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Effects of Hormonal Withdrawal on Prostate Cancer Progression

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Philadelphia College of Osteopathic Medicine

Biomedical Sciences

Effects of Hormonal Withdrawal on Prostate Cancer Progression

A thesis in Biomedical Research by Jacqueline C. So

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Submitted in Partial Fulfillment of the Requirements for the Degree of MS

November 2016
We have read and examined this manuscript and certify that it is adequate in scope and quality as a thesis for this MS degree.

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Abstract

Prostate cancer, or prostatic carcinoma (PrCa), is the second leading cause of cancer-related deaths in men in the Western world. One in seven men are expected to be diagnosed with the disease in the United States. Suppressing androgen/androgen receptor pathway is a first-line therapeutic treatment known as Androgen Deprivation Therapy (ADT). Most patients respond well to ADT, but an overwhelming majority of PrCa progress to an advanced stage of PrCa independent of hormone, known as Castration Resistant Prostate Cancer (CRPC). There is currently no known cure for CRPC. In this study, the androgen-sensitive LNCaP cells served as an in vitro model of ADT. LNCaP cells were grown without hormone for twenty days and were collected every two days; the levels of AR protein and its target gene PSA mRNA were examined over this interval. Full-length AR protein levels appear to remain unchanged throughout the twenty days, but hormone withdrawal leads to lower PSA levels. In addition, we also observed the effects of hormonal withdrawal on cell morphology; LNCaP cells adapted a shape resembling neuronal cells with long and slender connections between them. Furthermore, the appearance of truncated AR isoforms occurred during the extended hormonal withdrawal. These findings suggest that hormonal withdrawal not only suppresses AR activity without affecting AR protein levels in prostate cancer cells, but also results in the morphological change.
Aims:

• To investigate androgen deprivation on LNCaP cells for twenty days
• To observe any morphological changes
• To decrease time interval between collections from preliminary laboratory work of five days to two days in attempt to intercept possible prostate cancer cell conversion into CRPC
• To evaluate changes in AR levels and activity, utilizing PSA mRNA as an indicator of AR activity
• To provide any information on long-term withdrawal effects in LNCaP cells which have been deprived of hormone for thirty, sixty, seventy, and seventy-four days
**Introduction:**

Among the various forms of cancer, prostate cancer (PrCa) is the most ubiquitous form diagnosed in men and the second-leading cause of cancer-related deaths. In the United States, one in seven men will be diagnosed with the disease (1). PrCa is currently a disease with no cure, but androgen deprivation therapy (ADT) is one of the first-line treatments (2). However, an overwhelming majority of PrCa progress to an advanced stage, termed castration resistant prostate cancer (CRPC). Prognosis is poor for men whose PrCa has progressed into CRPC (3) due to the ability of these prostate cancer cells to proliferate and survive despite being deprived of androgens that are normally fundamental to the cells in the prostate. At the crux of prostate cell proliferation is the Androgen Receptor (AR).
Figure 1 Androgen Receptor. As shown in figure 1, the AR gene is located in the X chromosome with 8 exons. The transcription factor formed by an amino-terminal transactivation domain (NTD) (exon 1), a carboxyl-terminal ligand-binding domain (LBD) (exons 4-8), a DNA-binding domain (DBD) (exons 2-3), and a hinge region between the DNA-binding DBD and the steroid binding LBD that participates in nuclear localization and degradation (4).

The androgen receptor (AR) is a nuclear transcription factor and plays an essential role in the development and maintenance of the prostate. AR gene is located on the X chromosome with 8 exons. This transcription factor is formed by an amino-terminal transactivation domain (NTD) (exon 1), a carboxyl-terminal ligand-binding domain (LBD) (exons 4-8), a DNA-binding domain (DBD) (exons 2-3), and a hinge region between the DNA-binding DBD and the steroid binding LBD that participates in nuclear localization and degradation (4). Androgen binds to the C-terminal LBD.
Androgen receptor signaling

Figure 2 AR signaling. AR is associated with heat shock proteins (HSP) in the cytoplasm when unbound. Upon binding of androgen, the AR undergoes a conformational change and translocates to the nucleus as a homodimer, wherein AR binds to AREs (Androgen Responsive Elements) and transcription follows.

Normally, AR is located in the cytoplasm, associated with Heat Shock Proteins (HSP). Upon binding of androgens, such as 5α-dihydrotestosterone (DHT) or testosterone, the AR undergoes a conformational change and translocates to the nucleus as a homodimer. In the nucleus, AR binds to promoter/enhancer sequences on AREs (Androgen Responsive Elements) and regulates gene expression transcriptionally. AR affects a multitude of downstream target genes.

One of the many well-documented downstream target genes of AR is prostate specific antigen (PSA), which can be used as a serum biomarker for PrCa (5-7). PSA is a proteolytic enzyme belonging to the kallikrein family of serine proteases.
and produced from the prostatic epithelium. Due to androgen receptor’s crucial role in the maintenance and development of the prostate, PSA levels have been used to reflect and monitor AR activity. PSA normally remaining within the prostate but passes through the gaps into the bloodstream in patients with prostate cancer (5). Thus, targeting AR pathway via androgen withdrawal is a first line treatment. Though hormone suppression increases PrCa patient survival for several years (8), the tumor will progress and eventually relapse to a metastatic, castration resistant phase.

Figure 3 Primary and redundant AR pathways. Primary pathway with androgen binding (DHT) and redundant pathways via IL-6R and IGF-1, EGF are depicted. Adapted from Lonergan PE and Tindall DJ (30).

AR nuclear translocation upon binding of androgens is the primary pathway, but androgen withdrawal can result in AR translocation independent of androgen via
alternative, redundant pathways (Figure 3; 9-11). Selective pressures encourage activity in interleukin-6 (IL-6) and insulin-like growth factor (INF-1) or epidermal growth factor (EGF) pathways. IL-6 is a pro-inflammatory cytokine circulating in elevated levels in patients with obesity as well as prior to Type II diabetes mellitus (Type II) onset. In addition, conflicting studies are available on Type II providing a protective effect on prostate cancer development, but pooled overall risk from a meta-analysis of forty-five studies (twenty-nine cohort studies and sixteen case-control studies) from 1971-2011 by Bansal D et al. showed decreased risk of prostate cancer in patients with Type II (12).

There appears to be a cross-talk between redundant AR pathways. In the absence of androgen, IL-6 drives prostate cancer cell proliferation (13) with evidence suggesting continued AR signaling. Furthermore, IL-6 administration has been shown to activate tyrosine kinase receptor EGFR and induce expression of PSA. The proto-oncogene ERBB2 (also known as CD340 or HER2/neu) was shown to be overexpressed in PrCa progression towards metastatic androgen independence (10). While IGF-1 has been shown to stimulate the natural proliferation of prostatic epithelial for normal maintenance and growth, an overexpression of IGF-1 appears linked to PrCa initiation while decreased levels are associated with CRPC (14). Similarly, EGF has a role like IGF-1 as a regulator of normal growth in prostate cell.
However, while overexpressed IGF-1 appears to encourage initiation of diseased prostate but not CRPC, overexpression of EGF seems to protect diseased prostatic cells from apoptosis by inducing BAD phosphorylation via two redundant signaling pathways: Ras/MEK and Rac/PAK1, resulting in phosphorylation on Ser 112 and 136, respectively (11). BAD (BCL-2 associated death promoter) is a pro-apoptotic protein of programmed cell death pathway regulators. Thus, BAD can be inactivated by phosphorylation, making either Ras/MEK or Rac/PAK1 pathways sufficient to protect cells from apoptosis, but this also means both pathways must be effectively inhibited in order to block abnormal prostatic cell growth. Essentially, circulating levels of IL-6 or IGF-1/EGF are found elevated in PrCa. Decreased levels of IGF-1, upregulated levels of EGFR (15), or increased levels of ERBB2 indicate androgen independent PrCa advancement towards CRPC utilizing the redundant AR pathways. Gioeli et al. and Price et al. also found constitutively active ERK/MAPK in advanced CRPC, providing hints that alternate pathways become implicated in the progression of PrCa towards CRPC (16, 17).

There is a need for further advancement in the context of CRPC therapies. Persistent AR nuclear translocation in the absence of hormone can drive prostate cancer cell proliferation and eventually become castration resistant. Current treatment of CRPC are two drugs: enzalutamide (an AR antagonist) and abiraterone (a CYP17A1 inhibitor). While Phase III clinical trials initially showed
a survival advantage in patients with CRPC under these two treatments, patients generally relapsed within a year of treatment, and AR appears to remain active in the relapsed tumors. In studies performed using LNCaP cells and xenografts, an increase in enzymes (such as CYP17A1) necessary for *de novo* steroid synthesis were found in CRPC sublines. While the increase in these enzymes could generate measurable levels of hormone, such changes have not been observed in clinical samples (18). However, recently updated results from the European Commission’s CORDIS (Community Research and Development Information Service) at the end of July 2016 addresses a shift towards investigating resistance to enzalutamide and abiraterone. Hoftland et al. utilized mice engrafted with human prostate cancer xenografts and ex vivo patient samples. The results of this ASPIRE-PC study (A novel Androgen Synthesis Pathway in treatment-REsistant Prostate Cancer) were presented at the European Congress of Endocrinology and found flux into an alternative adrenal androgen pathway active within prostate cancer cells that appear to depend on CYP17A1 activity (19, 20).

Identifying CRPC incidence by ethnicity and location has become somewhat problematic due to an inability to explain disparities despite adjusting for varying socioeconomic, demographic, and co-morbid factors. African-American (AA) men have the world’s highest incidence of PrCa and more than twice the risk of non-Hispanic men in presenting with CRPC. In 2001, Hoffman et al. (21) found
clinically advanced stages of the disease detected more frequently in AA men than both Hispanic and non-Hispanic white men. However, after adjusting for socioeconomic, clinical, and pathological factors, increased relative risk of advanced PrCa was found in Hispanic but not AA men. African-American men still had a statistically significant increased rate of advanced disease even with higher socioeconomic status. African-American men appear to present with more aggressive PrCa due to the highest overall proportion of poorly differentiated PrCa in all clinical stages except for T2 (tumors confined to the prostate with suspicious digital-rectal examinations but no evidence of metastases or positive scans).

A more recent study in 2015 by Spratt DE and Osborne JR assessed recently published SEER data (National Cancer Institute’s Surveillance, Epidemiology, and End Results Program) to analyze CRPC risk in all reported ethnicities and locations in the United States. African-American men to retain an independently increased risk for CRPC regardless of access to health care (23), which remains consistent with the results discussed by Hoffman et al. from 2001 (21). This finding also takes into account recent advances in PrCa treatment that were not previously available during Hoffman et al.’s 2001 study, including data from several landmark phase III randomized controlled trials—some of which were performed by European centers. Additionally, the authors acknowledge the importance of supporting centers serving large African-American populations,
such as the North Carolina-Louisiana Prostate Cancer Project and the US FDA Office of Minority Health (created in 2010) in order to prioritize directed efforts towards PrCa therapies in AA men.

Another study also released in 2015 by Bell KJL et al. performed a meta-analysis of all autopsy studies with systematic histological examinations of men who had no history of pre-existing prostate cancer—twenty-nine studies in more than twenty countries over a period of sixty years (24). Bell and colleagues reported similar findings to previously cited reports which also examined age-specific prevalence with respect to race, finding lowest prevalence in men of Asian descent, intermediate prevalence in men of European descent, and highest prevalence in men of African descent. Ultimately, the increased incidence of CRPC in African-American men remains unknown.

One possibility of higher incidence in advanced stage PrCa in newly-diagnosed African-American men is that PrCa is a disease of aging, and because men are generally not encouraged to begin screening until their fifties, African-American men may have an earlier onset of the disease than other racial groups and may not catch the cancer until it has already progressed for years. The American Cancer Society has specifically identified AA men as a high-risk group and recommends annual screenings beginning at age forty-five for men in this category (25). Whitney et al. (26) further attempt to differentiate between CRPC
in both a non-metastatic (localized) and metastatic form with respect to race. Whitney et al. conclude a lack of association between race and metastases once age of prostate cancer diagnosis is taken into account. However, although there appears to be no racial difference of worse outcomes in the later metastases, AA men are younger at the time of prostate cancer diagnosis, which alludes to the necessity of being diagnosed earlier than other races in order to undergo effective treatment to avoid progression towards metastases.

In considering general survival of CRPC cells, there are a variety of mechanisms: local androgen synthesis (maintenance of residual hormone concentrations within a tumor), AR amplification (AR overexpression which can increase sensitivity to low androgen levels), change in ratios between co-activators and co-repressors (changing the co-regulatory molecules which regulate ligand sensitivity and AR stability), AR mutations that can broaden ligand specificity, and alternative splicing to generate different forms AR variants (truncated isoforms). AR mutations are rare in untreated, early PrCa but common in patients with CRPC previously treated with a first-generation anti-androgen (27). Of the possible AR isoforms, splice variant AR-V7 (Figure 4) is the best studied.
The structural differences between full length AR and splice variant AR-V7 can be seen in Figure 4. AR-V7 is a shorter, fragmented version of the full-length AR with intact transactivation and DBDs but not the LBD. Since AR-V7 lacks the C-terminal LBD (AR\(\Delta\)LBD), it serves as a transcription factor in a ligand-independent manner (28, 29).

Since the phase of hormone withdrawal precedes the phase of cancer progression into metastasis (30), it is imperative to find a connection between ADT and CRPC development; in particular, withdrawing hormone on an androgen-sensitive PrCa cell line will provide meaningful clues towards this progression. In our own lab, preliminary work performed by Peter Wieczorek found a change in PSA mRNA levels (Figure 5).
Figure 5 PSA levels in hormone-withdrawn LNCaP cells for 30 days. GAPDH as internal control. RT-PCR performed by Peter Wieczorek.

The preliminary study found caveats in withdrawing hormone from cells dependent upon androgen, including discovering that the five-day observation intervals were too large to see detailed trends in the observed changes from days five to fifteen without hormone.

Figure 6 Neuroendocrine transdifferentiation. Two different neuroendocrine markers (ChrA and NFM) show a change in shape of LNCaP cells starved of hormone for five days. Controls in upper level of both A and B. Farach et al.

Farach et al. treated LNCaP cells with serum starvation (0.1% FBS) for five days. Neuroendocrine (NE) markers Chromogranin A (ChrA) and Neurofilament M (NFM) were used to show changes in morphology (31) with NE transdifferentiation—morphology resembling neural cells (Figure 6).
Thus, we decided to withdraw hormone from LNCaP cells for twenty days and collect cells every two days. In our study, we are interested in the effects of hormone withdrawal on morphological shifts, AR protein levels and activity (utilizing PSA mRNA as an indicator), as well as potential splice variants. To learn the effect of long-term withdrawal on LNCaP cells, we monitored the effect of hormone withdrawal for thirty, sixty, seventy, and seventy-four days.
**Materials and Methods:**

**Materials.** LNCaP cells were purchased from the American Type Culture Collection. RPMI 1640 media with phenol-red and L-glutamine, and RPMI 1640 media with L-glutamine but without phenol-red were purchased from Corning Cellgro. Fetal bovine serum and charcoal treated fetal bovine serum were purchased from Atlanta Biological. One hundred X antibiotic/antimycotic was purchased from Gibco Life Technologies. Trypsin (0.03%) was purchased from PromoCell. Ten cm plates were purchased from CellTreat. Cells were counted using a Countess ii machine. Cell counting chamber slides and Trypan Blue dye purchased from Invitrogen. RNeasy purification kits were purchased from Qiagen. Superscript II Reverse Transcriptase, iBlot, 0.1M DTT, 10mM dNTPs, qPCR, and PCR primers for PSA and GAPDH were purchased from Invitrogen. qPCR reagents were purchased from TaKaRa. Fast Western kit, BCA assay kit, and 96 well plates were purchased from Thermo Fisher Scientific. Precision Plus Streptactin HRP-conjugate and Protein WesternC Standard were purchased from BioRad. From Invitrogen, antibodies AR-N20, Beta-tubulin, and AR-C19 were also purchased. Antibody AR-V7 was purchased from Abcam. GoTaq Flexi DNA polymerase was purchased from ProMega. All purpose Hi-Lo DNA marker purchased from Bionexus, Inc. All media was kept at 4°C. Enzymes and primers were kept at -20°C. RNA, cDNA, and protein lysate were stored at -30°C.
**Cell Culture & Treatment.** LNCaP cells were cultured in 10cm petri dishes in 10mL of RPMI 1640 media with phenol-red and L-glutamate and supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic. Cells were maintained in a fisher scientific incubator at 37°C and 5% CO₂. When cells grew to 80% confluence, they were shifted to either media with regular FBS (RPMI 1640 media with phenol-red and L-glutamate, supplemented with 10% Fetal Bovine Serum and 1% antibiotic/antimycotic) or media with charcoal-treated serum (RPMI 1640 media with L-glutamate and without phenol-red, supplemented with 10% Charcoal-treated Fetal Bovine Serum and 1% antibiotic/antimycotic). Photos documenting of cell shape changes were taken under microscope with 4x, 10x, 20x, and 40x magnification using consistent settings across all days.
Cell Counting and collecting.

As depicted in Figure 7, collection periods begin with six plates of cells. Cells were collected every two days and split every other collection. The first plate is collected, serving as Day 0; five plates of cells remain. After two days, a second plate is collected, serving as Day 2. For sub-culturing, four plates remain after this collection and they were trypsinized, re-suspended and combined into a 50mL conical tube and centrifuged at 3000 rpm for five minutes. Excess media was aspirated and re-suspended into 12mL of growth media. Then, 2mL of cell suspension was combined with 8mL of growth media and plated to 10cm plates.
For each collection, cells were trypsinized and re-suspended in 3mL of their growth media. A portion of the re-suspended cells and Trypan Blue dye were combined in a 1:1 ratio of 20uL each onto the Countess cell counting chamber slide and analyzed in the Countess ii machine. The remaining re-suspended cells were centrifuged at 3000 rpm for 5 minutes. Media was aspirated with 1mL leftover, and the cells were re-suspended in the remaining 1mL media; 500uL of this new suspension was aliquoted to two eppendorf tubes. Transferred cells were centrifuged at 2500 rpm for minutes and excess media was aspirated and the pellets were stored at -30°C for Western blotting or RT-PCR.

**RNA Purification & RT-PCR.** RNA was extracted with an RNeasy kit from Qiagen as per manufacturer’s instruction. Total RNA concentrations were quantified using a Nanodrop 2000. After verifying the purity and concentration of the RNA, cDNA was prepared using 5µg of RNA by adding appropriate volumes of RNA, RGDD, and 10µM random primers (9 bp) to thin-walled PCR tubes; samples were then denatured at 65°C for 5 min to break hairpins and secondary RNA structures before cDNA generation. Mastermix including 1st Strand Buffer 5X, Reverse Transcriptase, 0.1M DTT, and 10mM dNTPs were added to each sample before undergoing cDNA generation in the thermocycler: 25°C for 10min., 42°C for 50min., and 70°C for 15min. cDNA generated from this process was then diluted five times with RNase-free water and used as a template for PCR. 5µlof cDNA was added to new, thin-walled PCR tubes in preparation for
adding the appropriate sub-mastermixes afterwards. Mastermix with 5X Green Buffer, GoTaq flexi DNA polymerase, RGDD, 25mM MgCl₂, and 10mM dNTPs were added to each sub-mastermix, which had either PSA (5’-AGCACCAGCCACCAACCT GC-3’) or GAPDH(5’-ACAGCCTCAAGATCAGCAA-3’) primers. The PSA primers were prepared from [100µM] stock, and the GAPDH primers were prepared from [50µM] stock. Primers were diluted to a working concentration of 10µM before being added to the sub-mastermixes, which were then distributed to the respective samples each with 1µg of cDNA and placed in the thermocycler: 29+ cycles of 95°C for 1min, 60°C for 1 min, 72°C for 1min.

**Gel Electrophoresis.** PCR samples were separated on a 1% agarose gel (10µg Ethidium Bromide). All-purpose Hi-Lo DNA ladder was used as a molecular weight marker, and the gel was run at 70-71V for 40 minutes.

**cDNA test.** To determine whether cDNA was successfully reverse transcribed, a cDNA test using gel electrophoresis was performed with cDNA samples of interest: 10uL of sample was combined with 5uL of 5X Green Buffer in a PCR tube, and all 15uL were pipetted into their respective wells. After the gel finished running, results were visualized using the QuantityOne software on a VersaDoc machine to detect smears, indicating successful reverse transcription.
Western Blot. Cell lysate was made from the cell pellet using RIPA (1M Tris-Cl, 5M NaCl, 100% NP40, 10% DOC, 10% SDS, 0.1M DTT, 0.1M PMSF, and 100X PIC) for 20 minutes on ice and centrifuged for 45 minutes at 15,000 rpm. Protein-rich supernatant was transferred to new tubes and the cell debris was discarded. Protein concentrations in the lysates were measured by BCA assays. Lysate with 50µg protein was prepared with appropriate amounts of 2X SDS and RGDD before denatured at 95°C for 5 minutes. Proteins in the lysates were separated by SDS-PAGE. 10µL Fast Western Precision Plus Protein Standard was loaded into a well. After the run-time finished, the gel was transferred to a nitrocellulose membrane using an iBlot. The membrane was briefly covered in Ponceau Red in order to detect protein and determine whether the protein successfully transferred. The blot was then blocked with Fast Western Antibody Diluent for 1 or more hours before adding the primary antibody (AR N-20, AR-C19, or AR-V7) in a dilution of 1:5000 and incubated overnight on a rocker at 4°C followed by three 15-minute washes with PBS-tween (0.1%). Afterwards, 0.55µL Precision Plus Streptactin HRP-conjugate, 0.5mL HRP conjugate, and 5mL diluent were added to the membrane for 10 minutes followed by washing three times with PBS-tween for 15-minutes per wash. Clarity Western ECL Substrate was used to visualize the blot via chemiluminescence. Quantity One software generated images through densitometry. The blot was washed and probed with β-tubulin to confirm equivalent loading. Before re-probing with AR-C19 and AR-V7, the blot was stripped.
Results:

Morphological changes on LNCaP hormone-withdrawn cells

Figure 8 LNCaP morphology in hormonal withdrawal. Morphological changes as observed in hormone-deprived LNCaP cells for twenty days. Control in upper left and bottom right corners (Day 0). Shifts in morphology become apparent on Day 8 without hormone. Representative slender processes as observed are boxed on Days 8 and 12 for comparison. All magnifications at 40x.

Figure 8 shows the changes in LNCaP shape without hormone when viewed at 40x for the duration of twenty days. On Day 0, LNCaP cells appear to have adherent and semi-adherent qualities. They grow as aggregates and form
multiple connections with surrounding cells to create a web-like cluster and cover any remaining spaces on the plate. After hormone withdrawing for 2 days, cells appear to have grown slower with some cell death apparent. Day 4 depicts the re-growth of the cells after being re-plated without hormone; the abundance of individual spindle shapes with robust connections is visibly less. Day 6 is observed two days after re-plating, and the shape of the connections begins to visually deviate from control. Remaining plates are combined and re-plated to generate the cells viewed on Day 8.

Changes in morphology become apparent on Day 8, where the growth appears to have dramatically reduced and the previously robust connections have become long and thin between the fewer cells which have proliferated. Day 10 sees retention in the shift towards these slender processes, and the change in morphology persists in the remaining days shown (Day 12, Day 14, Day 16, Day 18, Day 20) when compared with control. The overall formation of hormone-deprived LNCaP cells for twenty days follows a transition from being characteristically adherent with multiple attachments to all surrounding cells into a shape resembling neural cells with a single cell body and long, thin connections.
Four separate hormone-withdrawn LNCaP cells for twenty days were collected every two days, and the trend of morphological changes becoming apparent on the eighth day without hormone was observed in all collections. The cells also appeared to be less abundant on the eighth day without hormone, correlating to increased cellular death. However, the cells are observed to re-proliferate again following the eighth day. This unique observation was of particular interest, so the hormone-withdrawn experiment was repeated again along with a control set of LNCaP cells grown in media with regular FBS (not deprived of hormone).

The cells in the new repeated experiment were carefully monitored for a time period of fourteen days in order to observe whether the previously-viewed trend
of decreased abundance on Day 8 and the following re-proliferation with morphological changes was reproducible. We again observed the trend of dramatic cell death in the hormone-deprived cells on the eighth day (Figure 9). Of note, Day 8 is the first day following a re-plating of cells, but the growth of the hormone-withdrawn sets (“ADT”) is visibly slower than the growth of the control set (LNCaP cells grown and cultured in hormone, “FBS”). Day 12 also represents the cells after a re-plating from Day 10, but only in the hormone-withdrawn sets are morphological changes established. Withdrawing hormone on LNCaP cells resulted in immediate slower growth (depicted in Figure 9) and a change in morphology not found in LNCaP cells grown in media with regular FBS serum. The overall trend of changes in LNCaP shape is reproducible with hormonal withdrawal; the shape shifts from abundantly small cells with robust spindle formations connecting adjoining cells in a web-like manner to becoming physically similar to neural cells: larger cell body with elongated, slender projections.
**Effect of hormone deprivation on PSA levels in LNCaP cells**

Figure 10, A-B LNCaP PSA expression under hormonal withdrawal. A) PSA levels as representative of AR activity in hormone-withdrawn LNCaP cells for twenty days. GAPDH as internal control. 10uL of each sample was loaded into the wells. PSA levels for Day 18 and Day 20 were run on a separate gel with Day 0. 10uL of each sample was loaded into the wells. GAPDH as internal control. B) RT-PCR of all hormone-withdrawn LNCaP samples from a separate collection run on the same gel with smaller wells, provided for reference of the observed trend. 8uL of each sample was loaded into the wells. PSA levels decrease throughout the twenty days. GAPDH as an internal control. Considering GAPDH levels, there appears to be no difference between Day 0 and Day 2 in PSA expression, but the levels decrease for the remaining days of LNCaP androgen deprivation when compared to control.

Figure 10 (A-B) represents the reproducible trend observed in all twenty-day hormone-withdrawn collections. Our results suggest that hormone withdrawal leads to lower levels of PSA levels. Since one gel could only accommodate a DNA ladder and samples for Days 0 through 16, samples for Days 18 and 20 were run with a control on a separate gel; results from both gels are combined into one image (A). Another set of PCR products was run using smaller wells to include all samples (B). PSA expression does not appear to change much for the first two days without hormone, similar to what is viewed in the PSA mRNA levels seen in Figure 10-A. PSA is lower throughout the remaining days when compared to control.
Effects of hormonal withdrawal on AR protein levels:

Figure 11 LNCaP AR protein levels under hormonal withdrawal. Results are shown of androgen deprivation on AR protein levels in LNCaP cells for twenty days as analyzed via Western Blot with antibody for full-length AR (AR-N20). Beta-tubulin is used as a loading control. AR protein levels do not appear to change throughout the twenty days without hormone.

PSA is one of the well-established target genes of AR (18). Reduced PSA levels suggests a lower AR activity. We then decide to explore the possibility that reduced PSA levels are the results of reduced AR protein expression in the LNCaP cells. In order to determine AR levels, antibody AR-N20 was used to probe for full-length AR, which falls at 110 kDa. The intensities of these bands were determined using chemiluminescence before visualizing with QuantityOne software. Beta-tubulin was used as a loading control for all Western Blots. Figure 11 is representative of the trend observed in Western Blots for all twenty-day collections. AR protein levels appear to remain fairly consistent. It appears to be an increase in AR levels at Day 6; however, the level of Beta-tubulin suggests uneven proteins loaded for that sample relative to Day 4.
Possible Splice Variants

PSA mRNA levels appear to change throughout the twenty days (Figure 10, A-B), suggesting a change in AR activity even though the AR protein levels appear unchanged throughout the same time period (Figure 11). Thus, the Western Blot was stripped and re-probed with antibodies AR-C19 and AR-V7 to look for possible splice variants. Figure 12 shows that truncated isoforms of the AR were found at ~75kDa on Day 8 and Days 14 through 20 with both antibodies AR-C19 (A) and AR-V7 (B).

Figure 12, A-D Induced splice variants. When the blot was probed with antibody AR-C19, splice variants appeared at 75 kDa on Day 8, and Days 14 through Day 20. Full length AR can be seen at 110 kDa (A). Splice variants with the molecular weight of 75 kDa also appeared after stripping and probing the blot using antibody AR-V7 (B). Entire Western Blot probed with AR-N20 provided as a reference (C). Ponceau S Stain depicting total levels of protein, provided as a reference (D).

The finding of splice variants correlates to the morphology shifts observed in the hormone-withdrawn LNCaP cells during the same time points. AR-V7 does not
retain the C-terminal LBD, which allows this splice variant to serve as a transcription factor in a ligand-independent manner (independent of hormone). A surprising find was the induction of a splice variant which has retained the C-terminal domain, as recognized by Antibody AR-C19. The ~75 kDa range of the blot remains fairly unremarkable until Day 8, where a distinct band is viewed; this distinct band disappears to return on Day 14 and remains at a consistent level for the remaining duration. To ensure the observation of these distinct bands at the lower molecular weight, both the immunoblot using AR-N20 and Ponceau S Stain were examined for bands in the range of 75-80 kDa which may have stained greater than others. No bands are more apparent than the preceding or following bands in either the western blot with AR-N20 (C) or the Ponceau S Stain showing total levels of protein (D), suggesting the appearance of splice variants is not an artifact.
Effects of Long-term Withdrawal: Morphology

Figure 13 LNCaP Morphology in long-term hormonal withdrawal. LNCaP cells were maintained in hormone-starved serum for up to 74 days. Days 0 and 20 are provided as references. Morphological changes are maintained in the long-term withdrawal. All photos are at 40x magnification.

In addition to the twenty-day collections, LNCaP cells were also maintained in hormone-free media for up to seventy-four days to observe long-term effects of any further morphological changes (Figure 13). Cells were monitored and collected at Days 30, 60, 70, and 74. In Figure 12, Days 0 and 20 are provided as references of LNCaP morphological changes. Changes in shape of LNCaP cells with prolonged androgen deprivation did not revert back to control. As observed on Days 30, 60, 70, and 74, the continued proliferation of these hormone-deprived cells continued to consist of singular, round cell bodies with long projections. The cell bodies have become rounder with less projections stemming from each cell, but those projections have become visibly longer. The long-term hormone-withdrawn cells appear to cluster in a lattice framework with
needlepoint projections. Furthermore, the individual cell bodies of LNCaP are noticeably larger and more apparent than control.

**Long-term Withdrawal Effects on AR:**

![Image of Western Blot](image)

**Figure 14** AR protein level on Day 74. Western Blot in long-term androgen deprivation, probing with antibody AR-N20 for full-length AR, which falls at 110kDa. Day 0 as negative control; Beta-tubulin is used as a loading control. AR protein levels appear unchanged throughout the long-term observation (74 days without hormone).

A Western Blot was performed on long-term hormone-withdrawn LNCaP cells to determine whether AR protein levels changed (Figure 14). A sample from Day 0 serves as control, and a sample from Day 74 represents LNCaP cells growing for long-term hormone withdrawal. The Beta-tubulin for Day 74 is comparable to that at Day 0 and AR protein level appears unchanged.

**Long-term Withdrawal Effects on PSA Expression:**

PSA mRNA levels for days 30, 60, 70, and 74 without hormone are shown (Figure 15, A). Day 0 serves as the basal level. GAPDH for Days 70 and 74 are comparable to control, but the PSA expression is dramatically reduced. Faint bands can still be seen at Days 70 and 74, suggesting that though PSA mRNA
levels greatly decreased, it is still expressed in long-term hormonal withdrawal. These results indicate that though AR protein levels remain unchanged in long-term withdrawal (Figure 14), AR activity does change.

Figure 15, A-B PSA expression in long-term hormonal withdrawal. RT-PCR performed using samples from LNCaP cells deprived of hormone for 30, 60, 70, and 74 days (A). A cDNA test shows that the cDNA for long-term withdrawal samples were successfully reverse transcribed (B). Day 0 is the control. PSA mRNA indicates AR activity. GAPDH serves as an internal control.

To account for the absent PSA and decreased GAPDH bands for samples from Day 30 and Day 60 without hormone, a cDNA test was performed (Figure 15, B). The result was visualized using QuantityOne software on a VersaDoc machine. If cDNA was successfully reverse-transcribed, a smear would appear in the respective lanes showing the extent of fragmentation. Figure 15 (B) shows that cDNA for samples Day 30, Day 60, Day 70, Day 74, and Day 0 (control) were successfully reverse-transcribed.
Discussion:

Our project addresses the caveats from the preliminary work in studying the effects of hormone deprivation on hormone-sensitive LNCaP prostate cancer cells, using phenotypic observations for twenty days as our guide to narrowing the scope of when regular prostate cancer cells begin transitioning into castration resistant prostate cancer cells. We adjusted our cell culturing timeline and the number of cells plated. In order to account for slower LNCaP growth when deprived of hormone, we retained total cell growth from one phase and evenly distributed that for the next growth phase; this ensures having enough cells to be collected every two days for the duration of the project. However, we acknowledged there may be inseparable variables contributing to our results, such as cell density in addition to hormone deprivation.

The exact impact that cell density has on growth is unknown, but previous work by Sieh S. et al. studied the phenotypic characteristics of LNCaP cells allowed to grow up to certain densities in a bioengineered, hypoxic environment; utilizing their 3D models. Sieh et al. described the appearance of multiple “finger-like” structures in LNCaP colonies after hypoxic conditions for twenty-eight days (32). The study mentions additional previous work by Anderson et al. (33) describing adaptive tumor cells in 3D cell culture acquiring similar morphology under nutrient-depleted or hypoxic conditions. Thus, while our study fails to account for confounding variables with respect to effects from cell density, our morphological
observations are still comparable with those described by previous 3D modeling work which adjusted for LNCaP cell size or density. This reassures us that our results are still meaningful and can provide another perspective into LNCaP hormonal withdrawal when selecting for retaining total cell growth in culture.

To determine whether hormone-sensitive LNCaP cells appear to alter themselves during androgen withdrawal, we looked at the effects of AR levels and AR activity in these deprived cells using PSA mRNA levels as an indicator. In addition, four collections of hormone-withdrawn cells were collected every two days for twenty days, with observed morphological shifts in the LNCaP cells documented at 4x, 10x, 20x, and 40x magnifications; cell counts and concentrations were also performed.

Our findings are consistent with previous studies including preliminary work in our lab. Throughout the twenty-day project, we observed repeatable, sustained morphology phenotypically resembling neural cells in hormone-withdrawn LNCaP cells, consistent with changes in morphology found by other studies (31, 34, 35). We also observed changing levels of PSA consistent with our preliminary findings, and we were able to view changes occurring in the hormone-withdrawn cells more closely due to narrowed observation periods from five-day intervals to two. However, despite a change in PSA mRNA levels, we observed no stark differences in AR protein levels throughout the twenty days. We also found AR
truncated isoforms appearing at ~75 kDa on Day 8 without hormone when the blot was probed with antibodies against either AR-C19 or AR-V7.

The appearance of splice variants at the lower molecular weight after several days without hormone indicates the concurrent changes in AR with progression of PrCa cells. This finding suggests an alternative mechanism in AR transcriptional regulation of the target gene expression separate from using the full-length AR. This finding also warrants more investigation into discovering the connection between a splice variant which has retained the C-LBD and one which does not recognize the C-LBD; how these variants regulate gene expression with prolonged hormonal withdrawal in our study has yet to be determined, but the discovery of these AR truncated isoforms around a similar phase of hormone deprivation in LNCaP cells suggests that the PrCa has transitioned into CRPC, as indicated by several other studies (36-38).

The timing behind LNCaP transdifferentiation and the appearance of AR splice variants appears to be curiously synchronized in some manner, but the exact mechanisms behind them are still being explored by our lab and other studies. In our study, the proclivity of hormone-withdrawn LNCaP prostate cancer cells to transition into a castrate resistant state can be hinted by the appearance of splice variants. The induction of splice variants with prolonged androgen deprivation is particularly exciting because these findings are consistent with a fairly recent
study (2013) performed by Mudryj M. and Tepper CG, who describe truncated ARs lacking a regulatory LBD as a novel mechanism in evading androgen deprivation, as well as proteolysis of full-length AR as one mechanism that results in the expression of AR\(\Delta\)LBD (28). LNCaP cells normally do not exhibit detectable levels of the splice variant lacking the C-terminal LBD (AR\(\Delta\)LBD), but the findings of our project agree with those observed by previous studies, which found that C-terminally truncated AR isoforms could be induced in LNCaP cells (39), and androgen deprivation also was found to enhance levels of the C-terminally truncated AR isoform in LNCaP cells (40).

It is possible that redundant AR pathways involving IL-6, IGF-1, or EGF might have been selected over time, but antibodies selecting for activity in these pathways (such as Ser\(^{112}\) for the Ras/MEK cascade) would be necessary in order to confirm this suspicion. Furthermore, utilizing NE markers to monitor the appearance and retention of the NE phenotype would validate the transdifferentiation we observed, as well as allow us to more accurately coordinate the timeline of activities and cellular changes under androgen deprivation duress. Qiu et al. found IL-6 treatment and Kim et al. found activating EGFR via ERK/MAPK induced LNCaP NE differentiation (41, 42). However, despite suggestions by previous studies of PrCa cell NE transdifferentiation through multiple pathways, it is difficult to compare the clinical progression for underlying mechanisms due to transdifferentiated cells being shown to revert
back to their original phenotype upon the re-introduction of hormone (15, 34). Shen et al. induced NE differentiation within five days of LNCaP cells cultured in chronic hormone-deprived medium, the altered morphology predominating at ten days, and a complete transformation observed by twenty days. However, Shen and colleagues noted a reversal in the morphology when the medium was entirely replaced by that containing unmodified FBS and not a reversal when DHT was directly added into the hormone-deprived medium (43). Thus, in order to determine any form of molecular basis for NE differentiation, establishing PrCa cells which retain their NE characteristics to resemble terminally differentiated clinical PrCa cells becomes pertinent.

A comprehensive review by Yuan TC et al. (44) attempted to answer this conundrum after successfully establishing stable “NE-like” subclone of LNCaP cells after hormone deprivation. Even after three-months of re-culturing in medium containing androgens, the cells in the subclone did not revert back to their original phenotype and still express NE markers. Yuan and colleagues described these subclones as ideal model for characterization due to the resemblance of terminal transdifferentiation in clinical samples. They found a lack of AR or PSA expression and suggest factors in this differentiation to be induced through several alternate pathways, such as IL-6, EGFR, and especially Ras-ERK/MAPK. Relating Yuan and colleagues’ stable NE-like LNCaP cells to our tangential, prolonged study (hormone-deprived LNCaP cells for seventy-four
days), we saw a dramatic reduction in PSA expression but not AR. We did not reintroduce hormone into these cells to determine whether the morphological changes were stable, though we have reason to believe the resiliency of these changes as phenotypic observations at thirty days and beyond appeared even more dramatic. Perhaps a differing level of AR expression between Yuan et al.’s findings and ours can be related back to the effects of cell density, but we would have to repeat our extended period with varying densities to confirm this.

In summary, despite an unknown variable of cell density effects on our results, our repeatable morphological observations remain consistent with works performed by other studies showing LNCaP transdifferentiation to cells resembling the neural cell morphology. Furthermore, we observed long-term effects of androgen withdrawal on LNCaP cells. Passages of LNCaP cells which have been deprived of hormone for the following days are: six times for thirty days; eight times for sixty days; nine times for seventy days; and ten times for seventy-four days. We found that LNCaP cells were able to survive without hormone for seventy-four days while retaining the morphological changes. We also found a dramatic decrease in PSA mRNA levels for Days 70 and 74 compared to control, though PSA could still be expressed. However, there was no apparent change in AR protein levels comparing Day 0 to Day 74, suggesting a change in the activity as a possible mechanism for prolonged survival in the androgen-independent state.
In addition to utilizing neuroendocrine markers, such as ChrA, NFM, NSE (neuron specific enolase), NeuN (neuronal nuclear protein), or S-100 (astrocyte marker) to validate the morphological shifts we repeatedly observed, our future work will require furthering our tangential project: the long-term effects of androgen deprivation on LNCaP cells for seventy-four days. By studying the LNCaP cells for this extended duration repeatedly, we will be able to confirm that our observations with the extended findings are reproducible. In repeating this project at least once more, we will consider verifying the LNCaP cell line from the ATCC website and synchronizing the cells prior to commencing treatment.

To conclude, ADT is a widely-used therapeutic treatment for PrCa to prolong survival for several years, especially when combined with other therapies. However, hormone-refractory PrCa can occur, leading to CRPC through mechanisms poorly understood. In this study, our data imply that androgen deprivation not only generated a C-terminally truncated AR isoform, but that a splice variant with the C-domain retained may be possible as well. Therefore, withdrawing hormone in hormone-sensitive prostate cancer cells result in changes in morphology, induction of splice variants, and no overall change in AR protein levels but a change in AR activity.

Our findings warrant more work to validate and further elucidate the biochemical mechanisms. We have shown evidence of time-sensitive treatment of prostate
cancer cells undergoing androgen independence when the cells are allowed to
grow in total form, undeterred from possible mechanical selection biases since
total growth from each previous growth was retained and evenly distributed to
observe the next growth phase. It can be possible to over treat or extend a first
line of defense therapy beyond a beneficial window. In vitro, we have shown that
it is possible to do more harm to a potential patient when androgen withdrawal no
longer becomes effective on reducing prostate cancer cellular growth despite
lower levels of PSA as an AR indicator. Thus, we have found that solely using
PSA as a marker of PrCa level is unreliable once PrCa cells appear to have
established NE-like features, possibly retaining them in the long-term CRPC
state.

Our work hints at using NE markers at the onset of treatment and during in order
to determine whether the NE levels have changed. If the NE levels appear to
markedly increase in the prostatic epithelia or surrounding tissue, this may serve
as a more reliable indicator of CRPC progression and the necessity to change
treatments. So that the available options do not exhaust their effectiveness,
perhaps it will behoove clinicians to consider monitoring NE levels before
beginning ADT, then stopping ADT once NE levels rise. After stopping ADT,
clinicians may consider targeting the alternative AR-pathways.
Since there are currently no known primary prevention strategies for PrCa, pre-emptive clinical focus should be placed on early stage prevention strategies, such as education on the time-sensitive nature of hormonal withdrawal and how the age of presentation may be different for individuals depending on other potential comorbidities, such as obesity or diabetes (especially type II). The American Cancer Society recommends PrCa screening to begin at age forty-five for high risk groups and at age forty for higher risk groups (25), but perhaps patients who are obese or have diabetes should begin screening for PrCa in young adulthood instead of in their forties. Especially since African-American men have been found to be both younger at the time of initial PrCa diagnosis (26) and have obesity linked to PrCa (45, 46), clinicians and researchers would have a better likelihood of finding any racial differences in PrCa progression by comparing early stages and diagnosis.

The correlation between the appearance of splice variants with morphological shifts further emphasizes the important of identifying biomarkers that can help clinicians to choose the best treatment options for patients depending on androgen-independent phase, lifestyle, and possible comorbidities. Furthermore, it may be better to use different treatments for patients with diabetes, obesity, and even abnormal vertical growth (IL-6, JAK/STAT, IGF-1/EGF, Ras/MEK, Ras/Ack, P13K/Akt, Ras or MEK to Src, etc.). We found that LNCaP cells survive up to seventy-four days without hormone while maintaining morphological
changes concurrent with barely expressing PSA levels. This means that low PSA levels do not necessarily correlate to a cancer being in remission. Previous therapeutic options following CRPC were limited to second-line therapies with limited clinical benefit due to eventual relapse within a year or two (3, 8, 47). The data from this study suggest that new agents targeting the alternative AR signaling axis could be used in treating CRPC.
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