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## NF- $\kappa$ B and Androgen Receptor Variant 7 Induce Expression of SRD5A Isoforms and Confer 5ARI Resistance

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### Abstract

**BACKGROUND**—Benign prostatic hyperplasia (BPH) is treated with 5 $\alpha$ -reductase inhibitors (5ARI). These drugs inhibit the conversion of testosterone to dihydrotestosterone resulting in apoptosis and prostate shrinkage. Most patients initially respond to 5ARIs; however, failure is common especially in inflamed prostates, and often results in surgery. This communication examines a link between activation of NF- $\kappa$ B and increased expression of SRD5A2 as a potential mechanism by which patients fail 5ARI therapy.

**METHODS**—Tissue was collected from “Surgical” patients, treated specifically for lower urinary tract symptoms secondary to advanced BPH; and, cancer free transition zone from “Incidental” patients treated for low grade, localized peripheral zone prostate cancer. Clinical, molecular and histopathological profiles were analyzed. Human prostatic stromal and epithelial cell lines were genetically modified to regulate NF- $\kappa$ B activity, androgen receptor (AR) full length (AR-FL), and AR variant 7 (AR-V7) expression.

**RESULTS**—SRD5A2 is upregulated in advanced BPH. SRD5A2 was significantly associated with prostate volume determined by Transrectal Ultrasound (TRUS), and with more severe lower urinary tract symptoms (LUTS) determined by American Urological Association Symptom Score (AUASS). Synthesis of androgens was seen in cells in which NF- $\kappa$ B was activated. AR-FL and AR-V7 expression increased SRD5A2 expression while forced activation of NF- $\kappa$ B increased all three SRD5A isoforms. Knockdown of SRD5A2 in the epithelial cells resulted in significant reduction in proliferation, AR target gene expression, and response to testosterone (T). In tissue

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recombinants, canonical NF- $\kappa$ B activation in prostatic epithelium elevated all three SRD5A isoforms and resulted in in vivo growth under castrated conditions.

**CONCLUSION**—Increased BPH severity in patients correlates with SRD5A2 expression. We demonstrate that NF- $\kappa$ B and AR-V7 upregulate SRD5A expression providing a mechanism to explain failure of 5ARI therapy in BPH patients.

### Keywords

p65; inflammation; BPH; androgen receptor; androgen receptor variant 7

## INTRODUCTION

Benign Prostatic Hyperplasia (BPH) is the most common urologic disease in men over the age of 50 [1]. BPH is an expansion of the transition zone of the prostate that compresses the urethra, causing increased bladder outlet resistance. This is a cause of lower urinary tract symptoms (LUTS), which include difficult (straining, weak stream, sensation of incomplete emptying) and irritable voiding (frequency, urgency, urge incontinence) [2]. The severity of LUTS is assessed by patient reporting, captured in the American Urological Association Symptom Score (AUASS). There are many potential etiological factors contributing to BPH pathogenesis including metabolic syndrome, growth factors, hormone signaling, and inflammation [3–6]. The strong relationship between LUTS, diabetes, metabolic syndrome, and other risk factors is becoming more appreciated [7,8] suggesting links to the biology and treatment responses of BPH.

There are two major medical approaches for patients presenting with symptoms suggestive of BPH: alpha-adrenergic receptor antagonists ( $\alpha$ -blockers) [9] and steroid-5-alpha-reductase (SRD5A) that is blocked by 5 $\alpha$ -reductase inhibitors (5ARI) [10]. Alpha-blockers relax the muscle of the prostate and provide symptomatic relief. 5ARIs inhibit the conversion of testosterone (T) to the more potent dihydrotestosterone (DHT) by the SRD5A isoforms, resulting in a reduction of androgen receptor activity [11]. Inhibition leads to epithelial apoptosis, a decreased prostate volume, and thereby reduces LUTS, the risk of acute urinary retention, and the risk of prostate surgery [12,13]. However, a large proportion of patients either fail to respond or become resistant to current medical approaches over time, with some progressing to surgical intervention [13]. Given their age and comorbidities, these patients are often not ideal candidates for surgery [14,15]. Therefore, understanding treatment failure and developing new medical therapies appropriately targeted to these specific patient groups are desirable ways to move forward with precision medicine.

The eukaryotic nuclear factor-kappa B (NF- $\kappa$ B) transcription factor family regulates the expression of numerous genes [16]. NF- $\kappa$ B transcription factors are activated in response to a variety of stress signals, including cytokines and pathogens [17] and signaling occurs through canonical (p50/RelA) and non-canonical (p52/RelB) pathways, resulting in activation of overlapping sets of downstream genes. We have previously shown that elevated NF- $\kappa$ B activation in patients was associated with expression of, as well as its forced activation in cells resulted in an increase in androgen receptor full length (AR-FL) and variant 7 (AR-V7) expression [18].

COOH-terminal truncated variants of the AR (ARVs), including AR-V7, have been described in prostate cancer [19–26] and BPH [18,27]. These AR-Vs lack the ligand-binding domain of AR-FL. AR-Vs are constitutively active, driving AR-regulated transcription and promoting tumor progression under castrate conditions. Expression of AR-V7 is predictive of resistance to Enzalutamide and abiraterone acetate in prostate cancer patients [28]. Gao has reported that AR-Vs can be produced in response to the non-canonical NF- $\kappa$ B pathway, and we have shown that AR-Vs can be induced by the canonical NF- $\kappa$ B pathway [18,29,30]. Currently, however there is not an established mechanistic link between NF- $\kappa$ B and resistance to 5ARI therapy in BPH.

5ARI therapy targets the enzyme 5 $\alpha$ -reductase, of which there are three isoforms (SRD5A1, -2, and -3) [31–35]. SRD5A2 is the major 5 $\alpha$ -reductase isozyme in the prostate [20–22]. In prostate cancer increased expression of SRD5A1 and SRD5A2 predicts biochemical recurrence, metastatic potential, and reduced efficacy of androgen deprivation treatment [20,21]. SRD5A3 has been linked to glycosylation [34], hormone-refractory prostate cancer [36], and can be inhibited by both Dutasteride and Finasteride [33]. Gene polymorphisms in both SRD5A1 and SRD5A2 are linked to the biochemical recurrence of prostate cancer [31] and these polymorphisms could affect the production rate of DHT and the corresponding local exposure to androgens of androgen-responsive cells, thus undermining the basis of androgen deprivation strategies. Such a scenario is also possible in relation to BPH patient's response to 5ARI therapy, and we suspected that SRD5A levels could affect 5ARI failure in BPH. The goal of this study was to elucidate mechanisms of 5ARI resistance.

Our previous studies have demonstrated that both NF- $\kappa$ B and AR-V7 are associated with BPH severity, and further we demonstrated that NF- $\kappa$ B induces AR-V7 expression [18]. In this study, we investigated the relationship between activation of NF- $\kappa$ B and/or AR-V7 expression and the levels of SRD5A isoforms in BPH to understand resistance to 5ARI. We utilized human tissue samples, as well as benign human prostate epithelial and stromal cells, to test the consequences of NF- $\kappa$ B, AR-FL, and AR-V7 activation on SRD5A isoforms. Our results provide a mechanism that could contribute to 5ARI therapy failure in some patients. Our data suggest that approaches to inhibit NF- $\kappa$ B activation in combination with a 5ARI could have an impact in reducing treatment failure in BPH.

## MATERIALS AND METHODS

### Cells, Reagents, and Antibodies

BHPrS1 and NHPrE1 cell lines [37,38] were cultured and transduced with empty vector IKK2 (EV) or constitutively activated NF- $\kappa$ B through IKK2 (EE), over expression of AR-FL and AR-V7 (V7) as previously described [18]. Primary antibodies against 5 $\alpha$ -reductase 2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were purchased from Vector Laboratories (Burlingame, CA).

### Transient Knockdown Assays

NHPrE1 and BHPrS1 cells were seeded in 12 well plates and grown in charcoal stripped medium for 3 days until 80% confluent. One day before transfection, growth medium

switched to antibiotic-free. siRNA and Lipofectamine were diluted individually in serum-free RPMI1640, combined and then incubated at room temperature for 20 min to produce a siRNA-Lipofectamine 2000 complex. siRNA was then transfected into the NHPPrE1 and BHPPrS1 cells according to the manufacturer's protocol (Invitrogen). p65, SRD5A2, and non-targeting siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### Cell Viability Assay

Cell viability assay in charcoal stripped medium and was performed using CellTiter-Glo (Promega) according to manufacturer's instructions. NHPPrE1 cells were seeded at 2000 cells/well in a 96-well plate, grown over 3, 5, or 7 days in the presence and absence of testosterone, Finasteride, and/or abiraterone acetate. CellTiter-Glo measurements were taken at several time points to track cell survival.

### Determination of Androgen Synthesis by Cells

NHPPrE1 were grown in CS media for 5 days and cell pellet and media samples were collected in triplicates and analyzed in one analytical run for five androgens (testosterone [T], dihydrotestosterone [DHT], dehydroepiandrosterone [DHEA], androstenedione [ASD], and androsterone [AND]) using a modification of a validated high pressure liquid chromatographic (HPLC) assay with tandem mass spectrometric detection (LC/MS/MS) performed by the Bioanalytics, Metabolomics, and Pharmacokinetics (BMPK) Shared Resource at Roswell Park Cancer Institute (Buffalo, NY) as previously described [39].

### Prostate Tissue

Our Benign Prostate Tissue repository has been described previously [14,18,40]. As part of an Institutional Review Board (IRB)-approved protocol, fresh prostate specimens were collected from consented patients undergoing holmium laser enucleation of the transition zone of the prostate (HoLEP) for symptomatic BPH with LUTS, referred to here as "Surgical BPH" and as a control, a pathologist resected transition zone nodules from patients undergoing radical prostatectomy for small volume, low risk, clinically localized peripheral zone prostate cancer, referred to here as "Incidental BPH" as described previously. Twenty-nine Incidental and 36 Surgical patients, a subset of our previous study [18] were analyzed.

### Tissue Processing and Pathology

After gross pathological examination, all prostate samples used for this study was processed to maximize utility for multiple assays. This involved dividing samples, flash freezing in liquid nitrogen followed by storage at  $-80^{\circ}\text{C}$  until use, as well formalin fixation for paraffin embedding. Samples were reviewed by a pathologist (O.H.) to confirm histologic findings and to exclude those with any foci of cancer as previously described [18].

### Quantitative-Real-Time PCR and IHC

For quantitative real-time PCR (qPCR) 50mg flash-frozen tissue from patient of experimental sources was ground using a mortar and pestle in liquid nitrogen and RNA was extracted with Trizol (Ambion). Subsequently, 500 ng RNA was reverse transcribed into cDNA using RT2 First Strand Kit (Qiagen, Valencia, CA). qPCR was performed using IQ

SYBR Green Supermix (BioRad, Hercules, CA) and results were analyzed using BioRad CFX manager software. All results were calculated using  $C_t$  analysis and normalized to GAPDH expression. qPCR primers have all been previously described and are all human-specific (no cross reaction to rat or mouse sequences). AR-FL, AR-V7, PSA, and TMPRSS2 primers were described in [18], those for SRD5A1, -2, and -3 were described in [36].

Immunohistochemistry used 4  $\mu$ m sections which were de-waxed, rehydrated, and endogenous peroxidases were blocked with hydrogen peroxide. Sections were then boiled in citrate and cooled for 1 hr. Primary blocking was 15 min using Vector Elite reagents (Vector Laboratories). Primary antibodies were incubated for 30 min at room temperature at the following concentrations: SRD5A2 (1:25). Slides were washed in PBS. Biotinylated secondary antibodies (Vector Laboratories) were incubated for 30 min at room temperature after. Slides were incubated in ABC-HRP complex (Vector Laboratories Burlingame, CA) for 30 min at room temperature. Bound antibodies were then visualized by incubation with 3,3' diaminobenzidine tetrahydrochloride (liquid DAB, DAKO). Slides were then rinsed in tap water, counterstained with hematoxylin, and mounted.

### Tissue Recombinants and Subrenal Capsule Xenografting

Rat urogenital sinus mesenchyme (rUGM), the inductive mesenchymal cells surrounding the epithelial core of the urogenital sinus, was prepared from E18.5 embryonic fetuses as previously described [41–43]. To prepare tissue recombinants, rat UGM was mixed with human prostate epithelial cells as discussed in the text. The cell mixture was pelleted and resuspended in 50  $\mu$ l rat-tail collagen (pretitrated to pH 7.4). After polymerization, the collagen was overlaid with growth medium. After incubation overnight at 37°C, the tissue recombinants were grafted under the renal capsule of intact Athymic Nude-Foxn1nu mouse. Mice were castrated, then rUGM recombination with either NHPPrE1-EV, NHPPrE1-EE, or NHPPrE1-AR-V7 cell lines. For each of the three epithelial cell lines we used three mice each carrying two recombinant grafts in Groups 1 and 2 for a total  $n = 6$  grafts per cell line per group. Grafts were inserted under the kidney capsule, and then supplemented with a 25mg testosterone pellet for 2 weeks to allow graft establishment. After 2 weeks mice were separated into two groups. Group 1 received a new testosterone pellet, while Group 2 animals had their testosterone pellet removed and returned to castrate-conditions. Hosts were euthanized at 8 weeks after grafting a portion of these grafts were flash frozen, subjected to mortar and pestle disassociation and Trizol treatment to recover RNA. cDNA was generated using reverse transcription. The other portion was analyzed histologically.

### Statistical Analysis

Mann–Whitney tests were used for univariate comparisons of study characteristics between Surgical and Incidental patients. A two-sided  $P$ -value of 0.05 or less was considered statistically significant. A two-way ANOVA test was used for comparisons of characteristics between 5 $\alpha$ -reductase isoform 1, 5 $\alpha$ -reductase isoform 2, and 5 $\alpha$ -reductase isoform 3 gene expressions in xenografts. Correlation between SRD5A2 1–3 and TRUS and AUASS was evaluated by the determination of the Spearman correlation coefficient (GraphPad Prism).

## RESULTS

### SRD5A2 Is Upregulated in More Severe BPH and in Patients Exposed to 5ARI Therapy

Progressive BPH is commonly associated with inflammation [44]. We recently demonstrated that NF $\kappa$ B expression was significantly higher in Surgical compared to Incidental BPH and, additionally, was correlated with TRUS volume [18]. We wanted to determine whether SRD5A levels in these same patients were associated with symptomatic progression. An initial immunohistochemical analysis was performed to compare expression in clinically advanced Surgical BPH specimens versus Incidental BPH specimens. The data show a complex staining pattern in both groups with a wide variability within the two groups and overlapping patterns between the groups. Two examples are shown in Figure 1A, and it is important to emphasize that examples of these two patterns of expression both occur in Surgical and Incidental patient samples. In some samples staining is essentially localized only to epithelial cells, this pattern has been reported previously [45]. As expected, many samples in both Surgical and Incidental groups have additional staining of variable distribution and intensity in the stroma.

To quantitate overall SRD5A expression we utilized qPCR to determine mRNA levels. We showed a significant increase in SRD5A2 expression in the Surgical versus the Incidental BPH (Fig. 1B). SRD5A1 and SRD5A3 were present at low levels in these samples and were not significantly correlated with disease severity (data not shown).

We next investigated whether SRD5A2 expression was associated with increased prostate size and with increased LUTS. We analyzed patients whose AUASS (n = 61) and TRUS volume (n = 53) was included in our data set. There was a significant correlation linking both increased TRUS volume ( $P = 0.0353$ ) (Fig. 1C) and AUASS ( $P < 0.0001$ ) (Fig. 1D) with SRD5A2 expression. In addition, SRD5A2 expression was significantly higher ( $P = 0.0127$ ) in patients who had received 5ARI therapy (Fig. 1E). No significant link was found between either SRD5A1 or SRD5A3 mRNA levels and TRUS volume (data not shown). We examined the relationship between SRD5A2 mRNA levels and those for AR-V7 and AR-FL. We found a significant positive correlation between SRD5A2 and AR-V7 mRNA levels (Fig. 1F) and a weak but not significant negative correlation with AR-FL levels when all patients were considered (Fig. 1G). A subanalysis of this dataset (Fig. 1G, red squares) revealed that when the advanced Surgical patients only were considered this negative correlation became stronger (Spearman  $r = -0.4208$ ) and significant ( $P = 0.01$ ).

### Chronic Activation of NF- $\kappa$ B Results in Enhanced Expression of SRD5A Isoforms

We previously demonstrated that activated NF- $\kappa$ B can induce expression of AR-V7, providing a potential route for resistance to 5ARI [18]. To determine whether chronic activation of NF- $\kappa$ B resulted in increased expression of SRD5A isoforms and influenced cell growth and function, we used previously generated cell lines [18] and show that NHPRE1-EE, a human prostatic epithelial cell line which has activated NF- $\kappa$ B through IKK2, significantly upregulated all three SRD5A isoforms ( $P < 0.001$ ) when compared to control empty vector (EV) cells (Fig. 2A). Forced expression of AR-V7 or AR-FL showed significant upregulation of only SRD5A2 ( $P < 0.05$ ) (Fig. 2A). In a stromal cell line

BHPrS1, SRD5A2 ( $P < 0.001$ ) was upregulated in the BHPrS1-EE, BHPrS1-AR-V7, BHPrS1-AR-FL lines, and SRD5A3 ( $P < 0.05$ ) in BHPrS1-AR-V7 cells (Fig. 2B).

### Inhibition of NF- $\kappa$ B Abrogates SRD5A Expression

To confirm that the elevation of SRD5A in the NHPPrE1-EE or the BHPrS1-EE cells was due to the effects of NF- $\kappa$ B activation, we performed experiments aimed at silencing downstream effectors of this activity. Since the constitutive activation of NF- $\kappa$ B in the EE-transduced cells is regulated by a modified form of IKK2, we used two alternative approaches to suppress NF- $\kappa$ B signaling. We used silencing of NF- $\kappa$ B, in both the epithelial and stromal cell lines through siRNA targeting of p65, and by the allosteric inhibition of IKK2 with the chemical compound BMS-345541. Silencing of NF- $\kappa$ B by allosteric inhibition (Fig. 3A and C) or by siRNA targeting (Fig. 3B and D) significantly ( $P < 0.0001$ ) reduced SRD5A2 mRNA expression in both benign human prostatic epithelial and stromal cell lines suggesting a general mechanism that NF- $\kappa$ B can exert control over SRD5A2 expression.

### Chronic Activation of NF- $\kappa$ B Induces Testosterone Synthesis

To determine whether expression of activated NF- $\kappa$ B can induce cells to generate increased levels of T and DHT, we used two approaches. First, a pharmacological inhibitor of androgen synthesis, abiraterone acetate (Abi) was used [46,47], and second direct quantitation using high pressure liquid chromatographic assay with tandem mass spectrometric detection (LC/MS/MS) to measure the levels of five endogenous androgens. NHPPrE1 cells were grown in charcoal stripped serum (vehicle) or with 4 nM Abi for 5 days. Treatment with Abi in activated NF- $\kappa$ B (NHPPrE1-EE) cell line resulted in a significant reduction in cell viability suggesting that suppressing androgen synthesis reduced cellular proliferation (Fig. 4A). LC/MS/MS was used to quantitate testosterone (T), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), androstenedione (ASD), and androsterone (AND) in NHPPrE1 cell lines grown in CS serum over a 5-day period. As shown in Figure 4B, the NHPPrE1-EE, NHPPrE1-AR-V7, and NHPPrE1-AR-FL cell lines can produce T. However, the NHPPrE1-EE cell line had over an eightfold higher production of DHT when compared to NHPPrE1-EV, NHPPrE1-AR-FL, and NHPPrE1-AR-V7 cells. This is consistent with the increased expression of SRD5A isoforms shown in Figure 2. DHEA, ASD, and AND were below the limit of detection in all cell lines.

### Knockdown of SRD5A2 Causes a Reduction in Cell Viability and AR Target Gene Expression

To determine whether there is a contribution of SRD5A2 in maintaining cell viability, we knocked down expression of this isoform using siRNA and assayed for cell viability after 3 days and AR target gene expression after 12 hr. As shown in Figure 5A, neither T or DHT affected the assay in the control (EV) cells. Also, as expected, knockdown of SRD5A2 in NHPPrE1-EV cells had no effect on cellular viability in the presence of T or DHT. In the NHPPrE1-EE cells androgen synthesis resulted in an increase in cell viability in the non-targeting siRNA cells, while knockdown of SRD5A2 decreased cell viability ( $P < 0.0001$ ) in the presence of T when compared to a non-targeting siRNA. This effect was rescued by DHT, confirming the necessity for SRD5A activity to convert T to DHT to elicit a response

(Fig. 5B). In the NHPPrE1-AR-V7 cell line we saw that the effects of T and DHT were minimal in the both the non-targeting siRNA and in the knockdown of SRD5A2 with no significant effects on cell viability (Fig. 5C). When looking at AR target gene expression in the knockdown cell lines using qPCR and normalized against—EV controls, we saw that there was no effect on PSA and TMPRSS2 expression in NHPPrE1-EV in the presence or absence of T or DHT or in the face of siRNA knockdown of SRD5A2 (data not shown). In marked contrast NHPPrE1-EE cells expressed both PSA and TMPRSS2 and this expression was increased by exogenous testosterone and had a significant decreased ( $P < 0.01$ ) when SRD5A2 was suppressed with siRNA (Fig. 6A). In the NHPPrE1-AR-V7 cell line, there was no change in AR target gene expression with the knockdown of SRD5A2 (Fig. 6B).

### NF- $\kappa$ B Induces Resistance to Castration

To determine how NHPPrE1-EV, NHPPrE1-EE, and NHPPrE1-AR-V7 cells lines respond to androgen withdrawal in vivo we used a tissue recombination model. The epithelial cells lines were recombined with prostatic inductive rUGM and grafted under the renal capsule of castrated or castrated and testosterone supplemented nude mice. The treatment schema is illustrated in Figure 7A. Each group contained six sub-renal capsule grafts containing cell lines NHPPrE1-EV, NHPPrE1-EE, or NHPPrE1-AR-V7 with rUGM. As proof of principle that the castration and the T pellet in castrated mice regulated androgen target tissues, the host seminal vesicles (SV) and associated prostatic complex were dissected and examined. The marked reduction in the size of the SV and prostate complex confirmed that androgenic stimulation had been effectively removed in these mice (not shown).

H&E staining of grafts in mice maintained under continuous androgenic stimulation showed prostatic ductal development, with luminal and basal epithelial cells surrounding a lumen (Fig. 7B). Under castrate conditions NHPPrE1-EV and NHPPrE1-AR-V7 grafts failed to maintain epithelial glandular architecture. In contrast, grafts in which NF- $\kappa$ B is constitutively activated in the epithelium (the NHPPrE1-EE grafts) displayed glandular development ( $n = 4/6$ ) even under castrate conditions (Fig. 7B).

To examined biological activity in the human epithelial cell component of the grafts we used a series of human-specific qPCR primers. As shown in Figure 8A left, in the presence of testosterone, NHPPrE1-EE recombinants had a significant increase in SRD5A2 ( $P < 0.001$ ) and SRD5A3 ( $P < 0.01$ ) when compared to NHPPrE1-EV while NHPPrE1-AR-V7 recombinants had a significant increase in only SRD5A2 ( $P < 0.01$ ). Under castrate conditions, NHPPrE1-EE grafts showed a significant increase ( $P < 0.001$ ) in all three SRD5A isoforms while NHPPrE1-AR-V7 grafts only had a significant increase ( $P < 0.01$ ) in SRD5A2 (Fig. 8A right).

When looking at the AR target genes PSA and TMPRSS2 in these grafts, both NHPPrE1-EE and NHPPrE1-AR-V7 showed a significant increase ( $P < 0.001$ ) in gene expression in the presence of testosterone, as compared to NHPPrE1-EV controls (Fig. 8B left). PSA is not present in rat or mouse genomes and the TMPRSS2 primers used for these assays detect only the human gene, so detection is limited to mRNA derived from the human cells in the grafts. Under castrate conditions the NHPPrE1-AR-V7 recombination grafts showed a significant decrease in expression of these human AR target genes under castration



compared to androgenized conditions while the NHPRE1-EE grafts expressed similar levels of these AR target gene expression in the presence and absence of exogenous androgens (Fig. 8B right).

## DISCUSSION

Our goal in this study was to elucidate a mechanism by which BPH patients may become resistant to 5ARI treatment. We investigated the link between activation of NF- $\kappa$ B and SRD5A levels in BPH. In prostate cancer SRD5A1 and SRD5A2 predict biochemical recurrence and reduced efficacy of androgen deprivation therapy [31,32]. In a previous study [18], we demonstrated that both activation of NF- $\kappa$ B and subsequent induction of AR-V7, contributed to resistance to a 5ARI. Here we examine whether the induction of SRD5A expression could also be a player in this phenomenon. We used a previously described repository of human prostate transition zone tissues that represents a range of BPH/LUTS severity. Using these Surgical BPH samples, we saw that SRD5A2 Levels were significantly higher in patients whose disease had progressed and required surgical intervention when compared to TZ from patients with low AUASS (Incidental BPH). We also saw that SRD5A2 significantly correlated with increased TRUS volume and AUASS. TRUS measures the entire volume of the prostate and our group has shown that TRUS volumes of Surgical BPH patients were significantly increased when compared to Incidental BPH (reflecting a general increase in prostate size as the severity of the disease increases). The Surgical BPH patients had failed therapy for severe symptoms while the transition zone tissue from Incidental patients generally represented earlier stages of BPH with no cancer lesions in the TZ and low grade and limited cancer lesions in the peripheral zone. This suggests that any change in prostate volume can be primarily attributed to transitional zone volume. We also saw that SRD5A2 was significantly higher in BPH patients who had been exposed to a 5ARI therapy; although, this result could reflect that more advanced BPH patients are more likely to receive 5ARI therapy. In patient samples, SRD5A2 correlated with expression of AR-V7 (which we have shown can be a downstream consequence of inflammation). Increased levels of SRD5A2 correlate weakly with the down-regulation of AR-FL levels when all patients are examined. It is established that DHT activation of AR-FL feedbacks to down-regulate AR-FL mRNA [48,49]. Thus, the inverse relationship with SRD5A2 and AR-FL suggest higher levels of DHT are present in the prostatic tissue from these BPH patients. It is noteworthy that when only the more advanced Surgical patient samples are considered this negative correlation between SRD5A2 and AR-FL became significant ( $P=0.01$ ) and the Spearman correlation was much stronger. This may suggest some changes in dysregulation of androgen action in more advanced disease and that such a contribution becomes more important as BPH progresses. Since SRD5A2 is the primary enzyme in the prostate that converts T to DHT [31–33], its increased expression in more symptomatic patients could help to explain why these patients failed 5ARI therapy and progressed to surgery. Increased NF- $\kappa$ B and associated increased SRD5A2 provide a potential mechanism by which increases in SRD5A enzyme expression might swamp the suppressive action of the 5ARI. While many of these correlations are statistically significant, they are associated with relatively low r-values, suggesting that they represent a partial explanation of what is likely a more complex phenomenon.

To clarify the underlying molecular processes, a series of in vitro experiments was performed to examine the effects of NF- $\kappa$ B on SRD5A levels. We have previously shown that NF- $\kappa$ B is upregulated in the Surgical BPH patient samples and that NF- $\kappa$ B activation can induce AR-FL and AR-V7 expressions [18]. Here, we wanted to determine whether NF- $\kappa$ B activation could affect SRD5A expression in benign human prostatic cell lines. We show that NF- $\kappa$ B activation markedly upregulates SRD5A2 in epithelial and stromal cell lines, with much more modest effects on SRD5A1 and A3. AR-FL and AR-V7 overexpression in epithelial and stromal cell lines upregulated the SRD5A2 isoform. We used LC/MS/MS analysis to demonstrate that the NHPPrE1-EV, NHPPrE1-EE, NHPPrE1-AR-V7, and NHPPrE1-AR-FL cell lines can synthesize androgens, although the NHPPrE1-EE cells produced the highest levels of DHT, consistent with the large increase in SRD5A2 in these cells. The conversion of T to DHT is likely the most important component of androgen action in BPH patients as suppression of androgen synthesis is not part of the normal care of these patients and as such circulating levels of testicular androgens are normal. Suppression of NF- $\kappa$ B activity using siRNA or BMS-345541 resulted in a significant decrease in SRD5A2 expression by qPCR in the cells in which the pathway is forcibly activated. These results suggest that NF- $\kappa$ B induction of SRD5A isoforms, most significantly of SRD5A2, could help to explain why patients fail 5ARI therapy.

To better understand the relationship between synthesis of T, SRD5A expression, and 5ARI therapy, we used a tissue recombination grafting model, where NHPPrE1-EV, NHPPrE1-EE, and NHPPrE1 AR-V7 cell lines were combined with rUGM and grafted under the kidney capsule in the presence and absence of androgens for 6 weeks. Six weeks after castration the NHPPrE1-EE cell line was still able to maintain glandular epithelial development, showing luminal and basal cell arrangement and expressed AR-regulated genes. This is a parallel result to our report that NF $\kappa$ B activation in the mouse prostate results in a hyperplastic, non-malignant castrate resistant prostate in vivo [50]. This shows that under castrated conditions in vivo, activation of NF- $\kappa$ B can circumvent androgen ablation and supports a mechanism by which NF- $\kappa$ B can confer resistance to a 5ARI. Castration results in the absolute removal of testicular androgens whereas 5ARI therapy reduces the conversion of T to DHT. However, these experiments in castrated mice demonstrate the biological effect that chronic action of NF- $\kappa$ B can have on prostate growth. Activation of NF- $\kappa$ B, a consequence of chronic inflammation, can increase T synthesis, SRD5A expression, and DHT production resulting in the expression of androgen regulated genes in vivo. BPH is often thought of as a stromal disease with epithelial growth as a secondary consequence, a concept first described by McNeal and validated by Cunha et al. [43,51]. Paracrine interactions between the stroma and epithelium are important for the normal development of the prostate and also for the development of BPH [52–57]. Immune/inflammatory cells are a component of, and influence the behavior of, the stroma. Activation of NF- $\kappa$ B results in the upregulation of SRD5A enzymes resulting in increased conversion of T to DHT. This provides a mechanism by which inflammation plays a role in BPH progression and 5ARI failure.

In summary, the development of resistance to 5ARI therapy is likely complex and multifactorial. We have previously shown a link between NF $\kappa$ B activation and the expression of constitutively active AR-V7 that suggests on contributing mechanism to this resistance. The data presented suggest an additional link; whereby, aberrant activation of

NF- $\kappa$ B drives upregulation SRD5A isoforms which increases the local synthesis of DHT. Increased NF- $\kappa$ B activity and the synthesis of locally produced androgens and elevated levels of SRD5A would drive the activation of AR signaling. Linking together these pathways provides a fundamental mechanism that explains failure of 5ARI therapy for BPH patients. The common theme between these observations is that inflammation that is a common response to the comorbidities of BPH, notably obesity [58–61]. This insight can result in new approaches to combat BPH progression and 5ARI failure and devise approaches in precision therapy to treat individual BPH patients.

## Acknowledgments

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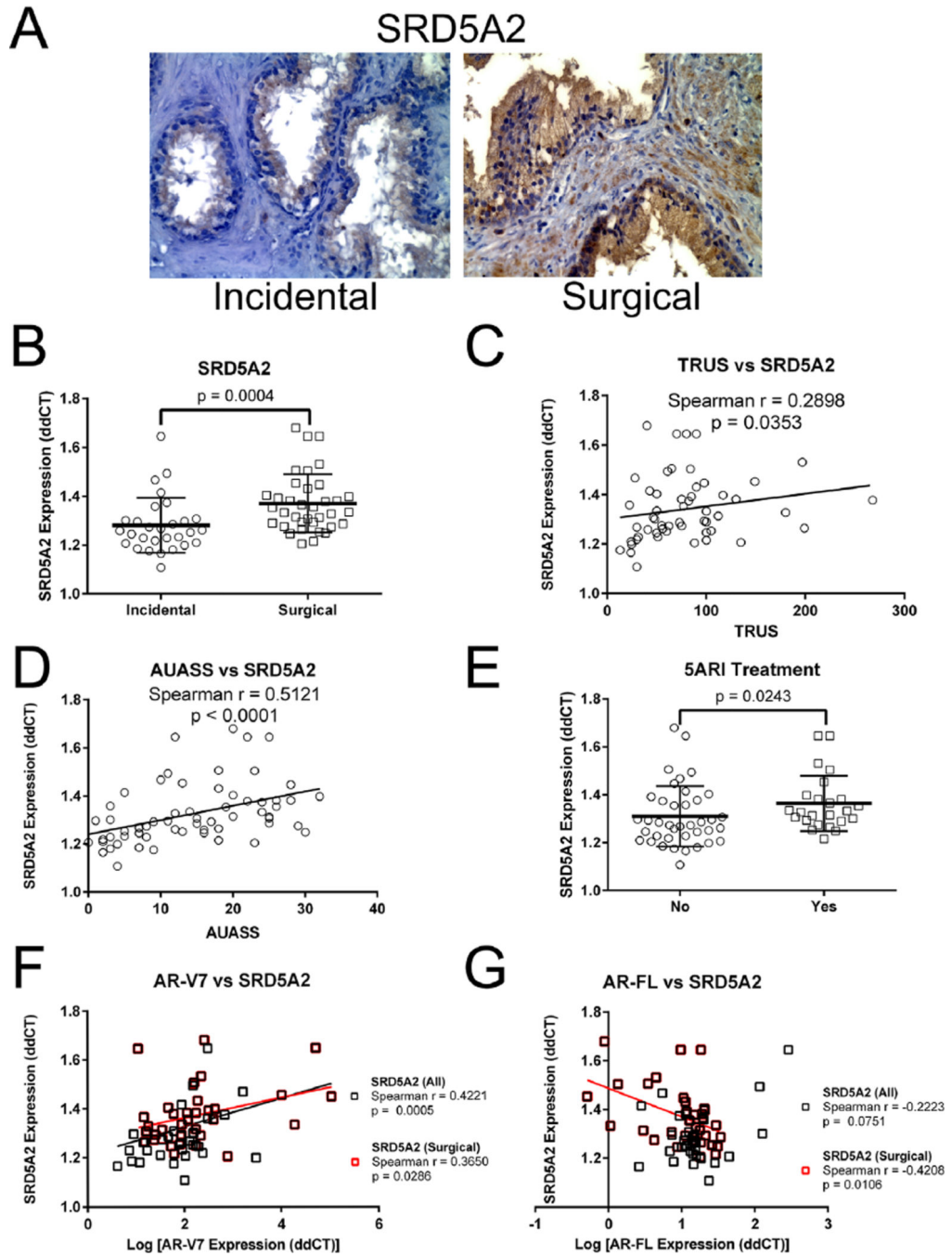
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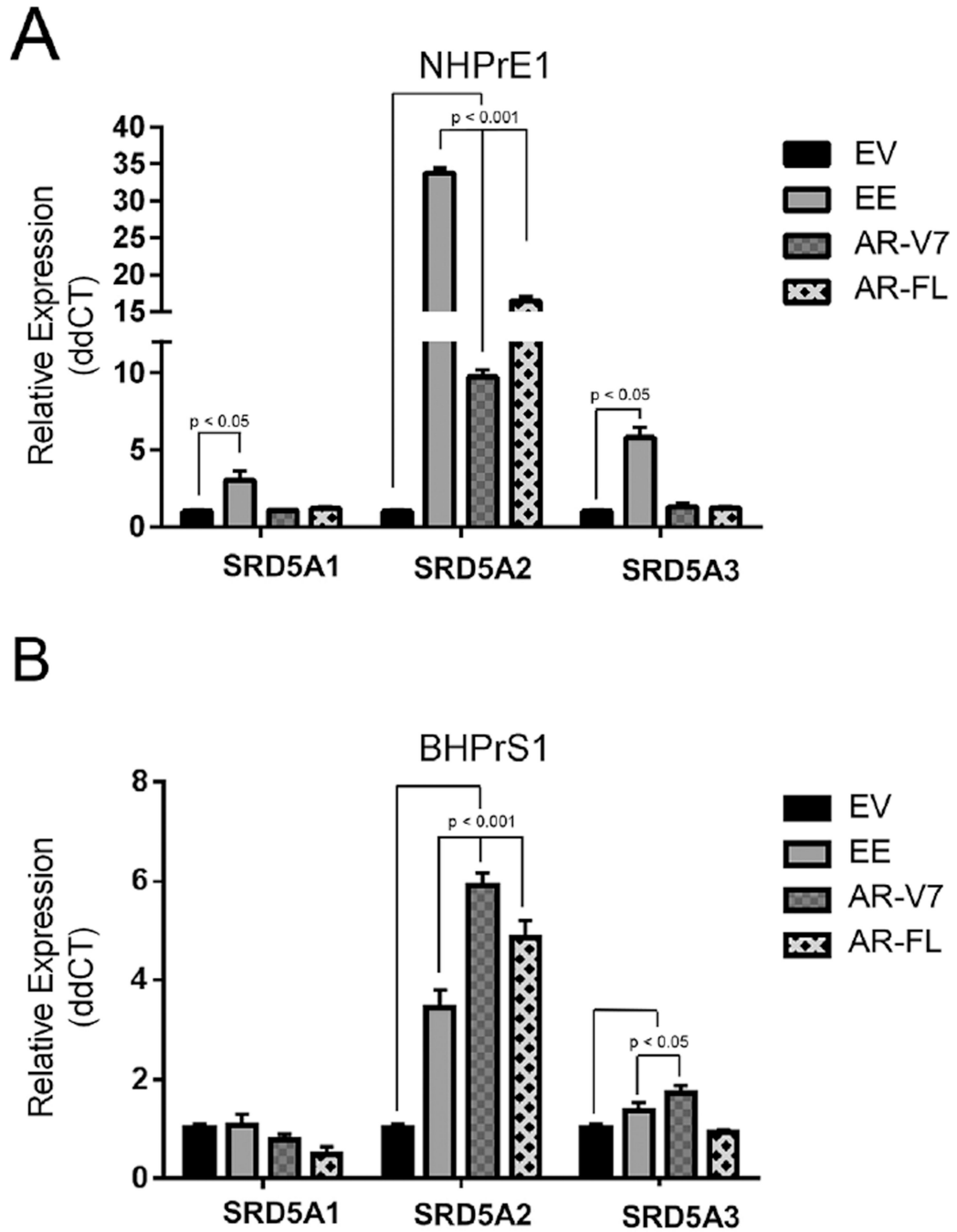
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**Fig. 1.** SRD5A expression in symptomatic BPH. Immunolocalization of SRD5A2 in incidental and surgical BPH samples. (A) SRD5A2. Consistent with the qPCR data shown in panel B there is a general increase in staining intensity in surgical versus incidental samples; however, there is significant variability and overlap in both intensity and pattern of staining in the groups. The examples shown illustrate an incidental sample where staining is essentially localized only to epithelial cells, this phenotype is common. However many samples, such as the surgical example shown have additional staining of variable distribution and intensity in

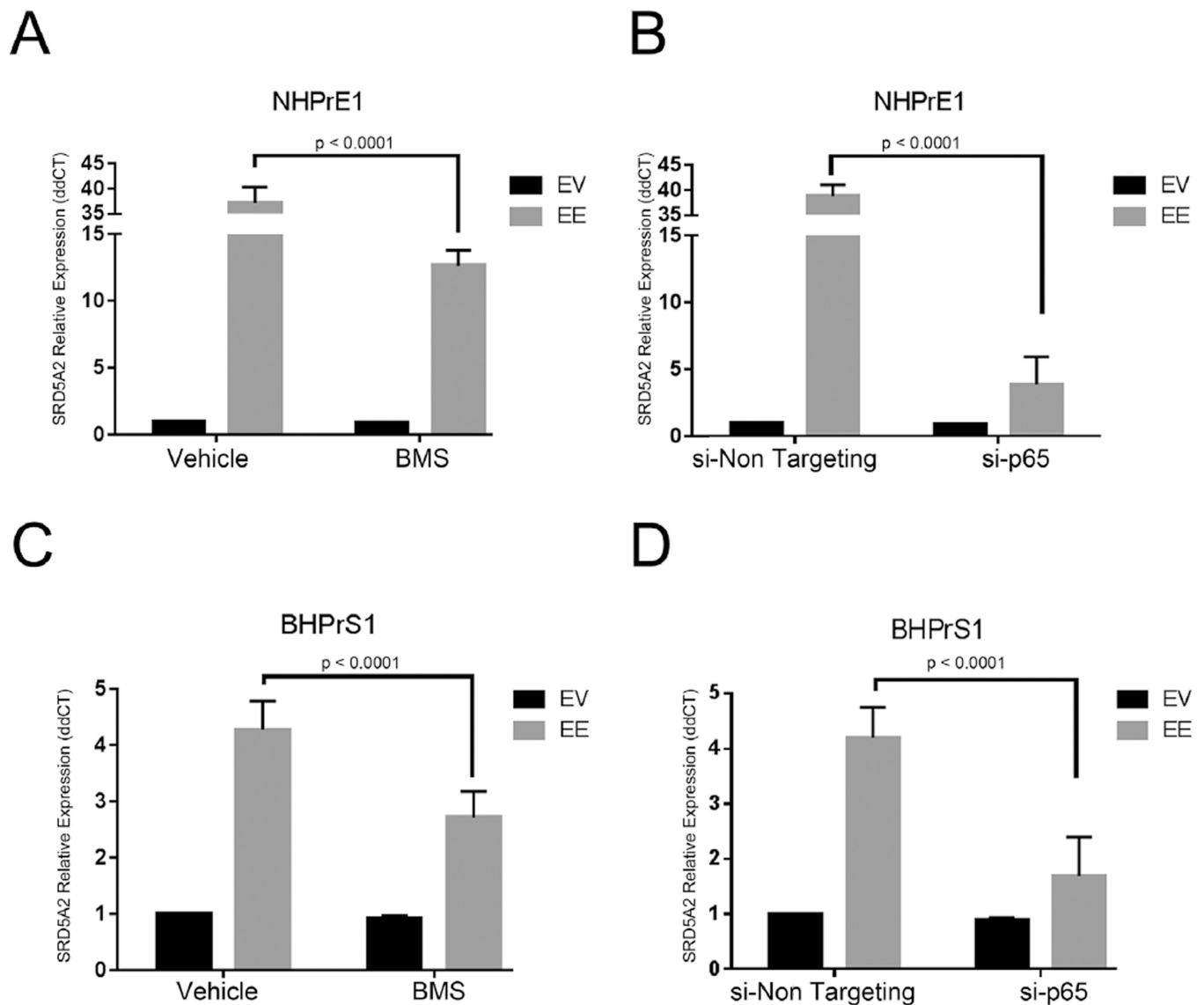
the stroma. **(B)** qPCR analysis of SRD5A2 expression in BPH, showing a significant increase in expression of SRD5A2 in the Surgical BPH (n = 36) cohort compared to Incidental BPH (n = 29). Bars are presented as standard deviation, *P*-value: non-parametric Mann–Whitney test. **(C)**. Spearman’s correlation coefficient analysis of SRD5A2 mRNA expression and TRUS volume showing a significant increase in SRD5A2 expression as TRUS volume increases (n = 53). **(D)** Spearman’s correlation coefficient analysis of SRD5A2 mRNA expression and AUASS showing a significant increase in SRD5A2 expression as AUASS increases (n = 61). **(E)** qPCR analysis of SRD5A2 expression in BPH patients. Patients treated with 5 $\alpha$ -reductase inhibitors (5ARI) displayed a significant increase in SRD5A2 expression versus patients not exposed to 5ARIs (n = 65). Bars are presented as standard deviation, *P*-value: non-parametric Mann–Whitney test. **(F)** Spearman’s correlation coefficient analysis of SRD5A2 and AR-V7 mRNA expression showing a significant increase in SRD5A2 expression as AR-V7 increases (n = 65). **(G)** Spearman’s correlation coefficient analysis of SRD5A2 shows a trend but no significant decrease of AR-FL mRNA expression n = 65.



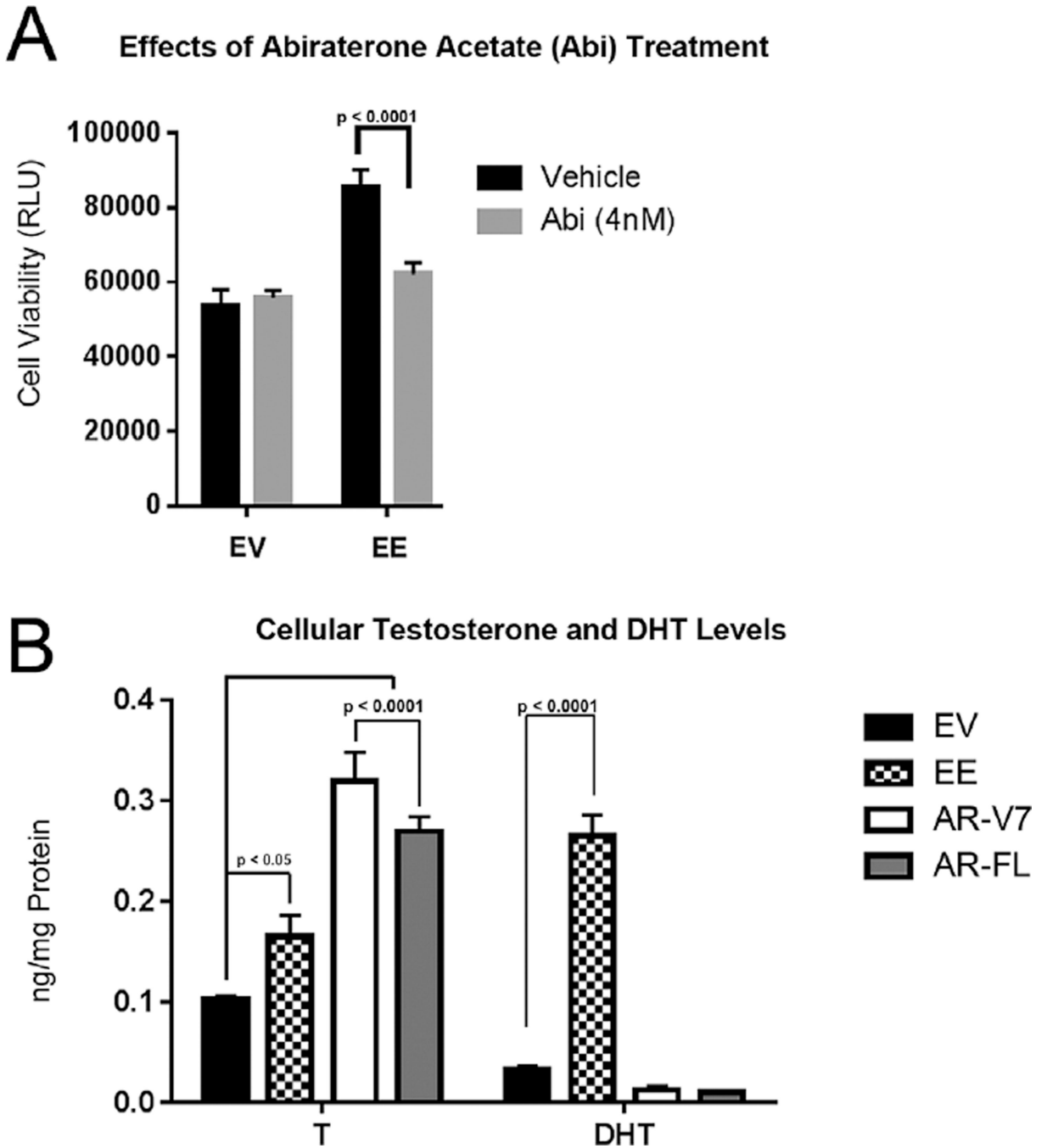


**Fig. 2.** NF- $\kappa$ B and AR can regulate SRD5A expression in human cell lines. qPCR analysis of SRD5A1, SRD5A2, and SRD5A3 expression in the benign human prostatic epithelial cell line NHPRE1 (**A**) and the benign human prostatic stromal cell line BHPPrS1 (**B**) transduced with empty vector (EV), constitutively active NF- $\kappa$ B (EE), overexpressed androgen receptor full length (AR), and androgen receptor variant 7 (V7). (**A**) Activation of NF- $\kappa$ B in NHPRE1 cells (NHPRE1-EE) resulted in a significant increase expression of all three SRD5A isoforms while expression of AR and AR-V7 (NHPRE1-AR-FL and NHPRE1-AR-

V7) resulted in a significant (and much attenuated) increase in SRD5A2 expression only. **(B)** BHPPrS1-EE, BHPPrS1-AR-FL, and BHPPrS1-AR-V7 demonstrated a significant increase in SRD5A2 expression. All experiments were performed three times with triplicate repetitions. Bars are presented as standard deviation, *P*-value: two-way ANOVA.



**Fig. 3.** NF- $\kappa$ B inhibition affects SRD5A2 expression. **(A)** Inhibition of NF- $\kappa$ B activation by BMS-345541 in NHPPrE1-EE and **(C)** BHPPrS1-EE cell lines resulted in a significant decrease in SRD5A2 expression. **(B)** Silencing of NF- $\kappa$ B using an siRNA towards p65 (si-p65) in NHPPrE1-EE and BHPPrS1-EE cells **(D)** Results in a significant decrease in SRD5A2 expression compared to a non-targeting siRNA (si-Non Targeting). Bar values are standard deviations. *P*-value determined using non-parametric Mann-Whitney test.



**Fig. 4.** NF- $\kappa$ B activation increases androgenic effects and DHT synthesis. (A) NHPRE1-EV (empty vector) and NHPRE1-EE (constitutively active NF- $\kappa$ B) cell lines were treated with 4 nM of Abiraterone acetate (Abi) to inhibit androgen synthesis. There was a significant decrease in viability of NHPRE1-EE over a five dayperiod when compared to vehicle. Bars are presented as standard deviation, *P*-value: two-way ANOVA (B) Measurement of two endogenous androgens (testosterone [T], and dihydrotestosterone [DHT]) in NHPRE1 cell lines grown in charcoal stripped serum over a five dayperiod. NHPRE1-EE cell line showed slightly

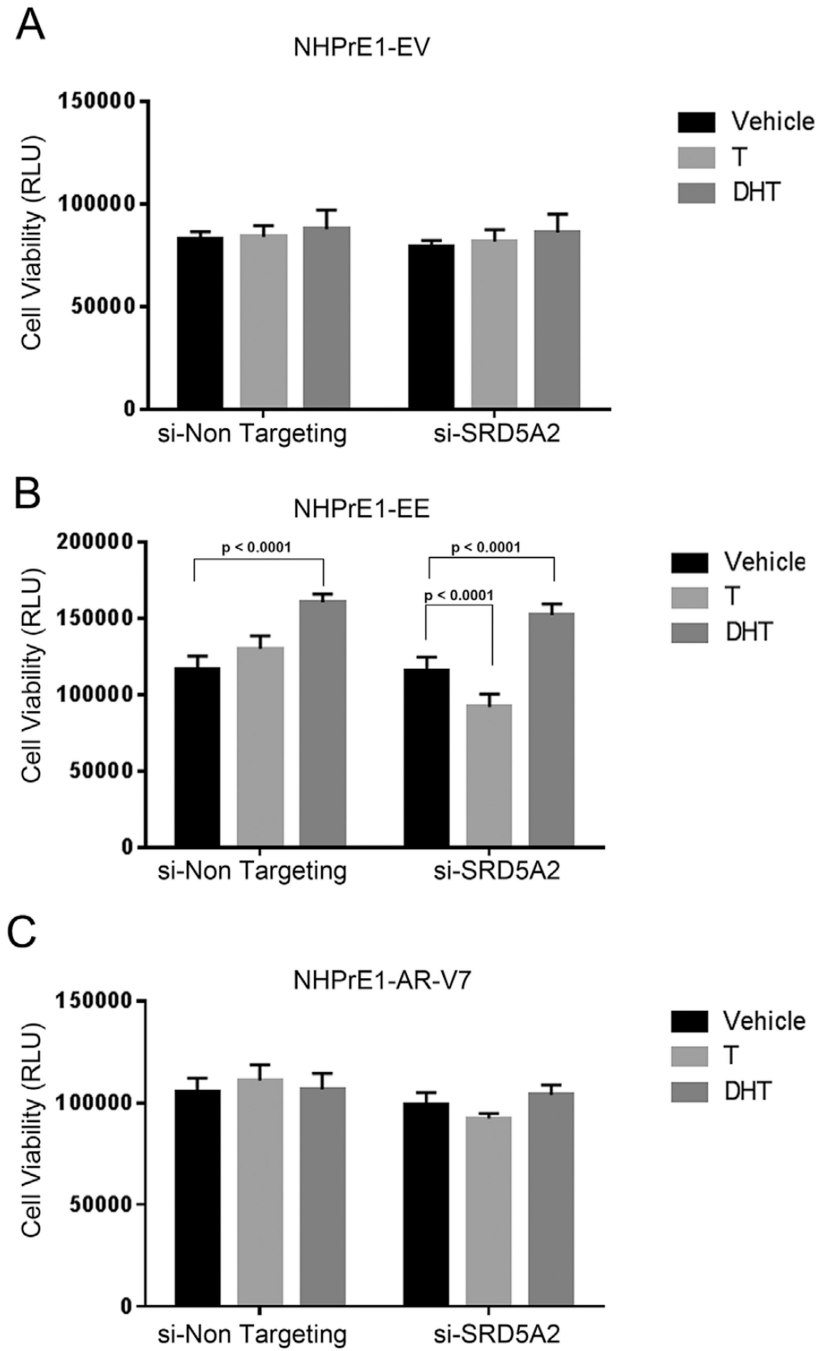
increased synthesis of T and a large increase in DHT when compared to NHPPrE1-EV (n = 3). Bars are presented as standard deviation, *P*-value: two-way ANOVA.

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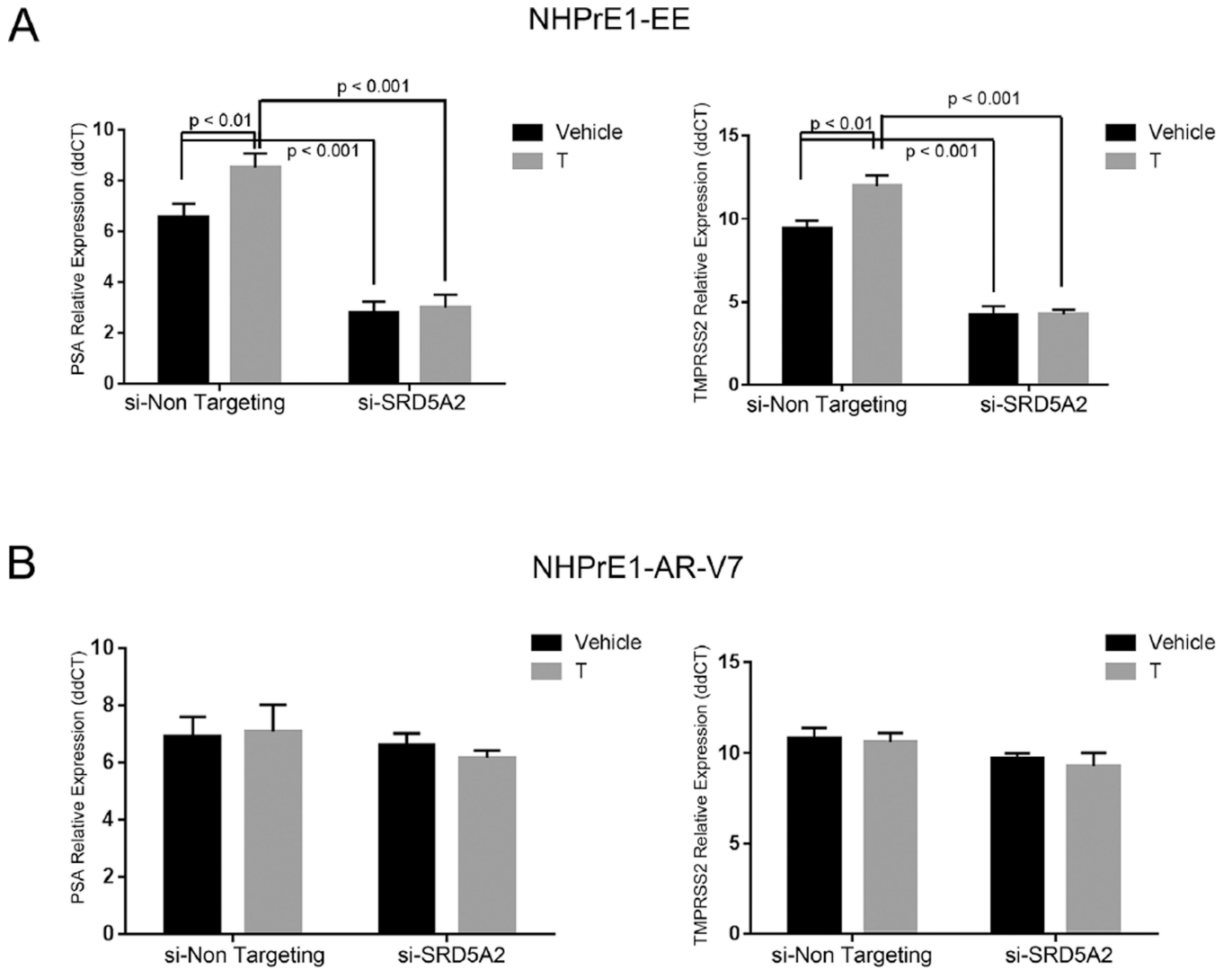
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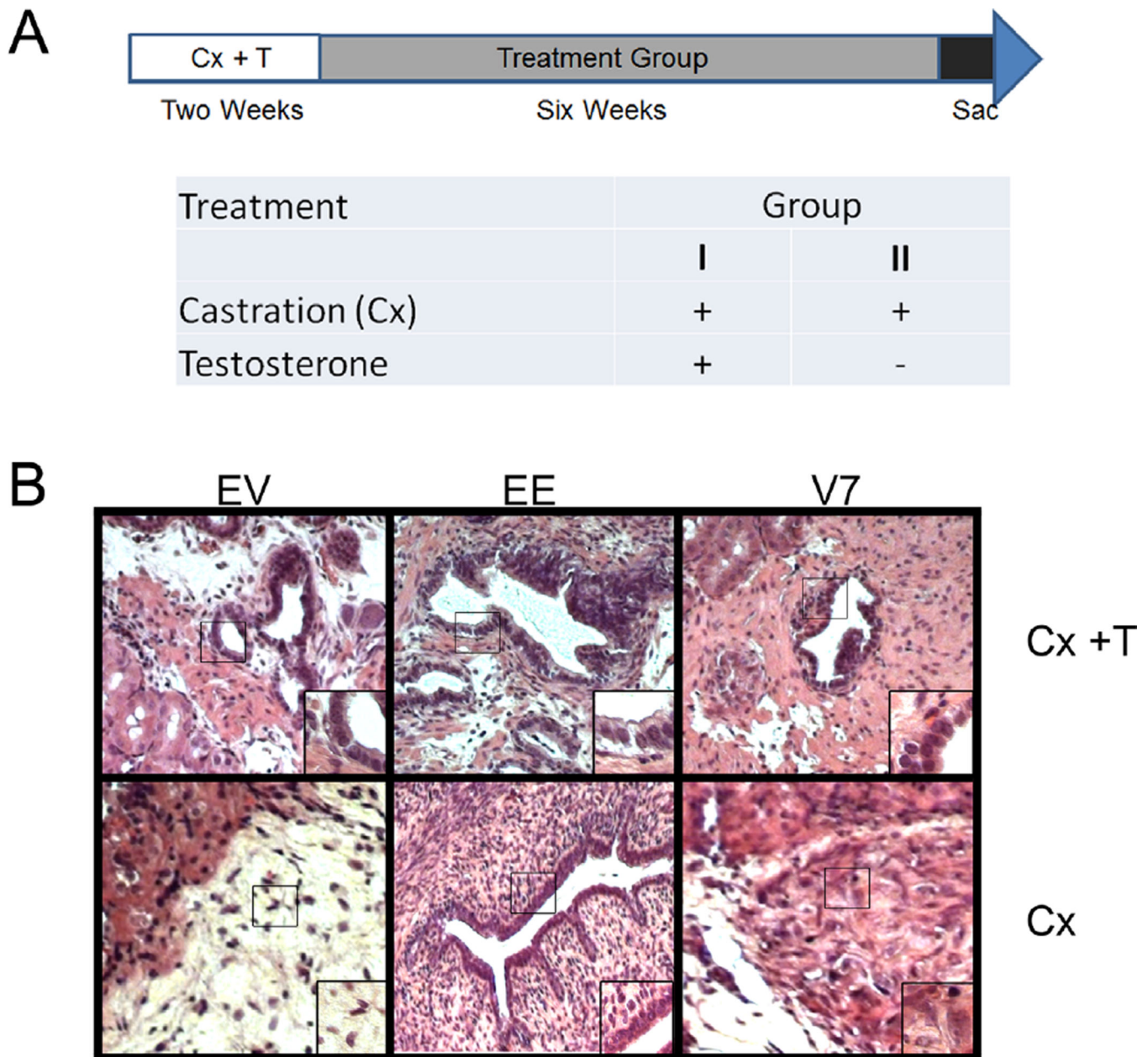
**Fig. 5.** Knock-down of SRD5A2 decreased viability in cells with activated NF- $\kappa$ B. NHPPr1-EV (empty vector control— **A** )-EE (constitutively active NF- $\kappa$ B—**B**), and -AR-V7 (expressing AR-V7—**C**) cell lines were transfected with siRNA corresponding to SRD5A2 (si-SRD5A2) or a control siRNA (si-Non Targeting) and incubated in the presence of vehicle (Ethanol), testosterone  $10^{-9}$  M (T) or dihydrotestosterone  $10^{-7}$  M (DHT). Cell viability was examined over a three day period. (**A**) Suppression of SRD5A2 had no effect on NHPPr1-EV viability in the presence or absence of androgens. (**B**) The control (si-Non Targeting) NHPPr1-EE

cell line responded to T and DHT and showed a significant ( $P < 0.0001$ ) increase in viability in the presence of DHT. Knock-down of SRD5A2 resulted in a significant ( $P < 0.0001$ ) decrease in cell viability even the presence of T but no change in the effects of DHT when compared to control cells. (C) NHPPrE1-AR-V7 cells line had no change in viability when SRD5A2 was knocked down even in the presence of androgens. All experiments were performed three times with triplicate repetitions ( $n = 9$ ). Bars are presented as standard deviation,  $P$ -value: two-way ANOVA.



**Fig. 6.** Knockdown of SRD5A2 results in a significant decrease in androgen receptor target gene expression in cells with active NF- $\kappa$ B but not in cells overexpressing AR-V7. NHPPrE1-EE (constitutively active NF- $\kappa$ B—**A**) and NHPPrE-V7 (expressing AR-V7—**B**) cell lines transfected with a control siRNA (control) or with siRNA corresponding to SRD5A2 (si-SRD5A2) were incubated in the presence of vehicle or testosterone (T). AR target gene expression was examined 12 hr later. Expression is relative to empty vector control cells that do not express androgen receptor. **(A)** qPCR of NHPPrE1-EE for AR target genes PSA (right) and TMPRSS2 (left) resulted in a significant increase in mRNA expression in the presence of androgens when compared to vehicle ( $P < 0.001$ ). SRD5A2 knock down cells had a significant decrease in PSA and TMPRSS2 expression even in the presence of androgens when compared to EE ( $P < 0.01$ ) and lost the ability to respond to androgens when compared SRD5A2 knock down vehicle treated. **(B)** qPCR in NHPPrE1-V7 cell lines for AR target genes PSA and TMPRSS2 resulted in no change in mRNA expression in the presence of testosterone. Bars are presented as standard deviation,  $P$ -value: two-way ANOVA. All experiments were performed three times with triplicate repetitions ( $n = 9$ ).





**Fig. 7.** Constitutively active NF- $\kappa$ B results in xenograft maintenance under castrated conditions. **(A)** Treatment schema for Athymic Nude-Foxn1nu mice. NHPPrE1-EV (empty vector control), NHPPrE1-EE (constitutively active NF- $\kappa$ B), and NHPPrE1-AR-V7 (overexpressing AR-V7) cells were recombined with rat urogenital sinus mesenchyme and grafted under the kidney capsule of mice. All mice were castrated and supplemented with testosterone (T) pellets for 2 weeks after grafting. After 2 weeks mice were separated into two groups. Group 1 (n = 9 mice) had a replacement T pellet and Group 2 (n = 9 mice) had their T pellet removed. Growth was assessed after 6 weeks and mice were euthanized. **(B)** Representative H&E images of recombinants containing NHPPrE-EV, NHPPrE-EE, and NHPPrE-AR-V7 cells. Under continuous androgenic stimulation these show glandular epithelial structures

that had developed. Following castration the NHPPrE1-EV and NHPPrE1-AR-V7 containing grafts, contain residual epithelium but no obvious retention of glandular structure, consistent with post castration regression in a recombinant. In contrast NHPPrE1-EE grafts show a maintenance of glandular architecture, representing protection from regression in the face of androgen ablation.

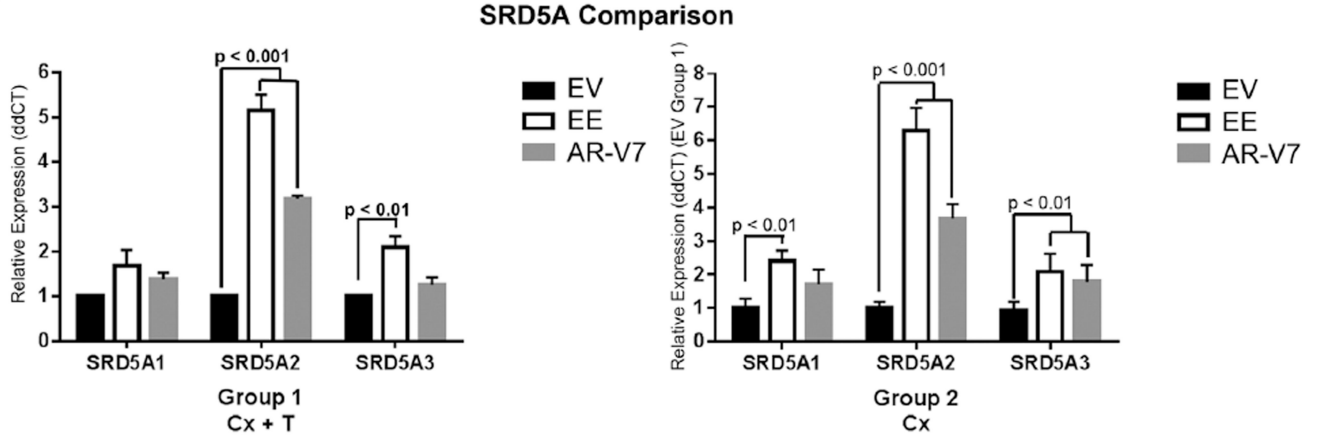
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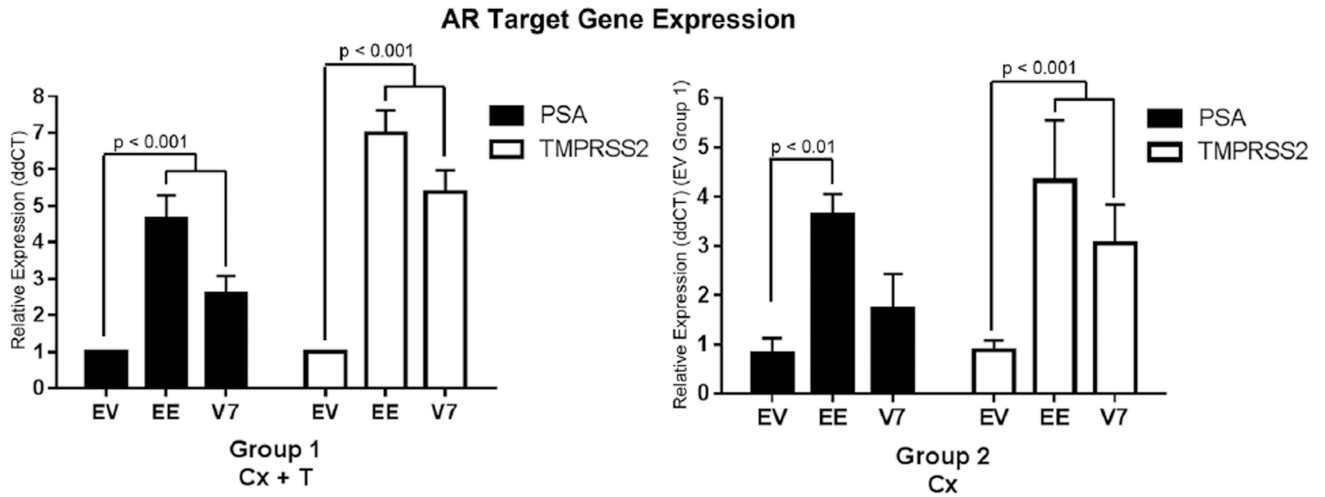
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A



B



**Fig. 8.** Constitutively active NF- $\kappa$ B results in significant increase of SRD5A and androgen receptor target gene expression under castrated conditions. qPCR analysis of mRNA expression of SRD5A isoforms (A) and AR target gene expression (B) were performed on tissue recombinant samples. Expression levels were separately standardized to the control level for each isoform and target gene. In the presence of androgens there was a significant increase in SRD5A2 and SRD5A3 ( $P < 0.01$ ) in NHPPrE1-EE (constitutively active NF- $\kappa$ B) grafts and in SRD5A2 ( $P < 0.001$ ) in NHPPrE1-AR-V7 grafts when compared to NHPPrE1-EV (right panel). When compared to NHPPrE1-EV grafts in the presence of androgens, NHPPrE1-EE grafts under castrated conditions resulted in a significant increase in all three SRD5A ( $P < 0.01$ ) isoforms, while NHPPrE1-AR-V7 grafts showed an increase in SRD5A2 ( $P < 0.01$ ) (left panel). (B) qPCR of AR target genes PSA and TMPRSS2 in the presence of androgens showed a significant increase in NHPPrE1-EE and NHPPrE1-V7 ( $P < 0.001$ ) grafts when compared to NHPPrE1-EV (right panel). When compared to AR target gene expression in NHPPrE1-EV grafts under castrate conditions, NHPPrE1-EE grafts under castrated conditions showed an increase in PSA and TMPRSS2 ( $P < 0.001$ ) expression while NHPPrE1-AR-V7

resulted in only a significant increase of TMPRSS2 ( $P < 0.01$ ) expression (left panel). Bars are presented as standard deviation,  $P$ -value: two-way ANOVA. All experiments were performed three times with triplicate repetitions ( $n = 9$ ).