes of the lethal disease.

1.7 Paternally Expressed Gene 10 (PEG10)

Paternally expressed gene 10 (PEG10) is a Tyr3/Gypsy family of retrotransposon that integrated into the mammalian genome about 120 million years ago by using its reverse transcription mechanism to convert its RNA into DNA then into the host genome [115]. It is mapped on chromosome 7q21.3 and is comprised of two exons separated by introns. Typical of viral genome, PEG10 also harbors two reading frames, RF1 and RF2, overlapped by 61 nucleotides and participates in -1 ribosomal frameshifting during translation. RF1 codes for the 100kDa

PEG10-RF1 protein where the N-terminus contains a coiled-coil domain and a zinc-finger domain in C- terminus, common characteristics observed in retroviral *gag* proteins [116]. RF2 codes for the 80kDa *pol*-like PEG10-RF1/2 fusion protein through the seven "slippery" nucleotide sequences, <u>GGGAAAC</u>TC, present in the RF1 and RF2 overlapping region [116]. The translated RF1/2 contains an aspartyl protease motif which is common in retroviruses for protein processing. This motif further generates a third protein, PEG10-cleaved N-terminus fragment (PEG10-CNF), which is 35kDa in size [117, 116]. Currently visualized PEG10 protein population consists of RF1 which make up about 75% of PEG10, RF1/2 which makes up 15%, and the remainder consists of PEG10-CNF [118] and their clear functions are not yet known.

PEG10 is observed to be predominantly expressed during embryonic development from day 9.5 to 16.5 especially in bone and cartilage forming tissues [119, 120]. It is also highly expressed in the cytotrophoblast layer of the placenta, and studies suggest PEG10 may be essential for trophoblast differentiation and uterine implantation [119, 120]. In adult humans, PEG10 is expressed in brain, kidney, lung, and testis [118].

Other studies have also found that PEG10 is highly expressed in hepatocellular carcinomas, although its expression is absent in normal liver cells. [121]. The knowledge regarding PEG10 function is still sparse, but increasing evidence suggests that PEG10 may be involved in cell proliferation and anti-apoptotic activity in hepatocellular carcinoma and B-cell acute and chronic lymphocytic leukemia. It has been observed to also interact with ALK1, a cell surface transmembrane receptor, to induce morphological changes in cells [121].

Recently, PEG10 has been identified in the post-castration model of patient-derived PCa in the transdifferentiation series model, as well as in clinical NEPC models suggesting that it may be a potential biomarker for NEPC emergence [84, 117].

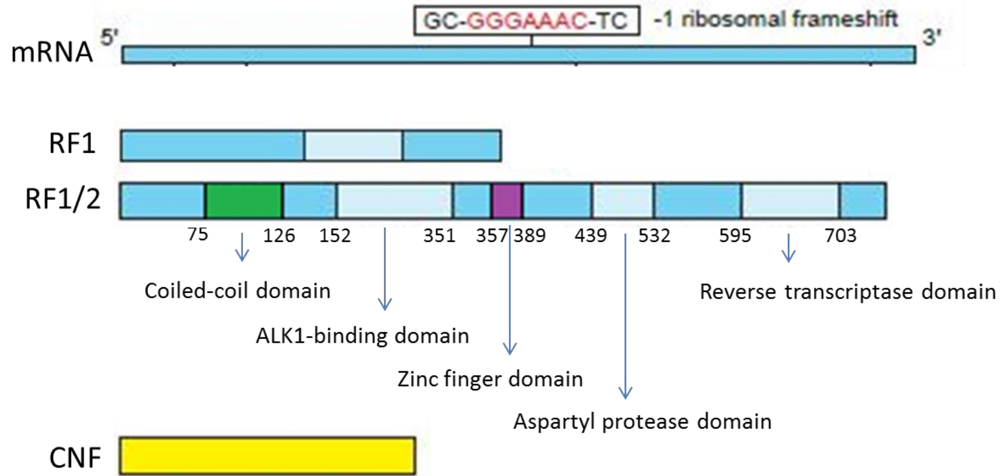


Figure 1. 8 Schematic diagram of PEG10 mRNA and protein domains (adapted from Akamatsu *et al* [117])
Reading Frame 1 (RF1) and Reading Frame 1/2 (RF1/2) are coded through -1 ribosomal frameshift. RF1/2 contains domains characteristic of retrovirus such as coiled-coil domain, zinc finger domain, and aspartyl protease domain. PEG10 also harbors a ALK1- binding domain. Cleavage of RF1/2 generates a 35kDa Cleaved N-terminal fragment (CNF) protein.

1.8 Enzalutamide CRPC-resistant Model

It is now clear that despite currently available regimes for prostate cancer treatment, resistance inevitably occurs and renders second-generation antiandrogens such as enzalutamide ineffective. Continued research on the mechanisms that confer resistance and identification of key markers is required for improved therapeutic strategies.

In order to study the mechanism behind enzalutamide resistance and progression to NEPC, our lab generated a unique model of enzalutamide-resistant (ENZ-R) CRPC cell lines through serial passages (Figure 1.9). Upon gene profiling we found that $42D^{ENZR}$ cell line was different from all other ENZ-R cells where a majority of its AR-regulated genes were downregulated (Figure 1.10a and c). Western blot analysis of the cell lines showed that while AR is expressed in the $42D^{ENZR}$ and $42F^{ENZR}$, their PSA level was very low (Figure 1.10b). When we analyzed the gene profiling data, we noticed an upregulation of NE markers including NCAM1, NSE, SYP, and CHGA compared to the enzalutamide-naïve CRPC cells (Figure 1.11a). This observation was further supported by upregulated mRNA levels of NE markers in both $42D^{ENZR}$ and $42F^{ENZR}$ cell lines from our qRT-PCR analysis (Figure 1.11b). We compared our data to NEPC patient data

collected by Beltran *et al.*, as well as gene profiling of NE transdifferentiation models by Akamatsu *et al.* (Figure 1.12) [117] and identified PEG10 gene to be highly upregulated (Figure 1.13). With supporting evidence from various studies that CRPC cells which underwent maximal androgen blockade can transdifferentiate and progress to NEPC, we questioned if the increase of PEG10 arises in response to treatment resistance and whether it is involved in NE differentiation.

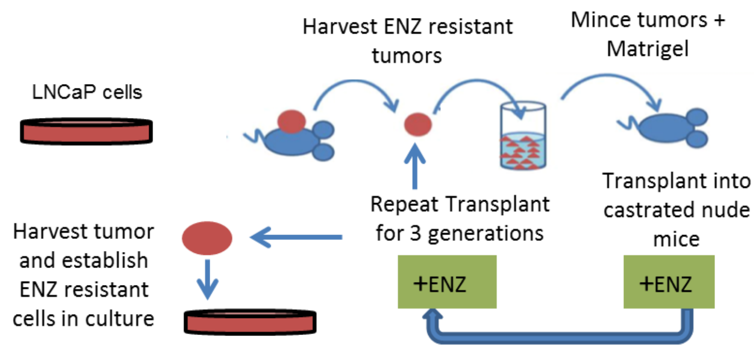
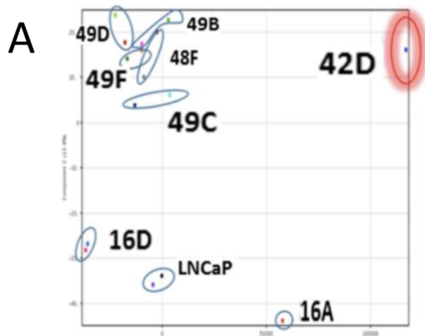
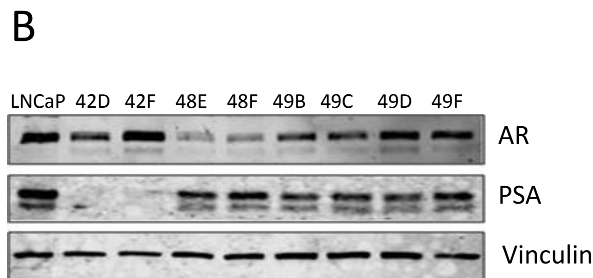


Figure 1. 9 Generation of ENZ-R cells (adapted from Kuruma *et al* [122])
LNCaP xenografts were excised and transplanted into castrated mice treated with 10mg/kg/daily dose of ENZ. Upon reaching maximal volume, the ENZ-resistant xenograft tumors were harvested into RPMI+10% FBS+ 10µM ENZ (103).





C

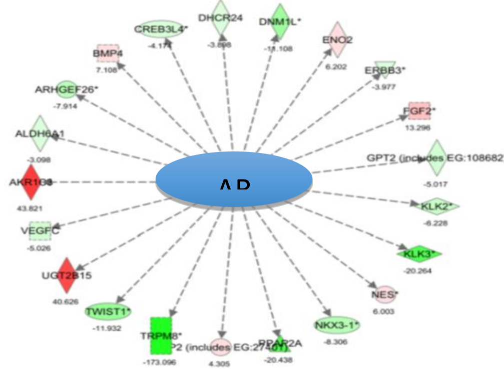
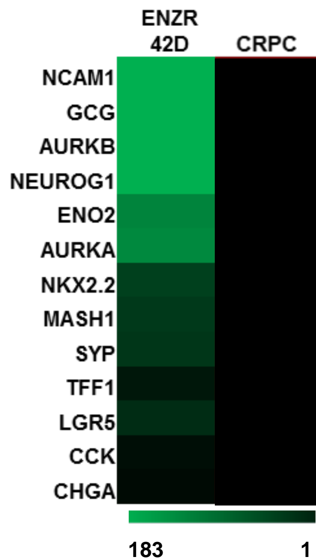


Figure 1. 10 Gene profile of 42D^{ENZR} and western blot of the cell lines

A. 42D^{ENZR} behaves differently from other ENZ-R cell lines, and B. Western blot analysis shows that 42D^{ENZR} and 42F^{ENZR} both express AR but expresses very low amount of PSA. C. AR-regulated genes are all down-regulated in 42D^{ENZR}.

A



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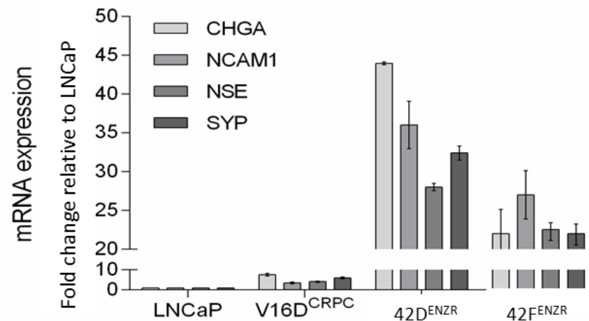


Figure 1. 11 Gene profiling analysis of 42D^{ENZR} and gene expressions in cell lines

A. Gene profiling analysis shows that 42D^{ENZR} expresses higher neuroendocrine markers than ENZ-naïve CRPC cell line. B. qRT-PCR analysis confirms the gene profile data that 42D^{ENZR} and 42F^{ENZR} express high levels of NE markers than the castration sensitive cell line LNCaP and castration resistant 16D cell line.

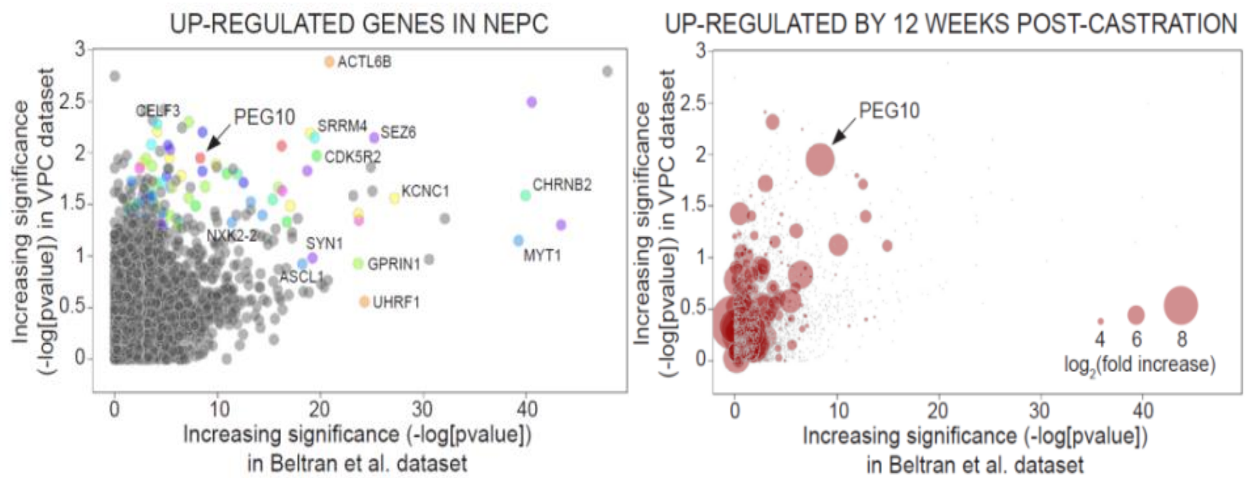


Figure 1. 12 Gene profiling of NE transdifferentiation models (adapted from Akamtsu *et al* [117])
Gene profiling reveals that PEG10 is highly expressed in patients' post-castration.

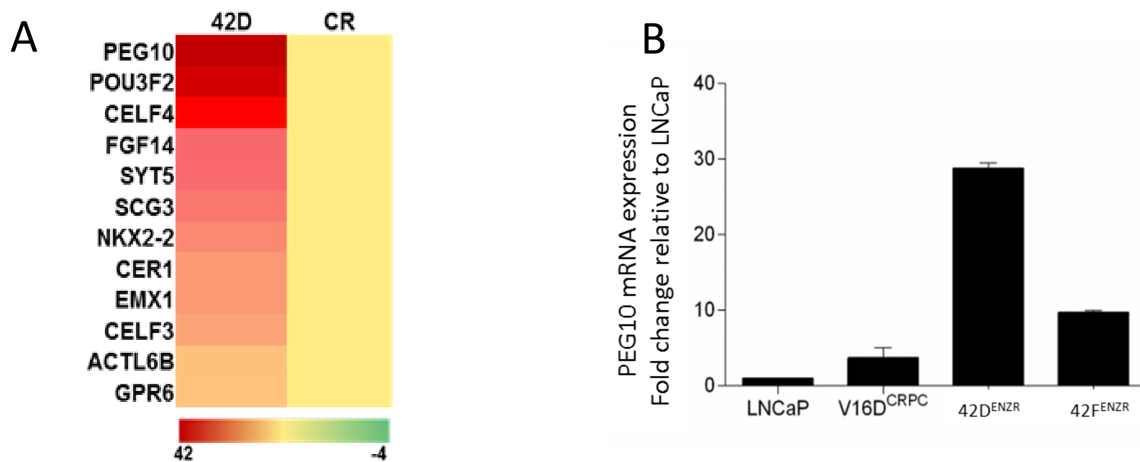


Figure 1. 13 Gene profiling analysis of 42D^{ENZR} and PEG10 gene expression in cell lines
A. Gene profiling analysis of 42D^{ENZR} shows an upregulation of PEG10 compared to CRPC cell line (Bishop *et al.*, in submission). B. qRT-PCR analysis confirms the upregulation of PEG10 mRNA level in 42D^{ENZR} in comparison to castration-sensitive and castration-resistant cell lines.

Buttyan *et al.* already showed that NE differentiation can be induced by hormone deprivation in CRPC cells [82, 123]. We confirmed this result as well and also observed that in such condition, PEG10 expression is upregulated in both mRNA and protein level (Figure 1.14).

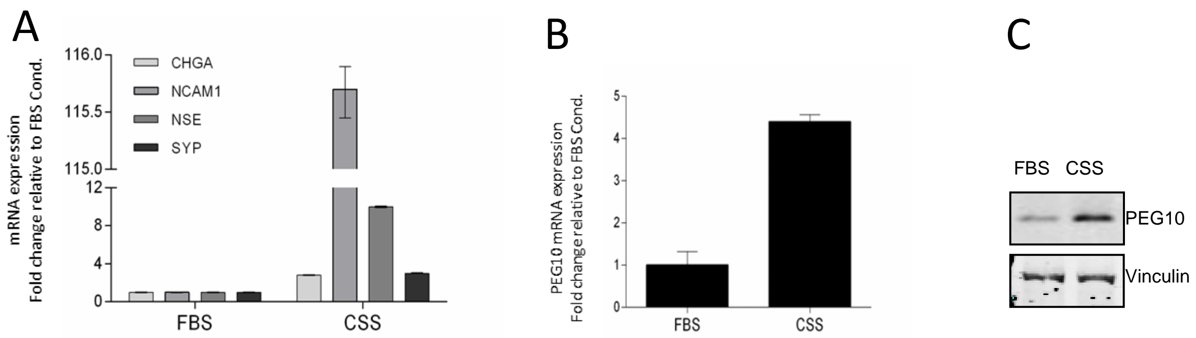


Figure 1. 14 Gene expressions and protein levels in castration-sensitive cell line LNCaP under CSS and FBS condition

A. qRT-PCR analysis shows that when LNCaP cells are cultured in charcoal-striped serum for 7 days the NE markers are upregulated in comparison to FBS. B. qRT-PCR analysis shows that under CSS condition, PEG10 is highly upregulated. C. Western blot analysis confirms that PEG10 protein is also upregulated in CSS condition in LNCaP.

Hypothesis:

AR negatively regulates PEG10 and PEG10 is required for the ENZ-induced emergence of NEPC from CRPC; targeting PEG10 will significantly inhibit NEPC tumor growth.

Aim #1: Investigate the molecular mechanisms by which AR activity negatively regulates PEG10 expression in CRPC and ENZ-R cell lines.

Aim #2: Investigate whether PEG10 is a driver or a target of NEPC progression in CRPC and ENZ-R cell lines.

Our data revealed that AR directly binds to the PEG10 promoter and inhibits PEG10 activity. ENZ inhibits AR activity which activates PEG10 with concomitant increase of NE markers.

To investigate if PEG10 is a NEPC driver, PEG10 was overexpressed in LNCaP cells and NE markers were evaluated. Our data revealed that overexpression of PEG10 alone did not increase NE markers and suggest that PEG10 alone is not a driver of NEPC.

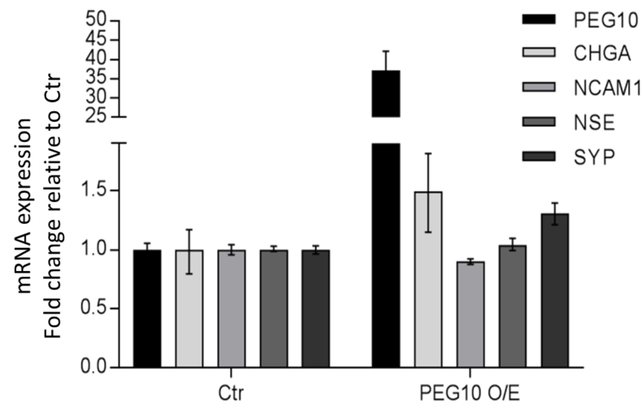


Figure 1. 15 Gene expressions in stable PEG10 overexpression LnCaP
qRT-PCR analysis shows that LNCaP with stably overexpressed PEG10 express no significant change in NE markers expression.

Importantly, we observed that transiently and stably knocking down PEG10 prevented ENZ-induced terminal NE markers expression. The knockdown of PEG10 did not activate AR pathway suggesting that there is no feed-forward loop between AR and PEG10, but a more

direct effect of AR on PEG10. Interestingly, PEG10 knockdown also resulted in reduced cell proliferation in both CRPC and ENZ-R cell lines *in vitro*, and attenuated tumor growth in *in vivo*.

This work has identified PEG10 as a potential therapeutic target for the patients who are progressing to NEPC. Data obtained in this thesis will help to better understand the mechanism involved in NEPC progression after second generation hormone therapy.

CHAPTER 2: PEG10 is an Androgen Receptor-regulated Gene Involved in Prostate Cancer Neuroendocrine Differentiation Following AR-targeted Therapies

2.1 Introduction

Prostate cancer (PCa) is one of the most commonly diagnosed malignancies in North America and is a leading cause of cancer mortality amongst men [124]. Androgen deprivation therapy (ADT) is an effective treatment for recurrent or metastatic prostate cancer. Nonetheless, treatment resistance is common and inevitably occurs following AR-axis targeting treatments. This is most commonly seen in the development of castration resistant PCa (CRPC) following ADT through medical or surgical castration. Studies indicate that persistent AR activity via canonical and non-canonical pathways continues to drive disease progression despite maximal androgen blockade (MAB) in CRPC. This highlights the adaptive nature of the AR-axis and other pro-survival pathways present in this disease state [125, 126].

Next generation AR antagonists such as enzalutamide have demonstrated an overall survival benefit in CRPC and highlight the ongoing “targetable” nature of the AR even in advanced disease [127]. Enzalutamide targets the ligand-binding domain (LBD) of the AR preventing its nuclear translocation and subsequent transcriptional activity. With the wider use of newer agents, 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 LBD [128, 129, 130, 131]. In other cases, CRPC cancers which behave in an aggressive and ultimately fatal manner after multi-agent AR-axis inhibition demonstrate a non-AR driven phenotype with neuroendocrine (NE) features absent in typical PCa and CRPC [132]. This has given rise to the term treatment-induced NE PCa (NEPC). The cellular origin of NEPC 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 and an increase in NE marker expression such as synaptophysin (SYP), chromogranin A (CHGA), neural

cell adhesion molecule (NCAM) and neuron-specific enolase (NSE) [103]. The loss of AR activity and acquisition of an NE phenotype has prompted investigation into the inhibitory role the AR may have in repressing an NE-like phenotype, a potential theory explaining the phenomenon of increasing aggressiveness of highly treated CRPC [101, 103].

Recently, our group described a human xenograft model that recapitulated transdifferentiation of adenocarcinoma to NEPC was used to identify molecular events associated with the NE phenotype in PCa [100, 117]. We identified paternally expressed, maternally silenced gene 10 (*PEG10*), an evolutionary conserved retrotransposon-derived gene located within an imprinted domain on chromosome 7q21 as a potential genetic driver of transdifferentiation. Further, the authors demonstrate that dynamic control of PEG10 expression is regulated by the AR and the E2F/RB pathways, and that PEG10 activity drives cell cycle proliferation and invasion in the context of TP53 loss.

Here, we demonstrate that the AR binds to the PEG10 promoter and acts to suppress PEG10 expression using a recently described cell model of non-AR driven ENZR ($42D^{ENZR}$, $42F^{ENZR}$) that displays NE features (Bishop *et al*., in submission). We characterize the changes in PEG10 and NE marker expression in LNCaP, $16D^{CRPC}$, $42D^{ENZR}$ and $42F^{ENZR}$ following androgen depletion and supplementation. We also silence PEG10 both *in vitro* and *in vivo* to further delineate its association with NE marker expression and tumour growth, respectively. PEG10 silencing affects cell cycle progression and proliferation, confirming the pro-growth properties of PEG10 [117]. Chromatin immunoprecipitation (ChIP) assays suggest that AR can bind the PEG10 promoter, however as opposed to the canonical AR-dependent gene PSA where AR promoter binding leads to gene expression, AR acts to repress PEG10 expression. Together, these findings demonstrate non-canonical AR activity whereby the AR acts to repress PEG10 expression, a gene that appears to be directly linked with NEPC progression. These findings also highlight the utility of our novel ENZR cell model, permitting evaluation of a novel mechanistic feature of NEPC progression. In the future, our models may potentially also serve as a platform for evaluation of future NEPC drug targets, which may include targeting of PEG10.

2.2 Material and Methods

2.2.1 Cell Culture and Transfection

PCa 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 10uM Enzalutamide (Selleck Chemicals). All cell lines were grown at 37°C in a humidified air atmosphere with 5% CO_2. For induction of AR activity experiments, R1881 (0-5nM) 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 cat#4392420 (Ambion) and custom designed Control siRNA 5'-AUCAAACUGUCAGCGCUG-3' (Dharmacon) were transfected twice using Oligofectamine (Invitrogen) at the final concentration of 20nM according to the manufacturer's protocol. The duration for the first transfection was 16 hours, and the duration of the second transfection 4 hours. Stable PEG10 knock-down cells were generated by transfecting PEG10 shRNA. Lentiviral Particles sc-152158-V (Santa Cruz) and Control vectors 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 2.3ug/ml puromycin (Gibco) containing media. Stable PEG10 knock-down cells were challenged with enzalutamide for 6 days prior to experiments to ensure knock-down of PEG10.

2.2.2 RNA Extraction and qRT-PCR Analysis

Total RNA was extracted using TRIzol (Invitrogen, CA). For qRT-PCR, extracted RNA was converted to cDNA by reverse transcription using M-MLV Reverse Transcriptase kit (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 for PEG10 (Table 1). The average change in threshold cycle (ΔCT) 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.

Gene Name	Forward Primer	Reverse Primer
PEG10	5' -GCGCCTGACGCAGGAA-3'	5'-CCACAGTAGAGGCACAGGTTCA-3'
AR	5'-TACCAGCTCACCAAGCTCCT3'	GCTTCACTGGGTGTGGAAAT-3'
PSA	5'-CACAGCCTGTTTCATCCTGA -3'	5'-AGGTCCATGACCTTCACAGC-3'
NCAM	GATGCGACCATCCACCTCAA-3'	5'-TCTCCGGAGGCTTCACAGGTA-3'
CHGA	5' -TCCAAGGCGCCAAGGA-3'	5'-CATCTTCAAAACCGCTGTGT-3'
NSE	5'- GAACTATCCTGTGGTCTCC -3	5'CGACATTGGCTGTGAACTTG -3'
SYP	5' -TCAGTTCCGGGTGGTCAAG-3'	5'-AAGACCCATTGCAGCACCTT-3'
CAPDH	5' -ACCCAGAAGACTGTGGATG -3'	5'-CAGTGAGCTTCCCGTTCAG -3'

Table 1.1 Primer sequences for selected gene used for quantitative PCR

2.2.3 Western Blot Analysis

Total protein from each of the cell lines was extracted using RIPA lysis buffer and the concentration was determined by using Pierce BCA Protein Assay Kit (Thermo Scientific). The protein was separated by SDS-PAGE and blotted to a nitrocellulose membrane. The membranes were probed with primary antibodies against PEG10 NBP2-13749 (Novus Biologicals) at 1/1000 dilution, 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 1/10000 was blotted as a loading control. IRDye secondary antibodies (LI-COR Biosciences) for the Odyssey Infrared Imaging System (LI-COR Biosciences) were used to detect protein bands. Alternately, HRP detected the bands with Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

2.2.4 Crystal Violet Proliferation Assay

3000 cells from each condition of the cell lines were plated onto 96-well plates in RPMI + 10% FBS + 2.3ug/ml puromycin, where ENZ-resistant cell line media was supplemented with enzalutamide 10µM. Over the span of 8 days, the cells were fixed with 1% glutaraldehyde and stained with crystal violet. Sorenson's buffer (0.1M sodium citrate+ 50% ethanol + dH_2O at

pH4.2) was used to solubilize the stain for absorbance measurement at 540nm 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.

2.2.5 Cell Cycle Analysis

Control and shPEG10 cells from each cell lines were cultured on a 6-well plate and harvested carefully with trypsin. After washing with PBS, the cells were fixed in cold 70% ethanol under vortex to minimize clumping and incubated in 4°C for 30 minutes. After fixing, the cells were washed 2 times in cold PBS and spun at 1000 rpm. 500uL solution containing 0.1% Triton X-100, 0.1mM EDTA disodium, 50ug/mL RNase A R-4875 (Sigma), and 50ug/mL propidium iodide P-4170 (Sigma) was added to stain the cells and incubated at 37°C for 40 minutes. Upon discarding the solution, the cells were suspended in 500µL PBS and were analyzed using the BD FACS CANTO II system.

2.2.6 Luciferase Assays

PEG10 promoter luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega) on Tecan's Infinite F500 Multimode Reader. LNCaP cells were plated onto 6-well plates and grown to 90% confluency, then transfected with a pGL2 vector containing the PEG10 promoter region (PEG10-prom-380; graciously provided by Dr. Andreas Lux at Mannheim University) and 1µg Renilla using Lipofectin (Invitrogen). Post-transfection, the cells were treated as indicated in RPMI + 10% FBS or RPMI + 10% CSS for 96 hours and luciferase activity was measured.

2.2.7 AR Chromatin Immunoprecipitation

All cell lines were cultured in RPMI + 10% CSS on 15cm plates with or without 1nM R1881 for 4 days. The sequence for the AR binding site on PEG10 promoter was adapted from Akamatsu *et al.*, 2015. 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 0.125M Glycine. Upon cell collection, nuclei were extracted and sonicated into 200bp fragments. The DNA-protein complex was immunoprecipitated with either 5µg 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, 2mM EDTA, 20mM Tris-HCl, 150mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X, 2mM EDTA, 20mM Tris-HCl, 500mM NaCl), LiCl buffer (250mM LiCl, 1% NP40, 1% sodium deoxycolate, 1mM EDTA, 10mM 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 promoter region [5'-CCCTCGGTAATCCCGTACTC-3' (forward), 5'-ACACCAACCGAAGTTGAAGC-3' (reverse)]. The PSA promoter primers [5'-TCTGCCTTTGTCCCCTAGAT-3' (forward), 5'-AACCTTCATTCCCCAGGACT-3' (reverse)] were used as a positive control for AR activity.

2.2.8 *in vivo* Tumor Formation

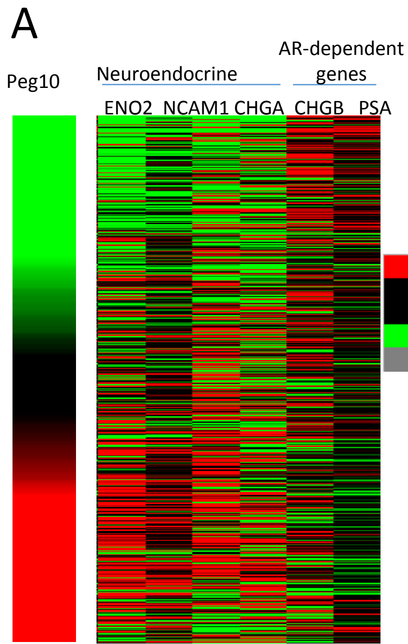
All animal studies were carried out in accordance with the recommendations of the Canadian Council on Animal Care and institutional guidelines. *In vivo* tumor formation of 42F cells stably transfected with either control or PEG10 shRNA were conducted via a sub-cutaneous inoculation of 2 million cells in the flank region of 4-6 week old male athymic nude mice post castration. Each experimental group consisted of 10 mice. Tumor volume was measured once a week (length x width x depth x 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.

2.3 Results

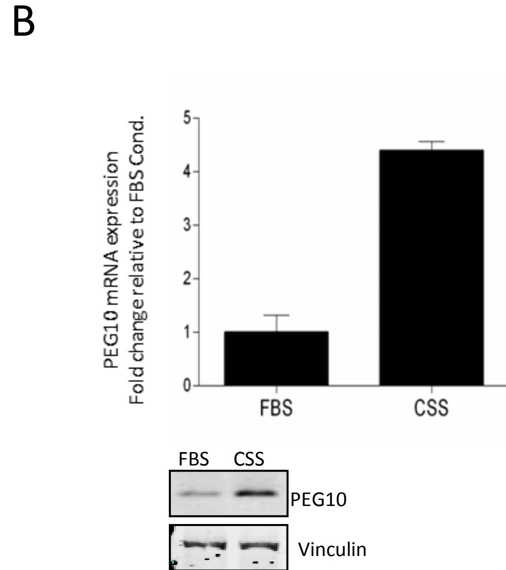
2.3.1 PEG10 and NE Markers Expression in Androgen-deprived LNCaP

An analysis of The Cancer Genome Atlas (TCGA) prostate cancer database demonstrates correlation of tumour PEG10 expression and representative markers of NE status; Neuron-specific Enolase 2 (ENO2), Neuronal Cell Adhesion Molecule (NCAM), Chromogranin A (CHGA) and B (CHGB) (figure 2.1A). In tumour samples enriched for PEG10 expression (red on heatmap), NE markers were consistently elevated. Tumours without PEG10 enrichment demonstrate no NE marker expression. Representative AR-dependent genes, PSA and FKBPS2, demonstrate a reciprocal relationship with PEG10 and NE marker status.

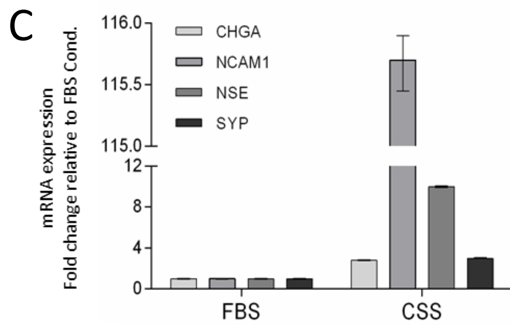
PEG10 upregulation occurs in LNCaP cells maintained in charcoal-stripped serum (CSS) media deplete of androgen for seven days, at the mRNA and protein levels (Figure 2.1B). Figure 2.1C demonstrates elevated NE marker status under similar conditions, especially of NCAM, which is upregulated over 100-fold at the mRNA level. Under androgen deplete conditions, canonical AR-signalling measured via PSA is decreased. Inhibition of AR-dependent signaling with 10uM enzalutamide (ENZ) results in increased PEG10 expression in LNCaP in a time-dependent manner up to seven days at both the mRNA and protein levels (Figure 1D). This effect of ENZ is accentuated in conditions of CSS, with highest PEG10 expression occurring after seven days of exposure to CSS and ENZ. NE marker response increases in both CSS and CSS + ENZ, similar to PEG10 expression (Figure 2.1E). However, maximal NE marker status response is seen after seven day exposure to ENZ alone (up to >80-fold for NCAM), and less marked for CSS and ENZ combination (15-20 fold for NCAM). PSA expression is completely abrogated in an early and sustained fashion following ENZ alone or ENZ + CSS combination.



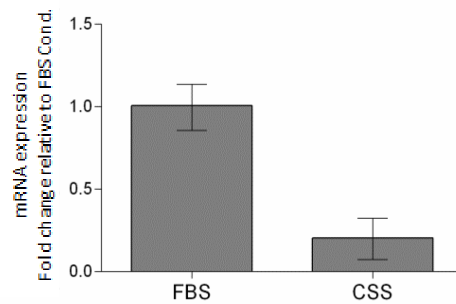
A
Peg10
Neuroendocrine
AR-dependent genes
ENO2 NCAM1 CHGA CHGB PSA
>1
0.25
-0.25
<-1
NA



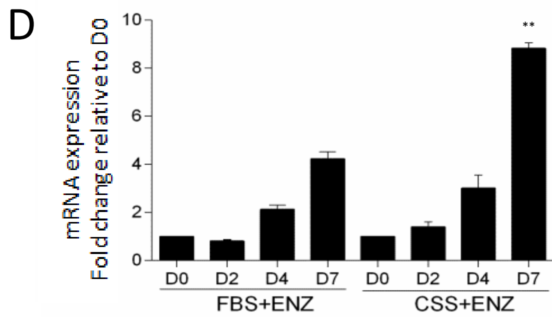
B
PEG10 mRNA expression
Fold change relative to FBS Cond.
FBS
CSS
PEG10
Vinculin



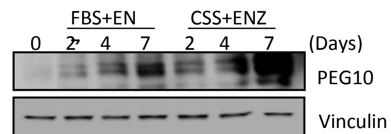
C
mRNA expression
Fold change relative to FBS Cond.
CHGA
NCAM1
NSE
SYP
FBS
CSS



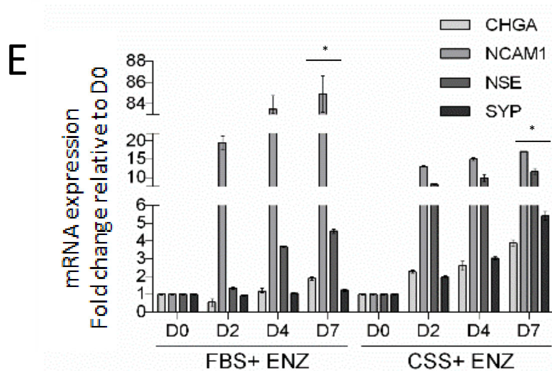
mRNA expression
Fold change relative to FBS Cond.
FBS
CSS



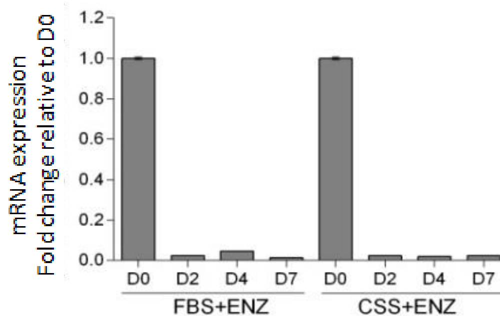
D
mRNA expression
Fold change relative to D0
D0 D2 D4 D7 D0 D2 D4 D7
FBS+ENZ
CSS+ENZ



FBS+EN
CSS+ENZ
0 2 4 7 2 4 7
(Days)
PEG10
Vinculin



E
CHGA
NCAM1
NSE
SYP
mRNA expression
Fold change relative to D0
D0 D2 D4 D7 D0 D2 D4 D7
FBS+ ENZ
CSS+ ENZ



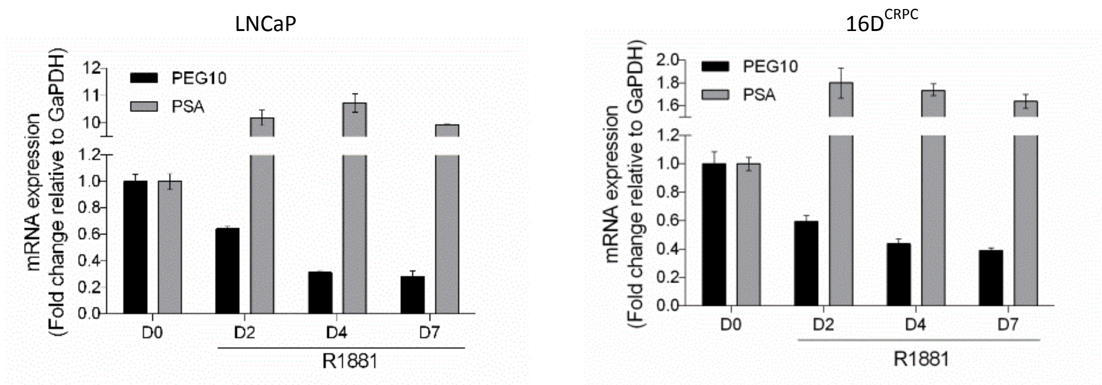
mRNA expression
Fold change relative to D0
D0 D2 D4 D7 D0 D2 D4 D7
FBS+ENZ
CSS+ENZ

Figure 2. 1 Positive PEG10 expression correlation with NE marker status in androgen-deprived LNCaP
A. Gene profile of PCa patient samples. PEG10 expression in prostate cancer is associated with NE marker expression and inversely correlated with AR-dependent gene expression. B. PEG10 expression increases in CSS media (androgen deplete) compared to the androgen replete FBS at both the mRNA and protein levels. C. NE marker expression is increased in LNCaP cultured in CSS media while PSA expression decreased, relative to androgen replete FBS. D. PEG10 expression increases at both the mRNA and protein levels in a time dependent fashion following enzalutamide therapy. Combination enzalutamide and CSS conditions result in a more pronounced increase in PEG10 expression. E. Corresponding to findings in D, increases in PEG10 expression due to conditions of ENZ and CSS results in multiple fold increases of all NE markers evaluated. As expected, the conditions of ENZ and CSS+ENZ result in abrogation PSA expression.

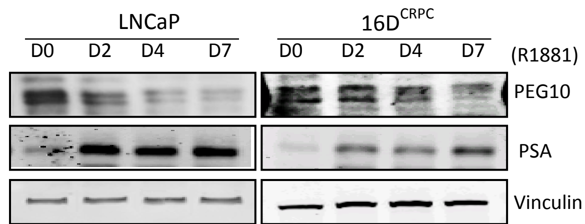
2.3.2 PEG10 and NE Markers Expression in Androgen -supplemented LNCaP and 16D^{CRPC}

In LNCaP or 16D^{CRPC}, androgen supplementation via 10nM R1881 reveals the inverse responses of PSA and PEG10. Addition of R1881 after seven days of androgen deprivation results in increase of PSA expression at both the mRNA and protein levels, while the PEG10 expression is reduced- both in a time dependent manner up to seven days (Figure 2.2A, B). PSA responses to androgen supplementation are more robust in LNCaP vs 16D^{CRPC}. Figure 2.2C demonstrates the NE marker response under conditions of androgen supplementation up to seven days. Expressions of all NE markers evaluated are markedly decreased following introduction of androgen in both LNCaP and 16D^{CRPC}. A PEG10-luciferase reporter assay using LNCaP demonstrates minimal activity in the presence of androgen (FBS ± R1881) (Figure 2.2D). However, with addition of ENZ, luciferase activity increases, and is highest with combination ENZ + CSS (8-fold increase with combination vs 6-fold with ENZ only). Chromatin immunoprecipitation (ChIP) for AR at the PEG10 and PSA promoters was performed, as both are known to harbor androgen responsive elements (AREs). Following androgen supplementation, AR binds to the promoters of both PEG10 and PSA in LNCaP and 16D^{CRPC} (Figure 2.2E).

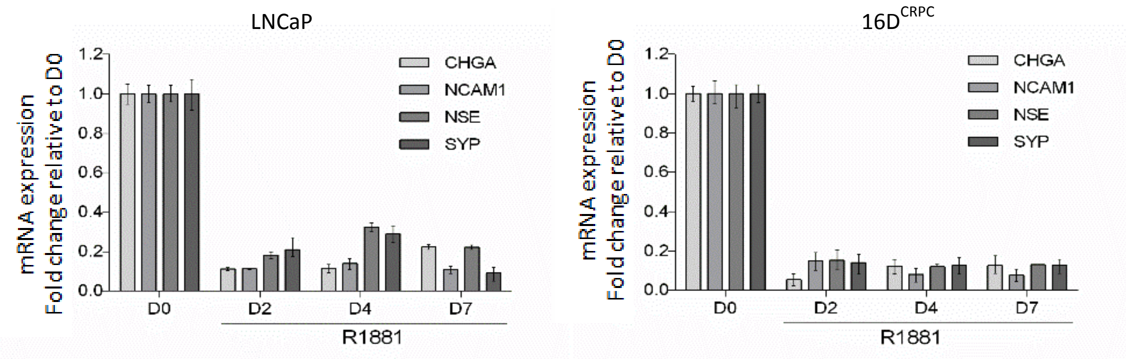
A



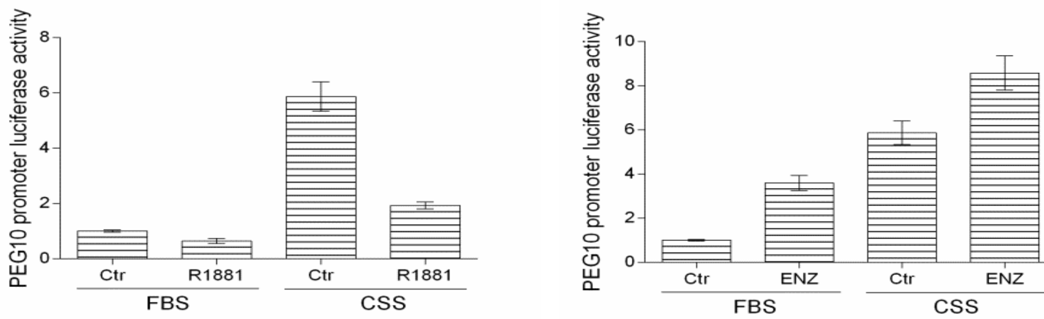
B



C



D



E

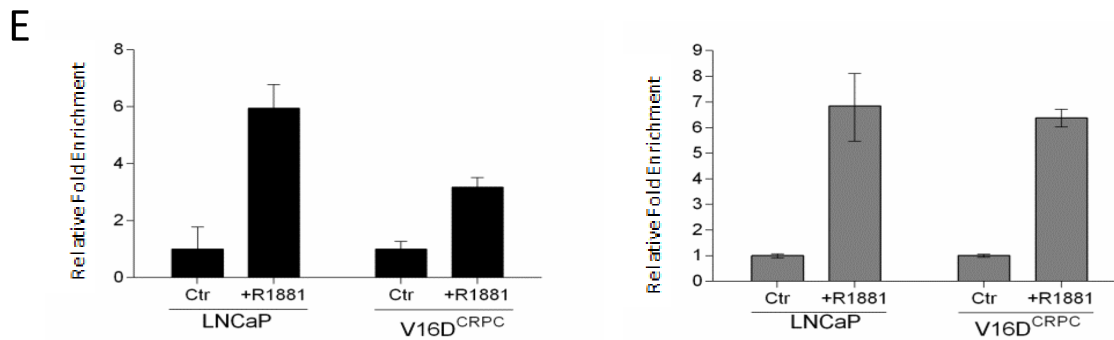


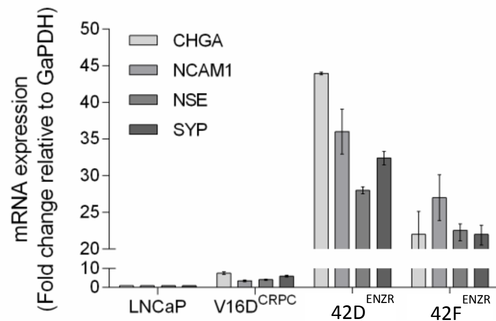
Figure 2. 2 PEG10 expression and NE markers status in androgen-supplemented LNCaP and 16D^{CRPC} (A-C), Androgen-depended AR binding on PEG10 (D,E)

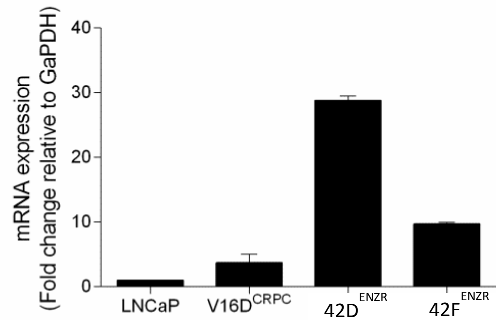
A. B. Reintroduction of androgen (10nM R1881) after 7 days of androgen deprivation recovers AR activity as shown by increasing levels of PSA. This is accompanied by a decrease in PEG10 expression at the mRNA and protein levels. C. Androgen supplementation significantly decreases NE marker status in LNCaP and 16D^{CRPC}. Decrease in NE marker expression is evident early and sustained with androgen supplementation up to seven days. D. In LNCaP with luciferase-PEG10 promoter construct, androgen supplementation results in decreased PEG10 promoter activity. PEG10 promoter activity is lowest when androgen concentration is highest (FBS + R1881) and highest in androgen-deplete CSS. Conversely, in conditions of androgen deprivation with 10uM ENZ, PEG10 promoter activity increases. Maximal PEG10 promoter activity corresponds to conditions of maximal androgen blockade (CSS+ENZ). E. Chromatin immunoprecipitation assay shows increased AR binding to the PEG10 promoter upon androgen supplementation in LNCaP and 16D^{CRPC}. As expected, androgen supplementation also increases binding of AR to the PSA promoter.

2.3.3 PEG10 and NE Markers Status in Enzalutamide-resistant (ENZ-R) Cell Lines

ENZR cell lines demonstrate a 20 to 40-fold increase in baseline NE marker expression, and a 10 to 30-fold increase in baseline PEG10 expression compared to LNCaP and 16D^{CRPC} (Figure 2.3A). 42D^{ENZR} has the highest baseline PEG10 expression which corresponds to the highest level of NE markers. In a similar fashion to previous figures, androgen supplementation in the ENZR cell lines reduces PEG10 expression while increasing PSA expression in a time dependent and dose dependent fashion (Figure 2.3B, Figure 2.4). Figure 2.3C demonstrates the corresponding loss of NE marker expression that occurs in ENZR cell lines following androgen supplementation. NE marker status in 42D^{ENZR} is attenuated to a greater degree than 42F^{ENZR}. ChIP assay for AR reveals increased binding of AR to the PEG10 and PSA promoters following androgen supplementation (Figure 2.3D).

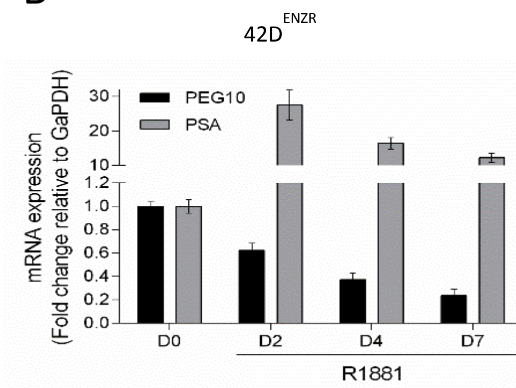
A



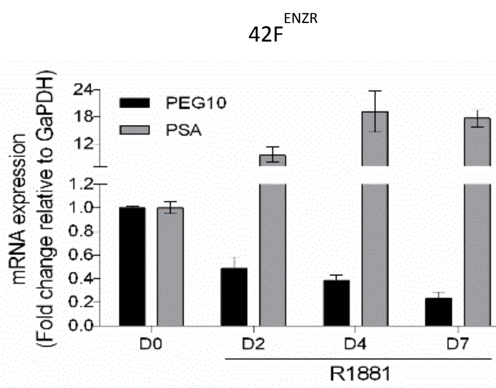


B

42D ENZR

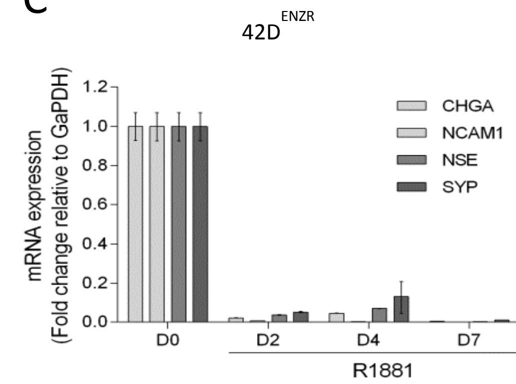


42F ENZR

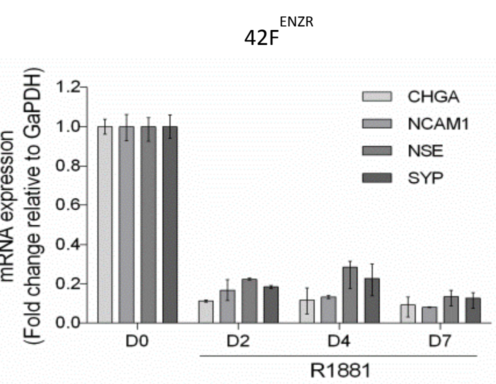


C

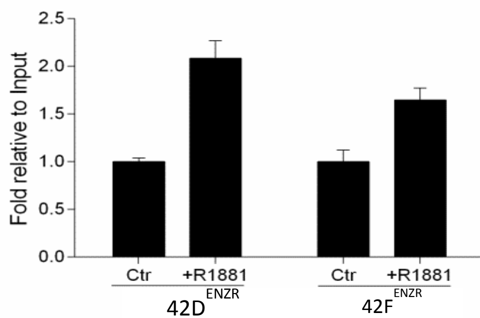
42D ENZR



42F ENZR



D



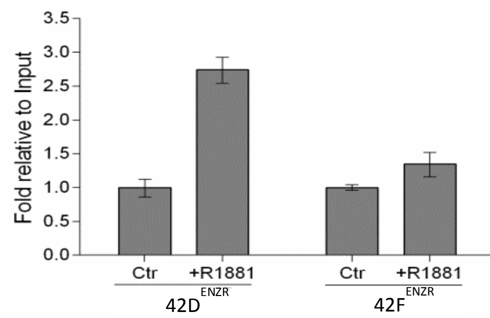


Figure 2. 3 PEG10 as an androgen regulated gene

A. PSA low, enzalutamide resistant cell lines 42D^{ENZR} and 42F^{ENZR} demonstrate elevated NE marker status at baseline, as well as increased PEG10 expression. B. Androgen supplementation in ENZR cell lines results in decreased PEG10 mRNA expression, but increases PSA expression over 10-fold in a sustained fashion over time. C. Correlating to findings presented in figure B., androgen supplementation in ENZR cell lines abrogates NE marker expression significantly. D. Chromatin immunoprecipitation (ChIP) demonstrates increased AR binding to the PEG10 promoter following androgen supplementation. R1881 supplementation also results in enhanced AR binding to the PSA promoter, serving as a positive control.

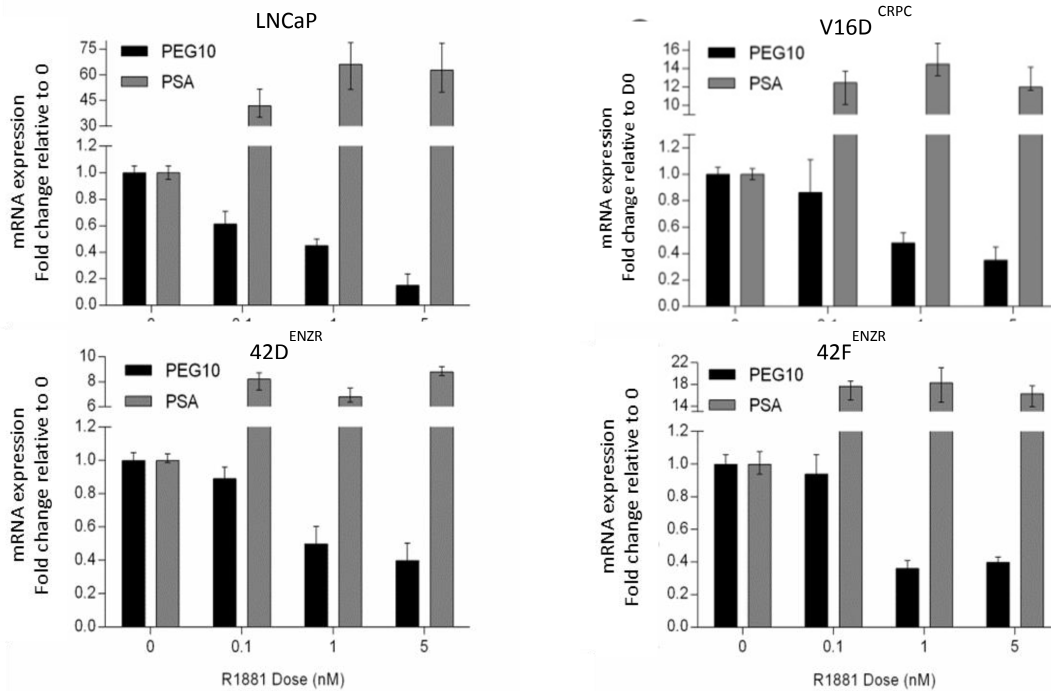


Figure 2. 4 Dose-dependent PEG10 response to R1881

Dose-dependent responses are measured following 72 hours of incubation with the various R1881 concentrations. Time-dependent response of PEG10 and PSA to R1881 is described in Figure 1 where a dose of 1nM is used.

2.3.4 The Role of PEG10 in Cell Cycle and Cell Proliferation

PEG10 knockdown was achieved via small-interference (siRNA) (Figure 2.5) and short-hairpin RNA (shRNA) (Figure 2.6). Figure 2.7 shows all shPEG10 clones we generated, with the most effective clones demonstrated in Figure2. 6A and B. shPEG10 results in effective silencing of PEG10 and abrogation of NE marker status in all cell lines evaluated. Across these lines, shPEG10 reduced cell cycle progression with increased G0/G1 and reduced G2/M (Figure 2.6C).

Proliferation measured via crystal violet assay in Figure 2.6D demonstrates significantly reduced proliferative ability of all cell lines tested following shPEG1

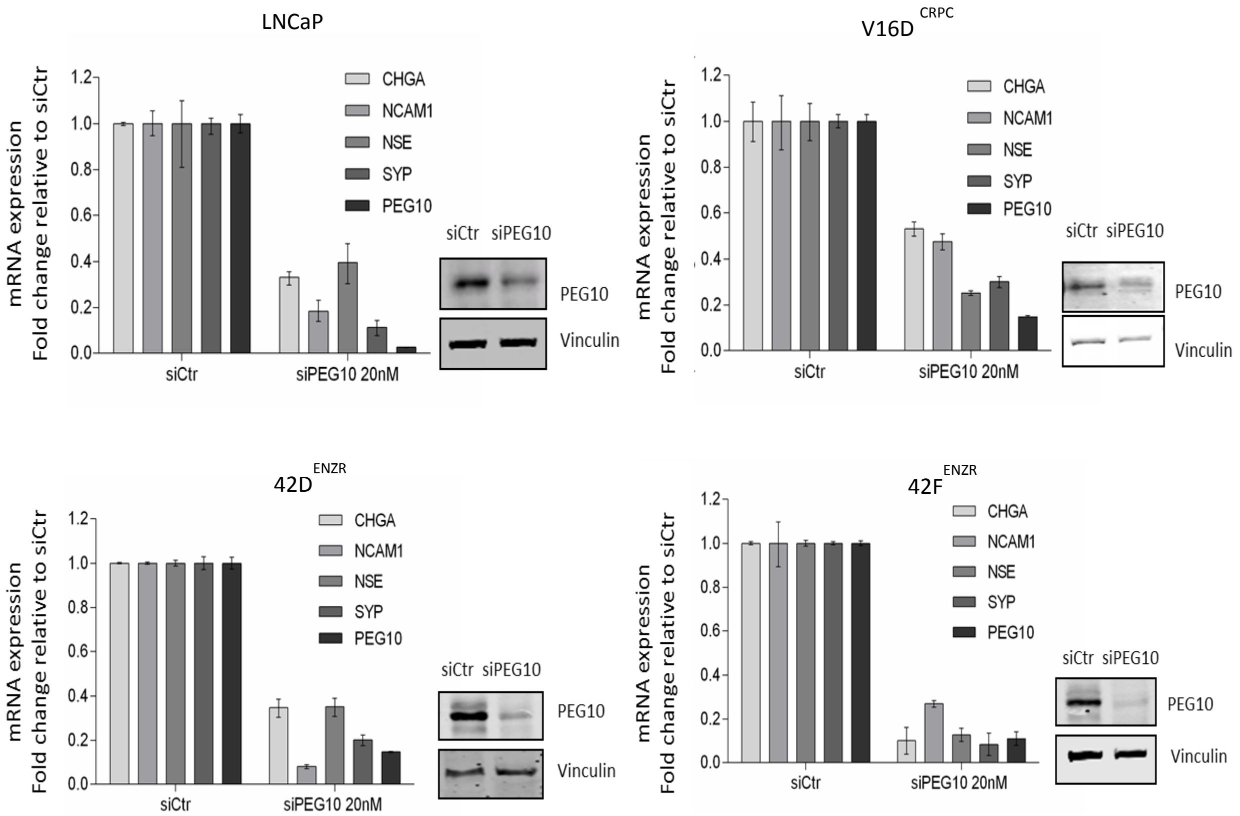
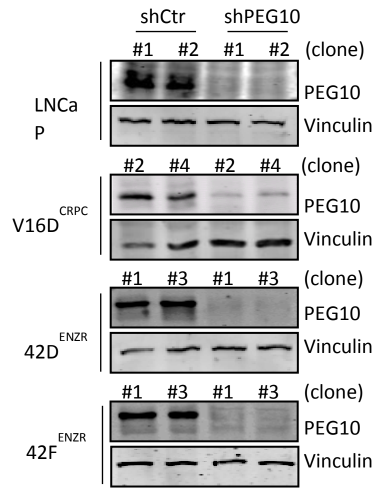


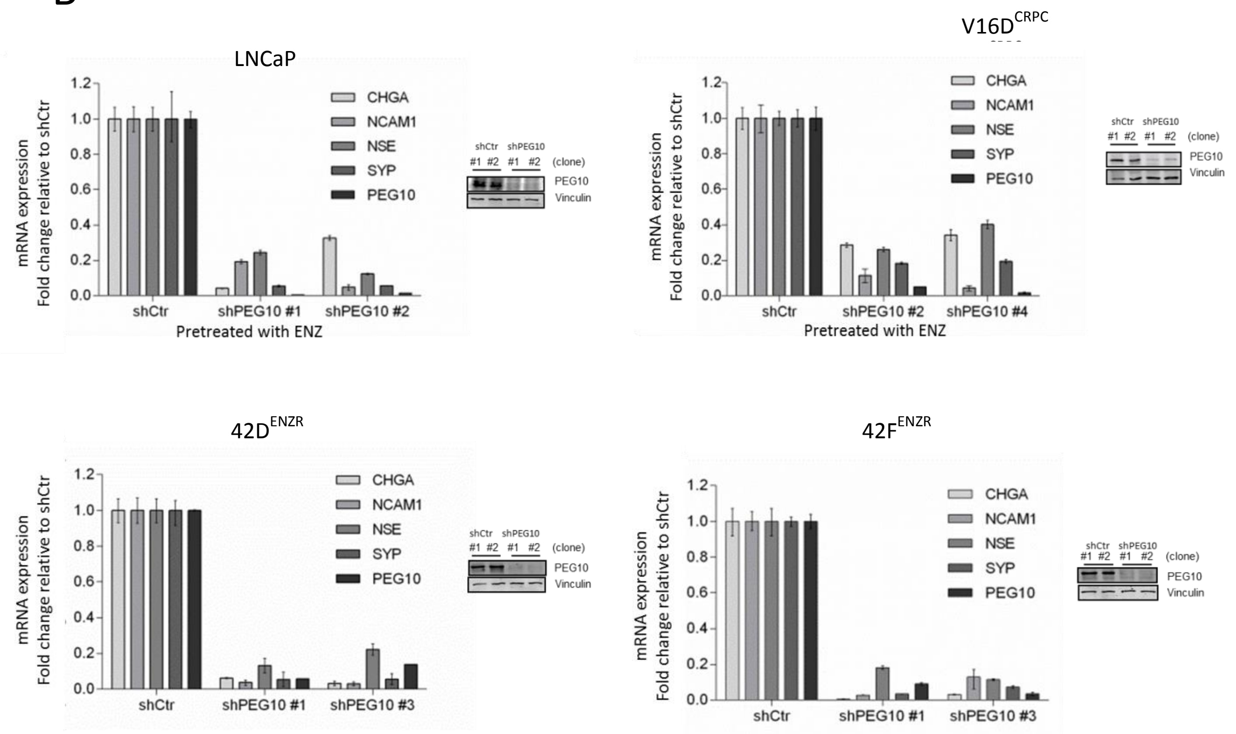
Figure 2. 5 Knockdown of PEG10 via siRNA *in vitro*

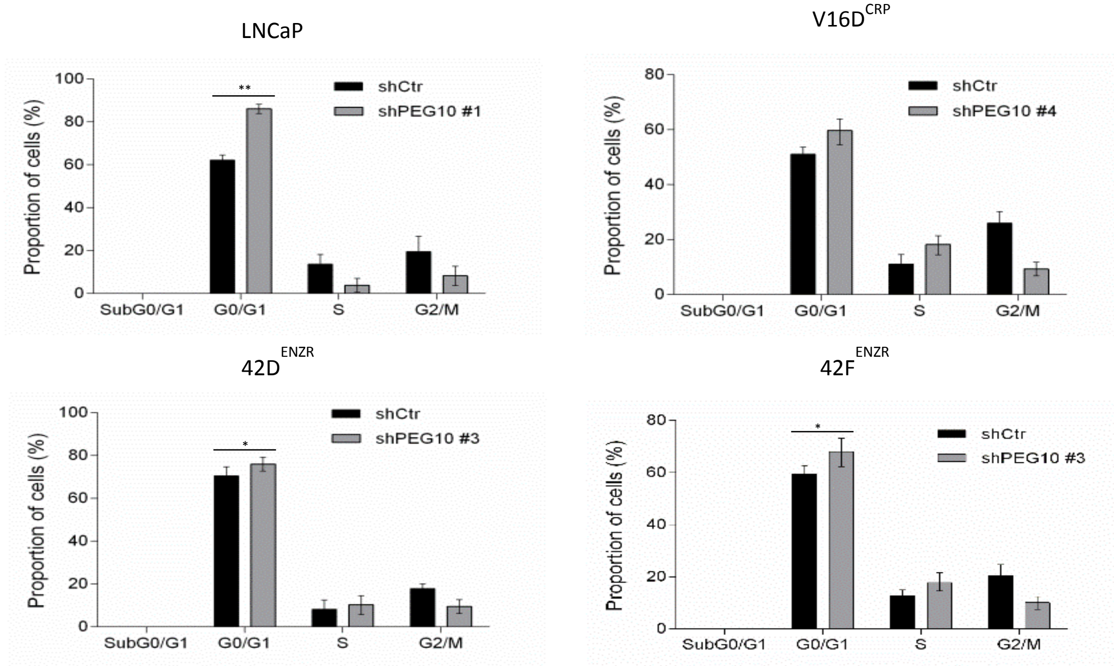
Knockdown of PEG10 was repeated several times using both short-interference and short-hairpin techniques. Both techniques achieve strong PEG10 knockdown.

A



B





D

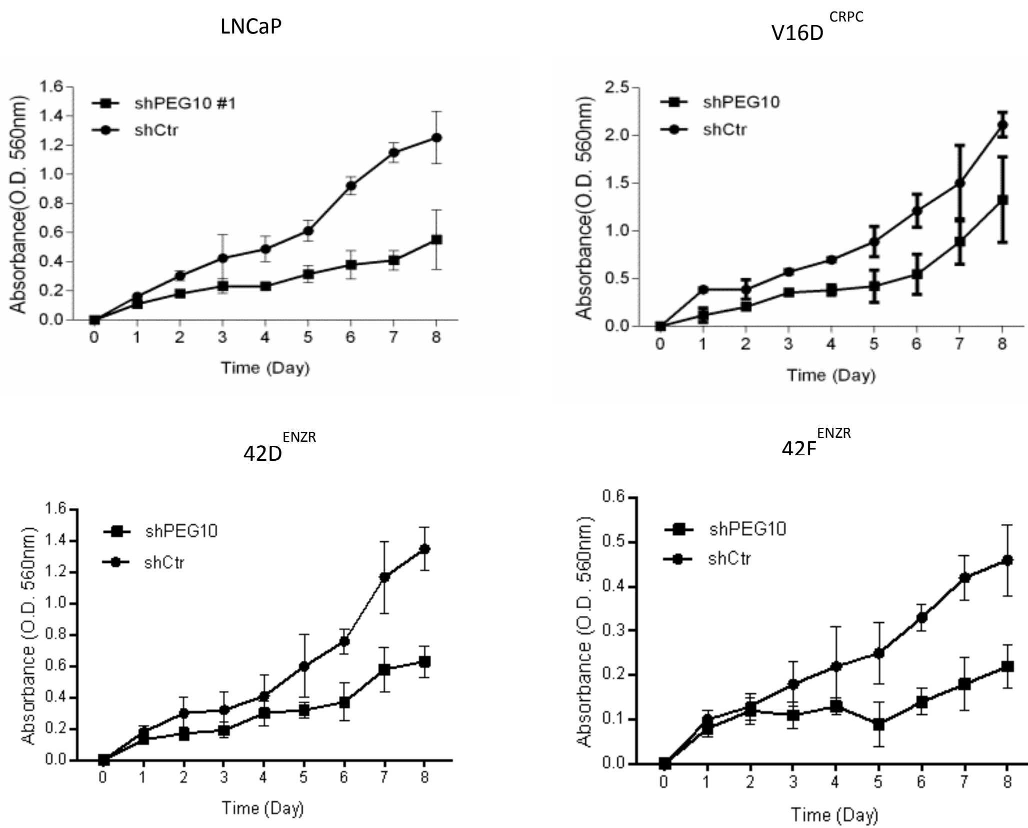


Figure 2. 6 The role of PEG10 on cell cycle control and proliferation in multiple cell lines
A. Stable PEG10 knockdown was achieved in multiple PCa cell types with shRNA (see supplemental figure 2 for siRNA results for all cell lines). B. Multiple shPEG10 clones each result in significant reduction in NE marker status in all evaluated cell lines compared to control. C. Cell cycle analysis reveals decreased mitosis and increased G0/G1 with shPEG10. D. Cell proliferation is significantly attenuated with shPEG10 across all cell lines.

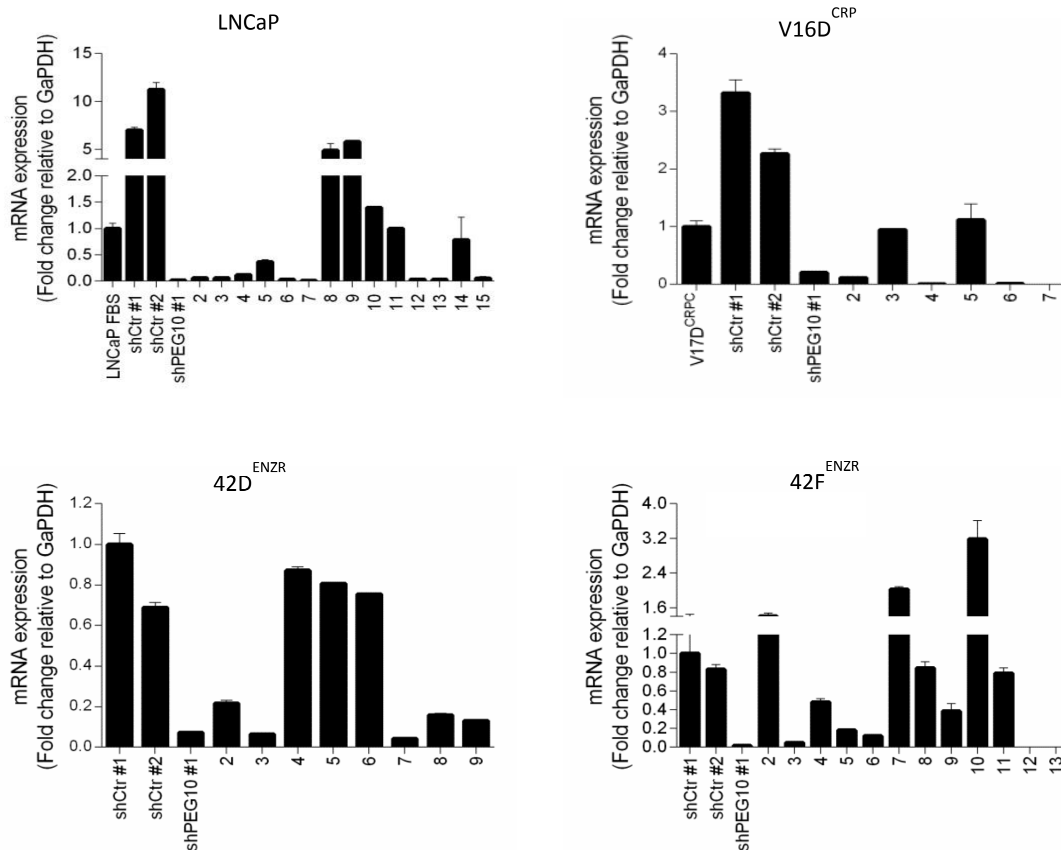


Figure 2. 7 Representative shPEG10 clones demonstrating variable knockdown of PEG10 activity
All shPEG10 clones resulting in the highest PEG10 knockdown were selected and used for analysis in figure 2.6 and for *in vivo* experiments in figure 2.8.

2.3.5 The Effect of Stable PEG10 Knockdown on *in vivo* Tumor Growth

A shPEG10 42F murine xenograft was established with significant attenuation of tumour growth compared to control (Figure 2.8A). Waterfall plot of tumour sizes between groups highlights four mice from the shPEG10 group which failed to grow any tumour beyond 10 weeks. Tumour tissue analysis confirms PEG10 knockdown and significantly reduced expression of CHGA, a representative NE marker.

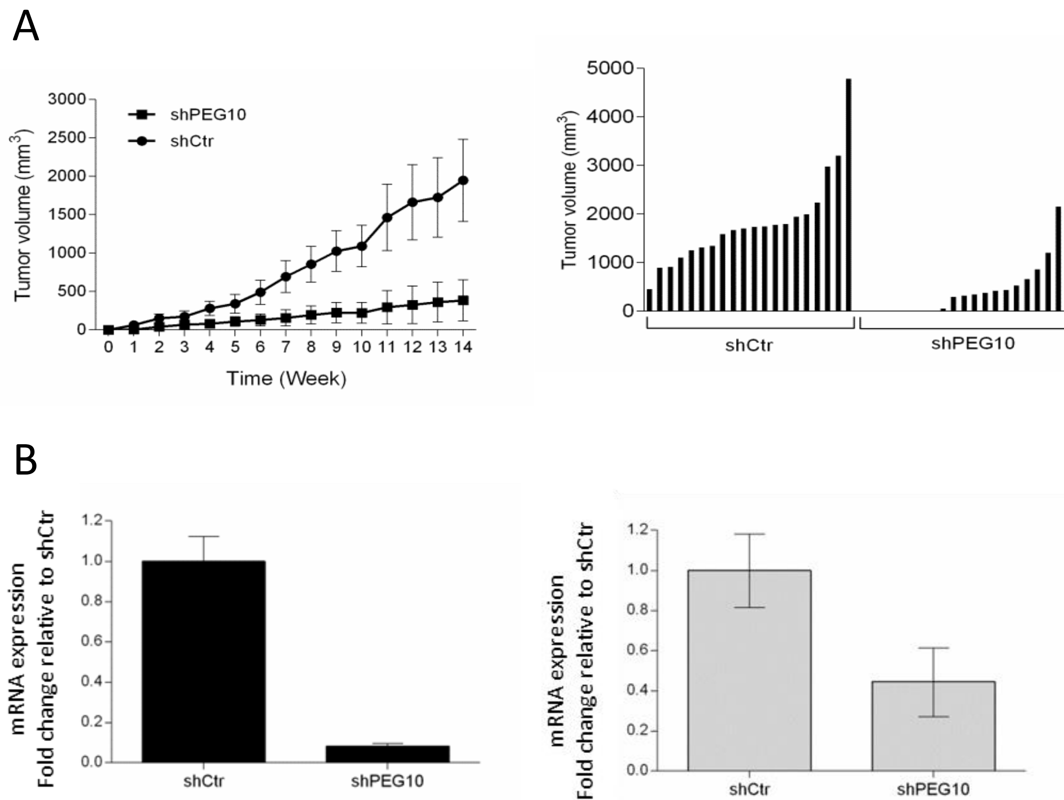


Figure 2. 8 Stable PEG10 knockdown in *in vivo*

A. $42F^{ENZR}$ xenograft with knockdown of PEG10 has significantly attenuated tumour growth compared to control. Waterfall plot demonstrates maximal tumour volumes between the two groups highlighting four animals in the shPEG10 group in which no tumour appeared. B. *In vivo* tissue analysis confirms knockdown of PEG10 and diminished expression of chromogranin A, a representative NE marker.

2.4 Discussion

There is a strong need for new therapies in CRPC, especially following multi-drug resistance and NE transdifferentiation which typically represents terminal disease. It has been suggested the actual prevalence of NEPC is under-recognized as routine biopsy sampling and/or confirmatory autopsy are typically not done in this population [103]. Increasing and earlier use of novel AR-axis directed therapies, including ENZ and abiraterone, has resulted in increased acquired resistance to these drugs, and increasing recognition of the NE phenotype [133]. While the mechanism by which neuroendocrine transdifferentiation occurs remains unclear, recent research has identified several key players in the NE transdifferentiation process. These include REST and SSRM4, nMyc and AURKA. While the exact mechanisms of how these genes govern the transdifferentiation process remains unknown, they present as attractive targets in clinical setting.

Early studies were pivotal in identifying that the expression of NE markers in PCa was inversely related to AR activity [83]. We therefore examined this in further detail with our cell models which recapitulate the cellular phenotype of advanced PCa (namely neuroendocrine features and enzalutamide resistance). Our study builds on previous studies that identified PEG10 as a driver of NEPC, and that PEG10 is negatively regulated by the AR [117]. Specifically, Akamatsu *et al*. demonstrated that PEG10 is a target during the neuroendocrine transdifferentiation process in the setting of TP53 and RB1 aberration and is derepressed by AR interference. We sought to investigate the relationship between AR signaling and PEG10 activity in prostate cancer cell lines that model enzalutamide resistance and display a NE phenotype, $42D^{ENZR}$ and $42F^{ENZR}$. Via ChIP assay in these cell lines, their parental line of origin (LNCaP), and the CRPC line $16D^{CRPC}$ that the AR binds the PEG10 promoter and that AR stimulation via synthetic androgen R1881 increased AR binding at the PEG10 promoter, with a resultant decrease in PEG10 gene activity and NE marker expression (Figure 2.2). These findings were observed in all cell lines evaluated. We identified several possible androgen responsive elements (ARE) in the promoter region of the PEG10 gene from publicly available data and hypothesized that AR binding acts as a repressor for PEG10 expression. AR ChIP at the PSA promoter served as an AR-dependent

positive control gene and indeed AR binding increased substantially at the PSA promoter following androgen supplementation, as expected. Combined with data demonstrating that terminal NE marker status is dependent on PEG10 activity, and that PEG10 expression decreased upon AR stimulation, these data strongly support the role of the AR as a negative regulator of PEG10 in our cellular model of NEPC. The role of the AR as a negative regulator has been shown for other genes involved in PCa progression including Sox2 [134]. Together, these findings support the clinical context of repeated and sustained inhibition of the AR-axis results in expression of AR-repressed genes, such as PEG10 which can then drive differentiation to a distinct disease phenotype in an AR-independent manner.

Akamatsu *et al* use the PC3 and DU145 lines as a model of NEPC phenotype. In these AR-negative lines, PEG10 expression was upregulated 250-fold for the highly metastatic PC3 line, and 25-fold for the less metastatic DU145 line, compared to LNCaP. Our modeling of ENZR represents another unique platform to model the NEPC phenotype, and we demonstrate that PEG10 activity is highly upregulated in these weaker AR-positive but ENZR lines. We found that $42D^{ENZR}$ had a 30-fold upregulation of PEG10 and the most pronounced NE marker status compared to LNCaP. The cell line $42F^{ENZR}$ demonstrated a 10-fold increase in PEG10 expression compared to LNCaP, with a less pronounced NE marker status compared to $42D^{ENZR}$ but increased compared to LNCaP. Further, we also demonstrated via ChIP assay that the canonically activated AR binds to the PEG10 promoter, even in the ENZR lines. These cell models may serve as a key platform to further investigate the transdifferentiation process, as the cells proliferate under pressure of ENZ, remain weakly AR positive, but also demonstrate markers of terminal NE differentiation associated with increased PEG10 expression.

Knockdown of PEG10 resulted in attenuation of NE marker status across high and low PEG10 expressing lines (Figure 2.6A and B). PEG10 silencing via shRNA techniques resulted in decreased cell proliferation and increased G0/G1 cell cycle (Figure 2.6 C and D). Figure 2.8 highlights the difference in *in vivo* growth in murine $42F^{ENZR}$ xenografts, where tumours with silenced PEG10 demonstrated significantly attenuated tumour growth. Waterfall plot of individual animal tumour growth in figure 2.8 B highlights that several animals inoculated with silenced PEG10 cell line failed to grow any appreciable tumour. These findings identify PEG10 as

a potential therapeutic target in advanced prostate cancers where NE differentiation or growth may be PEG10 dependent. Heat-map generated from PCa Genome Atlas samples confirmed the association between tumour PEG10 expression and NE marker expression in patient samples. (Figure 2.1 A). Utilizing *in vitro*, *in vivo* study design and corroborating our findings with human cancer databases, we have shown that PEG10 was one of the few genes that have been identified in multiple datasets as being upregulated during and following transdifferentiation [117, 103]. Figure 2.6 highlights the striking abrogation of NE marker status that is achieved with appropriate PEG10 knockdown *in vitro.* We also found that PEG10 knockdown affected cell cycle progression, with reduced G2/M phase. This effect on cell cycle control was also seen in human hepatocellular carcinoma models [135]. PEG10, although derived from a viral retrotransposon which has lost ability to self-splice, is required for placental development and murine PEG10 knockouts results in early embryonic lethality due to placental defects [136]. Interest in genes with restricted expression during placental development is increasing as a natural model of controlled invasion, mimicking the oncologic state [137]. Such genes may serve pivotal roles in driving coordinated invasion and immunosuppression 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.

This study adds to our understanding of the mechanisms involved in NEPC and the molecular underpinnings driving differentiation. We highlight the non-canonical role the AR plays in mediating a variety of cell pathways, namely its effect to repress the NE phenotype in prostate cancer. Limitations of our study include restricted characterization of PEG10 isoform type. It is recognized that full length and truncated PEG10 isoforms mediate different biological properties, namely proliferation and invasion, respectively. It is unclear the ratio of full length to truncated PEG10 in our study, and such characterization may serve to further elucidate the transdifferentiation process. For example, cell lines demonstrating enzalutamide resistance and an NE phenotype may become more dependent on a particular isoform type to maintain this phenotype. We also did not evaluate other cell lines of interest including the well described 22RV1 with a constitutively active AR due to loss of the ligand-binding domain, to evaluate AR PEG10 promoter interactions. Increasing variety in AR mutations is being described in advanced

clinical PCa, and whether certain mutations alter the repressive action of the AR at the PEG10 promoter (or other promoters for that matter) is unclear.

In conclusion, insight into NEPC, specifically its development from a conventional adenocarcinoma, is an increasingly recognized clinical scenario that needs further investigation. We have developed a cell model of this process which serves as a platform to characterize the drivers of the NE phenotype and can be used to characterize cell response to new treatments for this advanced PCa phenotype.

CHAPTER 3: Conclusion and Suggestions for Future Work

3.1 General Discussion and Conclusion

To this date, androgen deprivation therapy remains the only treatment option for advanced PCa. Due to the nature of the cancer cells to acquire resistance to the therapies however, the treatment only provides a short term survival benefit as the disease progresses to CRPC and NEPC.

The mechanism by which neuroendocrine transdifferentiation occurs remains unclear, but previous studies have identified an inverse relationship between the NE markers expressions and AR activity in PCa [83]. Akamatsu *et al.* also demonstrated that PEG10 as the target of neuroendocrine transdifferentiation in PCa, and in chapter 2 of this thesis we sought to investigate the role of PEG10 and its relationship with AR in our cell models that are representative of the ENZ-resistant CRPC displaying NE phenotype.

Through a series of experiments, we demonstrate that PEG10 expression is highly upregulated in ENZ-resistant models and that its expression can be manipulated through the control of AR activity. Our ChIP and luciferase activity data further confirm that AR binds to the PEG10 promoter to repress the transcription thereby inversely regulating the PEG10 expression.
Transient and stable knockdown of PEG10 in the models indicated decreased cell proliferation and reduced G2/M phase. These *in vitro* data are supported by our *in vivo* data, where PEG10 knockout mice demonstrated significantly attenuated tumor growth compared to the control mice.

Our cell lines represent a unique model where they express both ENZ resistance and NEPC phenotype. Through these cell lines, this thesis demonstrates PEG10 as a potential key marker for NE transdifferentiation. Furthermore, the results obtained from manipulating PEG10 expression suggests that progression to NEPC through NE transdifferentiation may be prevented or even reversed through AR and PEG10 targeting. These targets may open doors to a new therapeutic regime against NEPC.

3.2 Suggestions for Future Work

The work presented in this thesis demonstrated the mechanisms which AR negatively regulates PEG10 in Enzalutamide resistant prostate cancer setting. Targeting PEG10 expression using shRNA or siRNA reduces cell proliferation *in vitro* and attenuates tumor growth *in vivo*. Thus, PEG10 may be an excellent candidate as a therapeutic target to treat Enzalutamide-resistant CRPC patients who are progressing to NEPC.

However, PEG10 is not an easy molecule to target as it makes various isoforms under different conditions. Of these, some of the isoforms play a role in pro-survival while others play a role in pro-apoptosis. The state of when these isoforms are produced is not yet clearly understood.
Also, PEG10 does not have a pocket to target with a small molecule, and the fact that its crystal structure is not yet available for small molecule modeling makes it challenging to design readout.

Perhaps for the time being, targeting PEG10 with siRNA using drug delivery system or by anti-sense could be a useful strategy. Further studies on PEG10 at both transcriptional and translational level under various subpopulation of PCa would help contribute to the knowledge on this gene, thereby developing a therapy for NEPC.

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