The Association of Steroid 5-Alpha Reductase Type 2 and Estrogen Receptors in the Prostate

The Association of Steroid 5-Alpha Reductase Type 2 and Estrogen Receptors in the Prostate

Christina Sharkey

A Thesis in the Field of Biology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

May 2021

Abstract

Benign prostatic hyperplasia (BPH) continues to remain a significant health problem. Steroid 5α-reductase 2 (SRD5A2) is the predominant enzyme responsible for prostatic development and growth. Our previous study demonstrated that there is an "androgenic to estrogenic switch" when SRD5A2 is absent in the prostate gland. Here we wished to identify if the expression of estrogen receptors (ER) is associated with the level of SRD5A2 in the prostate. The transcript and protein expression levels of ER was determined in prostatic specimens collected from patients who underwent transurethral resection of the prostate. Using databases GTEx (Genotype-Tissue Expression, n=100), TCGA (The Cancer Genome Atlas, n=496), and Single-cell RNA sequencing data from StrandLab.net, the expression of ER was identified and correlated to *SRD5A2* in human prostate samples. Transcript and protein levels of *SRD5A2*, *ESR1*, and *ESR2* in *SRD5A2* transfected human prostatic stromal cell line (BHPrS1) and epithelial cell line (BPH1) were compared. ERα and ERβ were found to express variably in both the stroma and epithelium compartments. ERα was dominantly expressed in the myofibroblasts, and ERβ was mainly expressed in the endothelial cells in the prostate. *ESR1* was positively correlated with *SRD5A2* in human benign and malignant prostate tissues. The expression of ERα both in transcript and protein levels were promoted with the over-expression of *SRD5A2* in transfected BHPrS1 cells. Overall, this study demonstrated that the expression of ER is associated with SRD5A2 in the prostate.

Acknowledgments

First and foremost, I offer my most sincere gratitude to Dr. Aria Olumi and Dr. Zongwei Wang for the tremendous amount of mentorship throughout the entire thesis process. Thank you for this immensely valuable learning opportunity and the experience of a lifetime at Harvard. This study would not have been possible without your guidance every step of the way. I have gained invaluable research knowledge that I will treasure as I continue my research journey. Thank you to my colleague, Dr. Xingbo Long, for all the helpful assistance and advice.

A special thanks to Dr. James Morris, my research advisor, for your kindness and guidance, especially during the most challenging time of the pandemic.

Table of Contents

List of Figures

Chapter I.

Introduction

Benign Prostatic Hyperplasia

Benign Prostatic Hyperplasia (BPH) is the enlargement of the prostate that contributes to Lower Urinary Tract Symptoms (LUTS). LUTS include urinary frequency, urgency, intermittency, weak stream, straining during urination, and nocturia (McVary et al., 2011). Histologically, BPH is defined as a benign proliferation of the epithelial and stromal compartments of the prostate as well as smooth muscles, mainly in the transitional zone of the gland (McConnell 1991; Nicholson and Ricke, 2011), and in some cases may be associated with atrophy in the peripheral zone (Frost et al., 2019). A few proposed etiologies of BPH include epithelial-to-mesenchymal transition (EMT), changes in the male hormonal milieu, and progenitor-like epithelial cell types surrounding the urethra and peri-urethral prostate zones (Shao et al., 2014; Henry et al., 2018). However, the exact mechanism of BPH development remains unclear.

BPH Epidemiology

As men age, the prostate continues to grow on average 2.5% per year, peaking between 56-65 years old (Bosch et al., 2007; Loeb et al., 2009; Williams et al., 1999). With that, the prevalence of BPH increases as well. Whereas 20% of men have LUTS associated with BPH at age 50, 80% of men are affected between ages 70-80 (Berry, 1984). The World Health Organization estimates that around 210 million males (~6% of the world population) are affected by BPH (Figure 1). It has been estimated that one in

four men will experience LUTS associated with BPH in their lifetime (Lee et al., 2017). Due to differences in BPH disease definition and evaluation methods, an exact prevalence and incidence rate is challenging to conclude (Egan et al., 2016). However, there are a few populational studies that offer relative estimates. Analysis of the Integrated Health Care Information Solutions (IHCIS) database and The Medicare database showed that BPH is the fourth most common diagnosis in the US (Issa et al., 2006). Findings from multiple previous population surveys and cross-sectional surveys in different countries suggest that between 16-42% of men over the age of 40 report moderate-to-severe LUTS based on the International Prostate Symptom Score (IPSS) (Speakman et al., 2015). Using the large-scale EPIC survey to assess the prevalence of LUTS, Irwin et al. (2011) estimated that 44.7% of the world's male population would be affected by LUTS in 2018. In addition, 21.5 million men in the US are projected to have LUTS symptoms in 2025 (Litman & McKinlay, 2007). In terms of risk distributions among different races, Kristal et al. (2006) found that BPH risks were 41% higher for Black and Hispanic men than the White male population. Furthermore, there is a higher risk for BPH surgery in White men with Southern European ancestry (Platz et al., 2000).

BPH Risk Factors

There are many risk factors associated with BPH and LUTS. Previous literature has demonstrated a strong association of BPH/LUTS with prostate size, inflammation, prostate transitional zone fibrosis, hyperglycemia, insulin-resistance, hyperinsulinemia, diabetes, and obesity (Breyer and Sarma, 2014; Macoska et al., 2019; Muller et al., 2013; Simon et al., 2016; Xue et al., 2020). Some of the proposed mechanism of diabetes-related pathogenesis of BPH include overactivation of the sympathetic nervous system,

elevated activities of growth factors, changes in steroid sex hormones, and inflammation (Breyer & Sarma, 2014; Gul & Kaplan, 2019; McConnell et al., 2003). In a cross-sectional study in 2020 using the placebo-arm of the Prostate Cancer Prevention Trial (PCPT), Chadid et al., demonstrated that both total and free testosterone were positively associated with prostatic inflammation. Furthermore, total prostate volume, prostate-specific antigen (PSA), and post-void residual are key clinical predictors for the risk of BPH progression (Crawford, et al., 2006). Also, the risk of developing BPH/LUTS is 67% higher in men with a large prostate size, greater than 40 mL, than men with a small prostate size (Simon et al., 2016). Lastly, extensive research in both human and animal studies has supported that obesity and inflammatory mediators promote prostate growth that is associated with BPH (Muller et al., 2013; Parsons et al., 2013; Xue et al., 2020).

BPH Diagnosis and Treatments

The diagnosis of BPH involves endoscopic evaluations of bladder functions and the prostate's anatomy (McVary et al., 2011). The prostate size assessment is typically from transrectal ultrasound or previous radiological imaging, including CT abdomen/pelvis or MRI of the prostate (Welliver et al., 2019). IPSS or the American Urological Association-Symptom Index (AUA-SI) is used to evaluate the LUTS severity (McVary et al., 2011).

The current treatments for BPH/LUTS include conservative management, medical therapy with alpha-adrenergic-receptor antagonists (alpha-blockers), anticholinergic agents, 5-alpha reductase inhibitors (5ARIs), Phosphodiesterase type 5 (PDE5) inhibitors, or prostate reduction surgical interventions, including minimally invasive surgical

techniques and laser surgical procedures (Brock et al., 2013; McConnell et al., 2003; McVary et al., 2011; Roehrborn et al., 2015; Welliver et al., 2019; Parsons et al., 2020; Foster et al., 2018). Not all patients with BPH/LUTS seek urological consult until symptoms are bothersome enough to affect their quality of life. The burden of severe LUTS can impact multiple aspects of a patient's life, including sleep, work, sexual functions, and mental health (Speakman et al., 2015), in addition to the financial burden that may come with undergoing diagnostic testing (Kaplan et al., 2014). A majority of patients with mild to moderate symptoms prefer conservative management or watchful waiting and implement lifestyle modifications, such as fluid intake or diuretic substance restrictions, to reduce urinary symptoms (Kaplan, 2004; Parsons et al., 2020).

One of the main medical treatments for BPH is 5-alpha reductase inhibitors (5ARI), i.e., finasteride or dutasteride. The most commonly used 5ARI is finasteride, which inhibits 5-alpha reductase type 2 (aka Steroid 5-alpha reductase type 2: SRD5A2), an enzyme that facilitates the conversion from testosterone to dihydrotestosterone (DHT) (Gromley et al., 1992; Niu et al., 2011). Dutasteride inhibits both 5-alpha reductase type 1 and type 2 (Roehrborn et al., 2002) and reduces serum DHT and PSA (La Vignera et al., 2016; Sarkar et al., 2019). In a randomized, double-blinded clinical trial, dutasteride reduced DHT by 93.3% at 52 weeks compared to baseline, whereas finasteride reduced serum DHT by 72.7% (Amory et al., 2007). Long-term 5ARI therapy reduces the total prostate size by as much as 25% (Kaplan et al., 2008; Kim et al., 2018; Roehrborn et al., 2002; Roehrborn et al., 2004). Furthermore, symptom evaluation from an international randomized double-blind study found that combination of finasteride and a PDE5-inhibitor, tadalafil, resulted in significantly greater long-term treatment satisfaction than

finasteride alone (Roehborn et al., 2015). PDE5-inhibitor alone can also significantly improve IPSS at as early as one week of treatment (Porst et al., 2011).

The other medication class for BPH is alpha-blockers, which relaxes the smooth muscle at the bladder neck to relieve LUTS (McConnell et al., 2003). The Prospective European Doxazosin and Combination Therapy (PREDICT) trial performed across 90 European study centers found that doxazosin, an alpha-blocker, significantly improved maximum urinary flow rate and IPSS of BPH patients (Kirby et al., 2002). A cross-sectional study in 2019 showed that 49% of patients used predominantly alpha-blockers for BPH management in the Optum de-identified Clinformatics Data Mart (CDM) database (Welliver et al., 2019). Findings in the same study also suggest that transurethral resection of the prostate (TURP) was the most predominantly elected surgical intervention. While alpha blockers help to relieve the obstructive symptoms of LUTS associated with BPH, anticholinergics can be used to address the storage symptoms for patients with an overactive bladder (Athanasopoulos et al., 2003). Anticholinergics work by stabilizing detrusor activities, which results in improvements of frequency, urgency and nocturia (Athanasopoulos et al., 2003).

While long-term monotherapy of either 5ARI or alpha-blockers leads to significant LUTS improvement, the combination of both medication provided better outcomes. In the Medical Therapy of Prostatic Symptoms (MTOPS) study, data showed that the combination of 5ARI and alpha-blockers reduced the risk of overall LUTS progression by 66% compared to doxazosin or finasteride monotherapy (McConnell et al., 2003). Combination therapy significantly reduced the risk of clinical BPH progression in men with prostate volume greater than 25 mL (Kaplan et al., 2006).

While current medical therapy has been effective in improving LUTS and BPH patients' quality of life, the medication adherence rate of 5ARI is only 18% in the first year and decreases thereafter (Cindolo et al., 2014; Cindolo et al., 2015). Low adherence to BPH medication is significantly associated with undergoing BPH-related surgical procedures and can increase the risk of procedures by 29% (Nichol et al., 2009; Zhang et al., 2020). Furthermore, there is an increase in hospitalization for BPH-related adverse events despite medical therapy in the US (Stroup et al., 2011). Therefore, understanding the mechanism of medication resistance is vitally essential in the management of BPH.

SRD5A2 and Epigenetic Modifications

SRD5A2, the drug target of 5ARI, is the most prominent regulator of prostatic growth and development in the SRD5A family (Ge et al., 2015). There are three sub-types of 5-alpha reductases in human, with SRD5A2 being the most prominent in the prostate (Marks, 2004). SRD5A2 is responsible for synthesizing DHT, a major intraprostatic androgen (Marks, 2004; Ge et al., 2015). Congenital deficiency of SRD5A2 results in a phenotype termed “Guevedoces” or pseudohermaphrodites, where biological males are born with female genitalia until puberty, at which point the male genitalia develops (Marks, 2004). DHT is three to six times more potent than testosterone and is found to promote prostate growth associated with BPH when binding to androgen receptors (AR) (La Vignera et al., 2016; Figure 2). AR facilitates the intercellular communication between stromal and epithelial cells in the prostate (Chauhan, Mehta & Gupta, 2020). Epithelial-AR mainly regulates cellular homeostasis in the adult prostate, whereas stromal-AR activation limits epithelial cell growth in BPH patients (Chauhan, Mehta & Gupta, 2020).

Our previous research (Niu et al., 2011) found that 30% of the adult human prostate tissue do not express SRD5A2. Also, we found that 30% of the BPH specimens were methylated at the *SRD5A2* promoter region (Niu et al., 2011). These findings suggest that epigenetics, specifically DNA hypermethylation at the promoter region of the *SRD5A2* gene, leads to gene silencing and downregulation of protein expression (Niu et al., 2011; Kang et al., 2019). In 2015, our multivariate logistic regression analysis of 96 prostate samples derived from TURP found that BMI and age were significantly associated with DNA methylation at the *SRD5A2* promoter region (Bechis et al., 2015). Furthermore, *SRD5A2* promoter methylation is negatively correlated with SRD5A2 protein expression (Bechis et al., 2015). Bisulfite sequencing comparison of prostate samples with BPH and normal transitional zone control samples confirmed hypermethylation signals across genomic regions and promoter regions in the BPH tissues. Also, there was a negative association between promoter methylation and downstream gene expression (Liu et al., 2020). Therefore, substantial evidence supports the downregulation of SRD5A2 expression by hypermethylation at the *SRD5A2* promoter region and its association with BMI, age, and inflammation.

In addition to BMI, inflammatory mediators can also affect the *SRD5A2* promoter methylation status. Inflammatory mediators, specifically TNF-α and IL-6, regulate the *SRD5A2* promoter methylation via the activation of DNA methyltransferase I (DNMT1) in both BPH tissue and *in vitro* (Ge et al., 2015). DNMT1 is abundantly expressed in developing prostate and upregulated in castrated mice treated with testosterone (Joseph et al., 2018). Elevated DNMT1 activity promotes *SRD5A2* promoter methylation, leading to *SRD5A2* gene silencing (Figure 3) and the decrease of SRD5A2 protein expression. In

human prostatic stromal cells cultured in macrophage conditioned media, *SRD5A2* promoter methylation was significantly elevated, suggesting that proinflammatory mediators are associated with the *SRD5A2* hypermethylation at the promoter region (Xue et al., 2020).

Estrogen Receptors (ERs)

In the 30% of BPH patients with a lack of SRD5A2 expression, the estrogenic pathway is activated via the upregulation of aromatase and phosphorylated ERα (pERα) (Wang et al., 2017). In addition, TNF- α upregulates both aromatase and pERα (Figure 4). Aromatase is a key enzyme involved in the conversion of testosterone to estradiol (Da Silva and De Souza, 2019; Figure 2) and plays a crucial role in the elevation of androgen/ estrogen (testosterone/estradiol: T/E) ratio as men age (Ellem & Risbridger, 2010). Once testosterone is converted to estradiol, the ER signaling pathways regulate prostate growth upon binding to estradiol (Nicholson and Ricke, 2011). However, the molecular regulation of prostate growth via estrogenic pathways in the setting of BPH is unclear and requires further investigation.

The effect of estrogen on cell proliferation has been well established in many different research focuses, especially in breast cancer studies. In the prostate, administration of estrogen in primary prostatic stromal cells promotes cell proliferation and upregulates inflammatory mediators (Chen et al., 2020). Estrogenic effects are carried out by ER, divided into two major subtypes, alpha and beta (Nicholson et al., 2013). Estrogen receptor alpha (protein: ERα /gene: *ESR1*) carries a proliferative effect, whereas estrogen receptor beta (protein: ERβ/gene: *ESR2*) carries out proapoptotic

functions (McPherson et al., 2010; Nicholson et al., 2013). Many studies have investigated each receptor's role regarding their expression and association with androgens in the prostate.

The localization of ER remains controversial in the scientific literature. ERα is generally expressed in the stroma compartment, while ERβ is expressed in the basal and luminal cells of the epithelium (Bonkhoff, 2017; Hetzel et al., 2012; Leav et al., 2001; Song et al., 2016). However, both receptors' expressions were higher in the epithelium in BPH prostate tissue when compared to normal prostate samples (Nicholson et al., 2013; Royuela et al., 2001). Furthermore, multiple studies have found that ERα and ERβ express in both the stroma and epithelium of the prostate (Gangkak et al., 2017; Grindstad et al., 2016), rendering inconsistent findings in the literature.

ER may express differently depending on the prostate zone and the prostate size. While BPH mainly occurs in the central and transitional zones, prostate cancer is mostly found in the peripheral zone (Mahjoub et al., 2020; Laurent et al., 2014). Sehgal et al. (2019) have shown that ERα expression changes in location and cell type throughout the different stages and progression of prostate cancer. In addition, prostate size and the hormonal environment can also contribute to changes in ER expression. Zhang et al. (2016) found a reduction in ERα along with elevation of AR expression in patients with prostates larger than 80 mL, while ERβ expression did not change. Therefore, more robust research methods and quantitative protocols are needed to obtain consistent findings of ER expressions in the prostate.

The mechanism of ER Action

Unlike the ER expression, the mechanism of ER-mediated estrogenic pathways is relatively consistent in the previous studies. The ER mechanism of action can be carried out in two ways, ligand-dependent, where the receptors form dimers after binding to estrogen, or ligand-independent, where phosphorylation of ER can activate cascade pathways downstream (Prins and Korach, 2007).

In prostate cancer tissue, ERα has been found to express in stromal cells and regulate genes associated with tumor progression (Bonkhoff, 2017; Ellem & Risbridger, 2010). ERα is also associated with the induction of prostatic squamous metaplasia in the epithelium compartment of the prostate (Risbridger et al., 2001). Furthermore, research using prostate cancer cell lines has confirmed the role of ERα in EMT, one of the potential pathogenesis of BPH, where ERα reduces the E-cadherin expression and increases vimentin transcript expression upon estradiol treatment (Mishra et al., 2015). Investigation in nongenomic signaling through membrane-ER initiation shows that membrane-initiated ERα can activate AKT signaling pathway, which is responsible for cell proliferation and growth (Majumdar et al., 2019).

ERβ is known to exhibit tumor-suppressive properties in prostate cancer (Ellem & Risbridger, 2010). As an androgen-dependent transcription factor, the antiproliferative function of ERβ may be due to its negative regulation of AR expression (Chaurasiya et al., 2020). Investigation using the androgen-responsive prostate cancer cells found that ligand activation of ERβ leads to downregulation of AR activities (Chaurasiya et al., 2020). On the other hand, when AR is absent, ERβ inhibits AKT signaling and reduces cell migration in prostate cancer cells (Chaurasiya et al., 2007). However, the protein level of ERβ in prostate cancer cells is reduced upon exposure to AR inhibitors, such as

Enzalutamide, for primary prostate cancer and castration-resistant prostate cancer (Abazid et al., 2019), which suggests that the crosstalk between ERβ and AR needs further investigation. Different from ERα, membrane-initiated ERβ activates the MAPK signaling cascades which are responsible for cell proliferation and differentiation (Majumdar et al., 2019). Animal studies shows that administration of selective ERβ agonist in Aromatase KO mice can induce apoptosis in stromal cells and epithelium basal cells of the hyperplastic mouse prostate (McPherson et al., 2010), knockout of ERβ in the ventral lobe of the mouse prostate led to an increase in epithelial hyperplasia and overgrowth of the stroma, further confirming the tumor-suppressive functions of ERβ (Warner et al., 2020).

Research Aims and Hypothesis

Although ER mechanism of action has been investigated in prostate disease research, the association of ER and SRD5A2 has not been investigated. We hypothesized that the expression of ER is associated with SRD5A2 expression. The purpose of the study is to perform an in-depth analysis of the changes in ER expression in response to SRD5A2 changes. Using benign prostatic tissue derived from prostate surgeries, transfected human prostatic cell lines, RNA sequencing database from benign and malignant prostate samples, and single-cell RNA sequencing database from normal prostate cells, the transcript and protein expression of ER and SRD5A2 was determined and compared. The goal of the study is to determine the location of ER expression in both BPH tissue and prostatic cells and to analyze the relationship between ER and SRD5A2.

Given the controversies involved with the research in estrogen receptors in BPH, additional investigation will help elucidate the intricate relationship between the androgenic and estrogenic pathways. The implication of the study is to provide insight into the mechanism of the androgenetic to estrogenic switch. A clear understanding of the key players in the mechanism will help find new therapeutic targets for BPH patients who are resistant to 5ARI. Further investigation into the functions of estrogen receptors in the prostate, specifically in prostate development and growth regulation, is crucial for novel drug development.

Primary objective: To determine the association between SRD5A2 and ER in prostatic tissue and cells.

Specific Aim 1: To examine *SRD5A2, ESR1* and *ESR2* expression in benign and malignant prostate tissues

Methods: Pearson correlation analysis is preformed to analyze RNA sequencing data from GTEx and TCGA databases

Expected results: There will be expression of all three target genes in both prostate tissues types and that *SRD5A2* is associated with one of the ER subtypes

Specific Aim 2: To determine the location of ER expression in prostatic tissue and cells

Methods: Immunohistochemistry of benign prostatic tissue derived from TURP surgeries with semi-quantification of ER protein expression. Single-cell RNA sequence analysis of normal prostate cells and to

determine the primary cell type that each ER subtype is expressed in. Lastly, using immunocytochemistry to stain human stromal and epithelial cell lines for the target protein

Expected results: ERα is expected to mainly express in the stroma, while ERβ is expected to express mainly in the epithelium.

Specific Aim 3: To determine the association between *SRD5A2* promoter methylation and ER expression

Methods: Measure *SRD5A2* promoter methylation levels of each benign prostatic tissue from TURP surgeries. Semi-quantify ER protein expression using immunreactive scores and perform Pearson correlation analysis between methylation and expression

Expected results: A similar negative association as *SRD5A2* methylation and SRD6A2 protein expression is expected for *SRD5A2* methylation and ER protein expression.

Specific Aim 4: To determine the effect of SRD5A2 overexpression on ER expression using transfected human prostatic cell lines

Methods: Transfect human prostatic stromal (BHPrS1) and epithelial (BPH1) cell lines with *SRD5A2* or vector, and to compare *SRD5A2, ESR1* and *ESR2* transcript and protein expressions

Expected results: The changes in total and phosphorylated ER expression is associated with the overexpression of *SRD5A2*

Chapter II.

Material and Methods

Benign prostatic tissue samples were collected from patients with BPH that underwent TURP at Massachusetts General Hospital and Beth Israel Deaconess Medical Center (BIDMC) between 2016-2018. All research experiments were performed at BIDMC.

Single-cell RNA Sequencing Analysis

We used single-cell RNA Sequencing (sc-RNA seq) data library to analyze the percent and average expression of *SRD5A2*, *ESR1*, and *ESR2*. The database was created by Douglas Strand Lab (StrandLab.net, Henry et al., 2018) and consists of sequencing data of normal human prostates stratified by cell type and lineage. The cell quality control processing included singlet and doublet identification using R package DoubletFinder, removal of cells with high-stress signatures indicated by the percentage of mitochondrial content (threshold: >25%), and data filtering based on UMI counts, which measures the quality of cells. After data filtering and quality control, a total of 32,260 single cells were integrated and clustered into eight cell clusters. The cell clusters were displayed in a t-distributed stochastic neighbor embedding (tSNE) plot, including Luminal epithelial cells (LE), Basal epithelial cells (BE), Club epithelial cells (CE), Hillock epithelial cells (HE), Fibroblasts (Fib), Myofibroblasts (Myofibr/mFib), Leukocytes (Leu) and Endothelial cells (Endo). The RNA expression of *SRD5A2*, *ESR1*, and *ESR2* of each sub-cell population was graphical visualized as percentage and average expression.

RNA Sequencing Analysis

RNA sequencing analysis (RNA-seq) was performed using the Genotype-Tissue Expression (GTEx) cohort including fpkm data of 100 benign prostate tissue samples, and The Cancer Genome Analysis (TCGA) cohort including RNA-seq raw data and fragments trans per million (TPM) of 496 prostate cancer tissue samples. RNA expression of *SRD5A2*, *ESR1*, and *ESR2* was analyzed and compared in each cohort.

Methylation-PCR

The *SRD5A2* promoter methylation was measured and correlated with the expression of ERα, ERβ, and SRD5A2. Genome DNA was extracted from ~25mg of frozen benign prostatic tissue samples using QIAamp DNA mini kit (Qiagen, Hidlen, Germany). DNA concentration was determined using a full-spectrum NanoDrop spectrophotometer. MseI (New England BioLabs, Ipswich, MA) was used for restriction enzyme digestion of 4 µg of DNA samples. The incubation cycle was run on BioRad MyCycler for 5 hours at 37 ºC, 20 minutes at 65 ºC and 4 ºC overnight. The digested DNA was separated into methylated and unmethylated DNA using His-tagged MBD2b/MBD3L1 protein complexes in the MethylCollector Ultra kit (Active Motif, Carlsbad, CA). The separated DNA was purified using Chromatin IP DNA purification kit (Active Motif, Carlsbad, CA) before running qPCR. qPCR was performed using Powerup SYBR Green Master Mix (Applied Biosystems, Foster City, CA) in 20µL PCR reaction system. Cycles were run on a QuantStudio 6 Flex Real-Time system (Applied Biosystems, Foster City, CA). Cycling conditions included Hold stage (50ºC for 2 mins and 95ºC for 2 mins), PCR stage (55 cycles of 95 ºC for 15 seconds, 55 ºC for 15 seconds

and 72 ºC for 1 minute) and Melt Curve stage (95 ºC for 15 seconds, 60 ºC for 1 min and 95 ºC for 15 seconds).

PCR analysis was performed using standard curve for CT versus log DNA concentration generated specifically for *SRD5A2* DNA (Figure 5). Using the calculated DNA concentration of the methylated and unmethylated portions from the same sample, the methylation percentage was determined using Equation 1. Each test was triplicated and the average CT was used for the final analysis.

$$\%\, Methylation = \frac{DNA\ [\, methylated]}{DNA\ [methylated] + DNA\ [\, unmethylated]}$$

Equation 1: Calculation of DNA methylation level.

Immunohistochemistry (IHC)

A total of 18 samples were used to visualize the localization of ERα, ERβ, and SRD5A2 in prostate samples by IHC. The samples are the same as those in the methylation analysis. Slides of formalin-fixed and paraffin-embedded prostate tissue were deparaffinized by incubation in xylene and gradient concentrations of ethanol. Antigen was retrieved by IHC-specific steamer followed by 3% hydrogen peroxide incubation. The tissue was blocked using an Avidin blocking system (Vector Laboratories, Burlingame, CA). Primary antibodies were diluted in Biotin antibody diluting serum containing 5% goat serum in TBS with 2% Tween-20 (Vector Laboratories, Burlingame, CA) and incubated at 4 degree Celsius overnight. The following primary antibodies were used: SRD5A2 (1:200; Invitrogen, Carlsbad, CA), ERα (1:100; Invitrogen, Carlsbad, CA) and ERβ (1:100; Invitrogen, Carlsbad, CA).

Tissues were incubated in biotinylated secondary antibody for one hour at room temperature followed by avidin/biotin-based peroxidase system (Vector Laboratories, Burlingame, CA) treatment before DAB chromogen staining (Biocare Medical, Pacheco, CA). Sections were counterstained with Gill hematoxylin and dehydrated before imaging. The reagents used in the staining process were kept the same for all samples to ensure a consistent environment and accurate expression signals.

Slides were imaged with Olympus microscope and CellSense software. We captured field images with the stroma and epithelium sections of the tissue for comparison during imaging. Images were captured in multiple magnifications for visualization purposes. The final analysis was performed with images in 20X. A total of 16 fields (size: 400x400 pixel) were imaged for each slide and assigned a calculated average Immunoreactive Score (IRS). Each image was assigned a score between 0-4 for the percentage of positive staining and another score between 0-3 for the intensity of positive signals (Specht et al., 2015; Table 1). These individual scores of each image were multiplied to form a total IRS score (between 0-12), and 16 total IRS scores were averaged for each patient, which was used for the final statistical analysis. Staining intensity key was created for reference during the scoring process (Figures 21-23). In order to minimalize subjective bias, each sample was assigned a number, and the methylation information was not available at the time of scoring.

qPCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hidlen, Germany). cDNA was synthesized using High-Capacity RNA-to-cDNA kit (Applied Biosystems,

Foster City, CA). PCR was performed using Powerup SYBR Green Master mix (Applied Biosystems, Foster City, CA) on a QuantStudio 6 Flex Real-Time system (Applied Biosystems, Foster City, CA). Cycling conditions are the same as the methylation PCR protocol mentioned above. Primers used were as follows: SRD5A2, forward, 5'-GCAGTGTCTTAGTTGATGAG-3', reverse, 5'-TGTGTTATTAAAACCTGGC-3'; ESR1, forward, 5'-AATACCGCTAAAGCCAAAC-3', reverse, 5'-AGAGATTTCCAAGTTGCTG-3'; ESR2, forward, 5'-ACAGAGAGAGACACTGAAAAGG-3', reverse, 5'-AAAAAGGCCTTACATCCTTC-3'; GAPDH, forward, 5'AGGGGAGATTCAGTGTGGTG-3', reverse, 5'-GGCCTCCAAGGAGTAAGACC-3'. The $2^{-\Delta\Delta CT}$ method was used for data analysis and to determine fold changes in mRNA expression. Individual reaction CT was normalized to the housekeeping gene GAPDH.

Immunocytochemistry (ICC)

Human prostatic stromal cell line BHPrS1 and epithelial cell line BPH1 cells were grown on six-well cell culture plates and fixed using 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Cells were blocked with 5% BSA in PBS for one hour at room temperature and permeabilized using 0.1% Triton X-100 in PBS for 5 minutes at room temperature. Cells were incubated with primary ERα (1:100; Invitrogen, Carlsbad, CA), ERβ (1:100; Invitrogen, Carlsbad, CA), phosphorylated ERα (1:100; Abcam, Cambridge, MA), phosphorylated ERβ (1:100; Abcam, Cambridge, MA), and SRD5A2 (1:100; Abcam, Cambridge, MA) overnight at 4 degree Celsius and followed by secondary fluorochrome antibodies. Cells were mounted using SlowFade Gold antifade reagent with DAPI (Molecular Probes by Life Technologies). Representative images

were obtained using Olympus microscope and CellSense software. Images in multiple magnifications were obtained for visualization purposes.

Human prostatic cell transfection

BHPrS1 cells and BPH1 cells were seeded in six-well cell culture plates in RPMI-1640 medium (Gibco, Waltham, MA) with 10% FBS (Gibco, Waltham, MA) and 1% Penicillin streptomycin solution (Corning, NY). 1% of a lentiviral particle with *SRD5A2* (GeneCopoeia, Rockville, MD) and 0.1% of Polybrene were added to the wells after cell confluency reaches 70%. Cells were incubated at 37 degrees Celsius and 5% CO2 overnight. Fresh medium with 0.5% of puromycin was added 24 hours after the transfection and changed as needed every other day. Cells were maintained and transferred to culture flasks after cell confluency reaches 80-90%. Transfection efficiency was tested using qPCR and ICC.

Statistical Analysis

Transcript fold changes of *SRD5A2, ESR1* and *ESR2* were determined using analysis of variance, student's T-test and Mann-Whitney test as appropriate. Statistical significance was defined as $p<0.05$. Pearson correlation analysis was performed for transcript and protein expression correlation between *SRD5A2* and *ESR1/ESR2* using R software version 1.3.1056. All graphical visualization was generated using Prism 9 GraphPad.

Chapter III.

Results

The Expression of ER in Benign and Malignant Prostate Tissue

Genotype-Tissue Expression (GTEx) and The Cancer Genome Atlas (TCGA) databases were used to determine the correlation between *SRD5A2* and *ESR1/ESR2* RNA expressions. For the benign prostatic tissue, analysis of GTEx database (n=100) found a positive correlation between *SRD5A2* and *ESR1* (R=0.2636, p=0.008, Figure 6A) and no significant correlation between *SRD5A2* and *ESR2* (R=-0.0307, p=0.76, Figure 6B). For the malignant prostatic tissue, TCGA database analysis (n=496) showed a positive correlation between *SRD5A2* and *ESR1* (R=0.3867, p<0.001, Figure 7A), as well as *ESR2* (R=0.2947, p<0.001, Figure 7B). In both databases, the expression of *ESR1* was significantly higher than that of *ESR2* (p<0.001, Figure 6C and 7C).

ScRNA-seq database from StrandLab.net showed the various cell types in the human healthy prostate based on cell marker analysis (Figure 8A). *SRD5A2* is mainly expressed in fibroblasts and mildly expressed in basal epithelial cells (Figure 8B and 8E). *ESR1* is mainly expressed in myofibroblasts and mildly expressed in fibroblasts and hillock epithelial cells (Figure 8C and 8E). Lastly, *ESR2* is mainly expressed in endothelial cells (Figure 8D and 8E). These findings suggest that *SRD5A2* and *ESR1* are co-expressed in fibroblasts. Furthermore, *SRD5A2* transcript expression is associated with *ESR1* in the prostate.

The Expression of ER in BPH Tissue

To visualize the expression of ER subtypes in the prostate tissue, immunostaining was performed using 18 BPH tissue derived from TURP surgeries. First, various SRD5A2 protein expression levels were validated among the benign prostatic tissue (Figure 9). The protein expression of ERα varied among prostate samples (Figure 10A). IRS Semi-quantification showed that ERα is expressed in varying degrees in both the epithelium and the stroma compartments of the prostate (Figure 10B). Transcript expressions of *SRD5A2* and *ESR1* of the prostate tissues showed a significant positive association between *SRD5A2* and *ESR1* transcript expression (R=0.6797, p=0.0019, Figure 10C). *SRD5A2* transcript expression was then divided into high vs. low based on the cycle time (CT) value from qPCR. *ESR1* transcript expression in the high *SRD5A2* group was 3.2 folds higher than that in the low *SRD5A2* group (Figure 10D). Similar to ERa, ERβ is expressed in both compartments of the prostate at varying levels (Figure 11A and 11B). Correlation analysis of the transcript expression showed a significant positive correlation between *SRD5A2* and *ESR2* (R=0.7030, p=0.0011, Figure 11C). *ESR2* transcript expression in the high *SRD5A2* group was 3.9 folds higher than that in the low *SRD5A2* group (Figure 11D). Altogether, our findings suggest that ERα and ERβ express in both the stroma and epithelium of the prostate. Furthermore, *SRD5A2* is associated with *ESR1* and *ESR2* in prostate tissue with BPH.

The association between ER protein expression and *SRD5A2* promoter methylation

Previous research has demonstrated that epigenetic modification of the *SRD5A2* gene regulates the protein expression of the enzyme (Niu et al., 2011; Bechis et al., 2015). Since *SRD5A2* expression is associated with ER expression, the aim here is to

determine if there is an association that exists between *SRD5A2* promoter methylation and ER expression. The *SRD5A2* promoter methylation level was determined for each sample, and the IRS semi-quantification data of IHC was used for the correlation analysis. Among the prostate samples, there was an expected negative correlation between *SRD5A2* promoter methylation and SRD5A2 protein expression (R=-0.4341, p=0.05585, Figure 12A). Although ERα protein expression has a positive association trend with *SRD5A2* promoter methylation, it was not a significant association (R=0.2094, p=0.2069, Figure 12B). On the other hand, there was a significant association between ERβ protein expression and *SRD5A2* promoter methylation (R=0.3578, p=0.02336, Figure 12C). These findings suggest that *SRD5A2* methylation at the promoter region may affect ERβ expression in the prostate.

The ER Expression of Human Prostatic Cell Lines

To verify total and phosphorylated ERα and ERβ expression in the human prostatic cell lines, BHPrS1 and BPH1 cells were stained with corresponding antibodies. Phalloidin was used to visualize the cell structure and to help determine the location of ER expression in the respective cell lines. ERα is mainly expressed in the nucleus of both BHPrS1 and BPH1 cells, with mild expression in the cytoplasm of BHPrS1 cells (Figure 13). In addition, ERβ is only expressed in the nuclei of both cell types (Figure 13). Furthermore, pERα is expressed in the nuclei of BHPrS1 and BPH1 cells, with mild expression in the cytoplasm of BHPrS1 cells (Figure 14). pERβ is expressed in the nuclei of both cell types (Figure 14). Altogether, the findings confirm the nuclear expression of both ER subtypes in prostatic stromal and epithelial cells.

Plasmid Transfection of *SRD5A2* in Human Prostatic Cell Lines

In order to determine the potential association of *SRD5A2* and ER expressions *in vitro*, the *SRD5A2* overexpressed lentiviral particles were transfected into BHPrS1 and BPH1 cells. The transfection efficiency was validated via protein expression (ICC) and transcript expression (qPCR) of *SRD5A2*. ICC images demonstrated the overexpression of *SRD5A2* both in BHPrS1 (Figure 15A) and BPH1 (16A). Quantitative transcript measurements showed significantly elevated *SRD5A2* mRNA levels in BHPrS1 ($p<0.001$, Figure 15B) and BPH1 ($p<0.001$, Figure 16B). Taken together, the findings suggest the successful transfection of *SRD5A2* in cells.

The ER Expression in *SRD5A2* over-expressed Cells

After the SRD5A2 transfected BHPrS1 and BPH1 cell lines were established, ERα and ERβ transcript and protein expressions were compared. Total and phosphorylated ERα and ERβ were expressed in the nuclei of BHPrS1 and BPH1 cells (Figure 17A, 18A, 19A, and 20A). *ESR1* transcript expression was higher in the SRD5A2 overexpressed BHPrS1 ($p<0.0001$) and BPH1 ($p<0.0001$) than respective vector-transfected cells (Figure 17B and 18B). Furthermore, total ERα and phosphorylated ERα protein expressions were elevated in the SRD5A2 overexpressed BHPrS1 cells compared with the vector-transfected cells (Figure 17A). However, the elevation of ERα protein expression was not seen in the SRD5A2 overexpressed BPH1 cells, despite *ESR1* transcript elevation (Figure 18A).

ESR2 transcript expression decreased in the *SRD5A2* overexpressed BHPrS1 cells ($p<0.0001$) but increased in *SRD5A2* overexpressed BPH1 cells ($p<0.0001$) compared to

their respective vector-transfected cells (Figures 19B and 20B). However, total ERβ and phosphorylated ERβ (pERβ) protein expressions were elevated in *SRD5A2* overexpressed BHPrS1 cells, despite a reduction of *ESR2* transcript (Figure 19A). In addition, total ERβ and pERβ protein expressions appear to decrease in *SRD5A2* overexpressed BPH1 cells, despite the increase in *ESR2* transcript levels (Figure 20A). Altogether, the findings suggest that *ESR1* is mainly associated with SRD5A2 in the human prostatic stromal cell line. Overexpression of SRD5A2 may result in ERα enrichment in the prostate.

Chapter IV.

Discussion

Study Significance and Implications

SRD5A2 is an important enzyme that contributes to prostate development and growth. While 5ARI blocks the activity of SRD5A2, 30% of BPH patients do not express SRD5A2 and therefore is resistant to one of the main medical therapy for BPH (Niu et al., 2011). Our previous study has found that when SRD5A2 expression in the prostate is absent, the androgenic pathway for prostate growth switches to the estrogenic pathway (Wang et al., 2017). Specifically, in BPH patients who did not express SRD5A2, aromatase levels increased significantly, facilitating the process of converting testosterone to estrogen (Wang et al., 2017). Animal studies have suggested that increased aromatase expression is associated with prostate development, likely through the activation of ERα via estrogen synthesis (Morais-santos et al., 2018). However, the exact mechanism of the estrogenic pathway associated with BPH is unclear and requires further investigation. The current study investigated the association of SRD5A2 expression and ER expression in the prostate.

The novelty of this study is the in-depth evaluation of the two main estrogen receptors, ERα and ERβ, and their expression in both human prostate tissue and prostatic cell lines. As mentioned previously, there are controversies involved in the expression of estrogen receptors in the prostate. This study showed that ERα and ERβ are expressed variably in both the stromal and epithelial compartment of the prostate in BPH tissues. In addition, total and phosphorylated ERα and ERβ were expressed in the nucleus of both

prostatic stromal and epithelial cell lines. Previous study also found transcript expression of ERα and ERβ in BPH1 cells (Lau et al., 2000). Sc-RNA seq analysis demonstrated that *ESR1* expressed mainly in the myofibroblasts while *ESR2* expressed mainly in the endothelial cells, which is consistent with literature (Middletone et al., 2019).

The results from the analysis of large scale RNA seq databases (GTEx and TCGA) demonstrated a significant association between *SRD5A2* and *ESR1* in benign and malignant prostate tissues. Similar associations were identified when comparing *SRD5A2* expression both in transcript and protein levels with *ESR1* expression in both prostate tissues and BHPrS1 cell lines. The data suggest that ERα expression is associated with SRD5A2 expression in both prostate tissue and cells. RNA-seq analysis by Middleton et al. (2019) found a strong correlation between stromal gene signature and symptom scores in BPH samples (Middleton et al., 2019). Since *ESR1* is mainly associated with myofibroblast, a cell type in the stroma, there is a potential that *ESRI* is also associated with the progression of BPH/LUTS.

Consistent with previous findings from the lab, *SRD5A2* promoter methylation is negatively correlated with SRD5A2 protein expression. Although the analysis did not show a significant association between SRD5A2 promoter methylation with ERα protein expression, there was a significant association with ERβ. The finding indicates that an increase in *SRD5A2* promoter methylation leads to the reduction of the SRD5A2 protein expression, which is associated with an increase in ERβ protein expression. It is possible that since ERβ is an androgen-dependent transcription factor, which is more sensitive to the androgenic changes, it may have an association with epigenetic changes of the *SRD5A2*. For instance, when SRD5A2 expression is absent, there may be a reduction

in AR expression due to the decrease of DHT, which may trigger an elevation in ERβ to compensate. However, further experimentation with a larger BPH sample size is required to examine the mechanisms involved.

Overall, the most consistent finding throughout the study is the strong association between *SRD5A2* and *ESR1* through RNA-seq, transcript, and protein analysis in both prostatic tissue and prostatic cells. There is abundant evidence in the current literature that supports the proliferative functions of ERα and its potential role in BPH progression. Animal study with ERα knockout mice exposed to testosterone and E2 found no change in bladder mass and urinary retention compared to the enlargement found in the wild type or ERβ knockout mice (Nicholson et al., 2012), suggesting that ERα is necessary for bladder growth. Furthermore, administration of ERα antagonist selective estrogen receptor modulator (SERM) prevented BPH related bladder complications, whereas ERβ antagonist did not (Nicholson et al. 2014). The result of this study on ERα as a key therapeutic target for treatment of BPH is consistent with these findings in the animal studies.

Study Limitations

The study was performed with limited human prostatic tissue for the evaluation of transcript and protein expression of *SRD5A2, ESR1*, and *ESR2 in vivo*. Therefore, further investigation under the same experimental condition is needed with a larger sample size to support the findings presented in the current study. There was also limited information regarding to the medical history of the BPH patients. Since the samples are surgical specimens, there is an innate indication that LUTS did not improve. If the patients were on 5ARI, the length of the treatment period prior to surgery was unknown. Therefore, it is

difficult to assess if the patient was drug-resistant before proceeding with surgery. It's also possible that the severity of LUTS was bothersome enough that patients decided to proceed with surgical intervention without medical therapy. In the future, to combat this limitation, the SRD5A2 protein expression of tissue samples will be determined. We expect that patients with low to none SRD5A2 expression might be resistant to 5ARI treatment.

To obtain valid and accurate protein expressions in the tissue samples, choosing the optimal antibodies is crucial in the staining experiments. Since the expression of estrogen receptors are relatively low in the prostate compare to breast tissue, the positive control, there was some difficulty in distinguishing the true expression of the target in IHC staining. Furthermore, quantification of IHC is challenging because of the lack of well-established methods in quantifying protein expressions without subjective bias. Given the difficulty in protein quantification with IHC, in this study we selected to use a semi-quantification scoring system. Although the analysis was performed with attention to avoid subjective bias, the nature of a scoring system is not an unbiased quantification. Western blot analysis was unsuccessful due to the lack of specific and qualitative antibody for SRD5A2. Therefore, future research with an optimized research design for quantifiable protein levels is required to examine the relationships further.

Lastly, the technical process of DNA methylation-specific PCR during the separation of unmethylated and methylated samples is very error-prone. The design uses His-tagged recombinant MBD2b/MBD3L1 protein complexes that have a high affinity to MBD2b for CpG-methylated DNA magnetic beads to separate the methylated and unmethylated DNA, any loss of magnetic beads during the washing step leads to loss of

methylated DNA content and inaccurate CT values. Technical errors during the methylation step will lead to erroneous interpretation and analysis. One possible solution is to make a magnetic stand that is suitable for PCR tubes for the separation step. The magnetic stand will allow for stability during the washing step to eliminate or reduce content loss.

Despite the research limitations, this study is one of the few in-depth analyses of estrogen receptors and SRD5A2 in the setting of BPH. The study used multiple research approaches to confirm the association with *SRD5A2* and *ESR1*. The findings provide useful information regarding the interconnections in the male hormonal milieu.

Future Study

There are a few further directions to take from this study. The first logical follow-up study is to evaluate the effect of 5ARI on ER expression. Using the same prostatic cell lines, we can analyze ER expression upon 5ARI treatment. Since *SRD5A2* is associated with ERα, it can be expected that 5ARI could also reduce the expression of ERα. Dutasteride, an inhibitor of both SRD5A1 and SRD5A2, has been found to mediate ERβ-dependent apoptosis in BPH1 cells (Liu et al., 2016). In addition, prostatic inflammation is associated with a reduction of ERβ expression in the prostate of Rats (Mizoguchi et al., 2017). Rats with induced prostatic inflammation and treated with dutasteride exhibited restored ERβ expression (Mizoguchi et al., 2020). Therefore, 5ARI treatment may potentially change ER expression in the prostate.

Further research on the mechanism of prostate growth facilitated by ERα is also needed. One possible mechanism is the EMT pathways. A previous investigation in the

Rat model found that ERα colocalized with EMT markers, suggesting that ERα can activate EMT events in the prostate (Shao et al., 2014). In addition, ERα has also been shown to promote EMT in benign epithelial cell lines of BPH1 and RWPE1 when exposed to E2 (Shi et al., 2017). Based on the associations, there may be a possibility that SRD5A2 is indirectly correlated with EMT.

It might also be important to assess epithelial-stromal interactions in the setting of ER changes. Similar to differences in epithelial versus stromal-AR signaling, epithelial-ER signaling may differ from stromal-ER signaling as well (Chauhan, Mehta & Gupta, 2020). Using conditioned media of stromal cells treated with ER agonist or antagonist, the epithelial cell proliferation and ER expression may be regulated, which will propose the intercellular ER signaling communication within the prostate. In addition, co-culturing stromal and epithelial cells can also provide clues to ER functions in epithelial-stromal signaling *in vitro*. Understanding the ER expression and association within the prostatic cell network will better inform us of the underlying mechanisms of prostate growth.

Conclusions

ESR1 is positively associated with *SRD5A2* in both benign and malignant prostate tissues. While *ESR1* expression is mainly found in the myofibroblasts, *ESR2* expresses mostly in endothelial cells. In benign prostatic tissues, ERα and ERβ are expressed variably in both the stroma and epithelial compartment, and the transcript levels of ERα and ERβ are positively correlated with *SRD5A2*. In *SRD5A2* overexpressed stromal cells, the protein and transcript expression of *ESR1* is significantly associated with *SRD5A2*.

Altogether, our study supplies more evidence to support the hypothesis of androgenic to estrogenic switch in the prostate. Further assessing the role of ER will better inform us the potential mechanisms of 5ARI resistance in management of LUTS secondary to BPH.

Appendix

Table 1. Immunoreactive Scoring (IRS) semi-quantification system (Specht et al., 2015)

400 x 400 pixel IHC images were individually evaluated based on percentage of positively stained cells and the intensity of the staining to obtain IRS score (0-12).

A (Percentage of Positive cells)	**B (Intensity of staining)**	**IRS score (multiplication of A and B)**
0 = no positive cells	0 = no color reaction	0-1 = negative
1 = <10% of positive cells	1 = mild reaction	2-3 = mild
2 = 10-50% positive cells	2 = moderate reaction	4-8 = moderate
3 = 51-80% positive cells	3 = intense reaction	9-12 = strongly positive
4 = >80% positive cells	**Final IRS score (A x B): 0-12**	

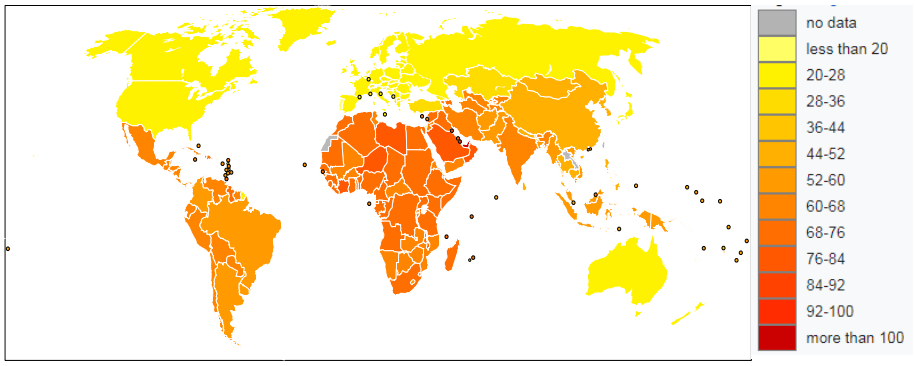

Figure 1: World Health Organization estimates for prevalence of BPH in 2004 (WHO).

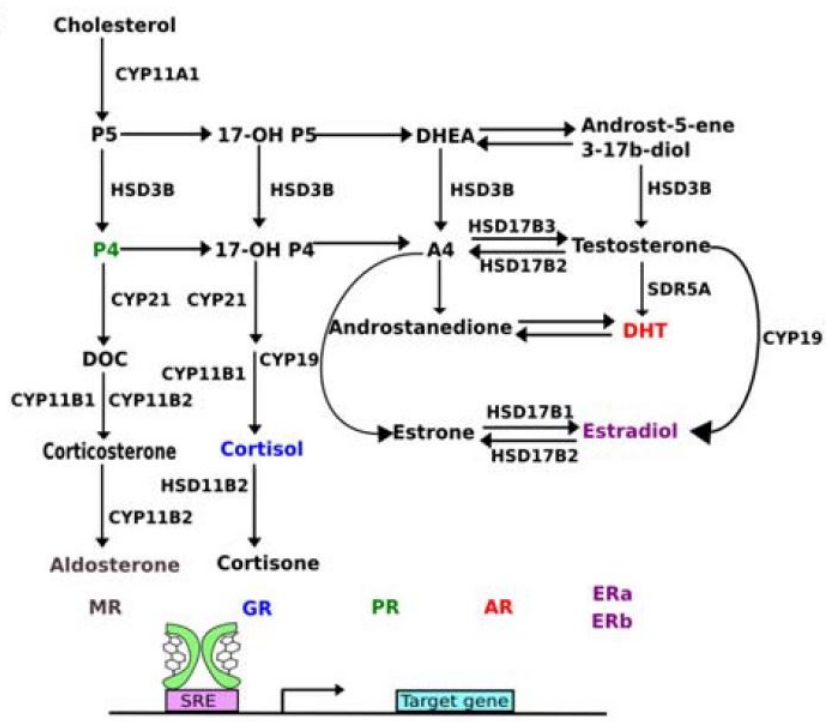

Figure 2: Human steroid sex hormone signaling pathways (Markov et al., 2009)

Steroid hormone signaling pathways and the enzymes that facilitates the corresponding conversions. Colored biomarkers are the hormones with its associated receptors.

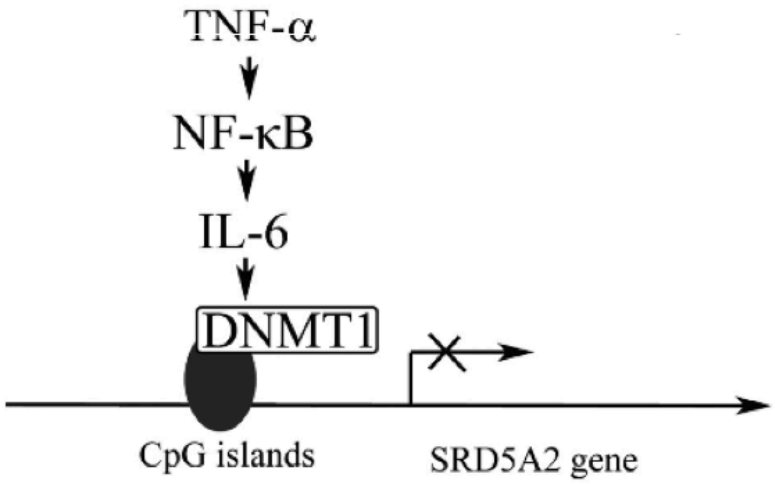

Figure 3. Schematic diagram depicting pathway of the activation of DNMT1 by inflammatory factors (Ge et al., 2015).

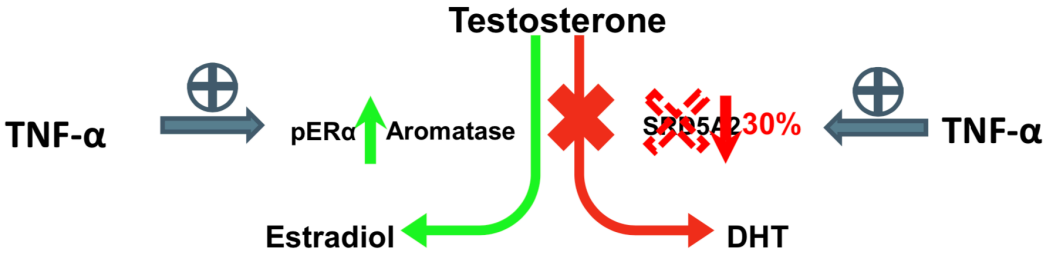

Figure 4: Schematic diagram depicting the "androgenic to estrogenic switch" (Olumi Lab).

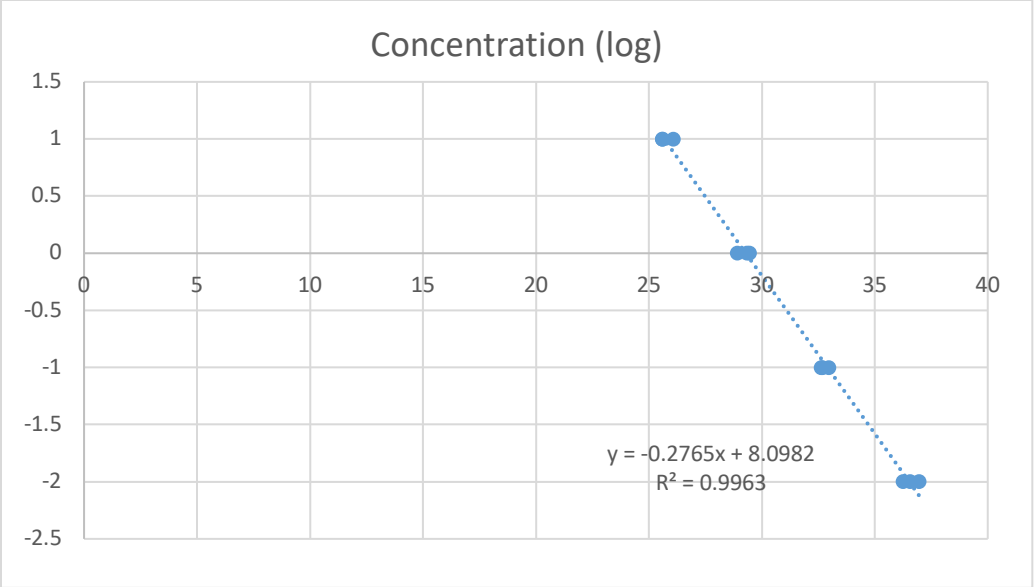

Figure 5: Standard curve plot for Methylation-PCR.

Equation was used to determine DNA concentration (y) from CT values (x). R^2 value was calculated in Microsoft Excel.

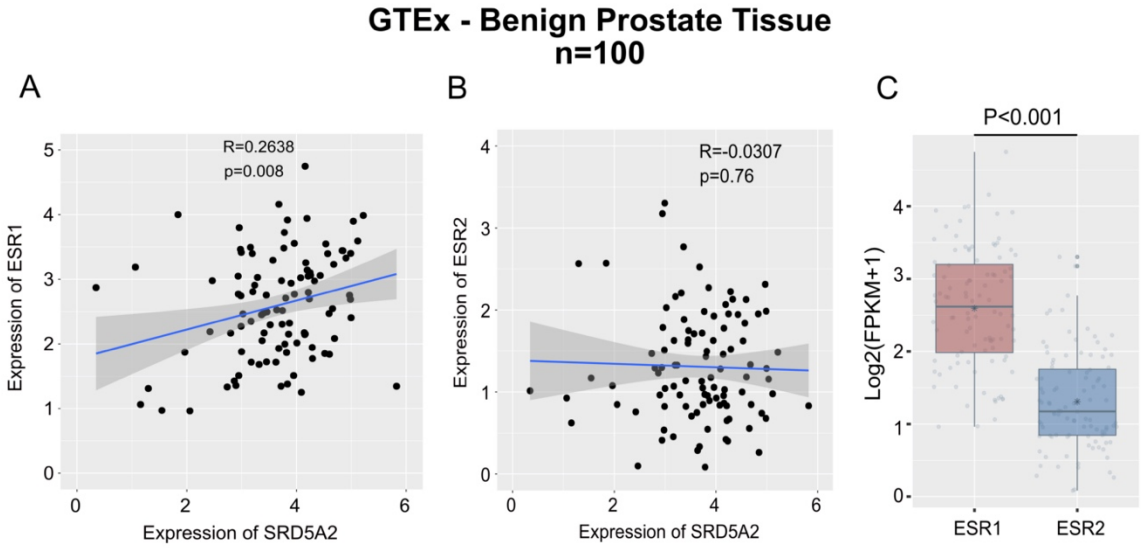

Figure 6: *SRD5A2* and *ESR1/ESR2* correlation plots from Genotype-Tissue Expression (GTEx) database

Correlation plots between SRD5A2 and ESR1 (A), ESR2 (B) RNA expression from GTEx database (n=100). Each black dot represents a single benign prostate tissue sample. Log expression of ESR1 and ESR2 was compared among the 100 samples (C).

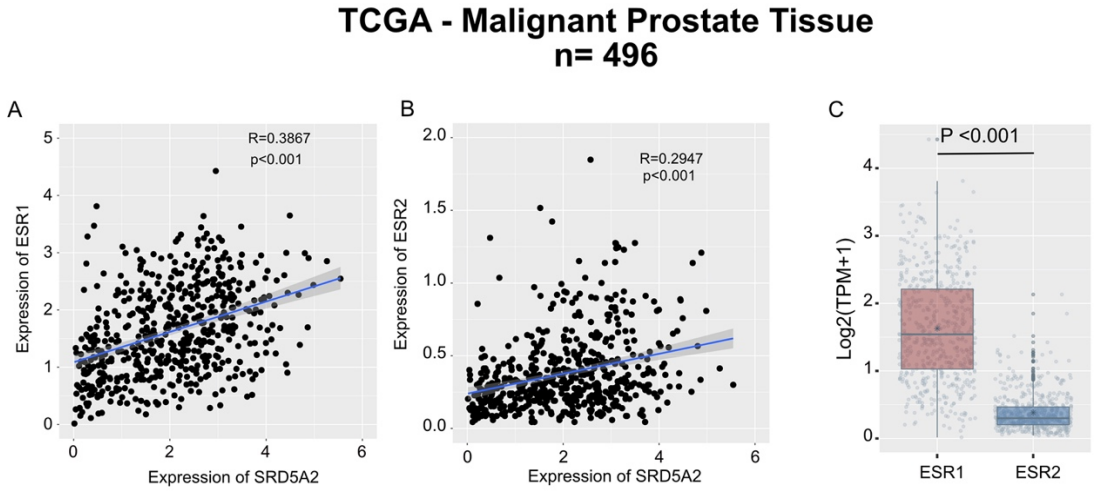

Figure 7: *SRD5A2* and *ESR1/ESR2* correlation plots from The Cancer Genome Analysis (TCGA) database

Correlation plots between SRD5A2 and ESR1 (A), ESR2 (B) RNA expression from TCGA database (n=496). Each black dot represents a single malignant prostate tissue sample. Log 2 expression of ESR1 and ESR2 was compared among the 496 samples (C).

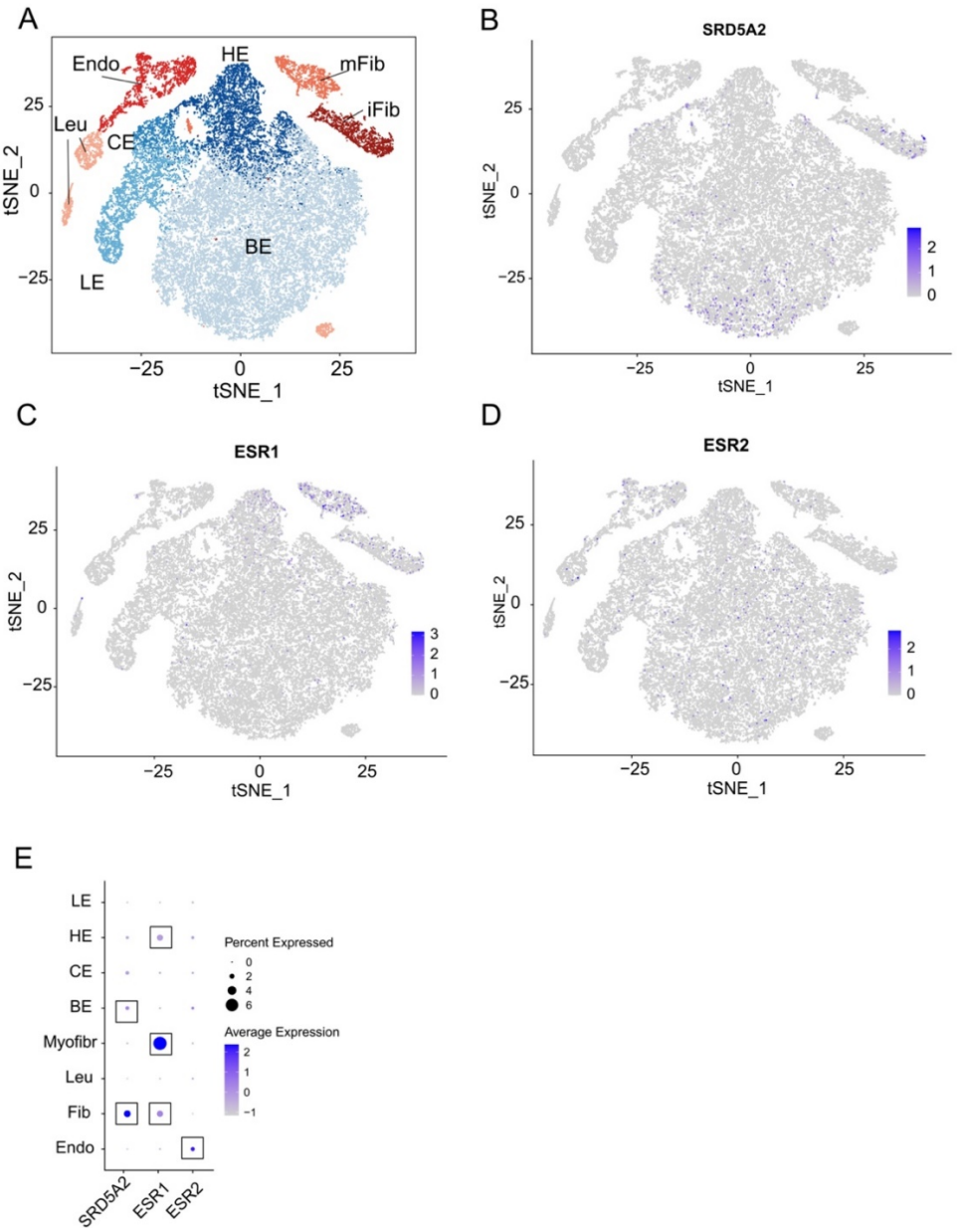

Figure 8: The cellular expression of *SRD5A2*, *ESR1* and *ESR2* in normal prostate.

The transcript expressions of SRD5A2 (B), ESR1(C) and ESR2 (D) were analyzed for subpopulation cells in Prostate. (A). Cell subpopulations include: Luminal epithelial cells (LE), Basal epithelial cells (BE), Club epithelial cells (CE), Hillock epithelial cells (HE), Fibroblasts (Fib), Myofibroblasts (Myofibr/mFib), Leukocytes (Leu) and Endothelial cells (Endo). (E) Average and present transcript expression of SRD5A2, ESR1 and ESR2. The data was extracted and analyzed from single-cell RNA sequencing database (StrandLab.net).

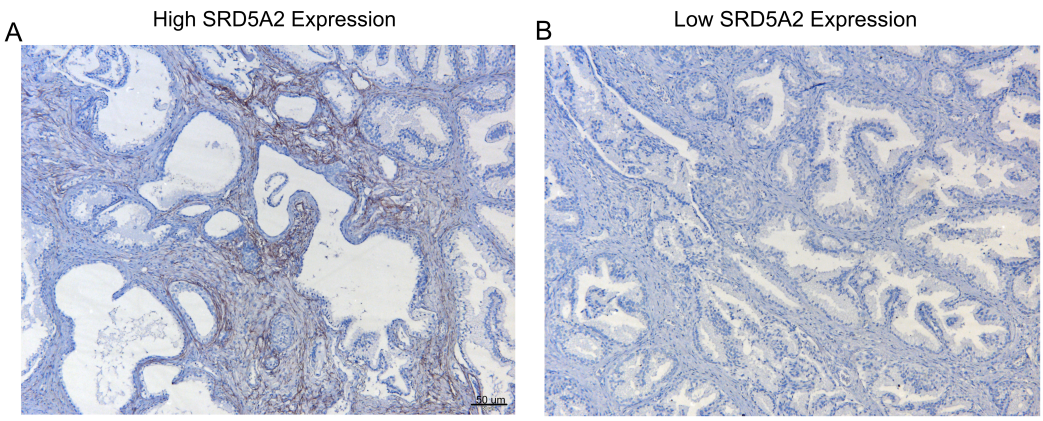

Figure 9: SRD5A2 expression in BPH tissue samples (n=18).

Immunohistochemistry representative images of high (A) versus low (B) protein expression of SRD5A2. Brown = SRD5A2 protein expression. Blue = Nucleus. Magnification: 10X

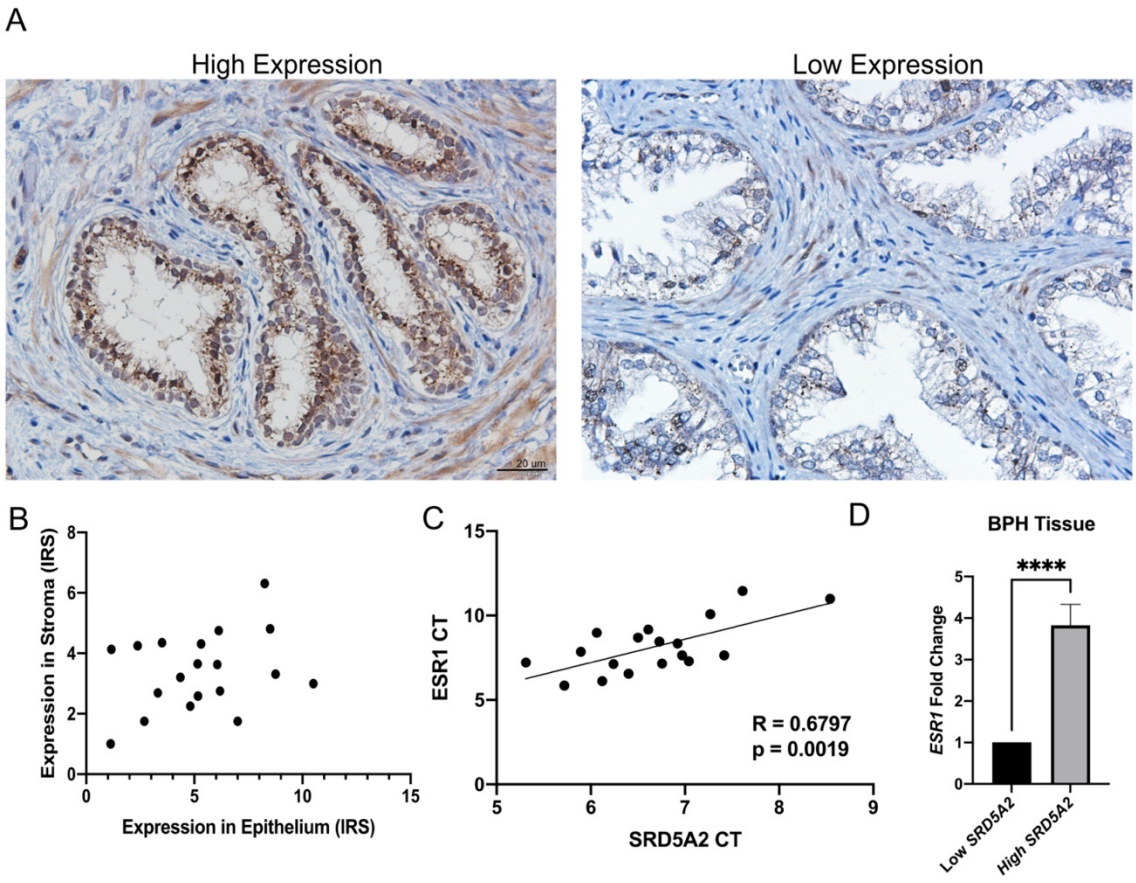

Figure 10: ERα expression in BPH tissue samples (n=18).

*Immunohistochemistry representative images of high (A: Left) versus low (A: Right) protein expression of ERα. Brown = ERα protein expression. Blue = Nucleus. Magnification: 40X. Average immunoreactive scores were plotted for each sample stratified by the stroma and epithelium compartment (B). Correlation plot of SRD5A2 and ESR1 was analyzed using cycle time (CT) values from qPCR (C). SRD5A2 expression was divided into low vs. high based on CT value (cut off for the group division was 6.5). ESR1 transcript expression was compared between the two SRD5A2 groups (D). ****: p<0.0001 as assessed by two-tailed Mann-Whitney test.*

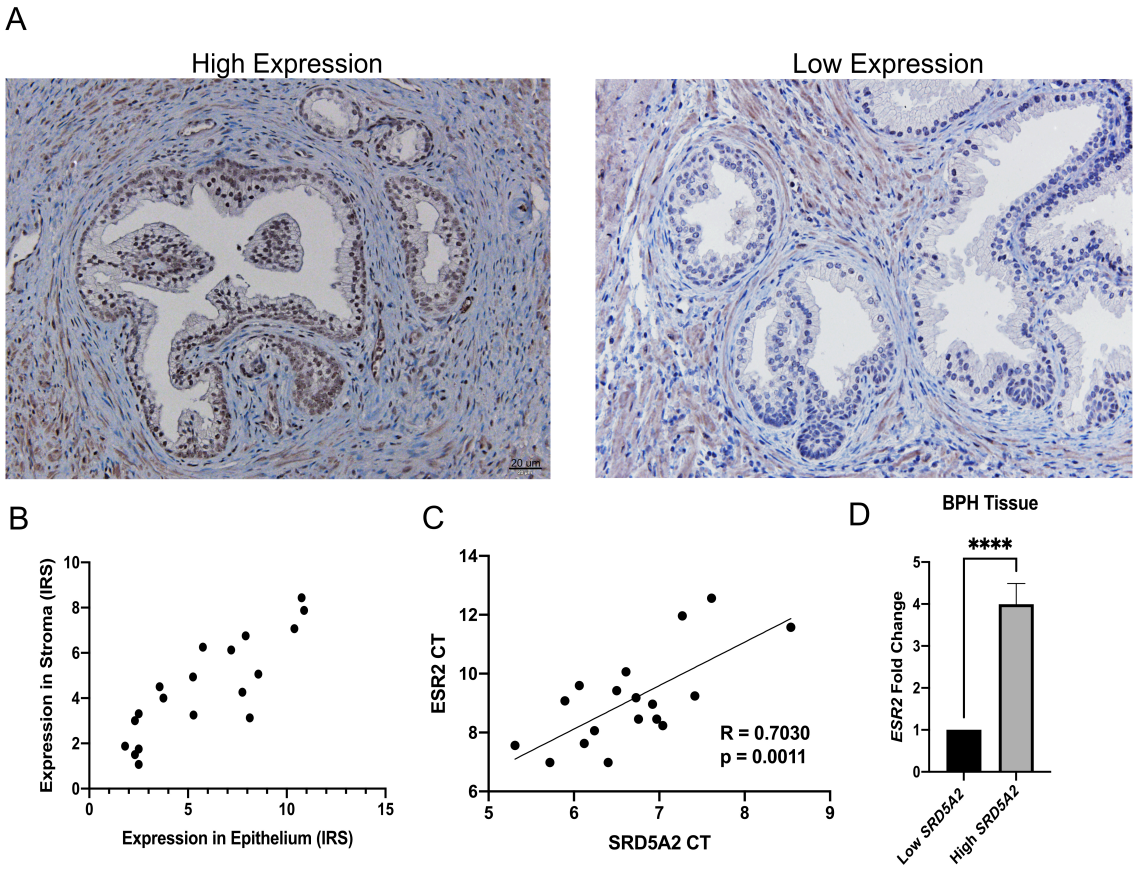

Figure 11: ERβ expression in BPH tissue samples (n=18).

*Immunohistochemistry representative images of high (A: Left) versus low (A: Right) protein expression of ERβ. Brown = ERβ protein expression. Blue = Nucleus. Magnification: 20X. Average immunoreactive scores were plotted for each sample stratified by the stroma and epithelium compartment (B). Correlation plot of SRD5A2 and ESR2 was analyzed using cycle time (CT) values from qPCR (C). SRD5A2 expression was divided into low vs. high based on CT value (cut off for the group division was 6.5). ESR2 transcript expression was compared between the two SRD5A2 groups (D). ****: p<0.0001 as assessed by two-tailed Mann-Whitney test.*

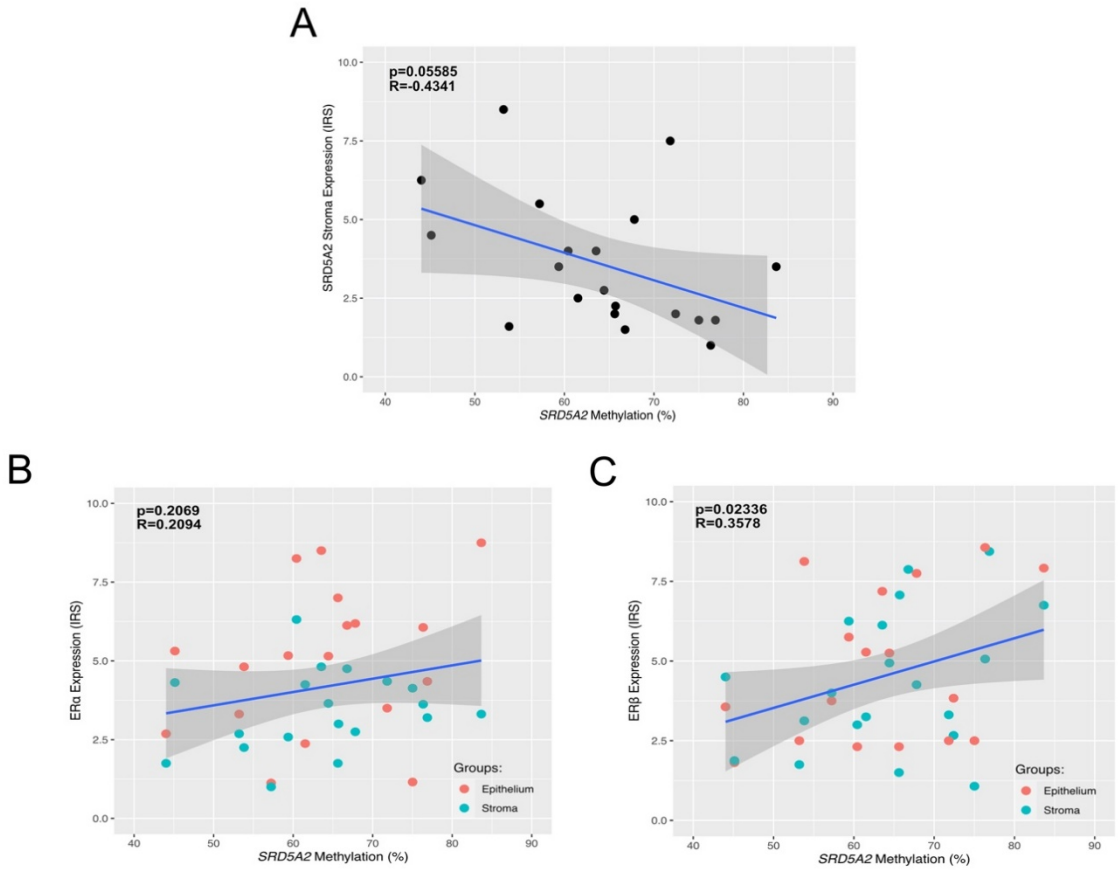

Figure 12: Correlation plots between SRD5A2 promoter methylation level and SRD5A2, ERα, and ERβ protein expressions (n=18).

SRD5A2 promoter methylation levels were determined and correlated with SRD5A2 protein expression in the stroma (A), ERα protein expression (B) and ERβ protein expression (C) in both the epithelium (red dots) and the stroma (green dots) compartment of the prostate. R value and p value were assessed by Pearson correlation test.

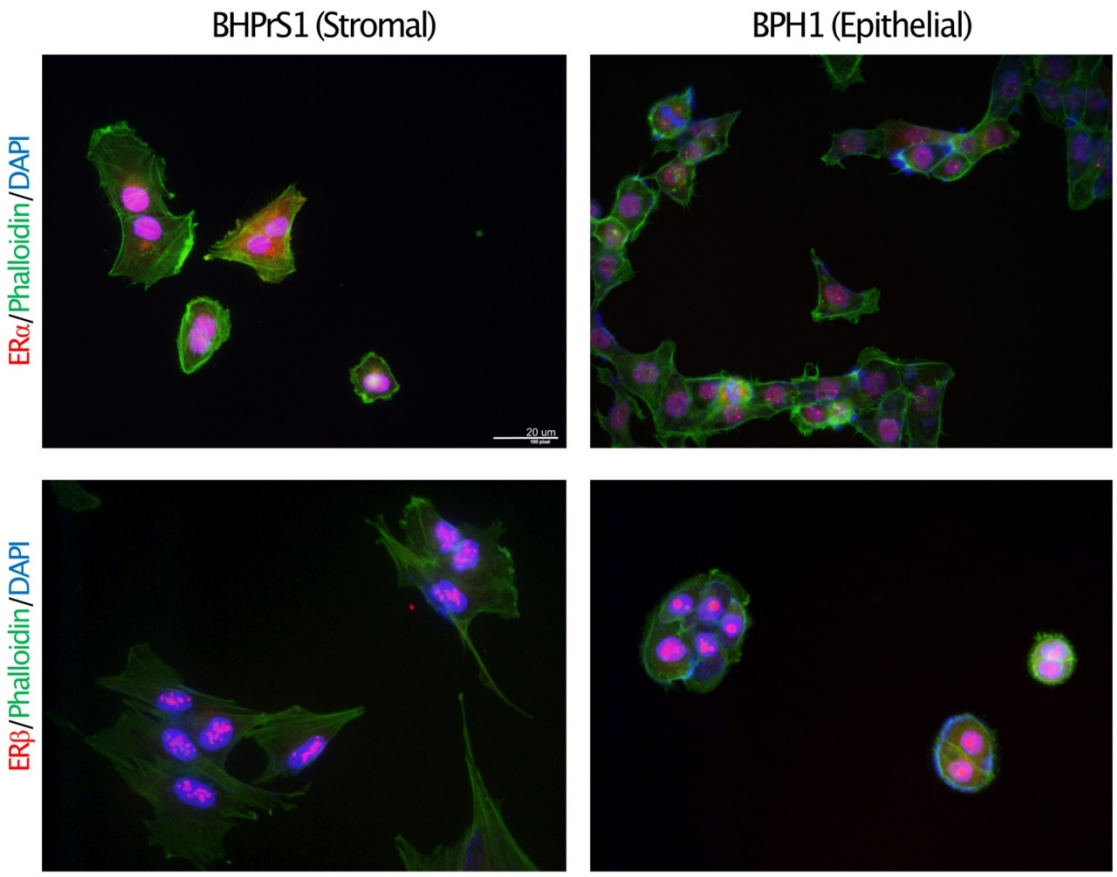

Figure 13: ERα and ERβ expression in BHPrS1 (stromal) and BPH1 (epithelial) cells.

Representative images of baseline ERα and ERβ protein expression in normal BHPrS1 and BPH1. Magnification 40X. Red = ERα or ERβ protein expression. Green = Phalloidin, actin filament marker. Blue = DAPI nuclei expression.

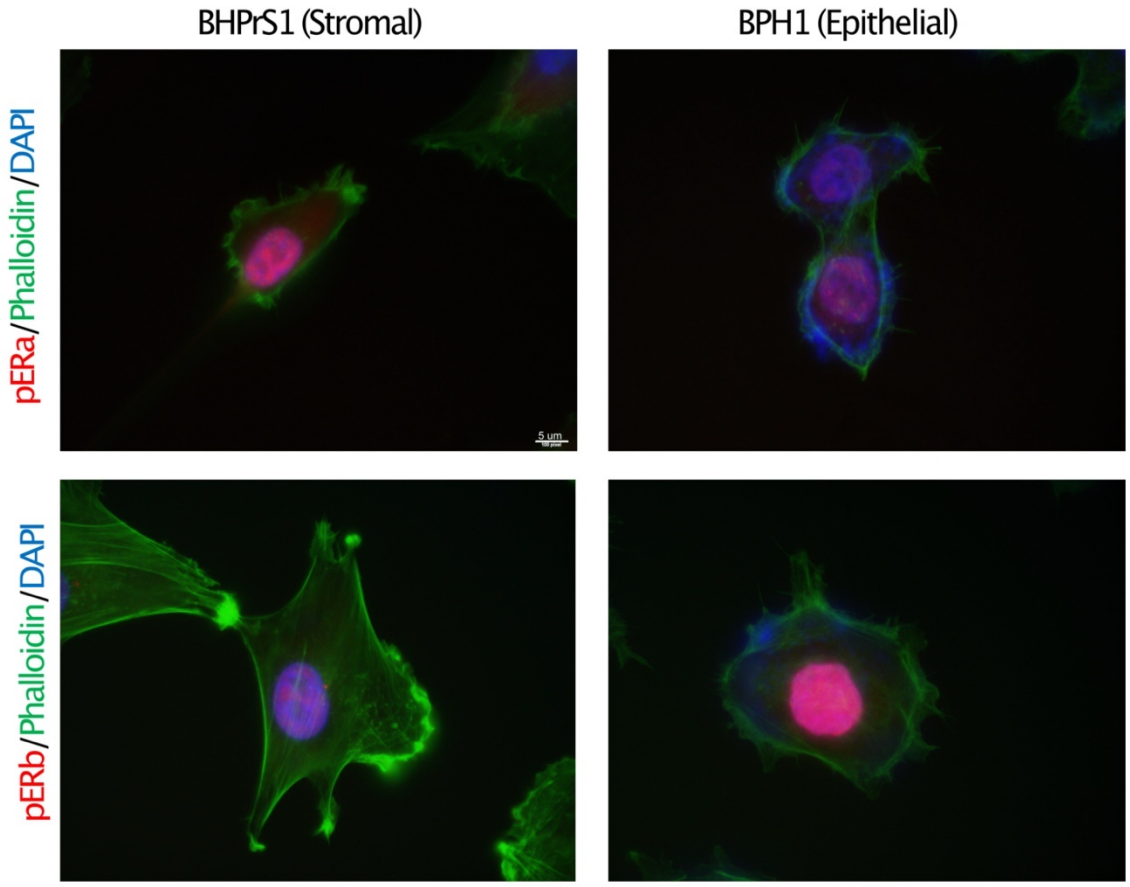

Figure 14: pERα and pERβ expression in BHPrS1 (stromal) and BPH1 (epithelial) cells.

Representative images of baseline pERα and pERβ protein expression in normal BHPrS1 and BPH1. Magnification 100X. Red = pERα or pERβ protein expression. Green= Phalloidin, actin filament marker. Blue = DAPI nuclei expression

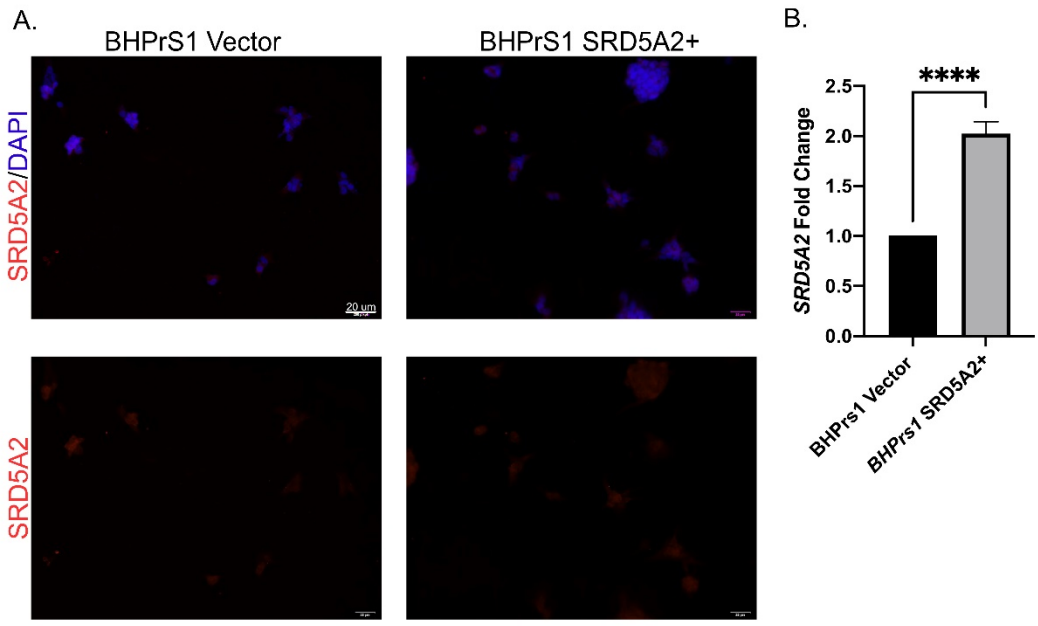

Figure 15: BHPrS1 (stromal) *SRD5A2* transfection efficiency.

*BHPrS1 cells were transfected with SRD5A2 lentiviral particle (SRD5A2+) or a negative vector. Representative image of SRD5A2 protein expression was captured after immunocytochemistry (A). Red = SRD5A2 protein expression. Blue = DAPI nuclei expression. Magnification 20X. SRD5A2 transcript expression was compared between the vector and SRD5A2 transfected BHPrS1 cells (B). ****: $p<0.0001$ assessed by two-tailed Mann-Whitney test.*

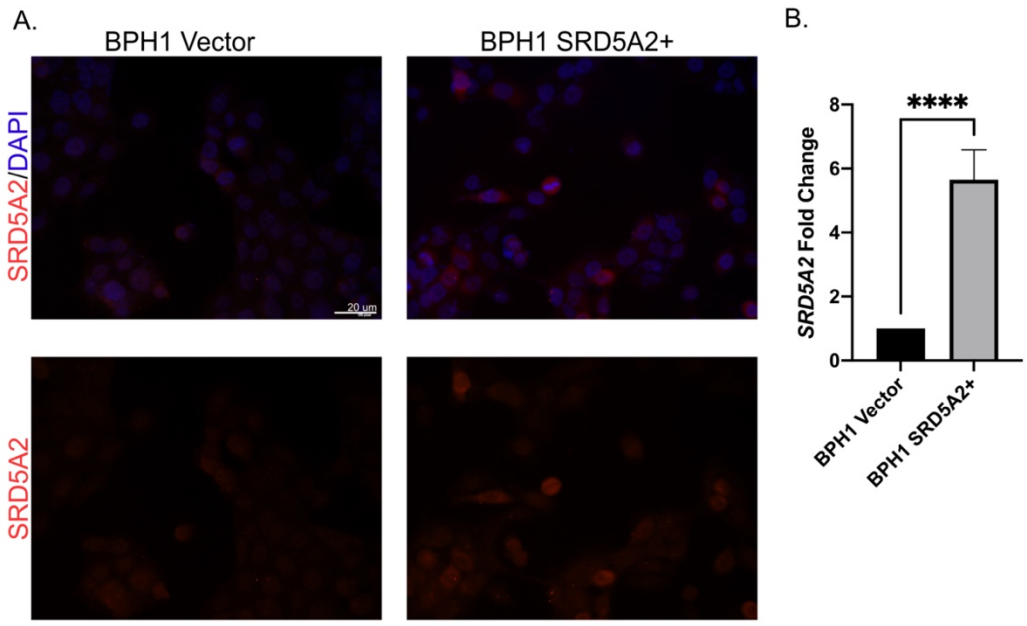

Figure 16: BPH1 (epithelial) *SRD5A2* transfection efficiency.

*BPH1 cells were transfected with SRD5A2 lentiviral particle (SRD5A2+) or a negative vector. Representative image of SRD5A2 protein expression was captured after immunocytochemistry (A). Red = SRD5A2 protein expression. Blue = DAPI nuclei expression. Magnification 40X. SRD5A2 transcript expression was compared between the vector and SRD5A2 transfected BPH1 cells (B). ****: $p<0.0001$ assessed by two-tailed Mann-Whitney test.*

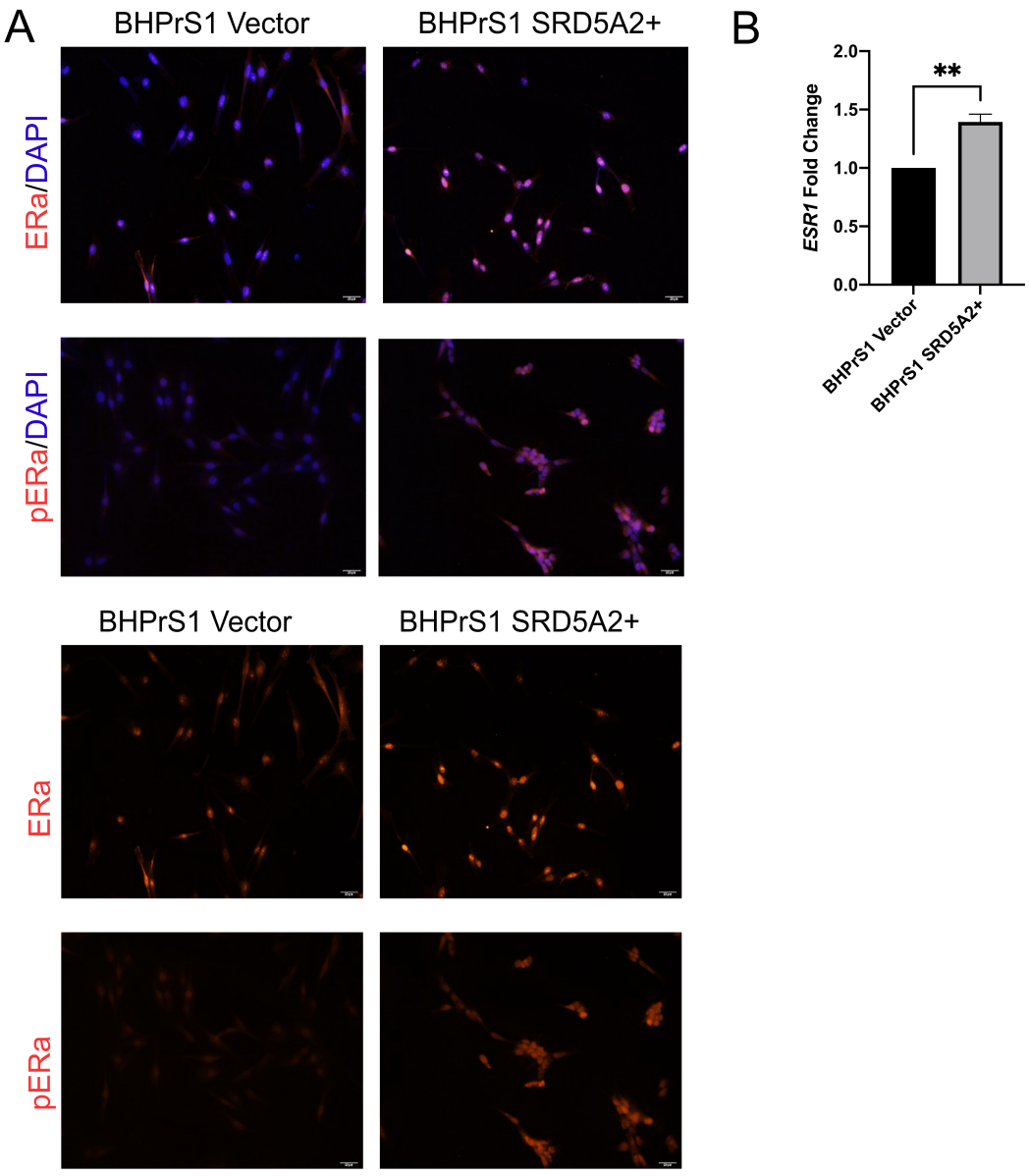

Figure 17: ERα/pERα expression in *SRD5A2* transfected BHPrS1 (stromal) cells.

*Representative images of total and phosphorylated ERα protein expression in vector and SRD5A2 transfected BHPrS1 cells (A). Red = ERα or pERα (labeled) protein expression. Blue = DAPI nuclei expression. Merged (top) and target (bottom) images are presented to clear visualization. Magnification: 20X. ESR1 transcript expression was compared between the vector and SRD5A2 transfected BHPrS1 cells (B). **: p<0.001 assessed by two-tailed Mann-Whitney test.*

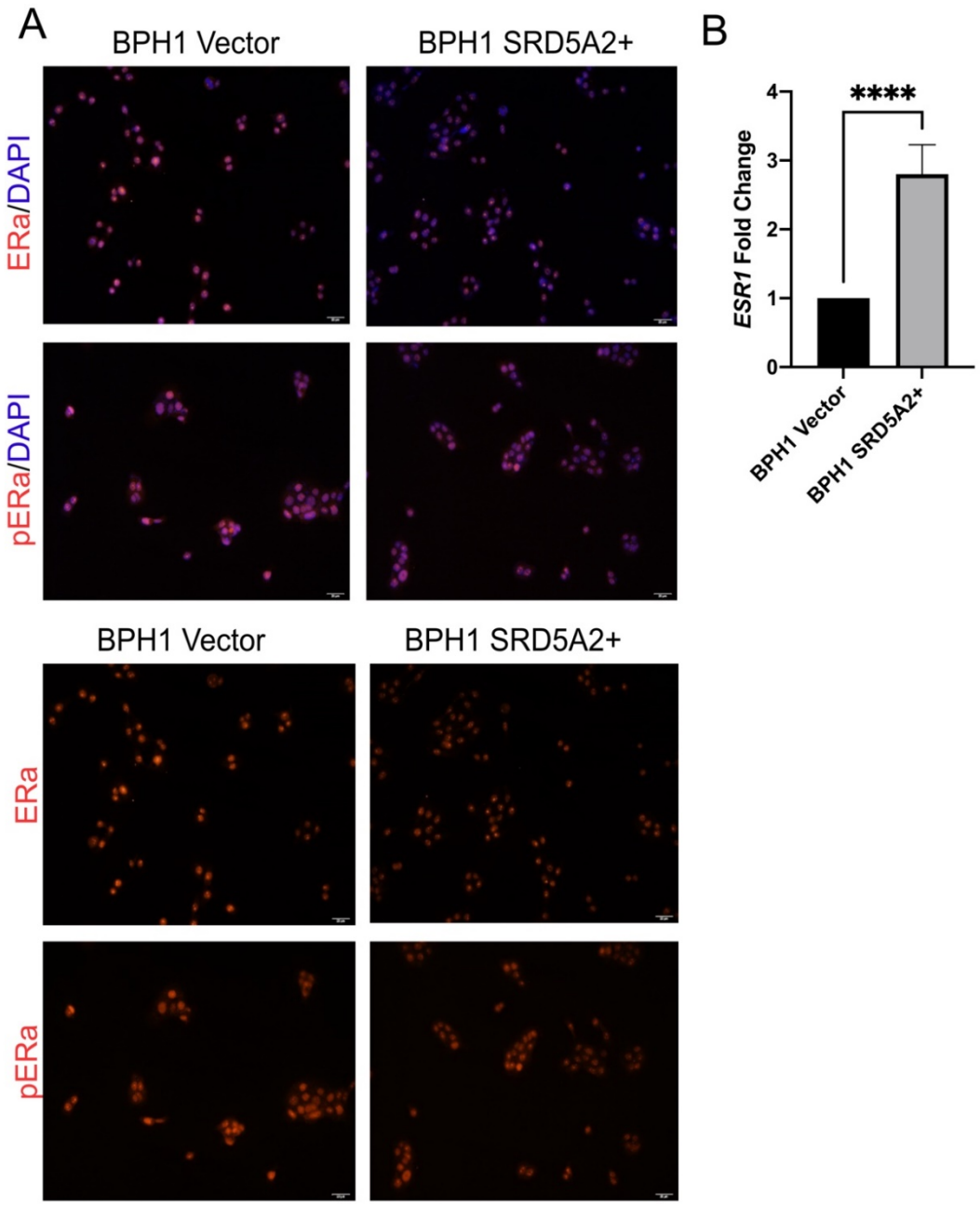

Figure 18: ERα/pERα expression in *SRD5A2* transfected BPH1 (epithelial) cells.

*Representative images of total and phosphorylated ERα protein expression in vector and SRD5A2 transfected BHPrS1 cells (A). Red = ERα or pERα (labeled) protein expression. Blue = DAPI nuclei expression. Merged (top) and target (bottom) images are presented to clear visualization. Magnification 20X. ESR1 transcript expression was compared between the vector and SRD5A2 transfected BHPrS1 cells (B). ****: $p<0.0001$ assessed by two-tailed Mann-Whitney test.*

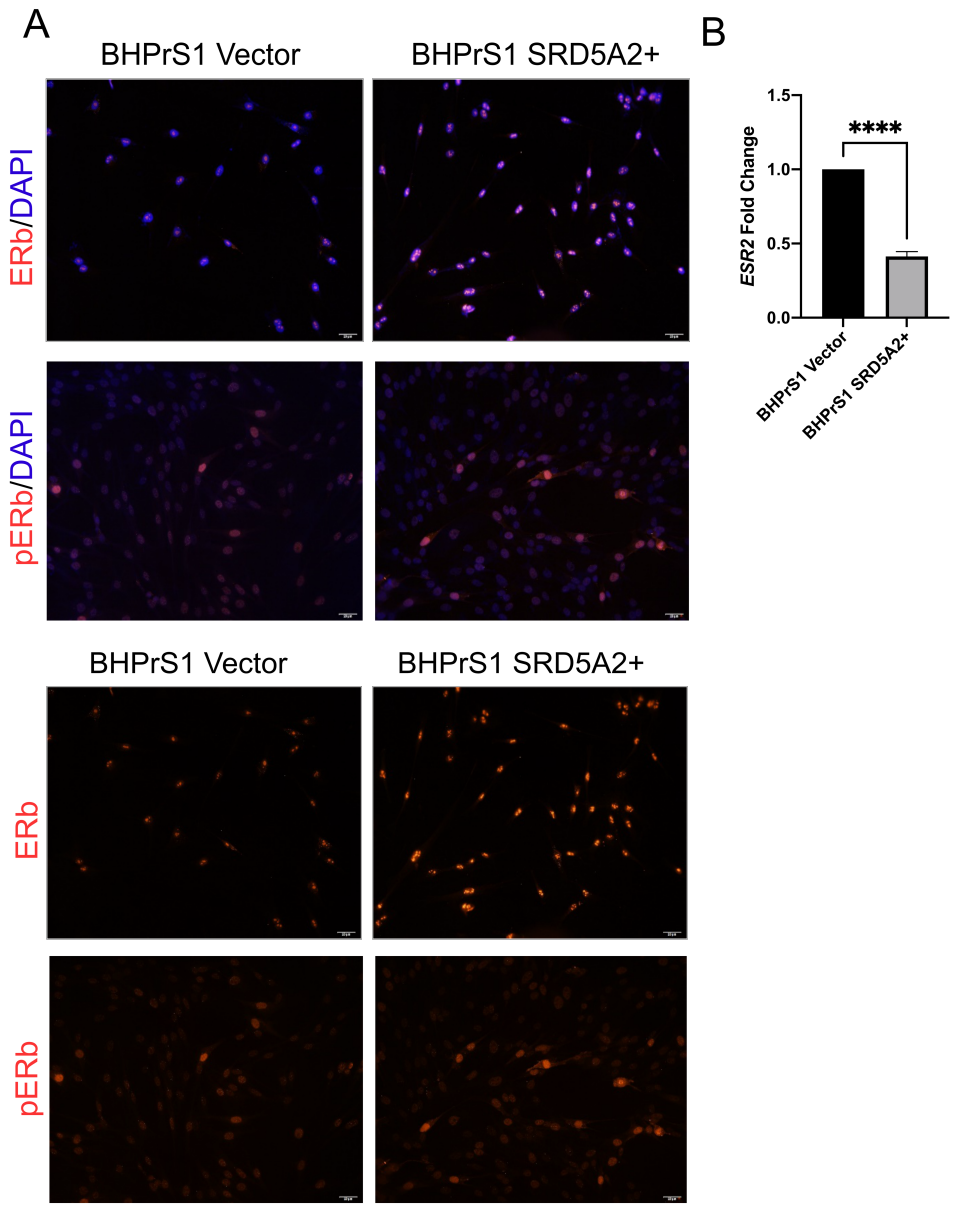

Figure 19: ERβ/pERβ expression in *SRD5A2* transfected BHPrS1 (stromal) cells.

*Representative images of total and phosphorylated ERβ protein expression in vector and SRD5A2 transfected BHPrS1 cells (A). Red = ERβ or pERβ (labeled) protein expression. Blue = DAPI nuclei expression. Merged (top) and target (bottom) images are presented to clear visualization. Magnification: 20X. ESR2 transcript expression was compared between the vector and SRD5A2 transfected BHPrS1 cells (B). ****: p<0.0001 assessed by two-tailed Mann-Whitney test.*

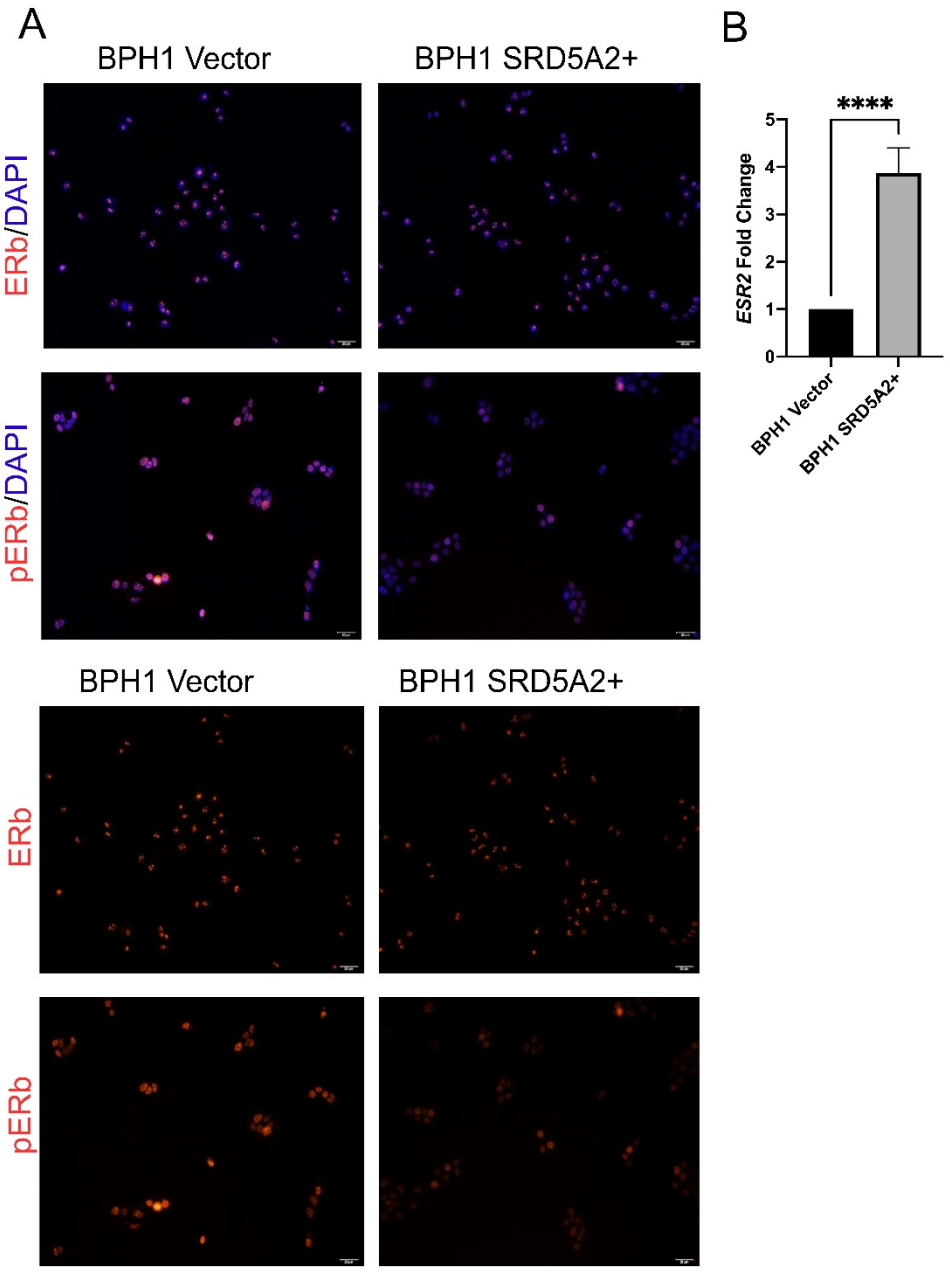

Figure 20: ERβ/pERβ expression in *SRD5A2* transfected BPH1 (epithelial) cells

*Representative images of total and phosphorylated ERβ protein expression in vector and SRD5A2 transfected BPH1 cells (A). Red = ERβ or pERβ (labeled) protein expression. Blue = DAPI nuclei expression. Merged (top) and target (bottom) images are presented to clear visualization. Magnification: 20X. ESR2 transcript expression was compared between the vector and SRD5A2 transfected BPH1 cells (B). ****: p<0.0001 assessed by two-tailed Mann-Whitney test.*

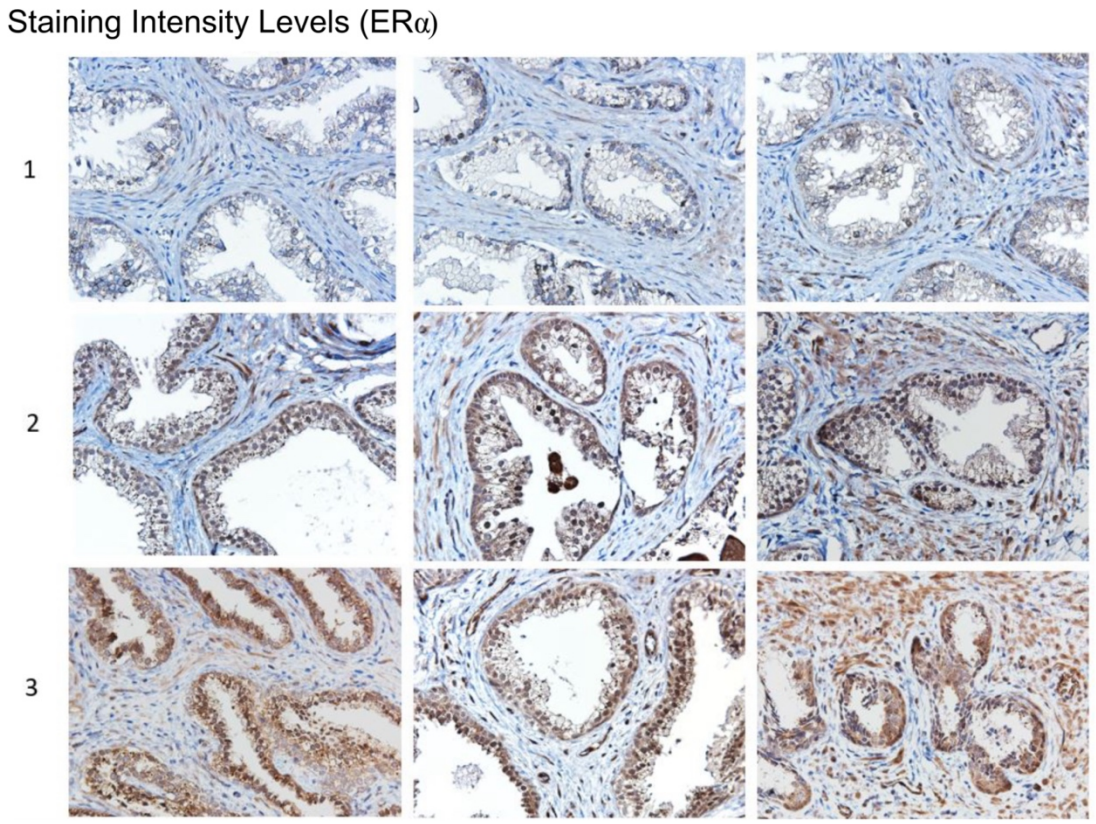

Figure 21: ERα staining intensity key for IRS

Staining intensity key used during the semi-quantification scoring process. All images of IHC staining of ERα was randomly screened, where representative images were chosen and divided into three different levels based on observed staining intensity in order to generate this key. Images include both the epithelium and stroma compartments. Magnification: 20X.

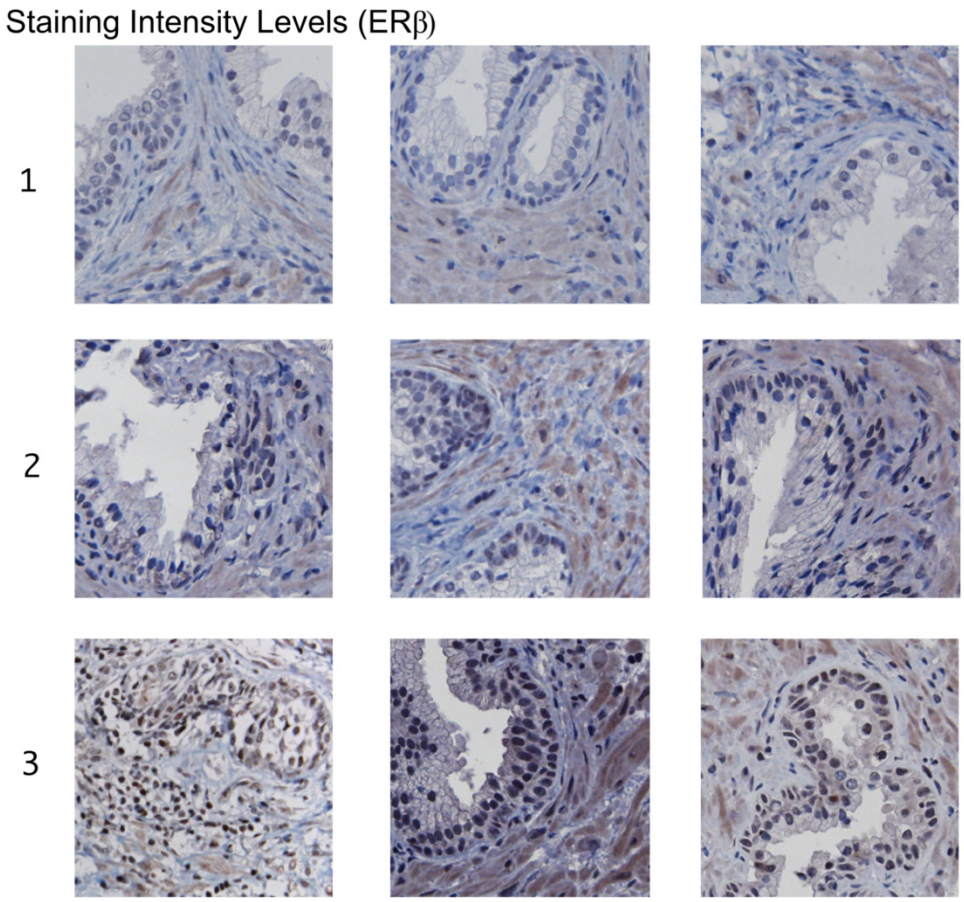

Figure 22: ERβ staining intensity key for IRS

Staining intensity key used during the semi-quantification scoring process. All images of IHC staining of ERβ was randomly screened, where representative images were chosen and divided into three different levels based on observed staining intensity in order to generate this key. Images include both the epithelium and stroma compartments. Magnification: 20X.

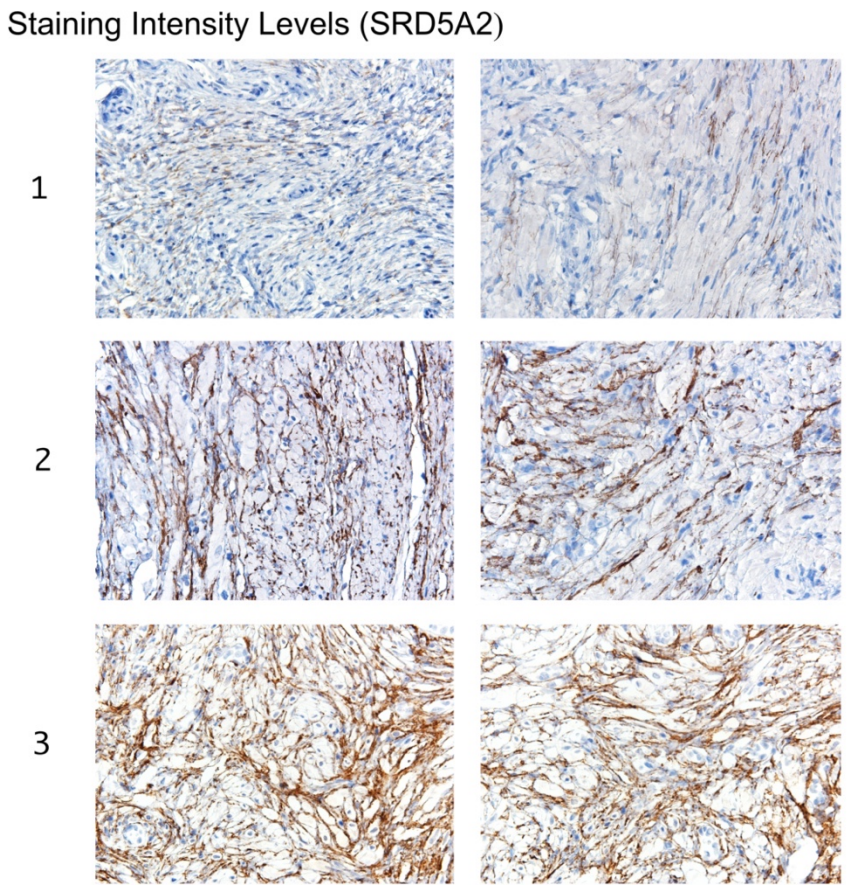

Figure 23: SRD5A2 staining intensity key for IRS

Staining intensity key used during the semi-quantification scoring process. All images of IHC staining of SRD5A2 was randomly screened, where representative images were chosen and divided into three different levels based on observed staining intensity in order to generate this key. Images only depict the stroma compartment as SRD5A2 was only expressed in the stroma. Magnification: 20X.

References

Abazid, A., Martin, B., Choinowski, A., McNeill, R. V., Brandenburg, L. O., Ziegler, P., Zimmermann, U., Burchardt, M., Erb, H., Stope, M. B. (2019). The androgen receptor antagonist enzalutamide induces apoptosis, dysregulates the heat shock protein system, and diminishes the androgen receptor and estrogen receptor beta1 expression in prostate cancer cells. *J Cell Biochem*, 120(10), 16711-16722. doi:10.1002/jcb.28929

Amory, J. K., Wang, C., Swerdloff, R. S., Anawalt, B. D., Matsumoto, A. M., Bremner, W. J., Walker, S.E., Haberer, L.J., Clark, R. V. (2007). The effect of 5alpha-reductase inhibition with dutasteride and finasteride on semen parameters and serum hormones in healthy men. *J Clin Endocrinol Metab*, 92(5), 1659-1665. doi:10.1210/jc.2006-2203

Athanasopoulos, A., Gyftopoulos, K., Giannitsas, K., Fisfis, J., Perimenis, P., Barbalias, G. (2003). Combination treatment with an alpha-blocker plus an anticholinergic for bladder outlet obstruction: a prospective, randomized, controlled study. *J Urol*, 169(6), 2253-2256. doi:10.1097/01.ju.0000067541.73285.eb

Bechis, S. K., Otsetov, A. G., Ge, R., Wang, Z., Vangel, M. G., Wu, C. L., Tabatabaei, S., Olumi, A. F. (2015). Age and Obesity Promote Methylation and Suppression of 5alpha-Reductase 2: Implications for Personalized Therapy of Benign Prostatic Hyperplasia. *J Urol*, 194(4), 1031-1037. doi:10.1016/j.juro.2015.04.079

Berry, S. J., Coffey, D. S., Walsh, P. C., & Ewing, L. L. (1984). The development of human benign prostatic hyperplasia with age. *J Urol*, 132(3), 474-479. doi:10.1016/s0022-5347(17)49698-4

Bonkhoff, H. (2018). Estrogen receptor signaling in prostate cancer: Implications for carcinogenesis and tumor progression. *Prostate*, 78(1), 2-10. doi:10.1002/pros.23446

Bosch, J. L., Tilling, K., Bohnen, A. M., Bangma, C. H., & Donovan, J. L. (2007). Establishing normal reference ranges for prostate volume change with age in the population-based Krimpen-study: prediction of future prostate volume in individual men. *Prostate*, 67(16), 1816-1824. doi:10.1002/pros.20663

Breyer, B. N., & Sarma, A. V. (2014). Hyperglycemia and insulin resistance and the risk of BPH/LUTS: an update of recent literature. *Curr Urol Rep*, 15(12), 462. doi:10.1007/s11934-014-0462-x

Brock, G., Broderick, G., Roehrborn, C. G., Xu, L., Wong, D., & Viktrup, L. (2013). Tadalafil once daily in the treatment of lower urinary tract symptoms (LUTS)

suggestive of benign prostatic hyperplasia (BPH) in men without erectile dysfunction. *BJU Int*, 112(7), 990-997. doi:10.1111/bju.12251

Chadid, S., Barber, J. R., Nelson, W. G., Gurel, B., Lucia, M. S., Thompson, I. M., Goodman, P.J., Stanczyk, F.Z., Parnes, H.L., Lippman, S.M., De Marzo, A.M., Platz, E. A. (2020). The association between serum sex steroid hormone concentrations and intraprostatic inflammation in men without prostate cancer and irrespective of clinical indication for biopsy in the placebo arm of the Prostate Cancer Prevention Trial. *Prostate*, 80(11), 895-905. doi:10.1002/pros.24023

Chauhan, G., Mehta, A., & Gupta, S. (2020). Stromal-AR influences the growth of epithelial cells in the development of benign prostate hyperplasia. *Mol Cell Biochem*, 471(1-2), 129-142. doi:10.1007/s11010-020-03773-z

Chaurasiya, S., Widmann, S., Botero, C., Lin, C. Y., Gustafsson, J. A., & Strom, A. M. (2020). Estrogen receptor beta exerts tumor suppressive effects in prostate cancer through repression of androgen receptor activity. *PLoS One*, 15(5), e0226057. doi:10.1371/journal.pone.0226057

Chaurasiya, S., Wu, W., Strom, A. M., Warner, M., & Gustafsson, J. A. (2020). Estrogen receptor beta regulates AKT activity through up-regulation of INPP4B and inhibits migration of prostate cancer cell line PC-3. *Proc Natl Acad Sci USA*, 117(42), 26347-26355. doi:10.1073/pnas.2007160117

Chen, B., Cao, D., Chen, Z., Huang, Y., Lin, T., Ai, J., Liu, L., Wei, Q. (2020). Estrogen regulates the proliferation and inflammatory expression of primary stromal cell in benign prostatic hyperplasia. *Transl Androl Urol*, 9(2), 322-331. doi:10.21037/tau.2020.02.08

Cindolo, L., Pirozzi, L., Fanizza, C., Romero, M., Tubaro, A., Autorino, R., De Nunzio, C., Schips, L. (2015). Drug adherence and clinical outcomes for patients under pharmacological therapy for lower urinary tract symptoms related to benign prostatic hyperplasia: population-based cohort study. *Eur Urol*, 68(3), 418-425. doi:10.1016/j.eururo.2014.11.006

Cindolo, L., Pirozzi, L., Sountoulides, P., Fanizza, C., Romero, M., Castellan, P., Antonelli, A., Simeone, C., Tubaro, A., de Nunzio, C., Schips, L. (2015). Patient's adherence on pharmacological therapy for benign prostatic hyperplasia (BPH)-associated lower urinary tract symptoms (LUTS) is different: is combination therapy better than monotherapy? *BMC Urol*, 15, 96. doi:10.1186/s12894-015-0090-x

Crawford, E. D., Wilson, S. S., McConnell, J. D., Slawin, K. M., Lieber, M. C., Smith, J. A., Meehan, A.G., Bautista, O.M., Noble, W.R., Kusek, J.W., Nyberg, L.M., Roehrborn, C. G. (2006). Baseline Factors as Predictors of Clinical Progression of Benign Prostatic Hyperplasia in Men Treated With Placebo. *Journal of Urology*, 175(4), 1422-1427. doi:10.1016/s0022-5347(05)00708-1

Egan, K. B. (2016). The Epidemiology of Benign Prostatic Hyperplasia Associated with Lower Urinary Tract Symptoms: Prevalence and Incident Rates. *Urol Clin North Am*, 43(3), 289-297. doi:10.1016/j.ucl.2016.04.001

Ellem, S. J., & Risbridger, G. P. (2010). Aromatase and regulating the estrogen:androgen ratio in the prostate gland. *J Steroid Biochem Mol Biol*, 118(4-5), 246-251. doi:10.1016/j.jsbmb.2009.10.015

Foster, H. E., Barry, M. J., Dahm, P., Gandhi, M. C., Kaplan, S. A., Kohler, T. S., Lerner, L.B., Lightner, D.J., Parsons, J.K., Roehrborn, C.G., Welliver, C., Wilt, T.J., McVary, K. T. (2018). Surgical Management of Lower Urinary Tract Symptoms Attributed to Benign Prostatic Hyperplasia: AUA Guideline. *J Urol*, 200(3), 612-619. doi:10.1016/j.juro.2018.05.048

Gangkak, G., Bhattar, R., Mittal, A., Yadav, S. S., Tomar, V., Yadav, A., & Mehta, J. (2017). Immunohistochemical analysis of estrogen receptors in prostate and clinical correlation in men with benign prostatic hyperplasia. *Investig Clin Urol*, 58(2), 117-126. doi:10.4111/icu.2017.58.2.117

Ge, R., Wang, Z., Bechis, S. K., Otsetov, A. G., Hua, S., Wu, S., Tabatabaei, S., Olumi, A. F. (2015). DNA methyl transferase 1 reduces expression of SRD5A2 in the aging adult prostate. *Am J Pathol*, 185(3), 870-882. doi:10.1016/j.ajpath.2014.11.020

Gormley, G. J., Stoner, E., Bruskewitz, R. C., Imperato-McGinley, J., Walsh, P. C., McConnell, J. D., . . . et al. (1992). The effect of finasteride in men with benign prostatic hyperplasia. The Finasteride Study Group. *N Engl J Med*, 327(17), 1185-1191. doi:10.1056/NEJM199210223271701

Grindstad, T., Skjefstad, K., Andersen, S., Ness, N., Nordby, Y., Al-Saad, S., Fismen, S., Donnem, T., Khanehkenari, M.R., Busund, L.T., Bremnes, R.M., Richardsen, E. (2016). Estrogen receptors alpha and beta and aromatase as independent predictors for prostate cancer outcome. *Sci Rep*, 6, 33114. doi:10.1038/srep33114

Gul, Z. G., & Kaplan, S. A. (2019). BPH: Why Do Patients Fail Medical Therapy? *Curr Urol Rep*, 20(7), 40. doi:10.1007/s11934-019-0899-z

Hetzl, A. C., Favaro, W. J., Billis, A., Ferreira, U., & Cagnon, V. H. (2012). Steroid hormone receptors, matrix metalloproteinases, insulin-like growth factor, and dystroglycans interactions in prostatic diseases in the elderly men. *Microsc Res Tech*, 75(9), 1197-1205. doi:10.1002/jemt.22049

Irwin, D. E., Kopp, Z. S., Agatep, B., Milsom, I., & Abrams, P. (2011). Worldwide prevalence estimates of lower urinary tract symptoms, overactive bladder, urinary incontinence and bladder outlet obstruction. *BJU Int*, 108(7), 1132-1138. doi:10.1111/j.1464-410X.2010.09993.x

Issa, M. M., Fenter, T. C., Black, L., Grogg, A. L., & Kruep, E. J. (2006). An assessment of the diagnosed prevalence of diseases in men 50 years of age or older. *Am J Manag Care*, 12(4 Suppl), S83-89. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/16551206

Joseph, D. B., Strand, D. W., & Vezina, C. M. (2018). DNA methylation in development and disease: an overview for prostate researchers. *Am J Clin Exp Urol*, 6(6), 197-218. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/30697577

Kang, P. M., Kim, Y. J., Seo, W. T., Kang, S. H., Kim, T. S., Chun, B. K., Seo, W.I., Jeong, J.Y., Chung, J. I. (2019). Correlation between 5-alpha reductase type 2 protein expression and methylation of 5-alpha reductase type 2 promotor gene of benign prostatic hyperplasia. *World J Urol*, 37(4), 709-718. doi:10.1007/s00345-018-2422-4

Kaplan, A. L., Agarwal, N., Setlur, N. P., Tan, H. J., Niedzwiecki, D., McLaughlin, N., Burke, M.A., Steinberg, K., Chamie, K., Saigal, C. S. (2015). Measuring the cost of care in benign prostatic hyperplasia using time-driven activity-based costing (TDABC). *Healthc (Amst)*, 3(1), 43-48. doi:10.1016/j.hjdsi.2014.09.007

Kaplan, S. A., McConnell, J. D., Roehrborn, C. G., Meehan, A. G., Lee, M. W., Noble, W. R., Kusek, J.W., Nyberg, L. M. (2006). Combination Therapy With Doxazosin and Finasteride for Benign Prostatic Hyperplasia in Patients With Lower Urinary Tract Symptoms and a Baseline Total Prostate Volume of 25 Ml or Greater. *Journal of Urology*, 175(1), 217-220. doi:10.1016/s0022-5347(05)00041-8

Kaplan, S. A., Roehrborn, C. G., McConnell, J. D., Meehan, A. G., Surynawanshi, S., Lee, J. Y., Rotonda, J., Kusek, J.W., Nyberg, L.M., Medical Therapy of Prostatic Symptoms Research, G. (2008). Long-term treatment with finasteride results in a clinically significant reduction in total prostate volume compared to placebo over the full range of baseline prostate sizes in men enrolled in the MTOPS trial. *J Urol*, 180(3), 1030-1032; discussion 1032-1033. doi:10.1016/j.juro.2008.05.004

Kaplan, S. A. (2004). AUA Guidelines and Their Impact on the Management of BPH: An Update. *Rev Urol*, 6 Suppl 9, S46-52. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/16985925

Kim, J. H., Baek, M. J., Sun, H. Y., Lee, B., Li, S., Khandwala, Y., Del Giudice, F., Chung, B. I. (2018). Efficacy and safety of 5 alpha-reductase inhibitor monotherapy in patients with benign prostatic hyperplasia: A meta-analysis. *PLoS One*, 13(10), e0203479. doi:10.1371/journal.pone.0203479

Kirby, R. S., Roehrborn, C., Boyle, P., Bartsch, G., Jardin, A., Cary, M. M., Sweeney, M., Grossman, E.B., Prospective European, Doxazosin, Combination Therapy Study, I. (2003). Efficacy and tolerability of doxazosin and finasteride, alone or in combination, in treatment of symptomatic benign prostatic hyperplasia: the Prospective European Doxazosin and Combination Therapy (PREDICT) trial. *Urology*, 61(1), 119-126. doi:10.1016/s0090-4295(02)02114-3

Kristal, A. R., Arnold, K. B., Schenk, J. M., Neuhouser, M. L., Weiss, N., Goodman, P., Antvelink, C.M., Penson, D.F., Thompson, I. M. (2007). Race/ethnicity, obesity, health related behaviors and the risk of symptomatic benign prostatic hyperplasia: results from the prostate cancer prevention trial. *J Urol*, 177(4), 1395-1400; quiz 1591. doi:10.1016/j.juro.2006.11.065

La Vignera, S., Condorelli, R. A., Russo, G. I., Morgia, G., & Calogero, A. E. (2016). Endocrine control of benign prostatic hyperplasia. *Andrology*, 4(3), 404-411. doi:10.1111/andr.12186

Lau, K. M., LaSpina, M., Long, J., & Ho, S. M. (2000). Expression of estrogen receptor (ER)-alpha and ER-beta in normal and malignant prostatic epithelial cells: regulation by methylation and involvement in growth regulation. *Cancer Res*, 60(12), 3175-3182. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10866308

Laurent, V., Guerard, A., Mazerolles, C., Le Gonidec, S., Toulet, A., Nieto, L., Zaidi, F., Majed, B., Garandeau, D., Socrier, Y., Golzio, M., Cadoudal, T., Chaoui, K., Dray, C., Monsarrat, B., Schiltz, O., Wang, Y.Y., Couderc, B., Valet, P., Malavaud, B., Muller, C. (2016). Periprostatic adipocytes act as a driving force for prostate cancer progression in obesity. *Nat Commun*, 7, 10230. doi:10.1038/ncomms10230

Leav, I., Lau, K. M., Adams, J. Y., McNeal, J. E., Taplin, M. E., Wang, J., Singh, H., Ho, S. M. (2001). Comparative studies of the estrogen receptors beta and alpha and the androgen receptor in normal human prostate glands, dysplasia, and in primary and metastatic carcinoma. *Am J Pathol*, 159(1), 79-92. doi:10.1016/s0002-9440(10)61676-8

Lee, S. W. H., Chan, E. M. C., & Lai, Y. K. (2017). The global burden of lower urinary tract symptoms suggestive of benign prostatic hyperplasia: A systematic review and meta-analysis. *Sci Rep*, 7(1), 7984. doi:10.1038/s41598-017-06628-8

Litman, H. J., & McKinlay, J. B. (2007). The future magnitude of urological symptoms in the USA: projections using the Boston Area Community Health survey. *BJU Int*, 100(4), 820-825. doi:10.1111/j.1464-410X.2007.07018.x

Liu, D., Shoag, J. E., Poliak, D., Goueli, R. S., Ravikumar, V., Redmond, D., Vosoughi, A., Fontugne, J., Pan, H., Lee, D., Thomas, D., Salari, K., Wang, Z., Romanel, A., Te, A., Lee, R., Chughtai, B., Olumi, A.F., Mosquera, J.M., Demichelis, F., Elemento, O., Rubin, M.A., Sboner, A., Barbieri, C. E. (2020). Integrative multiplatform molecular profiling of benign prostatic hyperplasia identifies distinct subtypes. *Nat Commun*, 11(1), 1987. doi:10.1038/s41467-020-15913-6

Liu, T. T., Grubisha, M. J., Frahm, K. A., Wendell, S. G., Liu, J., Ricke, W. A., Auchus, R.J., DeFranco, D. B. (2016). Opposing Effects of Cyclooxygenase-2 (COX-2) on Estrogen Receptor beta (ERbeta) Response to 5alpha-Reductase Inhibition in

Prostate Epithelial Cells. *J Biol Chem*, 291(28), 14747-14760. doi:10.1074/jbc.M115.711515

Loeb, S., Kettermann, A., Carter, H. B., Ferrucci, L., Metter, E. J., & Walsh, P. C. (2009). Prostate volume changes over time: results from the Baltimore Longitudinal Study of Aging. *J Urol*, 182(4), 1458-1462. doi:10.1016/j.juro.2009.06.047

Macoska, J. A., Uchtmann, K. S., Leverson, G. E., McVary, K. T., & Ricke, W. A. (2019). Prostate Transition Zone Fibrosis is Associated with Clinical Progression in the MTOPS Study. *J Urol*, 202(6), 1240-1247. doi:10.1097/JU.0000000000000385

Mahjoub, S., Baur, A. D. J., Lenk, J., Lee, C. H., Hartenstein, A., Rudolph, M. M., Cash, H., Hamm, B., Asbach, P., Haas, M., Penzkofer, T. (2020). Optimizing size thresholds for detection of clinically significant prostate cancer on MRI: Peripheral zone cancers are smaller and more predictable than transition zone tumors. *Eur J Radiol*, 129, 109071. doi:10.1016/j.ejrad.2020.109071

Majumdar, S., Rinaldi, J. C., Malhotra, N. R., Xie, L., Hu, D. P., Gauntner, T. D., Grewal, H.S., Hu, W.Y., Kim, S.H., Katzenellenbogen, J.A., Kasper, S., Prins, G. S. (2019). Differential Actions of Estrogen Receptor alpha and beta via Nongenomic Signaling in Human Prostate Stem and Progenitor Cells. *Endocrinology*, 160(11), 2692-2708. doi:10.1210/en.2019-00177

Markov, G. V., Tavares, R., Dauphin-Villemant, C., Demeneix, B. A., Baker, M. E., & Laudet, V. (2009). Independent elaboration of steroid hormone signaling pathways in metazoans. *Proc Natl Acad Sci U S A*, 106(29), 11913-11918. doi:10.1073/pnas.0812138106

McConnell, J. D. (1991). The pathophysiology of benign prostatic hyperplasia. *J Androl*, 12(6), 356-363. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/1722791

McConnell, J.D., Roehrborn, C.G., Bautista, O.M., Andriole, G.L.Jr., Dixon, C.M., Kusek, J.W., Lepor, H., McVary, K.T., Nyberg, L.M.Jr., Clarke, H.S., Crawford, E.D., Diokno, A., Foley, J.P., Foster, H.E., Jacobs, S.C., Kaplan, S.A., Kreder, K.J., Lieber, M.M., Lucia, M.S., Miller, G.J., Menon, M., Milam, D.F., Ramsdell, J.W., Schenkman, N.S., Slawin, K.M., Smith, J.A., Medical Therapy of Prostatic Symptoms (MTOPS) Research Group. (2003). The long-term effect of doxazosin, finasteride, and combination therapy on the clinical progression of benign prostatic hyperplasia. *New England Journal of Medicine*, 349(25): 2387-2398. doi:10.1056/NEJMoa030656

McPherson, S. J., Hussain, S., Balanathan, P., Hedwards, S. L., Niranjan, B., Grant, M., Chandrasiri, U.P., Toivanen, R., Wang, Y., Taylor, R.A., Risbridger, G. P. (2010). Estrogen receptor-beta activated apoptosis in benign hyperplasia and cancer of the prostate is androgen independent and TNFalpha mediated. *Proc Natl Acad Sci U S A*, 107(7), 3123-3128. doi:10.1073/pnas.0905524107

McVary, K. T., Roehrborn, C. G., Avins, A. L., Barry, M. J., Bruskewitz, R. C., Donnell, R. F., Foster, H.E., Gonzalez, C.M., Kaplan, S.A., Penson, D.F., Ulchaker, J.C., Wei, J. T. (2011). Update on AUA guideline on the management of benign prostatic hyperplasia. *J Urol*, 185(5), 1793-1803. doi:10.1016/j.juro.2011.01.074

Middleton, L. W., Shen, Z., Varma, S., Pollack, A. S., Gong, X., Zhu, S., Zhu, C., Foley, J.W., Vennam, S., Sweeney, R.T., Tu, K., Biscocho, J., Eminaga, O., Nolley, R., Tibshirani, R., Brooks, J.D., West, R.B., Pollack, J. R. (2019). Genomic analysis of benign prostatic hyperplasia implicates cellular re-landscaping in disease pathogenesis. *JCI Insight*, 5. doi:10.1172/jci.insight.129749

Mishra, S., Tai, Q., Gu, X., Schmitz, J., Poullard, A., Fajardo, R. J., Mahalingam, D., Chen, X., Zhu, X., Sun, L. Z. (2015). Estrogen and estrogen receptor alpha promotes malignancy and osteoblastic tumorigenesis in prostate cancer. *Oncotarget,* 6(42), 44388-44402. doi:10.18632/oncotarget.6317

Mizoguchi, S., Mori, K., Shin, T., Wang, Z., DeFranco, D. B., Yoshimura, N., & Mimata, H. (2020). Effects of dutasteride in a rat model of chemically induced prostatic inflammation-Potential role of estrogen receptor beta. *Prostate*, 80(16), 1413-1420. doi:10.1002/pros.24071

Morais-Santos, M., Werneck-Gomes, H., Campolina-Silva, G. H., Santos, L. C., Mahecha, G. A. B., Hess, R. A., & Oliveira, C. A. (2018). Basal Cells Show Increased Expression of Aromatase and Estrogen Receptor alpha in Prostate Epithelial Lesions of Male Aging Rats. *Endocrinology*, 159(2), 723-732. doi:10.1210/en.2017-00773

Muller, R. L., Gerber, L., Moreira, D. M., Andriole, G., Jr., Hamilton, R. J., Fleshner, N., Parsons, J.K., Freedland, S. J. (2013). Obesity is associated with increased prostate growth and attenuated prostate volume reduction by dutasteride. *Eur Urol*, 63(6), 1115-1121. doi:10.1016/j.eururo.2013.02.038

Nichol, M. B., Knight, T. K., Wu, J., Barron, R., & Penson, D. F. (2009). Evaluating use patterns of and adherence to medications for benign prostatic hyperplasia. *J Urol*, 181(5), 2214-2221; discussion 2221-2212. doi:10.1016/j.juro.2009.01.033

Nicholson, T. M., Moses, M. A., Uchtmann, K. S., Keil, K. P., Bjorling, D. E., Vezina, C. M., Wood, R.W., Ricke, W. A. (2015). Estrogen receptor-alpha is a key mediator and therapeutic target for bladder complications of benign prostatic hyperplasia. *J Urol*, 193(2), 722-729. doi:10.1016/j.juro.2014.08.093

Nicholson, T. M., Ricke, E. A., Marker, P. C., Miano, J. M., Mayer, R. D., Timms, B. G., vom Saal, F.S., Wood, R.W., Ricke, W. A. (2012). Testosterone and 17beta-estradiol induce glandular prostatic growth, bladder outlet obstruction, and voiding dysfunction in male mice. *Endocrinology*, 153(11), 5556-5565. doi:10.1210/en.2012-1522

Nicholson, T. M., & Ricke, W. A. (2011). Androgens and estrogens in benign prostatic hyperplasia: past, present and future. *Differentiation*, 82(4-5), 184-199. doi:10.1016/j.diff.2011.04.006

Nicholson, T. M., Sehgal, P. D., Drew, S. A., Huang, W., & Ricke, W. A. (2013). Sex steroid receptor expression and localization in benign prostatic hyperplasia varies with tissue compartment. *Differentiation*, 85(4-5), 140-149. doi:10.1016/j.diff.2013.02.006

Niu, Y., Ge, R., Hu, L., Diaz, C., Wang, Z., Wu, C. L., & Olumi, A. F. (2011). Reduced levels of 5-alpha reductase 2 in adult prostate tissue and implications for BPH therapy. *Prostate*, 71(12), 1317-1324. doi:10.1002/pros.21348

Parsons, J. K., Sarma, A. V., McVary, K., & Wei, J. T. (2013). Obesity and benign prostatic hyperplasia: clinical connections, emerging etiological paradigms and future directions. *J Urol*, 189(1 Suppl), S102-106. doi:10.1016/j.juro.2012.11.029

Parsons, J. K., Dahm, P., Kohler, T. S., Lerner, L. B., Wilt, T. J. (2020). Surgical Management of Lower Urinary Tract Symptoms Attributed to Benign Prostatic Hyperplasia: AUA Guideline Amendment 2020. *J Urol*, 204(4), 799-804. doi:10.1097/JU.0000000000001298

Platz, E. A., Kawachi, I., Rimm, E. B., Willett, W. C., & Giovannucci, E. (2000). Race, ethnicity and benign prostatic hyperplasia in the health professionals follow-up study. *J Urol*, 163(2), 490-495. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10647663

Porst, H., Kim, E. D., Casabe, A. R., Mirone, V., Secrest, R. J., Xu, L., Sundin, D.P., Viktrup, L., Lvhj study team (2011). Efficacy and safety of tadalafil once daily in the treatment of men with lower urinary tract symptoms suggestive of benign prostatic hyperplasia: results of an international randomized, double-blind, placebo-controlled trial. *Eur Urol*, 60(5), 1105-1113. doi:10.1016/j.eururo.2011.08.005

Prins, G. S., & Korach, K. S. (2008). The role of estrogens and estrogen receptors in normal prostate growth and disease. *Steroids*, 73(3), 233-244. doi:10.1016/j.steroids.2007.10.013

Risbridger, G., Wang, H., Young, P., Kurita, T., Wang, Y. Z., Lubahn, D., Gustafsson, J.A., Cunha, G. (2001). Evidence that epithelial and mesenchymal estrogen receptor-alpha mediates effects of estrogen on prostatic epithelium. *Dev Biol*, 229(2), 432-442. doi:10.1006/dbio.2000.9994

Roehrborn, C. G., Boyle, P., Nickel, J. C., Hoefner, K., Andriole, G., Aria, A., & Investigators, A. S. (2002). Efficacy and safety of a dual inhibitor of 5-alpha-reductase types 1 and 2 (dutasteride) in men with benign prostatic hyperplasia. *Urology*, 60(3), 434-441. doi:10.1016/s0090-4295(02)01905-2

Roehrborn, C. G., Casabe, A., Glina, S., Sorsaburu, S., Henneges, C., & Viktrup, L. (2015). Treatment satisfaction and clinically meaningful symptom improvement in men with lower urinary tract symptoms and prostatic enlargement secondary to benign prostatic hyperplasia: Secondary results from a 6-month, randomized, double-blind study comparing finasteride plus tadalafil with finasteride plus placebo. *Int J Urol*, 22(6), 582-587. doi:10.1111/iju.12741

Roehrborn, C. G., Marks, L. S., Fenter, T., Freedman, S., Tuttle, J., Gittleman, M., Morrill, B., Wolford, E. T. (2004). Efficacy and safety of dutasteride in the four-year treatment of men with benign prostatic hyperplasia. *Urology*, 63(4), 709-715. doi:10.1016/j.urology.2004.01.001

Royuela, M., de Miguel, M. P., Bethencourt, F. R., Sanchez-Chapado, M., Fraile, B., Arenas, M. I., & Paniagua, R. (2001). Estrogen receptors alpha and beta in the normal, hyperplastic and carcinomatous human prostate. *J Endocrinol*, 168(3), 447-454. doi:10.1677/joe.0.1680447

Sarkar, R. R., Parsons, J. K., Bryant, A. K., Ryan, S. T., Kader, A. K., McKay, R. R., D'Amico, A.V., Nguyen, P.L., Hulley, B.J., Einck, J.P., Mundt, A.J., Kane, C.J., Murphy, J.D., Rose, B. S. (2019). Association of Treatment With 5alpha-Reductase Inhibitors With Time to Diagnosis and Mortality in Prostate Cancer. *JAMA Intern Med*, 179(6), 812-819. doi:10.1001/jamainternmed.2019.0280

Sehgal, P. D., Bauman, T. M., Nicholson, T. M., Vellky, J. E., Ricke, E. A., Tang, W., Xu, Y., Huang, W., Ricke, W. A. (2019). Tissue-specific quantification and localization of androgen and estrogen receptors in prostate cancer. *Hum Pathol*, 89, 99-108. doi:10.1016/j.humpath.2019.04.009

Shao, R., Shi, J., Liu, H., Shi, X., Du, X., Klocker, H., Lee, C., Zhu, Y., Zhang, J. (2014). Epithelial-to-mesenchymal transition and estrogen receptor alpha mediated epithelial dedifferentiation mark the development of benign prostatic hyperplasia. *Prostate*, 74(9), 970-982. doi:10.1002/pros.22814

Shi, X., Peng, Y., Du, X., Liu, H., Klocker, H., Lin, Q., Shi, J., Zhang, J. (2017). Estradiol promotes epithelial-to-mesenchymal transition in human benign prostatic epithelial cells. *Prostate*, 77(14), 1424-1437. doi:10.1002/pros.23404

Simon, R. M., Howard, L. E., Moreira, D. M., Roehrborn, C., Vidal, A. C., Castro-Santamaria, R., & Freedland, S. J. (2016). Does Prostate Size Predict the Development of Incident Lower Urinary Tract Symptoms in Men with Mild to No Current Symptoms? Results from the REDUCE Trial. *Eur Urol*, 69(5), 885-891. doi:10.1016/j.eururo.2015.12.002

Song, L., Shen, W., Zhang, H., Wang, Q., Wang, Y., & Zhou, Z. (2016). Differential expression of androgen, estrogen, and progesterone receptors in benign prostatic hyperplasia. *Bosn J Basic Med Sci*, 16(3), 201-208. doi:10.17305/bjbms.2016.1209

Speakman, M., Kirby, R., Doyle, S., & Ioannou, C. (2015). Burden of male lower urinary tract symptoms (LUTS) suggestive of benign prostatic hyperplasia (BPH) - focus on the UK. *BJU Int*, 115(4), 508-519. doi:10.1111/bju.12745

Specht, E., Kaemmerer, D., Sanger, J., Wirtz, R. M., Schulz, S., & Lupp, A. (2015). Comparison of immunoreactive score, HER2/neu score and H score for the immunohistochemical evaluation of somatostatin receptors in bronchopulmonary neuroendocrine neoplasms. *Histopathology*, 67(3), 368-377. doi:10.1111/his.12662

Stroup, S. P., Palazzi-Churas, K., Kopp, R. P., & Parsons, J. K. (2012). Trends in adverse events of benign prostatic hyperplasia (BPH) in the USA, 1998 to 2008. *BJU Int*, 109(1), 84-87. doi:10.1111/j.1464-410X.2011.10250.x

Wang, Z., Hu, L., Salari, K., Bechis, S. K., Ge, R., Wu, S., Tabatabaei, S., Strand, D.W., Olumi, A. F. (2017). Androgenic to oestrogenic switch in the human adult prostate gland is regulated by epigenetic silencing of steroid 5alpha-reductase 2. *J Pathol*, 243(4), 457-467. doi:10.1002/path.4985

Warner, M., Wu, W. F., Montanholi, L., Nalvarte, I., Antonson, P., & Gustafsson, J. A. (2020). Ventral prostate and mammary gland phenotype in mice with complete deletion of the ERbeta gene. *Proc Natl Acad Sci U S A*, 117(9), 4902-4909. doi:10.1073/pnas.1920478117

Welliver, C., Feinstein, L., Ward, J. B., Fwu, C. W., Kirkali, Z., Bavendam, T., Matlaga, B.R., McVary, K.T., Urologic Diseases in America, P. (2020). Trends in Lower Urinary Tract Symptoms Associated with Benign Prostatic Hyperplasia, 2004 to 2013: the Urologic Diseases in America Project. *J Urol*, 203(1), 171-178. doi:10.1097/JU.0000000000000499

Williams, A. M., Simon, I., Landis, P. K., Moser, C., Christens-Barry, W., Carter, H. B., Metter, E.J., Partin, A. W. (1999). Prostatic growth rate determined from MRI data: age-related longitudinal changes. *J Androl*, 20(4), 474-480. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10452590

Xue, B., Wu, S., Sharkey, C., Tabatabaei, S., Wu, C. L., Tao, Z., Cheng, Z., Strand, D., Olumi, A.F., Wang, Z. (2020). Obesity-associated inflammation induces androgenic to estrogenic switch in the prostate gland. *Prostate Cancer Prostatic Dis*, 23(3), 465-474. doi:10.1038/s41391-020-0208-4

Zhang, H., Frendl, D. M., Wang, Z., & Olumi, A. F. (2020). High Real-World Medication Adherence and Durable Clinical Benefit in Medicare Patients Treated with 5-Alpha Reductase Inhibitors for Benign Prostatic Hyperplasia. *J Urol*, 204(2), 325-331. doi:10.1097/JU.0000000000001014

Zhang, P., Hu, W. L., Cheng, B., Zeng, Y. J., Wang, X. H., Liu, T. Z., & Zhang, W. B. (2016). Which play a more important role in the development of large-sized

prostates (>/=80 ml), androgen receptors or oestrogen receptors? A comparative study. *Int Urol Nephrol*, 48(3), 325-333. doi:10.1007/s11255-015-1181-z