The Role of Glucocorticoid Signaling in Prostate Cancer Health Disparities

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The Role of Glucocorticoid Signaling in Prostate Cancer Health Disparities

by

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A Dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy in Physiology

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Each person whose signature appears below certifies that this thesis/dissertation in his/her opinion is adequate, in scope and quality, as a thesis/dissertation for the degree Doctor of Philosophy.

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<tr>
<td>AA</td>
<td>African American</td>
</tr>
<tr>
<td>ACS</td>
<td>American Cancer Society</td>
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<tr>
<td>ACTH</td>
<td>andrenocorticotropic hormone</td>
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<tr>
<td>ADT</td>
<td>androgen deprivation therapy</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ARE</td>
<td>AR response element</td>
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<tr>
<td>CLU</td>
<td>clusterin</td>
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<tr>
<td>CpG</td>
<td>Cytosine-phosphate-Guanosine</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin releasing hormone</td>
</tr>
<tr>
<td>CTT</td>
<td>carboxyl-terminal-tail-terminal tail</td>
</tr>
<tr>
<td>CTX</td>
<td>cabazitaxel</td>
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<tr>
<td>Dex</td>
<td>dexamethasone</td>
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<tr>
<td>DFS</td>
<td>dense fine speckled</td>
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<tr>
<td>DHEA</td>
<td>dehydropiandrosterone</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
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<tr>
<td>DRE</td>
<td>digital rectal exam</td>
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<tr>
<td>DTX</td>
<td>docetaxel</td>
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<tr>
<td>EA</td>
<td>European American</td>
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<tr>
<td>Enz</td>
<td>enzalutamide</td>
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<tr>
<td>FKBP</td>
<td>FK506-binding protein</td>
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<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
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<tr>
<td>GRE</td>
<td>GR responsive element</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>GWAS</td>
<td>genome-wide association studies</td>
</tr>
<tr>
<td>HDGF</td>
<td>hepatoma-derived growth factor</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>HPT</td>
<td>hypothalamic-pituitary-testicular</td>
</tr>
<tr>
<td>IBD</td>
<td>integrase binding domain</td>
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<tr>
<td>LEDGF/p75</td>
<td>Lens Epithelium-Derived Growth Factor p75</td>
</tr>
<tr>
<td>LHRH</td>
<td>luteinizing hormone-releasing hormone</td>
</tr>
<tr>
<td>LMP</td>
<td>lysosomal membrane permeabilization</td>
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<tr>
<td>mCRPC</td>
<td>metastatic castration-resistant prostate cancer</td>
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<tr>
<td>nCLU</td>
<td>nuclear clusterin</td>
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<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
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<tr>
<td>NR3C1</td>
<td>nuclear receptor subfamily 3 group C</td>
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<tr>
<td>PCa</td>
<td>prostate cancer</td>
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<tr>
<td>PSA</td>
<td>prostate-specific antigen</td>
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<tr>
<td>psCLU</td>
<td>presecreted clusterin</td>
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<tr>
<td>PWWP</td>
<td>proline-tryptophan-tryptophan-proline</td>
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<tr>
<td>sCLU</td>
<td>secreted clusterin</td>
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<tr>
<td>SES</td>
<td>socioeconomic status</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis inducing ligand</td>
</tr>
<tr>
<td>U.S.</td>
<td>United States</td>
</tr>
<tr>
<td>USPSTF</td>
<td>U.S. Preventive Services Task Force</td>
</tr>
<tr>
<td>Zip</td>
<td>basic leucine zipper</td>
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11β-HSD2  11β-hydroxysteroid dehydrogenase-2
ABSTRACT OF THE DISSERTATION

The Role of Glucocorticoid Signaling in Prostate Cancer Health Disparities

by

Leanne W. Burnham

Doctor of Philosophy, Graduate Program in Physiology
Loma Linda University, June 2018
Dr. Carlos A. Casiano, Chairperson

African-American men are more likely to develop aggressive prostate cancer (PCa) and die from the disease than other ethnic groups. Glucocorticoid signaling is a contributing biological factor to worse PCa prognosis, and is emerging as a key driver of PCa progression in the absence of androgens. The mechanism involves glucocorticoids binding to glucocorticoid receptor (GR) and bypassing the androgen receptor (AR) signaling pathway to activate AR-target genes that promote tumor aggressiveness and therapy-resistance. This is problematic as African-American men have hypersensitive GR signaling and chronically-elevated levels of glucocorticoids linked to cumulative stressful life events. To explore the role of glucocorticoid signaling in PCa health disparities, this dissertation used a racially diverse pre-clinical model to examine the effects of GR activation on the expression of stress oncoproteins linked to tumor aggressiveness and therapy-resistance, specifically Lens Epithelium-Derived Growth Factor p75 (LEDGF/p75) and Clusterin (CLU). Results revealed a robust pattern of GR-induced upregulation of LEDGF/p75 and CLU in African-American (AA) PCa cells compared to European-American (EA) PCa cells. We also detected increased GR transcript expression in AA PCa tissues, compared to EA tissues, using Oncomine microarray datasets. In
addition, a trend towards elevated circulating LEDGF/p75 and CLU was observed in sera of AA patient samples. Taken together, these findings provide an initial framework for understanding the contribution of GR signaling to PCa health disparities.
CHAPTER ONE

INTRODUCTION

Prostate Cancer—Etiology, Stages, and Treatment Options

Cancer is a major worldwide health crisis and is the second leading cause of death within the United States (U.S.).\textsuperscript{1,2} Among the different types of cancer, PCa is most diagnosed in the U.S. and the second leading cause of cancer mortality.\textsuperscript{1} It is estimated that 164,690 U.S. men will be diagnosed and 29,430 men will die from PCa in 2018.\textsuperscript{1}

There are many underlying contributors to the development of PCa, but androgens (i.e. testosterone) are the necessary driving factor necessary for disease development.\textsuperscript{3} Testosterone is produced by 2 distinct populations of testicular Leydig cells during the fetal and adult developmental periods.\textsuperscript{3} At puberty, testosterone is converted to dihydrotestosterone (DHT) within the prostate stimulating prostate growth.\textsuperscript{3} An eventual homeostasis is achieved between prostatic cell differentiation and cell death resulting in a maintenance of appropriate prostate size.\textsuperscript{3} Although endogenous testosterone levels decrease as men age, prostatic cell proliferation increases resulting in prostatic disease including PCa.\textsuperscript{3} This suggests an age-related altered response of prostatic cells to androgens.\textsuperscript{3}

Because testosterone function is dependent upon binding to androgen receptors (AR), intraprostatic response of cells to androgens must depend on the expression and sensitivity of ARs.\textsuperscript{3} The important role of AR extends beyond normal prostate function and is an established main driver of prostate carcinogenesis and subsequent disease progression.\textsuperscript{3} Because of this, targeting androgen production and AR has been a mainstay of PCa treatment for over 7 decades.\textsuperscript{4} Dr. Charles Huggins initially demonstrated that
both surgical castration and estrogen administration resulted in regression of PCa metastasis. His pivotal findings pioneered the future development of many variations of androgen deprivation therapy (ADT). Both agonists and antagonists of luteinizing hormone-releasing hormone (LHRH) are typically used as a form of ADT meant to decrease endogenous testosterone production through the hypothalamic-pituitary-testicular (HPT) axis. There have also been anti-androgens developed that bind to AR and inhibit its activity including flutamide, bicalutamide, and nilutamide. Second-line anti-androgens Enzalutamide (Enz) and ARN509 have been developed for PCa patients who have failed LHRH agonists/antagonists or other first-line antiadrogens.

While ADT is initially successful in most PCa patients who choose this option, ADT-resistance is inevitable occurring within 18-24 months. It is important to note that prostatic epithelial cells demonstrate great plasticity in response to ADT giving rise to a highly heterogeneous co-existence of AR-positive and AR-negative cells. Once a PCa patient has developed ADT-resistance, he enters a disease stage referred to as metastatic castration-resistant prostate cancer (mCRPC) which is incurable. The only therapeutic strategies for mCRPC patients include taxane chemotherapy regimens docetaxel (DTX) plus prednisone followed by cabazitaxel (CTX) plus prednisone or palliative options. While DTX and/or CTX can extend life by a few months, chemoresistance develops and these treatments are not curative or long-lasting.

PCa is initially detected and diagnosed in patients through a series of screening procedures. Digital rectal examination (DRE) is performed manually by a physician typically in a primary care setting. The purpose of DRE is to detect abnormal size of the prostate as well as abnormal hard nodules. In most settings, the prostate-specific antigen
(PSA) blood test is a complimentary screening tool to the DRE. PSA is produced by the prostate and circulates at high levels in men with PCa.\(^{15}\) Circulating serum PSA levels are considered abnormal when detected above 4 ng/ml.\(^ {16}\) While the U.S. Food and Drug Administration approved the use of PSA testing in conjunction with DRE to screen asymptomatic men for PCa in 1994, the U.S. Preventive Services Task Force (USPSTF) issued a recommendation against PSA-based screening in 2012.\(^ {16}\) Conversely, the American Cancer Society (ACS) recommends PCa screening at age 50 for men at average-risk, 45 for men at high-risk, and 40 for men at higher-risk.\(^ {17}\) In addition, ACS recommends repeated annual PCa screening for men with PSA levels >2.5 ng/ml.\(^ {17}\) While the presence of high PSA is not always indicative of PCa, it is cause for follow-up investigation including prostate biopsy.

Upon biopsy, a pathologist is able to detect PCa and assign a Gleason score which is subsequently utilized by the patient’s physician to predict the aggressiveness of the cancerous cells.\(^ {18}\) The higher the Gleason score, the more likely the PCa will spread rapidly.\(^ {18}\) Gleason score ranges from 1-5 with an assignment of “1” representing small uniform glands.\(^ {18}\) A Gleason score of “3” represents moderately differentiated cells where more space is visualized between glands with distinct infiltration of cells from glands at the margins.\(^ {18}\) A Gleason score higher than “3” represents poorly differentiated and anaplastic cells with irregular masses of neoplastic cells and complete lack of glands.\(^ {18}\) Most PCa tumors score a grade of “3” or higher. Prostate biopsy results have revealed vast PCa phenotypic heterogeneity. The predominant histological subtypes found in prostate adenocarcinoma cells include luminal secretory, rare neuroendocrine, basal, and luminal epithelial cells.\(^ {19}\) And the trend towards heterogeneity continues.
within the luminal epithelial cellular subtype.\textsuperscript{19} Because of this heterogeneity, 2 grades are assigned to each patient by the pathologist to add to a final sum Gleason score typically ranging from 6-10. Because of the enormous heterogeneity observed within and among PCa patients there remains a monumental challenge to develop effective therapy for this malignancy. Current options, including radical prostatectomy and radiation, can be curative at early-stage diagnosis. However, treatment options for later-stage disease including the aforementioned ADT and chemotherapy are not long-lasting (Figure 1). This is problematic for men diagnosed with aggressive tumors or advanced PCa.
Figure 1. Stages and treatment options for PCa. There are a variety of treatment options available for PCa at each stage. PCa is curable if eradicated while localized. However, once tumor cells escape organ confinement, PCa is no longer curable and treatments are meant to extend life. ADT is a treatment option at any stage of PCa, and is a mainstay course recommended following biochemical recurrence. Castration-resistance occurs approximately 18-24 months after primary ADT initiation. Late-stage PCa can be treated with taxane chemotherapy as well as secondary ADT. Eventually chemoresistance develops and palliative care is provided at the terminal stage.
Prostate Cancer Health Disparities

AA men are 75% more likely to be diagnosed with PCa and 228% more likely to die from this malignancy than EA men.\textsuperscript{1} It is estimated that 1 in 8 EA men will be diagnosed with PCa in their lifetime.\textsuperscript{20} However, the estimation rate in AA men is 1 in 6 and we recently reported that 1 in 3 AA men had elevated circulating PSA in a study that recruited 414 AA men ranging from age 18-80.\textsuperscript{17,20} The biological characteristics of prostate tumors are exaggerated in AA men compared to EA men at time of diagnosis, including higher PSA levels, higher Gleason scores, and advanced tumor stage.\textsuperscript{21-23}

The cause of these inequalities are complex and include many contributing factors such as socioeconomic issues, biological and genetic determinants, diet, lifestyle, and access to healthcare.\textsuperscript{20,24-30} Social and economic disparities directly affect diet, lifestyle, access to healthcare, and therefore contribute greatly to differences observed in worse cancer outcomes.\textsuperscript{20,31-33} Income inequalities lead to increased exposure to risk factors such as barriers to high-quality cancer prevention, early detection, and cutting-edge treatment options.\textsuperscript{20,31-33} Educational achievement affects potential income and in 2014, 22\% of AAs had completed college while 36\% of EAs had completed college.\textsuperscript{20} That same year 26\% of AAs were living below federal poverty level compared with 10\% of EAs.\textsuperscript{20} Less education leading to lower income affects neighborhood placement, and lower socioeconomic neighborhoods are more likely to be targeted by marketing that promotes behaviors known to increase cancer risk.\textsuperscript{20} There is also less access to healthy fruits and vegetables as well as opportunities for physical activity.\textsuperscript{20} Socioeconomic status translates to overall survival rates for several reasons that include limited access to high-quality health care.\textsuperscript{32,34-36} Worse overall survival is compounded by the fact that
AAs are more likely to be diagnosed at a later stage. Later diagnosis limits treatment options and reduces the efficacy of those treatments. However, even when provided the same PCa treatment as EA men, AA men are more likely to experience delay in treatment administration and suffer greater postoperative complications. In addition, AAs are less likely to participate in clinical trials preventing them from exposure to cutting-edge options that could be more efficacious than widespread treatments. Comorbid conditions affecting the delivery of optimal treatment must also be taken into consideration, and these conditions including obesity, diabetes, and hypertension which are higher in AAs.

While socioeconomic factors largely contribute to cancer health disparities, we cannot rule out the role that genetic and biological determinants play as these disparities persist even after controlling for socioeconomic factors and access to care. PCa exhibits the highest reported heritability compared to any other cancer, and genomic differences between AA and EA men with PCa suggest genetic mediators are driving PCa health disparities. Although most family-based linkage studies of hereditary PCa have focused largely on European descent populations, a series of loci responsible for PCa have been identified for AA men including 12q24, 1q24-25, 2p16, 2p21, and 1p36. Additional linkage signals detected in AA pedigrees include 2p21, 11q22, 17p11, 22q12, and Xq21. Some loci have also been implicated in genome-wide association studies (GWAS) and confirmed in AA men including 12q24, 1q24-25, and 8q24. Given that loci detected by GWAS vs. family studies are not expected to be the same, evidence for the role of these congruent genes in PCa etiology is further strengthened. Genetic
variation on chromosome 8q24 has been consistently associated with PCa,\textsuperscript{60,62-65} and ethnic specific mutations and haplotypes have been reported in African populations.\textsuperscript{64,66}

In addition to inherited genomic factors linked to PCa, there are also somatic alterations that are associated with the development of this disease.\textsuperscript{62} For example, gene fusion TMPRSS2:ERG translocations have been found to be much lower in AAs (20\%) compared to EAs (62\%).\textsuperscript{67} AA and EA men also have significant differences in ERG expression.\textsuperscript{68} The prognostic value of TMPRSS2:ERG translocations and ERG expression is not clear, but the relationship of PCa risk factors differs by TMPRSS2:ERG translocation status.\textsuperscript{69-71} Therefore, the marker distribution may identify tumor heterogeneity underlying PCa etiology and outcome disparities.\textsuperscript{62,67} In addition, the association between obesity and worse PCa outcome has been found to vary by TMPRSS2:ERG translocation status.\textsuperscript{72} By default, TMPRSS2:ERG translocation status could drive tumor aggressiveness as AA men tend to have greater rates of obesity than EA men.\textsuperscript{62}

Gene expression and pathway profiles of PCa tumors have further confirmed prominent differences in tumor immunobiology between AA and EA men.\textsuperscript{54} Genes associated with autoimmunity and inflammation are differentially expressed in AA men including genes clustering in immune response, stress response, cytokine signaling, and chemotaxis pathways.\textsuperscript{54} Metastasis-promoting genes are also more highly expressed in AA men including autocrine mobility factor receptor, chemokine receptor 4, and matrix metalloproteinase 9.\textsuperscript{54} In a separate study, IL6, IL8, IL1B, CXCR4, and FASN were significantly expressed at higher levels in tissue of AA men with PCa compared to EA men.\textsuperscript{51} These are genes that have been associated with diet and lifestyle and are
associated with higher Gleason scores, AR regulation, aggressive PCa tumors, and metastasis. The differential expression of these genes is also consistent with metabolic syndrome associated with comorbid health conditions such as hypertension and obesity which AA men are more likely to suffer from.

Given that androgens drive PCa etiology and disease progression prior to mCRPC, several studies have explored differences in androgen production and AR signaling in AA men compared to EA men. AA men have been found to have higher testosterone levels than EA men. In addition, AA men have higher active 5-alpha reductase levels resulting in enhanced conversion of testosterone to the more potent DHT. The contrasting expression of epithelial and stromal AR in PCa tissue is emerging as a possible driver of castration resistance in patients receiving ADT. Studies exploring this possibility have observed that nuclear AR has found to be increased in AA PCa patients, but stromal AR are decreased in AA compared to EA PCa patients. A separate study examined the occurrence of AR mutations and polymorphisms and results revealed that AA PCa patients have higher frequency of germline and somatic mutations in AR. The expression of AR target genes such as PSA have also been found to be increased in AA PCa patients. Other AR target genes including RHOA, ITGB5, and PIK3CB are differentially expressed in tissue samples of AA PCa men compared to EA PCa men and are associated with properties of PCa tumor aggressiveness including increased invasion activity of cells.

Taken together, there is substantial evidence that AA men are genetically predisposed to develop PCa and with an aggressive tumor phenotype. In addition, the socioeconomic factors which amplify this predisposition towards disproportionate PCa
incidence and mortality cannot be overlooked. Substantial progress has been made towards addressing these PCa health disparities. However, there remains a need to continue biomedical research targeting AA men with PCa while also promoting equitable access to services for prevention, early detection, and high-quality treatments for this disease.

**African Americans and Stress**

African Americans are exposed to more cumulative lifetime stressors than other racial/ethnic groups and there is evidence that this detrimentally alters psychologic and physical health.\(^{92-94}\) Chronic stress leading to the dysregulation of endogenous cortisol production via the hypothalamic-pituitary-adrenocortical (HPA) axis in response to chronic stress is a major biological phenomenon that can enhance risk for metabolic disorders and cancer.\(^{92,94-96}\) The HPA axis regulates the human body’s response to stress.\(^{97,98}\) When the HPA axis is activated in response to stress, neurons in the paraventricular nucleus of the hypothalamus are triggered to release corticotropin-releasing hormone (CRH) and arginine vasopressin, which stimulate adrenocorticotropic hormone (ACTH) production and secretion from the anterior pituitary gland (Figure 2).\(^{97,99}\) ACTH circulation induces the synthesis and secretion of cortisol, a glucocorticoid, from the adrenal cortex (Figure 2).\(^{97,99}\) A classical endocrine negative feedback loop inhibits further release of CRH and ACTH in response to rising levels of cortisol (Figure 2). In this manner, a physiological homeostasis is maintained under normal conditions.\(^{97}\) In addition, the HPA axis tightly regulates glucose metabolism, the cardiovascular system, cell proliferation and survival, growth, cognition and behavior, immune function,
and reproduction directly through cortisol production.\textsuperscript{97} Cortisol is secreted diurnally, peaking after wakening when blood glucose levels are at the lowest and tapering throughout the day.\textsuperscript{92} This diurnal rhythm is unfortunately altered in response to chronically stressful situations (Figure 2).\textsuperscript{92,100} The stage is set for the initiation of pathogenic processes when cortisol levels are either elevated or cease to taper normally.\textsuperscript{92,101}
Figure 2. HPA axis regulation and negative feedback inhibition. The HPA axis tightly regulates the production of endogenous cortisol. The HPA axis is stimulated by the diurnal circadian rhythm as well as stressors. A normal negative feedback loop ensures physiological homeostasis to prevent excess cortisol production. (http://www.lapislight.com/wp/tag/hpa-axis/)
Chronic exposure to stressors is increased in individuals of lower socioeconomic status (SES) and there is a positive association between SES and stress in AA men.\textsuperscript{92,102} Decreased SES is defined by lesser income, education, or occupation and often results in increased exposure to environmental stressors leading to stress-related dysregulation of physiological systems and increased risk for disease.\textsuperscript{92,101,103} Even in AAs who have achieved high SES, racial disparities in health persist.\textsuperscript{104} For example, a poor lipid profile characterized by high triglycerides, LDL cholesterol, total cholesterol, and lower HDL cholesterol, actually increases in AAs as education increases.\textsuperscript{105} Considering all factors, AA men have the shortest life expectancy at 16 years less than EA women who benefit from the longest life expectancy.\textsuperscript{106}

SES and racial categories walk hand-in-hand and cannot be extricated from each other in the context of U.S. history.\textsuperscript{106,107} National data reveals that strikingly high levels of racial inequality in SES yet exist with little change over time and AAs continue to suffer disproportionately lower SES.\textsuperscript{106} For example, AAs have overall poverty levels that are two to three times higher than EAs.\textsuperscript{106} There is evidence that income does not increase in AAs commensurately as education increases, and AA men with a master’s degree earn $27,000 less than their EA counterparts.\textsuperscript{106} While the racial gap in education between AAs and EAs has narrowed over time, the relative pay of AA men declined by 10\% compared to EA men between 1979 and 1997, and AAs have 9 cents in wealth for every dollar of wealth that EAs have.\textsuperscript{108} Furthermore, employed AAs are more likely to be exposed to carcinogens and occupational hazards compared to others with matched education and job experience.\textsuperscript{108,109} Compounding these realities, AAs have less purchasing power as the costs of goods and services are highest in predominantly AA
Not surprisingly, the ensuing financial hardships translate to tangible stressors such as the inability to meet essential expenses, pay full mortgage or rent, or pay all utility bills.\(^\text{108}\)

Outside of the workplace, AAs are exposed to daily stressors within their segregated neighborhoods.\(^\text{106}\) The effects of segregated neighborhoods are vast and negatively impact the health of AA residents.\(^\text{110,111}\) For example, segregation limits access to quality education and limits socioeconomic mobility by impeding preparation for higher education and lucrative employment opportunities.\(^\text{110,111}\) Optimal health is also jeopardized in economically-disadvantaged segregated neighborhoods as nutrition suffers in the presence of higher cost, lower quality, and lesser availability of healthy foods.\(^\text{110,111}\)

Similarly, physical activity is reduced in the absence of recreational facilities amid safety concerns.\(^\text{110,111}\) Exposures to environmental toxins and poor-quality living conditions are also a reality in neighborhoods accustomed to institutional neglect and disinvestment.\(^\text{110,111}\)

Taken together, the stressors associated with lower income and issues arising from segregated neighborhoods are directly linked to elevated risk of illness and death, and greatly contribute to existing racial disparities in health.\(^\text{110,112}\) Individuals living in census tracts with elevated rates of unemployment and poverty have been shown to have diminished central nervous serotonergic activity leading to increased risk of depression and substance abuse.\(^\text{113}\) Adding to these stressors many AAs face on a daily basis are perpetual experiences of discrimination.\(^\text{106,114}\) An association has been firmly made between experiences of discrimination and adverse health effects including sexual dysfunction, less stage 4 sleep, increased abdominal fat, elevated hemoglobin A1c,
coronary artery calcification, increased uterine myomas, and cancer incidence.\textsuperscript{115}

Perpetuating the cycle of substance abuse, exposure to discrimination reduces health care seeking behaviors and increases tobacco, alcohol, and illicit drug use.\textsuperscript{115}

As AAs are disproportionately arrested by law enforcement and subsequently incarcerated than any other racial/ethnic group, recent studies are exploring the impact of these experiences on health.\textsuperscript{106,116,117} Incarceration is characterized as a “disorderly transitional” stressful event that is undesired and involuntary, and promotes lasting health effects including hypertension.\textsuperscript{117,118} Health is further compromised through a lifetime as incarceration, at any point and for any amount of time, places an individual on a trajectory of lower education, decreased job prospects, and income resulting in an increase of other stressors.\textsuperscript{116-118}

Social disparities in health are large, pervasive, and persistent in AAs and these inequalities in health reflect larger inequalities in society.\textsuperscript{106} It is important to carefully consider social risk factors to fully understand the biological processes shaping health disparities.\textsuperscript{106} SES has been shown to influence emotions and behaviors which subsequently alter cortisol levels.\textsuperscript{103} Lower SES is associated with greater perceived stress, depressive symptoms, negative affect, weak social networks and support, and sleep deprivation.\textsuperscript{92} Consequently, these factors are also linked to greater cortisol responses.\textsuperscript{119-122} A major study by Cohen et al. explored whether SES-associated dysregulation of cortisol diurnal rhythm is independent of race and occurs equally in AAs and EAs.\textsuperscript{92} This study reported that individuals with higher education and income had steeper declines in cortisol over the day in a diurnal slope resulting in lower cortisol levels in the evening.\textsuperscript{92} Yet after controlling for education and income, AAs were found
to wake with lower cortisol levels and sustain higher cortisol levels in the evening suggesting a dysregulation of normal cortisol diurnal rhythm that cannot be explained by differences in SES. The long-term consequences of this dysregulation are complicated and problematic for the health of AAs.

**Glucocorticoid Signaling—Normal vs. Aberrant**

Cortisol is an endogenous glucocorticoid produced by humans. Both cellular and pharmacological actions of glucocorticoids are mediated by the glucocorticoid receptor (GR). Alternative splicing produces 3’ UTR mRNA isoforms of GR—GRα, GRβ, and GRP. GRα shuttles between the cytoplasm and nucleus to regulate transcription, GRβ is localized in the nucleus and has a dominant negative effect on GRα upon forming GRα/GRβ heterodimers, and GRP has both a synergistic and antagonistic relationship with GRα. The GR gene nuclear receptor subfamily 3 group C member 1 (NR3C1) is regulated by complex promoter regions containing multiple transcription start sites and contains at least nine 5’ UTR first exons that are spliced to exon 2 (Figure 3). The alternative first exons are divided into two promoter regions, exon 1A and 1I, as well as a proximal region upstream of the translation start site containing exons 1B, 1C, 1D, 1E, 1F, 1H, and 1J, and each first exon is regulated by its own promoter (Figure 3).
Figure 3. GR isoforms. The GR is comprised of 9 exons. Alternative splicing of exon 9 at the 5’ end of the coding region leads to the formation of the classic GRα isoform and the dominant negative GRβ isoform. The multiple exon 1 variants that control tissue-selective gene expression are also shown. (Ito, Chung, and Adcock. J Allergy Clin Immunol. 2006.)
As GRα is the biologically relevant isoform, all references henceforth to GR will imply GRα. GR is a member of the nuclear receptor superfamily of ligand-dependent transcription factors. GR is made of three functional domains including an amino-terminal transactivation domain, a central DNA-binding domain, and a carboxy-terminal ligand-binding domain. There is a flexible hinge region that contains a nuclear localization signal between the DNA-binding domain and the ligand binding domain. It is within this flexible hinge region that genomic interactions occur. GR is intracellularly located within either the cytoplasm or the nucleus depending on the absence or presence of ligand. Glucocorticoid ligands of GR can be endogenous (cortisol) or synthetic and include hydrocortisone, dexamethasone (Dex), and prednisone. In the absence of ligand binding, GR resides in the cytoplasm as part of a large multi-protein complex including chaperone heat shock proteins hsp90, hsp70, and p23 as well as immunophilins of the FK506 family including FK506-binding protein (FKBP) 51 and FKBP52 (Figure 4). Upon ligand binding, a conformational change occurs releasing GR from the chaperone proteins and promoting nuclear localization of GR.
Figure 4. Translocation of GR. Upon binding to ligand, GR dissociates from chaperone proteins and translocates into the nucleus, where it regulates the transcriptional activity of GR-target genes positively and negatively either by binding to GREs located in the GR-target gene promoter region or by physically interacting with other transcription factors. After completion of changing the transcriptional activity of GR-target genes, GR is exported into the cytoplasm and is incorporated into the complex with chaperone proteins. (Ito T. Brain Immune Trends. 2010.)
Within the nucleus, GR homodimer binds to GR response element (GRE) DNA sequences within target genes. The consensus GRE sequence is comprised of two hexameric half-sites separated by a spacer of three nucleotides (AGAACAAnnTGTTCT). Once GR homodimers bind to GREs, chromatin is remodeled, co-regulators are recruited, and GR-induced transcription is initiated. In addition to activation of GR-target genes, GR also negatively represses genes. This occurs when GR binds to negative GREs with consensus sequence CTCC(n)0.2GGAGA and co-repressors are recruited. GR also mediates gene transcription via heterodimer binding interactions with other transcription factors.

Both the nature and intensity of cellular response to glucocorticoids is dependent upon a few factors including ligand dose and type, GR post-translational modifications, relative abundance of co-regulators, chromatin environment, and GREs of GR-target genes. Within peripheral tissues, GR stimulation is physiologically tightly regulated by 11β-hydroxysteroid dehydrogenase-2 (11β-HSD2) which enzymatically converts cortisol to its inactive form cortisone in humans. Loss of 11β-HSD2, mediated by the ubiquitin E3-ligase autocrine mobility factor receptor, within peripheral tissues results in sustained elevated cortisol and stimulates aberrant GR signaling.

GR signaling also plays a major role in carbohydrate, lipid, and protein metabolism. During periods of fasting, such as during sleep, glucocorticoids enable maintenance of physiological blood glucose levels by decreasing glucose uptake in muscle as well as stimulating hepatic glucose production via gluconeogenesis and glycogenolysis. Glucocorticoid excess leads to several adverse effects including impaired glucose homeostasis. This occurs specifically by enhancing hepatic
glucose output by inducing enzymes regulating gluconeogenesis including glucose-6-phosphatase, fructose-1,6-biphosphatase, and phosphoenolpyruvate carboxykinase.\textsuperscript{136,140} At the same time, glucocorticoids indirectly provide more substrates for gluconeogenesis via proteolysis in skeletal muscle and lipolysis in adipose tissue.\textsuperscript{136,141,142} Excess glucocorticoid production also leads to insulin resistance because translocation of the GLUT4 glucose transporter to the cell surface is reduced leading to decreased glucose uptake by muscle and adipose tissue.\textsuperscript{136,143,144}

GR signaling is triggered by a variety of physiological causes.\textsuperscript{97} Chronic stress resulting in sustained elevated glucocorticoid exposure throughout a lifetime has negative physiological consequences.\textsuperscript{92,94,97,106} Constant GRE binding induces local lasting changes in DNA methylation shaping subsequent responses to stressors and glucocorticoids.\textsuperscript{145-149} It is therefore plausible that chronic stress confers cumulative effects on DNA methylation sites with long-term epigenetic ramifications.\textsuperscript{94} Profound changes in DNA methylation are associated with aging-related diseases.\textsuperscript{150-156} Because of this, several DNA methylation-based predictors of aging have been recently developed.\textsuperscript{152,157-159} For example, a composite predictor comprised of 353 Cytosine-phosphate-Guanosine sites (CpGs) across the genome was shown to strongly correlate with chronological age across multiple human tissues.\textsuperscript{152} Several studies have used this predictor to calculate accelerated epigenetic aging, defined as the difference between DNA-methylation-predicted age and chronological age.\textsuperscript{160-163} This accelerated epigenetic aging has been consequently associated with cancer, obesity, PTSD, physical and cognitive decline, all-cause mortality, lower SES, and cumulative lifetimes stress.\textsuperscript{94,152,161,162} One study found that cumulative lifetime stress was associated with
accelerated epigenetic aging in AAs.\textsuperscript{94} This accelerated epigenetic aging was due to altered GR signaling marked by an increased number of epigenetic clock CpGs located within functional GREs, dynamic methylation changes following exposure to Dex, and dynamic regulation by genes with enriched association for aging-related diseases which neighbored these CpGs.\textsuperscript{94} These results support a model of stress-induced accelerated epigenetic aging mediated by the lasting effects of chronic stressor exposure and aberrant glucocorticoid signaling on the epigenome.\textsuperscript{94}

AAs also appear to have amplified GR signaling and increased glucocorticoid resistance.\textsuperscript{164} This was determined by a study exploring the role of body weight and body composition in insulin resistance and participants were treated with placebo or 4 mg Dex.\textsuperscript{164} Results revealed that AAs were significantly more hyperinsulinemic after Dex treatments than EAs, indicated by higher peak insulin and postprandial insulin.\textsuperscript{164} AAs were also found to be more insulin resistant as determined by fasting insulin and homeostatic model assessment.\textsuperscript{164} This hyperinsulinemia and increased insulin resistance in AAs was independent of body weight or composition suggesting that amplified GR signaling due to hyperactive GRs was more prevalent in the AA study participants.\textsuperscript{164} Taken together, there is a growing body of evidence that aberrant GR signaling occurs frequently within the AA population.

**Glucocorticoid Signaling and Prostate Cancer**

GR is recently emerging as a major driver of PCa progression.\textsuperscript{134,165-172} A pivotal study in 2013 identified induction of GR expression as a common feature of ADT-resistant PCa tumors using pre-clinical models and confirmed in patient samples.\textsuperscript{172} Their
findings established a mechanism of cellular escape from ADT-induced AR blockade via GR activating a set of classical AR-target genes thereby maintaining ADT-resistance in the absence of androgens.\textsuperscript{172} The implications of this initial finding sparked great interest as PCa patients are routinely administered synthetic glucocorticoids as co-therapy alongside ADT and taxane chemotherapy for palliative purposes to reduce adverse side effects.\textsuperscript{171,173-175} Furthermore, the mechanism behind the acquisition of ADT-resistance in PCa cells and the ability to progress in the absence of androgens had previously never been fully elucidated, and these findings paved the way for others to carefully investigate the role of GR.\textsuperscript{171}

There are many types of ADT available to PCa patients that employ various mechanisms of action, and glucocorticoids are co-administered to suppress ACTH resulting in reduction of adrenal androgenic precursors including dehydroepiandrosterone (DHEA) (Figure 5).\textsuperscript{171,176} Complimenting this effect are AR antagonists such as bicalutamide, flutamide, nilutamide, and Enz.\textsuperscript{171,177,178} In addition, drugs have been developed that block the androgen biosynthesis pathway by inhibiting necessary enzymes such as cytochrome P450 and 17α-hydroxylase/17,20-lyase including ketoconazole and abiraterone.\textsuperscript{179-182} Taken together, these antiandrogen agents block both androgen production and action by targeting the HPA and HPT axes as well as PCa intratumoral androgen synthesis (Figure 5).\textsuperscript{171}
Figure 5. Mechanisms by which ADT blocks androgen synthesis and action. The HPT (hypothalamic-pituitary-testicular) axis controls testicular androgen synthesis via luteinizing hormone (LH). In addition, the HPA regulates androgen precursor synthesis via ACTH. Once synthesized, testosterone and DHT bind to AR in the prostate. Upon heterodimerization, AR translocates to the nucleus and interacts with AREs of AR-target genes. Drugs that reduce androgen synthesis and signaling are used clinically to induce androgen deprivation in PCa patients. These drugs include GnRH inhibitors that block LH action, glucocorticoids that inhibit CRH release from the hypothalamus and ACTH from the pituitary, and ketoconazole and abiraterone that inhibit 17-a-hydroxylase activity inhibiting adrenal and testicular androgen steroidogenesis. Abiraterone is also able to block intratumoral synthesis of androgens in PCa cells. In addition, 5-a-reductase inhibitors block conversion of testosterone to DHT, and AR antagonists such as Enz interfere with androgen-AR binding thereby blocking AR signaling. (Narayanan S, Srinivas S, and Feldman D. *Nature Reviews Urology*. 2016.)
While ADT is highly effective in producing an initial period of PCa regression, mCRPC eventually develops characterized by rapidly rising serum PSA levels even though circulating testosterone levels are in the typical castration range (<50 ng/dl).\textsuperscript{183-187} This means that AR-target genes are operating in the absence of androgen to stimulate PCa cell survival, growth, and PSA secretion.\textsuperscript{171} To understand the prospect of GR bypassing the AR signaling pathway and directly activating AR-target genes, the similarities between AR and GR must be dissected. AR and GR belong to the same intracellular receptor family of transcriptional regulators, and the DNA binding domains of AR and GR are highly conserved with an 80\% match in amino acid sequence.\textsuperscript{171,188,189} Similar to GREs, AR response elements (AREs) in the promoter regions of AR-target genes are composed of a 15 base pair binding sequence comprised of two hexamer half-sites and separated by a 3 base pair spacer.\textsuperscript{190} This similarity allows GR to interact with ARE and alter the expression of AR-target genes in the absence of androgens.\textsuperscript{172}

These GR-AR interactions in the context of PCa present a major clinical dilemma because the effects of glucocorticoids are both beneficial and harmful to patients.\textsuperscript{166-168,170-172} Although the natural human glucocorticoid is cortisol (hydrocortisone), synthetic glucocorticoids prednisone and Dex are routinely used therapeutically.\textsuperscript{171} Synthetic glucocorticoids have much higher potency than cortisol in activating GR; for example, 4 mg of prednisone and 0.75 mg of Dex provide the physiological equivalent of 20 mg of cortisol.\textsuperscript{171} Within the clinic, prednisone is used in doses of 5-10 mg once or twice per day and Dex is used in doses at 0.75-1 mg once or twice per day.\textsuperscript{171} When co-administered with taxane chemotherapy for PCa, their potent anti-inflammatory
properties counteract pain, nausea, lack of appetite, fatigue, hypersensitivity, and fluid retention.\textsuperscript{191-195}

While the benefits of glucocorticoid co-administration to PCa patients have been established, there is growing evidence both pre-clinically and in clinical trials that GR signaling may also be detrimental.\textsuperscript{134,165-172,196} One study found that GR expression is initially reduced in primary PCa tissue, but is restored in metastatic lesions.\textsuperscript{168} This group also found that GR blockade by RNAi or chemical inhibition impaired the proliferation and 3D spheroid-forming capabilities of PCa cell lines.\textsuperscript{168} There is also evidence that GR is increased in DTX-resistant PCa cell lines and tissue from patients who have been treated with DTX, and GR antagonists were able to re-sensitize the PCa cells to DTX.\textsuperscript{169} A separate study reported that patients who relapse with PCa biochemical recurrence and have high GR, experience shortened progression-free survival.\textsuperscript{168} Another study demonstrated that Enz treatment induced GR expression in both PCa cell lines as well as patient tissue samples.\textsuperscript{172} Perhaps most disturbing are the reports that PCa patients enrolled in clinical trials have worse outcome in terms of overall survival when receiving glucocorticoids compared to patients not receiving glucocorticoids.\textsuperscript{167,171,197} This trend was observed in the AFFIRM phase 3 clinical trial evaluating the use of Enz as well as in the COU-AA-301 phase 3 clinical trial in which patients were randomized to prednisone plus abiraterone after failing taxane chemotherapy.\textsuperscript{171,198}

The mechanism fueling these adverse outcomes observed in PCa cells and patients treated with glucocorticoids points toward GR bypass of AR signaling pathway.\textsuperscript{172} ADT has been found to elevate PCa cellular GR content leading to activation of specific AR-target genes by GR, and this activation was achieved by a glucocorticoid-
GR complex independent of either androgen or AR. While researchers of that study identified 52 common overlapping genes out of 105 AR signature genes and 121 GR signature genes, several canonical AR-target genes were found to be regulated by GR which included PCa key players KLK3 encoding for PSA and TMPRSS2. Also, GR expression is normally repressed in PCa cells in the presence of AR, however this study demonstrated that AR blockade removes this GR inhibition and stimulates GR amplification.

Whether GR drives ADT-resistance by activating AR-target genes or activating an independent transcriptome that also drives therapy resistance, the argument is becoming very clear that GR plays a major role in the progression of mCRPC. There remains an urgent need however to further elucidate genes driven by GR signaling that are specifically associated with ADT-resistance while also identifying precise genes that have been linked to taxane chemotherapy. This is critical to our understanding of mechanisms by which GR may induce therapy resistance, and the identification of therapeutic targets.

The Role of LEDGF/p75 in Cancer and Chemoresistance

LEDGF/p75 (also known as PC4 and SFRS1 interacting protein [PSIP1] and dense fine speckled autoantigen of 70 kD [DFS70]) was initially identified as a growth factor critical for the proliferation of lens epithelial cells, however subsequent studies have demonstrated that LEDGF/p75 is activated during the cellular response to stress as a ubiquitous nuclear transcription co-activator with oncogenic functions. This protein has garnered increasing attention due to its relevance to cancer, autoimmunity, eye
diseases, and HIV-AIDS.\textsuperscript{201,202} LEDGF/p75 has been firmly established over the last decade as a critical cellular factor for the integration of the human immunodeficiency virus 1 (HIV-1) as LEDGF/p75 facilitates this integration of HIV-1 into host integrin through interaction with the HIV integrase.\textsuperscript{203-209} LEDGF/p75 specifically promotes cellular survival against various environmental stressors including oxidative stress, radiation, heat, serum starvation, and cytotoxic drugs.\textsuperscript{199-202,210-219} The role of LEDGF/p75 in PCa is under investigation as it has been shown to contribute to DTX-resistance, and also has been shown to be the target of autoantibody responses in a subset of PCa patients.\textsuperscript{199,201,220,221}

The \textit{PSIP} gene encodes various splice variants of LEDGF/p75 and has been mapped to chromosome 9p22.\textsuperscript{200} The most commonly recognized splice variants are LEDGF/p75 and its shorter variant LEDGF/p52 which are both members of the hepatoma-derived growth factor (HDGF) family characterized by a highly conserved proline-tryptophan-tryptophan-proline (PWWP) amino acid sequence motif (Figure 6).\textsuperscript{201,212,222} The \textit{PSIP1/LEDGF} gene is made up of 15 exons and 14 introns, with LEDGF/p75 encoded by exons 1-15 (530 amino acids) and LEDGF/p52 encoded by exons 1-9 and 24 nucleotides of intron 9 (333 amino acids). Both LEDGF/p75 and LEDGF/p52 share the amino (N)-terminal residues 1-325 including the PWWP domain (residues 1-98) (Figure 6).\textsuperscript{200,212,222,223} However, the intron-derived carboxyl C-terminal tail-terminal tail (CTT, amino acid residues 326-333) is present in LEDGF/p52 but is absent in LEDGF/p75 (Figure 6).\textsuperscript{224}
**Figure 6.** Two splice variants of *PSIP/LEDGF*, LEDGF/p75 and LEDGF/p52 (Ochs RL et al. *Clin Exp Med.* 2016.)
There is evidence to suggest the role of the PWWP domain in DNA binding, transcriptional repression, and methylation.\textsuperscript{212,222,223,225} When LEDGF/p75 has interacting proteins bound to its C-terminus, the PWWP domain facilitates the chromatin recognition, and locking of this protein.\textsuperscript{226} There is also evidence that LEDGF/p75 binds to transcriptionally active regions in the chromatin because the PWWP domain specifically recognizes trimethylated histone H3K36.\textsuperscript{227} Other important structural features of the N-terminus of LEDGF/p75 includes three charged domains (CR1, CR2, CR3), a nuclear localization signal (NLS) (residues 148-156), a TAT-like sequence within the NLS, and a basic leucine zipper (zip) sequence overlapping with a helix-turn-helix region (residues 154-175) (Figure 6).\textsuperscript{205,214,228} This zip sequence also overlaps with two AT-hooks (residues 178-183) which functions as binding to the DNA minor groove and modifying DNA architecture to facilitate accessibility of promoters to transcription factors.\textsuperscript{227} The NLS and AT-hooks within the tripartite region are sufficient for chromatin binding.\textsuperscript{228} LEDGF/p75 is able to preferentially bind to active transcription sites in the negatively supercoiled DNA over unconstrained DNA due to CR2, also referred to as the supercoiled DNA recognition domain (residues 200-336).\textsuperscript{229} A non-specific DNA-recognition domain also exists in the region comprised of residues 137-206.\textsuperscript{229}

The C-terminus region of LEDGF/p75 (residues 339-442) has been identified as the HIV integrase binding domain (IBD).\textsuperscript{205} The IBD entirely overlaps the epitope recognized by human autoantibodies recognizing LEDGF/p75, and these autoantibodies have been shown to be produced by healthy individuals as well as patients with different inflammatory conditions including PCa.\textsuperscript{201,230} Both the C-terminus and the N-terminus of LEDGF/p75 are utilized for transcription and stress survival functions via interacting
with chromatin-binding proteins or binding to specific stress gene promoter regions.\textsuperscript{207,231-236} Large random coiled regions containing disordered regions, implicated in DNA and RNA recognition, modulation of protein binding, and regulation of protein lifetime are also found in secondary amino acid sequence structures in the C-terminus of LEDGF/p75.\textsuperscript{237} A secondary structure consisting of N-terminal β-strand and α-helix was also predicted by PSIPRED protein structure prediction server V2.1 and PHDsec programs.\textsuperscript{212}

The interaction of LEDGF/p75 with multiple proteins or DNA to form an interactivactome suggests its role in multiple cellular processes including proliferation, growth, differentiation, cell survival, and cell death.\textsuperscript{201} More specifically, LEDGF/p75 upregulation has been shown to protect against an augmented state of oxidative stress induced by agents including ultraviolet B irradiation, hydrogen peroxide, alcohol, hyperthermia, nutrient deprivation, and some chemotherapeutic drugs.\textsuperscript{211-214,216-219,238-241} Our group reported that, during caspase-dependent cell death, LEDGF/p52 is cleaved by caspase-3.\textsuperscript{224} We determined that apoptosis can be induced when LEDGF/p52 or other splice variants lacking most of the PWWP domain are overexpressed in PCa cell lines.\textsuperscript{224} In a separate study, we showed that LEDGF/p75 cleavage by caspases-3 and -7 during apoptosis abrogated its pro-survival functions and accelerated cell death under starvations conditions.\textsuperscript{212}

LEDGF/p75 is also emerging as a stress oncoprotein driving carcinogenesis when cells upregulate this protein to evade cell death, induce growth signals, and upregulate other oncogenic proteins promoting pro-survival functions including cellular repair, DNA damage protection, lysosomal stability, evasion of proteolysis, and angiogenesis.\textsuperscript{217,218,242-}
When cells are exposed to damaging agents that induce thermal or oxidative stress, LEDGF/p75 is upregulated and binds to stress response elements and heat shock elements in target gene promoter regions. As a transcription co-activator, LEDGF/p75 enhances the transactivation of antioxidant genes including albumin, thyroid peroxidase, superoxide dismutase 3, cytoglobin, and antioxidant protein2/peroxiredoxin 6 to assist cells in evading cell death through the reduction of oxidative damage. In addition to this mechanism of action, LEDGF/p75 enhances the transactivation of HSP27 and αβ-crystallin which are anti-apoptotic proteins that respond to stress-related cellular damage by inhibiting caspase-3 activation. The vascular endothelial growth factor C, a prosurvival protein involved in angiogenesis and metastasis, is also transactivated by LEDGF/p75.

Aberrant LEDGF/p75 expression is critical to human leukemogenesis and this protein was reported as upregulated in chemoresistant blasts obtained from patients with acute myeloid leukemia (AML). Several studies have demonstrated that a chromosomal translocation associated to leukemogenesis, t(9;11)(p22;p15), leads to a fusion of nucleoporin 98 protein N-terminus to LEDGF/p75 C-terminus. LEDGF/p75 is also a key co-factor of the mixed lineage leukemia fusion complexes, and interacts with menin-MLL complexes targeting the homeobox HOX genes associated with stem cell self-renewal thereby exploiting migrating cancer progenitors.

In terms of PCa, our group previously reported that 22.3% of patients from a Loma Linda University cohort had circulating serum antibodies that reacted with LEDGF/p75 compared to 6.7% of normal patient controls. This finding is consistent with a separate study from our group demonstrating elevated LEDGF/p75 expression in
PCa tissues compared to normal adjacent tissue. Others have also confirmed this elevated frequency of anti-LEDGF/p75 autoantibodies, contributing to the growing body of literature suggesting altered LEDGF/p75 expression and function contributes to tumor aggressive properties. The ability of LEDGF/p75 to serve as a PCa detection tool has also been explored, and the inclusion of LEDGF/p75 into panels of tumor-associated antigens for serum autoantibody profiling improved the predictive frequency in PCa patients compared to PSA blood tests.

The role of LEDGF/p75 in cancer progression, including PCa, is continuing to be defined and our group was the first to document that elevated LEDGF/p75 protein expression was detected in clinical PCa tumor and benign prostatic hyperplasia tissues as compared to their corresponding normal tissues. We also reported that LEDGF/p75 is overexpressed in several human cancers, with statistically significant upregulation of transcript in prostate, colon, thyroid, and breast cancers, and statistically significant upregulation of protein in prostate, colon, thyroid, liver, and uterine tumors. In addition to LEDGF/p75 upregulation in cancer malignancies, increased expression of LEDGF/p75 is associated with chemoresistance. One study reported increased LEDGF/p75 transcript levels in AML blast from chemoresistant patients. Increased survival was observed in AML cells ectopically (plasmid-mediated) overexpressing LEDGF/p75 compared to cells expressing the empty vector when both cells were treated with daunorubicin or cAMP analogs, suggesting that increased LEDGF/p75 expression contributes to chemoresistance in AML.

Genetic knockdown (siRNAs) of LEDGF/p75 has been shown to result in increased caspase-independent cell death due to lysosomal membrane permeabilization
Conversely, ectopic overexpression of LEDGF/p75 protected MCF-7 cells against LMP-increasing agents such as siramesine, etoposide, doxorubicin, and TNF but not against staurosporine which is a classical inducer of apoptosis. LEDGF/p75 is also reported as necessary for effective DNA double strand break repairs. Complementing these studies, our group has explored the link between LEDGF/p75 overexpression and chemotherapy resistance as it pertains to PCa. We reported that DTX induces a caspase-independent cell death through lysosomal destabilization and cathepsin B activation in PCa cells. Using PC3 and RWPE-2 PCa cell lines, we found that cells with stable plasmid-mediated overexpression of LEDGF/p75 were DTX-resistant and exhibited stable lysosomes compared to cells transfected with an empty vector. We speculated that LEDGF/p75 may protect against lysosomal membrane permeabilization (LMP) but not against mitotic catastrophe or apoptotic cell death when we also observed that LEDGF/p75 overexpression did not abrogate DTX inhibition of microtubule depolymerization or cell death induced by TNF-related apoptosis inducing ligand (TRAIL).

In separate studies, we also showed that PCa cells grown in the presence of DTX and selected for their resistance to this drug, have high endogenous levels of this protein compared to the parental, drug-sensitive cell lines. More recently, we also demonstrated that these DTX-resistant cells overexpressing LEDGF/p75 were not only resistant to DTX, but also exhibited resistance to other taxane drugs used in clinical settings such as PTX and CTX. While we have demonstrated the role of LEDGF/p75 in chemoresistance, it has yet to be explored whether this protein is either AR or GR regulated.
The Role of Clusterin in Cancer, ADT-Resistance, and Chemoresistance

CLU, also known as testosterone-repressed prostate message, apolipoprotein J, and sulphated glycoprotein-2, is a secretory heterodimeric disulfide-linked glycoprotein (449 amino acids) found in all human fluids and widely expressed in many tissues including brain, ovary, testis, liver, heart, lung, breast, and prostate.\textsuperscript{264-267} CLU is involved in a variety of biological processes such as tissue remodeling, lipid transport, cell-cell interactions, sperm maturation, and apoptosis.\textsuperscript{268-274} In addition, CLU is also expressed in many cancers including breast, prostate, ovarian, pancreatic, and renal.\textsuperscript{275-279} CLU appears to play a particularly important role in PCa as high levels of CLU correlate with Gleason score and CLU levels increase following both ADT and chemoresistance.\textsuperscript{280-282} For this reason, the contribution of CLU to PCa tumor aggressiveness and therapy resistance continues to be investigated.

Secreted CLU (sCLU) is a glycosylated protein (76-80 kD) and appears as two different protein bands by immunoblot, one full-length uncleaved 60 kD protein as well as another 40 kD $\alpha$ and $\beta$ protein.\textsuperscript{267,283} sCLU expression is initiated when the first AUG codon of full-length CLU mRNA is translated into the 49 kD sCLU precursor protein (Figure 7a).\textsuperscript{267,283,284} Following translation, a leader signaling sequence directs sCLU to the endoplasmic reticulum and then transported towards the Golgi where it is cleaved at the $\alpha$ and $\beta$ site and heavily glycosylated (Figure 7b).\textsuperscript{267} The resultant sCLU is 80 kD consisting of $\alpha$ and $\beta$ peptides linked together by five disulfide bonds (Figure 7).\textsuperscript{267} This form appears at 40 kD by immunoblot.
Expression of CLU in Malignant Tumours and its Potential Role as a Prognostic Marker

Changes in sCLU expression have been documented in breast, oesophageal and skin cancers (as above), against which sCLU might contribute. However, there is little evidence that sCLU has significant biological activity in human and animal cells. A distinct feature of sCLU is its intracellular localization and elevated sCLU protein expression in the cytoplasm of unstressed cells. It becomes the mature form once it is transported to the nucleus. (Shannan B et al. Cell Death and Diff 2006.)

Figure 7. Generation of sCLU and nCLU. (a) sCLU precursor is translated from the first AUG codon of the full-length CLU mRNA, while nCLU precursor is obtained by alternative splicing from the second in-frame AUG codon of the full-length CLU mRNA. (b) sCLU precursor is transported into the rough ER and undergoes cleavage and extensive glycosylation while being transported to the Golgi apparatus. This results in sCLU of 80 kD with five disulfide bonds between the α and β subunit that is secreted outside the cell. nCLU does not undergo cleavage or glycosylation and resides in the cytoplasm of unstressed cells. It becomes the mature form once it is transported to the nucleus. (Shannan B et al. Cell Death and Diff 2006.)
In contrast, nuclear CLU (nCLU) is an initially synthesized protein of 49 kD and is synthesized from a second in-frame AUG codon resulting from an alternatively spliced nCLU mRNA; this alternative splicing eliminates exon II and by default the sCLU start AUG and signaling leader peptide (Figure 7).\textsuperscript{267} nCLU is localized to the cytoplasm of normal cells and does not undergo \(\alpha/\beta\) cleavage or extensive glycosylation (Figure 7b).\textsuperscript{267} (15) Under conditions of cellular damage, nCLU is post-translationally modified resulting in a mature 55 kD pro-apoptotic protein that is translocated from the cytoplasm to the nucleus.\textsuperscript{267,283} nCLU has been established as pro-apoptotic and capable of inhibiting cell growth and survival.\textsuperscript{283,285-288} Conversely, sCLU exerts cytoprotective properties and tumor cell survival is associated with loss of nCLU and overexpression of sCLU, and there is evidence that tumor growth is related to a pattern shift in isoform production.\textsuperscript{289}

Regarding cell death, p53 which is an activator of the apoptotic cascade can suppress both basal as well as radiation-induced sCLU expression in tumor cells.\textsuperscript{288} One study revealed that loss of functional p53 resulted in loss of nCLU function.\textsuperscript{267} CLU also appears to be regulated by B-MYB, a transcription factor involved in cell survival, proliferation, and differentiation.\textsuperscript{290} CLU has also been found to regulate NF-\(\kappa\)B which is also involved in cell survival, motility, proliferation, and transformation.\textsuperscript{267} The implications of loss of CLU expression in cells dependent upon NF-\(\kappa\)B activity for proliferation or chemoresistance could plausibly lead to tumor progression.\textsuperscript{267} There is also evidence that CLU is cell cycle dependent; CLU overexpression in PCa cells led to increased accumulation of cells at the \(G_0/G_1\) cell cycle phase resulting in slow down of cell cycle progression, reduced DNA synthesis, DNA damage accumulation, and
accelerated PCa progression.\textsuperscript{291} In addition, CLU appears to play an important role in DNA repair.\textsuperscript{292} nCLU protein binds to Ku70 to form a trimeric complex with Ku80 which are two components of the DNA-dependent protein kinase (DNA-PK) complex involved in nonhomologous DNA double-strand break repair.\textsuperscript{287,293} CLU overexpression reduces the binding activity of Ku70/Ku80 to DNA ends.\textsuperscript{287} Furthermore, nCLU prevents nonhomologous end joining by releasing Bax from the cytoprotective Ku70-Bax complex and through inhibition of Ku70/Ku80 end binding activity.\textsuperscript{267,294}

Matrix metalloproteinases (MMPs) are responsible for degradation of most extracellular matrix components, and MT6-MMP produced by neutrophils is believed to be important for invasion and migration of cells to inflammatory sites.\textsuperscript{295} CLU may act as a MT6-MMP negative regulator demonstrating an important role for this protein in cell matrix formation, cell membrane remodeling, and morphological tissue modulation.\textsuperscript{267} In a study overexpressing CLU (plasmid-mediated) in a PCa cell line, membrane damage was inhibited in the presence of hydrogen peroxide compared to cells transfected with empty vector.\textsuperscript{296}

Progression of PCa to castration-resistance requires an upregulation of anti-apoptotic genes following ADT, and CLU expression has been linked to ADT-resistance for over a decade.\textsuperscript{282} There is also evidence that PCa cell transformation at early stages requires CLU silencing through chromatin remodeling.\textsuperscript{297} An early study observed a 17-fold increase in CLU expression in malignant tissue from PCa patients who underwent ADT compared to untreated patients implying that CLU is upregulated in cells as an adaptive cell survival response.\textsuperscript{282} Several others have employed a variety of molecular techniques to elucidate the mechanism by which this CLU upregulation occurs in PCa
cells. Using the PC3 cell line, researchers found that co-treatment with siRNA targeting CLU in combination with paclitaxel significantly enhanced the cytotoxic effect of paclitaxel. In a separate study, both transcript and protein of CLU increased with androgen treatment in a time- and dose-dependent manner, and AR antagonist bicalutamide inhibited this increase in CLU. The first intron of the CLU gene contains putative AREs and was confirmed by chromatin immunoprecipitation and reporter assays to be both bound by AR and transactivated. When PCa cells were treated with Enz, CLU protein levels significantly increased as Enz-resistance developed; and CLU knockdown enhanced the cell growth inhibitory effects of Enz. This increase in CLU was also observed in a dose- and sequence-dependent manner when AR was specifically targeted with AR antisense in PCa cells. A combination of Enz plus CLU silencing was able to significantly reduce AR nuclear translocation and transcriptional activity of AR as well as accelerated proteasome-mediated AR degradation.

In addition to CLU’s role in ADT-resistance, CLU has been shown to be instrumental in the acquisition and maintenance of chemoresistance. The gold standard treatment for PCa is taxane chemotherapy, namely DTX, and CTX, and these agents induce cell death by stabilizing microtubules resulting in mitotic catastrophe. Taxanes are also highly effective in disrupting bcl-2 phosphorylation thereby reversing its anti-apoptotic function. However, the beneficial effects of taxane chemotherapy are short-lived and eventually PCa patients develop chemoresistance, and CLU plays a major role. There is evidence that CLU overexpression helps to create a chemoresistant phenotype. For example, LNCAP PCa cells are highly sensitive to taxane paclitaxel, but when transfected with CLU, LNCAP cells were able to withstand
paclitaxel and avoid apoptotic cell death. Similar studies using PC3 cells demonstrated that CLU blockade with specific siRNA re-sensitized cells to paclitaxel. In vivo studies in nude mice reported that parental human LNCAP tumors quickly regressed following castration and paclitaxel treatment, but CLU overexpressing LNCAP continued to grow. A complementary study was performed using Shionogi tumors characterized as CLU-positive; CLU antisense used in conjunction with paclitaxel was highly effective in tumor shrinkage. Similar studies have also been conducted in renal carcinoma, breast, and lung cancer pre-clinical cellular models and the results have mirrored what has been described in PCa cells. Given that CLU confers survival advantage to cancer cells and is easily induced by therapeutic agents, CLU targeting remains under investigation as a plausible clinical option to overcome drug resistance. Because of these findings at the benchside, clinical trials were recently developed to co-target CLU in combination with ADT or taxane chemotherapy for PCa patients.

While CLU has been established as a driver of ADT-resistance and chemoresistance in PCa cells and patient samples, the potential regulation of CLU by GR in the absence of AR function has yet to be explored.
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CHAPTER TWO

THE 22RV1 PROSTATE CANCER CELL LINE CARRIES MIXED GENETIC ANCESTRY: IMPLICATIONS FOR PROSTATE CANCER HEALTH DISPARITIES RESEARCH USING PRE-CLINICAL MODELS

Abstract

Understanding how biological factors contribute to prostate cancer (PCa) health disparities requires mechanistic functional analysis of specific genes or pathways in pre-clinical cellular and animal models of this malignancy. The 22Rv1 human prostatic carcinoma cell line was originally derived from the parental CWR22R cell line. Although 22Rv1 has been well characterized and used in numerous mechanistic studies, no racial identifier has ever been disclosed for this cell line. In accordance with the need for racial diversity in cancer biospecimens and recent guidelines by the NIH on authentication of key biological resources, we sought to determine the ancestry of 22RV1 and authenticate previously reported racial identifications for four other PCa cell lines. We used 29 established Ancestry Informative Marker (AIM) single nucleotide polymorphisms (SNPs) to conduct DNA ancestry analysis and assign ancestral proportions to a panel of five PCa cell lines that included 22Rv1, PC3, DU145, MDA-PCa-2b, and RC-77T/E. We found that 22Rv1 carries mixed genetic ancestry. The main ancestry proportions for this cell line were 0.41 West African (AFR) and 0.42 European (EUR). In addition, we verified the previously reported racial identifications for PC3 (0.73 EUR), DU145 (0.63 EUR), MDA-PCa-2b (0.73 AFR), and RC-77T/E (0.74 AFR) cell lines. Considering the mortality disparities associated with PCa, which disproportionately affect African American men, there remains a burden on the scientific...
community to diversify the availability of biospecimens, including cell lines, for mechanistic studies on potential biological mediators of these disparities. This study is beneficial by identifying another PCa cell line that carries substantial AFR ancestry. This finding may also open the door to new perspectives on previously published studies using this cell line.

Introduction

African American (AA) men and other men of African ancestry have the highest incidence and mortality rates of prostate cancer (PCa) in the world, resulting in the highest reported cancer health disparity. We use the term “African American” recognizing that while race is a social construct, racial classification remains extremely useful for describing general patterns of national health and health disparities, as most U.S. health data are reported by self-identified race.\(^1\,^5\) Recent studies have provided compelling evidence in support of the notion that PCa health disparities result from the interplay of multiple factors, including biological/genetic factors.\(^6\,^8\) For instance, several recent studies have reported genomic differences between AA men with PCa and Caucasian or European American (EA) with PCa, suggesting a potential role for biological mediators in driving PCa mortality disparities.\(^9\,^{13}\) Understanding how these mediators contribute to increased PCa mortality in AA men requires mechanistic functional studies in pre-clinical cellular and animal models of PCa.

Given the inherent difficulties in conducting mechanistic studies in primary PCa cells, including transfection issues and high degree of molecular variability as these primary cells propagate in vitro,\(^14\,^{16}\) it is essential to have available a well-characterized,
racingly diverse, patient-derived cohort of immortalized cell lines representing different stages of PCa. Unfortunately, there is currently a lack of racial diversity in the human PCa cell lines that are commercially available for research. While there is over a dozen of commercially available human prostate cell lines of European ancestry that are representative of various prostate phenotypes such as normal and different PCa stages, there are only three cell lines identified as having African ancestry (Table 1).\textsuperscript{17-29} This limits the scope of \textit{in vitro} studies addressing mechanistic events involving potential biological factors associated with PCa health disparities.
## Table 1. Commonly Used Commercially Available Prostate Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Site of Origin</th>
<th>Race/ Ethnicity</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>22Rv1</td>
<td>Primary xenograft</td>
<td>Not Available</td>
<td>ATCC</td>
<td>Sramkoski RM et al. 1999</td>
</tr>
<tr>
<td>DU145</td>
<td>Brain metastasis</td>
<td>EA/ White</td>
<td>ATCC</td>
<td>Stone KR et al. 1978</td>
</tr>
<tr>
<td>E006AA-hT</td>
<td>Primary tumor</td>
<td>AA/ Black</td>
<td>ATCC</td>
<td>Koochekpour S et al. 2014</td>
</tr>
<tr>
<td>LAPC-3</td>
<td>Prostate xenograft</td>
<td>Not Available</td>
<td>ATCC</td>
<td>Klein KA et al. 1997</td>
</tr>
<tr>
<td>LAPC-4</td>
<td>Lymph node metastasis</td>
<td>Not Available</td>
<td>ATCC</td>
<td>Klein KA et al. 1997</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Lymph node metastasis</td>
<td>EA/ White</td>
<td>ATCC</td>
<td>Horoszewicz JS et al. 1983</td>
</tr>
<tr>
<td>MDA-PCa-2a</td>
<td>Bone metastasis</td>
<td>AA/ Black</td>
<td>ATCC</td>
<td>Navone NM et al. 1997</td>
</tr>
<tr>
<td>MDA-PCa-2b</td>
<td>Bone metastasis</td>
<td>AA/ Black</td>
<td>ATCC</td>
<td>Navone NM et al. 1997</td>
</tr>
<tr>
<td>PC3</td>
<td>Bone metastasis</td>
<td>EA/ White</td>
<td>ATCC</td>
<td>Kaighn ME et al. 1979</td>
</tr>
<tr>
<td>PrEC</td>
<td>Normal human primary prostate epithelial cells</td>
<td>Variable</td>
<td>Lonza</td>
<td>Not applicable</td>
</tr>
<tr>
<td>PrSC/WPMY-1</td>
<td>Normal prostate/stroma</td>
<td>EA/ White</td>
<td>ATCC, Lonza</td>
<td>Webber MM et al. 1999</td>
</tr>
<tr>
<td>PWR-1E</td>
<td>Normal prostate</td>
<td>EA/ White</td>
<td>ATCC</td>
<td>Webber MM et al. 1996</td>
</tr>
<tr>
<td>PZ-HPV-7</td>
<td>Normal prostate/epithelial</td>
<td>EA/ White</td>
<td>ATCC</td>
<td>Weijerman PC, et al. 1994</td>
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<tr>
<td>VCaP</td>
<td>Vertebral metastasis</td>
<td>EA/ White</td>
<td>ATCC</td>
<td>Loberg RD et al. 2006</td>
</tr>
<tr>
<td>WISH-PC2</td>
<td>Neuroendocrine xenograft prostate small-cell carcinoma</td>
<td>EA/ White</td>
<td>ATCC</td>
<td>Pinthus JH et al. 2008</td>
</tr>
<tr>
<td>WPE1-NA22</td>
<td>Prostate</td>
<td>EA/ White</td>
<td>ATCC</td>
<td>Webber MM et al. 2001</td>
</tr>
<tr>
<td>WPE1-NB11</td>
<td>Normal prostate</td>
<td>EA/ White</td>
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<td>Webber MM et al. 2001</td>
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<td>WPE1-NB26</td>
<td>Normal prostate</td>
<td>EA/ White</td>
<td>ATCC</td>
<td>Webber MM et al. 2001</td>
</tr>
</tbody>
</table>

AA, African American; ATCC, American Type Culture Collection; EA, European American
Research into molecular and genetic mechanisms underlying PCa mortality disparities will be greatly advanced by the availability of pre-clinical cellular models of AA prostate tumors representing different stages of the disease. There is a growing awareness of the need for biospecimens from AA populations that could be used for biomedical research, and an effort has been made by PCa researchers to develop AA prostate cell lines for cancer health disparities research. For example, RC-77N/E and RC-77T/E is a pair of African American prostate cell lines that were recently developed by one of the co-authors in this study (CY), and are available for research upon request. While we recognize that the use of cell lines in cancer research has limitations, and that determinants of PCa health disparities may not be conclusively identified via in vitro studies, the use of racially diverse PCa cell lines in mechanistic studies may provide important clues into contributing biological mediators.

The 22Rv1 human prostatic carcinoma cell line was derived from a xenograft of CWR22R cells serially propagated in mice after castration-induced regression. A number of biological characteristics were specified when 22Rv1 was derived, including epithelial lineage, DNA content, and cytogenetic data. The donor patient clinical information provided for the parental cell line CWR22R was the diagnosis of Stage D primary prostatic carcinoma with Gleason score of 9 advanced to osseous metastasis. While conducting studies using 22Rv1 cells we noticed that no racial identifier was ever released for this cell line or the parental CWR22R, and no subsequent DNA ancestry analysis has been conducted to identify its ethnicity. Recently, the National Institutes of Health established guidelines concerning the authentication of key biological resources, including cell lines, in order to ensure the identity and validity of the resource.
This authentication applies to racial identity if the cell line is being used in biological studies relevant to cancer health disparities. In this context, we sought to determine the ancestry of 22Rv1 and authenticate, using genetic ancestry analysis, a few previously racially identified cell lines used in PCa research. These included the PCa cell lines MDA-PCa-2b and RC-77T/E, previously reported as Black or AA,\textsuperscript{22,30} and the PC3 and DU145 cell lines, previously reported as Caucasian or EA.\textsuperscript{18,23}

To determine the ancestry of the 22Rv1 cell line we used a set of Ancestry Informative Markers (AIMs) validated as single nucleotide polymorphism (SNP) genotypes for population structure analyses.\textsuperscript{35} PC3, DU145, MDA-PCa-2b, and RC-77T/E cell lines were included in the study to further validate their previously reported ethnicities and serve as controls for our SNP genotypes.\textsuperscript{18,22,23} Our analysis confirmed predominant European (EUR) ancestry in PC3 and DU145 cells as well as predominant West African (AFR) genetic ancestry in the MDA-PCa-2b and RC-77T/E cells. However, the 22Rv1 cell line was found to carry substantial AFR genetic ancestry. The racial classification of 22Rv1 as a mixed ancestry cell line with substantial AFR genetic ancestry expands diversity within the existing pool of human PCa cell lines. This finding may offer a new perspective on previously published studies using this cell line.

**Materials and Methods**

**Cell Culture**

Cell lines were purchased from American Type Culture Collection (ATCC) and grown in a humidified incubator with 5% CO\textsubscript{2} at 37°C. Cells were routinely tested for mycoplasma contamination using MycoAlert\textsuperscript{TM} PLUS Mycoplasma Detection Kit (Lonza,
Cat. # LT07). PC3 (ATCC, Cat. # CRL-1435), DU145 (ATCC, Cat. # HTB-81), and 22Rv1 (ATCC, Cat. # CRL-2505) cell lines were cultured in RPMI 1640 medium (Corning, Cat. # 10-040-CV) supplemented with 10% fetal bovine serum (Corning, Cat. # 35010CV), penicillin-streptomycin (Corning, Cat. # 30001Cl), and gentamicin (Gibco, Cat. # 15710064) as recommended by the supplier. MDA-PCa-2b (ATCC, Cat. # CRL-2422) cell line was cultured in F-12K medium (ATCC, Cat. # 30-2004) supplemented with 20% fetal bovine serum (Corning, Cat. # 35010CV), cholera toxin (Sigma-Aldrich, Cat. # C8052), epidermal growth factor (Sigma-Aldrich, Cat. # E4127), \( \alpha \)-phosphoethanolamine (Sigma-Aldrich, Cat. # P0503), hydrocortisone (Sigma-Aldrich, Cat. # H0888), selenious acid (ACROS Organics, Cat. # AC19887), bovine insulin (Sigma-Aldrich, Cat. # I6634), and penicillin-streptomycin as recommended by the supplier. 0.2% Normocin (Invivogen, Cat. # ANT-NR-1) was added to the medium for PC3, DU145, 22Rv1, and MDA-PCa-2b cell lines to prevent contamination by mycoplasma, bacteria, or fungi. The RC-77T/E cell line was cultured with keratinocyte serum-free medium (K-SFM) with L-glutamine supplemented with bovine pituitary extract and recombinant epidermal growth factor 1-53 (Life Technologies, Cat. # 17-005-042). Fungizone (0.4%, Gibco, Cat. # 15290018) was added to this medium to prevent contamination by yeast or multicellular fungi. To ensure proper cell attachment to culture plates, RC-77T/E cell line was cultured in 1% collagen-treated dishes (100mm x 20mm) (Life Technologies, Cat. # A1064401).
DNA Extraction

DNA was extracted using the QIAamp DNA mini kit (Qiagen, Valencia, CA, Cat. # 51104) following the manufacturer’s protocol. Briefly, cells were grown in a monolayer at confluency no greater than $5 \times 10^6$. Medium was aspirated and cells were washed with Dulbecco’s phosphate-buffered saline (PBS) (Corning, Cat. # 21030CM) and then trypsinized with 0.25% trypsin (Corning, Cat. # 25-053-C1) to detach them from the culture flask. Cells were then collected in appropriate medium, transferred to a 1.5 ml microcentrifuge tube, and centrifuged for 5 minutes at 300 $\times$ g. Supernatant was removed and cell pellet was resuspended in PBS to a final volume of 200 µl. After this, 20 µl Proteinase K and 200 µl of Qiagen buffer AL were added and the sample was incubated for 10 min at 56°C. Then, 200 µl of ethanol was added, and DNA was purified using the columns provided in the kit according to the manufacturer's instructions. The samples yielded approximately 6 µg of DNA with an $A_{260}/A_{280}$ ratio of 1.7-1.9. The samples were diluted in distilled water and stored at -20°C. Samples were shipped to the UCLA Sequencing and Genotyping Core, Department of Human Genetics, David Geffen School of Medicine, University of California, Los Angeles for subsequent SNP genotyping.

Selection of Ancestry Informative Marker (AIM) SNPs

We used 29 Ancestry Informative Marker (AIM) SNPs validated in a previous study involving determination of admixture proportions in human continental populations. We selected the smallest subset of 24 AIMS and 5 additional SNPs from the previous study, as a cost-and time-effective strategy, to determine the ancestry of the above-mentioned PCa cell lines of interest. All the 29 selected SNPs exhibited large
allele frequency differences between four continental populations - AFR, EUR, Amer-Indian (AMI), and East Asian (EAS) in the previous study. We confirmed the allele frequency differences between these ancestral groups for each of the 29 AIM SNPs using the NCBI dbSNP database.

**SNP Genotyping**

SNP genotyping was done using the Fluidigm Biomark HD system (South San Francisco, CA) at the UCLA Genotyping core facility. SNPtype™ assays and reagents for each of the SNPs were purchased from Fluidigm. DNA samples and assays were loaded onto GT 96*96 Dynamic array and processed per Fluidigm protocol. The genotyping calls were made using Fluidigm SNP genotyping software. Each DNA sample was analyzed in triplicate to ensure accuracy.

**DNA Ancestry Analysis**

Data for the current study was analyzed as part of a larger project involving 1078 DNA samples. Markers showing different alleles at very high frequency in distinct putative parental populations were used to efficiently distinguish different populations and for the inference of ancestry membership proportions. In this study, we used 29 selected unlinked AIM SNPs as 29 loci for the STRUCTURE software version 2.3.4, for providing an estimation of genetic ancestry. Allele frequencies for the 29 AIM SNPs are provided in Table 2. The software was installed and run locally after importing the SNP data for 1078 DNA samples in the form of a matrix where the data for samples were in rows and the loci were in columns (.txt file). For the determination of true K or the
assumed number of populations, length of the Burnin period used was 5,000, the number of Markov Chain Monte Carlo (MCMC) replicates after the Burnins was 50,000 and K was set for 1-10 with 3 replicates for each K. True K was determined to be equal to 2~3 by L(K) (log likelihood of each K) method, using Structure Harvester with a zipped version of the results from STRUCTURE, as an input file. After the determination of true K, STRUCTURE was run three times, without any prior population assignment for an admixture population using 100,000 as the length of the Burnin period and 500,000 as the number of MCMC replicates after the burnins, for K=2 and K=3. The missing value was represented as -9, the maximum value of ALPHA (ALPHAMAX) was 10.0 and the SD of proposal for updating ALPHA was 0.025, while rest of the parameters used were default. The three clusters obtained as an output from STRUCTURE were assigned EUR, AFR, and AMI ancestries by crosschecking the allele frequencies for AIMs of multiple DNA samples within each cluster against those found in NCBI dbSNP. The bar plot was generated by STRUCTURE for visualization of ancestral proportions in each sample. PC3 and DU145 cells along with MDA-PCa-2b and RC-77T/E cells served as controls for EUR and AFR ancestries, respectively, and aligned well with the results where K=3.
<table>
<thead>
<tr>
<th>dbSNP ID</th>
<th>Chromosome Number</th>
<th>Nucleotide Change</th>
<th>Allele 1 frequency</th>
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</thead>
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<td>18</td>
<td>G/A</td>
<td>0.89 0.09</td>
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<td>rs9530435</td>
<td>13</td>
<td>T/C</td>
<td>0.79 0.07</td>
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<td>T/C</td>
<td>0.73 0.10</td>
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<td>rs7554936</td>
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<td>T/C</td>
<td>0.34 0.99</td>
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<td>rs772262</td>
<td>12</td>
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<td>0.06 0.87</td>
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dbSNP, NCBI Single Nucleotide Polymorphism database; EUR, European; AFR, West African
Results

Informative Ancestry Markers for Distinguishing Ancestral Proportions

An initial subset of 24 AIM SNPs was selected from a validated larger set of 128 SNPs previously identified to define critical genome candidate regions and used to characterize samples from diverse population groups (Table 2). This subset of 24 AIMs contained specific SNPs capable of distinguishing the following four continent populations: EUR, AFR, AMI, and EAS. Five additional AIMs from the validated larger set of 128 AIMs were added to the smaller subset of 24 AIMs, bringing our total quantity of AIMs used for DNA ancestry analysis to 29 (Table 2).

Determination of Ancestral Proportions of Prostate Cancer Cell Lines

Each PCa cell line analyzed in our study possessed heterogeneous ancestral proportions (Figure 8, Table 3). However, for each cell line where ATCC provided a racial classification, we observed a predominant ancestral proportion that was consistent with what was previously reported. For instance, PC3 and DU145 cell line samples were included in our analysis as EUR controls, and MDA-PCa-2b and RC-77T/E as AFR controls based on their previously reported racial classification. PC3 is classified by ATCC as Caucasian and was found to carry 0.73 proportion of EUR ancestry. This cell line was also found to carry 0.19 AFR and 0.08 AMI ancestral proportions. DU145 is also classified by ATCC as Caucasian and was found to carry 0.63 proportion of EUR ancestry. Interestingly, DU145 was also found to carry 0.28 AFR and 0.09 AMI ancestral proportions. MDA-PCa-2b is classified by ATCC as Black and was found to carry 0.73 proportion of AFR ancestry as well as 0.13 AMI and 0.14 EUR ancestral proportions.
RC-77T/E is not available through ATCC, however it was reported by one of the authors of this study (C. Yates) as an AA cell line, and our results confirmed this. RC-77T/E was found to carry 0.74 proportion of AFR ancestry as well as 0.09 AMI and 0.17 EUR ancestral proportions. To date, there has never been a racial classification assigned to 22Rv1. Our results indicated the following ancestral proportions for 22Rv1: 0.41 AFR, 0.17 AMI, and 0.42 EUR. Therefore, 22Rv1 should be considered as a mixed ancestry cell line.
**Figure 8.** DNA Ancestry Analysis Reveals Ancestral Proportions of Prostate Cancer Cell Lines. DNA was isolated and extracted from five PCa cell lines and submitted to the UCLA genomics core for SNP genotyping. Cell lines included EUR controls PC3 and DU145, AFR controls MDA-PCa-2b and RC-77T/E, and the racially ambiguous 22Rv1. DNA samples were analyzed in triplicate to ensure accuracy. The bar plot shown in this figure was generated using STRUCTURE for visualization of the ancestral proportion in each sample. Subsequent analysis of population substructure was also conducted using STRUCTURE, a model-based clustering software able to detect the underlying genetic population among a set of individuals genotyped at multiple markers with the help of a bayesian approach. Using STRUCTURE, we were able to compute the proportion of the genome of the cell lines originating from each inferred population. Blue: AFR ancestry; Green: AMI ancestry; Red: EUR ancestry.
Table 3. Ancestry Proportions in Panel of Prostate Cancer Cell Lines

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<td></td>
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</tr>
<tr>
<td>PC3</td>
<td>0.19</td>
</tr>
<tr>
<td>DU145</td>
<td>0.28</td>
</tr>
<tr>
<td>MDA-PCa-2b</td>
<td>0.73</td>
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<td>RC-77T/E</td>
<td>0.74</td>
</tr>
<tr>
<td>22Rv1</td>
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</table>

Samples were analyzed in triplicates to ensure accuracy.
AFR, African ancestry; AMI, Ameri-Indian ancestry; EUR, European ancestry.
Discussion

In concordance with recent NIH guidelines requiring authentication of cell lines, this study sought to determine the racial identity of 22Rv1 PCa cells since racial classification/ethnicity for this cell line has never been reported to ATCC or previously published.\textsuperscript{17,34} It has been our experience in personal communication with other groups that that 22Rv1 is widely considered as a EA PCa cell line, and we had no reason to explore this assumption until we began incorporating various EA and AA cell lines in our studies.\textsuperscript{36-38} We initially conducted a thorough literature search and contacted ATCC to determine if there was any indication of racial identification for either 22Rv1 or its parental cell line CWR22R. In addition, we communicated with key authors of the initial reports on the characterization of both cell lines to determine if there was any racial identification linked to the tumor biospecimen from which these cell lines were derived.\textsuperscript{17,34} Unfortunately, these efforts did not provide any indication as to the racial classification of these cell lines. This lack of information prompted us to conduct DNA ancestry analysis on 22Rv1 cells and provide this information to the PCa research community and the scientific community at large. We felt this was a very important issue given the need for a comprehensive panel of racially diverse patient-derived cell lines for mechanistic studies on potential biological mediators of PCa mortality disparities.

SNP genotyping and subsequent DNA ancestry analysis were chosen as appropriate methods to determine the ancestry for 22Rv1. Other investigators have shown that differences in allele frequencies can be used to identify continental population groups.\textsuperscript{39,40} This is because several studies have demonstrated that large differences in allele frequencies between two or more continental populations can be attributed to many
individual SNPs distributed throughout the genome. In this study, 29 AIM SNPs were chosen from a validated larger set of 128 SNPs previously identified by others to define critical genome candidate regions and used to characterize samples from diverse population groups. Differences in population structure were addressed by extending genetic associations to minority groups that include extensive admixture between continents. This genetic association extension was essential to evaluate ethnic disparities in disease risk among multiethnic and admixed populations as a large number of whole genome association studies have evaluated populations of predominantly EUR ancestry.

Our analysis revealed that the 22Rv1 PCa cell line carries AFR genetic ancestry, with almost equal proportions for AFR and EUR ancestry (41% and 42%, respectively). While this study authenticates the previously reported racial identification of several PCa cell lines (PC3, DU145, MDA-PC-2b, and RC-77T/E), it establishes for the first time the ancestral composition of 22Rv1. Because 22Rv1 was derived from a xenograft of CWR22R which was serially propagated in mice, it would be appropriate to also assign this mixed ancestral racial classification to CWR22R. The implications of 22Rv1 and CWR22R carrying AFR genetic ancestry are far reaching as genetic factors associated with AFR ancestry have been recently linked to prostate tumor aggressive properties. Further studies are needed to determine if these cell lines carry any of these factors. In addition, our findings open the door for closely examining previous studies using 22Rv1 and CWR22R cells under the new perspective that these cell lines carry almost equal proportions of AFR and EUR genetic ancestry.
We suggest that 22Rv1 and CWR22R can be added to the current roster of PCa cell lines that are classified as Black, AA, or carry substantial AFR genetic ancestry, including MDA-PCa-2b, RC-77N/E, and RC-77T/E. Alternatively, they could be classified in a separate category as racially mixed ancestry cell lines. We cannot rule out the possibility that CWR22R was originally derived from a Hispanic male of Caribbean heritage as the proportions of AFR, AMI, and EUR genetic ancestry found in the 22Rv1 cell line are common in men from this geographical region. For example, recent studies have highlighted the high tri-hybrid admixture (Native American, European, West African) of the Puerto Rican population. Knowledge that 22Rv1 carries substantial AFR ancestry would be beneficial to efforts to expand the selection of racially diverse cell lines for in vitro mechanistic studies focusing on potential determinants PCa mortality disparities that could be attributable to genetically- and racially-driven tumor biological differences. It is important to note that while this study contributes to the increasing diversity of PCa cell lines, thus far all available AA PCa cell lines are derived from patients with metastatic disease and tumors with functional androgen and glucocorticoid receptors. There remains a critical need for AA PCa cell lines derived from metastatic androgen-independent tumors (e.g. equivalent to the EA PC3 and DU145). This would be important for mechanistic studies on different disease stages using AA PCa cell lines.

Conclusions

The field of PCa health disparities research continues to advance, with recent attention to potential biological determinants or mediators of these disparities that could
be mechanistically examined using a panel of racially diverse, patient-derived cell lines. The identification of 22Rv1 as a mixed ancestry PCa cell line carrying substantial AFR genetic ancestry provides an additional resource to advance molecular/cellular research in the context of PCa health disparities. While there still remains a deficit in the availability of AA PCa cell lines and clinical biospecimens, the trend towards acquiring additional specimens appears to be growing. The knowledge to be gained from mechanistic studies incorporating biospecimens (cell lines plus tumor samples) from AA men will not only have the potential to reduce the mortality disparities associated with this malignancy but also benefit the field of PCa research in general.

Acknowledgements

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References


CHAPTER THREE
GLUCOCORTICOIDS INDUCE STRESS ONCOPROTEINS ASSOCIATED WITH THERAPY-RESISTANCE IN AFRICAN AMERICAN AND EUROPEAN AMERICAN PROSTATE CANCER CELLS

Abstract

Glucocorticoid receptor (GR) is emerging as a key driver of prostate cancer (PCa) progression and therapy resistance in the absence of androgen receptor (AR) signaling. Acting as a bypass mechanism, GR activates AR-regulated genes, although GR-target genes contributing to PCa therapy resistance remain to be identified. Emerging evidence also shows that African American (AA) men, who disproportionately develop aggressive PCa, have hypersensitive GR signaling linked to cumulative stressful life events. Using racially diverse PCa cell lines—MDA-PCa-2b, 22Rv1, PC3, and DU145—we examined the effects of glucocorticoids on the expression of two stress oncoproteins associated with PCa therapy-resistance, Clusterin (CLU) and Lens Epithelium-Derived Growth Factor p75 (LEDGF/p75). We observed that glucocorticoids upregulated LEDGF/p75 and CLU in PCa cells, most robustly in cell lines of substantial African ancestry, MDA-PCa-2b and 22Rv1. Blockade of GR activation abolished this upregulation. We also detected increased GR transcript expression in AA PCa tissues, compared to European American (EA) tissues, using Oncomine microarray datasets. These results demonstrate that glucocorticoids upregulate the therapy resistance-associated oncoproteins LEDGF/p75 and CLU, and suggest that this effect may be enhanced in AA PCa. This study provides
an initial framework for understanding the contribution of glucocorticoid signaling to PCa health disparities.

**Introduction**

For decades, androgen deprivation therapy (ADT) has been a mainstay of treatment for advanced prostate cancer (PCa).\textsuperscript{1-3} The mechanism of action of ADT involves the lowering of serum testosterone or competitively blocking the binding of androgens to androgen receptor (AR). However, this therapy is not curative, as several studies have conclusively demonstrated that prostate tumors develop ADT-resistance.\textsuperscript{1,2} Glucocorticoid receptor (GR) signaling has recently been shown to drive ADT-resistance via its ability to bypass the AR pathway blockade and directly restore activation of AR-target genes in addition to activating an independent transcriptome that also drives therapy resistance.\textsuperscript{1,4-9}

A pressing implication is that glucocorticoid therapy presently administered to PCa patients as a standard of care could be detrimental under certain clinical conditions.\textsuperscript{1,9-11} For example, there is evidence that glucocorticoids promote PCa progression in patients whose tumors express GR, and that men who receive glucocorticoids concomitantly with the second-line ADT drug enzalutamide have significantly worse survival.\textsuperscript{4,6} However, a clinical dilemma exists as glucocorticoids confer many palliative benefits to patients who often suffer from debilitating side effects of their treatment.\textsuperscript{12}

Similarly, the importance of glucocorticoid co-therapy also extends to taxane-based chemotherapeutic regimens for patients with metastatic castration-resistant PCa.
(mCRPC). The taxane drugs docetaxel (DTX) and cabazitaxel (CTX) can extend patient survival, however, they are also not curative because patients eventually develop resistance to these drugs.\textsuperscript{13,14} Glucocorticoids are commonly co-administered with taxanes to mitigate side effects of chemotherapy such as nausea, vomiting, and inflammatory reactions. Of concern, however, is the recent evidence pointing to the possible contribution of GR signaling to the acquisition of taxane resistance in breast and prostate cancers.\textsuperscript{15,16} These recent studies indicated that simultaneous exposure of the cancer cells to DTX and the clinically relevant glucocorticoid dexamethasone (Dex) led to increased resistance to DTX.

While the ability of GR to activate AR-target genes in the context of mCRPC has been demonstrated,\textsuperscript{1,4-11} there is a need to identify specific genes driven by GR signaling that have been previously linked to taxane chemotherapy. This is critical to our understanding of mechanisms by which GR may induce taxane resistance, and the identification of potential therapeutic targets. We hypothesized that stress oncoproteins that are upregulated in the context of standard PCa treatments and that promote therapy resistance may be upregulated by GR signaling. As a first step in evaluating this hypothesis we focused on the contribution of GR signaling to the expression of the stress oncoproteins Clusterin (CLU) and Lens Epithelium-Derived Growth Factor p75 (LEDGF/p75), previously shown to be upregulated in response to standard PCa therapies, including taxane therapy.\textsuperscript{17-26} CLU is an AR-regulated, anti-apoptotic protein that is upregulated in PCa, particularly following ADT, as well as several other cancers.\textsuperscript{18,20,22,27,28} CLU has two isoforms that result from two transcriptional start sites; nuclear CLU is pro-apoptotic and sequestered in the nucleus whereas secreted CLU
(sCLU) is ultimately secreted following post-translational modifications and cleavage into two distinct alpha and beta peptides held together by disulfide bonds.\textsuperscript{18,19,29} Before cleavage, sCLU exists in the cytoplasm as a pre-secreted form (psCLU) and both forms contribute to DTX resistance.\textsuperscript{19,21}

Similar to CLU, LEDGF/p75 also promotes taxane resistance in PCa cells, albeit by a different mechanism. Our group and others have demonstrated that LEDGF/p75 is a stress response transcription co-activator upregulated in PCa as well as other cancers that promotes cellular survival in the presence of chemotherapeutic drugs.\textsuperscript{25,26,30-34} While CLU inhibits drug-induced apoptosis by preventing mitochondrial membrane permeabilization,\textsuperscript{19,21,22} LEDGF/p75, acting as a stress transcription co-activator, transactivates stress response and anti-oxidant genes, and promotes resistance to oxidative stress-induced necrosis and DTX-induced caspase-independent lysosomal cell death.\textsuperscript{25,26,30,31} In a recent study, we showed that depletion of LEDGF/p75 in DTX-resistant mCRPC cells partially resensitized the cells to DTX treatment.\textsuperscript{26} In addition, other groups have shown that downregulation of LEDGF/p75 reduced cancer cell proliferation, migration, tumorigenicity, and sensitized cancer cells to anti-tumor drugs.\textsuperscript{35-38}

The implications of glucocorticoid-activated GR signaling upregulating oncoproteins associated with tumor progression and therapy resistance are far-reaching and have the potential to impact PCa patients who may have elevated levels of endogenous cortisol or a propensity for hypersensitive GR signaling. For example, individuals exposed to chronic stressful life events tend to have increased cortisol levels, which have been positively associated with increased exposure to psychosocial
stressors.\textsuperscript{39-41} This could be problematic for African American (AA) men, who bear a disproportionate burden of incidence and mortality of PCa, compared to European American (EA) men,\textsuperscript{42-46} and have been shown to have increased cortisol production directly linked to cumulative stressful life events.\textsuperscript{40} In addition, there is evidence of dysregulated GR signaling due to hypersensitive GRs in AA men.\textsuperscript{41,47} Recent studies demonstrated that glucocorticoid treatment, which is included in PCa therapy regimens,\textsuperscript{12,48,49} induced dynamic changes in CpG methylation as well as transcription of neighboring genes within an AA cohort, and disease enrichment analysis of Dex-induced genes revealed associations with aging-related diseases including cancers.\textsuperscript{41}

It is well established that AA men are diagnosed with a more aggressive PCa phenotype attributable to the interplay between a number of factors including socioeconomic status, access to healthcare, diet and lifestyle, and biological contributors.\textsuperscript{42,43,45,46,50-62} However, it has yet to be explored whether the endogenously elevated glucocorticoid signaling in AA men that has been linked to cumulative stressful life events plays a role in the aggressive PCa phenotype and mortality disparities observed in this population. In this overall context, we examined the contribution of GR signaling to upregulation of LEDGF/p75 and CLU in a racially diverse panel of PCa cell lines, and explored the transcript expression of GR in racially diverse PCa tissues.

\textbf{Materials and Methods}

\textit{Cell Lines, Antibodies, and Reagents}

All cell lines were purchased from the American Type Culture Collection (ATCC) and grown in a humidified incubator with 5\% CO\textsubscript{2} at 37\textdegree. In accordance with
recent guidelines by the NIH on authentication of key biological resources, cells were authenticated utilizing Short Tandem Repeat (STR) profiling against the ATCC STR database (ATCC, Cat: ATCC 135-XV). Cells were routinely tested for mycoplasma contamination using MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza, Cat:LT07). PC3 (Cat:CRL-1435), DU145 (Cat:HTB-81), and 22Rv1 (Cat:CRL-2505) cell lines were cultured in RPMI 1640 medium (Corning, Cat:10-040-CV) supplemented with 10% fetal bovine serum (Corning, Cat:35010CV), penicillin-streptomycin (Corning, Cat:30001Cl), and gentamicin (Gibco, Cat:15710064) as recommended by the supplier. MDA-PCa-2b (Cat:CRL-2422) cell line was cultured in F-12K medium (ATCC®, Cat:30-2004) supplemented with 20% fetal bovine serum (Corning, Cat:35010CV), cholera toxin (Sigma-Aldrich, Cat:C8052), epidermal growth factor (Sigma-Aldrich, Cat:E4127), o-phosphoethanolamine (Sigma-Aldrich, Cat:P0503), hydrocortisone (Sigma-Aldrich, Cat:H0888), selenious acid (ACROS Organics, Cat:AC19887), bovine insulin (Sigma-Aldrich, Cat:I6634), and penicillin-streptomycin as recommended by the supplier. 0.2% Normocin (Invivogen, Cat:ANT-NR-1) was added to the medium for PC3, DU145, 22Rv1, and MDA-PCa-2b cell lines to prevent contamination by mycoplasma, bacteria, or fungi.

Cortisol (Sigma-Aldrich, Cat:H0888) and Dex (Sigma-Aldrich, Cat:D4902) reconstituted in ethanol were used at 10 nM concentrations as GR agonists while mifepristone (Mif) (Sigma-Aldrich, Cat:M8046) reconstituted in ethanol was used at 100nM concentration as a GR antagonist. DHT (Cat: D073) reconstituted in ethanol was used at 1 nM and 10 nM concentrations as AR agonists while enzalutamide (Enz) (HY-70002 Medchem Express) reconstituted in ethanol was used at 1 μM as an AR antagonist.
In all experiments incorporating cortisol, Dex, Mif, DHT, or Enz, charcoal-stripped fetal bovine serum (Atlanta Biologicals, Cat:S11650) was used in order to selectively remove hormones while avoiding non-specific loss of other serum components. For experiments exceeding 24 hours, media was replaced every 24 hours to ensure that cells would have consistent exposure to glucocorticoids.

The following commercially-acquired antibodies were used: rabbit polyclonal anti-LEDGF/p75 (1:1000, Bethyl Laboratories Inc., Cat:A300-848A), mouse monoclonal anti-GR (1:1000, BD Biosciences, Cat:611226), mouse monoclonal anti-clusterin α-chain (1:1000, Millipore, Cat:05-354), rabbit monoclonal anti-AR (1:1000, Cell Signaling, Cat:5153S), rabbit monoclonal anti-β-actin (1:5000, Cell Signaling, Cat:5125), rabbit polyclonal anti-α/β-tubulin (1:1000, Cell Signaling, Cat:2148S).

**Immunoblotting Procedures**

Immunoblotting was performed as described previously. Briefly, equal amounts of protein from whole cell lysates (5 μg for MDA-PCa-2b and 22Rv1; 20 μg for PC3 and DU145) were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, NuPAGE 4–12%, Thermo Fisher Scientific, Cat:NP0321BOX) and transferred into polyvinyl difluoride membranes (Millipore, Cat:IPFL00010). Membranes were blocked in 5% dry milk prepared in TBS-T buffer (20 mM Tris- HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20). Membranes were then probed individually with primary antibodies and corresponding secondary antibodies and washed several times with TBS-T between each antibody application. Enhanced chemiluminescence (ECL) was used to detect immunoreactive protein bands. For this, the ECL Western Blotting Substrate
(Thermo Fisher Scientific Pierce, Cat:32106) was added to the antibody-protein surface of each membrane, followed by incubation for 4 minutes. Membranes were then transferred to autoradiography cassettes and exposed to autoradiography films for different lengths of time to ensure accurate detection of immunoreactive protein bands. Protein bands from at least 3 independent experiments for each treatment were quantified using ImageJ Software. The ratios of CLU or LEDGF/p75 protein bands to the loading control bands (tubulin or β-actin) were normalized to one in control, untreated samples. This was then used to calculate the fold-upregulation of CLU and LEDGF/p75 in the treated samples. Depending on the cell line, we used either tubulin or β-actin as loading controls since we observed in initial experiments that glucocorticoids induced β-actin in a cell line-dependent manner.

**Quantitative Real-Time PCR**

Quantitative Real-Time PCR (qPCR) was performed as described previously. Briefly, Total RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen, Cat:74134). The iScript cDNA synthesis kit (BioRad, Cat:1708891) was used to reverse transcribe RNA (0.5 μg) into cDNA. qPCR was performed using the MyiQ real-time PCR detection system with primers using iQ SYBR Green Supermix (BioRad, Cat:1708882) following manufacturers’ recommendations. Primer sequences for LEDGF/p75, Clusterin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed using Primer 3 software. The forward sequence for LEDGF (5’ to 3’) was TGCTTTTCCAGACATGGTTGT and reverse sequence (5’ to 3’) was CCCACAAAACAGTGAAAAGACAG. The forward sequence for Clusterin (5’ to 3’) was
CTCTACTCTCCGAAGGGAATTGTC and reverse sequence (5′ to 3′) was CGGGCTGCCTGTGCAT. GAPDH mRNA was used for normalization and the forward sequence (5′ to 3′) was GAGTCAACGGATTTGGTCGT and reverse sequence (5′ to 3′) was TTGATTTTGGAGGGATCTCG. Primers were commercially synthesized by Integrated DNA Technologies (IDT). Data was normalized to values of corresponding untreated controls and analyzed in at least three independent experiments, each in triplicates.

**RNA Interference-Mediated Knockdown of GR in PCa Cells**

To achieve transient knockdown of GR in our cellular models, commercially-available specific short inhibitory Trisilencer-27 RNAs (Origene, Cat:SR301960) corresponding to Locus ID: 2908 were used as described previously.33 Cells were transfected in a pooled knockdown with 10 nM of each siRNA Trisilencer-27/Dicer-Substrate duplex using oligofectamine (Invitrogen, Cat:12252011) following manufacturer's instruction. A universal scrambled negative control siRNA duplex (Origene, Cat:SR30004) was used as a negative control. Quantification of protein bands in immunoblots was performed as indicated above.

**Cell Migration**

In order to evaluate the migratory response of PC3 and DU145 cells following exposure to Dex, a scratch wound healing assay was performed. Cells were seeded to confluency and grown for 24 hours in RPMI 1640 medium supplemented with 10% charcoal-stripped FBS, with or without 10 nM dexamethasone. Wound areas were
generated using a 200 µl pipette tip by scratching the cell surface confluent monolayer. Migration of cells into the wound areas was visually tracked using an Olympus IX70 microscope equipped with SPOT RT3 Imaging System and phase contrast images were captured at 0 and 24 hours. The wound recovery rate of migrating cells obtained in 6 independent experiments was measured using ImageJ Software.

**Identification of GR Binding Sites**

The consensus for the GR binding sites was created using WebLogo. The 30 sequences (15 base long GR binding sites) used as input are shown in Supplementary Table S1. The same sequences were used to create a letter probability matrix (Supplementary Table S2). The CLU and LEDGF/p75 promoters were scanned for putative GR binding sites with this matrix, using FIMO within the MEME Suite 4.12.0. For the identification of GR binding half sites a PERL script was used that was developed in-house. Promoters were searched for 6 base long half sites with appropriate degeneracies, the search was then expanded on either the 5' or the 3' end depending upon the half-site that was detected.

**Bioinformatics Analysis of Oncomine Cancer Gene Microarray Database**

For analysis of mRNA expression of GR, encoded by the NR3C1 gene, in PCa and normal prostate tissues, we selected 10 datasets from the Oncomine database (Compendia Biosciences; Ann Arbor, MI; www.oncomine.org). These datasets, derived from gene microarray analyses of PCa and normal prostate tissues, provide fold-change data for gene expression with $P$ values calculated by Oncomine using Student’s $t$-tests.
Two out of the 10 datasets, Wallace and Taylor, included ethnicity/race data. The Wallace dataset included 41 prostate tissue specimens from AA men and 46 from EA men while the Taylor dataset included 24 AA PCa and 115 PCa EA specimens in addition to 2 Asian and 5 Hispanic PCa tissue samples. 29 normal adjacent tissue samples were racially pooled. This allowed us to compare the NR3C1 transcript expression between tissues from AA and EA patients.

**Statistical Analysis**

SPSS Statistics Software V22.0 and GraphPad Prism 6 were used for statistical analyses. Differences between treatment groups were analyzed using unpaired Student’s t-test. P values below 0.05 were considered statistically significant. All differences highlighted by asterisks were statistically significant as encoded in figure legends ($^*P < 0.05; \, **P < 0.01; \, ***P < 0.001; \, ****P<0.0001$).

**Results**

**Activated AR Signaling Upregulates LEDGF/p75 and CLU in 22Rv1 Cells**

The GR bypass hypothesis posits that GR takes over the regulation of AR-target genes after treatment of advanced PCa with primary and secondary ADT. Although CLU is known to be androgen regulated, there are no studies determining that LEDGF/p75 expression is also stimulated by androgen exposure in PCa cells. To explore this, we examined the protein expression levels of LEDGF/p75 and CLU in androgen responsive 22Rv1 PCa cells following exposure to dihydrotestosterone (DHT) and the secondary ADT drug enzalutamide (Enz). We treated cells with either 1 nM DHT, 10 nM...
DHT, or 1 μM Enz for 24 hours, and collected total lysates of both treated and untreated
cells to perform immunoblotting analyses using antibodies specific for either LEDGF/p75
or CLU. We observed increased protein levels of both LEDGF/p75 and CLU in cells
treated with either 1 nM or 10 nM DHT, which was attenuated by exposure to 1 μM Enz
(Figure 9). These results were consistent with the previously reported upregulation of
CLU in PCa cells by androgens, and also provided evidence for androgen induction of
LEDGF/p75 in PCa cells.
Figure 9. Androgen-induced upregulation of LEDGF/p75 and CLU in 22Rv1 cells. Cells were treated for 24 hours with 1 nM DHT, 10 nM DHT, or 1 μM Enz. Untreated (U) cells were used as controls. Whole cell lysates were prepared for Western blotting and probed with anti-LEDGF/p75 and anti-sCLU antibodies. Results from two independent experiments were quantified with ImageJ. Results reveal a trend of increased LEDGF/p75 and CLU expression in 22Rv1 cells treated with DHT. Enz attenuated LEDGF/p75 and CLU expression. All blots represented within panels are derived from the same gel.
"Activated GR Signaling Upregulates LEDGF/p75 and CLU Protein Expression in PCa Cells, Most Robustly in Cell Lines Carrying Substantial African Ancestry"

We measured the protein expression of LEDGF/p75 in a racially diverse panel of cell lines expressing GR—i.e. MDA-PCa-2b (AA), 22Rv1 (AA/EA), PC3 (EA), and DU145 (EA)—following exposure to glucocorticoids, and compared the modulated expression to matched untreated control cells. In a recent study we validated the previously reported African ancestry of the MDA-PCa-2b cell line, as well as the reported European ancestry of the PC3 and DU145 cell lines, using a panel of 29 validated Ancestry Informative Markers. Through this ancestry analysis, we also established that the 22Rv1 cell line, whose racial ancestry origin had not been previously reported, had mixed genetic ancestry, with substantial African ancestry. We treated these cell lines with either 10 nM cortisol or 10 nM dexamethasone (Dex) for up to 48 hours, and collected total lysates of treated and untreated cells for immunoblotting analyses using an antibody specific for LEDGF/p75. We observed increased LEDGF/p75 protein expression in MDA-PCa-2b, 22Rv1, and PC3 cells treated with glucocorticoids (Figure 10A-C), with the most robust upregulation observed in MDA-PCa-2b cells and the highest statistical significance achieved in both the MDA-PCa-2b and 22Rv1 cell lines. Both cortisol and Dex treatments led to decreased protein expression of LEDGF/p75 in DU145 cells (Figure 10D). Interestingly, while 22Rv1 cells upregulated LEDGF/p75 protein expression after 24 hours of glucocorticoid exposure, MDA-PCa-2b, PC3, and DU145 cells required 48 hours of exposure to glucocorticoids to observe significant changes in LEDGF/p75 expression. Since we consistently found that Dex treatment induced a more robust modulation of LEDGF/p75 protein expression than
cortisol in our PCa cellular models, all subsequent experiments were conducted with Dex, a highly specific and potent GR agonist.\textsuperscript{65}
Figure 10. Glucocorticoids robustly increase LEDGF/p75 protein expression in PCa cells with substantial African ancestry. Cells were treated every 24 hours with 10 nM or 100 nM Dex or cortisol for up to 48 hours. Untreated (U) cells were used as controls. Whole cell lysates were prepared for Western blotting and probed with anti-LEDGF/p75 antibodies. Results from at least 3 independent experiments were quantified with ImageJ as described in the Methods section. Results reveal increased LEDGF/p75 expression in MDA-PCa-2b (A), 22Rv1 (B), and PC3 (C) cells treated with glucocorticoids. Unpaired t-test statistical analysis revealed that LEDGF/p75 induction in cell lines with substantial African ancestry (MDA-PCa-2b and 22Rv1) achieved the highest statistical significance. *p<0.05, **p<0.01, ***p<0.005. All blots presented within the panels are derived from the same gel.
Next, we treated our racially diverse panel of cell lines with 10 nM Dex and collected total lysates of treated and untreated cells to determine the upregulation of CLU using an antibody specific for the α-chain of CLU capable of detecting both psCLU and sCLU. While conducting immunoblotting and exposing the membranes to autoradiography film, we consistently observed a very intense upregulation of sCLU protein in the MDA-PCa-2b and 22Rv1 cell lines, which carry substantial African ancestry, compared to the EA PC3 and DU145 cell lines, which expressed relatively low levels. This prevented us from accurately quantifying sCLU protein expression levels in these cell lines and comparing them with those in PC3 and DU145. Because of this, in subsequent experiments we loaded only 5 µg of total protein in individual lanes of gels for MDA-PCa-2b and 22Rv1 cells, compared to 20 µg of total protein for PC3 and DU145 cells. After adjusting the protein loading amounts to better match band intensity across cell lines, we continued to observe a statistically significant increase in sCLU protein expression in MDA-PCa-2b and 22Rv1 cells, compared to PC3 cells, despite loading 75% less protein in the individual lanes (Figure 11A-C). In agreement with the results with LEDGF/p75, the most robust sCLU upregulation was consistently observed in the cell lines with substantial African ancestry—MDA-PCa-2b and 22Rv1 (Figure 11A-B), whereas treatment of DU145 cells with Dex did not alter the expression of CLU (Figure 11D). Interestingly, both ps-CLU and sCLU were modestly upregulated by Dex in PC3 cells (Figure 11C).
Figure 11. Glucocorticoids robustly increase CLU protein expression in PCa cells with substantial African ancestry. Cells were treated every 24 hours with 10 nM Dex for up to 48 hours. Untreated (U) cells were used as controls. Whole cell lysates were prepared for Western blotting and probed with anti-CLU antibodies recognizing both pre-secreted and secreted CLU (psCLU and sCLU). Results from at least 3 independent experiments were quantified with ImageJ as described in the Methods section. Results reveal increased sCLU expression in MDA-PCa-2b (A), 22Rv1 (B), and PC3 (C) cells treated with Dex. Unpaired t-test statistical analysis revealed that CLU increase in cell lines with substantial African ancestry (MDA-PCa-2b and 22Rv1) achieved the highest fold change compared to untreated controls. *p<0.05, **p<0.01. All blots represented within panels are derived from the same gel.
**LEDGF/p75 and CLU Transcript Levels Increase in Response to Activated GR Signaling**

We next determined if the observed elevation in protein expression of LEDGF/p75 and CLU in cells exposed to 10 nM Dex also occurred at the transcript level. For this we conducted qPCR analysis of LEDGF/p75 and CLU transcripts in MDA-PCa-2b, 22Rv1, PC3, and DU145 cells exposed to Dex, using the same experimental conditions described above. Consistent with our immunoblotting results, we observed a significant increase in LEDGF/p75 and CLU transcript expression in MDA-PCa-2b, 22Rv1, and PC3 cells exposed to 10 nM Dex, compared to untreated cells, with significant decrease in transcript expression in DU145 cells (Figure 12A-D).
Figure 12. Glucocorticoids increase transcript levels of LEDGF/p75 and CLU in PCa cells. Cells were treated every 24 hours with 10 nM Dex for up to 48 hours. Untreated (U) cells were used as controls. Total RNA was extracted from MDA-PCa-2b, 22Rv1, PC3, and DU145 cells. Results from at least 3 independent qPCR experiments revealed LEDGF/p75 and CLU transcript levels increased in MDA-PCa-2b (A), 22Rv1 (B), and PC3 (C) cells exposed to 10 nM Dex. LEDGF/p75 and CLU transcript levels decreased in DU145 (D) cells. Unpaired t-test statistical analysis was used to assess statistical significance (*p<0.05, ***p<0.001, ****p<0.0001).
Pharmacological Inhibition of GR Reduces Glucocorticoid-Induced Protein Expression of LEDGF/p75 and CLU in 22Rv1 Cells

Given that our initial experiments demonstrated glucocorticoid-induced upregulation of LEDGF/p75 and CLU expression in MDA-PCa-2b, 22Rv1, and PC3 cells, it was necessary to further explore if GR contributes to this upregulation. For these experiments, we inhibited GR function using the GR antagonist mifepristone (Mif). To overcome the inducing effects of 10 nM Dex, we used a concentration of 100 nM Mif. We treated 22Rv1 and PC3 cells with 10 nM Dex in the presence or absence of 100 nM Mif in order to compare the effect of GR inhibition on the Dex-induced upregulation of LEDGF/p75 and CLU. Immunoblotting analysis showed that 100 nM Mif attenuated the upregulation of LEDGF/p75 and sCLU protein expression induced by 10 nM Dex in 22Rv1 cells (Figure 13A). However, this significant attenuation was not observed for LEDGF/p75 or psCLU in PC3 cells (Figure 13B).
Figure 13. Pharmacological inhibition of GR attenuates glucocorticoid-induced protein expression of LEDGF/p75 and CLU in 22Rv1 cells. 22Rv1 and PC3 cells were co-treated with 10 nM Dex and 100nM Mif for up to 48 hours. Untreated cells were used as controls. Whole cell lysates were prepared for Western blotting analysis and probed with anti-GR, anti-LEDGF/p75, and anti-sCLU antibodies. Unpaired t-test statistical analysis revealed that Mif attenuated LEDGF/p75 and CLU expression in 22Rv1 cells (A). However, this attenuation was not observed in PC3 cells. (B). (*p<0.05)
**GR Knockdown Reduces Protein Expression of LEDGF/p75 and CLU in PCa Cells**

Recognizing that Mif is not entirely specific for GR since it has been shown by others to be a GR agonist under certain conditions,\(^6^6\) we sought to specifically target GR to determine if it plays a more direct role in the glucocorticoid induction of LEDGF/p75 and CLU. For these experiments we transiently knocked down GR using a pool of short inhibitory RNAs (siRNAs) specific for GR in 22Rv1 and PC3 cells. These siRNAs target the Nuclear Receptor Subfamily 3 Group C Member 1 (\(NR3C1\)) gene, which encodes GR. Transient knockdown of GR with siRNAs (si-GR) in 22Rv1 and PC3 cells led to significant depletion of LEDGF/p75 and CLU proteins compared to cells transfected with scrambled duplex siRNA (si-SD) control (Figure 14A-B). Taken together, these results suggested a direct contribution of GR to glucocorticoid-induced upregulation of LEDGF/p75 and CLU in PCa cells.
**Figure 14.** GR knockdown attenuates LEDGF/p75 and CLU protein expression in PCa cells. Transient knockdown of GR was generated in 22Rv1 and PC3 cells (si-GR). Gel electrophoresis using whole cell lysates of si-GR compared to the scrambled control (si-SD control) were probed with anti-GR, anti-LEDGF/p75, and anti-sCLU antibodies. Unpaired t-test statistical analysis revealed that knockdown of GR in 22Rv1 (A) and PC3 (B) cells attenuated the protein expression of LEDGF/p75 and CLU (*p<0.05, **p<0.01, ***p<0.005). All blots represented within panels are derived from the same gel.
Glucocorticoid Signaling Modulates PCa Cell Migration

Both glucocorticoid treatment and LEDGF/p75 depletion have been shown previously to decrease the migration of DU145 cells and breast cancer cells. This led us to examine the ability of glucocorticoids to alter the migration rate of PC3 and DU145 cells using scratch wound healing assays. We chose these two cell lines because of their ability to form a confluent monolayer, in contrast with the MDA-PC-2b and 22RV1 cell lines, which typically grow in clusters. After creating a wound area and treating cells with 10 nM Dex, we captured images of the cell cultures at 0 hours and 24 hours in order to track cell migration in treated versus untreated cells. We observed a marked and significant increase in the rate of migration of Dex-treated PC3 cells, compared to untreated cells, such that closure of the wound area occurred at 24 hours (Figure 15A). However, the opposite effect was observed in DU145 cells, as the rate of migration was significantly reduced in Dex-treated cells compared to untreated cells (Figure 15B).
Figure 15. Glucocorticoid signaling modulates PCa cell migration. Phase contrast images of scratch wound healing assays used to measure the migration of PC3 and DU145 cells were captured with an Olympus IX70 microscope. PC3 and DU145 cells treated with 10 nM Dex or untreated controls at 0 and 24 hours after the scratches were made at the same point. Unpaired t-test statistical analysis revealed that treatment with 10 nM Dex increases PC3 cell migration (A) but decreases DU145 cell migration (B). Scale bar=200 µm; (**p<0.01, ****p<0.0001).
GR Binding Sites Identified in Promoter Regions of LEDGF/p75 and CLU

To further explore the possibility that GR regulates the glucocorticoid induction of LEDGF/p75 and CLU in PCa cells, likely by binding to the promoter regions of these genes, we conducted a PubMed literature survey of GR promoter binding sites as well as an in-silico promoter analysis. These resulted in a comprehensive list of GR binding sites (Table 4) that have already been experimentally validated. The 49 previously identified GR binding sites ranged from 7 to 35 bases in length (Table 4), with 30 of these being 15 bases long. We used the sequences of these 30 GR binding sites to build a consensus GR binding element using WebLogo (Figure 16).

From the consensus sequence, it could be derived that the GR binding site in most cases consists of two half-sites, which are six base inverted repeats, separated by 3 degenerate bases (Figure 16). We observed that the last three bases (4-6) of the first (5') half-site and the first three bases (10-12) of the second (3') half-site are highly conserved (Figure 16). To determine if a similar 15 base long putative GR binding site is present in the promoters of LEDGF/p75 and CLU, we scanned these promoters with a letter probability matrix (Table 5) derived from their sequences, using FIMO, a webtool, within the MEME Suite 4.12.0. Putative GR binding sites were detected in both CLU (AGCACAGGAAGTATT; \(P<0.0001\)) and LEDGF/p75 (GAAACCCTACGTCCC; \(P<0.0008\)) promoters, in the reverse strands between 132-146 bases and 611-625 bases, respectively (Figure 16; Table 6).

We also observed from the list of experimentally validated GR-binding sites (Table 4), that a half-site is sufficient to bind GRs and the two half-sites may sometimes be separated by four instead of three bases. Taking these into consideration we
scanned the two promoters for the half-sites with the help of an in-house PERL script and identified at least two half-sites in each of the two promoters (Table 6). This *in-silico* analysis suggested that there are multiple putative GR binding sites within the LEDGF/p75 and CLU promoters to which GR might bind and regulate the expression of these genes.
Table 4. GR Binding Sites

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Table 5. Letter Probability Matrix

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**Figure 16.** GR binding sites in LEDGF/p75 and CLU promoter regions. The GR binding consensus derived from experimentally validated sites is aligned with the putative GR binding sites within the promoters of CLU and LEDGF/p75. Light gray shading indicates the conserved half-sites.
### Table 6. Putative GR Binding Sites Within LEDGF/p75 and CLU Promoter Regions

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Analysis of Cancer Gene Microarray Datasets Reveals Race/Ethnicity-Related Differential Expression of GR Transcript in PCa Tissues

Next, we sought to examine GR expression (NR3C1 gene) in human PCa tissues. Transcript expression of NR3C1 in PCa tissues, compared to normal prostate tissue, was analyzed using 10 PCa gene microarray datasets from the Oncomine database. NR3C1 was consistently downregulated in prostate tumors compared to normal prostate tissues in 9 of the 10 datasets (Figure 17A). The magnitude of the fold-decrease was modest, with only 5 datasets showing >1.5-fold downregulation and the remaining 4 datasets with >1-fold decrease. However, the P values were <0.001 in 7 datasets, indicating that GR downregulation was highly significant in PCa tissues compared to normal tissues.

Most of the Oncomine datasets examined above have no racial/ethnic identifiers. However, two datasets, Wallace and Taylor, had gene expression data for PCa tissues from different racial/ethnic groups. When grouping the NR3C1 transcript expression data by race/ethnicity, box-plots of the log2 median-centered intensity in the Wallace dataset showed that the median value was slightly higher in prostate tissues from AA men (3.065) as compared to EA men (2.993) (Figure 17B). The Taylor dataset displayed a much higher log2 median-centered intensity NR3C1 transcript in AA (0.589) PCa tissues compared to EA (0.272) and Asian (-0.134) PCa tissues. Interestingly, the NR3C1 median expression levels in AA PCa tissues was slightly lower than that in PCa tissues from Hispanic patients (0.64) in the Taylor dataset. Unfortunately, we did not have access to racial identifiers for the Hispanic patients. It is noteworthy that the Wallace dataset grouped the normal adjacent prostate tissues with prostate tumors for the race/ethnicity-based analysis, while the Taylor dataset grouped the normal adjacent and PCa tissues...
separately, which may account for the differences in \textit{NR3C1} expression between AA and EA PCa tissues observed in these two datasets.
Figure 17. Expression of GR/NR3C1 transcript between clinical prostate cancer tissues specimens from AAs and EAs in Oncomine. Transcript expression levels of GR/NR3C1 in prostate tumors versus normal prostate tissues was derived from cancer gene microarray datasets in Oncomine database. Individual dataset names appear in the legend boxes at the right (A). Box-plots from the Wallace and Taylor datasets were used from Oncomine gene microarray database (B). Fold-changes and corresponding $P$-values for the differences in gene expression between PCa and normal prostate tissues were obtained from Oncomine. The number of samples in each dataset is different, therefore higher fold change does not always correspond to statistical significance. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. 
Discussion

Understanding the mechanism(s) by which GR signaling activates AR-target genes in the absence of androgens has high relevance to the development of effective treatments for advanced PCa. The downstream effects of this GR bypass include promotion of tumor aggressiveness as well as resistance to standard PCa therapies such as ADT and potentially taxane chemotherapy. While key stress oncoproteins associated with increased PCa cell survival in the presence of environmental stressors such as chemotherapeutic drugs have been identified and at times targeted in pre-clinical and clinical studies (e.g., CLU, LEDGF/p75, HSP27, PRDXs), there remains a need to understand the potential contribution of glucocorticoid signaling to the activation and upregulation of these proteins. With this goal in mind, this study was designed to examine the contribution of glucocorticoid signaling to the expression of CLU and LEDGF/p75, two stress oncoproteins previously established as key contributors to therapy resistance in various cancer types. The roles of CLU and LEDGF/p75 in the acquisition and maintenance of resistance to standard PCa therapies have been established; however, this study is the first to implicate GR signaling in their upregulation in PCa cells. Multiple recent studies have linked GR signaling to PCa disease progression, but to our knowledge none of these studies have compared GR signaling events in racially diverse pre-clinical cellular models, or explored GR expression in racially diverse PCa tissues.

In this study, we observed that exposure to glucocorticoids led to upregulation of CLU and LEDGF/p75 transcript and protein levels in three out of four PCa cell lines, and that GR blockade attenuated this effect. Moreover, the most robust upregulation was
observed in the two PCa cell lines with substantial African ancestry, MDa-PCa-2b, and 22Rv1. Although many investigators have assumed for years that 22Rv1 is a EA PCa cell line, we recently conducted a DNA ancestry analysis on this cell line using a panel of 29 validated Ancestry Informative Markers, and reported that it is a mixed ancestry PCa cell line comprised of 0.41 West African, 0.42 European, and 0.17 Ameri-Indian ancestral proportions.\textsuperscript{63,64}

Our finding that CLU expression is highly responsive to glucocorticoids in MDA-PCa-2b and 22Rv1 cells is underscored by our decision to decrease substantially the amount of total protein from these two cell lines that were loaded in individual lanes of gels for immunoblotting. For instance, when we loaded 20 \( \mu \)g of protein per lane from MDA-PCa-2b and 22Rv1 cells, CLU protein expression was so dramatically elevated that we were unable to differentiate individual lanes within the blots, even after decreasing the exposure time. Conversely, CLU protein expression was relatively lower in PC3 and DU145 cells when individual lanes in gels were loaded with 20 \( \mu \)g of protein from these cells. The observed high endogenous expression of CLU in the MDA-PCa-2b and 22Rv1 cell lines could suggest that downstream effects of GR signaling such as the upregulation of therapy-resistance associated genes may be exaggerated in AA PCa patients. This would be consistent with the emerging notion that GR signaling is enhanced in the AA population.\textsuperscript{41,47}

Interestingly, we observed downregulation of LEDGF/p75 and CLU in DU145 cells exposed to glucocorticoids, which was consistently opposite from the results observed in MDA-PCa-2b, 22Rv1, and PC3 cells. This was expected as other investigators have reported that GR activation decreases the aggressive properties of the
DU145 cell line while its blockade reverses this effect.\textsuperscript{65,73-75} The impact of activated GR signaling on PCa cells includes the promotion of tumor aggressiveness properties leading to worse overall patient survival.\textsuperscript{4} Tumor aggressiveness develops when normal cellular functions are altered and established hallmarks of cancer such as increased cell migration are induced.\textsuperscript{76} We observed that Dex treatment significantly increased the migration rate in PC3 cells but not that of DU145 cells, suggesting a cell-type dependent effect. These results are consistent with our observation that GR induced downregulation of LEDGF/p75 in DU145 cells, and previous reports that LEDGF/p75 depletion or treatment with Dex decreased the migration rate of DU145 PCa cells.\textsuperscript{35,67} Several potential mechanisms have been proposed to explain these DU145-specific effects, including relatively higher GR expression compared to other PCa cell lines, which could lead to immediate downregulation of GR and shutdown of GR signaling upon exposure to glucocorticoids.\textsuperscript{65,75}

The ability of glucocorticoids to upregulate LEDGF/p75 and CLU in PCa cell lines appears to be mediated by GR. This was confirmed by our observation that pharmacological or genetic blockade of GR led to significant downregulation of LEDGF/p75 and CLU expression. Although it remains to be established that GR binds directly to and activates promoter regions of these two genes, our \textit{in-silico} analysis confirmed the presence of multiple putative GR binding sites within both LEDGF/p75 and CLU promoter regions, which would allow for direct regulation by activated GR.

Recent studies have demonstrated that GR expression is reduced in primary PCa tissues but increases in metastatic lesions, particularly in patients who have received DTX therapy.\textsuperscript{6,15} Since these analyses were conducted mostly in PCa tissues from EA
patients, we asked whether primary PCa tissues from AA men also express reduced levels of GR. Our analysis of 10 cancer gene microarray datasets from the Oncomine database revealed that GR (encoded by \textit{NR3C1} gene) transcript was consistently downregulated in prostate tumors compared to normal prostate tissues, in agreement with the previous reports.\textsuperscript{6,15} However, when we focused our analysis on GR expression based on racial classification, we observed differences between AA and EA PCa patients. Both the Wallace and Taylor datasets, which contain gene expression data from AA PCa tissues, revealed higher median values of GR in AA prostate tissues compared to EA prostate tissues. Unfortunately, there was no information in the datasets regarding the chemotherapy treatment status of the tissue donors. Nevertheless, these findings are consistent with the premise that AA men with PCa may have enhanced intratumoral GR signaling.

We speculate that the recently documented hyperactive GR signaling occurring in AA men\textsuperscript{41,47} could exacerbate the upregulation of LEDGF/p75 and CLU in PCa cells. It is possible that chronically elevated cortisol levels, increased GR levels, and hyperactive GR signaling sustained in AA men over time could prime them to develop aggressive PCa tumors. In addition, this enhanced GR signaling could induce a robust expression of oncoproteins associated with therapy-resistance, including LEDGF/p75 and CLU, leading to poor response to conventional treatments in AA PCa patients.

The results of this study also complement the growing body of literature suggesting that glucocorticoid co-administration with PCa therapies including ADT may potentially lead to worse overall patient survival.\textsuperscript{1,4,6,9-11} By upregulating oncoproteins associated with resistance to ADT and taxane chemotherapy, activated GR signaling may
promote the proliferation and migration of highly aggressive PCa tumor cells with enhanced therapy resistance capabilities. For instance, our findings that GR activation increased CLU could offer insights into why recent clinical trials targeting CLU with an antisense oligonucleotide-based drug, Custersin, in combination with taxane chemotherapy in advanced stage PCa patients were ineffective. It is plausible that CLU and other pro-survival proteins were activated by GR as patients in both study arms received glucocorticoid co-therapy to mitigate side effects.

The implications of our findings are far-reaching as GR is emerging as a key driver of PCa tumor aggressiveness, especially in the absence of AR signaling. Since our results demonstrate that LEDGF/p75 and CLU are upregulated in the absence of androgen via GR, future combinatorial therapies co-targeting AR, GR, and stress oncoproteins could potentially confer greater overall survival to patients with advanced PCa. In addition, given that AA men display an enhanced physiological response to glucocorticoids as well as disproportionate PCa incidence and mortality, further studies are needed to better elucidate the relationship between GR signaling and PCa tumor aggressiveness specifically in this racial/ethnic group.

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References


CHAPTER FOUR
PHYSICIAN CONSULTATIONS, PROSTATE CANCER KNOWLEDGE, AND PSA SCREENING OF AFRICAN AMERICAN MEN IN THE ERA OF SHARED DECISION-MAKING

Abstract

African American (AA)/Black men are more likely to develop aggressive prostate cancer (PCa), yet less likely to be screened despite guidelines espousing shared decision-making regarding PCa screening and prostate-specific antigen (PSA) testing. Given the documented racial disparities in PCa incidence and mortality, engaging interactions with physicians are especially important for AA/Black men. Thus, this study evaluated occurrence of physician-patient conversations among AA/Black men, and whether such conversations were associated with PCa knowledge. We also quantified the serum PSA values of participants who had, and had not, discussed testing with their physicians. Self-identified AA/Black men living in California and New York, ages 21-85, donated blood and completed a comprehensive socio-demographic and health survey (n=414). Less than half (45.2%) of participants had discussed PCa screening with their physicians. Multivariate analyses were used to assess whether physician-patient conversations predicted PCa knowledge after adjusting for key socio-demographic/economic and health care variables. Increased PCa knowledge was correlated with younger age, higher income and education, and having discussed the pros and cons of PCa testing with a physician. Serum PSA values were measured by ELISA. Higher-than-normal PSA values were found in 38.5% of men who had discussed PCa screening with a physician and 29.1% who had not discussed PCa screening. Our results suggest that physician-
AA/Black patient conversations regarding PCa risk need improvement. Encouraging more effective communication between physicians and AA/Black men concerning PCa screening and PSA testing has the potential to reduce PCa health disparities.

**Introduction**

Prostate cancer (PCa) is the most commonly diagnosed male cancer in the U.S., and African American (AA) men are 2.5 times more likely to die from this malignancy than European American (EA) men.\(^1,2\) Early detection through timely screening and optimal treatment options improve overall survival, yet AA and other Black men of African ancestry are not as likely to receive these health advantages as EA men.\(^1-6\) With the implementation of the Affordable Care Act, the gap in access to high-quality health care, timely diagnosis, and optimal treatment has narrowed between AAs and EAs.\(^7\) However, even under equal access to health care, disparities in PCa treatment and screening options still persist.\(^7-9\)

PCa diagnosis involves prostate-specific antigen (PSA) screening.\(^1,10-12\) Circulating serum PSA levels are considered abnormal when detected above 4 ng/ml.\(^10\) While the U.S. Food and Drug Administration approved the use of PSA testing in conjunction with digital rectal examination (DRE) to screen asymptomatic men for PCa in 1994, the U.S. Preventive Services Task Force (USPSTF) issued a recommendation against PSA-based screening in 2012.\(^10\) This recommendation was based on the assumption that, for most men, screening has no net benefit or the harms may outweigh the benefits.\(^10\) The recommendation influenced the current American Academy of Family Physicians’ overarching stance to “not routinely screen for PCa using a PSA test or DRE”. However, decreased screening differentially affects “patient populations under
consideration” which includes AA men. This is because the USPSTF report acknowledged that no firm conclusions about the benefits-to-harm ratio of PSA screening can be drawn in AA men due to their limited representation in the clinical trials that supported the recommendation against PSA screening. A more recent study that used Surveillance, Epidemiology, and End Results (SEER) data to investigate survival disparities between AA (n=23,782) and EA (n=188,937) men comparing pre-PSA testing era to current-PSA testing era provided a compelling case for continued aggressive PSA testing for AA men. Additionally, frequent and early PSA testing has been suggested for AA men in order to reduce racial disparities in PCa mortality.

Previous USPSTF guidelines recognized that before offering PSA screening, shared decision-making should occur through an engaged physician-patient conversation that enables informed choice based on patient preferences. The American Academy of Family Physicians currently advises that physicians offering PSA screening be “prepared to engage in shared decision-making that enables an informed choice by patients”. The American Cancer Society (ACS) also encourages informed decision-making and recommends PCa screening at age 50 for men at average-risk, 45 for men at high-risk, and 40 for men at higher-risk. ACS includes AA men in the high-risk category and recommends repeated annual PCa screening for men with PSA levels >2.5 ng/ml. While informed decision-making is the current recommendation for PCa screening, recent studies highlight that AA men may not be making informed decisions about PCa screening. This is largely due to patients having limited knowledge of PCa screening and providers either not offering sufficient up-to-date information or not asking patients about their preferences. Therefore, there is a need for more engaging interactions
regarding PCa screening between physicians and patients, especially for AA and other Black men of African ancestry, who are more likely to develop aggressive end-stage PCa at an earlier age. Knowledge of PCa and screening among AA/Black men may therefore play a critical role in reducing PCa health disparities.

AA/Black men in the U.S. comprise a heterogeneous population that includes both native- and foreign-born individuals, and nativity can affect individual health outcomes.\(^{14,15}\) Our survey data from a cohort of self-reported AA/Black men in two U.S. geographical regions focused on assessing men’s knowledge of PCa in light of clinical provider interactions. We explored factors potentially influencing PCa knowledge among AA/Black men, including whether their physicians had discussed PCa screening with them. Additionally, PSA values of participants were assessed to demonstrate the real-life value of PSA screening in this high-risk population.

**Materials and Methods**

**Participant Cohort**

Cross-sectional data were collected via Project C.H.A.N.G.E (Changing Health for Adult Men with New and Great Experiences), in Riverside, CA in 2013 and Brooklyn, NY in 2014. Recruited through community outreach, a convenience sample of adult men either donated blood or completed a 141-item health survey, or both, after written informed consent. While all study participants self-identified as Black, some participants further self-identified as AA and others as Caribbean Black or African. For discussion purposes we grouped them under the general term of AA/Black. This study
was conducted under approval of Loma Linda University Institutional Review Board (OSR#5110343).

**Serum Collection**

Blood was drawn by licensed staff and collected in red top vials. Collected blood rested at room temperature for 30 minutes to allow clotting. Serum was separated from blood cells by centrifugation, transferred to polypropylene tubes, and transported in dry ice for permanent storage at -80°C.

**PSA ELISA**

Human PSA ELISA Kits were purchased from Abnova (Taoyuan City, 320 Taiwan, catalog #KA0208). The 96-well ELISA plates were pre-coated with goat anti-PSA antibody for serum PSA detection. Following completion of the health fairs, sera from study participants who donated blood samples were added to the wells and circulating PSA was allowed to bind to the immobilized antibody. Wells were washed to remove unbound PSA. Monoclonal anti-PSA-horseradish peroxidase conjugate was then added to each well and allowed to bind PSA. Wells were washed and TMB (3,3’,5,5’ tetramethylbenzidine) reagent (provided in kit) was added to each well followed by incubation. Color development was interrupted with Stop Solution (provided in kit), and absorbance was measured by spectrophotometer at 450 nm, with PSA concentration directly proportional to color intensity. PSA values were calculated from a standard curve generated using PSA standards provided with the kit. PSA measurements were performed.
in duplicates for all serum samples. To ensure IRB compliance, individual PSA values were de-identified and not disclosed to study participants.

**Statistical Analysis**

Socio-demographic, socio-economic, and health care variables were evaluated using validated items from LaVeist and Deibert national surveys. Age was assessed as a continuous variable ranging from 21 to 85 years. Income was originally a categorical variable with 23 groups. We created a continuous distribution of income by constructing a new variable in which we estimated the midpoint of each group. We estimated the lowest group (0 to $5000) at $1000 and the top income group (more than $350,000) at $750,000. The resulting approximate income distribution was skewed. To minimize the skew, we took the log of the distribution and used this log transformation in our analyses. Education was coded as a categorical variable with three levels: high school graduate or below; some college or associate’s degree; and college graduate and above. To measure participant trust of health care providers and organizations, an 18-item adapted version of the LaVeist Medical Mistrust Index was used. Items were summed and normalized to the original four-point Likert scale, with higher scores indicating higher mistrust. Knowledge of PCa was assessed using 11 items modified from Deibert et al.’s scale. For each true/false question, a correct response was coded with a value of “1”, while an incorrect response or unanswered item was coded with a “0”. All items were then summed. Thus, a higher score represents higher PCa knowledge. Respondents who did not complete any knowledge questions or other relevant, nearby survey sections were excluded from the analysis (n=3). Categorical variables with yes/no responses included whether participants
had health insurance, were told by a physician that they had PCa, or ever discussed the pros and cons of PCa screening with a physician. Descriptive analysis was performed to explore distributions and describe the sample (Table 7). Multivariate analysis was conducted with the following variables: age, education (college graduate and above as the reference group), log income, health insurance, medical mistrust, ethnicity, whether a physician had told the respondent that he had PCa, and whether a physician had discussed with the respondent the pros and cons of testing. Hierarchical models were developed, but only the final model is presented (Table 8). Prior to analysis, data were screened for linearity, normality, and homoscedasticity. Except for income, no transformations were made. Based on Mahalanobis testing, no outliers were excluded. Statistical analysis was performed using IBM SPSS 23.
Table 7. Descriptive Statistics\textsuperscript{a}

<table>
<thead>
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<th>Variables</th>
<th>Range</th>
<th>Mean or Percentage</th>
<th>Standard Deviation</th>
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<td><strong>Dependent Variable</strong></td>
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<td></td>
<td></td>
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<td>Prostate Cancer Knowledge</td>
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</tr>
<tr>
<td><strong>Demographic Variables</strong></td>
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<tr>
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<td>0 – 100</td>
<td>38.6</td>
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<td>0 – 100</td>
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<tr>
<td>Medical Mistrust Scale</td>
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<td>Diagnosed with Prostate Cancer</td>
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<td>Doctor Discussed Screening Pros/Cons</td>
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<tr>
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<tr>
<td>Had Digital Rectal Exam</td>
<td>0 – 100</td>
<td>34.3</td>
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Note: a. n= 414
### Table 8. Multivariate Modeling of Predictors of PCa Knowledge

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<th>Standard Error</th>
<th>95% CI</th>
<th>p Value</th>
<th>Referent</th>
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<td>.487776</td>
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<td>-.0403466</td>
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<td>.2826495</td>
<td>-1.538938</td>
<td>.4271609</td>
<td>0.001 College graduate and above</td>
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<tr>
<td>Some college or Associate’s degree</td>
<td>-.730</td>
<td>.2417114</td>
<td>-1.20496</td>
<td>.254209</td>
<td>0.003 College graduate and above</td>
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<td>.1945511</td>
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<td>.2307852</td>
<td>-.4609131</td>
<td>.4468605</td>
<td>0.976 No health insurance</td>
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<td>.2631934</td>
<td>-.2114296</td>
<td>.8238191</td>
<td>0.245 Not applicable</td>
</tr>
<tr>
<td>Told has prostate cancer</td>
<td>-.535</td>
<td>.5159006</td>
<td>-1.549288</td>
<td>.4799626</td>
<td>0.301 Yes, told has prostate cancer</td>
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<tr>
<td>Discussed pros/cons of testing</td>
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<td>.2312566</td>
<td>.0272432</td>
<td>.9368714</td>
<td>0.038 Discussed pros/cons</td>
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<td>2.745492</td>
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<td>R-square</td>
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Results

Univariate Analyses

Only participants who provided written consent, donated blood, and completed the survey were included in our analyses (n=414). Men with a previous prostate cancer diagnosis (16/414, 3.9%) were included in analyses. We evaluated ethnicity in two groups: U.S.-born (163/414) AA men and foreign-born (251/414) Black men living in the U.S. Within the foreign-born group, 85.8% (215/251) of participants were from the Caribbean West Indies. Descriptive characteristics of study participants are shown in Table 7. Within the cohort, 45.2% (187/414) of participants reported having spoken with their physicians about the pros and cons of PCa screening.

Multivariate Analyses

Regression analysis (Table 8) assessed the relative contribution of correlates on PCa knowledge. Results indicate that PCa knowledge was inversely associated with age and positively associated with income. Compared to those with a high school degree or less, men who had a college degree or above, or had some college education or Associate’s degree, reported higher PCa knowledge scores. Importantly, after adjusting for the variables identified in Table 7, men who discussed the pros and cons of testing with their physicians reported higher PCa knowledge. Non-significant variables included not having health insurance, level of medical mistrust, having been told he has PCa, ethnicity, and the length of stay in the U.S. of the foreign-born participants (data not shown).
PSA Values in the Context of PCa Screening Conversations with Physicians

ELISA was used to quantify serum PSA levels in men participating in the study. Average PSA levels increased with age, with levels in men in their 30s averaging 0.8 ng/ml and men in their 80s averaging 16.4 ng/ml (Figure 18). Results revealed that 12.1% (50/414) of participants had higher-than-normal PSA levels when using the conventional cutoff >4 ng/ml. Of these, 38.0% (19/50) had never discussed the pros and cons of PCa screening with a physician (Figure 19). Additionally, 9.4% (39/414) of all the men in the study cohort had detectable PSA levels between 2.5-3.9 ng/ml, and 48.7% (19/39) of these had not discussed the pros and cons of PCa screening with a physician (Figure 19). Further, 11.8% (49/414) of total participants had detectable PSA levels between 1.5-2.49 ng/ml, and of these, 57.1% (28/49) had never discussed the pros and cons of PCa screening with a physician (Figure 19). Thus, 33% (138/414) of all participants had PSA values above 1.5 ng/ml, and of these, 47.8% (66/138) had not discussed PCa screening with their physicians. The remaining 66.7% (276/414) had PSA levels <1.5 ng/ml (Figure 19).
Figure 18. PSA values of AA/Black study participants differentiated by reported normal cutoffs. Serum PSA levels in sera of the study participants were determined by ELISA. As expected, average PSA levels increased with age, with PSA of men in their 30s averaging 0.8 ng/ml and PSA of men in their 80s averaging 16.4 ng/ml. We identified participants who had higher-than-normal PSA in the context of differing numerical values for what is considered higher-than-normal PSA levels for AA men. While the conventional cutoff for higher-than-normal PSA levels is 4 ng/ml, the American Cancer Society currently advises repeat screening for men with PSA levels greater than 2.5 ng/ml, and one study suggests 1.5 ng/ml as a predictor for PCa in AA men. Our results revealed that 33.3% of study participants had higher-than-normal PSA levels. Of these, 12.1% of participants (50/414) had PSA levels >4 ng/ml, 9.4% (39/414) had detectable PSA levels between 2.5-3.9 ng/ml, and 11.8% (49/414) had detectable PSA levels between 1.5-2.49 ng/ml.
Figure 19. PSA values of study participants who had discussed PCa screening with their physicians vs. those who had not. Diagram illustrating the percentage of participants with PSA values considered as high-risk separated into groups by those who had discussed the pros and cons of PSA testing versus those who had not. Of the total study participants, 54.8% (227/414) had never discussed the pros and cons of PSA testing with their physicians. Of these, 29.1% (66/227) had higher-than-normal PSA values as determined using the three cutoff values defined in Figure 18. Conversely, 45.2% (187/414) of study participants had discussed the pros and cons of PSA testing with their physicians. Of these, 38.5% (72/187) had higher-than-normal PSA values as determined using the three cutoff values defined in Figure 18.
Discussion

In light of the disproportionately high disparities in PCa incidence and mortality affecting AA/Black men, recent recommendations espouse earlier screening for this group.\textsuperscript{11,12} USPSTF guidelines are also currently undergoing a process of revision, although a full recommendation statement has not been finalized. The current draft under consideration includes a level C recommendation that clinicians inform men ages 55-69 about the potential benefits and harms of PSA-based screening for PCa, with a recommendation against PSA-based screening in men 70 years and older. A widespread endorsement of clinician conversations regarding PCa screening would be beneficial as studies have shown that a better understanding of PCa by AA/Black men is critical for reducing these disparities since knowledge of this disease strongly influences informed decision-making.\textsuperscript{8}

Several factors are associated with PCa knowledge among AA/Black men, including physician consultation. An important component of physician conversations with patients regarding PCa screening involves discussing the potential benefits and harms of testing.\textsuperscript{8} Ideally, these conversations should culminate in increased PCa patient knowledge to help steer choices regarding screening and treatment.\textsuperscript{13} However, AA/Black men are less likely to receive sufficient information from their physicians about PSA testing to make an informed decision.\textsuperscript{13}

Recognizing these concerns, this study evaluated the occurrence of physician-patient conversations within an AA/Black men cohort and assessed whether this translated into an increase in PCa knowledge and PSA testing. Our study represents a novel step in that it focuses specifically on AA/Black men. Additionally, it assesses
whether having discussed PCa screening with a physician is associated with higher PCa knowledge. Our study approach was also unique in that the responses were aligned with newly quantified PSA levels. We identified participants who had higher-than-normal PSA, recognizing that PCa experts assign differing cutoff values for what is considered higher-than-normal PSA levels for AA/Black men. For instance, while the conventional cutoff for higher-than-normal PSA is 4 ng/ml, ACS now recognizes PSA >2.5 ng/ml as reason for repeat annual screening, and one study suggests >1.5 ng/ml for AA men.\textsuperscript{10,16} Therefore, we determined the percentage of men under these PSA cutoff values separately. This study is also the first to report high-risk AA/Black men with higher-than-normal PSA values who had yet to discuss the pros and cons of PCa testing with their physicians. While elevated PSA does not inevitably predict PCa, these findings were distinctive as it has been reported that 1 in 4 AA/Black men will be diagnosed with PCa, yet 1 in 3 of our AA/Black participants had elevated PSA levels, which could be indicative of underlying PCa. We cannot rule out, however, that elevated PSA levels in some participants may be unrelated to PCa.

Multivariate analysis revealed that PCa knowledge increased as age decreased, as income and education increased, and in men who had discussed the pros and cons of testing with their physicians. This suggests that increased physician interaction with less-educated and lower-income men is critical, given that these groups are less likely to access health care or navigate their discussions with physicians as easily as their peers with higher income and education. Our findings also suggest that AA/Black men in their 40s may not have the knowledge they need to consider their high risk for PCa while making a decision about screening.
As expected, PSA values in our AA/Black male cohort increased with age. Approximately one-third of participants had PSA values that could be considered higher-than-normal; however, over half of the men had never discussed the pros and cons of PSA testing with their physicians. This is problematic as this high-risk population should be well-informed about PCa risk and screening options. Our results reveal that the physician-patient conversations may not be occurring frequently enough in a population with existing higher-than-normal PSA values, which includes AA/Black men under 40 years old. However, we recognize that there are no current recommendations for PCa screening for men in their 20s and 30s.

A limitation of this study is that participants were not instructed to accurately identify the type of physician or healthcare provided they interacted with (e.g. family physician, urologist, nurse practitioner, etc), which may have impacted the emphasis placed on a PCa screening conversation. Because of this limitation, we did not explore measures of physician competency that may have impacted the efficacy of these conversations. Another potential limitation is that the study was conducted within a church-based (Seventh-Day Adventist) population and urban areas of California (Riverside) and New York (Brooklyn), which are likely to attract men who are more aware about their health and about cancer prevention. To counterbalance the potential confounding factor of religion on survey responses as well as increase community involvement, we also recruited non-church affiliated men through local Black-owned barbershops for our Riverside event and from community organizations in Brooklyn. Nevertheless, consistent with previous findings, faith-based organizations are promising venues for health promotion in AA/Black communities. We also experienced during the
course of our study that these organizations provide an excellent venue and mechanism for the recruitment of AA/Black men for community-based participatory research.

Encouragingly, the physician-patient conversations that are occurring regarding PCa screening appear to be effective, as verified by subsequent PCa knowledge assessment. There is room for improvement, however, as we found that for many men who exhibited high PSA values and had discussed with their physicians the pros and cons of PCa screening, these discussions did not translate to actual PSA testing in 24% of this subgroup of men. This study further highlights the continued need for effective communication between physician and patient regarding prostate health and PCa screening and for better provider education about the special needs of AA men, which may not have been adequately addressed under existing procedural recommendations.

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References


CHAPTER 5
SELECTED UNPUBLISHED DATA

Patient Sera Samples Used in ELISAs

Sera from patients with PCa were obtained from Loma Linda University (LLU) Cancer Center Biospecimen Laboratory, Bioserve (Beltsville, MD), LLU Medical Center, and the serum bank in the LLU Center for Health Disparities and Molecular Medicine. Normal human sera were obtained from the serum bank in the Cancer Autoimmunity Research Laboratory of The University of Texas at El Paso (UTEP) and used as controls. These non-PCa sera were collected during annual health examinations in adults who had no obvious evidence of malignancy or autoimmune disease. We collected additional non-PCa sera under approval of LLU Institutional Review Board (OSR#5110343) during community Black Men’s Health Fairs held in Riverside, California, and Brooklyn, New York organized under Project C.H.A.N.G.E. (Changing Health in Adults with New and Great Experiences), a LLU community outreach initiative comprised of a transdisciplinary team of scientists and health care professionals.

Enzyme-Linked Immunosorbent Assays (ELISAs)

Commercially-available ELISA kits were used to quantify circulating LEDGF/p75 (MyBioSource, Cat:MBS706164) and sCLU (Boster, Cat:EK0914) according to manufacturers’ instructions. 96-well plates provided within the kits were pre-coated with capture antibody specific for either LEDGF/p75 or sCLU. Briefly, human sera were added to the wells and circulating LEDGF/p75 or sCLU was allowed to
bind to the immobilized antibody. Wells were washed to remove unbound LEDGF/p75 or sCLU. Anti-LEDGF/p75 or anti-sCLU horseradish peroxidase conjugate was then added to each well and allowed to bind to LEDGF/p75 or sCLU. Wells were washed and TMB (3,3’,5,5’ tetramethylbenzidine) reagent (provided in kit) was added to each well followed by incubation. Color development was interrupted with Stop Solution (provided in kit), and absorbance was measured by spectrophotometer at 450 nm, with LEDGF/p75 or sCLU concentration directly proportional to color intensity. LEDGF/p75 and sCLU values were calculated from a standard curve generated using standards provided with the kits. LEDGF/p75 and sCLU measurements were performed in duplicates for all serum samples.

**LEDGF/p75 and CLU May Circulate at Higher Levels in Sera of AA Men Compared to EA Men**

We used ELISAs specific for either LEDGF/p75 or CLU to quantify circulating levels of the proteins in the sera of AA and EA PCa patients. We also included normal AA and EA patient controls. There was no statistically significant difference found in levels of circulating LEDGF/p75 between AA and EA PCa patients, between normal and PCa patients, or between AA and EA normal patients (Figure 20). However, there were 3 outlier patients whose circulating LEDGF/p75 levels greatly exceeded the levels of all other patients. All 3 of these outlier patients were AA PCa patients. In terms of CLU, there were statistically significant differences in circulating levels within sera of our patient samples (Figure 21). Interestingly, there was no statistically significant difference between circulating CLU levels between AA PCa patients and AA normal patients.
Rather, every AA normal patient was found to have CLU circulating at a level which matched levels expected to be found in PCa patients.
Figure 20. Elevated circulating LEDGF/p75 observed in AA PCa patients.
Figure 21. Elevated circulating CLU observed in AA normal men matching levels observed in AA and EA PCa patients. (*p<0.05, ***p<0.001).
**Dex transactivates the CLU promoter**

Our observations led us to further investigate if Dex plays a direct role in transactivating the promoters of CLU and LEDGF/p75. To determine this, a luciferase transcription reporter assay was performed in a preliminary experiment using PC3 cells. Cells were transfected with either pGL4.10-Vec, pGL4.10-CLU, or pGL4.10-ledgf/p75. PC3 cells untreated with Dex but transfected with pGL4.10-CLU or pGL4.10-ledgf/p75 displayed significant increase in promoter transactivation, as measured by relative light units of luciferase activity, when compared to cells transfected with pGL4.10-Vec (Figure 22). More importantly, PC3 cells treated with 10 nM Dex and transfected with pGL4.10-CLU displayed significant increase in promoter transactivation, as measured by relative light units of luciferase activity, when compared to cells transfected with pGL4.10-Vec. PC3 cells treated with 10 nM Dex and transfected with pGL4.10-ledge/p75 displayed a slight increase in promoter transactivation, as measured by relative light units of luciferase activity, when compared to cells transfected with pGL4.10-Vec, however the increase was not significant. Results from this experiment are preliminary and will be repeated in the future in order to report statistical significance in terms of fold change.
Figure 22. 10 nM Dex robustly transactivates CLU promoter in luciferase-based transcription reporter assay using PC3 cells. A slight increase in transactivation was observed in LEDGF/p75 promoter. (*p<0.05, ***p<0.001, ****p<0.0001).
CHAPTER 6
OVERALL DISCUSSION

PCa is the most commonly diagnosed cancer and the second leading cause of cancer death among men in the U.S.\(^1\) For 80 years, it has been established that androgens are needed to drive PCa development and progression, and for this reason ADT drugs targeting androgen synthesis and AR have been a mainstay treatment for PCa patients.\(^2\)

While ADT is successful in most patients who choose this option, ADT-resistance is inevitable and PCa progresses to mCRPC.\(^3\) Taxane chemotherapy is available to mCRPC patients in the form of DTX and CTX, but these treatments are not long-lasting or curative.\(^4-6\) Once chemoresistance inevitably occurs, there are no further treatments available and palliative care is the only remaining option to improve patient comfort.\(^7\)

Because PCa cells escaping organ-confinement develop castration-resistance and chemoresistance in response to PCa treatments, there is an urgent need to identify new molecular targets that may confer greater benefit and increase longevity.\(^8\)

Until recently, the mechanism behind the acquisition of ADT-resistance in PCa cells and the ability to progress in the absence of androgens had not been fully elucidated. Pivotal studies within the last five years have proposed that, in the absence of androgens and functional AR, activated GR can bypass the classical AR pathway and bind AREs within the promoter regions of AREs thereby transactivating canonical AR-target genes associated with tumor aggressiveness.\(^9-14\) Complementing these reports were studies that demonstrated increased GR expression within ADT-resistant and chemoresistant pre-clinical PCa cellular models and patient samples.\(^10,15\) The implications of these findings were troublesome as PCa patients are routinely administered synthetic glucocorticoids as
co-therapy alongside ADT and taxane chemotherapy for palliative purposes to reduce adverse side effects.\textsuperscript{12,16-18} Unfortunately, patients co-administered glucocorticoids with Enz or abiraterone had worse overall survival outcomes in two separate clinical trials.\textsuperscript{12,19}

Under normal cellular conditions, GR signaling is activated when glucocorticoids bind to GR, GR translocates the nucleus, and binds to GREs transactivating GR-target genes.\textsuperscript{20} In the context of ADT-resistance, however, GR appears to activate both GR- and AR-target genes.\textsuperscript{10}

The prospect of GR driving advanced PCa progression is potentially amplified in AA men who, alongside suffering a disproportionate rate of PCa incidence and mortality, are also reported to have amplified GR signaling and excessive cortisol.\textsuperscript{1,21-23} There is evidence that chronic stress throughout the lifetime of AAs leads to dysregulation of endogenous cortisol production via the HPA axis, and this HPA dysregulation is well documented to enhance risk for metabolic disorders and cancer.\textsuperscript{22-25} The HPA axis is activated in response to stress, and there is a positive association between stress and lower SES in AA men.\textsuperscript{22,26} Decreased SES is defined by lesser income, education, or occupation and often results in increased exposure to environmental stressors leading to stress-related dysregulation of physiological systems and increased risk for disease.\textsuperscript{22,27,28} While lower SES is associated with dysregulation of cortisol diurnal rhythm, AAs also wake with lower cortisol levels and sustain higher cortisol levels in the evening suggesting a dysregulation of normal cortisol diurnal rhythm that cannot be explained by SES.\textsuperscript{22} The long-term consequences of this dysregulation are complicated and problematic for the health of AAs,\textsuperscript{23,29,30} and the overall role of altered GR signaling in the context of PCa health disparities has not been previously explored.
The cause of PCa health disparities documented in AA men are complex and include many contributing factors such as socioeconomic issues, biological and genetic determinants, diet, lifestyle, and access to healthcare.\textsuperscript{31-38} The compounding effect of these factors lead to AA men being diagnosed with exaggerated biological characteristics of PCa including higher PSA levels, higher Gleason scores, and advanced tumor stage.\textsuperscript{39-41} Men are more likely to be diagnosed with more advanced stages PCa when proper screening protocols are not observed.\textsuperscript{42} Deciding when and whom to screen for PCa has been a topic of hot debate since screening recommendations were drastically altered in 2012 in favor of significantly reduced PSA testing.\textsuperscript{43} While a caveat encouraging conversations between physicians and AA men regarding shared-decision making was provided within the USPSTF grade D recommendation against PSA testing, this study explored whether these conversations were occurring as well as effective.

We recruited over five hundred men to participate in Black Men’s Health Fairs in Riverside, CA and Brooklyn, NY and complete a comprehensive socio-demographic and health survey as well as donate blood for research purposes.\textsuperscript{44} Disturbingly, we found that less than half (45.2\%) of participants had ever discussed the pros and cons of PCa screening with their physicians. We then adjusted for key socio-demographic/economic and health care variables and performed multivariate analyses to assess whether physician-patient conversations predicted PCa knowledge.\textsuperscript{44} We found that increased PCa knowledge was correlated with younger age, higher income and education, and having discussed the pros and cons of testing with a physician.\textsuperscript{44} These results suggested that although many physicians were not having important shared-decision making conversations with their AA patients, physicians who did discuss PCa screening with
their AA patients were communicating effectively.\textsuperscript{44} When we analyzed existing circulating PSA values by ELISA in these same participants, we detected higher-than-normal levels in 38.5% of men who had discussed PCa screening with a physician and 29.1% of men who had not discussed PCa screening.\textsuperscript{44} Taken together, there is room for improvement as a third of the AA study participants had elevated circulating PSA, a biomarker used in PCa screening, yet many of these men had never discussed PCa screening with their physicians.\textsuperscript{44} Our results highlight the need for improved screening practices among AA men at high-risk of developing aggressive PCa.\textsuperscript{44}

Given the reports of enhanced GR signaling in this racial/ethnic group in conjunction with emerging studies documenting the important role of GR in PCa tumor aggressiveness and therapy-resistance, this study also sought to explore the potential relationship. Understanding how biological factors contribute to health disparities requires mechanistic functional analysis of specific genes or pathways in pre-clinical cellular models of this malignancy. To accomplish this task, it is essential to have available a well-characterized and racially diverse patient-derived cohort of immortalized cell lines representing different stages of PCa. Unfortunately, there is currently a lack of racial diversity in the human PCa cell lines that are commercially available for research, thereby limiting the scope of \textit{in vitro} addressing mechanistic events involving potential biological factors associated with PCa health disparities.\textsuperscript{45} Recognizing the importance of well-characterized and authenticated cell lines to ensure validity of published reports, the NIH recently established guidelines concerning the authentication of key biological resources including cell lines.\textsuperscript{45} This authentication also applies to racial identity for studies exploring biological determinants of health disparities. In this context, we verified
the racial identity of several cell lines essential to our studies while also establishing for the first time that popular cell line 22Rv1, assumed to be EA for nearly two decades, carries mixed genetic ancestry with substantial AFR composition.\textsuperscript{45}

To determine our findings, we used a validated set of 29 SNP genotypes referred to as AIMs and frequently used for population structure analyses.\textsuperscript{45,46} All the 29 selected SNPs exhibited large allele frequency differences between four continental populations—AFR, EUR, AMI, and EAS—and these differences were confirmed using the NCBI dbSNP database.\textsuperscript{46} SNP genotyping conducted at the UCLU Genomics Core revealed that PC3 and DU145 cell lines, originally reported to be EA, carried majority EUR ancestry.\textsuperscript{45} In addition, MDA-PCa-2b and RC-77T/E cell lines, reportedly derived from AA patients, carried majority AFR ancestry.\textsuperscript{45} The 22Rv1 cell line, long considered to be EA, was found to carry the following ancestral proportions: 0.41 AFR, 0.42 EUR, and 0.17 AMI.\textsuperscript{45} Our valuable finding has provided an additional resource to advance molecular research in the context of PCa health disparities.\textsuperscript{45}

Once we validated the racial identifiers of each cell line required for our mechanistic studies, we continued our quest to explore the relationship between GR signaling and PCa health disparities. More specifically, we examined the ability of activated GR to upregulate two key stress oncoproteins associated with PCa therapy-resistance—LEDGF/p75 and CLU—and observed race-dependent differential expression. To complement studies suggesting that activated GR bypasses the AR signaling pathway and activates AR-target genes\textsuperscript{10,12}, we first determined and validated that LEDGF/p75 and CLU are regulated by androgens. Using AR agonist DHT and AR antagonist Enz, we treated 22Rv1 cells which are AR-positive and -sensitive and
observed that both LEDGF/p75 and CLU are regulated by androgens. Our findings support other studies that have confirmed androgen-regulation of CLU.\textsuperscript{47-49}

We next treated a racially diverse panel of PCa cell lines with GR agonists cortisol and Dex to determine the ability of activated GR to upregulate LEDGF/p75 protein expression. LEDGF/p75 was upregulated in three out of four PCa cell lines with the most robust fold change in protein expression observed in MDA-PCa-2b and 22Rv1 cells with substantial AFR ancestry. This same GR-mediated upregulation was observed with CLU, and MDA-PCa-2b and 22Rv1 cells continued to the trend measured in LEDGF/p75 with heightened intensity of CLU upregulation compared to EA cells. After determining that activated GR upregulates LEDGF/p75 and CLU protein expression in PCa cells, and most robustly in cells with substantial AFR ancestry, we assessed transcript levels under the same experimental conditions. We found that activated GR increased LEDGF/p75 and CLU transcript levels in three out of the four cell lines.

To further explore if GR contributes to LEDGF/p75 and CLU upregulation, we pharmacologically inhibited GR with Mif. Using 22Rv1 and PC3 cells, we observed an attenuation of LEDGF/p75 and CLU in 22Rv1 cells, but this same attenuation was not observed in PC3 cells. Recognizing that Mif is not entirely specific for GR and has been shown by others to be a GR agonist under certain conditions,\textsuperscript{50} we sought to specifically target GR using transient knockdown with siRNAs specific for NR3C1. This genetic blockade of GR led to significant depletion of LEDGF/p75 and CLU in both 22Rv1 and PC3 cells.

We also examined the ability of glucocorticoids to modulate the migration rate of PCa cells and chose PC3 and DU145 cells because of their abilities to form a confluent
monolayer. We performed scratch wound healing assays after treating cells with Dex and observed significantly increased migration in PC3 cells, yet significantly decreased migration in DU145 cells. Our consistent observations that GR decreased migration in DU145 cells in conjunction with repeated downregulation of LEDGF/p75 and CLU in these cells when treated with glucocorticoids is consistent with other reports demonstrating that high levels of basal GR in DU145 cells could lead to immediate downregulation and shutdown of GR signaling upon exposure to glucocorticoids.\textsuperscript{51,52}

To further explore the possibility that GR regulates the glucocorticoid induction of LEDGF/p75 and CLU in PCa cells, likely by binding to the promoter regions of these genes, we performed \textit{in silico} analysis suggesting that there are multiple putative GR binding sites within the LEDGF/p75 and CLU promoters to which GR might bind and regulate the expression of these genes.

Upon determining that activated GR is able to upregulate stress oncoproteins associated with PCa therapy-resistance—LEDGF/p75 and CLU—in PCa cells differentially dependent upon race, we conducted Oncomine analysis to identify transcript GR expression in primary prostate tissues from AA and EA men. We observed that, consistent with other reports, that GR transcript was consistently downregulated in PCa tumors compared to normal prostate tissues. However, when we focused our analysis on GR expression based on racial classification, we observed higher median values of GR in AA prostate tissues compared to EA prostate tissues. Our findings were consistent with the premise that AA men with PCa may have enhanced intratumoral GR signaling.

We were also able to measure circulating levels of LEDGF/p75 and CLU using serum samples from AA and EA men. Using ELISA we determined that, while there was
no statistically significant difference found in levels of circulating LEDGF/p75 between AA and EA PCa patients, between normal and PCa patients, or between AA and EA normal patients, there were 3 AA PCa outlier patients whose circulating LEDGF/p75 levels greatly exceeded the levels of all other patients. In terms of CLU, every AA normal patient was found to have circulating CLU at levels which matched both AA and EA PCa patients. Further studies including more patient samples are needed to further support our preliminary findings. However, our results suggest that AA men have elevated circulating LEDGF/p75 and CLU, which may be attributed to amplified GR signaling, priming them to develop more aggressive PCa tumors as well as acquire resistance to standard PCa therapies.

Taken together, the implications of our findings are far-reaching as GR is emerging as a key driver of PCa tumor aggressiveness.⁹,¹²,⁵³ Our results indicate that this effect may be amplified in AA men, and could provide a rationale for increased tumor aggressiveness and worse outcome observed in this racial/ethnic group. Our findings also complement the growing body of literature suggesting that glucocorticoid co-administration with PCa therapies including ADT may potentially lead to worse overall patient survival.⁹,¹⁰,¹²,⁵³-⁵⁵ By upregulating oncoproteins associate with resistance to ADT and taxane chemotherapy, activated GR signaling may promote the proliferation and migration of highly aggressive PCa tumor cells with enhanced therapy resistance capabilities. Since our results demonstrate that LEDGF/p75 and CLU are upregulated in the absence of androgen via GR, future combinatorial therapies co-targeting AR, GR, and stress oncoproteins could potentially confer greater overall survival to patients with advanced PCa. There remains a need to further elucidate genes driven by GR signaling...
that are specifically associated with ADT-resistance as well as chemoresistance. Given that AA men display an enhanced physiological response to glucocorticoids as well as disproportionate PCa incidence and mortality, further studies are needed to better elucidate the relationship between GR signaling and PCa tumor aggressiveness specifically in this racial/ethnic group.
References


CHAPTER 7

METHODOLOGY

The contents of this chapter are for the purpose of instructing current and future laboratory members on the methodology utilized in this project. This section will provide key details and insights that are important to reproduce the studies presented in this dissertation and to troubleshoot common problems that could be encountered in follow-up studies.

Cell Culture

Each cell line has a specific protocol for growing conditions. Please refer to ATCC Culture Methods section for current instructions. For this study, PC3, DU145, and 22Rv1 use the following culture conditions. Upon arrival of fresh cells from ATCC or when thawing previously frozen stocks, thaw cells in warm water bath (37°C) without submerging to prevent contamination. Once thawed, immediately pipette full volume of cell suspension into T25 flasks pre-warmed with 5mL media. Incubation conditions are 95% air, 5% carbon dioxide, and 37°C. Cells will proliferate and attach quickly. The following day, remove media (in order to remove DMSO present in freezing media) and replace with fresh media. Once cells are confluent, expand cells to a T75 flask. The media required for these cell lines consists of RPMI-1640 with 10% FBS. We also supplement the 500 mL media bottle with 100 µL of gentamycin and 1000 µL of normocin to prevent bacterial and fungal contamination. The subculture ratio for expansion is 1:2 with media renewal every 2 to 3 days. To freeze cells in liquid nitrogen, use complete growth media supplemented with 5% DMSO.
MDA-PCa-2b cells have unique growing conditions. Upon arrival of fresh cells from ATCC or when thawing previously frozen stocks, thaw cells in warm water bath (37° C) without submerging to prevent contamination. Once thawed, immediately pipette full volume of cell suspension into T25 flasks pre-warmed with 5mL media. Incubation conditions are 95% air, 5% carbon dioxide, and 37°C. Cells will proliferate very slowly for the first 3 weeks. This cell line never reaches full confluency and the morphology appears as grapelike clusters rather than a uniform monolayer. After one week, expand cells from one T25 to two T25 flasks. A week later, expand cells from 2 T25 flasks to 4 T25 flasks. The following week expand 4 T25 flasks to 1 T75 flask. At that point cells can be expanded from 1 T75 into 2 T75 flasks. The media required for this cell line consists of F12-K with 20% FBS. We also supplement the media with cholera toxin (Sigma Aldrich, Cat:C8052), epidermal growth factor (Sigma Aldrich, Cat:E9644), phosphoethanolamine (Sigma Aldrich, Cat:P0503), hydrocortisone (Sigma Aldrich, Cat:H0135), selenious acid (Sigma Aldrich, Cat:229857), and insulin (Sigma Aldrich, Cat:I0516).

The media preparation for MDA-PCa-2b is tedious and specific and should be meticulously adhered to as follows. Remove 100 mL of F12K media (500 mL bottle) and aliquot into two 50 mL tubes inside the sterile hood. Add 100 mL FBS to the F12K media (500 mL bottle) inside the sterile hood. Add penicillin-streptomycin (either 5 mL of 10,000 units or 10 mL of 5,000 units). Take two 50 mL tubes of removed F12K media to lab bench outside of sterile hood. Aliquot 10 mL of media into new 15 mL “master mix” tube. Dissolve 0.5 mg of cholera toxin (1 mg/mL) in 500 µL of media (use extra media aliquoted from 50 mL tubes) (store in 4° C for future use). Add 12.5 µL of dissolved
cholera toxin into 15 mL “master mix” tube. Dissolve 0.1 mg epidermal growth factor in 250 μL of media (use extra media aliquoted from 50 mL tubes) (store in 4°C for future use). Add 12.5 μl of dissolved epidermal growth factor into 15 mL “master mix” tube. Add 250 μL of insulin (0.005 mg/mL) into 15 mL “master mix” tube. Dissolve 0.01 g of selenious acid (45 mM) in 10 mL of media (use extra media aliquoted from 50 mL tubes). Add 2.9 μL into 15 mL “master mix” tube. Dissolve 1 mg of hydrocortisone (100 pg/mL) in 10 mL of media (use extra media aliquoted from 50 mL tubes) (store in 4°C for future use). Add 0.5 μL into 15 mL “master mix” tube. Dissolve 0.01 g of phosphoethanolamine in 10 mL of media (use extra media aliquoted from 50 mL tubes). Add 352 μL into 15 mL “master mix” tube. Take 15 mL “master mix” tube back into sterile hood and prepare to filter the contents of the “master” tube into the 500 mL bottle of F12K. You will need a sterile disposable syringe that can hold 10 mL of liquid as well as a disposable sterile syringe filter capable of screwing onto the disposable syringe.

Inside the sterile hood, remove the lid of the 500 mL F12K bottle and remove the syringe from its packaging. Then remove the seal of the disposable sterile syringe filter. Remove the sterile syringe filter and carefully balance on the exposed opening of the 500 mL F12K bottle. Carefully pour the contents of the “master mix” tube into the empty plastic container that housed the sterile syringe filter. Draw up the contents of the “master mix” tube that are now within the plastic container into the syringe. Dispense the entire syringe contents through the filter into the F12K media bottle. To ensure that all bacteria have been filtered out, aliquot 5 mL newly made complete media into empty T25 flask and allow to grow at least 48 hours to rule out contaminated media. Make sure media is
not contaminated prior to adding thawed cells. The subculture ratio for expansion is 1:2 with media renewal every 2 to 3 days. These cells cannot be frozen and rethawed.

_Treatment of PCa Cells with AR- and GR-Binding Drugs_

In our experiments, PCa cells were treated with different AR- and GR-binding drugs at different concentrations for up to 48 hours. To ensure reproducibility and efficiency, drugs should be handled following the instructions given by the manufacturer. For example, to prepare 10nM Dex, dissolve the drug in solid form in the vehicle agent ethanol. It is important to know what solvent to use to dissolve each drug considering their solubility properties. The solubility of Dex in ethanol is 25mg/ml. The molecular weight of Dex is 392.46. To prepare 10 nM stock, adhere to the following steps. It is important to dilute each drug in serial dilutions to maintain proportion of drug to media and equal distribution of particulate. Dissolve 0.4 g of Dex in 10 mL of ethanol (or 0.2 g of Dex in 5 mL of ethanol) for final 100 mM stock concentration. Divide in aliquots of 20 mL and freeze the aliquots in -80°C not in use to ensure stability. From the 100 mM Dex stock, dissolve 10 mL of 100 mM stock solution into 10 mL of charcoal-stripped media for final 100 mM solution. From the 100 mM Dex solution, dissolve 10 mL into 10 mL of charcoal-stripped media for final 100 nM solution. From the 100 nM solution, dissolve 1000 mL into 10 mL of charcoal-stripped media for final 10 nM solution. Charcoal-stripped FBS is required when supplementing media in order to remove trace endogenous hormone levels.

For experiments using cortisol or Dex exceeding 24 hours, media was replaced every 24 hours to ensure that cells would have consistent exposure to glucocorticoids.
The following drugs were obtained from these companies: Dex (Sigma-Aldrich, Cat:D4902), cortisol (Sigma-Aldrich, Cat:H0888), Mif (Sigma-Aldrich, Cat:M8046), DHT (Sigma-Aldrich, Cat: D073), Enz (HY-70002 Medchem Express), and charcoal-stripped FBS (Atlanta Biologicals, Cat:S11650).

**Immunoblotting Procedures**

The following commercially-acquired antibodies were used for our studies: rabbit polyclonal anti-LEDGF/p75 (1:1000, Bethyl Laboratories Inc., Cat:A300-848A), mouse monoclonal anti-GR (1:1000, BD Biosciences, Cat:611226), mouse monoclonal anti-clusterin α-chain (1:1000, Millipore, Cat:05-354), rabbit monoclonal anti-AR (1:1000, Cell Signaling, Cat: 5153S), rabbit monoclonal anti-β-actin (1:5000, Cell Signaling, Cat:5125), rabbit polyclonal anti-α/β-tubulin (1:1000, Cell Signaling, Cat:2148S).

Whole cell lysates were prepared as follows. To collect cells, we detached them from the surface of tissue culture flasks or plates using 1 mL of trypsin. Cell suspensions were collected in 5 mL plastic tissue culture tubes and stored on ice. We then spun the cells for 4 minutes in a clinical centrifuge at 4,000 RPM, discarded the supernatant carefully, and washed the pellet with PBS by additional centrifugations. This step was repeated 2 times to eliminate any residual trypsin and media. This is important because albumin is highly abundant in medium and may interfere with electrophoresis and immunoblotting if not adequately removed. We dissolved the pellet in Laemmlli sample buffer containing a protease inhibitor cocktail (LSB:CPI, volume depends on size of pellet but typically we started with 100μl of this reagent for cells collected from two 6-
well plates seeded at 120,000 cells per well). Cells were sonicated on ice to disrupt cellular structures. The lysates were stored at -80°C to preserve protein integrity.

Immunoblotting was performed following this procedure. Equal amounts of protein from whole cell lysates were loaded into individual wells of 4-12% polyacrylamide gradient gels (SDS-PAGE, NuPAGE, Thermo Fisher Scientific, Cat:NP0321BOX). Protein concentration was determined utilizing the DC protein assay kit from BioRad following the manufacturer’s instructions. To observe and compare changes in protein expression between cell lines, only 5 µg-10 µg should be loaded onto wells to avoid saturating the chemiluminescence signal in immunoblots. For other applications, 15 µg to 20 µg is ideal but the sensitivity of the antibody should also be considered. Proteins were separated by SDS-PAGE and transferred onto polyvinyl difluoride membranes (PVDF) (Millipore, Cat:IPFL00010) in a NuPAGE electrophoresis system by Thermo Fisher Scientific.

To prepare the samples after calculating the desired protein concentration, we diluted the appropriate protein volume in 5 µl NuPAGE LDS sample buffer (4X) and 2 µl of NuPAGE reducing agent (10X). Final volume, typically 20 µl, was achieved utilizing deionized water. The buffers utilized were purchased from the manufacturer. The protein separation was done in MOPS SDS running buffer and 500 µL of antioxidant for 90 minutes at 175 volts. Other members of the lab perform protein separation for 60 minutes. However, for the purpose of further separating the proteins of interest in this study, the migration time was extended to 90 minutes. The protein transfer was done in transfer buffer diluted to a concentration of 1X (the buffer comes in a 20X concentration) with 10% methanol and 70 µL of antioxidant for 90 minutes at 25 volts.
To visualize and confirm complete protein transfer, membranes were rocked slowly at room temperature for 20 minutes in ponceau S stain. Membranes were then cut at appropriate molecular weight to allow individual antibody probing. Membranes were blocked in 5% dry milk prepared in TBS-T buffer (20 mM Tris- HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20) for 1 hour. Blocking was done rocking slowly at room temperature. Membranes were then probed individually with primary and allowed to rock overnight in the cold room at 4°C. The next morning, membranes were washed 3 times for 10 minutes with TBS-T and corresponding secondary antibodies were added to membranes which rocked at room temperature for 2 hours. The dilution ratio varies between antibodies and should be done following manufacturer's instructions and in-house optimization. For example, when utilizing a rabbit anti-LEDGF/p75 antibody by the company Bethyl (1:1000, Bethyl Laboratories Inc., Cat:A300-848A), we incubated with primary antibody at a 1:1000 dilution overnight rocking in the cold room. We then washed with TBS-T 3 times, changing TBS-T every 10 minutes. Then, we applied the appropriate secondary antibody for 2 hours in a wet chamber. After incubation, we repeated the washing cycles. Enhanced chemiluminescence (ECL) was used to detect immunoreactive protein bands. For this, the ECL Western Blotting Substrate (Thermo Fisher Scientific Pierce, Cat:32106) was added to the antibody-protein surface of each PVDF membrane, followed by incubation for 4 minutes. Membranes were then transferred to autoradiography cassettes and exposed to autoradiography films for different lengths of time to ensure accurate detection of immunoreactive protein bands.
RNA Interference-Mediated Knockdown of GR in PCa Cells

To achieve transient knockdown of GR in our cellular models, commercially-available specific short inhibitory Trisilencer-27 RNAs (Origene, Cat:SR301960) corresponding to Locus ID: 2908 were used. Cells were transfected in a pooled knockdown with 10 nM of each siRNA Trisilencer-27/Dicer-Substrate duplex using oligofectamine (Invitrogen, Cat:12252011) following manufacturer's instruction. A universal scrambled negative control siRNA duplex (Origene, Cat:SR30004) was used as a negative control. To seed the cells in 6 well plates on day 1, we used antibiotic-free media, which ensure that antibiotics do not interfere with the uptake of the siRNA oligos. We also prepared the oligofectamine-siRNA complexes in serum-free media prior to adding to cells plated in 6 well plates on day 2 since serum interferes with the formation of these complexes. Cells were harvested on day 4 (48 hours following transfection).

Quantitative real-time PCR

When working with RNA, clean workspace with ethanol and RNase Eliminator. Total RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen, Cat:74134). Briefly, cells reconstituted in RNAprotect Cell Reagent are thawed at room temperature and then centrifuged for 5 minutes at 7000 RPM. Remove the supernatant completely by pipetting and loosen the pellet by flicking the microcentrifuge tube. Add 600 μL Buffer RLT Plus and dissolve the pellet completely by vortexing. Homogenize the lysate with 25G needle and 1 mL syringe 5 times. Transfer the homogenized lysate to a gDNA Eliminator spin column placed in a supplied 2 mL collection tube and centrifuge for 30 seconds at 10,000 RPM. Discard the column and save the flow through. Add 1
volume of 70% ethanol to the flow-through and mix well by pipetting. Transfer up to 700 μL of the sample to an RNeasy spin column placed in a supplied 2 mL collection tube and centrifuge for 15 seconds at 10,000 RPM. Discard the flow-through. Add 700 μL Buffer RW1 to the RNeasy spin column and centrifuge for 15 seconds at 10,000 RPM. Discard the flow through. Add 500 μL Buffer RPE to the RNeasy spin column and centrifuge for 2 minutes at 10,000 RPM. Place the RNeasy spin column in a new supplied 1.5 mL collection tube. Add 30-50 μL RNase-free water directly to the spin column membrane and centrifuge for 1 minute at 10,000 RPM to elute the RNA.

The iScript cDNA synthesis kit (BioRad, Cat:1708891) was used to reverse transcribe RNA (0.5 μg) into cDNA in duplicates. Briefly, prepare enough assay master mix for all reactions by adding all required components in the following order (per reaction): 1. 4 μL iScript Reaction Mix, 2. 0.5 μg RNA template, 3. 1 μL iScript Reverse Transcriptase, 4. Nuclease-free water (variable, add total volume 20 μL – other reagents listed 1-3.) Mix the assay master mix thoroughly and dispense equal aliquots into wells of 96-well qPCR plate. Seal 96-well plate with adhesive film (BioRad, Cat: MSB1001). Program BioRad thermal cycling protocol on the real-time PCR instrument.

qPCR was performed using the MyiQ real-time PCR detection system with primers using iQ SYBR Green Supermix (BioRad, Cat:1708882) following manufacturers’ recommendations. Primer sequences for LEDGF/p75, Clusterin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed using Primer 3 software. The forward sequence for LEDGF (5’ to 3’) was TGCTTTTCCAGACATGGTTGT and reverse sequence (5’ to 3’) was CCCACAAACAGTGAAAAAGACAG. The forward sequence for Clusterin (5’ to 3’) was
CTCTACTCTCCGAAGGGAATTGTC and reverse sequence (5′ to 3′) was CGGGCTGCCTGTGCAT. GAPDH mRNA was used for normalization and the forward sequence (5′ to 3′) was GAGTCAACGGATTTGGTCGT and reverse sequence (5′ to 3′) was TTGATTTTGGAGGGATCTCG. Primers were commercially synthesized by Integrated DNA Technologies (IDT). When preparing primer mix, add 8 µL reverse primer, 8 µL forward primer, 64 µL nuclease-free water. Thaw iQ SYBR Green Supermix, cDNA, and primers in ice and vortex. For each reaction, prepare 12.5 µL iQ SYBR Green Supermix, 10.5 µL deionized water, 1 µL cDNA, and 1 µL primer mix (forward and reverse combined) to a total volume of 20 µL.

**ELISA**

It is important to follow the approved safety protocol when handling human sera. First, protective goggles (or eyeglasses), double gloves, and lab coat must be worn at all times. Protective and absorbent cover should be placed over the bench to ensure that if any serum is spilled, it does not contaminate the work area. Pipet tips that were used to pipet human sera should be place in a container with bleach to decontaminate them before discarding in approved container. We preferably thawed human sera by placing the tubes in ice a few hours before use. If sera must be thawed immediately, put the tube in 37°C bath but return to ice immediately after thawing. If possible, aliquot serum samples to minimize thawing and freezing cycles, which may inactivate serum autoantibodies and other proteins.

Each ELISA Kit used in our studies are purchased with complete instructions provided. It is important to follow each manufacturers’ instructions as reagents,
antibodies, incubation times, and required sera volume vary. For example, human PSA ELISA Kits were purchased from Abnova (Taoyuan City, 320 Taiwan, catalog #KA0208). The 96-well ELISA plates were pre-coated with goat anti-PSA antibody for serum PSA detection. Sera were added to the wells in duplicates and circulating PSA was allowed to bind to the immobilized antibody. Wells were washed to remove unbound PSA. Monoclonal anti-PSA-horseradish peroxidase conjugate was then added to each well and allowed to bind PSA. Wells were washed and TMB (3,3’,5,5’ tetramethylbenzidine) reagent (provided in kit) was added to each well followed by incubation. Color development was interrupted with Stop Solution (provided in kit), and absorbance was measured by spectrophotometer at 450 nm, with PSA concentration directly proportional to color intensity. PSA values were calculated from a standard curve generated using PSA standards provided with the kit.

**Cell Migration**

In order to evaluate the migratory response of PC3 and DU145 cells following exposure to Dex, a scratch wound healing assay was performed. Cells were seeded to confluency in 6-well plates and grown for 24 hours in RPMI 1640 medium supplemented with 10% charcoal-stripped FBS, with or without 10 nM dexamethasone. Wound areas were generated (3 per well) using a 200 µl pipette tip by scratching the cell surface confluent monolayer. Migration of cells into the wound areas was visually tracked using an Olympus IX70 microscope equipped with SPOT RT3 Imaging System and phase contrast images were captured at 0, 6, 12, 18, and 24 hours. When capturing 0 hour images, it is necessary to draw circles using permanent marker around area captured with
microscope on plastic lid of 6-well plate. These circles are the landmark to use for subsequent image captures to ensure identical wound areas are compared every 6 hours. Frequent images were captured in order to track migration prior to complete closure of the wound areas. The wound recovery rate of migrating cells was measured using ImageJ Software.

**Luciferase Reporter Assay**

To conduct future luciferase reporter assays in the lab, it was necessary to expand plasmids containing the promoter regions of LEDGF/p75 and CLU provided to us from collaborators. The following instructions outline the steps taken to prepare for luciferase reporter assay.

Prior to working with bacteria for transformation, it is important to prepare LB broth and ampicillin plates. Make LB broth according to manufacturer instructions. We used Fisher (Cat: BP1427-2) 20g LB powder/1 Liter ddH2O. Make broth in flask large enough for steam overflow in autoclave (i.e. 1000 mL LB broth in 2000 mL Erlenmeyer flask). Cover flask with foil and place autoclave tape on top. Autoclave on liquid/liquid setting for pre-set 20 minutes. Store flasks at room temperature to use within 48 hours. Once LB broth is made, ampicillin plates can be made. The formula to make the gel base for the ampicillin plates is 20 g LB + 15 g agar/Liter. Place LB and agar in Erlenmeyer flask and then fill with ddH2O. Stir the mixture with magnetic stir bar for 15 minutes. Remove stir bar, cover flask opening with foil, and place autoclave tape on top. Autoclave mixture on liquid/liquid setting at pre-set 20 minutes. Place flask into sterile
hood and allow flask to cool to warm to the touch (not room temperature as you do not want mixture to coagulate before pouring). Place sterile cell culture dishes into sterile hood. For 1 L of LB broth plus agar add 0.1 g ampicillin making final concentration of 100 μg/mL. Add ampicillin to cooling Erlenmeyer flask. Pipette 25 mL mixture per cell culture plate. When pipetting, only remove plate lid slightly to allow space for pipetting. Leave plates with the top slightly open to dry. When dry, close and put into labeled bag with gel side of plates facing up. Put plates into 4°C cold room. Empty coagulated contents of Erlenmeyer flask into trash and then clean flask.

Once the ampicillin plates are made, bacterial transformation with plasmids can begin. Label Eppendorf tubes (i.e. empty vector, CLU P1, CLU P2). Add 75uL TE buffer to tubes. Cut plasmids stored on filter paper into 1/4th (e.g. 1 slice of a 4-slice circular pizza). Place 1/4th circle of plasmid submerged into TE buffer in tubes and vortex for 5 seconds. Leave at room temperature. Place chemically competent bacteria cell tubes into ice (one tube per plasmid). Label tubes while kept in ice. Set timer for 15 minutes. Place pre-made ampicillin plates upside down at room temperature. When timer goes off, flick the bottom of plasmid tubes. Pipette up 5 uL plasmid liquid and transfer into chemically competent bacteria cell tubes. Pipette up and down without shaking the tube. Keep in ice 30 minutes. Place tubes in hot water bath 42.8°C in Hughes lab for 90 seconds. Place tubes in ice bucket 2 minutes. Add 1000 uL pre-made LB broth into tubes and shake. Place tubes in 37°C non-sterile incubator for 15 minutes. (Incubator by the cold room). Centrifuge tubes 5000 RPM for 2 minutes. Pipette approximately 100 uL of mixture and drop onto pre-made ampicillin plate. Use sterile plastic streaking wand and transfer cells by rubbing wand back and forth distributing cells throughout the plate. Store plates
upside down in 37°C non-sterile incubator overnight. The next morning, check plates to ensure bacteria colonies have grown. Place parafilm around the edges of the plates and place upside down in the cold room until the end of the day when bacteria can be seeded in tubes for expansion.

To expand bacterial growth, be sure to have autoclaved sterile toothpicks, culture collection tubes with lids, and pair of tweezers. Conduct this procedure later in the day to allow for overnight incubation/shaking. Perform procedure at open flame. Each collection tube needs 3mL LB broth plus ampicillin; therefore, make mastermix with total volume LB broth and ampicillin needed for experiment and vortex to mix. Aliquot 3mL mixture per tube needed for experiment. (Need 3 tubes per bacterial plate). Flame tweezer point for 10 seconds and pick up toothpick with tweezer near flame. Softly “scrape” approximately 6 bacterial colonies (or until visible collection of bacterial colonies at the tip of the toothpick). Place toothpick with colonies colony-side-down into LB broth/ampicillin tubes. Place tubes on shaker overnight 225 RPM at 37°C. (Shaker in autoclave/ice room). The next morning, collect cryovials of 500 µL bacterial growth and add 500 µL of 50% glycerol in water for storage in -80°C for future use if needed.

DNA miniprep is the next step to isolate plasmid DNA. We used several kits and had the most efficient yield with QIAprep Spin Miniprep Kit (Qiagen, Cat:27106). Briefly, transfer the overnight bacterial culture into centrifuge tubes and pellet by centrifugation at 8,000 RPM for 3 minutes. Resuspend pelleted bacterial cells in 250 µL Buffer P1 and transfer to a microcentrifuge tube. Add 250 µL Buffer P2 and mix thoroughly by inverting the tube 5 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 minutes. Add 350 µL Buffer N3 and mix.
immediately and thoroughly by inverting the tube 5 times. Centrifuge for 10 minutes at 13,000 RPM. Apply the supernatant to the QIAprep spin column by pipetting. Centrifuge for 60 seconds and discard the flow through. Wash the QIAprep spin column by adding 0.5 mL Buffer PB and centrifuge for 60 seconds and discard the flow through. Wash the QIAprep spin column by adding 0.75 mL Buffer PE and centrifuge for 60 seconds and discard the flow through. Transfer the QIAprep spin column to the collection tube and centrifuge for 1 minute to remove residual wash buffer. Place the QIAprep column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 50 µL Buffer EB or water to the center of the QIAprep spin column, let stand for 1 minute, and centrifuge for 1 minute. Nanodrop plasmid sample to determine DNA concentration prior to sending to Eton Bio for sequencing for verification. Coordinate with Jo-Wen in Dr. Marino De Leon’s lab to schedule Eton Bio plasmid sequencing. Once plasmid sequence of expanded samples has been verified to match sequence of original plasmid, further expansion can occur.

To expand bacteria in preparation for DNA maxiprep kit, prepare one 500 mL LB broth inside 2000 mL Erlenmeyer flask per plasmid sample. At the end of the day, seed bacterial plates with plasmid-transformed bacteria prepared previously and stored in -80°C prepared in glycerol in cryovials. Follow previous instructions for overnight incubation. The next morning, place parafilm around the edges of the plates and store upside down in the 4°C cold room. At the end of the day, thaw bacterial plates in 37°C non-sterile incubator. Prepare a fresh ampicillin stock as previously described (0.2 g ampicillin powder reconstituted in 1 mL ddH2O). At open flame, dispense 250 µL ampicillin/ddH2O per 500 mL LB broth flask. Flame bacterial streaking wand, place tip of wand onto blank space of bacterial plate to cool off the tip to avoid killing bacteria.
Collect a few colonies until visible on the tip of the wand. Place streaking wand into Erlenmeyer flask until submerged in the LB broth/ampicillin flask. Keeping flask covered with foil, shake/rotate overnight at 37°C at 195 RPM. Remove flasks from shaker/rocker the next morning (not longer than 16 hours) and place in the cold room until conducting DNA maxiprep the same day.

Because we need to insert plasmids of interest into PCa cells, it is important to purchase an endotoxin-free maxiprep kit. For this reason, we used the Maxi Fast Ion Plasmid Endotoxin-Free Kit (IBI Scientific, Cat:IB47124). Briefly, transfer cultured bacterial cells to a 250 mL centrifuge bottle (bottles in M. De Leon Lab) and centrifuge at 3000 x g (centrifuge in Langridge Lab) for 15 minutes at room temperature to form a cell pellet. Discard the supernatant completely. During centrifugation, place a plasmid maxi column in a new 50 mL centrifuge tube and equilibrate the plasmid maxi column by adding 10 mL of PEQ Buffer. Allow the column to empty completely by gravity flow. Discard the flow-through and place the plasmid maxi column back in the 50 mL centrifuge tube and set aside. Add 10 mL of PM1 Buffer (with RNase A added) and 100 μL of I-Blue Lysis Buffer to a new 50 mL centrifuge tube. Mix by shaking gently.
Transfer the mixture to the 250 mL centrifuge tube containing the cell pellet and resuspend by vortex. Transfer the resuspended sample to a new 50 mL centrifuge tube. Add 10 mL of PM2 Buffer to the resuspended sample then mix gently by inverting the tube 10 times. Do not vortex to avoid shearing the genomic DNA. Let stand at room temperature for at least 2 minutes to ensure the lysate is homogeneous. Do not exceed 5 minutes. Add 10 mL of PM3 Buffer and mix immediately by inverting the tube 10 times.
Do not vortex to avoid shearing the genomic DNA. Centrifuge at 3,000 x g for 20 minutes at room temperature (centrifuge in M. De Leon Lab).

To begin endotoxin removal, invert PER Buffer bottle 3-5 times immediately prior to use. Transfer the supernatant to a clean 50 mL centrifuge tube. Add 3 mL of PER Buffer then mix by inverting 5-10 times. Incubate on ice for 30 minutes. Following ice incubation, transfer the mixture to the equilibrated Plasmid Maxi Column. Allow the column to empty completely by gravity flow. Discard the flow-through then place the Plasmid Maxi Column back in the 50 mL centrifuge tube. Wash the Plasmid Maxi Column by adding 30 mL of PW Buffer and allow the column to empty completely by gravity flow then discard the flow-through. Place the Plasmid Maxi Column in a clean 50 mL centrifuge tube then add 12 mL of PEL Buffer to elute the DNA by gravity flow. Discard the Plasmid Maxi Column once it has emptied completely. Add 9 mL of isopropanol to the eluted DNA. Mix the tube completely by inverting the centrifuge at 15,000 x g for 30 minutes at 4°C (centrifuge in M. De Leon Lab). Carefully remove the supernatant then wash the DNA pellet with 5 mL of 75% ethanol. Centrifuge at 15,000 x g for 10 minutes at 4°C. CAREFULLY remove the supernatant then air-dry the DNA pellet for 10 minutes. Once the pellet is dry add 500 μL-2mL (or a suitable volume) of fresh ddH2O collected immediately from water dispenser to ensure the pH is >8. Ambient ddH2O can quickly cause acidification. Place the tube in a 60°C water bath for 5-10 minutes to dissolve the pellet. We created this water bath by microwaving glass beaker of water for 1 minute and monitoring temperature with thermometer. Store the dissolved pellet in 20°C.
Prior to transfecting PCa cells with DNA plasmids, the plasmids need to be reconstituted or dried to a 1 μg/1 μL stock concentration. To accomplish this, we used a vacuum (2nd floor Mortensen Hall) to evaporate excess water to better concentrate the plasmid once nanodrop revealed plasmid samples were too dilute. Nanodrop was repeated every 15 minutes of vacuuming until optimal concentration was reached. To continue with transfection, we used TransIT-X2 Dynamic Delivery System (Mirus, Cat:MIR6000). Briefly, plate cells at 15,000 cells/well in 3 mL of complete growth media per well using 96-well plate (opaque, white tissue-culture plate to minimize cross-talk between wells and absorption of emitted light during luciferase reporter assay). Culture overnight at cells should be >80% confluent on day of transfection. Warm TransIT-X2 to room temperature and vortex gently. Place 10 μL/well of serum-free media in a sterile tube. Add 0.1 μL/well DNA plasmid to sterile tube and mix gently by pipetting. Add 0.3 μL/well TransIT-X2 to sterile tube and mix gently by pipetting. Incubate at room temperature for 30 minutes. Add TransIT-X2:DNA complex mixture drop-wise to different areas of the well. Gently rock the plate for even distribution of complexes and incubate 24 hours. At 24 hours, spike in 10 nM of Dex per well. To do this, begin with 100 mM Dex stock and mix 1 μL Dex in 1 mL charcoal-stripped media for final concentration of 100 μM. From 100 μM Dex stock, mix 10 μL Dex in 10 mL charcoal-stripped media for final concentration of 100 nM. From 100 nM Dex stock, spike in 10 μL to each well. Incubate an additional 24 hours to prepare for luciferase reporter assay.

To conduct luciferase reporter assay, we used ONE-Glo Ex system (Promega, Cat:E8110). Briefly, equilibrate plates to room temperature. Bring ONE-Glo Ex reagents to room temperature and mix together immediately prior to use. Add 80 μL of ONE-Glo
Ex to each well and incubate samples for at least 3 minutes mixing on an orbital shaker (300-600 RPM) (shaker in M. De Leon Lab). Measure firefly luminescence using SpectraMax settings specific for luminescence measuring all wavelengths.

**Statistical Analyses**

SPSS Statistics Software V22.0 and V23.0 and GraphPad Prism 6 were used for statistical analyses for these studies. Differences between treatment groups were analyzed using unpaired Student’s t-test. When comparing multiple groups, ANOVA with Bonferroni correction was used. P values below 0.05 were considered statistically significant. All differences highlighted by asterisks were statistically significant as encoded in figure legends (*P < 0.05; **P < 0.01; ***P < 0.001; ****P<0.0001).

Additional protocols and experimental details can be found in the laboratory notebooks stored in the Casiano Laboratory, located in the first floor of Mortensen Hall in Loma Linda University, Loma Linda, CA. All electronic files pertaining this dissertation work are stored in the Casiano Laboratory computers and the Casiano Laboratory external drive memory under the folder Leanne Burnham Casiano Lab Files.