THE ROLE OF NUCLEAR FACTOR KAPPA B IN BENIGN PROSTATIC HYPERPLASIA

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CANCER BIOLOGY

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Dissertation under the direction of Professor Simon W. Hayward, Ph. D.

Benign prostatic hyperplasia (BPH) is a common, progressive chronic disease. Inflammation is associated with prostatic enlargement and resistance to 5α-reductase inhibitor (5ARI) therapy. Activation of the nuclear factor-kappa B (NF-κB) pathway is linked to both inflammation and ligand-independent prostate cancer progression. Most patients initially respond to 5ARI therapy; however, failure is common.

To address why patients fail therapy we used transition zone tissue samples from patients with a wide range of American Urological Association symptom score (AUASS) from incidental BPH in patients treated for low grade, localized peripheral zone prostate cancer to advanced disease requiring surgical intervention. NF-κB activation and androgen receptor variant (AR-V) expression were quantified. To further investigate these pathways, human prostatic stromal and epithelial cell lines were transduced with constitutively active or kinase dead forms of IKK2 to regulate canonical NF-κB activity, AR-FL, and AR-variant 7 (AR-V7).

We determined that canonical NF-κB signaling was found to be upregulated in late versus early stage BPH. Elevated expression of AR-V7 was found in advanced BPH samples. Expression of AR-V7 significantly correlated with the patient AUASS. Forced

activation of canonical NF-κB in human prostatic epithelial and stromal cells resulted in elevated expression of both AR-FL and AR-V7, with concomitant ligand-independent activation of AR reporters. Activation of NF-κB and over expression of AR-V7 in human prostatic epithelial cells maintained cell viability in the face of 5ARI treatment. To understand why NF-κB and AR-V7 maintained viability within 5ARI treatment we examined the levels of 5α-reductase enzymes (SRD5A1, SRD5A2, SRD5A3). We determined that SRD5A2 is upregulated in more advanced BPH. SRD5A2 was significantly associated with AUASS and patients on a 5ARI. AR-FL and AR-V7 expression increased SRD5A2 expression whereas forced NF-κB activation increased all SRD5A isoforms.

In summary, activation of NF-κB and AR-V7 in the prostate is associated with increased disease severity. Increased BPH severity in patients correlates with SRD5A2 expression. *De novo* synthesis of androgens and AR-V7 expression is inducible in human prostate cells by forced activation of NF-κB. NF-κB and AR-V7 upregulate SRD5A2 resulting in resistance to 5ARI treatment, suggesting a potential mechanism by which patients may become resistant to 5ARI therapy.

Dedicated to my loving and supportive family.

Especially to my late and loving grandmothers, Alvena Austin and Maxine Toney, who always taught me the value of education, sacrifice, determination, and hard work. Who also taught me never to quit when times get tough. They passed during the completion of my Ph.D. studies, but their memories remain in my heart and with me every day. This was for you grandmas.

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PREFACE

Treatment and diagnosis of benign prostatic hyperplasia (BPH) afflicts millions of middle to late age men, men over 60, word wide and is considered a one of the important topics in urology. BPH is considered as a nonmalignant hyperplasia of both the epithelial and stromal compartments within the transitional zone of the prostate. When men present with BPH there is usually a relationship with lower urinary tract symptoms (LUTS) due to the progression of prostatic growth. Although BPH is not life threatening, it is a clinical manifestation that reduces the quality of life for that patient over many years. Due to patients presenting with LUTS due to BPH and associated complaints, BPH has a high economic burden. Treatment of BPH is usually watchful waiting, life style changes, and/or medical and surgical intervention. To treat patients medically they are either put on individual or combinational treatment of α-blockers and/or 5 α-reductase inhibitors (5ARIs). Patients who do not respond and/or fail therapy are then treated surgically to circumvent symptoms. However, these patients that progress to surgery are significantly older in nature and are not prime candidates for surgery due to their age. Therefore, identifying why these patients fail therapy is of particular importance.

The first part of the thesis describes how patients fail therapy by comparing the activation of NF-κB and androgen receptor activation, and its variants, in transition zone tissue samples from patients with a wide range of American Urology Association Symptom Score (AUASS) from incidental BPH in patients treated for low grade, localized peripheral zone prostate cancer to advanced disease requiring surgical intervention. It also describes how chronic activation of NF-κB induces androgen receptor variant activation as well as

resistance to 5ARIs. The second part of the thesis describes mechanistically how and why patients fail therapy by looking specifically at the 5 α-reductase enzymes and if they differ in our incidental BPH versus surgical intervention BPH. It also looks at how chronic activation of NF-κB induces 5 α-reductase enzyme expression and is responsible for castrate resistant growth *in vivo*.

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5-AR	5α-reductase
5ARIs	5α-reductase inhibitors
Abi	Abiraterone Acetate
ABP	Acute Bacterial Prostatitis
ADT	Androgen Deprivation Therapy
AIS	Androgen Insensitivity Syndrome
AND	Androsterone
APC	Antigen Presenting Cells
AR	Androgen Receptor
AREs	Androgen Response Elements
AR-FL	Androgen Receptor full-length
ARv	AR variants
AR-V7	AR splice variant 7
ASD	Androstenedione
AUASS	American Urologic Association Symptom Score
BMI	Body Mass Index
BPH	Benign Prostatic Hyperplasia
CBP	CREB Binding Protein
CBPr	Chronic Bacterial Prostatitis
CK	Cytokeratin
COX2	Cyclo-oxygenase 2

CPPS	Chronic Pelvic Pain Syndrome
CRPC	Castrate Resistant Prostate Cancer
CS	Charcoal Stripped FBS
DAMPs	Damage-associated Molecular Patterns
DBD	DNA Binding Domain
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
EE	IKK2 Constitutively Active
EMT	Epithelial to Mesenchymal Transition
EV	Empty Vector
FBS	Fetal Bovine Serum
HPLC	High Pressure Liquid Chromatographic
IKK	IκB kinase
IL	Interleukin
IκB	Inhibitor of NF-κB
IκBα	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells Inhibitor Alpha
KD	IKK2-Kinase Dead
LBD	C Terminal Ligand Binding
LPS	Lipopolysaccharide
LUTS	Lower Urinary Tract Symptoms
MAPKK	Mitogen Activated Protein Kinase Kinase

MEKK1	Mitogen Activated Protein Kinase/ERK Kinase Kinase 1
MetS	Metabolic Syndrome
NF-κB	Nuclear Factor-kappa Beta
NIDDM	Non-insulin Dependent Diabetes Mellitus
NIH	National Institutes of Health
NIK	NF-κB Inducing Kinase
NLR	NOD (Nucleotide-Binding Oligomerization Domain Protein)-like Receptors
NLS	Nuclear Localization Signal
NTD	N-Terminal Domain
PALT	Prostate Associated Lymphoid Tissue
PAMPs	Pathogen Associated Molecular Patterns
PCa	Prostate Cancer
PSA	Prostate-Specific Antigen
qPCR	Quantitative Real-Time PCR
RDH	Rel Homology Domain
ROS	Reactive Oxygen Species
rUGM	Rat Urogenital Mesenchyme
T	Testosterone
T2DM	Type 2 Diabetes Mellitus
Tfm	Testicular Feminized Mice
TLR	Toll-like Receptors

TNF	Tumor Necrosis Factor
TURP	Transurethral Resection of the Prostate
VEGF	Vascular Endothelial Growth Factors
WT	Wild Type

CHAPTER I

Introduction

Overview

Benign prostatic hyperplasia (BPH) is an important cause of morbidity in the adult male population and is the most common symptomatic noncancerous condition in humans [1]. Clinically, BPH results in urethral constriction with a consequent slowing of urinary flow rates and an inability to properly empty the urinary bladder. Left untreated, this condition can ultimately lead to complete acute urinary retention and death. In practice, in the Western world, BPH is not a life-threatening condition. However, it is a condition with significant morbidity and consequent associated healthcare costs.

BPH etiology remains incompletely understood, however, it is believed to be dependent on the bioavailability of testosterone (T), dihydrotestosterone (DHT) and the androgen receptor (AR) and is often associated with prostatitis [2-6]. AR signaling contributes to BPH by increasing epithelial and stromal cell growth [7-11]. Recent studies have indicated that the AR can also be expressed as C-terminal truncated variants, called AR-variants (ARv), through alternative RNA splicing [12-18]. Lacking the ligand-binding domain of full-length AR (AR-FL), ARv are constitutively active in driving AR-regulated transcription and promoting tumor progression, even under castrate conditions [14, 19, 20]. Although a

number of ARv have been described in prostate cancer (PCa) cell lines and xenografts, AR splice variant 7 (AR-V7) is the most commonly expressed ARv in human tissues. The levels of AR-V7 are correlated with increased risk of biochemical relapse and shorter survival time in prostate cancer patients [13].

Activation of the nuclear factor-kappaB (NF-κB) pathway is a downstream consequence of a variety of stresses including inflammation [21], which is widespread in BPH [22, 23]. NF-κB is a nuclear transcription factor that responds to a variety of stimuli, including cytokines and stress and which in turn influences the immune and inflammatory pathways. Altered NF-κB regulation is associated with inflammation and a host of human disease processes [24, 25]. NF-κB signaling occurs through both canonical (p65) and non-canonical, or alternative (p100/p52) pathways resulting in the activation of overlapping sets of genes. Our laboratory has recently determined that in human BPH samples the canonical NF-κB pathway is activated in both the epithelial cells and stromal cells of BPH nodules [26]. This finding, along with studies from the Blackwell and Yull groups at Vanderbilt, demonstrated the separate and important roles for the two NF-κB pathways in mammary developmental and disease processes and underlines the need to more fully understand these processes in BPH [27-30]. Asymptomatic inflammatory prostatitis associated with BPH is a common but a

poorly understood phenomenon [31, 32]. The complexity of acute and chronic inflammatory processes seems to play a role in the development of BPH.

This chapter sets the historic and scientific foundation for understanding the impact of NF-κB on BPH development and progression. We introduce the prostate, NF-κB, and BPH and treatments associated with BPH. I then describe how inflammation plays a role in BPH and end with my hypothesis and dissertation goals.

The Prostate

Anatomy and Function

The prostate is a male reproductive organ, located just below the bladder and in front of the rectum (Figure 1). The function of the prostate gland is to secrete enzymes, lipids, and metal ions into the ejaculatory fluid produced by the seminal vesicles to nourish and protect sperm. The prostate enhances male fertility by producing fluid containing, zinc, citric acid, fructose, and prostate-specific antigen (PSA). In humans, the prostate is divided into three zones: central, transitional, and peripheral [33]. The central zone surrounds the ejaculatory ducts. The peripheral zone surrounds the distal urethra and is the main site of PCa. The transitional zone surrounds the proximal urethra and is the main site of BPH.

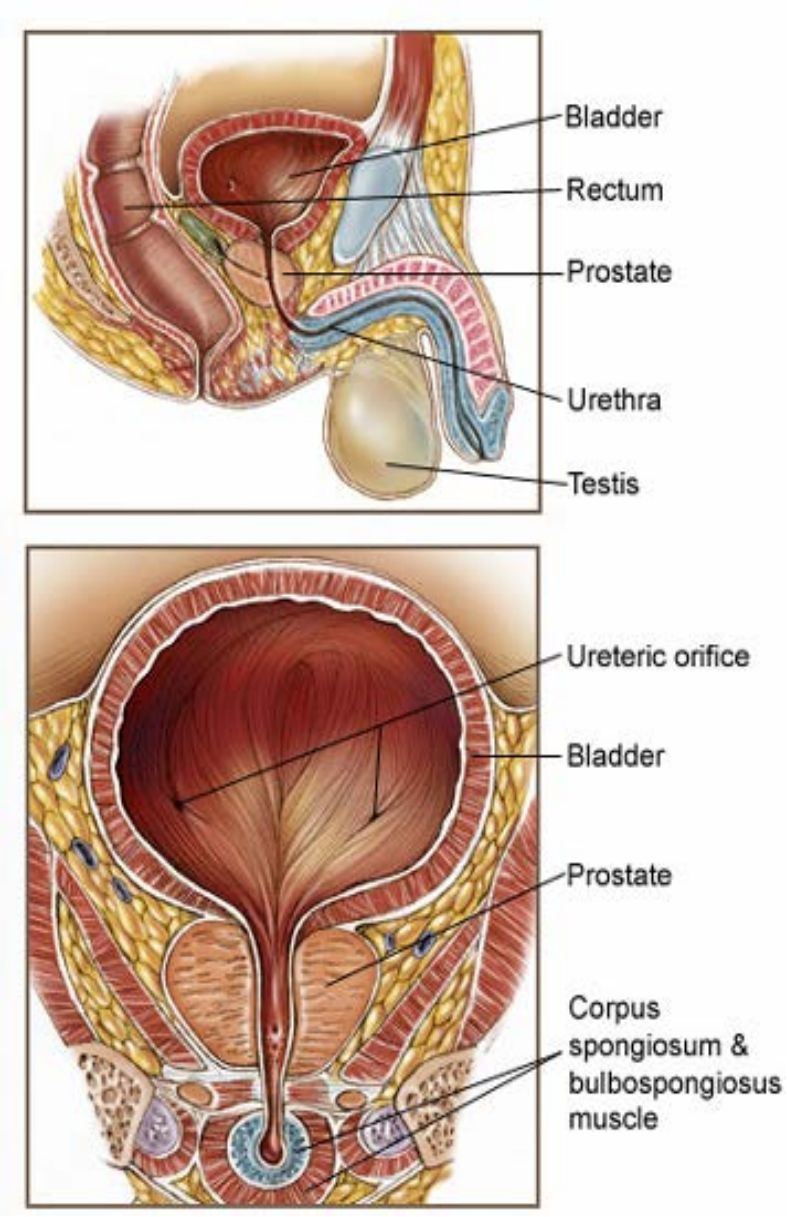


Figure 1: Anatomy of the Prostate (From 2003 American Society of Clinical Oncology)

Prostate Development

In mammals, the developing testes release androgens which are responsible for male characteristics. The release of androgen causes the Wolffian ducts, urogenital sinus, and the urogenital tubercle to differentiate into the epididymis/vas deferens, prostate, and penis [34]. Androgens released by the testes are required for the formation and growth of the prostate. The prostate develops by a process of branching morphogenesis [35], beginning as an epithelial bud that invades the surrounding mesenchyme while projecting dividing epithelial tubes away from the site of initiation. Growth of the ductal network of the prostate occurs during the prepubertal stage and happens with different rates: highest in the distal region and lowest in the proximal region closest to the urethra [34, 35].

The glandular structure of the prostate consists of organized secretory epithelia surrounded by a basement membrane and fibromuscular stroma [36] (Figure 2). Homeostasis of the prostate requires paracrine signals acting between epithelium and stroma to maintain growth quiescence. Human prostatic stroma is made up of a mixture of smooth muscle cells and fibroblasts [37-40]. The stroma of the prostate surrounds the gland and is responsible for producing growth factors for epithelial development [38, 41, 42]. The prostate gland is formed by the epithelium which contains luminal/secretory cells, basal cells, and neuroendocrine cells [43]. The luminal/secretory cells express the androgen

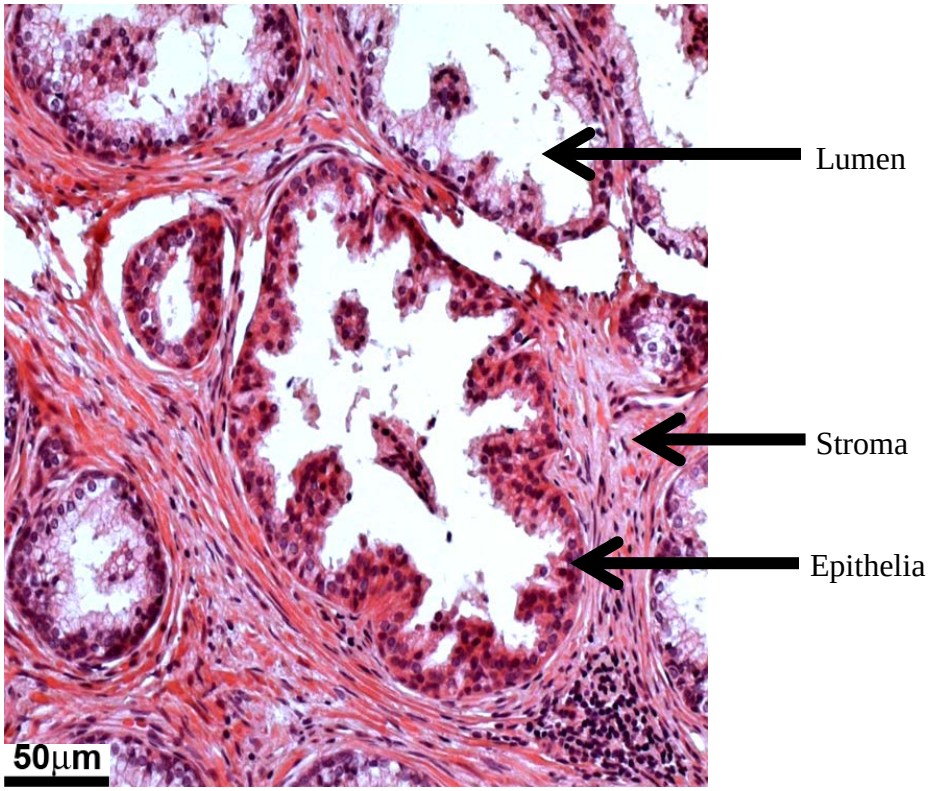


Figure 2: Glandular Structure of the Prostate. H&E of patient prostate WD-1183. Secretory pseudostratified epithelium, comprising of columnar cells and basal cells which are supported by a fibroelastic stroma. The lumen (is the inside space of the epithelial structure) which collects the prostatic fluid which is needed for sperm support during fertilization.

receptor (AR), cytokeratin (CK) 8, CK18, and are positive for PSA [43, 44]. The luminal/secretory cells are androgen dependent for survival and terminal differentiation. Basal cells are located between the basement membrane and the luminal/secretory cells. Basal cells express markers such as p63 and CK5 and CK14 [43, 45]. Neuroendocrine cells express chromogranin A. They do not express AR or PSA, and their function in the normal prostate is not fully understood [43]. Epithelial development and proper differentiation is necessary for proper prostatic development and function. This development of the prostate requires androgens and these stromal and epithelial interactions play a role in prostate homeostasis, and later in life contribute to BPH [46-48].

Androgens and the Androgen Receptor

Androgens

Androgens are male sex hormones responsible for the phenotypic characteristics of male sexual development. The major circulating androgen in the male body is testosterone (T). T is derived from cholesterol and is secreted by the testes and adrenal glands (Figure 3). T is converted to DHT by the enzyme 5α-reductase (5-AR) [49]. When T and DHT bind to AR it creates an AR-ligand complex that leads to transactivation. This complex then translocates to the nucleus where activation of androgen response elements (ARE's) in gene

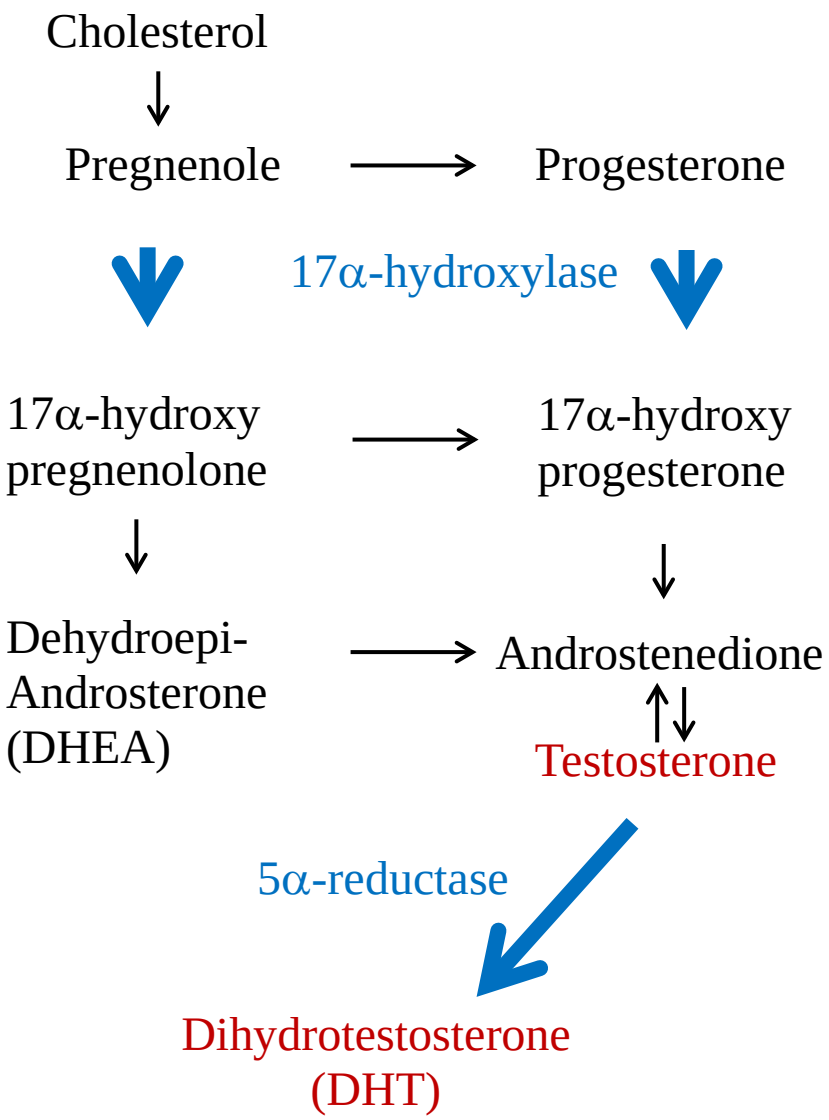


Figure 3: Androgen Synthesis Pathway

promoters occurs. The binding of DHT-AR has a three-fold lower disassociation rate than T-AR, which makes DHT-AR binding more stable [50, 51] and has 10-fold higher AR signal transduction than T-AR.

5α-reductase Isozymes

The 5-AR family contains three isozymes, 5-AR1, 5-AR2, and 5-AR3 [52, 53]. These are microsomal nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzymes. They all have similar gene structures with *SRD5A1*, *SRD5A2*, and *SRD5A3* all having five exons and four introns [54]. 5AR-1 and 5-AR2 are expressed throughout human life [55]. In early gestation 5-AR1 is located in fetal scalp and nongenital skin at low levels and 5-AR2 is located in the external genital skin [56, 57]. In adults 5-AR1 is located in nongenital skin, liver and at low levels in the prostate, genital skin, epididymis, seminal vesicles, testis, adrenal gland and kidney. 5AR-2 is expressed in the prostate, genital skin, epididymis, seminal vesicles, and liver [55, 56, 58]. 5AR-3 is expressed in very low levels in brain, kidney, lung, and pancreas and has been linked to castrate resistant prostate cancer (CRPC) progression [53, 59]. In the human prostate specifically, 5AR1/2 are located in the epithelial and stromal cells, while 5AR2 is predominant in stromal cells [52, 58], and 5-AR3 in basal epithelial cells [59].

Androgen Receptor

Androgen function is dependent on signaling through the androgen receptor (AR), which is a member of the nuclear receptor superfamily [60-62]. AR is organized into three domains, a N-terminal domain (NTD), a DNA binding domain (DBD), and a C-terminal ligand binding (LBD) (Figure 4). The DBD and LBD and highly conserved, however the NTD sequence varies among species [63-65]. The NTD controls the majority of AR transcriptional activity. AR is expressed in the cytoplasm of the cell when its ligand is not present. Once the ligand binds, AR translocates to the nucleus, dimerizes, and binds to DNA at consensus sequences known as AREs. Within this AR-DNA complex, coactivator proteins associate and corepressors dissociate from the complex. AR-DNA complex then leads to gene expression in genes present with AREs. Such target genes include PSA, TMPRSS2, and NKx3.1 [66-68].

The prostate is an androgen dependent organ, and androgens regulate its growth, development, and maintenance [69-71]. In early development of the prostate, a pioneer of the field, Gerald Cunha, explored how AR can induce the differentiation and branching morphogenesis of the prostate [41, 72-74]. Cunha et al demonstrated the need for androgens to stimulate epithelial development and growth by interacting with stromal AR [74].

Spontaneous AR mutants with an androgen insensitivity phenotype have been known as testicular feminized (Tfm) mice. Tfm mice lack a vas deferens,

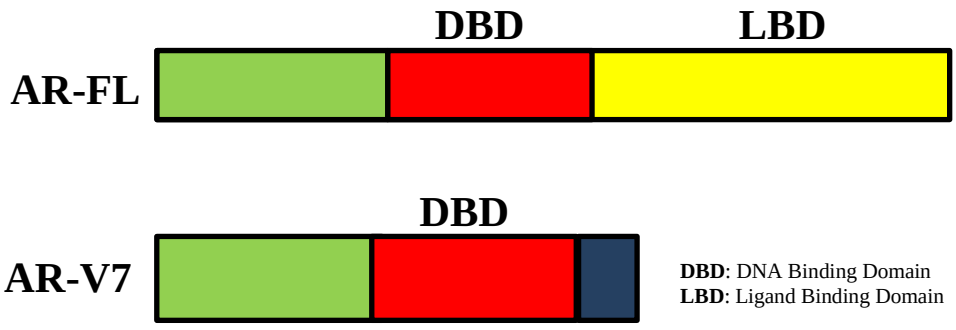


Figure 4: The Androgen Receptor and Androgen Receptor Variant 7

epididymis, and male accessory sex organs which make them a valuable experimental model to test AR mediated sex differentiation. Early studies using the Tfm mice demonstrated the need for the AR expression in both the epithelia and stroma for maturation of the adult prostate [74, 75]. In tissue recombination experiments where the epithelium was knocked out for AR and combined with wild-type (WT) stroma with expression of AR, the ducts that formed did not fully mature because they lacked the secretions indicative of a functional prostate. Also, in tissue recombination where the stroma was AR deficient and the epithelium expressing WT, resulted in a vagina like phenotype. This study showed that stromal AR is necessary for proper duct formation by the epithelium and epithelial AR is required for mature functional prostatic ducts. These studies demonstrated the importance of AR expression in the stroma and epithelium and the importance of androgen signaling in the prostate and its requirement in proper prostate development [74].

AR gene defects can prevent the development of normal male characteristics in 46, XY individuals and result in androgen insensitivity syndrome (AIS) also known as Tfm [74, 76-78]. AIS is the partial or complete inability of cells to respond to androgens and is an X-linked condition. AR gene mutations have four sources: single point mutations resulting in substitutions or premature stop codons, nucleotide insertions/deletions resulting in frameshifts,

complete/ partial deletion of the gene, or intronic mutations that affect AR RNA splicing [79].

In humans AIS is characterized by a range of phenotypes from individuals with subtle undervirilization, infertility, to individuals with complete feminization who exhibit normal breast development and female external genitalia [78]. Tfm mice have an external female phenotype with small abdominal testes [80] and are completely insensitive to androgens [81]. These animals make a valuable experimental model to test AR mediated sex differentiation because they lack a vas deferens, epididymis, and male accessory sex organs.

Recent studies involving AR demonstrated that it plays key roles in many pathways such as differentiation [74], proliferation [34], apoptosis [82], and inflammatory/immune response [83] and AR can be regulated by NF-κB [18, 84-86]. Jin et al transfected a PCa cell line (LNCaP) with four constructs: 1) the AR responsive reporter vector ARR_2-PB-Luc vector (which does not respond directly to NF-κB), 2) adenoviral vector expressing a dominant inhibitor, 3) nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) of the NF-κB pathway (IκBα-DN), or 4) control (empty adenovirus) to determine how both NF-κB expression and neuroendocrine differentiation contribute to prostate cancer progression [85]. They showed that neuroendocrine secreted neuropeptides increased AR activity more in the control than the IκBα-DN through the NF-κB pathway. The authors also demonstrated using a knockout model of IκBα [87],

that constitutive NF-κB signaling prevents the mouse prostate from regressing under castration and maintains prostatic epithelial cell proliferation [85]. These results indicate that activation of NF-κB is sufficient to maintain androgen independent growth of prostate and prostate cancer by regulating AR action.

In another study detailing AR regulation, Zhang et al demonstrated in a PCa cell line model (LNCaP) that NF-κB activates the AR promoter, endogenous AR expression, and that NF-κB inhibition decreases endogenous AR expression, activity, and AR promoter binding [85, 88]. They showed by transfecting LNCaP cells with the AR promoter-reporter construct, hARp-Luc, with increasing amounts of human p65, human p50, a combination of p50 and p65, or IκBα, that over expression of p65, or the combination of p65 and p50, activated the AR promoter in a dose-dependent manner. Expression of p50 alone slightly inhibited the human AR promoter activity and over expression of IκBα inhibited the AR promoter.

Androgen Receptor Variants

AR can be expressed as constitutively active androgen receptor variants (ARv) [12, 89]. ARv contain the NTD and DBD but lack the LBD (Figure 4). Conventional androgen deprivation therapy (ADT) inhibits androgen-dependent activation of AR. However, the presence of ARv provides a mechanism for cells to circumvent ADT. As of today at least 20 different ARv have been identified either at the mRNA or protein level in PCa samples, cell-lines of PCa, and BPH

samples [12-17, 90-94]. Among the 20, AR-variant 7 (AR-V7) is the most commonly expressed [13] and as such it is often chosen as a model of research. Since ARv are constitutively active and lack the LBD provide compelling mechanisms for BPH to circumvent 5α-reductase (5ARI) treatment. ARv have been extensively studied in prostate cancer [13-15, 95, 96]. These previous studies have demonstrated that ARv play a role in tumor progression to castrate resistant prostate cancer (CRPC) which is a more aggressive phenotype of PCa. However, BPH progression and ARv have not been linked. This study will focus on how AR-V7 expression leads to BPH treatment failure.

Role of Androgen Receptor Variants

Studies of ARv have primarily been focused on PCa and its role in tumor progression to CRPC. Evidence suggests that there is a relationship between ARv expression and epithelial to mesenchymal transition (EMT) [96]. In a study by Collard et al, EMT markers were analyzed in LNCaP cells. Overexpression of AR-FL or AR-V7, and transfection of ARv into these cells resulted in an increase of EMT markers, such as vimentin, N-cadherin, Snail and Zeb1 relative to cells transfected with AR-FL [96]. Another study using a transgenic mouse model, which overexpressed AR-V7, showed upregulation of autocrine and paracrine growth factors such as transforming growth factor ß2, and insulin like growth factor 1, as well as EMT associated genes, *TWIST, VIM, SNAI1, and CDH2* [97]. In a different study, using *in silico* sequence analysis, Hu et al demonstrated that

elevated mRNA expression of AR-V7 in hormone-naïve primary prostate cancer specimens was associated with increased biochemical recurrence following prostatectomy [14]. These studies suggest that ARv are involved in PCa progression, associated with EMT markers, and could play a role in PCa to CRPC progression. Therefore, this study will look at how ARv plays a role in BPH progression.

Inflammation

Inflammation is a complex multifaceted defense response of the immune system, for the neutralization of a foreign body and to reestablish the normal tissue structure and function [98]. Inflammation is characterized by redness, swelling, pain, and sometimes failure of function of a specific tissue. It is mediated and regulated by inflammatory cytokines and chemokines and can be subdivided into acute and chronic inflammation.

Cytokines and Chemokines

Cytokines are derived from antigen presenting cells (APCs) and mononuclear phagocytic cells are effective in promoting resident tissue damage and cellular infiltrate. One pathway of cytokine production is the processing of antigens by APCs, presented to T-helper cells and by monocytes through the use of pattern recognition receptors that recognize components of pathogens but not host cell [99]. One such receptor is the lipopolysaccharide (LPS) receptor, which

is responsible for the identification of pathogens from nonpathogenic proteins to which the immune system may become exposed to. The predominant cytokines are tumor necrosis factor (TNF), interferons, and several members of the interleukin (IL) family such as IL-1, IL-6, IL-8, IL-12, IL-15, IL-18, and IL-23 [99, 100].

The role of chemokines is to control cellular migration to areas of injury or infections. There are three subfamilies of chemokines, CXC, CC, and CX3C [99, 101]. Specific chemokines bind to specific receptors, with little to no cross-subfamily receptor interaction. Chemokine receptors are located on the cell membrane, contain a seven-transmembrane motif, and have chemokine specificity. These receptors are CXCR1 to 6, CCR1 to 11, and CX3CR1 [99, 102].

Acute and Chronic Inflammation

The basic level of the immune response is the acute immune response, which is triggered by tissue injury or infection and involves the delivery of leukocytes to the site of injury [103]. Acute inflammation, characterized by the activation of toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization domain protein)-like receptors (NLRs) triggered by microbial infections [104], are recognized by tissue resident macrophages and mast cells, leads to the production of inflammatory mediators (cyto/chemokines) which executes a local inflammatory effect. The primary leukocytes for acute

inflammatory responses are neutrophils [105]. Neutrophils kill the invading agent by releasing their granules which have toxic contents [106, 107]. The release of these granules does not distinguish between host and microbial agents, therefore surrounding tissue damage is unavoidable [106, 107]. Resolution of acute inflammation is controlled by tissue-resident and recruited macrophages [108] that play a crucial role in the initiation of wound healing/tissue repair [108, 109].

If the pathogen is not eliminated by the acute inflammatory response, the immune response changes to a chronic inflammatory response, with a switch from neutrophils to macrophage and T-cell infiltrates [103, 110]. The characteristics of this chronic inflammatory response are dependent on either the class of T-cells present, a persistent pathogen, or tissue damage from autoimmune responses. If the chronic inflammatory response fails to eliminate the pathogen, then a granuloma is formed [103, 111].

Proinflammatory cytokines are responsible for increasing the inflammatory response while anti-inflammatory cytokines reduces this response. Chronic inflammation has been associated with many different diseases such as cancer, diabetes, cardiovascular disorders, neurological, and pulmonary diseases [112-116]. Proinflammatory cytokines, like chemokines, are known to cause chronic inflammation. Several proinflammatory genes, for example, TNF and members of its superfamily, IL-1α, IL-1β, IL-6, IL-8, IL-18, chemokines, VEGF, MMP-9, and COX-2, play critical roles in the control of apoptosis, angiogenesis,

proliferation, invasion, and metastasis. Overexpression of transcription factors like NF-κB, which becomes constitutively active, is principally responsible for the expression of these proinflammatory genes [117-119].

Nuclear Factor kappa B

NF-κB was first discovered and found to play a role in the immune response as a constitutively nuclear transcription factor in mature B cells that are bound to an element in the kappa immunoglobin light chain enhancer [120]. Shortly afterwards, the same authors, found that NF-κB consisted of a complex of two subunits with molecular weights of 50 kD (p50) and 65 kD (p65), and was present in most cell types in an inactive cytoplasmic form bound to an inhibitor protein termed IκB[121]. Five members of the mammalian NF-κB/Rel proteins have been identified, c-Rel, NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65 (RelA), and RelB [122]. These are characterized by the Rel homology domain (RHD), and an N-terminal region. These proteins share a RHD, which mediates DNA binding, dimerization, and interactions with specific inhibitory factors, the IκBs, which retain NF-κB dimers in the cytoplasm [123]. Two of the proteins, NF-κB1 (p105) and NF-κB2 (p100), contain multiple copies of the so-called ankyrin repeat at their C-termini. Processing of these proteins leads to the production of the p50 and p52 subunits.

Activation of NF-κB is mediated by various receptors located on the extra and intracellular membrane. Significant knowledge about NF-κB activation came from studying the activation of receptors in the IL-1 and TNF-α class. Cytokines such as IL-1β, TNF-α, can act in a paracrine as well as an autocrine manner to activate the NF-κB activity through their cognate receptors on the cell surface. Toll like receptors (TLRs) belonging to the IL receptor family are a group of membrane anchored receptors that activate NF-κB in response to specific pathogen associated molecular patterns (PAMPs) and many pro-inflammatory cytokines [124-126].

NF-κB activation is a tightly controlled process and regulates the proteolysis of inhibitory of NF-κB activity (IκB) proteins [127]. After stimulation of receptors that have PAMPs or damage-associated molecular pattern molecules (DAMPs), a rapid signal transduction process occurs that leads to the activation of IκB kinase (IKK). Activated IKK phosphorylates IκBα, predominantly via the action of IKKβ, triggering its polyubiquitination and proteasomal degradation and inducing the nuclear translocation of associated NF-κB subunits. NF-κB complexes are capable of shuttling between the nucleus and the cytoplasm, and therefore, masking of the p65 nuclear localization signal (NLS), combined with the effect of IκB nuclear export signal, results in steady-state cytoplasmic

localization of NF-κB dimers, thus preventing DNA binding. Proteasomal degradation of IκBα induces nuclear localization of NF-κB [123, 128].

NF-κB is one of the first lines of defense against threats to the health of the organism. Activation of NF-κB has been reported to occur rapidly in response to an extremely wide range of stimuli, including cytokines, growth factors, bacterial products, viral infection, receptor ligands, and some pharmaceutical drugs and chemicals [129]. The primary targets of activation of NF-κB are the IκB proteins. IκB proteins contain three family members, α, β and y, which all contain two conserved serine residues within their N-terminal domain, phosphorylation of which, in response to most activators, targets them for polyubiquitination and subsequent degradation by the 26S proteasome [130]. The IκB proteins are targeted by the IKK complex. IKK contains three family members α(1), β(2), and γ (also known as NEMO or IKKAP1) [84]. The importance of NF-κB was demonstrated using p65, IKK2, or NEMO knockout experiments in mice. p65 null mice were embryonically lethal as a result of extensive liver apoptosis and cells derived from these mice show enhanced sensitivity to TNF-induced cell death [131]. IKK2 and NEMO [132, 133] null mice also die of extensive liver apoptosis, and cells derived from these animals show profound defects in NF-κB activation.

NF-κB can be activated by the IKK complexes. IKK kinases include members of the mitogen-activated protein kinase kinase (MAPKK) family, such as NF-κB-inducing kinase (NIK) and mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1), 2 and 3[130], members of the atypical protein kinase C family [134], and Akt/protein kinase B [135, 136]. Transcription of NF-κB target genes takes place when it translocates to the nucleus (Figure 5), and gains access to the promoters and enhancers of specific genes. NF-κB response and expression occurs in virtually all cell types and responds to a variety of activators, and therefore the genes activated by NF-κB will also vary depending on cellular location [137]. p65 transcriptional activity has been shown to interact with components of the TFIID complex and TFIIB. p65 also recruits histone acetyltransferase activity to promoters by interacting with p300 and CREB-binding protein (CBP) [137, 138]. The interaction of p300 and CBP provides another indirect mechanism of regulating p65 because these coactivators are regulated by various signaling pathways and the cell cycle [139, 140].

NF-κB plays a crucial role in many inflammatory diseases (Table 1), Alzheimer's disease, and cancer [24, 25, 141]. In normal cells, many negative-feedback loops exist to ensure that activation of NF-κB, particularly p65-containing complexes is self-limiting. One example is the expression of IκBα which is induced by the activation of NF-κB that allows for its resynthesis after

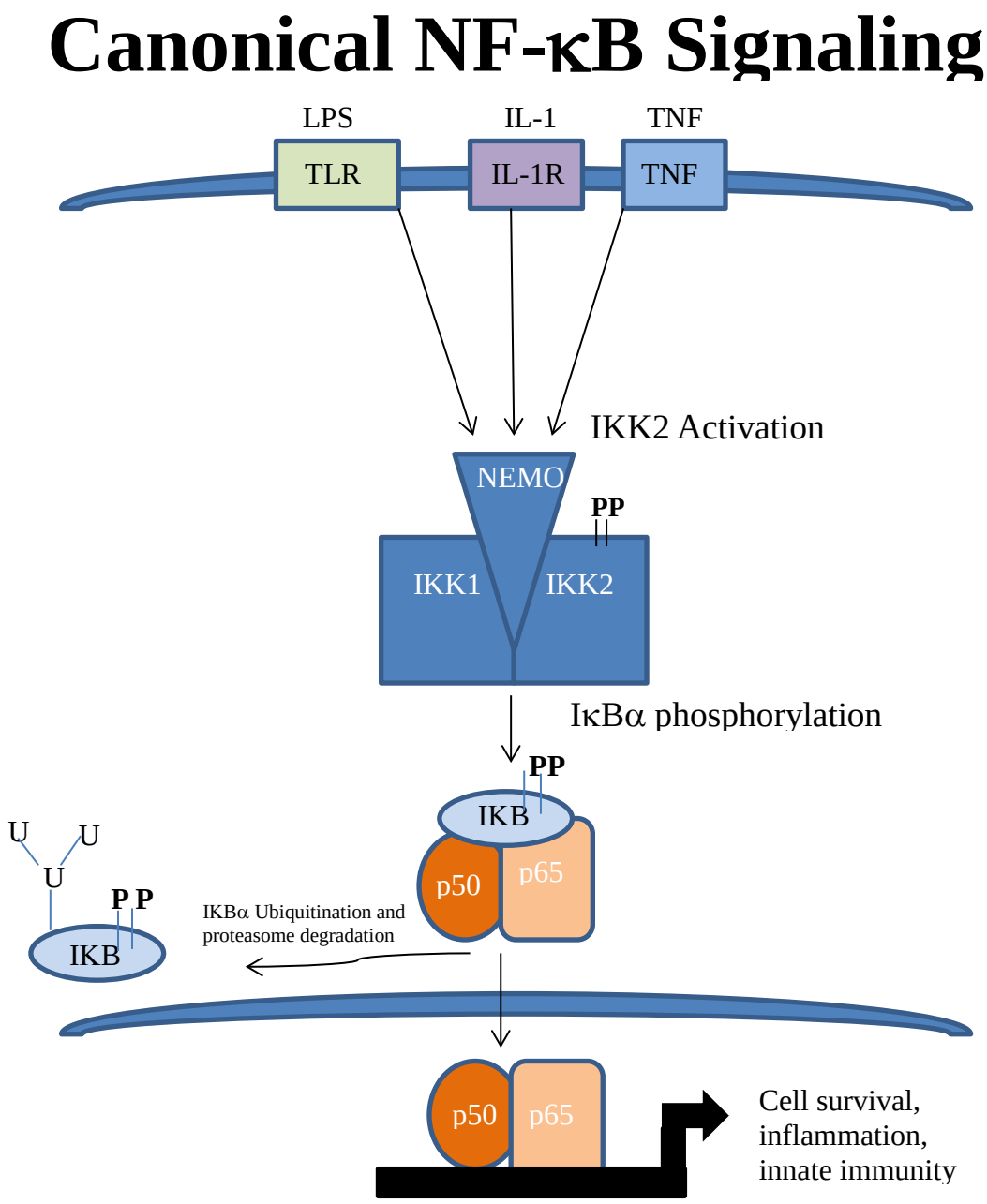


Figure 5: Canonical NF-κB Signaling

Type of NF-κB alteration	Disease
Mutation or truncation of IκB	Hodgkin's disease (IκBα and IκBϵ)
Constitutive IκB kinase activity	Hodgkin's disease
	Childhood acute lymphoblastic leukemia
NF-κB gene rearrangement, amplification, or overexpression	Various lymphomas, myelomas and leukemias
	Various carcinomas and adenocarcinomas
Other cases of aberrant nuclear NF-κB activity	Rheumatoid arthritis
	Asthma
	Atherosclerosis
	Alzheimer's disease
	Various carcinomas
	Melanoma
Impaired NF-κB activation	Ataxia telangiectasia
	Systemic lupus erythematosus

Table 1: Some of the Diseases Associated with Abnormal NF-κB Activity

degradation due to phosphorylation by IKK2, [142] and IκBα contains nuclear localization and export sequences [143]. Another intracellular mechanism for NF-κB regulation is the A20 protein, which is induced by NF-κB and also feeds back to inhibit it [144]. Extracellular mechanisms also exist to inhibit NF-κB, for example IL-10 [145] and cyclooxygenase 2 (COX-2) [146]. The anti-inflammatory cytokine IL-10 has been shown to inhibit IKK activity and NF-κB DNA binding and COX-2 activity, resulting in the synthesis of cyclopentanone prostaglandins, which have been shown to inhibit IKK activity [146].

NF-κB can also contribute to tumorigenesis by regulating apoptosis and cellular proliferation by providing tumors its anti-apoptotic signals [131, 147, 148]. In this study we investigate the association of immune inflammation and AR expression in human BPH samples. Wu et al showed that the total prostate volume was significantly higher in specimens with infiltrating inflammatory cells including B or T lymphocytes than in those without, suggesting that the immune inflammatory process may contribute to development of BPH [149]. In this study, we will look at how chronic inflammation influences AR and ARv expression and activity.

Benign Prostatic Hyperplasia

BPH is the most common urologic disease in men over the age of fifty [1]. Men diagnosed with BPH usually present with lower urinary tract symptoms (LUTS). LUTS can be treated either with medication or surgery for severe LUTS [150-153]. BPH is characterized by a low mortality rate and high morbidity and is considered an important health issue [154] due to its high cost to manage. BPH can be divided into two phases: pathological and clinical. The pathological phase is asymptomatic and is associated with nodular hyperplasia of the prostate [155]. The clinical phase occurs when the enlarged prostate compresses the urethra, which results in the resistance of urine flow. BPH is often associated with inflammation and consists of two components: dynamic and static [156]. The dynamic component affects the stromal tissues and is due to tension in smooth muscle within the prostate. Alpha-1 adrenergic receptors mediate the dynamic process [157-162]. The static phase involves the epithelium which is affected by androgen induced proliferation [163]. The symptoms of the clinical phase lead to terminal dribbling, feeling of incomplete emptying of the bladder, anxiety, sleep disturbance and sexual dysfunction, which has a negative impact on the quality of life (QoL) [164-166].

The origin of BPH is not clearly defined and several hypotheses have been proposed to explain the causes of its development. The first is based on the role of androgens and growth factors. Prostate cells are able to convert about 90% of T to

DHT using the enzyme 5-AR. DHT binds to AR with higher affinity than T, and it appears to act directly by stimulating protein synthesis, differentiation and prostate cell growth [167, 168]. The binding of DHT to its receptor further stimulates the transcription and translation of specific DNA segments, coding for growth factors (e.g., epidermal growth factor: EGF; insulin like growth factor: IGF), leading to abnormal prostate cell proliferation [166]. The second idea about BPH development is based on the presence of a small percentage of androgen-independent prostate cells that can self-renew in androgen-deficient conditions [155]. The third concept concerns the interactions between stroma and epithelium. Both the epithelium and stroma are able to convert T into DHT, allowing the production of various growth factors (fibroblast growth factor 2: FGF-2; transforming growth factor beta: TGFβ), responsible for proliferation, apoptosis and secretory activities of both stromal and epithelial tissues [155, 169].

BPH is not only viewed as a hydraulic problem but also as a metabolic problem. Recent studies have provided striking evidence that metabolic syndrome (MetS) and/or its individual components help drive the development of BPH [170]. Links between BPH and type 2 diabetes mellitus (T2DM) have been noted as far back as 1968 [171, 172] and recent reports have linked T2DM and hyperinsulinemia as potential risk factors of BPH [173-175]. In worldwide studies, obesity, and more importantly visceral obesity, was found to be comorbid with BPH [176-179] and a recent meta-analysis reported a positive association

between body mass index (BMI) and BPH [180]. It can be concluded that each individual factor of MetS can be associated with BPH/LUTS progression. Hammarsten et. al. investigated the relationship between prostate volume and individual MetS components in roughly 160 men with BPH. They demonstrated that T2DM, hypertension, and obesity were all risk factors for BPH development [181].

Treatment of BPH has evolved considerably over the last thirty years. Prior to the early 1990s, symptomatic BPH was commonly treated surgically by transurethral prostatectomy (TURP). Surgery declined significantly in the 1990's due to medical therapy using non-selective α-blockers. Around this time, 5α-reductase inhibitors (5ARIs) were also introduced and combination therapies of α-blockers and 5ARIs have now become safe and effective treatments for many men with BPH [182].

The current therapeutic approaches for the treatment of BPH consist of: watchful waiting, pharmacological therapy, and surgery. The choice of treatment should be decided together with the patient and should be individualized, according to his personal preference and disease severity [183]. The main aim is to relieve symptoms of BPH and to improve patient's QoL.

Watchful waiting (i.e., periodic clinical visitation) and changes in lifestyle are recommended for patients with mild symptoms who are not at risk of acute urinary retention [184]. In the case of worsening of symptoms pharmacological

therapies are proposed. The two main classes of drugs are α1-antagonists (α-blockers - prazosin, doxazosin, terazosin, alfuzosin, tamsulosin, silodosin) and 5ARI (Finasteride, Dutasteride), often effectively used in combination as a recommended option for the treatment of patients at risk of BPH progression.

The target of α-blockers is the dynamic component (muscle tension) of LUTS and is considered as the mainstay of therapy [185]. α-blockers improve LUTS by inhibiting the effects of the α-adrenoreceptors of the prostate. There are two classes of α-receptors (α1 and α2) and three subtypes of α1-receptor (1a, 1b, and 1d). The α1a receptors are the main receptors located in the stroma of the prostate and represent the major target for medical therapy [186]. α-blockers increase the volume and the stream force, improving symptoms and consequently patient's QoL.

5ARIs reduce the static component (increased prostate tissue mass) causing androgen reduction. 5ARIs inhibit the enzyme, 5α-reductase, from converting T to DHT. This results in apoptosis and shrinkage of the prostate. 5ARIs are more effective in patients with a significant enlargement of the prostate gland [187]. In the worst cases of symptomatic BPH, invasive surgery (open prostatectomy: OP) or minimally invasive procedures (transurethral resection - TURP, laser ablation endoscopy) may be required. The type of surgery is based on the size of the prostate, the presence of painful symptoms, and/or other

concomitant diseases. OP is considered as the most effective as well as the most invasive procedure [163]. Holmium laser enucleation of the prostate (HoLEP) is another treatment for symptomatic BPH. It involves using a laser to precisely remove the whole obstructive portion of the prostate in fragments. It has been to shown to be just as effective at reducing the symptoms of BPH as TURP [188] and has been shown to be an effective alternative [189].

TURP is the most common surgical method for the treatment of BPH. It has been considered the "gold standard" in patients with BPH because this surgical approach allows an immediate removal of the obstruction and long-term improvement of the symptoms. However, TURP can lead to complications (e.g. incontinence, erectile dysfunction, bleeding) and many of the patients for this type of surgery are older (<70) [190-192]. α-blockers are frequently prescribed for BPH treatment and have a quick action of onset, usually within 3 to 5 days [193]. However these drugs alone fail to shrink the prostate and are often insufficient to eliminate symptoms [194]. 5ARIs have greater effect in reducing prostate volume and data indicate the combination of a 5ARI and α-blocker leads to the best symptomatic response [182, 195, 196].

The Medical Therapies of Prostate Symptoms (MTOPS) trial [182], which was a long term, double blind trial (mean follow up 4.5yrs) involving roughly 3000 men, compared the effects of placebo, doxazosin (α-blocker), finasteride (5ARI) and combination therapy on measures of the clinical progression of BPH.

The trial determined that roughly one third of patients failed combination therapy, had higher inflammation, and progressed to surgery. This finding was confirmed in a subgroup analysis of 8,224 men in the Reduction by Dutasteride of Prostate Cancer Events (REDUCE) trial, which indicated that histologic inflammation was present in more than 78% men and that the severity of LUTS and the intensity of inflammation were related [197]. Further, inflammation in the prostate increased significantly with the increased prostate volume and age [198-200]. In another study, it was demonstrated that T-cell-derived cytokines induced hyper-proliferation of BPH-derived stromal cells [200, 201]. Kramer et al (2003) demonstrated that, a similar pattern of cytokines seen in autoimmunity and chronic inflammatory diseases are present in BPH tissue. This study will focus on how chronic inflammation can contribute to therapeutic failure of a 5ARI. The clinical data cited above suggest that androgen/AR signaling as well as an inflammatory response play a key role in the development of BPH and that targeting either the inflammatory response and/or androgen/AR signaling could be a therapeutic approach to BPH.

The prostate contains several classes of immunocompetent cells (lymphocytes, macrophages, and granulocytes) that are physiologically resident and termed human prostate-associated lymphoid tissue (PALT). PALT activates the acute inflammatory response and it is believed that this is succeeded by chronic inflammation with the help of hormonal/metabolic derangements, or by

exposure to other environmental and dietary factors [202]. Activated PALT stimulates and recruits the proliferation of other immunocompetent cells and leads to an up-regulation of proinflammatory cytokines and chemokines [203]. It has been shown that prostatic stromal cells act as targets of bacterial or viral TLR agonist and later progress as antigen-presenting cells (APC), and play a crucial role in the induction of the inflammatory process [204]. TLRs increase the recruitment of inflammatory cells and also influence the growth of prostatic cells [205]. In addition, TLR activation leads to the production of proinflammatory cytokines (IL-6) and chemokines (IL-8 and CXCL10) that are capable of recruiting leukocytes and neutrophils and promoting cell hyperplasia [204, 206, 207].

Chronic inflammation causes hypoxia which is followed by the release of reactive oxygen species (ROS) and nitric oxide. This oxidative stress triggers the release of arachidonic acid, which is converted to prostaglandins by cyclooxygenases. These prostaglandins play a role in the regulation of cell proliferation [208]. Hypoxia also induces the release of VEGF, which stimulates neoangiogenesis and fibroblast differentiation, which helps promote BPH [209]. Chronic inflammation has, as a consequence, significant tissue damage, followed by slow wound healing and scarring, which is the initiator of BPH-specific nodules. Chronic inflammation of the prostate correlates with BPH and LUTS [197, 198, 201].

Prostatitis

An inflammatory condition of the prostate is known as prostatitis and was recognized and defined by the National Institutes of Health (NIH) [3-6, 210] in 1999. Currently, prostatitis remains more prevalent in men less than 50 years old and NIH classifies prostatitis in four categories: acute (ABP) and chronic bacterial prostatitis (CBPr), chronic pelvic pain syndrome (CPPS) formerly known as chronic nonbacterial prostatitis, and asymptomatic inflammatory prostatitis [210]. ABP is the least common of the four types. ABP is the easiest form to diagnose and treat. However if ABP is left untreated can be a life-threatening condition because bacteria can overwhelm the body's defense mechanisms and lead to sepsis, which is a life-threatening emergency [4]. CBPr is the swelling and inflammation of the prostate gland that develops slowly. Chronic prostatitis is the most common form, and is not age related. CPPS is differentiated from CBPr by bacterial cultures, because CPPS is negative and CBPr is positive. CPPS is divided into two classes: the presence or absence of inflammation. In the inflammatory form, urine, semen, and prostatic fluid is positive for inflammatory cells but not bacteria and in the non-inflammatory form there are no inflammatory cells present in this fluid [4, 211, 212]. Asymptomatic inflammatory prostatitis is defined as inflammatory cells present in their prostate fluid and semen without pain or discomfort [4]. Although a clear link between prostatitis and development

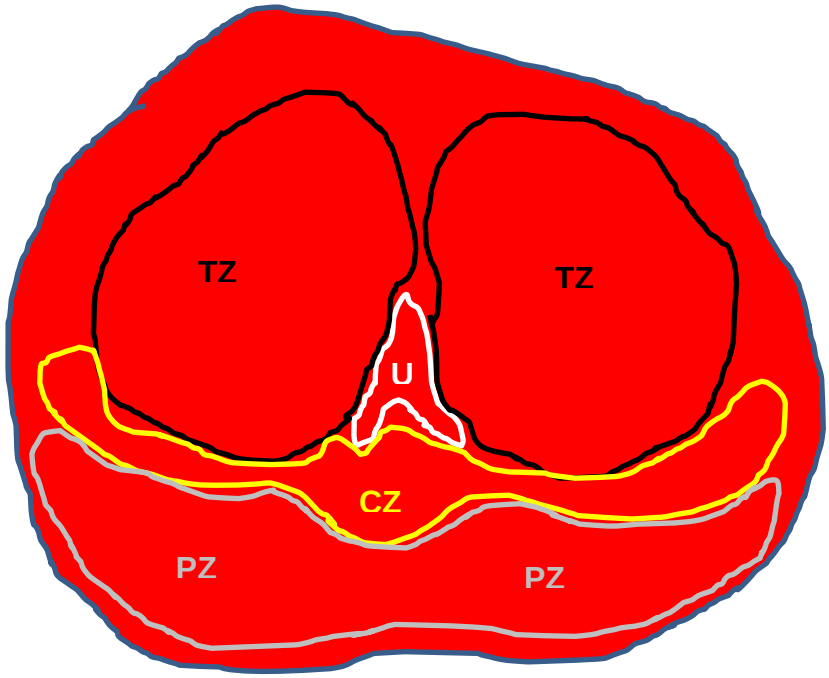
of BPH has not been definitively shown, prostatitis has been significantly associated with BPH and PCa [213, 214].

Benign Prostatic Hyperplasia and Prostate Cancer

BPH and PCa are both chronic diseases with long evolution, characterized by slow progression and early precursor lesions. The precursor of BPH is the micronodular hyperplasia of the transition zone, which progress to macroscopic enlargement and is accompanied by clinical symptoms late in progression. PCa develops from early precancerous focal lesions, most commonly situated in the peripheral zone, at the dorsal and dorso-lateral sides of the prostate [215] (Figure 6). Despites these differences, BPH and PCa share common traits, they are androgen dependent [215] and both are frequently associated with chronic inflammation [197]. One of the main questions of the study is to determine if there is a relationship between chronic inflammation and BPH progression.

Summary and Dissertation goals

People are living longer and living healthier lives. According to the World Health Organization (WHO), 601 million people worldwide were older than 65 in 2015. We must proactively face the health issues of the elderly because by 2030 the projected number of people above the age of 65 will be 1 billion. BPH/LUTS represents a significant, bothersome disease among aging men. Historically, BPH was considered a “normal” consequence of the aging process, and the effects of



U: Urethra
TZ: Transitional Zone
CZ: Central Zone
PZ: Peripheral Zone

Figure 6: Zones of the Prostate. PZ: The sub-capsular portion of the posterior aspect of the prostate gland that surrounds the distal urethra. CZ: This zone surrounds the ejaculatory ducts. TZ: The transition zone surrounds the proximal urethra and is the region of the prostate gland that grows throughout life and is responsible for the disease of benign prostatic hyperplasia.

symptomatic BPH where only dealt with by medical or surgical intervention. BPH is now considered an inevitable disease of the aging population [216]. BPH is complex disorder that involves a metabolic component that may begin early in life. Evidence suggests that several modifiable factors play a role in the progression of BPH such as MetS, obesity, and prostate inflammation. The relationships between MetS and prostate inflammation are likely to be the same in young and old men, but chronic exposure to elevated inflammation may also contribute to the disease. Preventing the development of the disease even from the asymptomatic phase should be the basis for designing a resilient program for BPH therapy.

In men with mild to moderate symptoms, lifestyle approaches may be sufficient to reduce BPH progression. However for those in whom lifestyle changes are unsuccessful, combinational therapy of an α-blocker and 5ARI are used to improve symptoms. Additional surgical and minimally invasive procedures provide a larger and often durable response. Surgical interventions are mainly indicated for men with more severe symptoms or those who do not adequately respond to combination treatment.

The goal of this dissertation is to better understand how and why inflammation controls progression of BPH and why increased inflammation in BPH is also associated with therapeutic failure. Many studies have focused on how inflammation controls cancer progression and how ARv contributes to

castrate resistant prostate cancer. However, this is the first study to date to investigate how NF-κB can control AR and ARv expression in BPH. We hypothesize that chronic activation of NF-κB results in a profile of epithelial and stromal changes that contribute to focal benign glandular expansion. This thesis work examines how NF-κB can induce AR and ARv expression, NF-κB is associated with increased symptomatic BPH, and NF-κB induces resistance to 5ARIs. In chapter 3 we explore *in vitro,* how chronic expression of NF-κB induces AR-V7 expression and how this expression is responsible for 5ARI failure. Chapter 4 evaluates, *in vivo,* how chronic expression of NF-κB can induce 5ARI failure and induce development of BPH. Our results show that chronic activation of NF-κB induces growth and proliferation of benign prostatic epithelial cells, induce AR-FL and AR-V7 expression and that this expression is responsible for 5ARI therapy failure. This suggests that inhibiting NF-κB and thus AR-V7, in combination with current co-therapies, could be a possible strategy to suppress the development of BPH.

CHAPTER **II**

Material and Methods

Cells, reagents, and antibodies

BHPrS1 human prostate stromal cells [217] were cultured in RPMI 1640 (Gibco, Grand Island, NY), 5% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (Gibco). NHPrE1 prostate epithelial cells [218] were cultured in DMEM/F12 (Gibco) containing either 5% complete fetal bovine serum (FBS) or 5% dextran-coated charcoal-stripped FBS (CS) (Atlanta Biologicals), 1% penicillin/streptomycin (Gibco), 10ng/ml epidermal growth factor (Sigma Aldrich, St. Louis, MO), 1% insulin-transferrin-selenium (ITS) (Gibco), and 0.4% bovine pituitary extract (Atlanta Biologicals). Primary antibodies against p65, acetyl-p65 (K310) were purchased from Cell Signaling (Beverly, MA), AR (N-20 and C-19) from Santa Cruz Biotechnology (Santa Cruz, CA), AR-V7 from Precision Antibody (Western blotting) (Columbia, MD) and Abcam (IHC)(Cambridge, MA), and phospho-p65 (S276) from Abcam. 5α-reductase 1 and 2 from Santa Cruz Biotechnology, and 5α-reductase 3 from Abcam (Cambridge, MA). Secondary antibodies were purchased from GE Healthcare (Pittsburg, PA).

Matrigel culture

Single cell suspensions of NHPrE1 cells in monolayer culture medium containing 2% growth factor reduced Matrigel (BD Bioscience, Oxford, UK) were seeded at 2000 cells/well in 48 well plate containing 200 µL of medium containing 4% growth factor reduced Matrigel, 5% charcoal- stripped FBS (CS-FBS in DMEM/F12 medium with or without DHT (10^{-8} M). Cells were incubated at 37°C with replacement of the growth medium containing 4% growth factor reduced Matrigel with/without DHT at day 3. Medium was then changed every 2 days. NHPrE1 cells were extracted from the Matrigel using cell recovery solution (BD Biosciences). Extracted NHPrE1 cells were then dissociated to a single cell suspension using enzymatic disaggregation (0.25% Trypsin EDTA, Sigma).

Viral transduction

The pCFG5-IEGZ retroviral vector, gift from Martin Leverkus (Heidelberg, Germany) containing complementary DNA inserts of IKK2-Empty Vector (EV), IKK2-constitutively active (EE), IKK2-kinase dead (KD) (gifts from Dr. Timothy Blackwell, Vanderbilt), AR-FL and V7 (gift from Dr. Ganesh Raj, University of Texas Southwestern) were used for infection of NHPrE1 and BHPrS1 prostatic epithelial and stromal cells as previously described [219-221]. Briefly, the amphotropic producer cell line φNX was transfected with 10µg of the retroviral vectors by calcium phosphate precipitation. To select transfected producer cells,

0.5μg/ml zeocin (IKK2) (Life Technologies) or blasticidin (AR-FL, V7) (Life Technologies) was added to the culture medium for 3 days to obtain >95% green fluorescent protein-positive producer cells. Cell culture supernatants containing viral particles were generated by incubation of producer cells with DMEM containing 10% FBS overnight. Following filtration at 45μm (Millipore Billerica, MA), culture supernatant was added to NHPrE1 or BHPrS1 cells seeded in six-well plates 24 hours earlier in the presence of 1μg/ml polybrene. After 5 days recovery of bulk-infected cultures, FACS analysis for green fluorescent protein expression and western blot analysis was performed on expanded polyclonal cells to confirm ectopic expression of the respective molecules. Cell lines used were the IKK2-empty vector (NHPrE1-EV and BHPrS1-EV), IKK2-kinase dead (NHPrE1-KD andBHPrS1-KD), or IKK2-constitutively active, (NHPrE1-EE and BHPrS1-EE).

Transient transfection assay

The NGL vector [a NF-κB responsive reporter vector that has *luciferase* and green fluorescent protein (*GFP*) reporter genes [222]] was a gift from Dr. Timothy Blackwell (Vanderbilt), ARR_2PB-Luc vector [an AR-responsive reporter vector that does not respond directly to NF-κB [223]], and pGL3-PSA-PSAE1-Luc reporters [224] along with p65 and AR siRNA purchased from Cell Signaling (Beverly, MA), Life Technologies (Carlsbad, CA), and Santa Cruz Biotechnology

(Santa Cruz, CA) respectively. The transfection efficiency was determined by co-transfecting pRL-CMV containing the Renilla luciferase reporter gene (Promega, Madison, WI). Luciferase activity was determined using the Promega Corp luciferase assay system 24 h after transfection. The values plotted represent the mean of at least three individual samples ± SD.

Cell viability assay

Cell viability assay was performed using CellTiter-Glo (Promega) according to manufacturer's instructions. NHPrE1 cells were seeded at 2000 cells/well in a 96-well plate, grown over 5 and 7 days in the presence and absence of testosterone and Finasteride. CellTiter-Glo measurements were taken at several time points to track cell survival.

Tissue Recombinants and Subrenal Capsule Xenografting

Rat UGM, the inductive mesenchymal cells surrounding the epithelial core of the urogenital sinus, was prepared from E18.5 embryonic fetuses as previously described [225-227]. To prepare tissue recombinants, rat UGM was mixed with human prostate epithelial cells as discussed in the text. The cell mixture was pelleted and resuspended in 50 μl rat-tail collagen (pretitrated to pH 7.4). After polymerization, the collagen was overlaid with growth medium. After incubation overnight at 37°C, the tissue recombinants were grafted under the renal capsule of

intact Athymic Nude-Foxn1nu mouse. Hosts were euthanized at eight weeks after grafting as noted in Results. A portion of these grafts were flash frozen, subjected to mortar and pestle disassociation and then subject to Trizol treatment to recover RNA. We then performed reverse transcription to obtain cDNA

Determination of Androgen Synthesis by Cells

NHPrE1-AR-FL cells were grown in CS media for 5 days and cell pellet and media samples were collected in triplicates and analyzed in 1 analytical run for 5 androgens [testosterone (T), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), androstenedione (ASD), and androsterone (AND)] using a modification of a validated high pressure liquid chromatographic (HPLC) assay with tandem mass spectrometric detection (LC/MS/MS) performed Bioanalytics, Metabolomics and Pharmacokinetics (BMPK) Shared Resource as previously described [228].

Prostate Tissue

As part of an Institutional Review Board (IRB)-approved protocol, fresh prostate specimens were collected from 46 consented patients undergoing holmium laser enucleation of the transition zone of the prostate (HoLEP) for symptomatic BPH with LUTS, referred to here as ‘Surgical BPH’. In the majority of cases, these patients had failed medical therapy with an α-blocker, a 5ARI, or both (see Table

2). As a control, a pathologist resected transition zone nodules from 53 patients undergoing radical prostatectomy for small volume, low risk, clinically localized peripheral zone prostate cancer (referred to here as 'Incidental BPH') at Vanderbilt University Medical Center (Nashville, TN) as previously described [200]. Low risk prostate cancer was defined as clinical stage T1c, pathologic stage T2a, pre-operative PSA < 10 ng/ml, Gleason Score 3+3=6 or lower, and cancer present in less than 5% of the specimen isolated to the peripheral zone. For all patients, demographic and clinical data was collected including medication history, past medical history (including hypertension, hypercholesterolemia, and NIDDM), demographic and anthropomorphic measurements such as height and weight, and LUTS using the self-reported American Urologic Association Symptom Score (AUASS).

Tissue processing and pathology

After gross pathological examination, all prostate samples used for this study were stored at 4°C and processed within 24 hours. Processing of samples involved flash freezing in liquid nitrogen followed by storage at -80°C until use, as well formalin fixation for paraffin embedding. Samples were reviewed by a pathologist to confirm histologic findings and to exclude those with any foci of cancer.

Quantitative-real-time PCR, Western blot and IHC

For quantitative real-time PCR (qPCR) 50mg flash-frozen tissue was ground using a mortar and pestle in liquid nitrogen and RNA was extracted with Trizol (Ambion) from 46 surgical BPH and 53 incidental BPH specimens. Subsequently, 500ng RNA was reverse transcribed into cDNA using RT^2 First Strand Kit (Qiagen, Valencia, CA). qPCR was performed using IQ SYBR Green Supermix (BioRad, Hercules, CA) and results were analyzed using BioRad CFX manager software. All results were calculated using ΔΔCt analysis and normalized to GAPDH expression. Primer sequences are listed in Table 2

For Western blotting, approximately 50mg of flash frozen human prostate tissue was ground in liquid nitrogen using a mortar and pestle. Protein was extracted with 2% SDS buffer. 30μg protein was run on pre-made 10% polyacrylamide gels (Life Technologies). Primary antibodies were incubated in 5% BSA in TBST overnight at 4°C followed by incubation in secondary antibodies and development using ECL. Membranes were stripped and re-probed with an antibody against β-actin (Sigma).

Immunohistochemistry was performed as previously described [229]. Briefly, 5μm sections were de-waxed, rehydrated and endogenous peroxidases were blocked with hydrogen peroxide. Sections were then boiled in citrate and blocked in 5% serum for 1hr. Primary antibodies were incubated overnight at 4°C at the following concentrations: p-p65-S276 (1:200), AR (N-20 1:200)(C-19

1:50), AR-V7 (1:100)(Abcam). Biotinylated anti-mouse or -rabbit secondary antibodies (DAKO Carpentaria, CA) were incubated for 60min at room temperature after slides were washed for 1hr in PBS. Slides were incubated in ABC-HRP complex (Vector Laboratories Burlingame, CA) for 30min. Bound antibodies were then visualized by incubation with 3,3' diaminobenzidine tetrahydrochloride (liquid DAB, DAKO). Slides were then rinsed in tap water, counterstained with hematoxylin, and mounted.

Immunofluorescence

Tissues were fixed with 4% paraformaldehyde, permeablized with 0.5% Triton X-100 and incubated with 1% BSA to block non-specific binding. Tissues were then incubated with rabbit anti-phospho-p65 (276) (Abcam) and mouse anti-wide-spectrum cytokeratin (DAKO) and were visualized with anti-rabbit 594-rhodamine-conjugated or anti-mouse FITC-conjugated secondary antibodies (Life Technologies) and nuclei were visualized with DAPI (Vector Laboratories).

Image analysis

Immunostained tissue images were captured using a high throughput Leica SCN400 Slide Scanner automated digital image system from Leica Microsystems. Whole slides were imaged at 20X magnification to a resolution of 0.5 µm/pixel. Tissue cores were mapped using Ariol® Review software. The numbers of

positive (brown) and negative (blue) nuclei were determined by analysis of the high-resolution images in the Ariol® software. Fluorescent, immunostained tissue slides were imaged on an Ariol SL-50 automated slide scanner (Leica Biosystems). Tissue cores were imaged at 20X magnification to a resolution of 0.323 μm/pixel. The software used in this work, CellProfiler 2.0, is an open source package for Windows (available from the Broad Institute at www.cellprofiler.org). The software uses a pipeline (available upon request from the authors) of modules designed to automatically identify, quantify, and export the area-shape measurements of cells stained for wide spectrum cytokeratin, phosphorylated p65, and DAPI (nucleus). The pipeline also saves a resultant image with each cell detected (outlined in color). Analysis included total cell count to determine NF-κB positive epithelial cells (wide spectrum cytokeratin, p65-S276 and DAPI) or NF-κB positive stromal cells (p65-S276 and DAPI).

Statistical Analysis

Chi-square and the Wilcoxon-Rank Sum tests were used for univariate comparisons of study characteristics between surgical and incidental patients. Primary analyses of differences in NF-κB, AR, or AR-V7 expression across incidental and surgical groups were performed within a linear regression model that allowed us to adjust for differences in age, body mass index (BMI), and Non-insulin dependent diabetes mellitus (NIDDM) between groups. Additional

regression analyses investigated the age- and group-adjusted associations between NF-κB, AR, and AR-V7 levels with BMI, NIDDM, and 5ARI and α-blocker use. Tissue markers were natural log transformed prior to analysis to normalize these distributions as necessary to meet model assumptions, and adjusted mean biomarker values were then back transformed such that geometric means are reported. A two-sided *p*-value of 0.05 or less was considered statistically significant. A One-Way ANOVA test was used for comparisons of characteristics between IKK2-empty vector (EV), IKK2-kinase dead (KD), and IKK2-constitutively active (EE) gene expression. A Wilcoxon-Rank Sum test was used when comparing inhibition of NF-κB in EE to EV. Correlation between AR-FL, AR-V7, and AUASS and TRUS volume was evaluated by the determination of the Spearman correlation coefficient (GraphPad Prism).

	All pts (n=98)		Incidental (n=53)		Surgical (n=45)		
	Median	25^{th}, 75^{th}	Median	25^{th}, 75^{th}	Median	25^{th}, 75^{th}	p
Age (years)	63.5	58.0, 70.0	60.0	55.0, 65.0	67.0	62.0, 72.0	**<0.01**
BMI	28.6	25.4, 31.2	28.4	25.6, 31.9	28.7	25.3, 30.3	0.57
AUASS	12	5, 21	7	3, 12	20	15, 25	**<0.01**
PSA (ng/ml)	5.1	4.2, 7.3	4.8	4.3, 6.5	5.6	3.8, 9.9	0.62
Prostate Volume	60	41, 98	43	30, 57	94	70, 130	<**0.01**
NF-κB-Stromal	5.3	2.5, 10.7	4.6	1.7, 10.0	6.0	4.1, 10.8	**0.04**
NF-κB-Epithelial	13.4	10.4, 18.7	11.9	9.3, 14.3	16.6	13.2, 23.7	**<0.01**
AR-FL mRNA	13.0	5.4, 18.4	11.6	5.4, 17.3	14.7	5.9, 19.7	0.66
AR-V7 mRNA	86.5	15.3, 205.6	32.4	5.1, 106.5	176.5	62.0, 425.2	**<0.01**
	n	%	n	%	n	%	p
BPH TX							
α blocker only	16	16.8%	5	9.6%	11	25.6%	**<0.01**
ARI5 only	6	6.3%	3	5.8%	3	6.7%	
5ARI + α-	29	30.5%	4	7.7%	25	58.1%	
None	44	46.3%	40	76.9%	4	9.3%	
Comorbidity							
NIDDM	21	21.4%	11	20.8%	10	22.2%	0.86
BMI ≥ 30	37	37.8%	22	41.5%	15	40.5%	0.41
AUASS ≥8	64	68.1%	25	47.2%	39	95.1%	**<0.01**

Table 2: Patient medical records were used to determine Age, BMI, AUASS, PSA, Volume, BPH TX and Comorbidity. Stromal and epithelial NF-κB activation was determine by quantitated immunofluorescent protein expression. AR-FL and AR-V7 mRNA expression was determine by qPCR. One participant with missing age was dropped from all analyses. Missing data: AUASS (n=4; all in surgical group), PSA (n=13; all in Surgical group), Volume (n=17; 10 in Incidental and 7 in Surgical groups), alpha-blocker (n=1), ARI5 (n=2). TX: Treatment. p-value : two-sided Wilcoxon Rank Sums test or Chi-squares test comparing differences between Incidental and Surgical groups.

Primer set	Oligonucleotidsequence	Annealing temp. (°C)	Amplification cycles
AR^{FL} (V1 and V2)-Fw	ACATCAAGGAACTCGATCGTATCATTGC	60	55
AR^{FL} (V1 and V2)-Rev	TTGGGCACTTGCACAGAGAT		
AR-V1,V2, V3, V4-Fw (AR-P1/P2/P3-Fw)	TGTCACTATGGAGCTCTCACATGTGG	60	55
AR-V1,V2, V3, V4-Rev (AR-P1-Rev)	CACCTCTCAAATATGCTAGACGAATCTGT		
AR^{-567es}-Fw	TGCTGGACACGACAACAA	60	40
AR^{-567es}-Rev	GCAGCTCTCTCGCAATCA		
V7 (AR-P1/P2/P3-Fw)	TGTCACTATGGAGCTCTCACATGTGG	60	60
V7 (AR-P3-Rev)	CTGTGGATCAGCTACTACCTTCAGCTC		
PSA-Fw	GCAGTCTGCGGCGGTGTTCT	58	55
PSA-Rev	GCGGGTGTGGGAAGCTGTGG		
TMPRSS2-Fw	GCACAGCCCACTGTGGTCCC	58	55
TMPRSS2-Rev	CAGAGTAGGCCAGCGGCCAG		
p63-Fw	TTTGTCTGTGTGCTCTGGGA	55	55
p63-Rev	ACTGCCCTGACCCTTACATC		
GAPDH-Fw	TGCACCACCAACTGCTTAGC	55	55
GAPDH-Rev	GGCATGGACTGTGGTCATGAG		

Table 3: Primer Sequences

CHAPTER III

NF-κB and Androgen Receptor Variant Expression Correlate with Human BPH progression

Introduction

BPH is the most common urologic disease in men over the age of fifty [1]. Comorbidities of BPH include age, systemic inflammation, autoimmune and inflammatory disease, and individual components of metabolic syndrome [22, 23, 230, 231]. There are many potential etiological factors contributing to BPH pathogenesis such as disruption of growth factor and hormone signaling, inflammation, fibrosis, and sympathetic nerve activity [232-235]. Consistent with concepts discussed by other investigators [236, 237] we have recently demonstrated that gene expression profiles of advanced BPH show marked similarities with conditions such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease suggesting the possibility of an autoimmune/inflammatory component to the disease process [200].

There are two major medical approaches for patients presenting with symptoms suggestive of BPH: alpha-adrenergic receptor antagonists (α-blockers that decrease smooth muscle tone [194] and 5ARI that reduce the enzymatic conversion of testosterone to dihydrotestosterone, resulting in apoptosis and a decrease of around 25% in total prostate volume [238]. Combination treatment

with these therapies was shown to provide a significant reduction in the risk of symptomatic progression. However, nearly 20% of patients display serious adverse complications to these medications and many patients either fail to respond or become resistant over time, with 5-7% progressing to surgical intervention [239]. Given the age and comorbidity profile of this population, many of these patients are not good candidates for surgery [240]. The variability in clinical responses to existing BPH therapies highlights the need to better understand the molecular basis of BPH progression with a view to developing new therapies appropriately targeted to specific patient groups [94].

NF-κB transcription factor family regulates the expression of a large variety of genes involved in inflammatory and immune responses as well as cellular growth and development [21]. NF-κB transcription factors are activated as a response to a variety of stress signals, including cytokines and pathogens. Activation of NF-κB proteins is tightly regulated, and inappropriate activation of these signaling pathways has been linked to autoimmunity, chronic inflammation, and various cancers [24, 25]. NF-κB signaling occurs through canonical (p50/RelA) and non-canonical (p52/RelB) pathways resulting in activation of overlapping sets of downstream genes.

AR has been shown to be expressed in the form of C-terminal truncated variants (ARv) in prostate cancer [12-14, 16-18, 241]. These ARv lack the ligand-binding domain of full-length AR. ARv can be constitutively active, driving AR-

regulated transcription and promoting tumor progression, even under castrate conditions [14, 19, 20, 242]. Expression of AR variant 7 (AR-V7) in circulating prostate tumor cells has been shown to be predictive of resistance to enzalutamide (which interacts with the ligand binding domain of the AR) and abiraterone (which depletes androgen levels) [19]. Gao et al have reported that non-canonical NF-κB signaling induces the expression of ARv in prostate cancer [243]. We have reported that ARv are also induced by canonical NF-κB signaling resulting in castrate-resistant prostate cancer (CRPC) [244]. Inhibition of NF-κB expression results in ARv down-regulation and restores sensitivity of CRPC to anti-androgens [244]. Baseline prostate volume is the most reliable predictor of therapeutic failure of BPH and LUTS progression [245] and is most commonly targeted by 5ARI therapy; therefore, our goal is to understand the potential mechanisms of 5ARI resistance. Currently, there is not an established link between ARv expression and resistance to 5ARI therapy in BPH.

In this chapter, we investigated the activation of NF-κB and AR-V7 in BPH. We compared human tissue samples from patients who underwent surgery for moderate to severe BPH/LUTS to a cohort of patients with, mildly symptomatic BPH incidental to radical prostatectomy for prostate cancer. We utilized benign human prostate epithelial and stromal cells, to test the presence and consequences of NF-κB activation on AR and AR-V7. To our knowledge, this is the first study to link chronic activation of NF-κB signaling in BPH to

increased AR-V7 expression. This provides the basis for a mechanism that could explain why certain patients with BPH fail 5ARI therapy. Our previous study with CRPC demonstrated that inhibition of NF-κB and AR-V7 expression restores responsiveness to medical therapy [246], suggesting that targeting both NF-κB and AR could have an impact in reducing failure of treatment for BPH.

Results

Activation of NF-κB signaling is associated with clinical progression of BPH

Progressive BPH is commonly associated with inflammation [197], and indeed has molecular similarities to other autoimmune inflammatory conditions [200]. The NF-κB pathway is a molecular program that plays an important role in the regulation of inflammation. An unbiased high resolution scanning analysis of immunofluorescent nuclear phospho-p65 localization (indicating canonical NF-κB signaling) was performed to compare NF-κB activation in 46 clinically advanced surgical BPH specimens versus 53 incidental BPH specimens. As illustrated in a representative example in Figure 7A, and quantified in Figure 7B there is significantly higher nuclear phospho-p65 in the more advanced surgical samples compared to incidental BPH. Nuclear p65 levels were also higher within the epithelial vs. stromal compartment in both clinical groups, and the epithelial

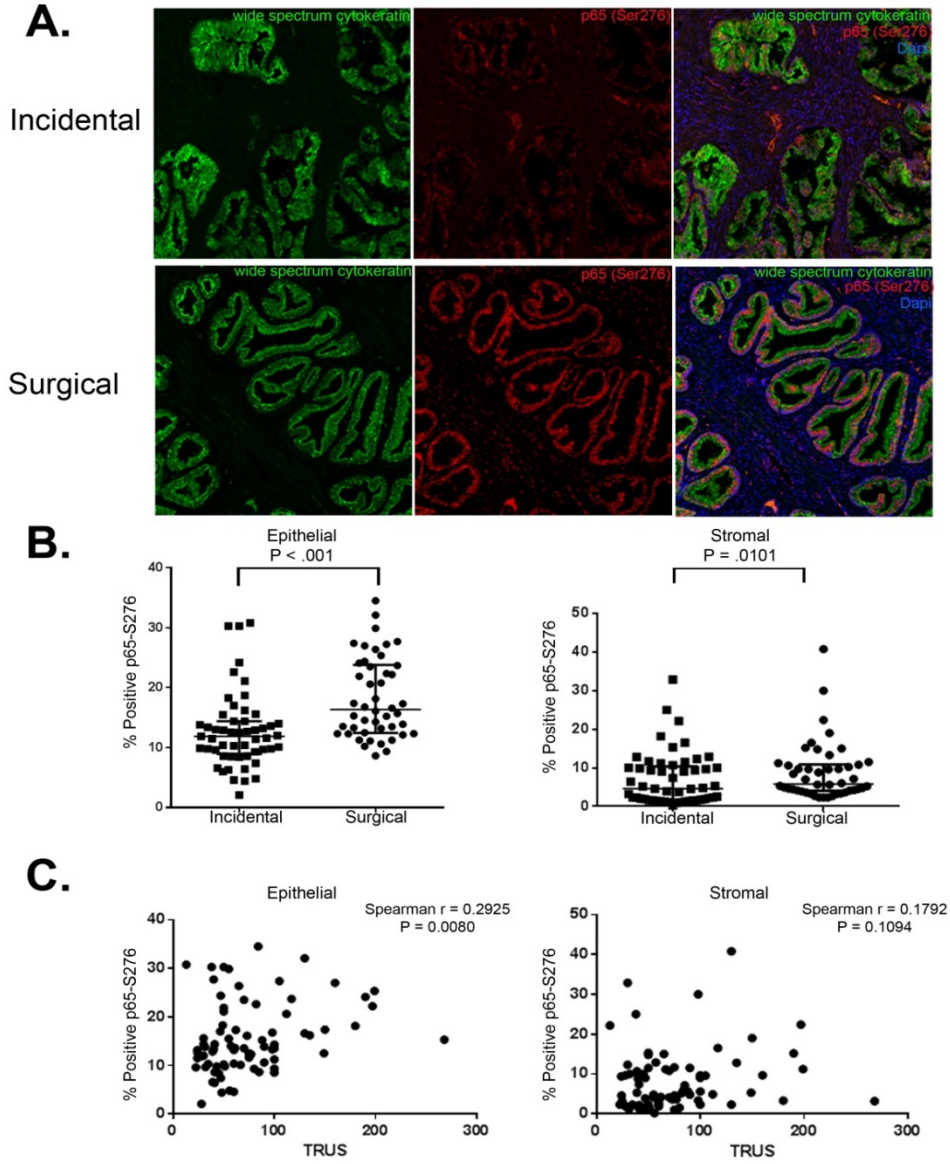


Figure 7. NF-κB expression is significantly higher in symptomatic BPH. Representative images of immunofluorescence staining for the epithelium using wide spectrum cytokeratin (green) and activated NF-κB (p65-S276 red) in 99 BPH patients. **A.** Examples of immunofluorescence for wide spectrum cytokeratin (green), p65-S276 (red), and DAPI (blue) in incidental and surgical BPH. **B.** Quantitative analysis of immunofluorescence staining of 53 incidental and 46 surgical samples for p65-S276 positivity in wide-spectrum cytokeratin-positive epithelium and wide spectrum cytokeratin-negative stroma showing a significant increase in NF-κB activation in both the epithelium and stroma of surgical versus incidental patients. **C.** Spearman's correlation coefficient analysis of NF-κB activation (p65-S276) in wide-spectrum cytokeratin-positive epithelium showing a significant correlation between NF-κB expression and TRUS volume and wide spectrum cytokeratin-negative in the stroma and TRUS volume. Bars are presented as medians, p-value: two-sided Wilcoxon Rank Sums test.

compartment in surgical BPH patients displayed the highest p65 levels ($P < 0.01$). We next investigated whether activated NF-κB expression was associated with increased LUTS. We examined all incidental and surgical BPH patients (n=99), and analyzed patients whose TRUS (TransRectal UltraSound) volume measurements were included in our data set (n=81). We looked at NF-κB activation by compartment and saw that epithelial NF-κB activation significantly correlated with TRUS volume while there was no significant correlation between stromal NF-κB activation and TRUS volume. (Figure 7C).

BPH progression is associated with increased expression of AR-V7

Recent studies have linked NF-κB activation to androgen receptor expression and in particular to the expression of AR variants, in castrate-resistant prostatic cancer [88, 243, 246]. Given that most advanced BPH patients are refractory to 5ARI therapy, we examined the expression of both full length and variant forms of the AR in surgical and incidental BPH patient samples. When compared to incidental BPH patients, surgical BPH patients were significantly older, had higher AUASS scores, larger prostate volumes, and were more likely to have taken a 5ARI or an α-blocker (Table 2).

Immunohistochemical analysis with an N-terminal AR antibody did not show differences by clinical group (Figure 8, Table 2). This was confirmed using

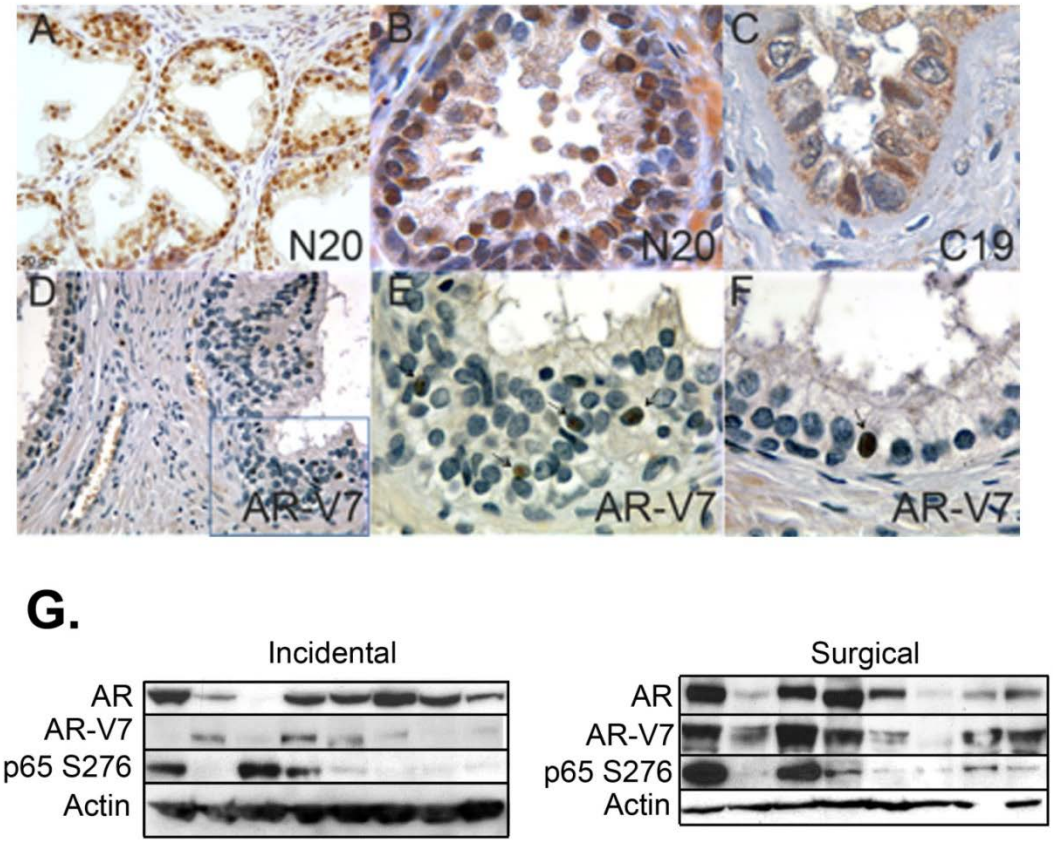


Figure 8. AR expression in BPH. **A.** An immunohistochemical stain using an antibody against AR N20 that should stain both AR-FL and AR-V demonstrates nuclear positivity in most epithelial cells lining the prostate glands. Some cells show focal positivity in the cytoplasm. **B.** A higher power view reveals strong intranuclear positivity (denoted by brown staining) admixed with cells showing both nuclear and cytoplasmic staining. **C.** Immunohistochemical staining using an AR C19 antibody that should recognize only AR-FL demonstrates somewhat lower levels of nuclear positivity as compared to N20 stained sections. Strong nuclear positivity appeared to be more common in elongated nuclei, while negative cells had larger round nuclei and finely stippled chromatin. **D and E.** Low (D) and high (E) power photomicrographs of a more proliferative region of the prostate showing AR-V7 expression. Several cells have nuclear positivity (arrows) while other cells are clearly negative. **F.** AR-V7 positivity varied among glands and in those appearing more indolent with a single layer of epithelial and basal cells, only an occasional AR-V7 positive cell could be identified (arrow). **G.** Western blot analysis of 10 Incidental and 10 randomly selected Surgical patients against AR-FL (N-20), AR-V7, p65-S276, and actin, showing higher expression of AR-V7 in surgical patients compared to incidental.

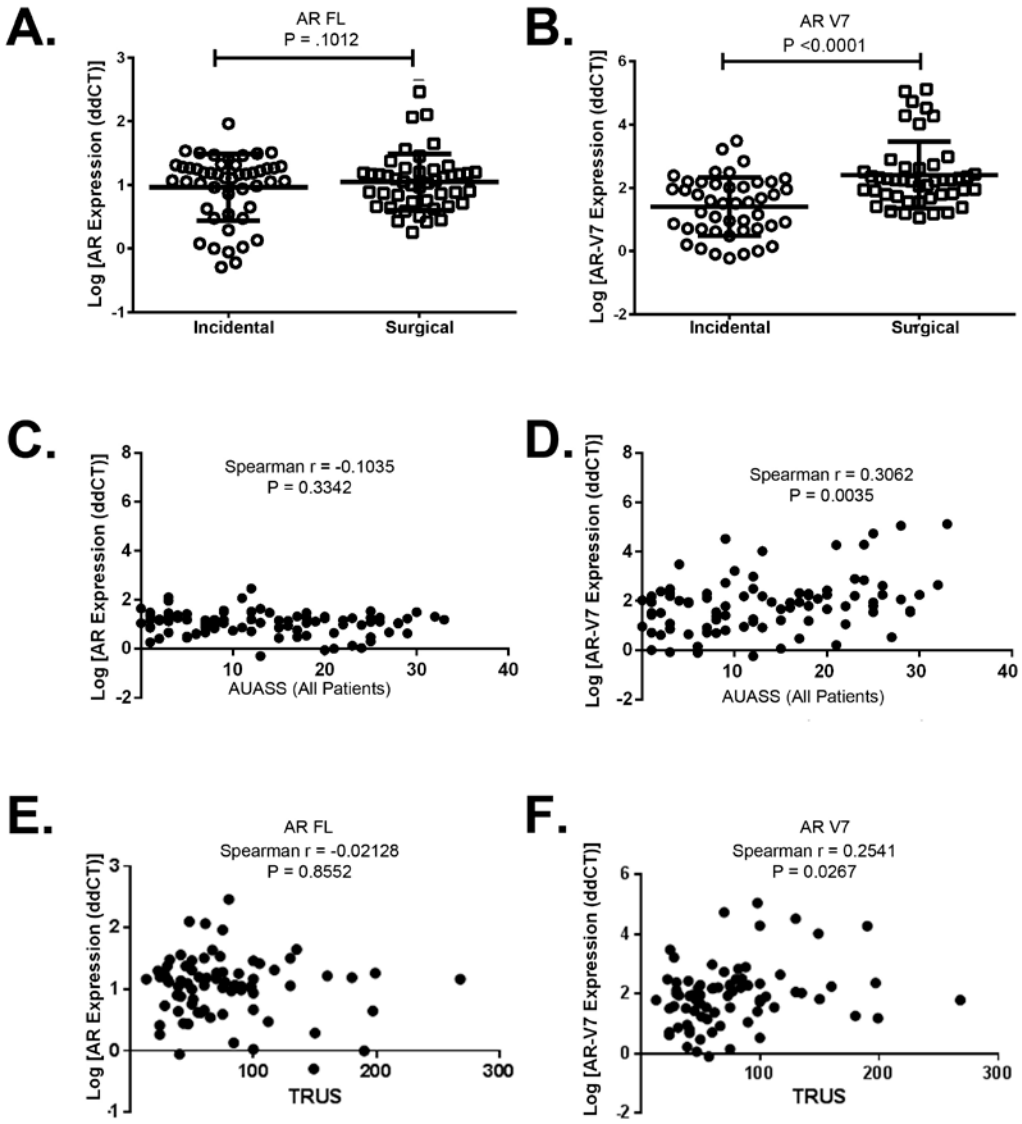


Figure 9. AR-V7 expression is significantly higher in advanced BPH. **A.** qPCR analysis of AR-FL showed no significant difference between cohorts. **B.** qPCR analysis of AR-V7 showed there is a significant increase in expression in the surgical cohort. **C.** Spearman's correlation coefficient analysis of AR mRNA expression and AUASS showing no correlation. **D.** Spearman's correlation coefficient analysis of AR-V7 mRNA expression and AUASS showing a significant correlation between increased AR-V7 mRNA expression and AUASS. **E.** Spearman's correlation coefficient analysis of AR mRNA expression and TRUS volume showing no correlation. **F.** Spearman's correlation coefficient analysis of AR-V7 mRNA expression and TRUS volume showing a significant correlation between increased AR-V7 expression and TRUS volume.

qPCR with primers for full length AR (Figure 9A). Following previous studies [94, 97], we examined the expression of a series of AR variants (V1, V2, V3, V4, V567^{es}, and V7). Only AR-V7 was expressed at detectible levels. qPCR quantitation of AR-V7 showed a significant increase (Figure 9B) ($P < 0.001$) in the surgical vs. incidental BPH samples. Immunohistochemistry was used to localize both AR-FL and to confirm the presence of nuclear AR-V7 protein using a specific antibody for this variant and was confirmed using Western blotting (Figure 8G).

AR-V7 correlates with AUASS scores and TRUS volume

We next investigated whether AR-FL or AR-V7 mRNA expression was associated with increased LUTS. We examined all incidental and surgical BPH patients (n=99), and analyzed patients whose AUASS score was included in our data set (n=89). While there was no significant correlation between AR-FL expression and AUASS (Figure 9C), there was a significant correlation between increased AUASS and AR-V7 expression ($P < 0.0035$) (Figure 9D). To use a non-subjective measurement to determine the association between AR-FL and AR-V7 mRNA expression with increased LUTS, we used TRUS volumes that were included in our data set (n=81). While there was no significant correlation

between AR-FL expression and TRUS volume (Figure 9E), there was a significant correlation between increased TRUS volume and AR-V7 expression (Figure 9F)($P = 0.0267$).

Inflammation is associated with increased AR expression

To determine whether adjacent inflammation is associated with increased AR and NF-κB expression we performed immunohistochemistry for inflammatory cells, AR, and activated NF-κB (p-p65). Inflammatory markers such as CD3ε and CD4 were used to determine naïve T-lymphocytes and T-helper lymphocytes respectively. As shown in representative images in Figure 10, epithelial cells adjacent to areas with low inflammation (Figure 10A) had lower expression levels of AR and activated NF-κB. High areas of inflammation (Figure 10B) correlated with increased expression of AR and NF-κB. incidental disease samples displayed relatively low levels of AR expression and NF-κB activation while surgical BPH patients displayed increased expression of AR and NF-κB in inflamed areas.

NF-κB activation and decreased AR expression are associated with NIDDM

Given that obesity and non-insulin dependent diabetes mellitus (NIDDM) is associated with BPH risk [178, 216], we examined whether NF-κB and AR-V7 expression were associated with BMI or NIDDM (Table 3). NF-κB expression

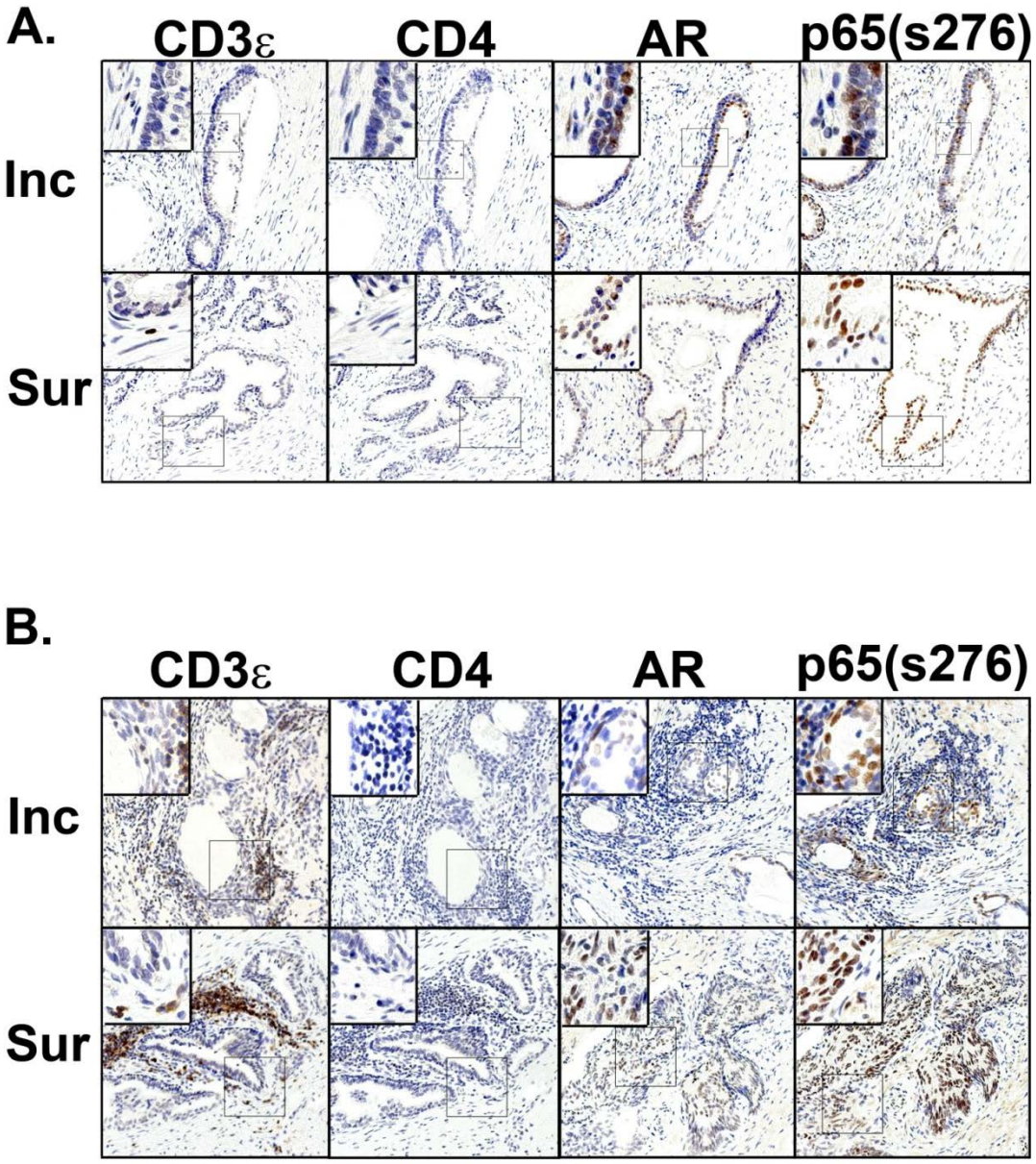


Figure 10. AR expression is associated with adjacent inflammation. CD3ε was used to stain naïve T-cells and CD4 was used to stain T_h-cells. Representative images from immunohistochemical analysis of CD3ε, CD4, AR, and phospho-p65 (S276) in incidental (top) n = 53 and surgical (bottom) n = 46 BPH of (**A)** non-inflamed by negative CD3ε expression (**B**) and inflamed areas by positive CD3ε expression.

was significantly elevated in both the stroma and epithelium of diabetic versus non-diabetic patients (Table 3). Obesity, defined as a BMI > 30, was not associated with NF-κB activation after controlling for NIDDM. Patterns were similar within incidental and surgical BPH groups, with no significant difference in epithelial or stromal cell NF-κB activity with either high or low BMI. In contrast, AR-FL expression was significantly lower in surgical BPH patients with NIDDM, while differences in AR-V7 expression levels were not significantly associated with BMI or NIDDM.

Since androgen ablation can lead to the expression of ARv in prostate cancer [20], we also examined whether medication used to treat BPH was associated with NF-κB activation, AR-FL or AR-V7 expression (Table 4). There was a significant increase in AR-V7 in patients with a history of α-blocker use (Table 4), whether in incidental or surgical BPH groups. While this result certainly warrants future investigation, we note that α-blockers are the front line therapy for BPH symptoms and this may simply reflect an underlying biology of the disease where patients with high levels of AR-V7 may be more likely to show symptoms that are treatable with α-blockers.

		All Pts		Incidental		Surgical	
		Mean[#]	P	Mean[##]	P	Mean[##]	P
NF-κB-S	BMI < 30	6.7	0.489	6.4	0.78	7.2	0.19
	BMI ≥ 30	7.6		5.9		9.7	
	NIDDM	10.5	**<0.01**	10.7	**<0.01**	10.5	0.07
	No NIDDM	4.8		3.5		6.6	
NF-κB-E	BMI < 30	16.1	0.18	12.8	0.13	20.2	0.78
	BMI ≥ 30	17.9		15.5		19.7	
	NIDDM	22.9	**<0.01**	19.5	**<0.01**	25.7	**<0.01**
	No NIDDM	12.6		10.2		15.4	
AR-FL	BMI < 30	9.0	0.54	11.8	0.99	7.4	0.64
	BMI ≥ 30	10.5		11.8		8.7	
	NIDDM	7.8	0.11	11.8	0.99	5.1	**0.03**
	No NIDDM	12.3		11.8		12.7	
AR-V7	BMI < 30	53.1	0.09	17.1	0.17	167.2	0.46
	BMI ≥ 30	127.7		44.7		308.6	
	NIDDM	67.3	0.50	28.4	0.95	140.8	0.31
	No NIDDM	100.8		26.9		366.4	

Table 4: Patient medical records were used to determine BMI and NIDDM. Stromal (S) and Epithelial (E) NF-κB activation was determine by quantitated immunofluorescent protein expression. AR-FL and AR-V7 mRNA expression was determine by qPCR. # adjusted geometric mean: NF-κB and AR variables were natural log transformed prior to analysis, and p values derive from analysis with each marker as the dependent variable in a linear model that included group, BMI, NIDDM, and age. Biomarkers were then back transformed to produce adjusted mean values. Thus, p-values represent the likelihood of a difference in biomarker value between each category, adjusted for the other parameters in the model. ## Similar approach, but models run separately within each diagnostic group.

Marker	Drug(s)		All	Mean Inci	Surg
NF-κB-S	5ARI only		4.9	5.3	3.9
	α blocker only		5.3	2.0	9.7
	5ARI + α-blocker		5.2	3.1	7.1
	None		6.2	5.1	3.4
		P	0.90	0.26	**0.04**
NF-κB-E	5ARI only		12.4	12.7	12.2
	α blocker only		15.3	11.8	19.4
	5ARI + α-blocker		13.2	7.1	17.5
	None		14.5	11.9	14.9
		P	0.64	0.30	0.24
AR-FL	5ARI only		14.1	4.4	31.2
	α blocker only		13.7	21.3	10.2
	5ARI + α-blocker		11.5	43.7	8.1
	None		9.1	9.9	16.2
		P	0.69	**0.01**	0.24
AR-V7	5ARI only		22.9	1.8	150.9
	α blocker only		450.8	142.5	1290.5
	5ARI + α-blocker		92.4	60.4	212.2
	None		45.9	20.0	51.9
		P	**0.01**	0.06	0.11

Table 5: Patient medical records were used to determine medication history. Stromal and Epithelial NF-κB activation was determine by quantitated immunofluorescent protein expression. AR-FL and AR-V7 mRNA expression was determine by qPCR. Control for group and age; Group specific analyses control for age; p-values – omnibus test of significant variability within the group being tested, simply indicating whether or not there is a significant association between drug use and biomarker. Specific mean values may be based on only a handful of subjects. Check n values from table 2. Early associations between 5ARI and NF-κB were due to confounding by differences in 5ARI use between groups. Once group differences are controlled for, 5ARI is no longer associated.

Activation of NF-κB upregulates AR-FL and AR-V7 expression in benign prostate epithelial and stromal cells

Activation of ARv by NF-κB has previously been described in prostate cancer but not in BPH. To determine whether chronic activation of NF-κB results in increased expression of AR and influenced cell growth and function, we retrovirally transduced benign human prostatic NHPrE1 (epithelial) and BHPrS1 (stromal) cells [217, 218], using empty vector (EV), kinase dead IKK2 (KD), or constitutively active IKK2 (EE) retroviral constructs. KD will block NF-κB activity while EE will increase NF-κB activity. Cells were grown in 5% charcoal-stripped FBS (CS) in the presence/absence of 10^{-8} M DHT. Activation of NF-κB signaling was assessed using the NGL reporter, a plasmid with an NF-κB responsive element coupled to GFP/Luciferase. We confirmed the successful transduction and increased expression of NF-κB signaling in NHPrE1 cells (Figure 11A) and BHPrS1 cells (Figure 11B). As expected, the background level of NF-κB activity was suppressed by the KD construct and enhanced by the constitutively active EE form of IKK2. The presence or absence of DHT had no significant effect on the activation of NF-κB.

Having established that the transductions were effective, we used the ARR_2PB-Luc reporter that responds to activation of the AR but has no binding sites for NF-κB. Both NHPrE1 and BHPrS1 showed significant activation of the

reporter construct when NF-κB signaling was constitutively activated even in the absence of androgens (Figures 11C and D). Stromal cells have a stronger basal reporter activity and a smaller induction of the reporter with DHT without the expression of NF-κB, likely reflecting basal expression of low levels of AR in culture.

NHPrE1 epithelial cells, which do not normally express AR *in vitro,* showed a clearer induction of signal. Addition of DHT increased the activity of the androgen-driven reporter in both epithelial and stromal cells (Figure 12C and D). We repeated this experiment using a PSA promoter driven luciferase reporter. This construct contains AR responsive elements and also an NF-κB responsive element, previously identified in the PSA gene [247]. Activation of NF-κB drove increased PSA promoter activity that was only slightly increased by the addition of androgens (Figure 11E). These data demonstrate that cells in which NF-κB is active are able to activate AR reporters in the absence of ligand, but retain an additional and greater ligand-driven response.

We examined the expression of AR variants V1, V2, V3, V4, V567^{es}, and V7 in the retrovirally transduced cells lines. Consistent with our observations in human tissue samples, the only significant ($P < 0.01$) increase in expression of variants was in AR-V7 (Figure 12A, Figure 13A and B). We performed Western blot analysis to examine the relationship between NF-κB activity and the

expression of AR-FL and AR-V7. This Western blot analysis (Figure 13C and D) confirmed the luciferase reporter data shown in Figure 11, showing increased phosphorylation of p65 in cells with constitutive activation of NF-κB (EE) and suppression of total and phosphorylated p65 by the kinase dead (KD) construct. Both AR-FL and AR-V7 proteins were upregulated in cells in which NF-κB signaling was constitutively activated. To evaluate the biological effects of NF-κB on proliferation, we performed growth assays. Constitutive activation of NF-κB in prostate epithelial and stromal cells was associated with an increase in proliferation starting by three days in culture in the absence of androgens, compared to the empty vector and kinase dead NF-κB (Figure 13E and F).

To determine whether the increased proliferation was due to NF-κB or AR-FL we used a cell viability assay based on quantitation of ATP, using cellular metabolism as a surrogate for cell number. AR expression was knocked down in NHPrE1-EV and NHPrE1-EE cell lines. Cells were grown in charcoal-stripped (CS) serum in vehicle or in the presence of 10^{-7} M Testosterone (T). There was a significant increase in proliferation in EE ($P < 0.001$) and EE-AR knockdown cells ($P < 0.003$) when compared to EV and EV-AR knockdown respectively (Figure 12B). This highlights that the increased proliferation is due to both the expression of NF-κB and expression of AR and increased proliferation when NF-κB and AR are coexpressed.

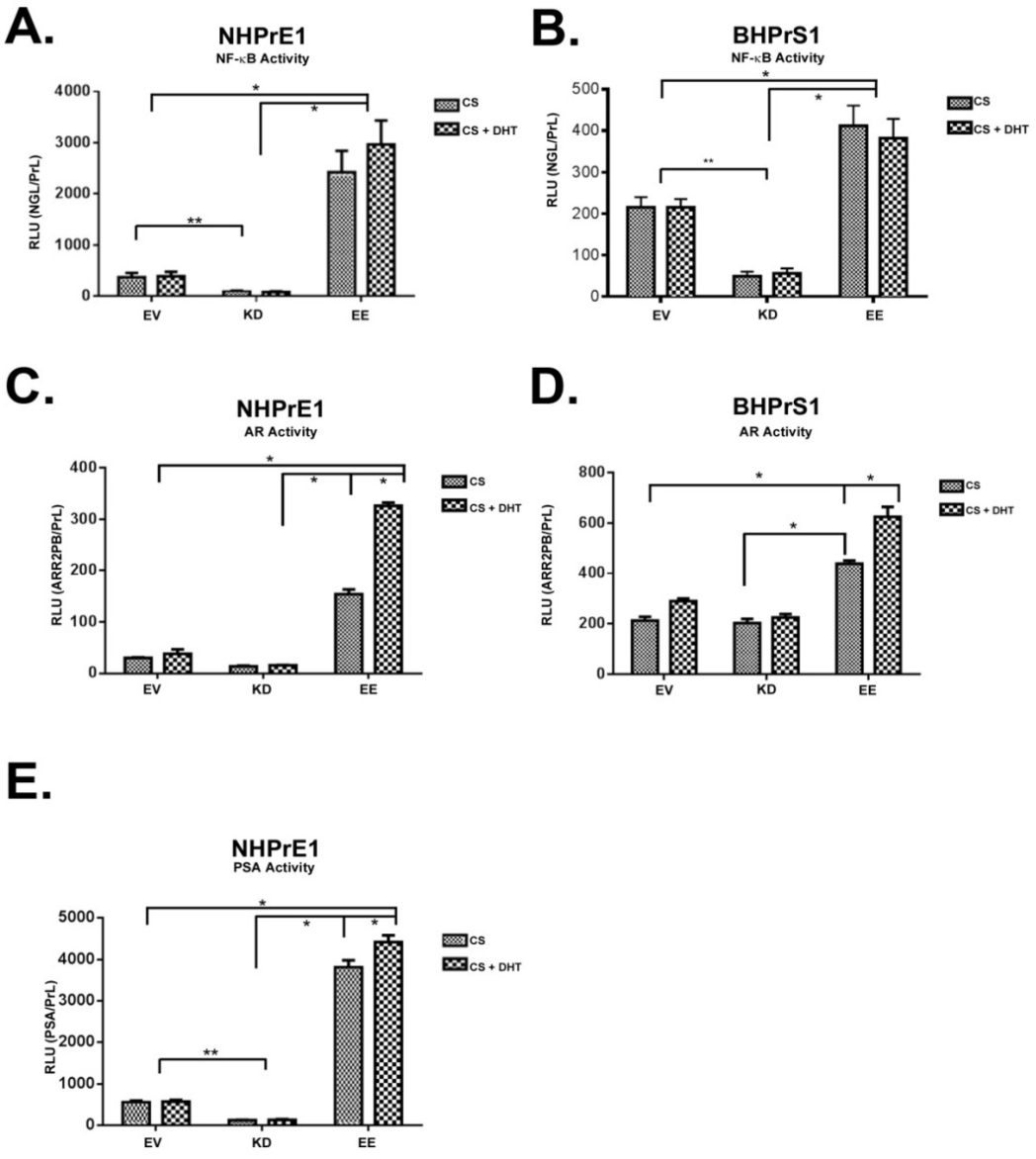

A.
NHPrE1
NF-κB Activity
RLU (NGL/PrL)
4000
3000
2000
1000
0
EV
KD
EE
CS
CS + DHT
B.
BHPrS1
NF-κB Activity
RLU (NGL/PrL)
500
400
300
200
100
0
EV
KD
EE
CS
CS + DHT
C.
NHPrE1
AR Activity
RLU (ARR2PB/PrL)
400
300
200
100
0
EV
KD
EE
CS
CS + DHT
D.
BHPrS1
AR Activity
RLU (ARR2PB/PrL)
800
600
400
200
0
EV
KD
EE
CS
CS + DHT
E.
NHPrE1
PSA Activity
RLU (PSA/PrL)
5000
4000
3000
2000
1000
0
EV
KD
EE
CS
CS + DHT

Figure 11. Chronic activation of NF-κB increases AR activity. NF-κB reporter activity (NGL) vector was transduced into benign epithelial (NHPrE1) and stromal (BHPrS1) cell lines to determine NF-κB activity plus/minus 10^{-8}M DHT after cell lines were transduced with IKK2-empty vector (EV), kinase dead (KD), or constitutively active (EE) constructs. **A.** NF-κB activity in NHPrE1-EE cell line was significantly increased when compared to -EV and the NHPrE1-KD cell line showing a significant decrease in activity when compared to -EV. **B.** NF-κB activity in BHPrS1-EE cell line was significantly increase when compared to -EV and BHPrS1-KD cell line showing a significant decrease in activity when compared to -EV. **A-B** cell lines were reported as relative luciferase units (RLU) normalized to the transfection control Renilla luciferase reporter (pRL) plus/minus 10^{-8} M dihydrotestosterone (DHT) in charcoal stripped serum (CS). **C-D.** AR activity was significantly increased in -EE cell lines when compared to -EV and -KD in both the presence and absence of 10^{-8}M DHT. **C-D.** AR reporter activity (ARR_2PB) in **(C)** NHPrE1 and **(D)** BHPrS1 **E.** Prostate specific antigen (PSA) promoter activity was significantly increased in NHPrE1-EE when compared to -EV and NHPrE1-KD PSA activity was significantly decreased when compared to -EV. Error bars are presented as mean +/- SD. Significant differences are compared to EV and are indicated in the graph. p-value: One-Way ANOVA. (* = $P < 0.01$, ** = $P < 0.05$).

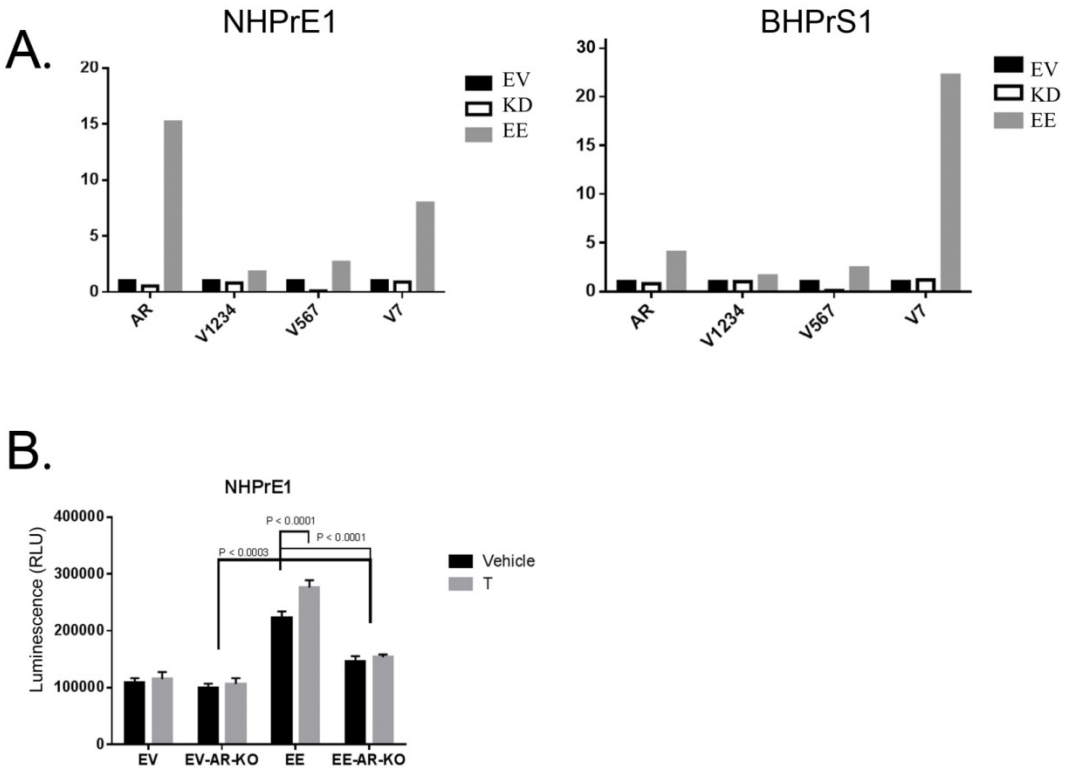


Figure 12. Activation of NF-κB induces AR-FL and AR-V7 expression. **A.** qPCR of AR-variants in the NHPrE1-EV, KD, and EE normalized to GAPDH in NHPrE1-EV. Showing significantly higher gene expression of AR-V7 in the NHPrE1-EE compared to NHPrE1-EV, NHPrE1-KD. **B.** AR-FL was knocked down in NHPrE1-EV and NHPrE1-EE cells where using siRNA (AR-KO) towards AR-FL. NHPrE1-EE-AR-KO had a significant decrease in viability when compared to NHPrE1-EE in the absence/presence of T. NHPrE1-EE-AR-KO showed a significant increase in viability when compared to NHrE1-EV-AR-KO in the presence/absence of T. p-value: Two-Way ANOVA.

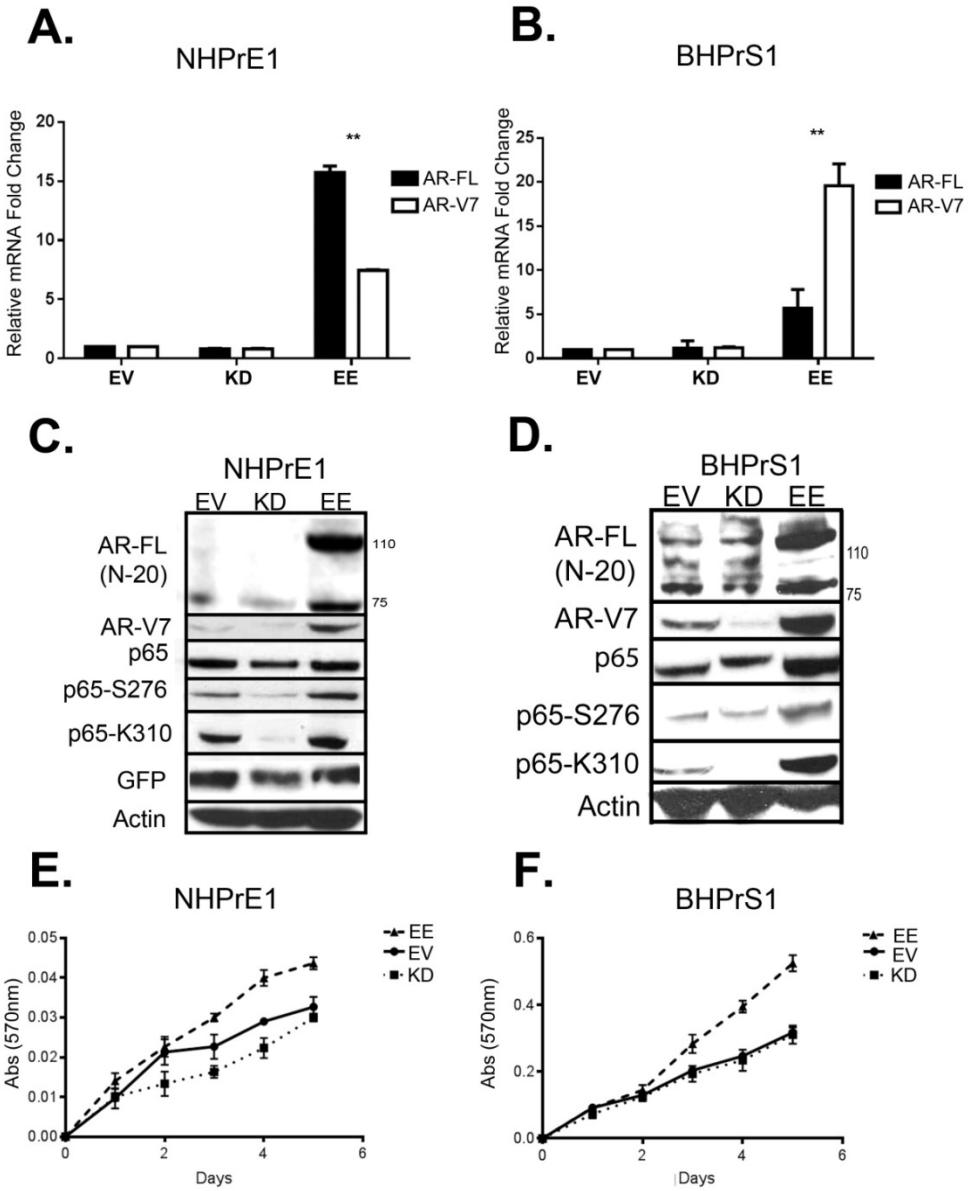


Figure 13. Chronic activation of NF-κB induces AR and AR-V7 expression in benign epithelial and stromal cells. To determine whether transduction of the IKK2 construct affected AR-FL and/or AR-V7 expression at the message and protein level we performed quantitative real time PCR (qPCR), western blot analysis and assessed proliferation in transduced cell lines. qPCR analysis of **(A)** NHPrE1 and **(B)** BHPrS1 cell lines of AR-FL and AR-V7 mRNA expression normalized to -EV, showing a significant increase in AR-FL and AR-V7 expression in -EE cell lines when compared to -EV. Western blot analysis of showing increased expression of AR/ARV (N-20), AR-V7, p65, p65-S276, p65-S536, and p65K310 in the -EE cell line when compared to -EV in **(C)** NHPrE1 and **(D)** BHPrS1 cell lines. Quantitative analysis of growth is made by a crystal violet assay using absorbance at 570 nm in **(E)** NHPrE1, showing a significant increase in growth in the -EE cell line and a significant decrease in growth in the -KD cell line when compared to EV, and **(F)** BHPrS1-EE cell line showing a significant increase in growth compared to -EV. Error bars are presented as means +/- SD. Significant differences are compared to EV and are indicated in the graph p-value: One-Way ANOVA. (*= $P < 0.05$), **=$P < 0.01$)

Chronic Activation of NF-κB induces resistance to a 5ARI

NHPrE1-V7 and NHPrE1-AR-FL were established by drug selection following retroviral transduction with the AR-V7 (V7) and AR-FL overexpression constructs, respectively. To examine whether chronic activation of NF-κB affected cellular response to a 5ARI, we assessed total metabolic activity. NHPrE1-EV, NHPrE1-KD, NHPrE1-EE, NHPrE1-V7, and NHPrE1-AR-FL cells were grown in charcoal-stripped (CS) serum in the presence/absence of 10^{-9} M Testosterone (T), or 10^{-9} M T combined with 10^{-7} M Finasteride (5ARI) or ethanol vehicle as control over a seven day period (Fig 14A-E). T is used rather than DHT since we are accessing the ability of 5ARI to block the conversion of T to DHT. Overall, the activation of NF-κB and overexpression of AR-V7 were resistant to a 5ARI, as no decrease in viability was detected in response to a 5ARI.

NHPrE1-AR-FL cells had a significant increase in viability in the presence of testosterone over vehicle and had a significant decrease in the presence of a 5ARI ($P < 0.05$) (Figure 14A). This highlights that these cells are responsive to a 5ARI. NHPrE1-EE cells had an increase in cell viability at three days with the addition of testosterone; however, the addition of a 5ARI did not significantly change overall metabolic activity (Figure 14B). Testosterone alone or in combination with the 5ARI did not affect viability of NHPrE1-V7 cells (Figure 14C). As expected, NHPrE1-EV (Figure 14D) and -KD (Figure 14E) cells did not respond to a 5ARI as these cells express little to no AR.

To examine the effects of chronic activation of NF-κB on epithelial cell function, NHPrE1 cell lines were transfected with the ARR_2PB-Luc reporter. This reporter responds to activation of the AR but, unlike the PSA promoter, has no binding sites for NF-κB. NHPrE1-EE and NHPrE1-V7 showed similar increased activation of the reporter construct in the absence of T. As expected, testosterone increased this signal in the NHPrE1-EE cells (which express elevated AR-FL in addition to AR-V7), but not in the NHPrE1-V7 cells. Cells in which only the AR-FL was overexpressed showed a response to testosterone that, as expected, could be down-regulated by the addition of 5ARI (Figure 14F). The NHPrE1-EV and NHPrE1-KD cell lines express little to no AR in 2D culture and therefore would not be expected to respond to the presence of androgens or a 5ARI. These data suggest that chronic activation of NF-κB can induce expression of AR-V7, and that the expression of AR-V7 can confer resistance to the effects of a 5ARI.

To determine if NHPrE1-EV cells express the enzyme 5α-reductase isoforms -1, -2, and -3 (SRD5A1, -2, -3) we performed qPCR of NHPrE1-EV cells grown in charcoal-stripped (CS) serum to determine the relative quantities of mRNA expression. As shown in Figure 16A, NHPrE1-EV cells express all three isoforms; however, SRD5A2 is expressed significantly higher than SRD5A1 and SRD5A3 ($P < 0.0001$). To determine if NHPrE1-AR-FL cells produce *de novo* synthesis of androgens we used two different approaches. One method used a pharmacological inhibitor to androgen synthesis: abiraterone acetate (Abi), in

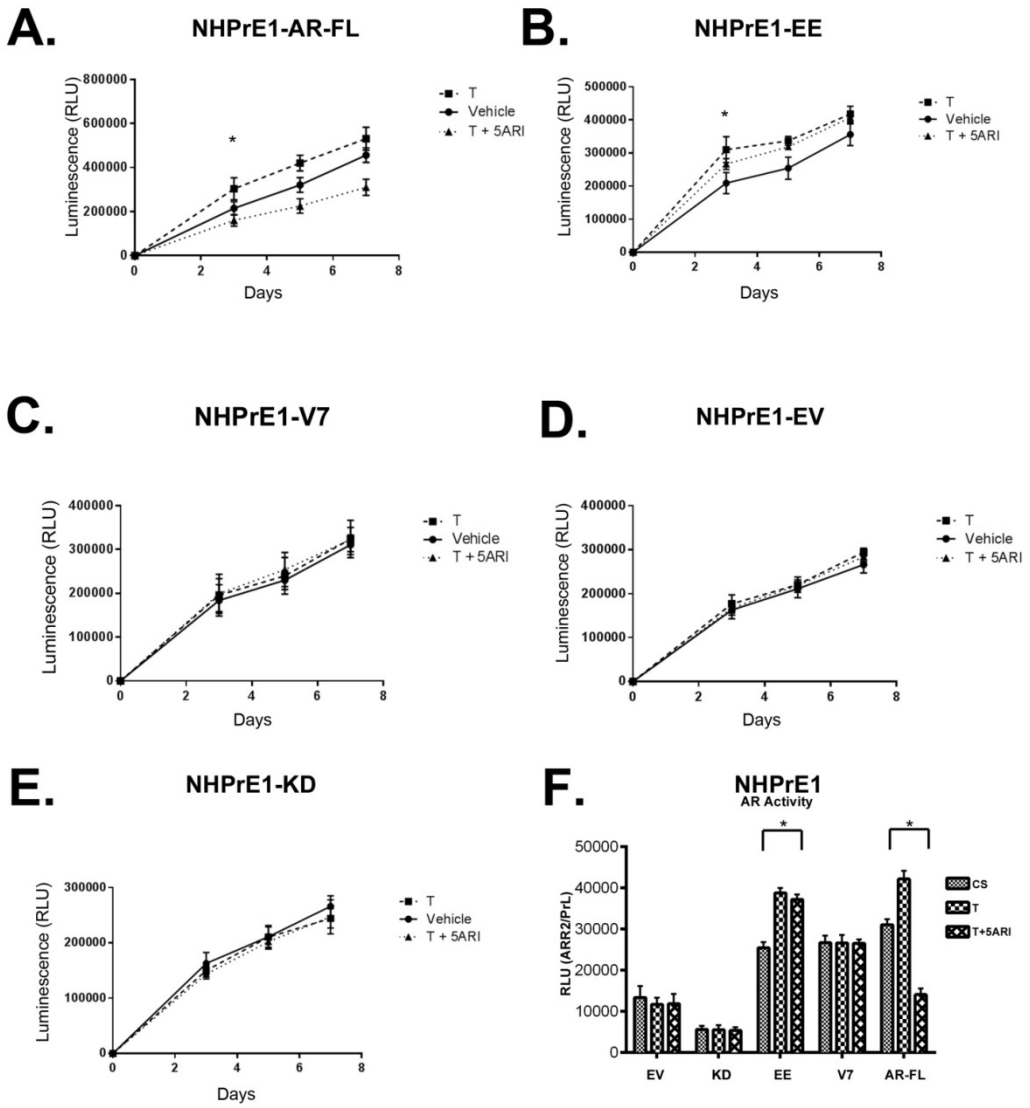


Figure 14. Chronic activation of NF-κB induces increased cell viability in response to a 5ARI. (**A-E)** NHPrE1 cell lines where treated with plus/minus 10^{-9} Testosterone (T) and 10^{-7} M Finasteride (5ARI). **A.** NHPrE1-AR-FL when compared to vehicle had a significant increase in viability in the presence of T and a significant decrease in the addition of T and a 5ARI. **B.** NHPrE1-EE cells had a significant increase in viability in the presence of plus/minus T and 5ARI when compared to vehicle. **C-E.** NHPrE1-V7,-EV, and -KD cell lines had no significant difference in viability in the presence of plus/minus T and 5ARI. **F.** Indicating that AR activity was significantly increased in NHPrE1-EE cells plus/minus T and 5ARI and AR activity was significantly decreased in NHPrE1-AR-FL cell in in the presence of T and 5ARI. NHPrE1-AR-V7, -EV, and -KD had no significant change in AR activity plus/minus T and 5ARI. Error bars are presented as mean +/- SD. Significant differences are compared to EV and are indicated in the graph. p-value: One-Way ANOVA. (* = $P < 0.01$).

relation to vehicle, and the second method used was high pressure liquid chromatographic (HPLC) to detect the levels of 5 endogenous androgens. As shown in Figure 15B, NHPrE1-AR-FL cells grown in CS or CS with Abi treatment resulted in significantly reduced cell viability across a range of concentrations, although concentrations higher than 6μM became toxic to the cells. Also, 5 endogenous androgens [testosterone (T), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), androstenedione (ASD), and androsterone (AND)] were quantitated in NHPrE1-AR-FL cells grown in CS serum over a 5 day period. Analysis of NHPrE1-AR-FL showed that these cells can produce T and DHT (Figure 15C). ASD, AND, and DHEA were below the lower limit of quantitation. This demonstrates that NHPrE1-AR-FL cells can manufacture limited amounts of androgens.

Chronic Activation of NF-κB increases 3D growth

To determine whether chronic activation of NF-κB affected growth in a 3D organoid culture model that we previously described [248], NHPrE1_EV, NHPrE1-KD, and NHPrE1-EE cells were seeded in triplicate in 48 well plates in the absence or presence of DHT (Figure 16). Figure 17A shows a significant ($P < 0.01$) increase in the number of NHPrE1-EE cells in the presence of DHT. AR-FL and AR-V7 expression in the NHPrE1-EE cells normalized to NHPrE1-EV (Figure 17B) showed a significant increase ($P = 0.002$) in AR-FL expression and

a significant reduction (P = 0.001) of AR-V7 expression 24 hours after treatment with DHT. PSA and TMPRSS2, two genes transcriptionally regulated by AR, have a significant increase in expression (P = 0.01 and 0.012 respectively - Figure 17C) in the presence of DHT. These data suggest that chronic activation of NF-κB is able to significantly induce AR activity in a ligand-independent manner. A concomitant reduction in the relative expression of p63 (a marker of basal phenotype prominent in 2D culture) in the presence of androgens reflects increased differentiation and organization of the organoids in 3D culture (Figure 16).

Inhibition of NF-κB abrogates AR signaling

To better understand the specific role of canonical NF-κB signaling in regulating AR expression in benign prostate cells, we performed a series of experiments aimed at abrogating critical downstream effectors. Since the constitutive activation of NF-κB in the EE-transduced cells is regulated by a modified form of IKK2, we used two alternative approaches to suppress NF-κB signaling. We used silencing of NF-κB, through siRNA targeting of p65, and the allosteric inhibition of IKK2 with the chemical compound BMS-345541. We determined whether these approaches had an effect on AR-FL expression or the proliferation of

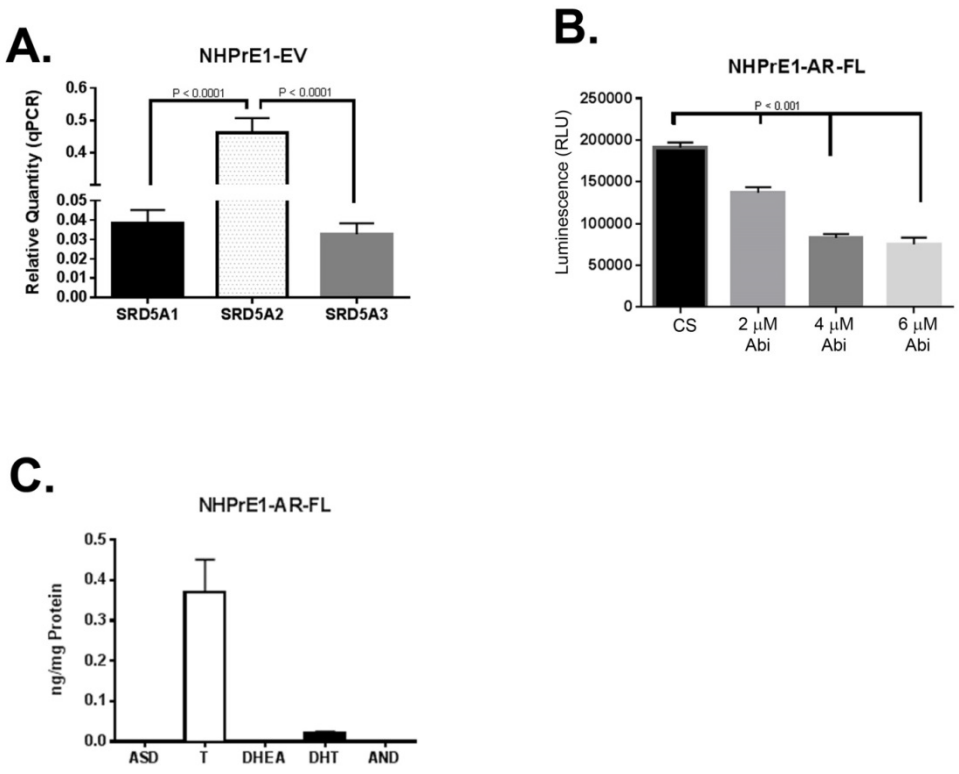


Figure 15. NHPrE1 cells express the 5α-reductase (SRD5A) isoforms and produce androgens. **A.** qPCR of NHPrE1 cell lines showing relative quantity when compared to GAPDH showing expression of all three SRD5A isoforms and also shows a significant increase in SRD5A2 expression when compared to SRD5A1 and SRD5A3. **B.** NHPrE1-AR-FL cell line treated with increasing amounts of abiraterone acetate (Abi) to inhibit androgen synthesis. There was a significant decrease in viability over a 5 day period when compared to vehicle (CS). **C.** Measurement of 5 endogenous androgens [testosterone (T), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), androstenedione (ASD), and androsterone (AND)] in NHPrE1-AR-FL cells grown in CS serum over a 5 day period. NHPrE1-AR-FL produced T and DHT. ASD, AND, and DHEA was below the lower limit of quantitation. p-value: One-Way ANOVA.

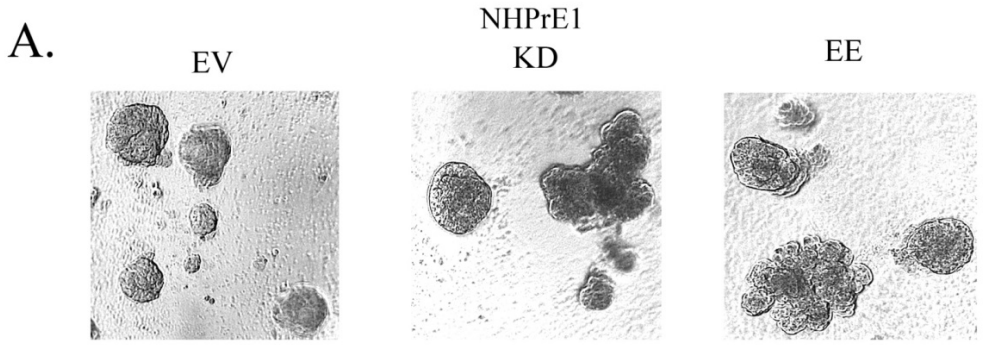


Figure 16. Activation of NF-κB increases growth in Matrigel. **(A)** NHPrE1-EV, KD, EE embedded in Matrigel over a 10 day period. Representative images are from day 10 treated with 10^{-8}M DHT showing larger colony growth when compared to -EV.

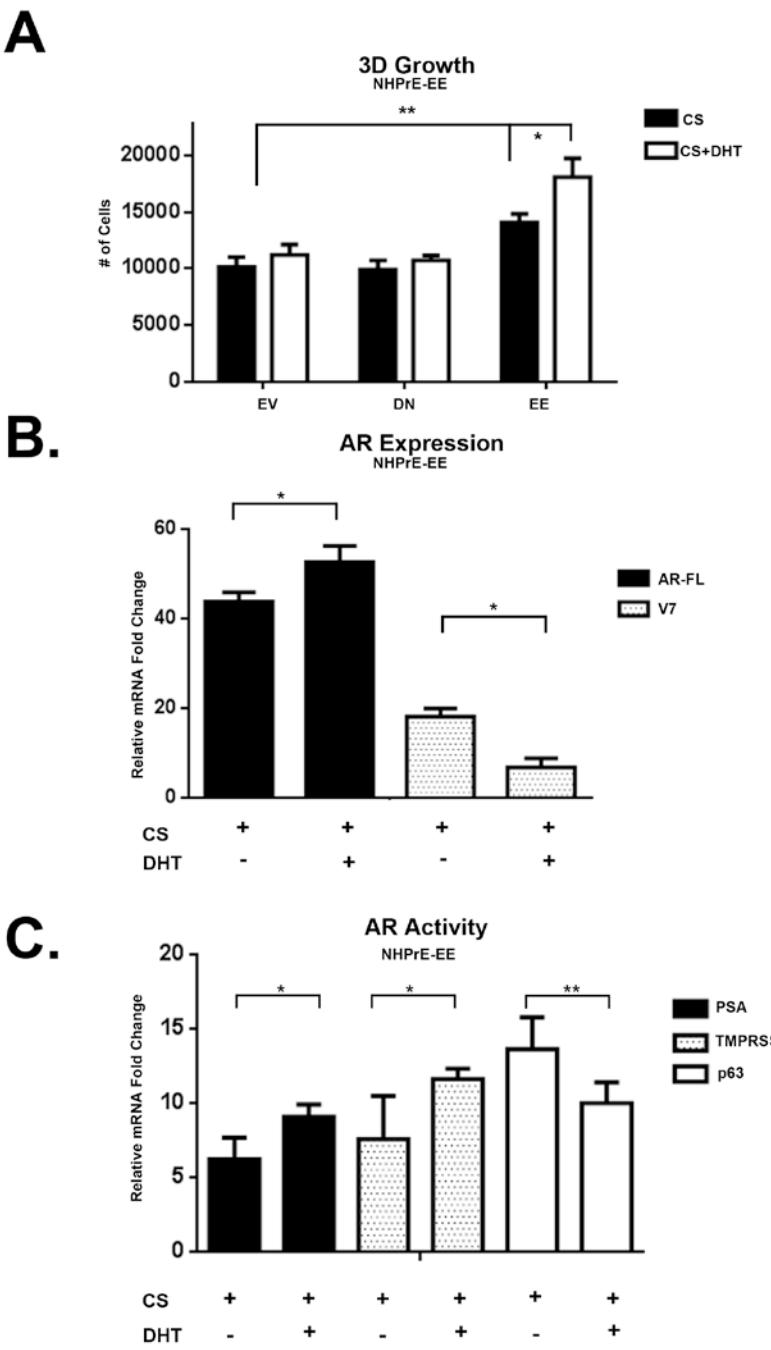

A
3D Growth
NHPrE-EE
**
*
CS
CS+DHT
# of Cells
20000
15000
10000
5000
0
EV
DN
EE
B.
AR Expression
NHPrE-EE
*
*
60
40
20
0
Relative mRNA Fold Change
AR-FL
V7
CS + + + +
DHT - + - +
C.
AR Activity
NHPrE-EE
*
*
**
20
15
10
5
0
Relative mRNA Fold Change
PSA
TMPRSS2
p63
CS + + + + + +
DHT - + - + - +

Figure 17. 3D Growth of NHPrE1-EE induces AR expression. **A.** Quantitative analysis of NHPrE1 cells grown in Matrigel over a ten day period in absence or presence of DHT (10^{-8} M) showing a significant increase in growth in NHPrE1-EE in the presence/absence of DHT when compared to -EV. **B.** qPCR of AR-FL and AR-V7 expression in NHPrE1-EE cells in the absence or presence of DHT (10^{-8} M) normalized to NHPrE1-EV showing a significant increase in AR-FL mRNA expression in the presence of DHT and a significant decrease in AR-V7 mRNA expression in the presence of DHT. **C.** AR activity in NHPrE1-EE was significantly increased in the presence of DHT. AR activity was analyzed by qPCR for the induction of PSA and TMPRSS2. p63, was used as basal cell differentiation marker and was significantly decreased in the presence of DHT when normalized to NHPrE1-EV GAPDH. Error bars are presented as means +/- SD. Significant differences are compared to NHPrE1-EV and are indicated in the graph (A: p-value: One-Way ANOVA, B and C: p-value: Two way ANOVA (*= $P < 0.01$), **=$P < 0.05$)

NHPrE1-EE cells and controls (Figure 18 and 19). As shown in Figure 18, inhibition of NF-κB by p65 siRNA or BMS-345541 (25 μM) was sufficient to reduce p65 phosphorylation (and total p65 in the case of the siRNA approach). Significantly, inhibition of p65 by siRNA (Figure 18E) or BMS-345541 (Figure 18F) was sufficient to reduce AR-FL and AR-V7 protein levels. siRNA knockdown or chemical inhibition of NF-κB also decreased AR-FL and AR-V7 mRNA expression as determined by qPCR (Figure 19A). While use of the BMS inhibitor resulted in an approximately 50% reduction in NGL luciferase expression, use of p65 siRNA resulted in almost complete abrogation of NGL activity (Figure 19B). We also found that suppression of NF-κB by either method reduced AR action in the absence of DHT (reflecting a reduction in ARv levels and associated constitutive activity) using the PSA promoter luciferase reporter (Figure 19C). Next we assessed whether inhibition of NF-κB in NHPrE1-EE cells had an effect on proliferation. As shown in Figure 19D, significant ($P < 0.05$) inhibition of the proliferation of NHPrE1-EE cells was seen by 3 days in culture and was maintained until the end of the experiment at 5 days. These data confirm that chronic activation of NF-κB in prostate epithelial cells can regulate proliferation through expression of AR-FL and AR-V7.

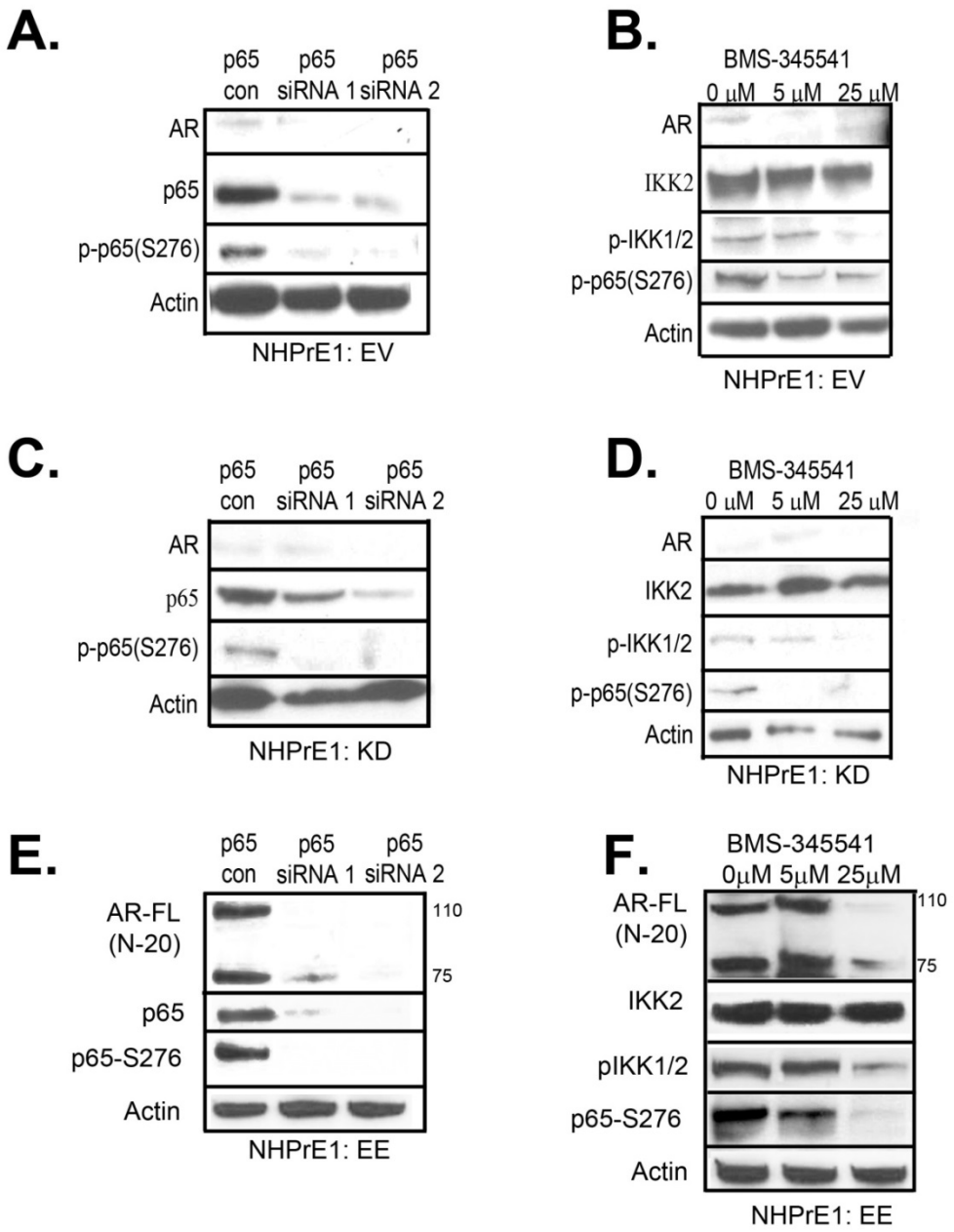


Figure 18. Inhibition of NF-κB decreases AR-FL and AR-V7 protein expression. (**A,C,E**) Western blot confirming knockdown of NF-κB in NHPrE1-EV,-KD and -EE cell lines by transfection of p65-siRNA (20 μM). Showing decreased p65 protein expression and activity (p-p65 S276) (**B,D,F**) Western blot confirming the inhibition of NF-κB in NHPrE1-EV, -KD, and -EE by a highly selective IKK2 inhibitor BMS-345541 (5 and 25 μM). IKK2 expression is not changed with the addition of the BMS-345541, however the phosphorylation of IKK2 and p65 activity (p-p65 S276) were decreased.

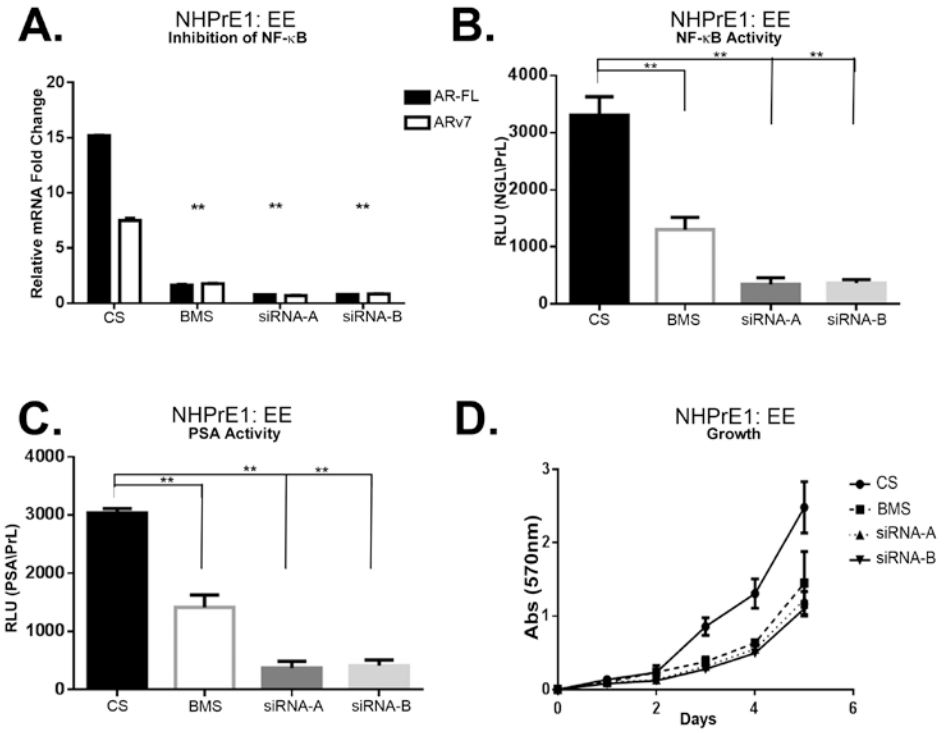


Figure 19. Inhibition of NF-κB decreases AR-FL and AR-V7 mRNA expression and AR activity. **A.** qPCR of NHPrE1-EE for AR-FL and AR-V7 transfected with p65-siRNA or incubated with BMS-345541 showing a significant decrease in both AR-FL and AR-V7 mRNA expression. AR-FL and AR-V7 are normalized to NHPrE1-EV in charcoal stripped media (CS). (**B)** NF-κB activity in NHPrE1-EE was significantly decreased when transfected with p65-siRNA or treated with BMS-345541 when compared to NHPrE1-EE cell grown in CS. Reported as RLU normalized to pRL. **C.** AR activity was significantly decreased when transfected with p65-siRNA or incubated with BMS-345541. Reported as prostate specific antigen (PSA) normalized to pRL. **D.** Quantitative analysis of growth was significantly decrease when transfected with p65-siRNA or incubated with BMS-345541. Analysis by crystal violet at absorbance 570 nM of NHPrE1-EE cell lines transfected with p65-siRNA or incubated with BMS-345541. Error bars are presented as means +/- SD. Significant differences are compared to CS and are indicated in the graph. p-value: One-Way ANOVA (** = $P < 0.01$).

Discussion

Benign prostatic hyperplasia and associated LUTS represent a common health problem resulting in significant morbidity and associated patient care costs [1, 249]. The most common medical approaches for patients with BPH/LUTS are α-adrenergic blockers and 5α-reductase inhibitors. While often initially effective in relieving LUTS, many patients see a slow and continued progression of their disease [239]. As a result, even though the routine use of 5ARIs and α-adrenergic inhibitors has reduced the number of patients that undergo surgical intervention, surgery remains common with approximately 260,000 such interventions performed annually [250]. These patients are usually older, often with comorbidities such as obesity and diabetes, and thus are often not prime candidates for surgery. An understanding of the underlying biological factors that result in disease progression should lead to new approaches to help prevent resistance to therapy with subsequent disease progression and the need for surgical intervention.

BPH has long been considered to be a product of androgen action upon an aging prostate. However, longitudinal epidemiological studies from numerous patient cohorts that controlled for related hormone levels have shown that androgen levels are unlikely to be solely responsible [251]. Separate mechanisms are almost certainly in play in many patients [216, 252]. Furthermore, comorbidities associated with inflammation appear to play an important role in

disease progression [253] potentially contributing to cell proliferation and resistance to current therapies. There is broad recognition in the field that BPH represents a variety of pathologies, which may lead to prostatic inflammation, increased prostate volume and therapeutic resistance [197, 254]. Baseline prostate volume and PSA levels are the most reliable clinical predictors of acute urinary retention and surgical intervention for BPH/LUTS [245] and 5ARIs are the most widely used therapy for treating prostatic enlargement. Accordingly, our goal is to understand the mechanisms that cause resistance to 5ARI therapy.

We have developed a repository of human prostate transition zone tissues representing a range of BPH/LUTS severity. A previous analysis of this resource demonstrated that increased expression of AP-1 transcription factors was associated with advanced disease [200]. Consistent with observations from other groups, our data suggested a possible autoimmune inflammatory component to BPH [236, 237].

In this chapter we examined the activation of the p65/RelA, the canonical NF-κB pathway, as well as the expression of constitutively active AR variants in BPH and BPH-derived cell lines. Increased activation of canonical NF-κB was seen in both the epithelial and stromal cells of human BPH and was increased in more advanced disease. Since NF-κB activation has also been linked to the expression of ARv in prostate cancer [243, 246], we examined the expression of a panel of ARv in BPH. Significant expression of AR-V7 mRNA and protein was

found while the other AR variants were either not expressed or were detected at very low levels. We also saw increased expression of AR-FL associated with canonical NF-κB activation adjacent to areas of inflammation compared to areas lacking inflammation. To the best of our knowledge, this is the first report on the expression of AR variants in human BPH and suggests a mechanism for escape from growth regulation by 5ARI therapy.

Analysis of the clinical samples revealed a number of interesting correlations in detection of NF-κB and AR-V7. We determined that patients with more advanced disease showed higher levels of both epithelial and stromal nuclear p-p65 (NF-κB activation) and also increased AR-V7 transcript levels. We determined that increased AR-V7 levels positively correlate with AUASS and TRUS volume. We also determined that increased activation of epithelial cell NF-κB correlates with increased TRUS volume. This suggests a linkage between disease progression, activation of NF-κB and expression of AR-V7. We hypothesized that obesity would also be linked to these same outcomes, but based on a binary analysis (BMI < 30 vs. BMI ≥ 30) there was no significant relationship between obesity and either NF-κB or AR-V7 expression after adjusting for differences in NIDDM prevalence between groups. In contrast, NIDDM was positively associated with NF-κB expression. Past studies have found direct associations between diabetes and BPH progression [255]. This

might suggest that specific forms of systemic stress exert distinct influences in the pathogenesis of BPH, and reinforces the idea that, although prostatic hyperplasia is associated with both obesity and diabetes, these conditions may act through separate or overlapping mechanisms [23]. We also saw an association between α-blocker treated patients as having increased NF-κB activation and AR-V7 expression. Conflicting reports in literature suggest that α-adrenergic receptors are both pro-inflammatory [256-258] and anti-inflammatory [259-261]. Our data would be consistent with the latter scenario, that α-adrenergic receptors are anti-inflammatory and that patients on an α-blocker would have NF-κB activation increased. This could also support our observations of increased AR-V7 levels in this patient population. The role of α-blocker to alter inflammation needs to be elucidated further.

We performed a series of 2D and 3D *in vitro* experiments to investigate whether activation of NF-κB can result in the expression of both AR full length (AR-FL) and AR-V7 in benign epithelial and stromal cell lines. The findings were consistent in epithelial and stromal cell lines in that the constitutive activation of NF-κB resulted in the coordinate expression of both AR-FL and AR-V7. This is consistent with observations in prostate cancer where both forms of the androgen receptor are regulated together by the NF-κB pathway [246].We next performed a series of *in vitro* experiments to determine if NF-κB and specifically AR-V7 can

induce resistance to a 5ARI. These findings demonstrated that forced activation of NF-κB and over expression of AR-V7 are able to induce resistance to a 5ARI. We also showed by HLPC LC/MS/MS analysis that NHPrE1-AR-FL cells can undertake androgen synthesis. This could suggest another mechanism by which BPH patients could fail 5ARI therapy. Stromal cells are an important component of BPH, which is often considered to be a disease of the stromal tissue with epithelial growth as a secondary consequence. This follows the early observations of McNeal who suggested the reawakening of fetal mesenchymal potential contributed to the development of BPH, a concept subsequently validated by Cunha and colleagues in rodent and tissue recombination models [227, 262]. The activation of NF-κB presented here is consistent with the idea that inflammation plays a role in BPH progression as well as providing a potential molecular mechanism of resistance to 5ARIs via activation of AR-V7 expression. AR-V7 has been shown to modulate expression of a number of tumor-promoting autocrine/paracrine growth factors in prostate cancer [97]. However, it is likely that this is a multifactorial process in which underlying comorbidities play a complex role. There may well be a number of alternative and possibly complementary pathways involved in this process. For example, our previous observations that AP-1 transcription factors (molecules known to be related to several immune/inflammatory conditions) are highly enriched in symptomatic BPH [200]. AP-1 transcription factors are post-transcriptionally regulated by

upstream factors such as NF-κB, JNK, ERK, and p38 [263] and therefore activation of NF-κB can upregulate not only AR-FL and AR-V7 but also AP-1 factors which can serve as AR co-factors to regulate transcription, which could lead to the development and progression of therapy-resistant BPH. These pathways might be interrelated, with no individual change (for example, androgen synthesis, activation of stress factors, expression of AR-V7) being sufficient to allow escape from therapy but that these individual changes may be additive and allow for eventual regrowth of the prostate in the face of therapy.

In summary, the observations presented here provide a potential mechanism to explain the previously observed links between prostatic inflammation and 5ARI resistance [197], whereby aberrant activation of NF-κB drives AR-V7 expression resulting in ligand independent activation of AR resulting in 5ARI resistance. BPH is a complex condition and there are likely multiple pathways in play in individual patients relating to common comorbidities including diabetes, obesity and metabolic syndrome. An understanding of the stress pathways active in individual patients may provide a route to appropriately tailor therapy and avoid the profile of resistance and subsequent progression to surgery that is seen in many patients.

CHAPTER IV

NF-κB and Androgen Receptor Variant 7 Expression induces expression of SRD5A isoforms and confers resistance to 5ARI treatment

Introduction

Benign Prostatic Hyperplasia (BPH) is the most common urologic disease in men [1]. BPH is an expansion of the transition zone of the prostate that compresses the urethra, causing increased bladder outlet resistance. This is a cause of LUTS, which include difficult (straining, weak stream, sensation of incomplete emptying) and irritable (frequency, urgency, urge incontinence) voiding [264]. There are many potential etiological factors contributing to BPH pathogenesis such as metabolic syndrome (MetS), growth factor, hormone signaling, and inflammation [181, 232, 234, 252]. Understanding the biology and treatment of BPH has become increasingly important as the strong relationship between LUTS, diabetes, metabolic syndrome, and other risk factors is more appreciated [265, 266].

There are two major medical approaches for patients presenting with symptoms suggestive of BPH: α-blockers [194], and 5ARI [238]. α-blockers relax the muscle of the prostate and provide symptomatic relief. 5ARIs inhibit the conversion of T to the more potent DHT resulting in a reduction of androgen receptor activity [267]. Inhibition leads to epithelial apoptosis, a decreased

prostate volume, and thereby reduces LUTS, the risk of acute urinary retention, and the risk of prostate surgery [182, 268]. However, nearly 20% of patients either fail to respond or become resistant over time, with some progressing to surgical intervention [182]. Given their age and comorbities these patients are often not ideal candidates for surgical intervention [200, 240]. Therefore, understanding treatment failure and developing new medical therapies appropriately targeted to these specific patient groups are desirable ways to move forwards.

NF-κB transcription factor family regulates the expression of numerous genes and we have previously shown in chapter III that forced activation of NF-κB resulted in an increase in AR-FL and AR-V7 expression [26].

There are three isoforms of the enzyme 5α-reductase (SRD5A1, -2, and -3) [269-273]. These enzymes convert T to DHT, which is the most active androgen in the prostate [267]. The AR can be expressed as COOH-terminal truncated variants (ARv) in prostate cancer [12-18, 97] and BPH [26, 274]. These ARv lack the ligand-binding domain of AR-FL. ARv are constitutively active, driving AR-regulated transcription and promoting tumor progression under castrate conditions. Expression of AR-V7 is predictive of resistance to enzalutamide and abiraterone acetate in prostate cancer patients [19]. Gao has reported that ARv can be produced in response to the non-canonical NF-κB pathway, and we have shown that ARv can be induced by the canonical pathway

of NF-κB [26, 243, 246]. Currently, there is not an established link between NF-κB and resistance to 5ARI therapy in BPH.

The primary mode of action of 5ARIs is to inhibit the enzyme 5α-reductase from converting T to DHT [12], where DHT has a higher binding affinity to AR than T [13]. SRD5A2 is the major SRD5A isozyme in the prostate [20-22]. In prostate cancer it has been shown that increased expression of SRD5A1 and SRD5A2 predicts biochemical recurrence of prostate cancer and metastatic potential, and reduced efficacy of androgen deprivation treatment [20, 21]. We suspected that SRD5A levels could affect progression of BPH to fail medical therapy. The goal of this study was to try to elucidate mechanisms of 5ARI resistance.

Works described in chapter III have demonstrated that NF-κB induces AR-V7, which in turn is associated with increased BPH severity [26]. In this chapter, we investigated whether the activation of NF-κB and/or AR-V7 expression could increase expression of SRD5A isoforms in BPH. We utilized human tissue samples, as well as benign human prostate epithelial and stromal cells, to test the consequences of NF-κB, AR-FL, and AR-V7 activation on SRD5A isoforms. Our results provide a mechanism that could explain why certain patients with BPH fail 5ARI therapy. Our data suggest that inhibition of NF-κB

activation in combination with a 5ARI could have an impact in reducing failure of treatment in BPH patients.

Results

SRD5A1 and SRD5A2 are upregulated in Surgical BPH patients

Progressive BPH is commonly associated with inflammation [197] and as demonstrated in chapter III, that NF-κB expression was significantly higher in surgical compared to incidental BPH and correlated with TRUS volume [26]. We wanted to determine whether SRD5A levels in these patients were associated with symptomatic progression. An initial analysis of SRD5A isoforms was performed to compare activation in 46 clinically advanced surgical BPH specimens versus 53 incidental BPH specimens. As illustrated in representative immunofluorescent images in Figure 20A, B, and mRNA expression quantified in Figure 21A there is significantly higher expression of SRD5A1 ($P = 0.0259$) and SRD5A2 ($P < 0.0001$) in the more advanced surgical samples. In contrast, SRD5A3 ($P = 0.0427$) mRNA was significantly lower in surgical compared to incidental samples.

SRD5A2 correlates with TRUS volume

We investigated whether SRD5A1, SRD5A2, and SRD5A3 expression was associated with increased prostate size and with increased LUTS. We analyzed patients whose AUASS (n=72) and TRUS volume (n=68) was included in our data set. There was a significant correlation linking both increased TRUS volume ($P = 0.0379$) (Figure 21B middle) and AUASS ($P < 0.0001$) (Figure 21C middle) with SRD5A2 expression. There was no correlation between TRUS volume and AUASS with SRD5A1 and SRD5A3 (Figure 21B/C).

SRD5A2 is significantly higher in patients on a 5ARI treatment

To determine the expression levels of SRD5A isoforms and 5ARI treatment we examined all patients in our data set and determined that SRD5A2 expression was significantly higher ($P = 0.0127$) in patients who had received 5ARI therapy. SRD5A3 was significantly lower ($P = 0.0372$) in patients on a 5ARI (Figure 21D).

Chronic activation of NF-κB results in enhanced expression of SRD5A isoforms

Chapter III demonstrated that activated NF-κB can induce expression of AR-V7, providing a potential route for resistance to 5ARI [26]. To determine whether chronic activation of NF-κB resulted in increased expression of SRD5A isoforms

and influenced cell growth and function, we used generated cell lines described in chapter III [26] and show that NHPrE-EE, a human prostatic epithelial cell line which has activated NF-κB through IKK2, significantly upregulated all SRD5A isoforms ($P < 0.001$) when compared to control empty vector (EV) cells (Figure 22A). Expression of AR-V7, AR-FL showed significant upregulation of only SRD5A2 ($P < 0.01$) (Figure 22A). In a stromal cell line BHPrS1 SRD5A2 was upregulated in the –EE, -ARV7 and AR-FL lines, however expression of the other two isoforms was unaffected (Figure 22B).

Chronic activation of NF-κB induces *de novo* testosterone synthesis

To determine whether expression of activated NF-κB can induce cells to undertake *de novo* androgen synthesis, we used two different approaches. One method was a pharmacological inhibitor to androgen synthesis: abiraterone acetate (Abi) [275, 276], and the second method was high pressure liquid chromatography (HPLC) tandem mass spectrometric detection (LC/MS/MS) to detect the levels of 5 endogenous androgens. As shown in Figure 23A, cells grown in charcoal stripped serum (vehicle) or with 4 nM Abi for five days. Treatment with Abi in activated NF-κB (NHPrE1-EE) cell line showed a

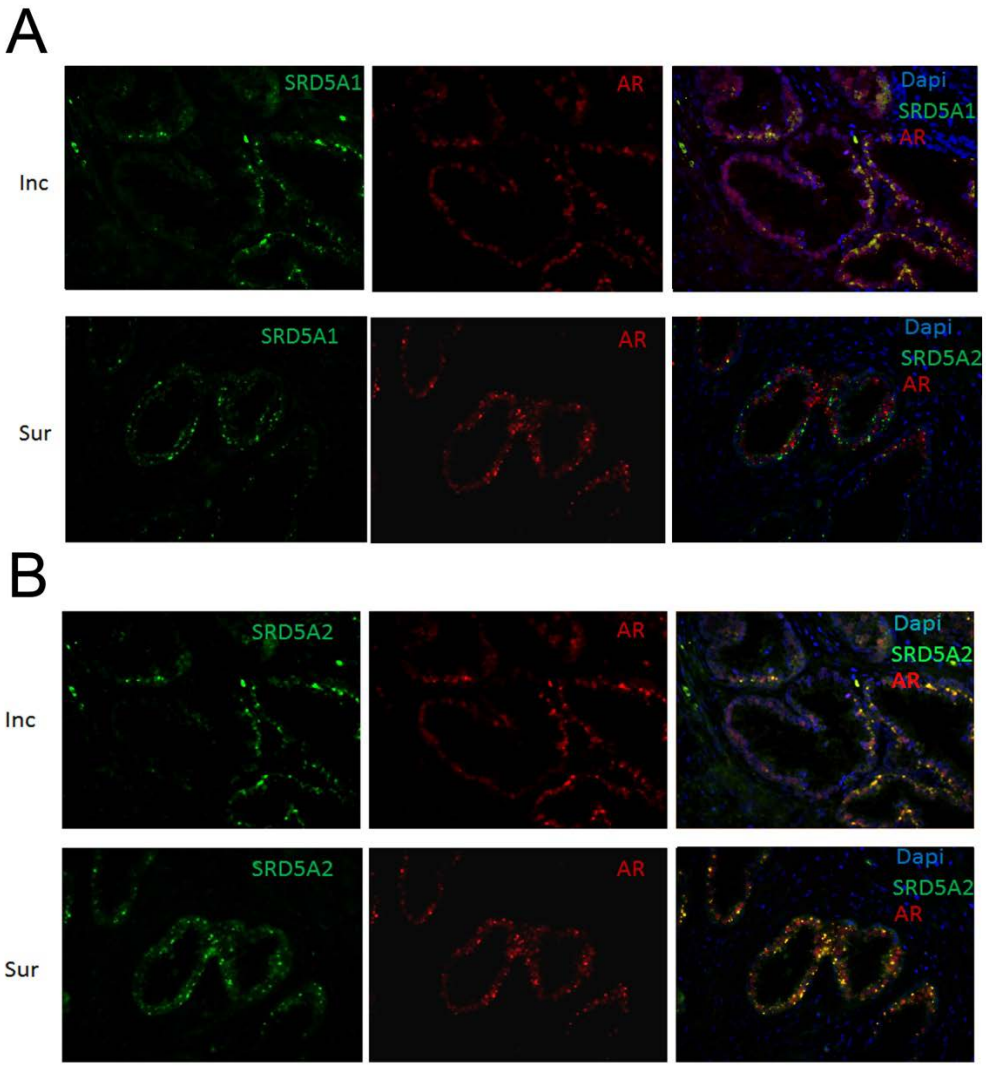


Figure 20. SRD5A expression in symptomatic BPH. Representative images of immunofluorescence staining for SRD5A1, SRD5A2, AR-FL, and immunohistochemistry staining for SRD5A3 in 40 BPH Patients. **A/B.** Examples of immunofluorescence for SRD5A1 and SRD5A2 (green), AR-FL (red), and DAPI (blue) in Incidental and Surgical BPH.

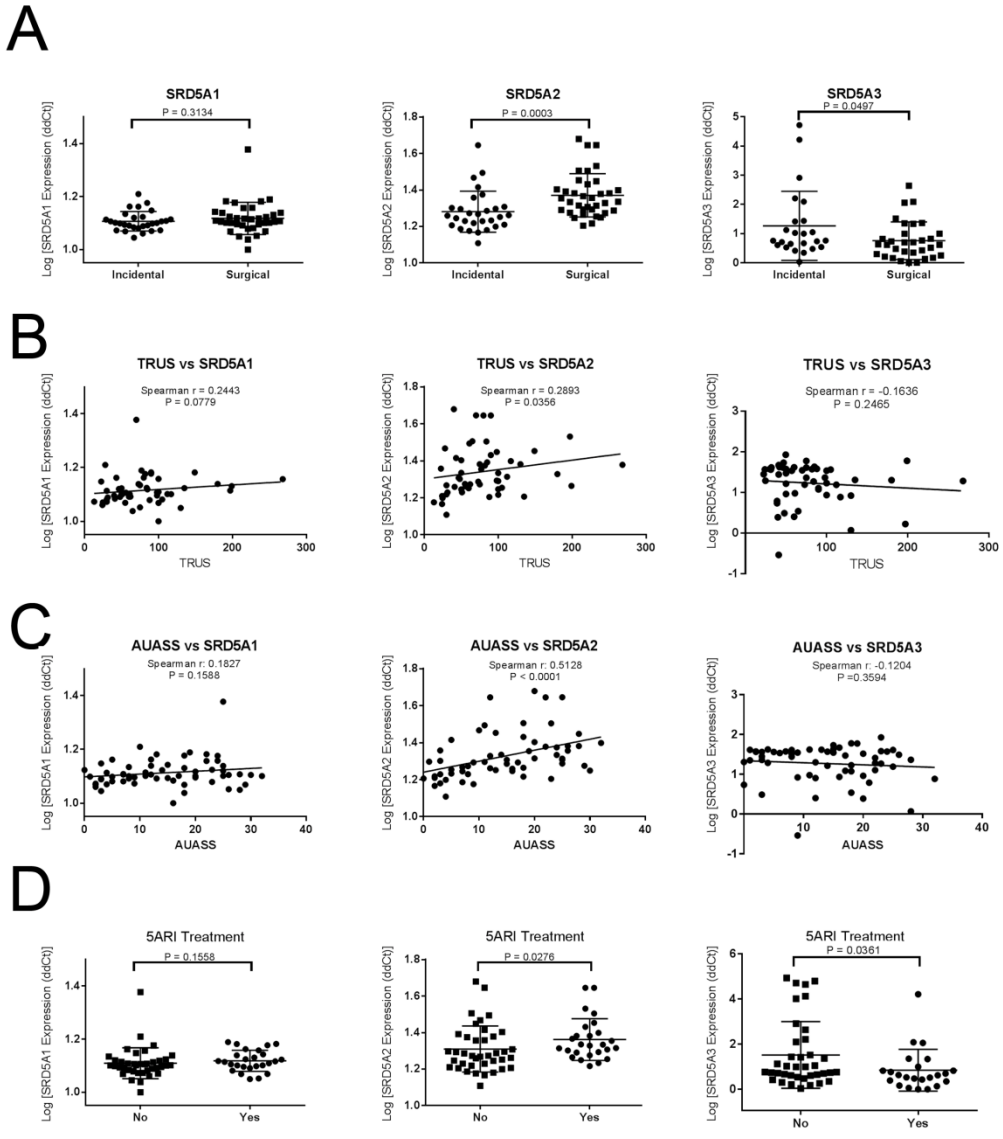

A
SRD5A1
P = 0.3134
Log [SRD5A1 Expression (ddCt)]
Incidental
Surgical
SRD5A2
P = 0.0003
Log [SRD5A2 Expression (ddCt)]
Incidental
Surgical
SRD5A3
P = 0.0497
Log [SRD5A3 Expression (ddCt)]
Incidental
Surgical
B
TRUS vs SRD5A1
Spearman r = 0.2443
P = 0.0779
Log [SRD5A1 Expression (ddCt)]
TRUS
TRUS vs SRD5A2
Spearman r = 0.2893
P = 0.0356
Log [SRD5A2 Expression (ddCt)]
TRUS
TRUS vs SRD5A3
Spearman r = -0.1636
P = 0.2465
Log [SRD5A3 Expression (ddCt)]
TRUS
C
AUASS vs SRD5A1
Spearman r: 0.1827
P = 0.1588
Log [SRD5A1 Expression (ddCt)]
AUASS
AUASS vs SRD5A2
Spearman r: 0.5128
P < 0.0001
Log [SRD5A2 Expression (ddCt)]
AUASS
AUASS vs SRD5A3
Spearman r: -0.1204
P =0.3594
Log [SRD5A3 Expression (ddCt)]
AUASS
D
5ARI Treatment
P = 0.1558
Log [SRD5A1 Expression (ddCt)]
No
Yes
5ARI Treatment
P = 0.0276
Log [SRD5A2 Expression (ddCt)]
No
Yes
5ARI Treatment
P = 0.0361
Log [SRD5A3 Expression (ddCt)]
No
Yes

Figure 21. SRD5A2 expression correlates with BPH progression. **A.** qPCR analysis of SRD5A1, SRD5A2, and SRD5A3 expression in BPH, showing a significant increase in expression of SRD5A2 and decrease in SRD5A3 expression in the surgical BPH (n=46) cohort compared to incidental BPH (n=53). Bars are presented as standard deviation, p-value: non-parametric Mann-Whitney test. **B.** Spearman's correlation coefficient analysis of SRD5A1, SRD5A2, and SRD5A3 mRNA expression and TRUS volume showing a significant increase in SRD5A2 expression as TRUS volume increases. There is no correlation between SRD5A1 and SRD5A3 expression (n=68). **C.** Spearman's correlation coefficient analysis of SRD5A1, SRD5A2, and SRD5A3 mRNA expression and AUASS showing a significant increase in SRD5A2 expression as AUASS increases. There is no correlation between SRD5A1 and SRD5A3 expression (n=72). **D.** qPCR analysis of SRD5A1, SRD5A2, and SRD5A3 expression in BPH patients who had been exposed to 5α-reductase inhibitor (5ARI) treatment displaying a significant increase in SRD5A2 expression when on a 5ARI and a significant decrease in SRD5A3 expression when on 5ARI therapy (n=90). Bars are presented as standard deviation, p-value: non-parametric Mann-Whitney test.

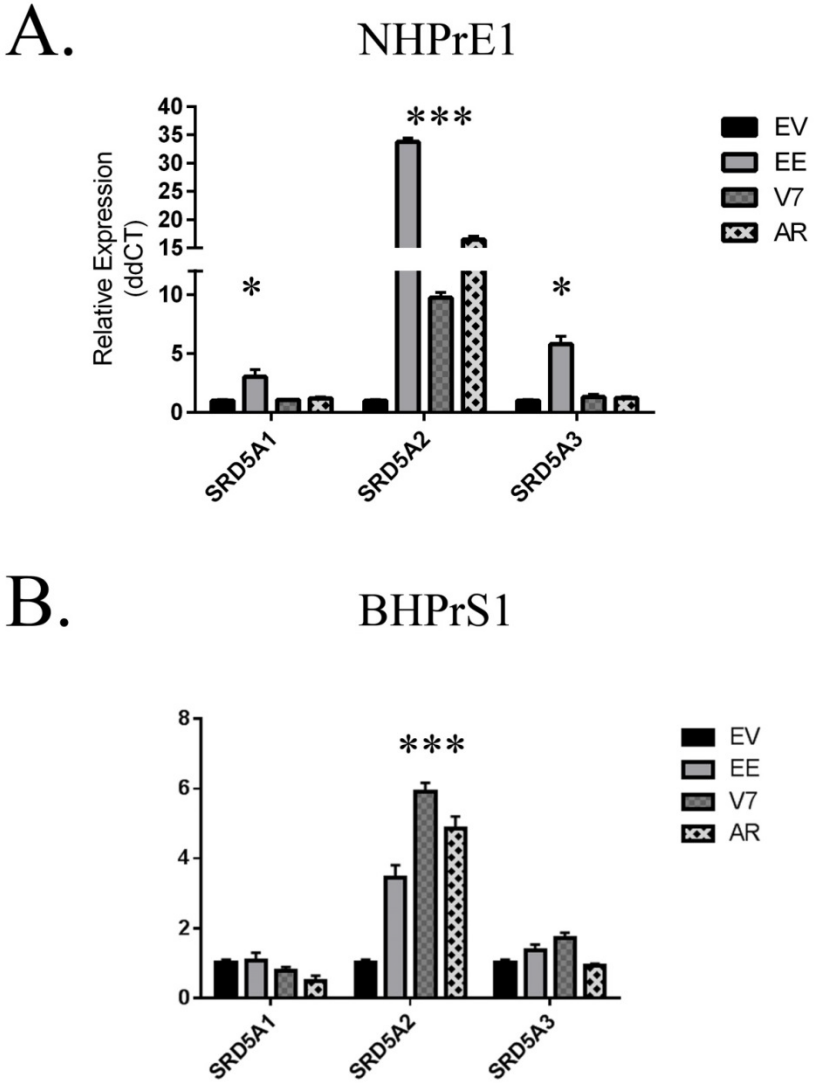


Figure 22. NF-κB significantly increases SRD5A expression. qPCR analysis of SRD5A1, SRD5A2, SRD5A3 expression in our benign epithelial cell line (**A.**) and our benign stromal cell line (**B.**) transduced with empty vector (EV) ,constitutively active NF-κB (EE), overexpressed androgen receptor full length (AR), and androgen receptor variant 7 (V7). **A.** NHPrE1-EE resulted in a significant increase expression of all three SRD5A isoforms while NHPrE1-AR and NHPrE1-V7 resulted in a significant increase in SRD5A2 expression. **B.** BHPrS1-EE, BHPrS1-AR and BHPrS1-V7 resulted in a significant increase in SRD5A2 expression. All experiments were performed three times with triplicate repetitions. Bars are presented as standard deviation, p-value: 2way ANOVA. * $P < 0.05$, *** $P < 0.001$.

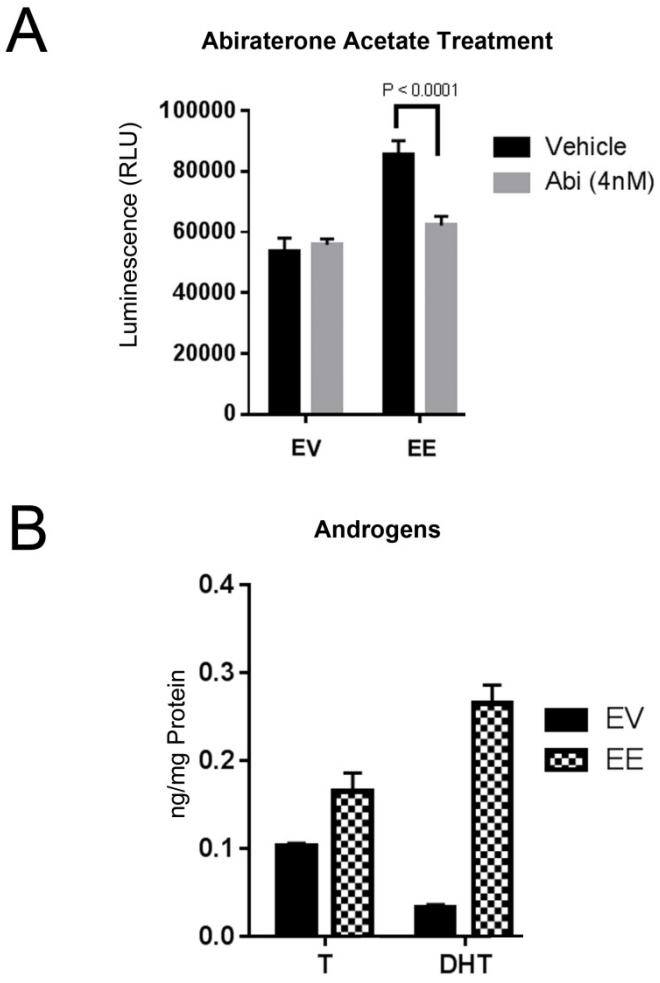


Figure 23. NF-κB increases *de novo* androgen synthesis. **A.** NHPrE1-EV (empty vector) and NHPrE1-EE (constitutively active NF-κB) cell lines treated with 4μM of Abiraterone acetate (Abi) to inhibit androgen synthesis. There was a significant decrease in viability in NHPrE1-EE over a 5 day period when compared to vehicle. Bars are presented as standard deviation, p-value: 2way ANOVA. **B.** Measurement of 2 endogenous androgens [testosterone (T), dihydrotestosterone (DHT)] in NHPrE1 cell lines grown in charcoal stripped serum over a 5 day period. NHPrE1-EE cell line showing slightly increased de novo synthesis of T and a large increase in DHT when compared to NHPrE1-EV (n=3).

significant ($P < 0.01$) reduction in cell viability (Figure 23A). HPCL tandem mass spectrometric detection (LC/MS/MS) method was used to quantitate testosterone (T), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), androstenedione (ASD), and androsterone (AND) in cell lines grown in CS serum over a 5 day period. As shown in Figure 23B, the NHPrE1-EE cell line can all produce T. However, the -EE cell line had a higher production of DHT when compared to NHPrE1-EV cells. This could be due to the increased expression of SRD5A isoforms as shown in Figure 22A. DHEA, AND, and ASD were below the limit of detection in all cell lines.

Knockdown of SRD5A isoforms causes a reduction in cell viability and AR target gene expression

To determine whether there is a contribution of SRD5A1, -2, and -3 in maintaining cell viability, we knocked down expression of the isoforms using siRNA and assayed for cell viability after 3 days and AR target gene expression after 12 hrs. As shown in Figure 24A, knockdown of any of the three SRD5A isoforms in the NHPrE1-EE cell line resulted in a significant decrease in cell viability and SRD5A2 and SRD5A3 knockdown resulted in a loss of the ability to respond to ligand. In the NHPrE1-V7 cell line we saw that knockdown for SRD5A1, SRD5A2, or SRD5A3 had no significant effect on cell viability (Figure 24B). This could be because NHPrE1-AR-V7 cell line does not express AR-FL

and the knockdown of each SRD5A isoform does not affect cellular viability. When looking at AR target gene expression in the knockdown cell lines using qPCR, we saw that NHPrE1-EE cells had a significant increase in PSA and TMPRSS2 gene expression in the presence of T (Figure 25A). In the NHPrE1-V7 cell line, there was no change in AR target gene expression with the knockdown of the SRD5A isoforms (Figure 25B).

NF-κB induces resistance to castration

To determine how NHPrE-EV, -EE and -V7 cells lines respond to androgen withdrawal *in vivo* we used a tissue recombination model. The epithelial cells lines were recombined with prostatic inductive rUGM and grafted under the renal capsule of castrated or castrated and testosterone supplemented nude mice. Treatment is illustrated in Figure 26A. Each cell line was recombined with rUGM in 3 mice in each group. The total number of grafts per cell line per group was 6. As proof of principle that the castration worked, the seminal vesicles and associated prostatic complex were dissected and compared. Seminal vesicles are androgen dependent organs and regress under castrate conditions as shown in Figure 26B. H&E staining of mice maintained under continuous androgenic stimulation displayed prostatic ductal development, with luminal and basal epithelial cells surrounding a lumen (Figure 26C). Under castrate conditions EV and AR-V7 grafts failed to develop epithelial glandular architecture. In

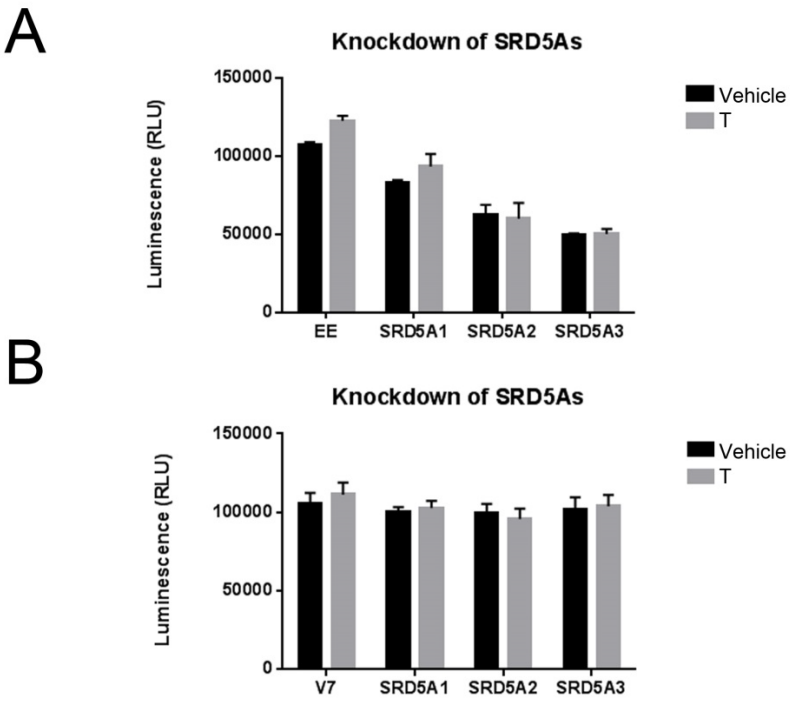


Figure 24. Knock-down of SRD5A2 results in significantly decreased cell viability. NHPrE1-EE (constitutively active NF-κB - **A**) and NHPrE-V7 (expressing AR-V7- **B**) cell lines were transfected with siRNA corresponding to SRD5A1, SRD5A2, and SRD5A3, incubated in the absence/presence of Testosterone (T) and cell viability was examined over a three day period. **A.** NHPrE1-EE cell line responded to T and showed a significant ($P < 0.001$) increase in viability. Knock-down of SRD5A1, SRD5A2, and SRD5A3 resulted in a significant ($P < 0.01$) decrease in cell viability even in the presence of androgens when compared to EE. SRD5A1 knockdown was still able to respond to T while SRD5A2 and -3 knock down cells did not respond to T. **B.** NHPrE1-V7 cells line had no change in viability when SRD5A1, SRD5A2, or SRD5A3 were knocked down even in the presence of androgens. All experiments were performed three times with triplicate repetitions (n=9). Bars are presented as standard deviation, p-value: 2way ANOVA

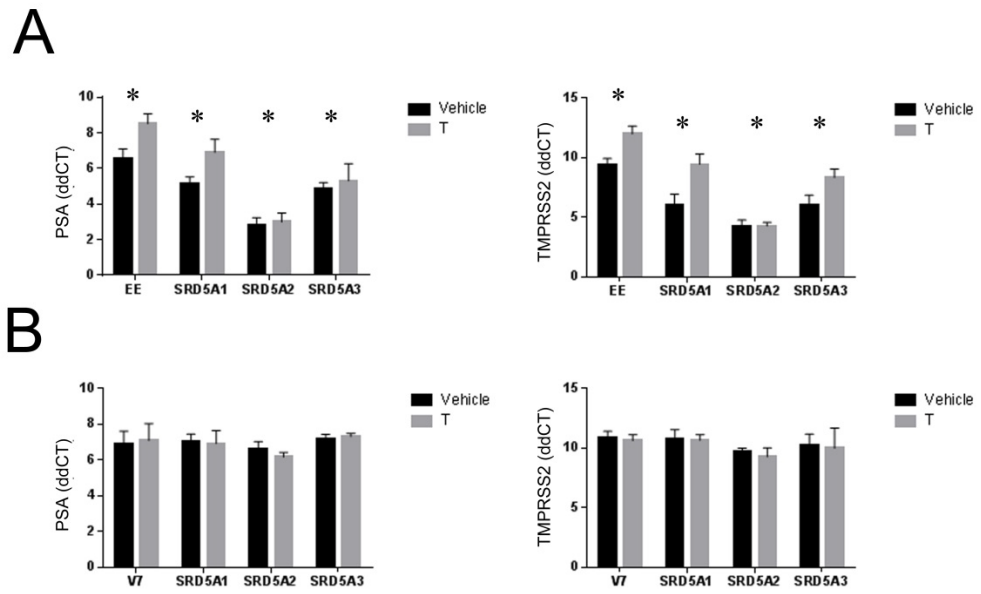


Figure 25. Knock-down of SRD5A2 results in a significant decrease in androgen receptor target gene expression in cells with active NF-κB but not in cells overexpressing AR-V7. NHPrE1-EE (constitutively active NF-κB - **A**) and NHPrE-V7 (expressing AR-V7 - **B**) cell lines transfected with siRNA corresponding to SRD5A1, SRD5A2, and ARD5A3 were incubated in the absence/presence of Testosterone (T). AR target gene expression was examined 12 hours later. **A.** qPCR of NHPrE1-EE for AR target genes PSA (right) and TMPRSS2 (left) resulted in a significant increase in mRNA expression in the presence of androgens when compared to vehicle ($P < 0.001$). SRD5A1 knock down resulted in a significant decrease in PSA and TMPRSS2 expression when compared to EE even in the presence of androgens ($P < 0.01$). SRD5A1 knock down cells were still able to respond to androgens when comparing PSA and TMPRSS2 expression to SRD5A1 knockdown vehicle ($P < 0.001$). SRD5A2 and SRD5A3 knock down cells had a significant decrease in PSA and TMPRSS3 expression even in the presence of androgens when compared to EE ($P < 0.01$) and lost the ability to respond to androgens when compared SRD5A2 and SRD5A3 knock down vehicle treated. **B.** qPCR inNHPrE1-V7 cell lines for AR target genes PSA and TMPRSS2 resulted in no change in mRNA expression in the presence of androgens when compared to vehicle or when SRD5A1, SRD5A2, or SRD5A3 were knocked down. Bars are presented as standard deviation, p-value: 2way ANOVA. All experiments were performed three times with triplicate repetitions (n=9)

contrast grafts in which NF-κB is constitutively activated in the epithelium (the -EE grafts) displayed glandular development (n = 4/6) (Figure 26D).

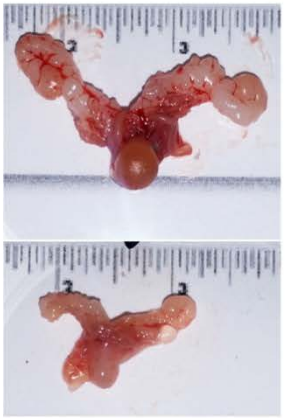
We examined SRD5A isoform levels in these grafts (n=6) by qPCR. As shown in Figure 27A left, in the presence of testosterone -EE recombinants had a significant increase in SRD5A2 and SRD5A3 when compared to -EV while -V7 recombinants had a significant increase in only SRD5A2. Under castrate conditions, -EE grafts showed a significant increase in all three SRD5A isoforms while -V7 grafts only had a significant increase in SRD5A2 (Figure 27A right).

When looking at the AR target genes PSA and TMPRSS2 in these grafts, both -EE and -V7 showed a significant increase in gene expression in the presence of testosterone, as compared to -EV controls (Figure 27B left) Under castrate conditions the -V7 recombination grafts showed a significant decrease in AR target gene expression under castration compared to androgenized conditions while the -EE grafts expressed similar levels of these AR target gene expression in the presence and absence of exogenous androgens (Figure 27B right).

A

Treatment	Group	
	I	II
Castration (Cx)	+	+
Testosterone	+	-

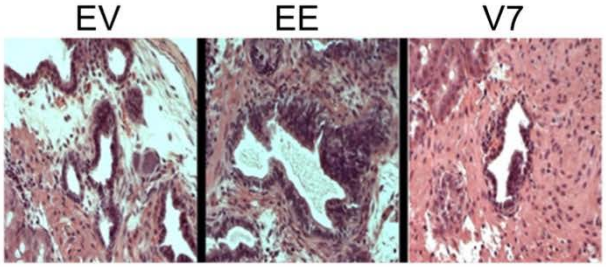
B



Cx +T

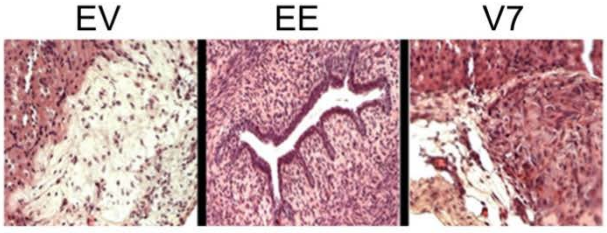
Cx

C



Cx +T

D



Cx

Figure 26. Constitutively active NF-κB results in xenograft growth under castrated conditions. **A.** Treatment diagram for Athymic Nude-Foxn1nu mice. NHPrE1-EV (empty vector control), NHPrE1-EE (constitutively active NF-κB), and NHPrE1-V7 (overexpressing AR-V7) cells where recombined with rat urogenital sinus mesenchyme and grafted under the kidney capsule of mice. All mice were castrated and supplemented with Testosterone (T) pellet for two weeks after grafting. After two weeks mice where then separated into two groups. Group 1 (mice n=9) had a replacement T pellet and Group 2 (mice n=9) had their T pellet removed to mimic 5ARI therapy. Growth was then assessed after six weeks and mice were euthanized. **B.** Representative images of seminal vesicles from the two groups. Seminal vesicles are androgen dependent organs and regressed under castrated conditions (Group 2). **C.** Representative H&E images of recombinants containing NHPrE-EV (n=6), NHPrE1-EE (n=6), and NHPrE1-V7 (n=6) cells in Group 1 displaying glandular development. D. Representative H&E images of NHPrE-EV (n=6), NHPrE1-EE (n=6), and NHPrE1-V7 (n=6) cells in Group 2 display stromal cells in NHPrE1-EV grafts, glandular development in NHPrE1-EE grafts, and no glandular development in NHPrE1-V7 grafts.

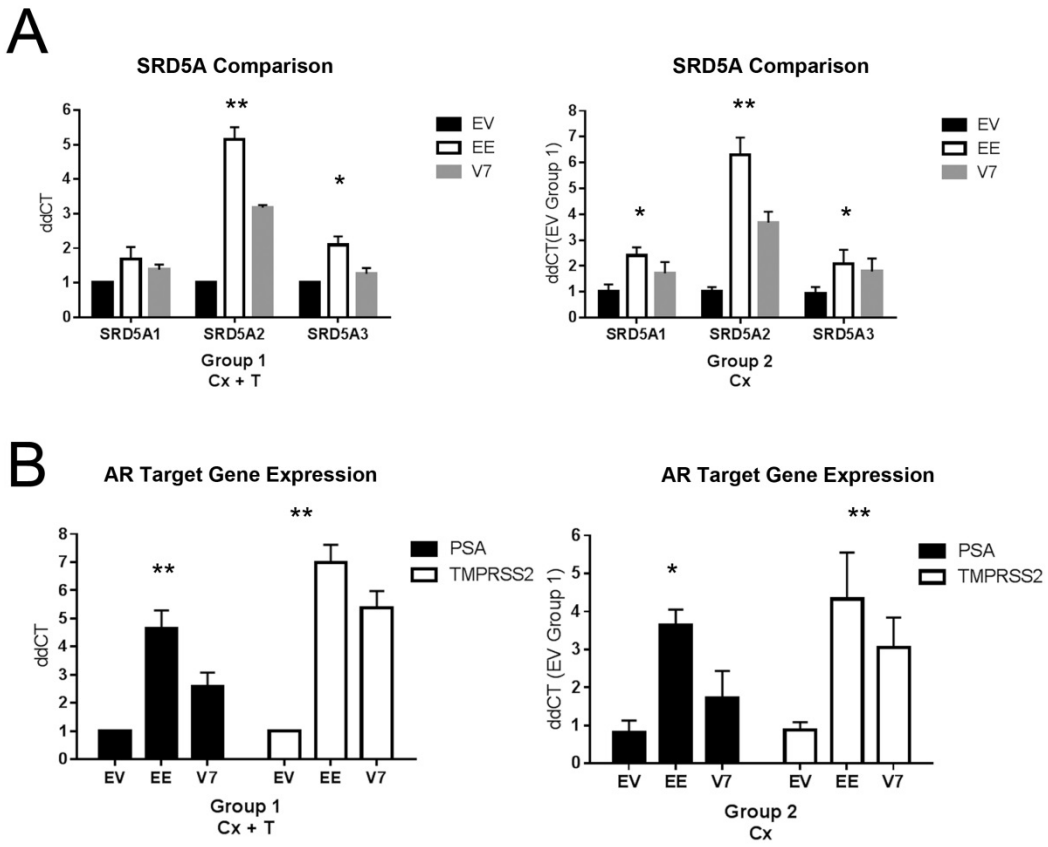


Figure 27. Constitutively active NF-κB results in significant increase of SRD5A and androgen receptor target gene expression under castrated conditions. All mice were castrated and supplemented with Testosterone (T) pellet for two weeks after grafting. After two weeks mice where then separated into two groups. Group 1 (mice n=9) had a replacement T pellet and Group 2 (mice n=9) had their T pellet removed to mimic 5ARI therapy. qPCR analysis of mRNA expression of SRD5A isoforms (**A**) and AR target gene expression (**B**) were performed on tissue recombinant samples. **A.** In the presence of androgens there was a significant increase in SRD5A2 and SRD5A3 ($P < 0.01$) in NHPrE1-EE (constitutively active NF-κB) grafts and in SRD5A2 ($P < 0.001$) in NHPrE1-V7 grafts when compared to NHPrE1-EV (right panel). When compared to NHPrE1-EV grafts in the presence of androgens, NHPrE1-EE grafts under castrated conditions resulted in a significant increase in all three SRD5A ($P < 0.01$) isoforms, while NHPrE1-V7 grafts showed an increase in SRD5A2 ($P < 0.01$) (left panel). **B.** qPCR of AR target genes PSA and TMPRSS2 in the presence of androgens showed a significant increase in NHPrE1-EE and NHPrE1-V7 ($P < 0.001$) grafts when compared to NHPrE1-EV (right panel). When compared to AR target gene expression in NHPrE1-EV grafts under castrate conditions, NHPrE1-EE grafts under castrated conditions showed an increase in PSA and TMPRSS2 ($P < 0.001$) expression while NHPrE-V7 resulted in only a significant increase of TMPRSS2 ($P < 0.01$) expression (left panel). Bars are presented as standard deviation, p-value: 2way ANOVA. * $P < .01$ ** $P < 0.001$) All experiments were performed three times with triplicate repetitions (n=9)

Discussion

Benign prostatic hyperplasia is a common chronic disease in the male population resulting in significant morbidity and associated patient care costs [1, 249]. The primary treatment options for men with BPH are α-blockers and 5ARIs. Combinational treatment with both classes of agent are more effect than individual therapies [182, 184], however within some patients the disease continues to progress and surgery is required to avoid acute urinary retention [182]. These patients who progress to surgery are usually older and, as a group, are not prime candidates for surgical intervention [200]. Therefore, we need to develop new approaches to either develop new therapies or combat resistance to existing medical approaches for the group of men who fail therapy and progress to surgery. Our goal in this study was to try elucidating a mechanism by which BPH patients become resistance to 5ARI treatment.

Testosterone is converted to the more biologically active androgen dihydrotestosterone (DHT) in the prostate. DHT is required for the normal development of the prostate, and is the major active androgen in both BPH and prostate cancer [277-279]. The conversion of testosterone to DHT is mediated by the 5α-reductase (SRD5A) family which is present in 3 isoforms [269-272]. In normal prostate, SRD5A2 is the predominant enzyme [269, 280], however in prostate cancer there is a switch from SRD5A2 to SRD5A1 becoming the predominant enzyme [280], and this switch can predict biochemical recurrence of

prostate cancer, metastatic potential, and reduced efficacy of 5ARIs [184, 269, 270, 280]. SRD5A3 has been linked to glycosylation [272], linked to hormone-refractory prostate cancer [53], and can be inhibited by both Dutesteride and Finasteride [271]. Audet-Walsh et al were the first study to show that gene polymorphisms in both SRD5A1 and SRD5A2 were linked to the biochemical recurrence of prostate cancer [269] and these polymorphisms in SRD5A genes could affect the production rate of dihydrotestosterone and the corresponding local exposure to androgens of androgen-responsive cells, thus undermining the basis of androgen deprivation strategies. Such a scenario is also possible in relation to BPH patient's response to 5ARI therapy. Uemura et al studied the enzymatic activity of SRD5A3 [53] and Cantagrel et al uncovered the enzymatic and biological function of SRD5A3 and demonstrate that SRD5A3 encodes a polyprenol reductase that is essential for N-linked glycosylation of proteins in yeast and mammals [273], while SRD5A1 and SRD5A2 only convert T to DHT [269, 270]. One surprising result that Cantagrel et al showed that contradicts Uemura et al is that SRD5A3 is not involved in steroid hormone formation or sexual development but instead plays a crucial role in the N-linked glycosylation of proteins. Uemura et al demonstrated that SRD5A3 is overexpressed, specifically in hormone-refractory (castrate resistant) prostate cancer cells, an androgen-independent phenotype. N-linked glycosylation facilitates the proper

folding and trafficking of proteins and therefore could have a secondary action in the secretion of proteins as well at reductase activity in prostate cancer.

In this chapter we investigated the link between activation of NF-κB and SRD5A levels in BPH. In prostate cancer SRD5A1 and SRD5A2 predict biochemical recurrence of prostate cancer and reduced efficacy in androgen deprivation therapy [269, 270]. In a chapter III [26], we demonstrated that both AR-V7, and to a greater extent, activation of NF-κB, induced resistance to a 5ARI. Here we examine whether 5ARI resistance could be driven by the induction of SRD5A expression. We used a repository of human prostate transition zone tissues from patients that represents a range of BPH/LUTS severity. Using these BPH samples, we saw that SRD5A1 and SRD5A2 levels were significantly higher in patients whose disease had progressed and required surgical intervention when compared to patients with incidental BPH that had undergone surgery for prostate cancer. In contrast SRD5A3 expression moved significantly in the opposite direction (lower in surgical patients). Dutasteride and Finasteride have been shown to have a higher affinity for SRD5A3 than -1 and -2 [271]. We saw that SRD5A2 significantly correlates with increased TRUS volume and AUASS while SRD5A3 was inversely correlated with increased TRUS volumes. We also saw that when BPH patients had been exposed to a 5ARI therapy, SRD5A2 was significantly higher and SRD5A3 was significantly reduced. Since SRD5A2 is the primary enzyme in the prostate that converts T to

DHT [269-271], its increased expression in more symptomatic patients could explain why these patients failed 5ARI therapy and progressed to surgery. Increased NF-κB and associated increased SRD5A2 provide a potential mechanism by which increases in SRD5A enzyme expression swamp the suppressive action of the 5ARI.

A series of *in vitro* experiments were performed to determine the effects of NF-κB on SRD5A levels. We have previously shown that NF-κB is upregulated in the surgical BPH patient samples and that NF-κB activation can induce AR and AR-V7 expression [26]. Here, we wanted to determine whether NF-κB activation could affect SRD5A expression in benign human prostatic cell lines. We show that NF-κB activation upregulates all three isoforms of SRD5A in epithelial cells and only SRD5A2 in a stromal cell line. Consistent with NF-κB activation, AR-FL and AR-V7 overexpression in epithelial and stromal cell lines both upregulated the SRD5A2 isoform. We also show by HLPC LC/MS/MS analysis that the NHPrE1-EE and -EV cell lines synthesize androgens although only the NHPrE1-EE cells produced measurable DHT. We also show that down-regulation of SRD5A2 and SRD5A3 using siRNA significantly reduced cell viability and the cells ability to respond to androgens. Taken together, this suggests that NF-κB induction of all SRD5A isoforms, but significantly SRD5A2, could explain why patients fail 5ARI therapy. This is supported by research showing that an increase

of SRD5A1 over SRD5A2 correlates with prostate cancer progression [281, 282] and that SRD5A3 expression correlates with progression to hormone-refractory (castrate resistant) prostate cancer [53]. The present study shows that the activation of NF-κB can induce synthesis of T and can also upregulate all SRD5A isoforms. This mechanism can explain, at least in part, why some BPH patients either never respond to, or ultimately fail 5ARI therapy.

To better understand the relationship between *de novo* synthesis of T, SRD5A expression, and 5ARI therapy, we used a tissue recombination grafting model, where NHPrE1-EV, -EE, and -V7 cell lines were combined with rUGM and grafted under the kidney capsule in the presence and absence of androgens for 6 weeks. We show that after six weeks of castration our NHPrE1-EE cell line was still able to undergo glandular epithelial development, showing luminal and basal cell arrangement and was able to turn on AR regulated genes. This shows that under castrated conditions *in vivo* activation of NF-κB can circumvent androgen ablation and supports a mechanism by which NF-κB can confer resistance to a 5ARI. BPH is thought of as a stromal disease with epithelial growth as a secondary consequence, a concept first described by McNeal and validated by Cunha and colleagues [33, 227]. Paracrine interactions between the stroma and epithelium are important for the normal development of the prostate and the development of BPH [38, 41, 42, 46-48]. Immune/inflammatory cells are a component of and influence the behavior of the stroma. Activation of NF-κB,

subsequent *de novo* synthesis of T, and upregulation of SRD5A enzymes as shown here provides a mechanism by which inflammation plays a role in BPH progression and 5ARI failure.

In summary, the data presented above provide a potential mechanism to explain the previously observed links between prostatic inflammation and 5ARI resistance [26, 197]; whereby, aberrant activation of NF-κB drives AR-V7 expression, *de novo* synthesis of T, and upregulation SRD5A isoforms. The AR-V7 is a constitutively active form of the androgen receptor while the increased synthesis of local androgens and elevated levels of SRD5A would drive the activation of AR-FL. Linking together these pathways provides a fundamental mechanism that explains failure of medical therapy for BPH patients. This insight can result in new approaches to combat BPH progress and 5ARI failure.

CHAPTER V

Discussion

BPH is the most common tumor-like disease of the aging male [1] and is believed to have an inflammatory etiology [197, 198, 201]. As BPH patients progress, they can be treated with medication or surgery for severe LUTS. BPH is considered a health issue because of the urethral constriction, co-morbidities that present with the disease, and its high cost to manage. To manage the disease, patients are put on medical therapies that consist of α–blockers and/or 5ARIs. While a number of patients initially respond, a third of these patients fail therapy, and will progress to surgical intervention to manage the disease [182]. Discovering the underlying factors and mechanisms that drive this resistance is of particular importance.

Comorbidities and BPH outcomes – a role for inflammation

Comorbidities such as T2DM (NIDDM) [173-175], increased obesity (BMI) [176-179], as well as inflammation [31, 32, 283, 284] are risk factors for, and/or present with, BPH. All three have also been associated with increased disease severity and therapeutic failure. T2DM is a type of insulin resistance and can be preceded by MetS. Research shows that abdominal obesity precedes diagnostic factors for MetS [285] in that the pro-inflammatory action of the

visceral fat (production of adipokines) is a predisposing factor for outcomes [286], such as insulin-insensitivity and hypertension. Visceral fat, which is associated with the aforementioned factors, could influence the progression of BPH. The relationship between obesity and insulin resistance is widely described [287-289]. The clinical consequences of insulin resistance include T2DM. Obesity is linked with T2DM and prostate volume has repeatedly been associated with obesity [181, 290]. This is of particular importance as increased prostate volume strongly predicts adverse clinical outcomes associated with BPH [252, 291]. It has also been shown that in men with BPH who present with T2DM [170] or men that are obese, the efficacy of 5ARIs such as Finasteride [292] and Dutasteride [293] decreases.

Epidemiological and histopathologic research provides clinical evidence of a correlation between metabolic disorders within MetS and the pathogenesis of BPH [216, 252]. The induction of inflammatory states (physical characteristics such as pain, swelling and molecular characteristics such as increased IL-1, -8 and TNF expression in the prostatic cells seems to be an important linking factor between BPH and metabolic disorders [202, 207, 294]. Obesity, a component of MetS, presents an inflammatory component and it is now widely agreed that obesity is also associated with a state of low-grade chronic inflammation [295, 296]. Recent studies have shown that measures of body fat positively correlate with serum levels of inflammatory proteins, and markers of abdominal obesity

(e.g. hip to waist ratio) seem to be more strongly associated with inflammatory markers than BMI [297, 298], which indicates that central obesity has greater impact on inflammation. Also, early studies suggested a link between BPH and inflammation, with the extent and severity of the inflammation corresponding to the magnitude of prostate enlargement [299, 300]. Moreover, analysis from The Medical Therapies of Prostate Symptoms (MTOPS) trial [182], confirmed by the Reduction by Dutasteride of Prostate Cancer Events (REDUCE) trial [197] demonstrated that increased inflammation was associated with an increase in therapeutic failure of a 5ARI.

A majority of observational studies suggest that inflammation is linked to the development of BPH and LUTS and correlates with treatment failure of 5ARI. The mechanisms underlying this relationship are still unclear. One potential explanation is that MetS, which promotes systemic inflammation, could mediate this connection. Inflammation has been implicated as a primary stimulus for prostate carcinogenesis, and it is possible that BPH represents a non-malignant proliferative pathway that is also promoted by inflammatory mediators [170].

Expression of NF-κB, a key pro-inflammatory transcription factor, in BPH

NF-κB is a key pro-inflammatory transcription factor. We used our repository of human prostate transition zone tissues representing a range of BPH/LUTS severity to determine NF-κB activation. The results from this study

indicate that chronic activation of NF-κB is associated with BPH progression. This is supported by previous work indicating that progressive BPH is associated with inflammation [182, 197]. We showed that as patients progress to surgery, either because of failed therapy or severe symptoms, total NF-κB nuclear expression increased when compared to incidental patients with lower symptom scores (Figure 7A). We also show that NF-κB activation (as determined by P-p65 nuclear localization) was significantly higher in the epithelial tissue when compared to stroma in both incidental and surgical patients. Also, the epithelial compartment in the surgical patients presented with the highest expression of NF-κB (Figure 7B). This, and a previous finding in our laboratory that demonstrated that increased expression of AP-1 transcription factors, which are pro-inflammatory, was associated with advanced disease [200], demonstrate that BPH progression can be linked to inflammation [26]. This is further supported by our findings that T2DM was positively associated with NF-κB expression and disease severity (Table 4). These findings are consistent with other work showing that inflammation is associated with disease severity, and that MetS associated with increased inflammation exacerbates the progression of BPH [170-175, 177] which is supported by Figure 7C. Identifying the mechanism by which MetS can influence BPH progression is very important in the development of new medical approaches to disease progression.

AR-V7 expression corresponds with BPH disease severity

Since NF-κB has been linked to ARv expression and the progression of CRPC [85, 243, 246], we wanted to determine whether ARv expression is also associated with BPH progression. We examined our repository for the expression of a panel ARv at the mRNA and protein level. While most AR variants were not expressed, or were detected a very low levels, I determined that AR-V7 corresponds to disease severity. Consistent with previous work, AR-FL expression was not significantly different between the two groups [200]. However, AR-V7 mRNA was significantly higher in surgical patients when compared to incidental (Figure 8B). We also determined that increased AR-V7 levels positively correlate with AUASS (Figure 8D) and prostate size (TRUS volume) (Figure 8F). We looked at prostate size because increased volume strongly predicts adverse clinical outcomes associated with BPH [182, 291]. This study suggests that AR-V7 expression could be a mechanism for escape from growth regulation by 5ARI therapy. This is the first report on the expression of AR variants in human BPH. Since it has been previously shown that ARv can induce resistance to 5ARIs in PCa [16, 96, 97], and that NF-κB activation is linked to progression of BPH [26] and CRPC [85, 243, 246], this suggests a linkage between disease progression, activation of NF-κB and expression of AR-V7.

NF-κB induces the expression of AR-FL and AR-V7

We performed a series of *in vitro* experiments to investigate the linkage between disease progression, activation of NF-κB and expression of AR-V7 in patient tissues and whether activation of NF-κB can result in the expression of both AR-FL and AR-V7 in benign epithelial and stromal cell lines. I determined that activation of NF-κB resulted in the expression of both AR-FL and AR-V7 in both epithelial and stromal cell lines (Figure 13 A/B). This finding is consistent with previous observations in PCa, where both forms of the androgen receptor are regulated together by the NF-κB pathway [246]. We also showed, using the ARR_2PB-Luc reporter (responds to activation of the AR but has no binding sites for NF-κB), that in the absence of androgens, chronic activation of NF-κB increased AR activity when compared to EV. The cells that have chronic activation of NF-κB had the ability to retain a ligand driven response in the absence of androgens. In the presence of androgens, these cells had a greater response when compared to no ligand (Figure 11C). In addition chronic activation of NF-κB resulted in increased growth of cells in the absence of testosterone when compared to control (Fig 13 E/F). We performed 3D cell culture, to recapitulate aspects of the living system, and saw that chronic activation of NF-κB can increase 3D colony growth (Figure 16 Figure 17A). When we silenced NF-κB, either by a pharmacological or a molecular approach, We were able to

abrogate AR expression at the mRNA and protein level (Figure 18 E/F) as well as decrease AR activity as monitored by the PSA promoter luciferase reporter (Figure 19C). This suggests that NF-κB induces the expression of AR-FL and AR-V7 and could therefore provide a potential mechanism of how and why patients fail therapy.

AR-V7 expression in vitro can confer resistance to a 5ARI

The data presented here are consistent with the idea that inflammation plays a role in BPH progression and therefore might also play a role in resistance to 5ARI. To investigate whether chronic activation of NF-κB affected cellular response to a 5ARI, we used a cell viability assay based on quantitation of ATP. I also established two new cell lines, NHPrE1-V7 and NHPrE1-AR-FL, using retroviral transduction with the AR-V7 (V7) and AR-FL overexpression constructs, respectively. Chronic activation of NF-κB resulted in a significant increase in cell viability in the presence of a 5ARI. NHPrE-V7 showed no change in viability in the absence or presence of a 5ARI, and NHPrE-AR showed a significant decrease in cell viability in the presence of a 5ARI (Fig 14 A-E). This was also confirmed by looking at AR activity using the ARR2PB-Luc reporter in that chronic activation of NF-κB demonstrated a significant increase in AR activity in the presence of 5ARI, NHPrE-V7 had no change in AR activity in the presence of a 5ARI, and NHPrE-AR showed a significant decrease in AR activity

in the presence of a 5ARI (Figure 14F). These data suggest that chronic activation of NF-κB can induce expression of AR-V7, and that the expression of AR-V7 can confer resistance to a 5ARI.

The role of 5α-reductase isoforms in BPH development and 5ARI treatment

5ARIs inhibit the enzyme, 5α-reductase (5-AR), from converting T to DHT. 5ARIs work by inducing apoptosis secondary to decreasing levels of DHT, thus shrinking the prostate and relieving symptoms of BPH. When 5ARI treatment is introduced and it fails to shrink the prostate, patients progress to surgery, representing a failure of therapy. To understand the mechanism underlying therapy failure, I examined 5α-reductase enzyme expression in our repository of BPH patient samples using immunohistochemistry. 5-AR is expressed in three isoforms, 5-AR1, 5-AR2, and 5-AR3. We determined that 5-AR1 and 5-AR2 isoforms have higher expression in the more severe surgical patients when compared to incidental patients while 5-AR3 is significantly higher in the incidental patients (Figure 20). This was supported by mRNA expression data demonstrating that the message is significantly higher in the surgical patients when compared to incidental (Figure 21A). We showed that 5-AR2 isoforms of 5-AR are positively associated with disease progression determined by AUASS and TRUS volume and also associated with increased prostate volume while 5-AR3 is negatively associated (Figure 21B). These data are supported by recent findings in

PCa suggesting that higher levels of 5-AR type 1 and type 2 predicts biochemical recurrence of PCa, metastatic potential, and reduced efficacy of 5ARIs [269, 270, 301, 302]. The data also suggest and are supported by previous work showing that 5-AR3 is expressed at higher levels than 5-AR1 and 5-AR2 [271]. Taken together this shows that 5-AR isoforms can play a significant role in BPH development since DHT is the major androgen active in the prostate.

When observing our repository of BPH patients, we found that when patients are on a 5ARI, there is no significant change in 5-AR1 mRNA expression. 5-AR2 mRNA is significantly increased when compared to patients that are not on therapy, and 5-AR3 expression is significantly lower in patients that are on a 5ARI treatment (Figure 21C). This is consistent with previous findings showing that 5ARI can decrease 5-AR3 expression and that Dutasteride has a higher affinity for 5-AR3 than the other isoforms [271]. Since our patient data do not specifically detail which 5ARI therapy was given, the non-specificity of which 5ARI the patient was on could be one possible explanation of why we see a significant decrease in 5-AR3 expression but not the other isoforms. The question still remains why 5-AR2 expression is significantly increased compared to patients not on a therapy. This could be because most patients that were on 5ARI treatments were in the surgical group, and we have shown that the surgical group of patients had a significantly higher expression of 5-AR2 or that NF-κB and/or V7 is driving the expression of 5-AR isoforms. Since we have already

shown the linkage between NF-κB/AR-V7 and disease progression, and we have linked NF-κB to AR-V7 expression, we wanted to determine if there is a linkage between NF-κB and AR-V7 expression and 5-AR levels. Such a linkage could provide a mechanism by which patients fail therapy and progress to surgery.

We examined 5-AR levels in our retrovirally-transduced cell lines using chronic activation (EE) of NF-κB, AR-FL and AR-V7 over-expression in both our epithelial and stromal cell lines. We determined that the epithelial NHPrE1-EE cell line had a significant increase in all three isoforms of 5-AR when compared to control. In our stromal cell line, BHPrS1-EE only 5-AR2 was significantly expressed. When looking at the relationship of 5-ARs and AR-V7, we saw that only 5-AR2 was significantly expressed in both our epithelial and stromal cell lines. In AR-FL only 5-AR2 was significantly upregulated in both our epithelial and stromal cell lines (Figure 22). These data suggest that chronic activation of NF-κB can increase all isoforms of 5-AR in the epithelium, while AR-V7 and AR-FL can induce the expression of 5-AR2, and that this increased expression of the enzymes can confer resistance to a 5ARI. We next examined how knockdown of AR-FL can affect 5-AR expression and growth. When looking in the NHPrE1-EE cell line, knockdown of AR-FL reduced growth when compared to EE control and also reduced expression of AR target genes. When AR was knocked down we also saw a significant decrease in all 5-AR isoforms in our EE cell line. This finding is supported by others who show that inhibition of

AR can affect growth of CRPC [303, 304]. In addition, knockout of AR reduced 5-AR isoform expression. This also highlights the effect AR-FL has on 5-AR expression.

Next we wanted to determine what specific role each isoform of 5-AR had in growth and AR target gene expression in response to T. When we knockout each specific isoform, and analyzed for growth and AR target gene expression we saw the greatest effect of 5-AR2 and 5-AR3 (Figure 24 and Figure 25). In the EE cell line, 5-AR3 knockdown affected the growth response, and its ability to respond to T (Figure 24A). In contrast the AR-FL overexpressing cell line could still respond to T but had a significant decrease in growth over a three day period. When NHPrE1-AR-FL, were knockout for 5-AR2, it significantly reduced growth and AR target gene expression such as PSA and TMPRSS2. Knockout of 5-AR1 only affected growth (Figure 24B). These data are consistent with data from the REDUCE and MTOPS trials that indicate that 5ARI treatment can reduce growth of the prostate [184, 197]. This is also supported by previous published data showing that each isoform plays a role in maintaining prostate enlargement [305]. This suggests that one possible reason for failure of 5ARI therapy is increased 5-AR expression induced by NF-κB.

We then wanted to determine the effects of recombination of rUGM with NHPrE1-EE and –V7 in BPH development, *in vivo*, using castrated mice with

supplement T. After 8 weeks we observed that the mice responded to castration by the regression of the host seminal vesicles (Fig 26B), an androgen dependent organ [306, 307]. We also saw that when mice were castrated, EE-expressing cells where still able to develop and express AR related genes such as PSA (Fig 26C). The graft volume was also increased in the EE and V7 cells. Since proper development of the prostate depends on both epithelial and stromal AR-FL, this could explain why we saw development of glandular structures and increased volume compare to V7, whereas V7 alone cannot support glandular formation in the absence of AR-FL protein (Fig 27C), but could support cellular growth through the activation of AR regulated genes. This could possibly be due to chronic expression of NF-κB inducing AR, AR-V7, and 5-AR expression, as discussed above potentially leading to escape of treatment by a 5ARI and continued growth.

Is *de-novo* DHT production an alternative mechanism of ARI resistance

We then wanted to determine if NHPrE1-EE, V7, and AR-FL cell lines produce de-novo DHT and determine if this could be an alternative mechanism of therapy resistance. Therefore we treated with abiraterone acetate (Abi), which inhibits androgen synthesis, to determine if these cells can produce de-novo DHT. NHPrE1-EV and AR had no significant difference in response to Abi, while V7 and EE cell lines showed a significant decrease in growth (Fig 24A). This

suggests that V7 and EE cell lines have the ability to undertake de novo synthesis of androgens. The finding was confirmed by the John Wilton group (Figure 23B) who analyzed NHPrE1-EV, -EE, V7- and AR-FL cell lines. This data showed that our –EE, -V7, and –AR-FL cell lines can undergo de novo androgen synthesis, where our –V7 and –AR-FL cell lines displayed a 1 fold increase when compared to –EE. However in our –EE cell line there was a 900 fold increase in DHT production when compared to –V7 and our –AR-FL cell lines. This could possibly be due to the fact that our –EE cell lines have a significant higher expression of all three 5α-reductase enzymes which could give a mechanism by which BPH patients can become resistant to 5ARI therapy. This could be supported by reports showing that treatment with a 5α-reductase inhibitor minimally impacts serum T, yet results in substantial reduction in intraprostatic DHT [308-310] and that chronic inflammation could supplement DHT synthesis by the upregulation of 5-AR isoforms. Further, increased DHT production is supported by reports showing that prostate tissue hormone levels obtained at the time of autopsy have demonstrated that DHT is the dominant androgen in the human prostate, consistent with the high levels of intraprostatic 5α-reductase [311, 312]. It is important to remember histologically over 90% of men have BPH at time of death, although not all display symptoms [313]. This data also suggest that 5-ARs may contribute to intracrine synthesis of testicular androgens which helps drive 5ARI failure.

Proposed Mechanism of Failure

We have shown by the data presented that NF-κB can induce the expression of AR-FL and AR-V7. I have also shown that NF-κB, AR-FL, and AR-V7 can induce the expression of SRD5A isoforms and that they can produce *de novo* androgen synthesis. Also, we have shown that NF-κB and AR-V7 can induce resistance to a 5ARI. The overall mechanism of 5ARI resistance in BPH is depicted in Figure 28, showing that expression of NF-κB can induce the expression of AR-V7 which can confer resistance to a 5ARI and that the upregulation of SRD5A isoforms by NF-κB and AR-V7 can convert endogenous T to DHT which can further add resistance to a 5ARI.

Future Directions – novel treatment approaches

Given the role of inflammation in the development and progression of BPH by the data shown here, it has been suggested that inhibition of inflammation would decrease the risk of prostatic disease. Nonsteroidal anti-inflammatory drugs (NSAIDs) have been used in prostate cancer and prostatic disease. While NSAIDs have been shown to reduce the symptoms of BPH [314-319] they do not reduce progression of the disease. This is consistent with findings on PCa that NSAID use does not reduce the risk of PCa [320, 321], but the use of NSAIDs could possibly increase the risk of PCa [322]. Therefore I would design a study looking

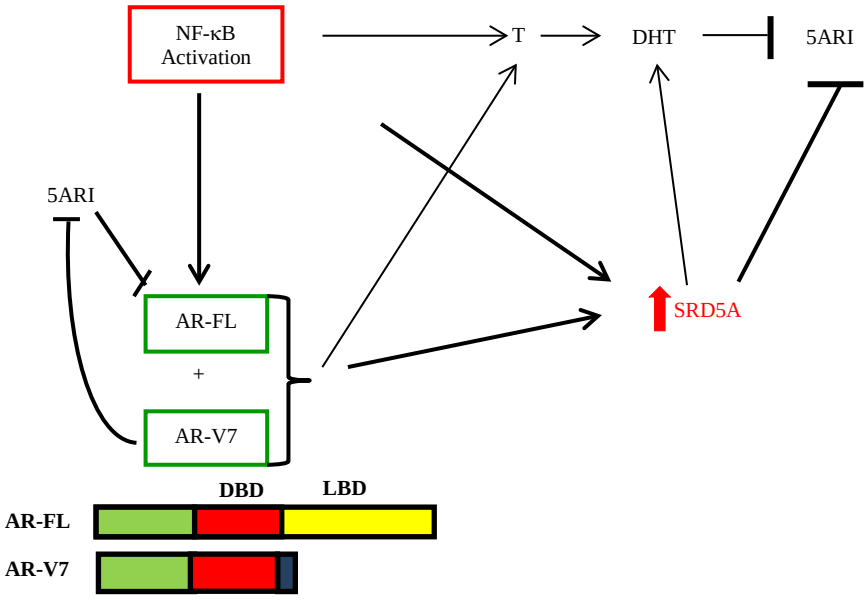


Figure 29: Proposed Mechanism for BPH Progression

at how the addition of low dose methotrexate can be combined with a 5ARI. Since we and others have shown that BPH could be an autoimmune related disease, the addition of methotrexate in therapy could address this. Low dose methotrexate treatment is given to patients with another autoimmune disease such as rheumatoid arthritis with great efficacy.

There are currently several proposed mechanisms for the anti-inflammatory effects of methotrexate on rheumatoid arthritis. Two primary mechanisms are that methotrexate inhibits proliferation of the cells responsible for synovial inflammation in rheumatoid arthritis [323, 324]. The second biochemical explanation is that methotrexate inhibits the synthesis of potentially toxic compounds that accumulate in chronically inflamed tissues [325, 326]. The efficacy of methotrexate is due to some combination of these mechanisms and is responsible for the potent anti-inflammatory effects. In the context of BPH, low dose methotrexate could be given to counteract the activation of inflammation and thereby resensitize the cells to 5ARI therapy. One of the possible problems with methotrexate treatment could be toxicity. Such toxicities include gastrointestinal toxicity, stomatitis, alopecia, marrow suppression, and liver function abnormalities [327]. However, folic acid or folinic acid supplementation has been used to circumvent this toxicity [327].

Future trials should focus on blocking multiple steroidogenic enzymes at once in men with BPH. Blocking several different steps in steroidogenesis

simultaneously may not allow BPH cells time to adjust to loss of androgen stimulation.

Another approach would be to look at the mechanism by which 5-AR isoforms are upregulated. According to Qiagen, a website used for the prediction of promoter binding sites, there could be indirect mechanisms by which NF-κB can regulate each specific isoform. Since, according to Qiagen, there are no specific NF-κB binding sites within any of the 5-AR isoform promoter regions, we should look at how NF-κB target genes could play a role. With respect to 5-AR1 and 5-AR2, there are specific promoter binding sites for PPAR-alpha and PPAR-gamma, both of which have binding sites for AP-1 transcription factors in their promoters. Our laboratory has previously shown that AP-1 transcription factors, such as Jun and Fos, are upregulated in severe symptomatic BPH patients [200], and these factors are regulated by inflammation. There are binding sites for estrogen receptor alpha (ERα) in 5-AR3. ERα has been shown to interact with NF-κB and AP-1 transcription factors [328-330]. Therefore, it would bring insight to the field to determine how chronic activation of NF-κB can affect these target genes indirectly and determine if chronic activation of NF-κB with an inhibitor or knock out of NF-κB target genes could reduce 5-AR levels. This could possibly allow for 5ARI resynthesis due to down regulation of 5-ARs.

Another approach, although more difficult, is development of next generation, highly specific 5ARI drugs specifically targeting 5-AR3. This approach could be an elegant way to treat BPH, since, from our data, 5-AR3 is responsible for the largest reduction in growth when compared to 5-AR1 and 5-AR2. Finasteride has a IC_{50} of 69nM, Dutasteride has a IC_{50} of 6nM and a much higher half-life than Finasteride and higher efficiency in inhibiting 5-AR1 and 5-AR2. This next generation, more selective 5ARI could be developed with lower $IC_{50,}$ greater half-life, and importantly inhibit all three 5-AR isoforms. Such drug development would be very complex and would require multiple disciplines, but the payoff would be substantial due to the advantages derived from an inhibitor that affects all three isoforms of 5-AR and has a greater half-life. The 5-AR system is an important player in human physiology and pathobiology. More work is needed to identify the biochemical characteristics and role of 5-AR3 in BPH and PCa.

Concomitant with age, the immune system becomes less effective at fighting off illness and is associated with autoimmune diseases. BPH incidence therefore increases with age. One could foresee that as our understanding of the immune system and other processes increases, we will develop preventative measures to boost the immune system as a way to combat and or eradicate BPH. Analogous to automobile maintenance, “immune system tune-ups” could keep the body in better shape to detect and prevent not only BPH but many other diseases.

As described in these studies, there is a multifaceted mechanism by which inflammation and MetS could contribute to BPH progression and 5ARI failure, such as the upregulation of AR-V7 and upregulation of all isoforms of 5-AR. As our knowledge expands, treatment and preventative care options will increase. Therefore, the future for BPH treatment holds promise.

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