DEVELOPMENT AND APPLICATION OF NEW SYSTEMS FOR MODELING MURINE PROSTATE DEVELOPMENT AND DISEASE

by
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Abstract

Animal models have been useful in the study of prostate biology and disease for decades, with continuous refinement and renewal to improve utility and comparative accuracy. This work describes two advances in murine models of prostate development and disease, and details the application of these new models to discover critical aspects of prostate biology.

The mechanisms driving epithelial differentiation, proliferation, and invasion during prostate development have been postulated to provide critical insight into similar processes occurring in prostate cancer. Investigations into gene function at the earliest stages of prostate development were limited by the lack of suitable methods for controlled, tissue specific deletion of genes in the urogenital sinus. To remedy this problem, we employed tamoxifen-dependent conditional mutagenesis and developed a method to rapidly and efficiently delete target genes in the developing prostate with precise temporal control. Using this method, we describe the absolute requirement for Wnt signaling through beta-catenin in the initial phase of lineage commitment during prostate morphogenesis.

Inflammation promotes cancer initiation and progression in a variety of organs, and has been linked epidemiologically to prostate cancer. Limitations in animal models of prostatitis have hindered efforts to produce definitive evidence that inflammation promotes cancer progression. Here, we characterize the immunologic and morphologic changes induced in the prostate by a new model of murine bacterial prostatitis. This model uses CP1, a strain of *Escherichia coli* recently isolated from a human and described in acute studies of murine pelvic

pain. CP1 induces chronic prostatitis lasting at least one year, with epithelial hyperplasia and immune cell infiltration. Using this model, we show chronic inflammation accelerates prostate cancer progression in the Hi-Myc model of murine prostate cancer and provide the first definitive link between prostate inflammation and prostate cancer progression.

A single process unites these two seemingly disparate projects: recognizing a gap in scientific knowledge, fashioning a model that can be used to fill this gap, and finally applying these models to uncover basic and translational aspects of prostate biology. Together, the data presented here expand the understanding of prostate biology and provide new tools for future inquiry.

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To my family,

Cary, Samantha, and John,

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I. Introduction

Animal models of prostate disease

Aging men experience prostate diseases at a high frequency. The incidence of lower urinary tract symptoms (LUTS), usually associated with benign prostatic hypertrophy or BPH, increases steadily with age, and almost half of men experience symptoms by 65 years of age [1]. Prostate cancer is similarly common, with autopsy studies reporting undiagnosed cases of prostate cancer present in more than 30 percent of men in their 50s [2]. Prostate inflammation, linked to both prostate cancer and BPH, may have an even higher incidence than either disease, and has been proposed to initiate or accelerate the development of both BPH and cancer. However, the natural history of these diseases remains poorly understood. Epidemiologic studies have found multiple genetic and environmental factors may affect prostate disease, from the general health effects of diet and obesity, to specific genetic risk factors, such as *HOXB13* mutations [3]. Despite these studies, differentiating indolent from lethal prostate cancer and identifying factors that contribute to each remains a challenge.

Answering these questions in human research is difficult because the necessary studies are often not feasible or would be unethical. For these reasons, a substantial portion of our understanding of prostate biology and disease stems from research using animal models. From dogs to rodents, these models rely on anatomical and physiological relevance to human disease, and have benefited from constant refinement. Historically, dogs were used extensively to study prostate hypertrophy and prostate cancer, as this is one of the few species in which these diseases spontaneously occur [4]. However, prostate disease in dogs takes years to develop, and occurs at a lower rate than humans [5]. The more recent development of genetically modified mice has led to an explosion in the use of mouse models of prostate disease. Mice develop spontaneous prostate disease at a very low rate, making them a "blank slate" on which to determine what role environmental and genetic manipulations play in the development of prostate

disease. The effects these factors have on the time course and penetrance of carcinogenesis and metastasis have been determined by transgenic mice, which provide predictable and reproducible models of prostate cancer.

Comparative anatomy of the mouse and human prostate

An understanding of the similarities and an appreciation of the differences between mouse and human prostate anatomy should support and guide the use of murine models of prostate biology. In both species, the prostate is an accessory sex organ that develops from the urogenital sinus (Fig. 1). The formation and maintenance of the prostate requires androgens, and the mature organ in both species is a lobular secretory gland with basal, luminal, and neuroendocrine cell types. However, significant differences in anatomy and histology should be recognized to appropriately interpret mouse studies.

In the embryo, prostate development is similar in the human and mouse, with distinct lobes arising from precise anatomic locations in the urogenital sinus. The human prostate rapidly adopts a compact structure with a thick fibromuscular stroma, while the mouse prostate retains a loose, lobular appearance with scant stroma (Fig. 2). Although not divided into lobes, the adult human prostate is divided into recognizable zones, which have a consistent anatomic arrangement and are histologically distinct. The lobes have differential susceptibilities to pathology, with BPH arising primarily in transition zone and cancer arising most in the peripheral zone [6]. Mouse prostate lobes are similarly distinct histologically (Fig. 3A&B), with distinct gene expression profiles and secretory products. Some rodent models of prostate disease show lobe predilection, but the pronounced lobe specific pathology in genetically engineered models of prostate

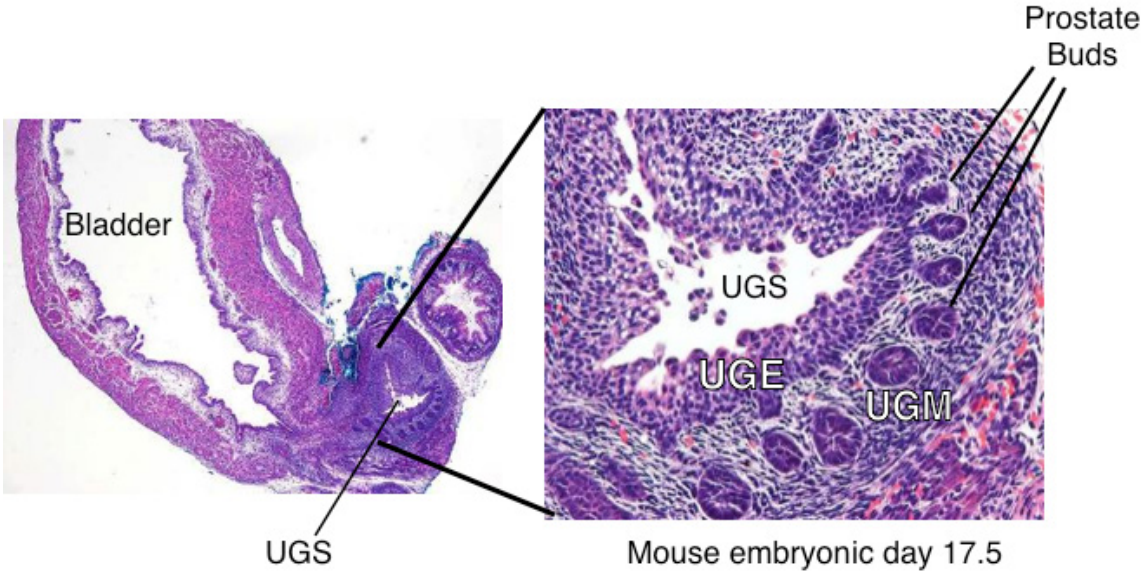

Figure 1. *Prostate Bud Formation in the Urogenital Sinus*

The urogenital sinus (UGS) is located caudal to the bladder. At embryonic day 17.5 (E17.5) the urogenital sinus epithelium (UGE) adopts a prostatic identity and invades into the urogenital sinus mesenchyme (UGM).

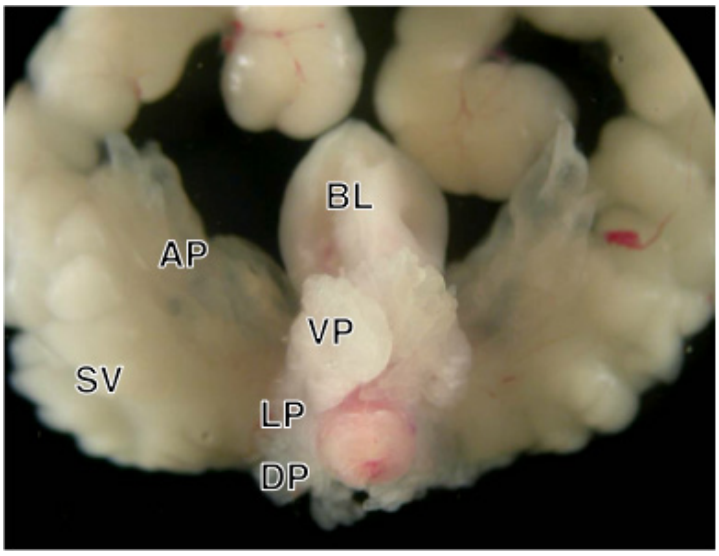

Figure 2. *Mouse Urogenital Tract Gross Anatomy*

The mouse prostate is divided into distinct bilaterally symmetric lobes named for their anatomic location, which arise from the urethra at the base of the bladder (BL). Anterior prostate (AP), ventral prostate (VP), lateral prostate (LP), and dorsal prostate (DP) are of urogenital sinus origin. The much larger seminal vesicles (SV) originate from the Wolffian ducts.

cancer may be attributed to differential transgene expression, rather than differences in biology. In fact, prostate pathology of any kind is extremely rare in the mouse, with spontaneous carcinoma described only in case reports, and epithelial hyperplasia or inflammation reported to occur in 1 percent and 5 percent of mice, respectively [7]. In both species, the majority of cells have a basal or luminal phenotype, but the relative number and distribution are somewhat different in the mouse. Whereas basal cells in the normal human prostate gland form a near continuous layer upon which the luminal cells rest, basal cells in the mouse form an incomplete layer and form a smaller proportion of the total epithelium (Fig. 3C).

Tools for mouse models of development and disease

A variety of genetically engineered lines of mice have been developed for use in studies of prostate development and disease. Traditional knockout or mutant lines of mice, arising spontaneously or through targeted homologous recombination, involve somatic mutations that delete or inactivate a single gene in all tissues. Although these mutations have been useful for the study of gene function, they can be of limited use in the prostate due to effects on other organs. Inactivation of tumor suppressors, such as p53, APC, or PTEN, results in tumor formation in other organs prior to the onset of significant prostate pathology. For developmental studies, homozygous gene deletion is often embryonic lethal prior to prostate morphogenesis. For these reasons, tools for conditional or tissue specific mutagenesis were developed.

Conditional mutagenesis is most commonly accomplished with the Cre-LoxP system. This system uses a bacterial recombinase that recognizes LoxP sites inserted into the genome, brings them together, and excises the intervening DNA. This system

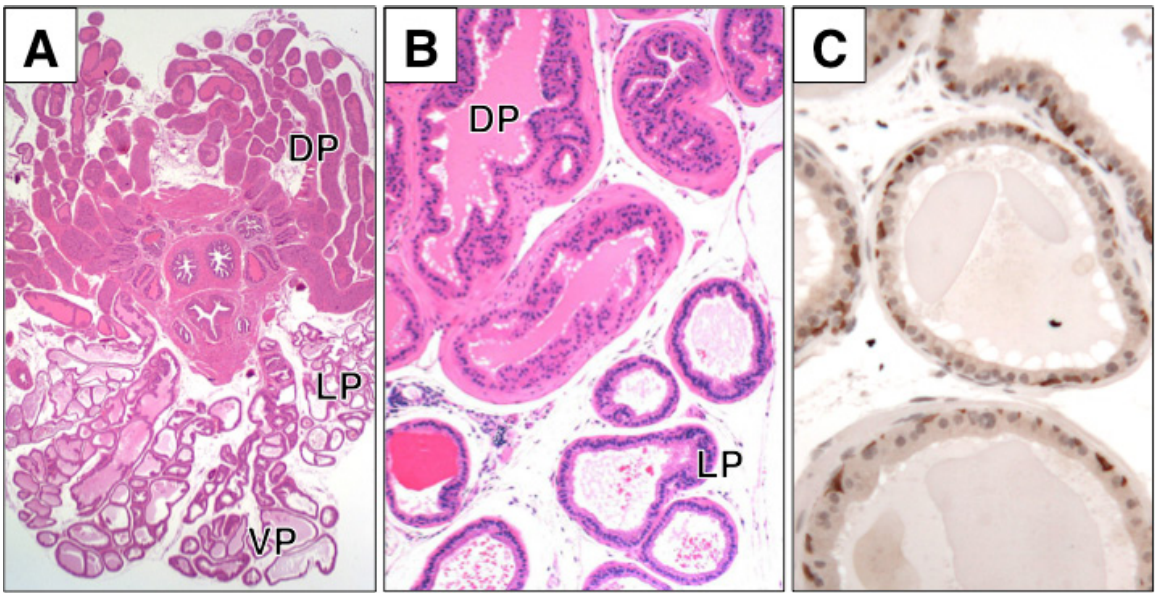

Figure 3. *Mouse Prostate Histology*

(A,B) Mouse prostate lobes can be recognized by their distinct histologic appearance. Glands from the dorsal prostate (DP) show moderate infolding, granular cytoplasm, and bright eosinophilic homogenous secretions. Glands from the lateral prostate (LP) have sparse infoldings and granular or particulate eosinophilic secretions, and ventral prostate (VP) glands have rare infoldings with large lumens and pale serous secretions. (C) Immunohistochemistry for keratin 14 (CK14) highlights an incomplete rim of basal cells adjacent to the basement membrane in a normal prostate gland.

can be used to delete a gene or activate a transgene if the intervening sequence contains a stop codon. This deletion can be controlled with expression of Cre by a prostate-specific promoter or with a drug dependent expression system. The most commonly used prostate specific promoters are Probasin [8], *Nkx3.1* [9], and *Hoxb13* [10]. Although these promoters drive expression specifically in the prostate, they all suffer from extra-prostatic expression. Additionally, these promoters drive Cre expression beginning at different times and in different, but overlapping, sets of epithelial cells (Table 1). Probasin-Cre is useful for carcinogenesis experiments as expression begins after postnatal day 14 and will not interfere with early prostate morphogenesis. However, this promoter drives epithelial Cre expression in a mosaic fashion, and shows strong expression in the stroma and seminal vesicles (Fig. 4). Furthermore, Probasin-Cre can be expressed in the ovary, and maternally inherited Cre may result in mosaic recombination [11]. *Nkx3.1* and *Hoxb13* promoters drive expression in embryogenesis, which is useful for developmental studies, but can preclude carcinogenesis studies if deletion of the targeted gene interferes with prostate morphogenesis. To remedy this, investigators have used conditional mutagenesis, which is dependent on drug treatment. The two most widely used methods involve tamoxifen-dependent Cre, and Tet-on/off systems. Both require drugs for activation, but Tet-on/off systems have the advantage of being reversible once Tetracycline treatment is withdrawn.

Mouse models of prostatitis

A variety of potential causes have been associated with prostatic inflammation in humans, including hormonal changes, dietary factors, autoimmune responses, and infectious agents [12]. To understand the mechanisms underlying chronic prostatitis, rodent models of prostate inflammation have been developed using many of these

Promoter	Prostate Expression	Cell Type	Timecourse	Androgen Dependent	Extra-prostatic Expression
Probasin	LP>DP>VP>>AP Mosaic	Luminal Stromal	D14-adult	Yes	Seminal Vesicles Infrequently in Other Sites
Nkx3.1	DP,LP>AP>VP Widespread	Multiple (embryonic) Luminal (adult)	E18-adult	Yes	Developing Somites (Embryonic)
Hoxb13	VP>LP,DP,AP Widespread	Multiple (embryonic) Luminal (adult)	E9-adult	No	Distal Colon (Adult) Tail, Spinal Cord (Embryonic)

Table 1. *A Comparison of Prostate-Specific Promoters*

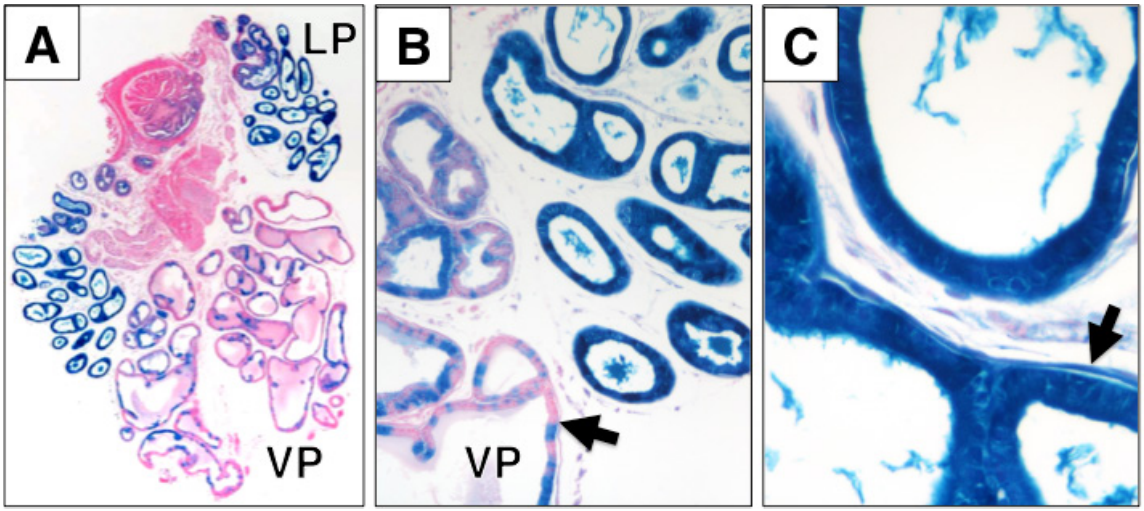

Figure 4. *Probasin Promoter Driven Cre Recombinase Expression*

(A,B) Probasin-Cre (PB-Cre4) R26R LacZ reporter mouse shows diffuse Cre expression in the lateral prostate (LP), but the arrow indicates sparse, mosaic expression in the ventral prostate (VP). (C) Cre expression is evident in the epithelium as well as stromal cells (arrow).

factors. Several strains of rats and mice have been identified with a high incidence of spontaneous nonbacterial prostatitis. The Lewis strain of rat in particular, has a 60-70% incidence of prostatitis as young as 12 weeks of age [13]. In mice, the Non-Obese Diabetic (NOD) strain of mice develops a high incidence of prostatitis [14]. This strain is particularly sensitive to autoimmune disease in many organs, and prostate inflammation is likely a manifestation of this phenotype. Hormones, especially estrogen, have been linked to autoimmune disease in women, and have been associated with an increased risk for prostate cancer [15, 16]. To study this association, estrogen has been used to initiate prostatitis in a rat model. Neonatal Wistar rats injected with 17-beta-estradiol followed by testosterone develop severe prostatitis [17]. Environmental exposures may also affect prostate carcinogenesis. Consumption of red meat has been associated with prostate cancer, and may act through the formation of heterocyclic amines in the cooking process [18]. In rats, exposure to PhIP, a heterocyclic amine formed in cooked meat, results in a high incidence of DNA damage and inflammation in the prostate [19]. Any source of tissue damage and inflammation can release prostate epithelial antigens in the context of immune stimulation and could potentially initiate an autoimmune response. Evidence supporting this hypothesis comes in the form of auto-reactive T cells in men with prostatitis [20]. Although this hypothesis is difficult to test in men, several rodent models of prostatitis have successfully induced prostatitis by manipulating the immune response. Thymectomy three day old mice alters T cell maturation and induces an autoimmune response in a variety of organs, including the prostate [21]. An autoimmune response specific to the prostate can be initiated by vaccination with whole prostate lysate, or purified prostate proteins [22-24]. Transfer of specific T cells into mice genetically engineered to express ovalbumin can generate autoimmune prostatitis, as in the POET model [25]. Infectious prostatitis has been studied in rats and mice primarily using isolates of *Escherichia coli*, but also with *Propionibacterium acnes* [26-28]. These models have evolved from direct surgical injection of bacteria into the prostate to less

invasive means of instillation via urethral catheter. However, the strains of *E. coli* most often used to initiate prostatitis are not prostate derived strains, but highly pathogenic strains isolated from septic patients [29].

5. Project Overview

The overall goal of this project was to understand the function of Wnt signaling in prostate morphogenesis, and to determine the effects of inflammation on prostate cancer progression.

Chapter I examines the function of beta-catenin and Wnt signaling in early prostate lineage commitment. Previous work in our lab identified the Wnt pathway as highly differentially expressed during prostate epithelial differentiation and invasion, but the function of this pathway in prostate development had not been described. A serious limitation to studying this, or any, pathway in prostate development was the lack of a well-characterized method to delete genes in a controlled fashion in the urogenital sinus. To answer this question, we adapted tamoxifen-dependent Cre mutagenesis to use in the embryonic urogenital sinus in order to rapidly and efficiently delete beta-catenin. Using this technique, we determined that Wnt signaling is critical in the initial cascade of signaling events initiated by androgens that result in undifferentiated urogenital sinus epithelium adopting prostatic identity and invading the surrounding mesenchyme.

Chapter II focuses on the role of prostate inflammation in prostate cancer progression. The CP1 model of prostatitis has been described in short-term experiments and in chronic pelvic pain, but long-term inflammation from this strain had not been previously described. We characterized the immune cell infiltrate and epithelial changes resulting from chronic inflammation. After adding CP1 induced-inflammation

to the Hi-Myc model of prostate cancer, we were able to provide the first direct evidence that prostate inflammation accelerates prostate cancer progression.

These data fill a void in the understanding of prostate biology with the potential for expanding our understanding of prostate carcinogenesis and cancer progression. In addition, we refine and describe two methods with wide application beyond the following studies.

II. Wnt Signaling Through Beta-catenin is Required for Prostate Lineage Specification

Wnt Signaling Though Beta-catenin is Required for Prostate Lineage Specification

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Abstract

Androgens initiate a complex network of signals within the UGS that trigger prostate lineage commitment and bud formation. Given its contributions to organogenesis in other systems, we investigated a role for canonical Wnt signaling in prostate development. We developed a new method to achieve complete deletion of beta-catenin, the transcriptional coactivator required for canonical Wnt signaling, in early prostate development. Beta-catenin deletion abrogated canonical Wnt signaling and yielded prostate rudiments that exhibited dramatically decreased budding and failed to adopt prostatic identity. This requirement for canonical Wnt signaling was limited to a brief critical period during the initial molecular phase of prostate identity specification. Deletion of beta-catenin in the adult prostate did not significantly affect organ homeostasis. Collectively, these data establish that beta-catenin and Wnt signaling play key roles in prostate lineage specification and bud outgrowth.

Introduction

The adult rodent prostate is an exocrine gland divided into distinct lobes, with each lobe consisting of branching ducts and blind ending tubules. In the embryo, this complex structure originates from a simple cone-shaped structure called the urogenital sinus (UGS), which is present in both males and females [30]. Between embryonic days 14.5 and 17.5 (E14.5 to E17.5) in males, rising levels of testicular androgens act directly on urogenital sinus mesenchyme to initiate prostate development [31]. In particular, androgens induce a variety of mesenchymal signals, which instruct the adjacent urogenital sinus epithelium (UGE) to adopt prostatic identity, invade the surrounding mesenchyme, and form solid tubular buds. Although androgens play a crucial role in initiating prostate organogenesis from the mesenchymal compartment, the subsequent signaling events driving lineage commitment and branching morphogenesis have not been fully defined.

Prostate bud formation at embryonic day E17.5 represents the first morphologic evidence of prostate development, but molecular evidence of prostate differentiation is present shortly after circulating androgen levels begin to rise. The molecular phase of prostate development is defined by the earliest known marker of prostatic differentiation, the homeobox gene *Nkx3.1*. *Nkx3.1* expression is first detectable in the UGS around E16, when androgen is present but prostate buds have yet to form [32, 33]. Although *Nkx3.1* expression is a reliable marker of prostatic differentiation, it is not required for prostate formation [32, 34]. This suggests other signaling pathways are responsible for prostate lineage commitment. Our previous work, complemented by several

other studies, have identified Wnt pathway related genes as significantly induced by androgens at the initiation of prostatic differentiation [35-38]. Moreover, we have shown a link between the signaling pathways active during early lineage commitment and pathways aberrantly activated in prostate pathology. Clearly defining the role for Wnt signaling in early prostate development will provide a foundation for future studies in prostate development, and may provide insights into prostatic disease.

Wnt signaling is critical for cell fate determination in a variety of organs outside of the prostate. For example, canonical Wnt signaling is required for specification of distal epithelial cells in the mouse lung [39], and is required for the initiation of mammary development [40]. During pancreas development, canonical Wnt signaling is required for exocrine lineage specification [41, 42]. In the prostate, investigations into roles for canonical Wnt signaling in embryonic prostate development are limited. Constitutively active beta-catenin induces hyperplasia and prostatic intraepithelial neoplasia (mPIN) if expressed in the embryo [43]. The non-canonical, beta-catenin independent, Wnt pathway has been shown to regulate bud positioning and outgrowth through examination of *Wnt5a* knockout mice [44, 45]. Postnatal manipulation of canonical Wnt signaling in organ culture of rat UGSs inhibits branching, increases proliferation, and alters differentiation of prostate epithelium [46]. Investigations of Wnt function in adult cells have shown, in addition to oncogenic effects, that activation of canonical Wnt signaling leads to a change in cellular differentiation, either preventing maturation or inducing transdifferentiation into squamous epithelium [43, 47, 48]. Overexpression of *Wif1*, an antagonist of Wnt signaling,

in recombinant tissue grafts prevents transdifferentiation of adult bladder transitional epithelial cells to prostate epithelial cells [49]. Thus, multiple lines of evidence indicate a potential role for canonical Wnt signaling in prostate lineage commitment, but the precise role of this pathway in early prostate organogenesis is undefined.

Canonical Wnt signaling is activated when a Wnt ligand binds a low-density lipoprotein receptor-related protein (LRP) and one of the frizzled (Fzd) transmembrane receptor family members [50]. These complex receptor-ligand interactions all funnel into a single transcriptional co-activator, beta-catenin, encoded by a single gene, *Ctnnb1*. Thus, deletion of *Ctnnb1* can effectively silence canonical Wnt signaling. Beta-catenin levels are strictly regulated through an elegant series of phosphorylation and degradation events. In the absence of Wnt ligand, cytoplasmic beta-catenin is phosphorylated and is subsequently ubiquitinated and targeted for proteasomal degradation [51, 52]. Wnt signaling inhibits phosphorylation of beta-catenin, allowing it to accumulate in the cytoplasm and translocate to the nucleus where it associates with TCF/LEF family transcription factors and regulates target gene expression. One such target is *Axin2*, a negative feedback regulator of Wnt signaling and a useful marker of canonical pathway activity, both generally [53] and in the prostate [54]. Unlike other Wnt targets that can also be induced by other pathways, *Axin2* transcription appears to be a specific indicator of canonical Wnt signaling [55].

Because Wnt signaling is broadly active in development, genetic modes to explore a role for canonical Wnt signaling in prostate lineage determination are challenging. Beta-catenin germline knockout mice show severe defects at

gastrulation and die at E7, and thus are not useful for assessing a prostate phenotype at E16 and later [56]. Furthermore, existing systems for conditional deletion of genes in the prostate, such as Nkx3.1-Cre and Probasin-Cre, are expressed after prostate development has begun, so are unsuitable for studying lineage commitment [57, 58]. We therefore adapted a method used previously to study kidney development [59] and accomplished controlled, conditional deletion of beta-catenin in early prostate development [60]. Tamoxifen-inducible Cre allows rapid and efficient deletion of target genes flanked by LoxP sites *in vivo* and *in vitro*. With this method, we demonstrate an absolute requirement for canonical Wnt signaling in lineage commitment and bud outgrowth.

Materials and Methods

Animals

All experimental procedures were approved by the Johns Hopkins Institutional Animal Care and Use Committee (IACUC). All mouse lines were maintained on a C57BL/6J background. Wildtype C57BL/6J, Ctnnb1$^{fl/fl}$, NOD.SCID, and Cre-ER^{T1} mice (Tamoxifen-inducible Cre inserted into Rosa26 locus) were obtained from Jackson Laboratories (Bar Harbor, ME, Stocks 664, 4152, 1303, and 4847). Probasin-Cre (Pb-Cre4) mice were obtained from NCI Mouse Repository (Frederick, MD). Nkx3.1-Cre mice were a gift from Michael Shen (Columbia University, New York, NY). For tamoxifen mediated deletion of beta-catenin, Cre-ER^{T1} mice were crossed with Ctnnb1$^{fl/fl}$ mice to generate breeder pairs homozygous for both mutant alleles. For in vivo deletion of beta-catenin, Pb-

Cre4 hemizygous, Ctnnb1$^{fl/fl}$ males were crossed with Ctnnb1$^{fl/fl}$ females. Nkx3.1-Cre heterozygous, Ctnnb1$^{fl/+}$ males were crossed with Ctnnb1$^{fl/fl}$ females to generate Nkx3.1-Cre heterozygous, Ctnnb1$^{fl/fl}$ embryos. Genotyping was performed using primer sets and protocols recommended by the source of each strain. DNA for PCR was isolated from tails (adult mice) or liver (embryos).

UGS organ culture and subcapsular renal grafts

Timed pregnant female mice were obtained from overnight mating, with the following day considered E0.5. The UGS was harvested from embryos at the specified ages (E15.5-E17.5) and sex was determined by gonadal inspection. UGSs were harvested into ice-cold serum free UGS media [DMEM-F12 (1:1) media (Invitrogen), supplemented with nonessential amino acids (Cellgro), ITS liquid media supplement (Sigma), Penicillin/Streptomycin (Invitrogen), 1 g/L D-glucose (Sigma), L-glutamine (Invitrogen), and 1x10^{-8}M dihydrotestosterone (Sigma-Aldrich)]. After harvest, UGSs were submerged in cold UGS media for 1 hour containing (Z)-4-hydroxytamoxifen (referred to in text as tamoxifen, Sigma-Aldrich), PKF118-310, or vehicle (ethanol and DMSO, respectively). Following treatment, the UGSs were cultured on 0.4 micron membranes (Millipore), oriented with the ventral surface up, overlying UGS media containing the indicated drugs or vehicle for 2-5 days. Media was changed every 48 hours. In all experiments, a minimum of 5 UGSs were treated per condition. For subcapsular renal grafts, UGSs and prostates were similarly harvested and cultured for 48 hours in the presence of tamoxifen or vehicle, then grafted under the kidney capsule of adult male NOD.SCID host mice and harvested after 4 weeks.

Histology, Immunohistochemistry (IHC), and Immunofluorescence

Tissues were fixed in formalin, routinely processed, embedded, and sectioned. For immunohistochemistry, the sections were deparaffinized and rehydrated before steaming in Target Retrieval Solution (Dako) for 40 minutes. For Nkx3.1 staining, slides were steamed for 40 minutes in EDTA pH 9. Endogenous peroxidases were quenched by peroxide treatment, and the slides were blocked for one hour with Serum Free Protein Block (Dako). Slides were incubated with antibodies directed against beta-catenin (BD Biosciences, #14), E-cadherin (Cell Signaling, #4065), AR (Santa Cruz, N-20), FoxA2 (Cell Signaling, D56D6), Nkx3.1 [61], or Hoxb13 [62], and visualized with ImmPRESS Polymer detection kit (Vector Labs). For two color immunohistochemistry, the procedure was repeated and visualized with Vector VIP Peroxidase Substrate. For immunofluorescence, Alexafluor conjugated secondary antibodies (Invitrogen) were applied after incubation with the primary antibody.

Real-time reverse-transcription PCR

To separate UGE and UGM, the UGSs were incubated in 1% Trypsin for 75 minutes and tissue compartments were separated by fine dissection as previously described [63]. Total RNA was isolated using RNeasy Mini-kit (Qiagen) and first strand cDNA was synthesized using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) according to manufacturer's directions. Quantitative real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) with oligonucleotides specific for *Hprt*, *Ctnnb1*, *Axin2*, *Nkx3.1*, *Ar*, *Cdh1*, *Fgf10*, *Tcf7*, *Foxa1*, and *Foxa2* (sequences listed in

Supplementary Table S1). Individual expression values were normalized by comparison to *Hprt*, and relative levels of mRNA expression were calculated using the delta-delta Ct method [64].

Immunoblotting

UGSs were lysed in NuPAGE LDS sample buffer and were fractionated on NuPAGE gels (Invitrogen). After transfer to PVDF membranes, the samples were blocked and incubated with antibodies directed against beta-catenin (BD Biosciences, #14) and GAPDH (Santa Cruz, Clone 6C5). Blots were developed using Odyssey IRDye (LI-COR Biosciences).

Results

Temporal and spatial pattern of Wnt signaling in the UGS

Multiple components of the Wnt pathway are induced by androgens in a temporal and spatial pattern that suggests a role for Wnt signaling in early prostate development [35-38]. To understand the relationship between Wnt signaling and prostate lineage specification, we measured the relative expression of *Axin2* and *Nkx3.1* mRNA. UGSs were harvested at E16.5 from male and female embryos and mRNA was isolated from enzymatically separated UGM and UGE. At this time point of prostate lineage specification, when molecular prostatic differentiation subsequent to androgen exposure is beginning but morphologic changes have not begun, *Axin2* transcript expression was significantly higher in males than in females. Furthermore, differential expression was limited to the UGE (Fig. 1A), suggesting a role for Wnt in specifying the prostate epithelial lineage. To refine the timing of these events, we cultured androgen-naive E15.5

UGSs with dihydrotestosterone (DHT), a potent androgen capable of inducing prostatic differentiation *in vitro* [65], and harvested UGE after 24 and 48 hours of androgen exposure. Compared to controls harvested before culture, UGE showed significantly increased *Axin2* transcript expression at both time points after androgen exposure, coincident with increased *Nkx3.1* expression (Fig. 1B). Together, these observations confirm that canonical Wnt/beta-catenin signaling is active in UGE during the earliest phases of prostate lineage specification, including time points before the emergence of prostate buds.

Conditional deletion of beta-catenin in the UGS

Because germline deletion of beta-catenin is embryonic lethal, we used a conditional Cre-LoxP system to delete *Ctnnb1*, the gene encoding beta-catenin. E15.5 UGSs from embryos homozygous for tamoxifen-inducible Cre ($CreER^{T1}$) and floxed beta-catenin ($Ctnnb1^{fl/fl}$)were cultured with 2 mM tamoxifen or vehicle for 48 hours, and immunoblots were used to determine the time course and efficiency of gene deletion [66, 67]. Beta-catenin protein levels began to decrease within 24 hours of tamoxifen exposure and were nearly undetectable by the 48 hour time point (Fig. 2A). As expected, canonical Wnt signaling was dramatically suppressed after beta-catenin deletion, indicated by marked reduction in *Axin2* and *Tcf7* mRNA extracted from whole UGS cultures 48 hours after tamoxifen exposure (Fig. 2C). Several other genes known to influence prostate development were unaffected by beta-catenin loss, including the beta-catenin binding partner E-cadherin (*Cdh1*) and known mediators of prostate development, such as androgen receptor (*Ar*), *Fgf10*, and *Foxa1* (Fig. 2C). In

contrast, expression of the transcription factor *Foxa2*, which has been shown to be induced by activated beta-catenin in the prostate [43, 68], was suppressed in the gene-deleted UGS compared to controls. Immunohistochemistry (IHC) confirmed uniform deletion of beta-catenin throughout the UGS and a marked decrease in FoxA2 expression (Fig. 2B). In the control group, FoxA2 expression closely matched Nkx3.1 expression at the UGE/UGM border and in the nascent buds. After beta-catenin deletion, Nkx3.1 was undetectable, and FoxA2 expression was limited to scattered individual cells. Androgen receptor, which is expressed throughout the UGS in both sexes before prostate development, continued to be expressed in both the UGE and UGM of beta-catenin deleted UGSs (Fig. 2B).

Loss of Wnt signaling prevents bud formation and prostate determination

In a well established *in vitro* organ culture system [69], wild type controls showed robust bud development, whereas tamoxifen-treated beta-catenin null UGSs showed little or no bud formation (Fig. 3A, B). To confirm that decreased budding was due to loss of canonical Wnt signaling and not a peripheral role of beta-catenin, we treated UGSs with the small molecule Wnt inhibitor PKF118-310. This drug targets the beta-catenin/TCF complex to disrupt Wnt signaling [70]. We observed a similar phenotype with both genetic and pharmacologic methods of Wnt inhibition, with significant decreases in bud number in both beta-catenin deleted and PKF118-310 treated UGSs compared to controls (Fig. 3A, B). Treatment of wildtype UGSs in organ culture with neither 2 mM

tamoxifen, the dose used for conditional deletion of beta-catenin, nor a log fold higher dose had any discernable effect on bud formation or growth (Fig. 3A, S1), indicating the observed effect is not from tamoxifen exposure, but loss of beta-catenin.

Several steps are required for prostate bud formation, most notably lineage commitment, followed by bud initiation and bud elongation [32]. We investigated the acquisition of prostate identity using immunohistochemistry for the prostate epithelial lineage marker *Nkx3.1*. Expression of *Nkx3.1* by immunohistochemistry was similar in control UGSs grown *in vitro* or *in vivo*, but beta-catenin deleted UGSs failed to express *Nkx3.1*, suggesting a failure of prostate lineage commitment (Fig. 2B).

Beta-catenin deletion prevents prostate development

Growth of UGSs as subcapsular renal grafts mirrors normal development *in vivo*, allowing assessment of long term prostate growth and maturation [69]. After four weeks of growth in a host mouse, tamoxifen-treated grafts were significantly smaller than vehicle-treated controls (Fig 4A, H, S2). Histology and immunohistochemical analysis showed the control grafts to be composed primarily of mature prostate ducts (Fig 4B-G). The prostatic epithelium in these grafts expressed normal levels of beta-catenin, Nkx3.1, Hoxb13, and AR (Fig 4D-G). The grafts from beta-catenin deleted UGSs were examined by two experienced pathologists (BWS, DMB) for histologic evidence of prostatic differentiation. The grafts were found to be disorganized, and did not contain

histologically recognizable seminal vesicle or prostatic structures (Fig 4I, J, S3). Immunohistochemistry showed a complete absence of beta-catenin and *Nkx3.1* expression in the tamoxifen-treated and grafted UGSs (Fig. 4K, L), confirming a lack of prostate differentiation. Although *Nkx3.1* is a robust marker of prostate lineage, its expression is regulated by androgens [71]. An androgen-independent marker of prostate identity can be found in *Hoxb13*. Although it is more widely expressed in the developing embryo, expression in adult tissues is limited to colon and prostate [72]. All grafted tissues remained AR positive (Fig. 4N), but *Hoxb13* expression was limited to scattered cells within areas of periurethral gland differentiation (Fig. 4M). The lack of significant prostate differentiation and failure of bud formation confirmed that canonical Wnt/beta-catenin signaling is required for prostate lineage specification.

After lineage commitment, prostate development can occur without beta-catenin

To determine if canonical Wnt signaling is continuously required for prostate lineage commitment or if it is only required during prostatic differentiation, we utilized methods to delete beta-catenin at time points after lineage commitment and bud initiation. The Nkx3.1-Cre mouse has been indispensible for studies of prostatic development and differentiation, but reporter-based studies of Cre activity show expression after E17.0 [58], and expression of floxed target genes may not be eliminated until after E18.5 [73]. We took advantage of this Cre expression timeline and compared deletion with our inducible system, giving loss of beta-catenin within 24-48 hours, to Nkx3.1-

Cre Ctnnb1$^{fl/fl}$ tissues, predicted to show complete deletion at a later time point. The Nkx3.1-Cre cross was embryonic lethal, possibly due to expression in the developing sclerotome [73, 74], but we were able to harvest E15.5 UGSs and culture them *in vitro*. In contrast to the inducible system, these UGSs were able to show evidence of prostatic differentiation by forming buds which lacked beta-catenin, but expressed Nkx3.1 (Fig. 5).

To further assess the timeline of beta-catenin dependent prostate differentiation, we allowed prostate development to proceed *in vivo* until birth, and harvested prostates from pups at postnatal day zero (P0), approximately five days after prostate development begins. These prostates were treated with vehicle or tamoxifen *in vitro* for two days to delete beta-catenin, and grown for four weeks as subcapsular renal grafts. When harvested, control grafts contained mature prostate tissue (Fig. 6 A-D). Tamoxifen-treated grafts showed scattered areas of prostatic differentiation and Nkx3.1 expression despite complete beta-catenin deletion (Fig. 6E-H). In addition to Nkx3.1, these grafts expressed basal and luminal markers (CK14, p63, CK8) in the expected pattern (Fig. S5). However, the SMA positive stroma was frequently discontinuous around the beta-catenin deleted glands.

In order to further shorten the window of active beta-catenin signaling, we harvested E15.5 female UGSs for culture with DHT for 24 hours to initiate prostatic differentiation before deleting beta-catenin with tamoxifen. Grown *in vitro*, UGSs harvested from male or female embryos at E15.5 show an absence of budding after beta-catenin deletion (Fig. S4). However, pretreatment of these grafts with DHT for 24 hours before tamoxifen treatment, or physiologic *in vivo*

exposure to DHT in male embryos harvested at E16.5, resulted in rudimentary bud formation despite beta-catenin deletion (Fig. S4). Grafts from these E15.5 UGSs pretreated with DHT before tamoxifen treatment contained occasional mature prostate glands which maintained Nkx3.1 expression despite the absence of beta-catenin (Fig 6I-L). Thus, these results indicate a window between E14.5-E16.5 where prostatic differentiation requires canonical Wnt/beta-catenin signaling (Fig. 6M).

Loss of beta-catenin in luminal adult epithelial cells does not affect glandular homeostasis

In addition to roles in canonical Wnt signaling, beta-catenin participates in adherens junctions by binding E-cadherin and may play a role in androgen signaling through interactions with AR [75, 76]. We sought to exclude the possibility that loss of beta-catenin would interfere with E-cadherin localization or androgen signaling. To accomplish this, we examined the prostates from 12 week old Probasin-Cre (Pb-Cre4) Ctnnb1$^{fl/fl}$ mice. Probasin-driven Cre expression is initiated postnatally, is active in the luminal epithelial cells, and should have no effect on prostate determination and initial branching. Because Probasin-Cre shows mosaic expression in the prostate, we were able to compare beta-catenin deleted and replete cells in the same gland. Both cell types showed normal histology and expression of E-cadherin and AR. Expression of the androgen-independent differentiation marker Hoxb13 was unchanged, as was the androgen-dependent Nkx3.1 (Fig. 7A-D). These data indicate that loss of beta-catenin in adult prostate cells does not significantly affect homeostasis and

suggest that androgen signaling and cellular adhesion sufficient to maintain mature prostate glands can occur in the absence of beta-catenin.

Discussion

A variety of Wnt pathway members have been shown to be expressed in the developing prostate in an androgen dependent fashion. However, the role of canonical Wnt signaling in early prostate development was not previously addressed. Here, we show that prostate lineage specification depends on canonical Wnt signaling through beta-catenin, and that this signal is required at the earliest stages of prostate lineage commitment. Deletion of beta-catenin or pharmacologic inhibition of canonical Wnt signaling blocks prostatic differentiation, as illustrated by a failure to initiate bud formation or form recognizable glandular tissue, and by undetectable levels of Nkx3.1 and greatly reduced Hoxb13 expression. Previous reports indicate a role for beta-catenin in epithelial differentiation and cell fate determination in other organs [39-42]. Our findings are consistent with these reports, and suggest that Wnt mediated cell-fate determination may be a widely conserved feature of branching morphogenesis.

Investigations into the genetic programs which control prostate morphogenesis are limited by available strategies for conditional mutagenesis. Germline knockouts and other mutant mice have proven useful for studying some pathways, but certain mutations result in early embryonic death or prevent normal formation of the UGS. Currently available models for conditional

mutagenesis, such as Cre-LoxP systems, are useful for studies of late embryonic prostate morphogenesis and adult homeostasis, but are activated too late in development to study the initial stages of prostate differentiation. We show that conditional deletion of genes in the developing prostate can be accomplished rapidly and efficiently with complete temporal control.

In addition to its role in canonical Wnt signaling, beta-catenin functions in cell-cell adhesion though participation in the adherens junction. In the prostate, loss of beta-catenin did not effect on E-cadherin expression or localization, and previous reports show no interruption of adherens junctions and cell-cell adhesion after loss of beta-catenin [77]. A pharmacologic inhibitor of Wnt signaling produced similar results to genetic deletion. Since the inhibitor affects transcriptional activity of beta-catenin through disruption of its association with Tcf/LEF family members, it should not affect adherens functions of beta-catenin. This result further supports the requirement for canonical Wnt signaling in prostate specification.

Previously demonstrated roles of Wnt signaling in the prostate include postnatal regulation of branching and stem cell maintenance [46, 54]. Our findings do not contradict these reports, as we focused on the role of canonical Wnt signaling in earlier prenatal events. Prostate tissue forming from beta-catenin deleted tissue was smaller than controls, but because we used in vitro organ culture and subcapsular renal grafts, we were unable to enumerate branch points. We cannot make determinations about the role of Wnt signaling in prostatic stem cell maintenance because many of these phenotypes are evident only after castration and regeneration cycling of adult mice. Just as we have

shown a short window of absolute requirement for canonical Wnt signaling in early prostate development, it is reasonable to hypothesize that the function of the Wnt signaling pathway can change during different phases of prostate maturation.

Prostate development is controlled by androgens, but estrogen and estrogenic endocrine disruptors have shown a variety of effects on prostate growth and branching prostate [78]. By using an inducible system for gene deletion that requires the presence of tamoxifen, a partial agonist of the estrogen receptor in mice, we were compelled to carefully control for potential effects of tamoxifen on prostate lineage specification and branching. Fortunately, no observable effects of tamoxifen on UGS organ culture were noted, even at one log higher concentration than those necessary to induce Cre recombination.

Androgen induced signals from the UGM to the UGE initiate prostate development [31]. So called "andromedins" are postulated to relay this signal and induce prostatic differentiation. A number of Wnt family members are expressed in the UGS in a manner consistent with a role in epithelial-mesenchymal interactions during prostate induction, and could function as andromedins [35, 36, 38]. Here we show that canonical Wnt signaling in the epithelium is induced after androgen exposure. Although we have determined that canonical Wnt signaling is required for prostate specification, the source and identity of the initiating Wnt ligand has yet to be determined. Because this inducible system deletes beta-catenin in both the epithelium and mesenchyme, we could not determine which compartment requires Wnt signaling. Although Nkx3.1-Cre expression is specific to the epithelium, the ability of prostatic

differentiation to occur after Nkx3.1-Cre mediated deletion of beta-catenin suggests that either the Wnt requirement is mesenchymal, or, as we hypothesize, that the requirement is epithelial, but transient, and abates prior to Nkx3.1-Cre expression. While it remains unclear whether UGE or UGM initiates Wnt signaling, we detect androgen induced Wnt signaling exclusively in the UGE.

An additional aspect of this pathway which remains undetermined is the precise role of Wnt/beta-catenin signaling in prostate lineage commitment, and the downstream mediators that carry out this role. In our study, the absence of identifiable prostatic structures in beta-catenin null tissue indicates an absolute requirement for this protein in prostatic induction. However, it is unclear if Wnt signaling is directly inducing prostatic lineage committment, or if it plays another, more permissive role, such as promoting survival or proliferation of committed prostate progenitors. A key to determining the function of Wnt signaling will be determining the downstream targets which are subsequently induced. Previous studies have indicated that *Foxa2* is positively regulated by activated Wnt signaling [43, 68]. Here we show the complement, that *Foxa2* expression is diminished after beta-catenin deletion. The timing and localization of *Foxa2* during prostate development, expression only during early morphogenesis, suggest it may play a role in the earliest stages of prostate lineage commitment. Our data confirm that *Foxa2* is regulated by Wnt signaling, suggesting a potential pathway which could mediate the transient requirement for Wnt signaling in prostate development.

In summary, we show a critical requirement for canonical Wnt/beta-catenin signaling in early prostate lineage commitment. To accomplish this, we

adapted a strategy for gene deletion to use in studies of prostatic differentiation and development that allows careful interrogation of gene function at the earliest stages of prostate development. Future studies should focus on the identity and source of the signaling molecules that initiate Wnt signaling in the UGE, and what downstream targets mediate this effect.

Acknowledgments

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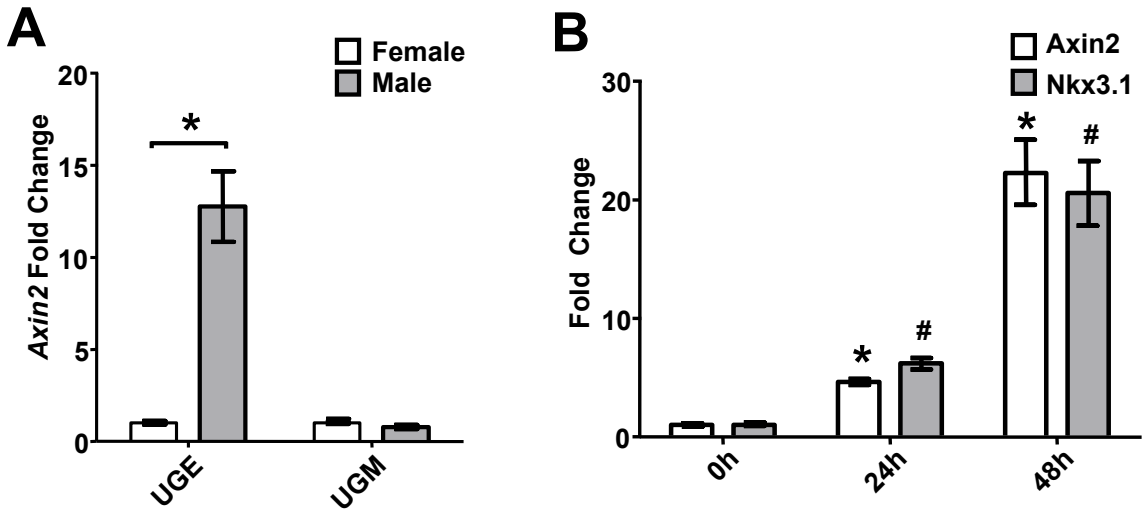

Figure 1: *Androgen induces Wnt signaling in UGE during prostate specification.* (A) qRT-PCR of male and female E16.5 separated UGE and UGM for *Axin2* expression normalized to respective female samples. Differential expression of *Axin2* was limited to the UGE ($p<0.01$). (B) E15.5 female UGSs cultured *in vitro* with DHT demonstrated a significant increase in *Axin2* expression at 24 and 48 hours ($p<0.01$) by qRT-PCR of separated UGE, coinciding with significant increases in *Nkx3.1* expression ($p<0.01$). Graphs represent mean ± standard error of the mean (SEM), p-values calculated using unpaired two-tailed Student's *t*-test with unequal variance.

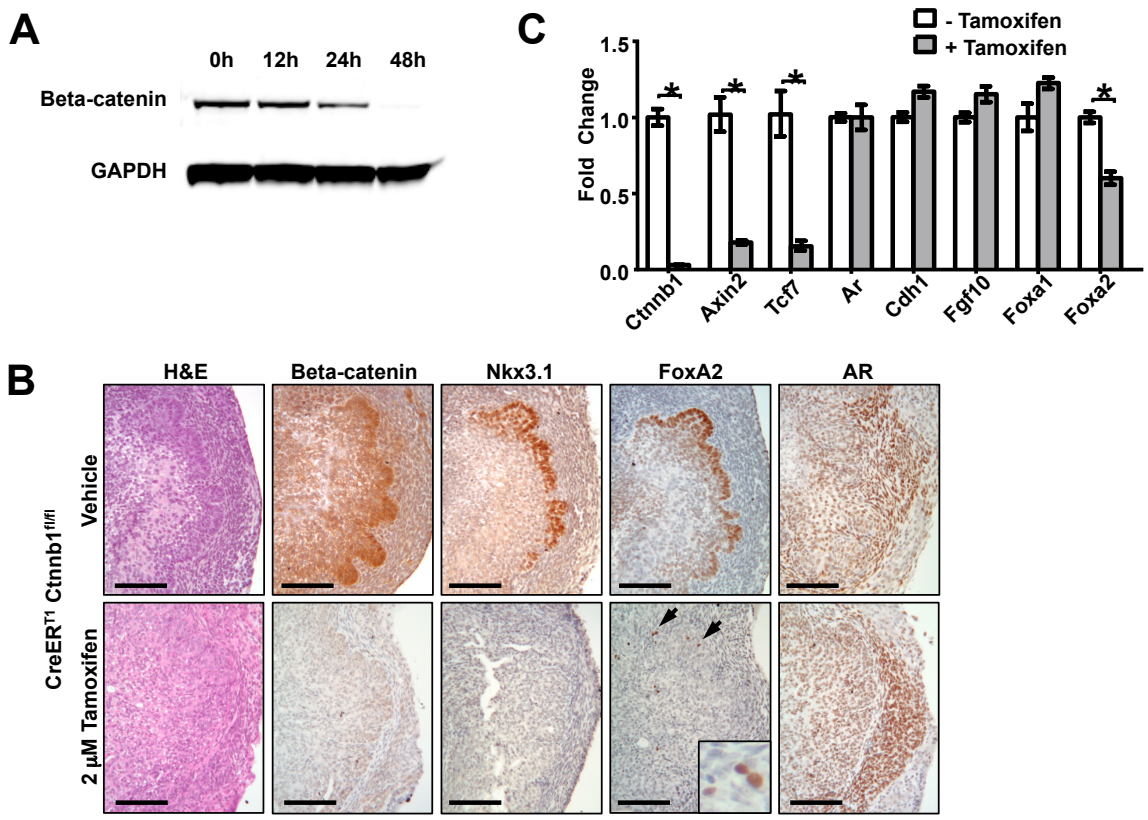

Figure 2: *Inducible knockout of beta-catenin is rapid and results in decreased Foxa2 expression.* (A) Immunoblot analysis of E15.5 CreER^{T1} Ctnnb$^{fl/fl}$ UGSs grown *in vitro* with 2 mM tamoxifen showed decreased beta-catenin protein level after 24 hours and loss within 48 hours. (B) H&E stain and IHC confirmation of beta-catenin deletion after 48 hours tamoxifen treatment (scale bar 100 micrometers). Nkx3.1 expression is undetectable, and FoxA2 expression is limited to scattered individual cells (arrows and inset). AR expression was similar between the two groups. Scale bars are 100 micrometers. (C) qRT-PCR analysis of whole UGSs after 48 hour tamoxifen treatment demonstrates decreased beta-catenin (*Ctnnb1*), *Axin2*, *Tcf7*, and *Foxa2* expression relative to vehicle treated controls ($p<0.01$). Graph represents mean ± standard error of the mean (SEM), p-values calculated using unpaired two-tailed Student's *t*-test with unequal variance.

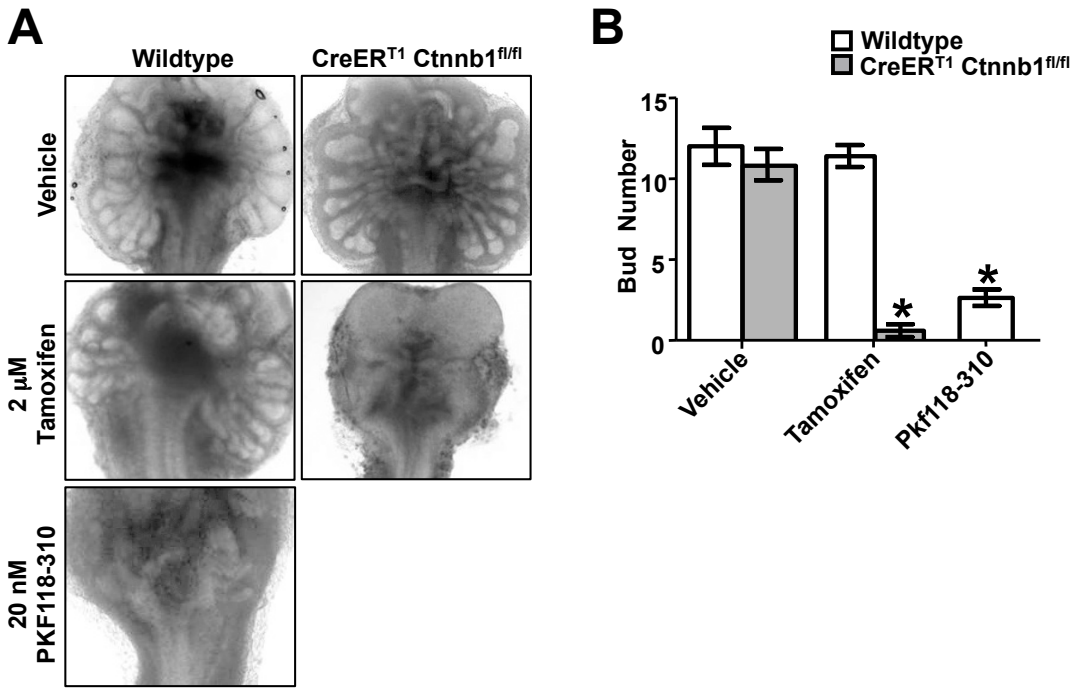

Figure 3: *Deletion of beta-catenin prevents prostate differentiation and bud formation in vitro*. (A) Wildtype (left column) and CreER^{T1} Ctnnb1$^{fl/fl}$ (right column) E15.5 UGSs cultured *in vitro* for five days with 2 mM tamoxifen, vehicle, or 20nM PKF118-310. (B) Quantitative analysis of bud formation *in vitro* demonstrates significantly reduced bud formation in beta-catenin deleted and inhibitor treated tissues compared to controls ($n=5$, $p<0.01$), but no difference in tamoxifen-treated versus vehicle treated wildtype UGSs ($n=5$, $p=0.663$). Graph represents mean ± standard error of the mean (SEM), p-values calculated using unpaired two-tailed Student's *t*-test with unequal variance.

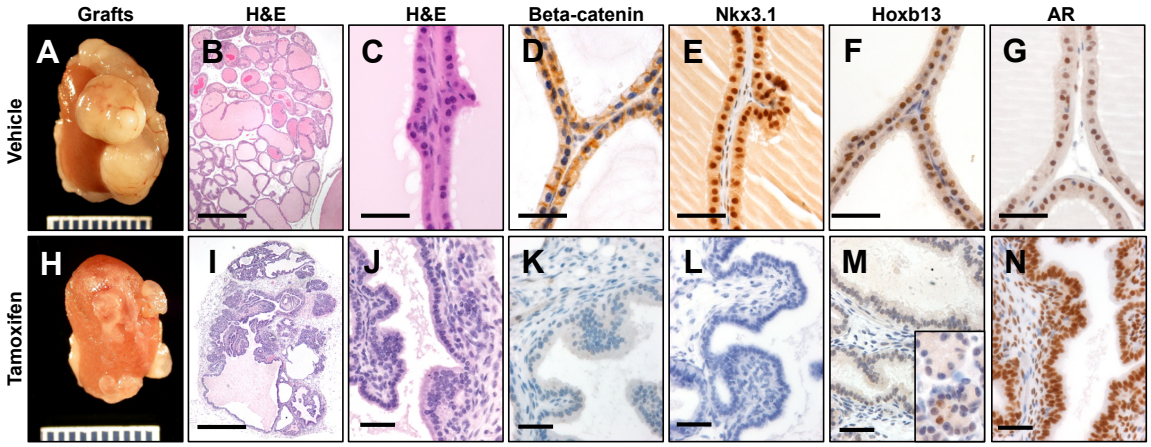

Figure 4: B*eta-catenin knockout grafts do not show prostatic differentiation*. Renal grafts from E15.5 CreER^{T1} Ctnnb1$^{fl/fl}$ UGSs grown after 48 hour *in vitro* culture with vehicle (A-G) or tamoxifen (H-N) before 4 weeks growth as subcapsular renal grafts. Vehicle treated grafts show normal prostate gland formation (B, C), and express beta-catenin (D), Nkx3.1 (E), Hoxb13 (F), and AR (G). Beta-catenin deletion resulted in smaller grafts compared to controls (A, H), and H&E staining demonstrates disorganized grafts without recognizable prostatic differentiation (I, J). IHC analysis showed loss of beta-catenin (K), and Nkx3.1 (L). Hoxb13 expression was absent in the majority of tissue (M), with expression limited to small foci of cells with periurethral gland differentiation (inset). Grafted tissue retains AR expression (N). Scale bars 500 micrometers in B, I. All others 50 micrometers.

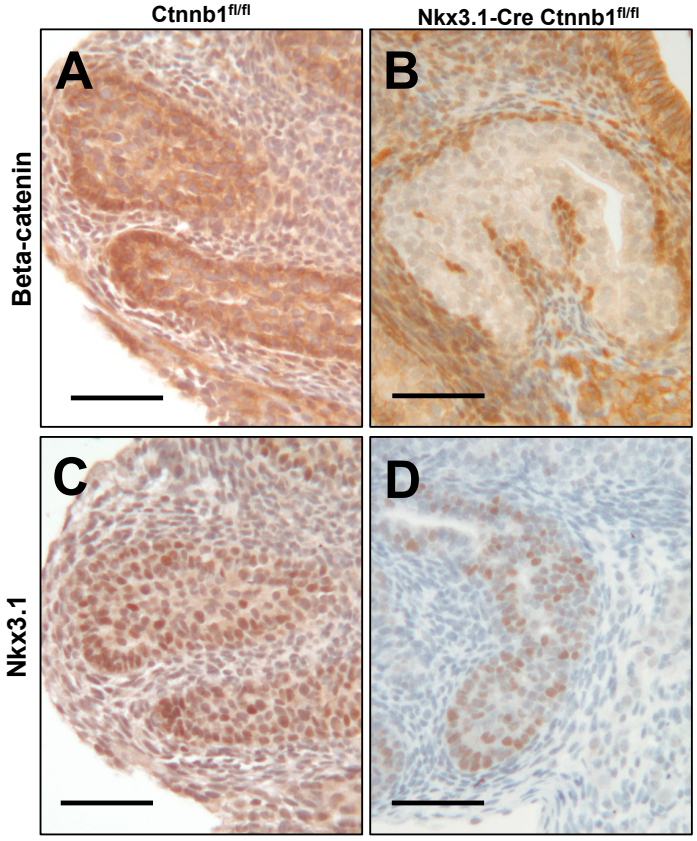

Figure 5: *Beta-catenin deletion after differentiation does not prevent bud formation.* Organ cultures of E15.5 Nkx3.1-Cre Ctnnb1$^{fl/+}$ and Ctnnb1$^{fl/fl}$ UGSs show normal beta-catenin (A) and Nkx3.1 (C) expression in control UGS prostate buds after 5 days in controls. Nkx3.1-Cre Ctnnb1 fl/fl mice form (B) beta-catenin deficient prostate buds that (D) maintain Nkx3.1 expression. Scale bars are 50 micrometers.

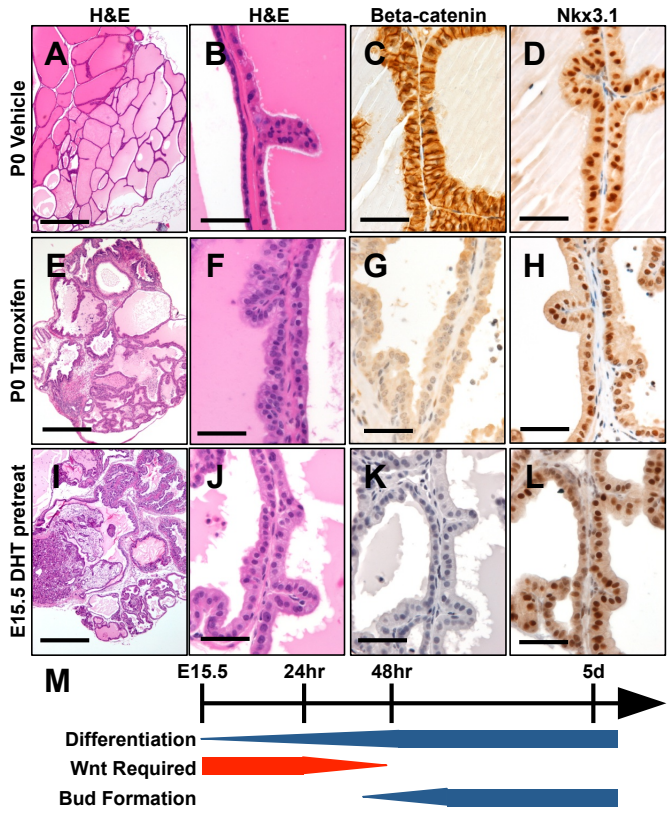

Figure 6: *After androgen action, prostate glands can form without beta-catenin.* Grafts from P0 CreER^{T1} Ctnnb1$^{fl/fl}$ prostates treated *in vitro* with vehicle (A-D) or tamoxifen (E-H) for 48 hours show normal development in controls (A-D). Tamoxifen treated grafts are smaller than controls, but show prostate gland formation (E,F) despite loss of beta-catenin (G), and retain Nkx3.1 expression (H). Grafts from E15.5 female UGSs pretreated with DHT for 24 hours before exposure to tamoxifen (I-L) show a small number of prostate glands (I, J) forming without beta-catenin (K) that retain Nkx3.1 expression (L). (M) Timeline depicting normal prostatic differentiation, bud formation, and the transient requirement for Wnt signaling on which these processes rely. Scale bars 500 micrometers in A, E, I. All others 50 micrometers.

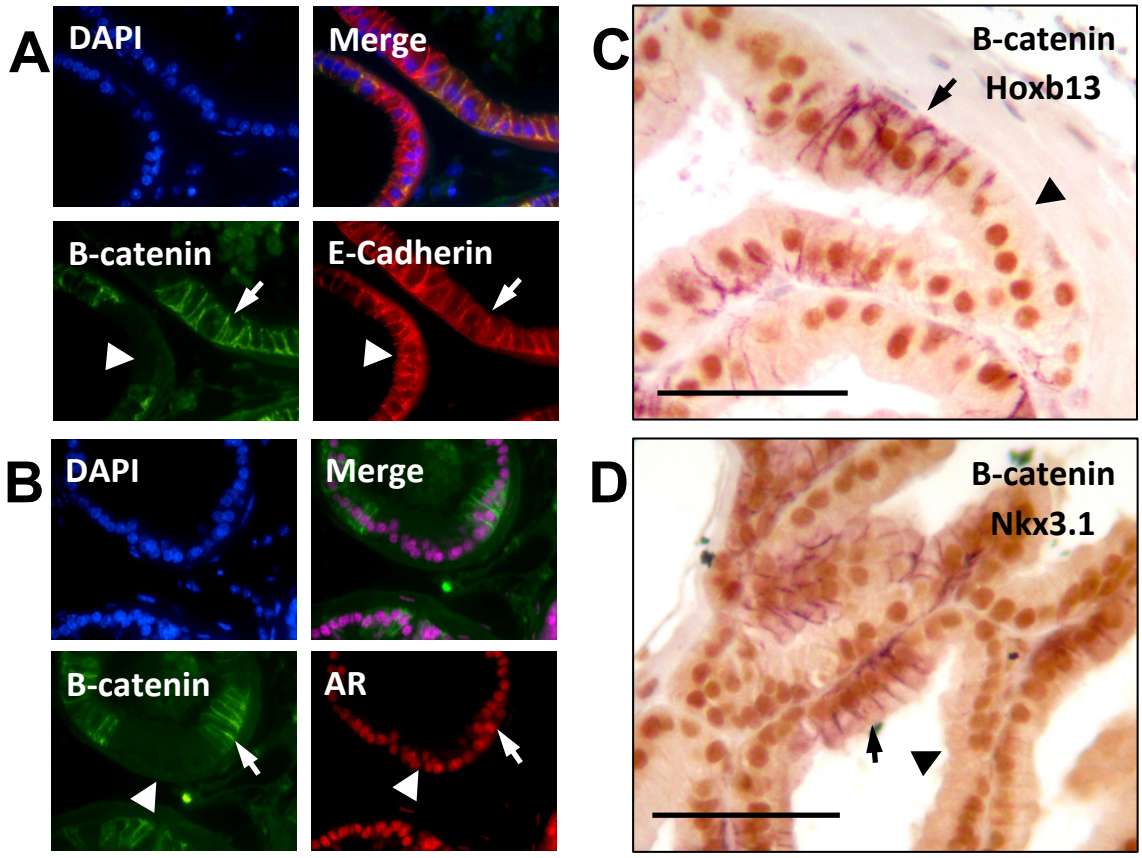

Figure 7: *Mature secretory luminal cells maintain prostatic identity after beta-catenin deletion*. Pb-Cre4 Ctnnb1$^{fl/fl}$ mice show mosaic deletion of beta-catenin in luminal prostate epithelial cells. In areas with beta-catenin expression (arrow) and in areas of beta-catenin deletion (arrowhead) epithelial cells retain normal expression and localization of E-cadherin (A) and Androgen receptor (B) by immunofluorescence. (C,D) Two color immunohistochemistry shows mosaic beta-catenin expression (purple) and normal expression of Hoxb13 (C), and Nkx3.1 (D) in brown. Scale bars are 50 micrometers.

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
Hprt1	TCCTGTGGCCATCTGCCTAG	GGGACGCAGCAACTGACATT
Ctnnb1	CTGCTCATCCCACTAATGTC	TTAACTACCACCTGGTCCTC
Axin2	GATGACGCCTGTGGAACCTG	CCAGACTATGGCGGCTTTCC
Nkx3.1	AAACGTGGCCTGTTGTCTCC	GCCGGGGTTTGCTAACTGAT
AR	GCTCCTGGATTCTGTGCAGC	AGGAAAGTCCACGCTCACCA
Cdh1	GGCGCACTACTGAGTTCCCA	GAGCAAACACTGAGCTCGGG
Fgf10	CCTGGAGATAACATCAGTGG	CAATGCCACATACATTTGCC
Foxa1	TGGAACAGCTACTACGCGGA	CGCTCGTGGTCATGGTGTT
Foxa2	GTATGCTGGGAGCCGTGAAG	TGCTCACGGAAGAGTAGCCC
Tcf7	GTCTACTCTGCCTTCAATCTG	CTGTGAACTCCTTGCTTCTG

Table S1: *Primers used for qRT-PCR*

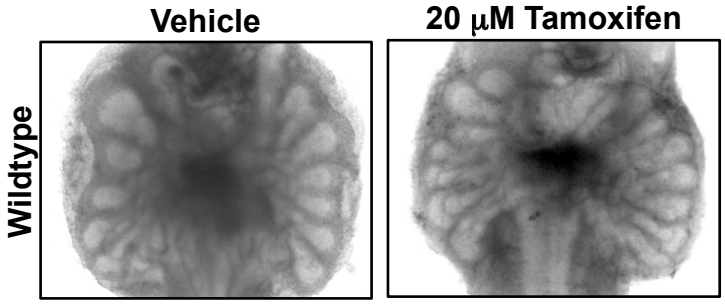

Figure S1: *Tamoxifen does not inhibit bud formation in vitro.* UGSs from E15.5 female embryos cultured for 5 days with vehicle or 20 mM tamoxifen formed similar numbers of buds.

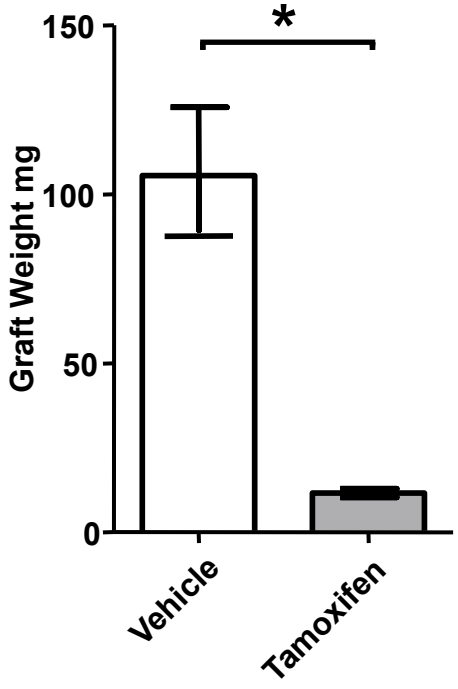

Figure S2: *Grafts from beta-catenin deleted UGSs are significantly smaller than controls.* Quantitative analysis of graft weights from E15.5 CreER^{T1} Ctnnb1$^{fl/fl}$ UGSs cultured *in vitro* for 48 hours with tamoxifen before grafting weighed significantly less than vehicle treated controls (n=8-12, $p<0.0001$). Graph represents mean ± standard error of the mean (SEM), p-values calculated using unpaired two-tailed Student's *t*-test with unequal variance.

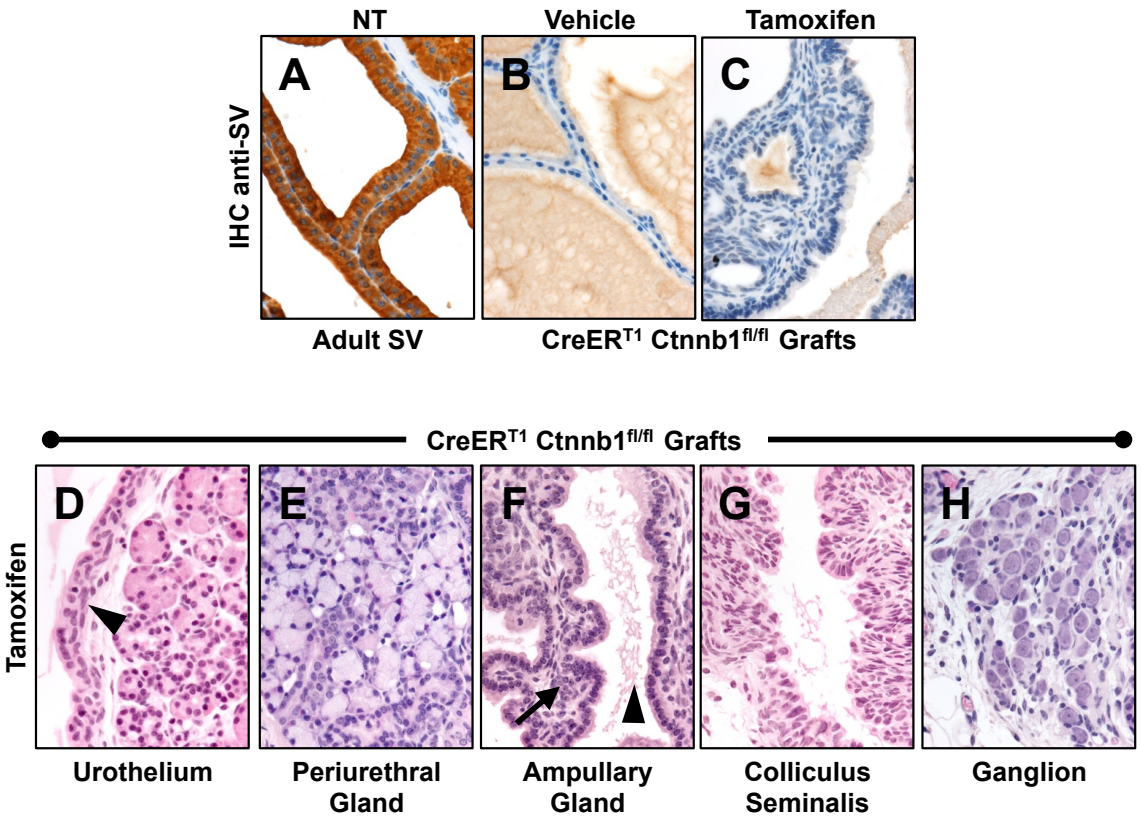

Figure S3: *Differentiation of beta-catenin deleted* UGSs. Wildtype adult seminal vesicle stains strongly with anti-SV antibody (A), while prostate glands from E15.5 CreER^{T1} Ctnnb1$^{fl/fl}$ UGSs cultured *in vitro* for 48 hours with vehicle before grafting are not SV positive (B). Tamoxifen treated grafts do not contain SV positive epithelium (C). Grafts from tamoxifen-treated, beta-catenin deleted UGSs were small and composed primarily non-prostatic tissues derived from, or adjacent to, the UGS such as: (D) urothelium (arrowhead), (E) periurethral glands, (F) ampullary glands with characteristic stroma (arrow) and "swiss cheese" secretions (arrowhead), (G) colliculus seminalis, and (H) ganglia.

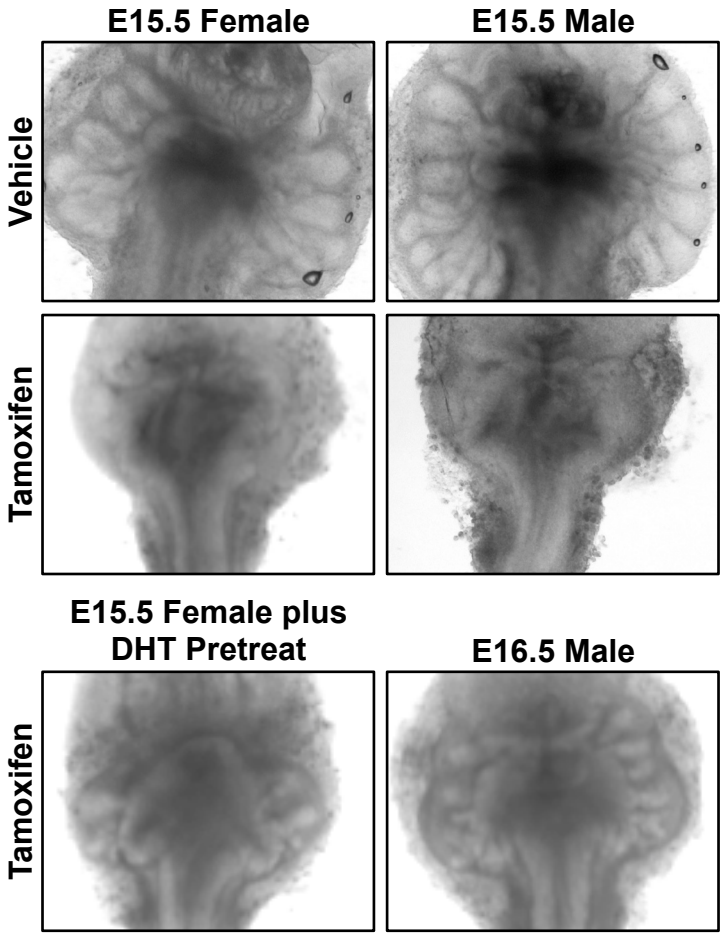

Figure S4: *Androgen exposure reduces the requirement for beta-catenin in bud formation.* Male or female E15.5 CreERT Ctnnb1$^{fl/fl}$ UGSs cultured with vehicle for 5 days show normal bud numbers (top row), but tamoxifen treatment to delete beta-catenin results in lack of bud formation (middle row). Pretreatment of female E15.5 UGSs for 24 hours with DHT before tamoxifen exposure, or *in vivo* exposure to testicular antigens of E16.5 males allows partial bud formation (bottom row) after 5 days in culture.

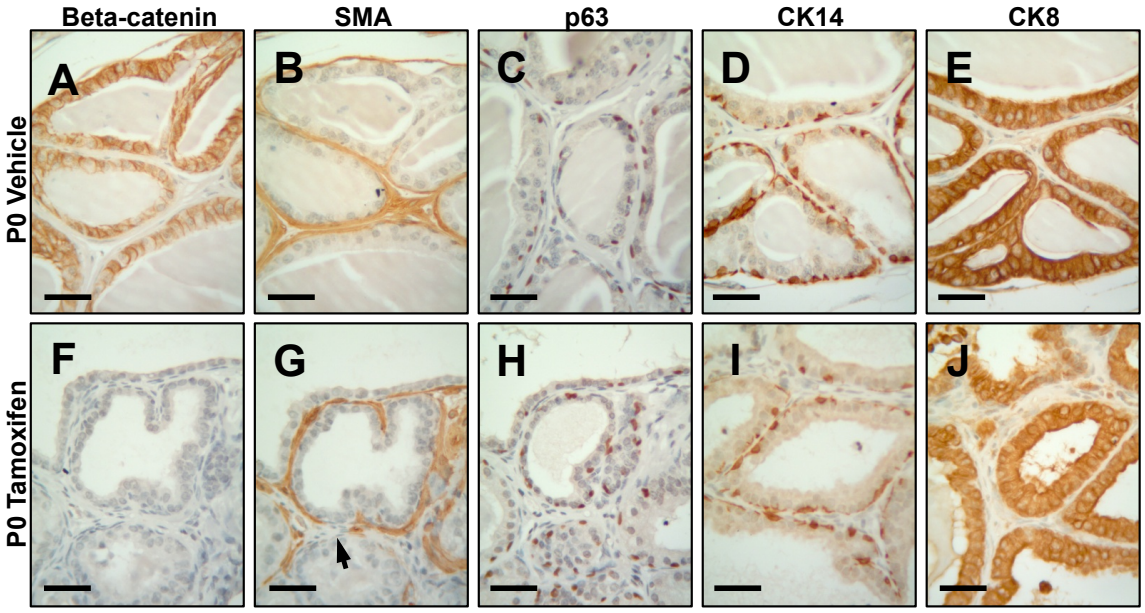

Figure S5: *Cellular differentiation in beta-catenin null glands.* Grafts from P0 CreER^{T1} Ctnnb1$^{fl/fl}$ UGSs prostates treated *in vitro* with vehicle (A-E) or tamoxifen (F-J) show normal expression and localization of (A) beta-catenin, (B) SMA, (C) p63, (D) CK14, and(E) CK8. Tamoxifen treated grafts (F-J) show loss of (F) beta-catenin, and (G) occasional incomplete smooth muscle capsules (arrow). (H) p63, (I) CK14, and (J) CK8 are normally expressed.

III. A Human Prostatic Bacterial Isolate Alters the Prostatic Microenvironment and Accelerates Prostate Cancer Progression

A Human Prostatic Bacterial Isolate Alters the Prostatic Microenvironment and Accelerates Prostate Cancer Progression

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Abstract

Inflammation is associated with several diseases of the prostate including benign enlargement and cancer, but a causal relationship has not been established. Our objective was to characterize the prostate inflammatory microenvironment after infection with a human prostate derived bacterial strain and to determine the effect of inflammation on prostate cancer progression. To this end, we mimicked typical human prostate infection with retrograde urethral instillation of CP1, a human prostatic isolate of *Escherichia coli*. CP1 bacteria were tropic for the accessory sex glands and induced acute inflammation in the prostate and seminal vesicles with chronic inflammation lasting at least one year. Compared to controls, infection induced both acute and chronic inflammation with epithelial hyperplasia, stromal hyperplasia, and inflammatory cell infiltrates. In areas of inflammation, epithelial proliferation and hyperplasia often persist despite decreased expression of androgen receptor (AR). Inflammatory cells in the prostates of CP1 infected mice were characterized at 8 weeks post-infection by flow cytometry, which showed an increase in macrophages and lymphocytes, particularly Th17 cells. Inflammation was additionally assessed in the context of carcinogenesis. Multiplex cytokine profiles of inflamed prostates showed distinct inflammatory cytokines were expressed during prostate inflammation and cancer, with a subset of cytokines synergistically increased during concurrent inflammation and cancer. Furthermore, CP1 infection in the Hi-Myc mouse model of prostate cancer accelerated the development of invasive prostate adenocarcinoma with 70% more mice developing cancer by 4.5 months of age. This study provides the first direct evidence that prostate inflammation accelerates prostate cancer progression, and gives insight into the

microenvironment changes induced by inflammation that may accelerate tumour initiation or progression.

Key Words: Prostate, Inflammation, Cancer, Mouse, Prostatitis

Introduction

Chronic inflammation is associated with increased cancer risk in a number of organs, including stomach, pancreas, colon, and lung [79]. A variety of epidemiologic and histopathologic evidence suggests prostate cancer risk also correlates with inflammation, but a causal relationship has been difficult to establish in human studies [12, 80-83]. One factor contributing to this difficulty is the high prevalence of clinically silent prostatitis. More than 77% of men have histologic evidence of prostate inflammation, but less than 10% of these men report symptoms of prostatitis [84]. A second confounding issue is detection bias. Men with symptomatic prostatitis are more likely to be diagnosed with prostate cancer because they are more likely to be treated by a urologist and subsequently screened for cancer [83]. These difficulties have driven an increased focus on animal models of prostate inflammation and prostate cancer [12].

Despite the high prevalence of prostatitis, the cause of this disease remains uncertain in most cases. Many factors have been proposed as the initiating cause of prostate inflammation, including viruses, bacteria, hormones, urinary reflux, and diet [12]. Bacteria can be isolated from only a minority of cases of prostatitis, but when a bacterial species can be cultured from prostatic secretions, the majority of these are *Escherichia coli* [85]. Numerous studies using non-culture based techniques have confirmed the presence of *E. coli* in additional cases of prostatitis by detecting bacterial DNA in inflamed prostates and in corpora amylacea [86-88]. Together, these data indicate that prostate infection by *E. coli* is an important, and potentially underreported, cause of chronic prostatitis.

Inflammation alters the prostatic microenvironment in multiple ways that may facilitate cancer initiation or progression [12]. Infiltrating leukocytes secrete

a variety of cytokines that promote prostate epithelial proliferation [89]. Release of reactive oxygen and nitrogen species can directly damage DNA [90]. Other inflammatory cells, especially macrophages, migrate through the stroma and can secrete proteolytic enzymes that degrade the extracellular matrix and may facilitate invasion or metastasis [91]. A variety of inflammatory cell types have been identified in human Proliferative Inflammatory Atrophy (PIA) and prostate cancer, and have been proposed to mediate many of these changes in the microenvironment. Among these are macrophages and T cells, particularly IL-17 secreting Th17 cells [92-94]. When tested in animal models of prostate and colon cancer, these cell types were found to promote carcinogenesis or tumour progression via STAT3 activation [92, 95]. Thus, multiple mechanisms have been postulated to promote cancer initiation or progression due to chronic prostatitis, but the relative contribution of each has not been established.

Animal models have been used to study prostatitis with a variety of methods to induce inflammation, including bacteria, hormone treatment, and immunization [17, 26, 27, 96]. Although previous reports describe reactive inflammatory changes and pre-invasive mouse prostatic intraepithelial neoplasia (mPIN) in mice with chronic prostatitis, the effect of prostatic inflammation on prostate cancer progression is unknown [27, 96-99]. We chose to use a recently developed model of bacterial prostatitis using the *E. coli* isolate CP1. This strain of bacteria differs from other reported bacterial models in that it was isolated from the prostate of a human, and has been shown to induce chronic prostatitis in a several mouse strains [100]. Previous analysis of prostatitis induced by CP1 demonstrated tropism for the prostate and induction of persistent inflammation in C57BL/6J mice despite the absence of detectable bacteria by culture after 28

days [100]. Because inflammation has been associated with multiple human prostatic diseases, we first characterized the long-term effects of inflammation from a human bacterial isolate on the prostatic epithelium and stroma. Additionally, as chronic inflammation has been linked to multiple cancers, including prostate cancer, we explored the influence of infection-associated inflammation on cancer progression in the Hi-Myc model of prostate cancer [101]. Here we show CP1 induces chronic inflammation characterized by an influx of macrophages and Th17 lymphocytes, and accelerates cancer progression in Hi-Myc mice. Additionally, we demonstrate distinct cytokine profiles induced by inflammation and cancer.

Materials and Methods

Mice

All experimental procedures were approved by the Johns Hopkins Institutional Animal Care and Use Committee (IACUC). Wild type C57BL/6J and FVB/NJ were obtained from Jackson Laboratories (Bar Harbor, ME, Stocks 664 and 1800). FVB-Tg(ARR2/Pbsn-MYC)7Key (Hi-Myc, Strain 01XF5) mice were obtained from NCI Mouse Repository (Frederick, MD). Genotyping was performed using primer sets and protocols recommended by the vendor. Genomic DNA for PCR was isolated from tails.

Bacterial Strain and Intraurethral Inoculation

CP1 is an *E.* coli strain of the B1 clonal group isolated from the expressed prostatic secretion (EPS) of a patient with chronic prostatitis [100]. Bacterial culture and transurethral inoculation were performed as previously described

[100, 102]. To infect mice, 10 mL of phosphate-buffered saline containing 1 x 10^8 cfu CP1 bacteria was introduced into the urethra of anesthetized mice by catheterization. Sterile saline was introduced in control animals in an identical fashion. All mice were inoculated with a single dose of CP1 at 8 weeks of age. Heat-killed bacteria were heated at 70°C for 30 minutes. Culture supernatant was prepared by centrifugation followed by 0.2 μm filtration. Lack of viable cells was confirmed for heat-killed bacteria and supernatant by zero colony growth on agar plates.

Histology and Immunohistochemistry

At indicated times, prostates were harvested and dissected to separate lobes, fixed in formalin, processed, embedded, sectioned and stained with hematoxylin and eosin (H&E). Inflammation and cancer were scored according to established guidelines in a blinded fashion [103]. For immunohistochemistry, slides were deparaffinised and rehydrated before steaming in Target Retrieval Solution (Dako), or EDTA pH8 (Invitrogen) for 40 minutes. Endogenous peroxidases were quenched with BLOXALL (Vector Labs), and the slides were blocked for one hour with Serum Free Protein Block (Dako). Slides were incubated with antibodies directed against CK8 (Covance), CK14 (Covance), p63 (Sigma), F4/80 (Abcam), CD3 (Abcam), SMA (Dako), Ki67 (Abcam), Phosphorylated STAT3 (Cell Signaling), Myc (Abcam), Laminin (Sigma), AR (Santa Cruz, N-20), Vimentin (Cell Signaling) and Nkx3.1 [61]. Staining was visualized with ImmPRESS Polymer detection kit and ImmPACT DAB (Vector Labs).

Flow Cytometry

To obtain prostate-infiltrating lymphocytes, prostate tissue was mechanically disrupted and incubated for 1 hour at 37°C in RPMI Media with Liberase DL (Roche Applied Science) following manufacturer's instructions. Lymphocytes were isolated using Ficoll-Paque Premium gradient centrifugation (GE Healthcare). For intracellular cytokines, lymphocytes were stimulated for 6 hours with PMA (100 ng/mL), ionomycin (500 ng/mL), and Golgi-stop (BD Biosciences) at 37°C. Intracellular staining was performed with the Ebioscience permeabilization buffer. The cells were analyzed by fluorescence activated cell sorting (FACS) analysis using a FACSCalibur flow cytometer (BD Biosciences). All antibodies were purchased from BD Bioscience except anti-FoxP3 (eBioscience). Data were analyzed with FlowJo software (Treestar Inc.).

Cytokine Assay

Tissues from saline treated C57BL/6J, saline treated FVBN/J, CP1 treated C57BL/6J, saline treated Hi-Myc, and CP1 treated Hi-Myc were harvested at 6 months of age (4 months post-inoculation). The prostate was frozen and stored at -80°C until the assay. Frozen tissue samples were homogenized in homogenization buffer (50 mM Tris-HCl, pH 7.2) containing Na_3VO_4 and a protease-inhibitor cocktail (Sigma-Aldrich) using an OmniTH homogenizer (Omni International, Marietta, GA). Following sonication, the homogenate was centrifuged at 2000g for 5 minutes. The resulting supernatants were collected as total prostate proteins and protein concentrations were measured using the Bio-Rad Protein Assay (Hercules, CA). To quantify cytokine levels, a multiplex mouse 20-plex cytokine immunoassay (Life Technologies), was used. Analytes

measured include GM-CSF, IFN-g, IL-1a, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, TNF-α, CCL2, CCL3, CXCL1, CXCL9, and CXCL10. Prostate protein samples were run in duplicate as previously described [104]. Analyte concentrations were quantified by fitting using a standard curve and normalized by total protein concentration.

Statistical Analysis

All experiments were performed using five or more mice in independent experiments. For comparisons between two groups, Student's *t* test was used. For multiple comparisons, One-way ANOVA with Tukey's multiple comparisons test was used (Graphpad Prism).

Results

CP1 induces chronic prostatitis with stromal and epithelial hyperplasia

We first sought to determine the duration and distribution of prostatitis that could be initiated by a single intraurethral exposure to CP1 bacteria. At 8 weeks of age, wild type C57BL/6J mice were inoculated with CP1 or sterile saline. Bacteria were delivered via catheter into the proximal urethra (Fig. S1). Prostate tissue was harvested 8 weeks, 6 months, and 1 year after inoculation and processed for histology. No inflammation above expected background was noted in saline treated control mice at any time point (Fig. 1A&E). CP1 consistently produced chronic prostatitis, with inflammation present in 89% (34/38) of mice at 8 weeks, 85% (17/20) of mice at 6 months and 40% (4/10) of mice after 1 year. After 8 weeks, mixed inflammatory cells multifocally infiltrated prostate stroma and glands, with reactive epithelial hyperplasia in inflamed glands (Fig. 1B&F)

characterized by multiple layers of epithelial cells, tufting, and cribriform changes. At later time points, inflammatory cells were still present, but glands were often lined by attenuated epithelium with more prominent stromal hyperplasia (Fig. 1C-D&G-H). Inflammation was most common in the anterior prostate, but was common in all lobes (Fig. 1I). The majority of mice (71%, 27/38) had inflammation present in two or more lobes after 8 weeks (Fig. 1J); however, inflammation was not uniformly distributed with inflamed and normal glands present in all mice. Viable intact bacteria are required during the initial inoculation to produce this sustained inflammatory response, as neither heat-killed bacteria nor filtered culture supernatant produced any inflammation (Fig. S2).

Consistent with previous reports of other models of chronic prostatitis, CP1 inflammation induces reactive hyperplasia in both basal and luminal compartments [27, 97]. Basal cells, marked by immunohistochemical staining for cytokeratin 14 (CK14) or p63, increased from a single, incomplete layer in control mice to a layer often two to three cells thick in areas of inflammation (Fig. 2A-D). Similarly, the luminal cell layer, marked by cytokeratin 8 (CK8) was thickened in inflamed areas (Fig. 2E&F). In addition to epithelial hyperplasia, marked stromal thickening was noted in inflamed glands, especially at later time points (Fig. 1 C-D). Prostate glands in saline treated controls were surrounded by a thin layer of smooth muscle (Fig. 2K), with minimal collagen and few vimentin positive stromal cells (Fig S3). Inflamed glands showed a disruption of the smooth muscle actin (SMA) positive stromal layer (Fig. 2L). The inflamed stroma was thickened by bands of collagen, indicated by blue staining with Masson's

trichrome histochemical stain, and vimentin positive reactive stromal cells (Fig. S3).

In addition to hyperplastic changes, inoculation of CP1 induced infiltration of numerous immune cells. Immunohistochemical analysis of inflamed prostates for the macrophage marker F4/80 showed marked infiltration of the stroma and glands 8 weeks post-infection (Fig. 2 G&H). Similarly, CD3+ T cells were present throughout inflamed areas of the prostates while rare in controls (Fig. 2 I&J). To further characterize the inflammatory infiltrate, dissociated prostates from wild type mice were analysed by flow cytometry 8 weeks post-infection to quantify inflammatory cells. The absolute number of lymphocytes and macrophages was significantly increased at this time point (Fig. 3A-C). The relative populations of T cells (including CD4+, CD8+, gd+) and their respective subtypes (IFN-g+ or IL-17+), CD4+ FoxP3+ cells (Treg), and CD49b+ cells (includes NK cells) were quantified. Significant increases in the relative proportion of gd T cells and CD4+ IL-17+ (Th17) cells were noted in inflamed prostates compared to saline treated controls (Fig. 3).

Chronic prostatitis promotes prostate cancer progression

CP1 inoculation produces marked epithelial and stromal hyperplasia with moderate atypia and occasional cribriform morphology that resembles mPIN, but no definitive foci of invasive cancer were identified at any time point. This indicates that CP1 infection alone is insufficient to initiate invasive cancer formation in wild type C57BL/6J mice. However, upregulation of multiple oncogenes and pro-oncogenic pathways suggested that CP1 induced inflammation could affect prostate cancer progression. Epithelial proliferation,

determined by immunohistochemical staining for Ki67, was dramatically increased at 8 weeks post-infection and remained elevated one year post-infection (Fig. 4A, E, I). Quantification of percent Ki67 positive epithelial cells showed a significant increase at 8 weeks (9.3±1.8%, $p<0.001$) and one year (10.9±3.2%, $p<0.001$) compared to controls (0.5±0.3%). In addition to increased proliferation, inflamed prostates showed widespread expression of MYC and activation of STAT3 (Fig. 4), both oncogenes shown to promote prostate cancer initiation or progression in mice and humans [101, 105-107]. Finally, inflammation induced fragmentation of the normally continuous prostate basement membrane, which could facilitate cancer invasion (Fig. 4D, H, L). Similar to previous reports, AR expression and the AR target NKX3.1 are diminished in areas of inflammation (Fig. 5) [27]. Luminal cells typically express high levels of nuclear AR and NKX3.1 (Fig. 5A&E). In castrated mice, AR levels decrease and nuclear localization is lost, with subsequent decrease in androgen dependent genes, such as Nkx3.1 (Fig. 5 B&F). In inflamed prostate glands, AR and Nkx3.1 expression are diminished (Fig. 5 C&G), but the epithelial cells remain highly proliferative (Fig. 5 D&H).

Because CP1-induced inflammation induces pro-oncogenic epithelial changes, we investigated its role in prostate cancer progression. We inoculated Hi-Myc mice with CP1 or sterile saline at 8 weeks of age. In this model of prostate cancer, mPIN develops at an early age and progresses to invasive cancer by 6 months of age in 100% of animals [108]. At 4.5 months of age (137±2 days), a minority of saline treated mice had foci of invasive cancer (43%, N=26/60). However, significantly more CP1 treated mice had invasive cancer (73%, N=22/30, $p=0.008$). Similar to wild type mice, Hi-Myc mice showed little expression of

phosphorylated STAT3 throughout tumourigenesis. In contrast, phosphorylated STAT3 expression was widespread in inflamed areas of CP1 treated Hi-Myc mice (Fig.6).

Inflammation and cancer have distinct cytokine profiles

The high incidence of both prostate inflammation and prostate cancer in humans makes it difficult to determine the individual effect of each on cytokine expression and immune cell infiltrates [93]. However, these processes can be separated in mouse models. We employed multiplex assays to compare cytokine levels in saline treated wild type mice, CP1 inoculated wild type mice, saline treated Hi-Myc mice, and CP1 inoculated Hi-Myc mice all at 6 months of age (4 months post infection, Fig. 7). Of the twenty cytokines and chemokines included in the multiplex ELISA, seventeen had significantly different expression levels among treatment groups. These cytokines could be separated into distinct profiles of those associated specifically with inflammation, with cancer, or only when inflammation and cancer were present concurrently (Table 1). Cytokines were considered to be associated with inflammation when expression levels were significantly increased in CP1 treated animals when compared to wild type and cancer groups. Cytokines were considered to be associated with cancer when expression levels were significantly increased in Hi-Myc mice compared to wild type and CP1 treated wild type mice.

Discussion

Inflammation contributes to cancer initiation and progression in a variety of organs, and has been shown to act directly by inducing genetic changes and

indirectly by altering the microenvironment through immune cell infiltrate and cytokine expression. Here, we show the first evidence that prostate inflammation accelerates prostate cancer progression and examine the complex inflammatory infiltrate and cytokine milieu associated with inflammation in the prostate glandular microenvironment.

CP1 Model of Prostatitis

In this report, we utilize the CP1 model of prostatitis, which has proven useful for investigations into mechanisms of prostatitis, chronic pelvic pain, and now prostate cancer. We feel this model has several distinct advantages. CP1 was isolated from prostatic secretions, and thus may more closely model typical human infectious prostatitis. Additionally, some infectious models of prostatitis require direct injection into the prostate, or project bacteria into prostate ducts by larger volume urethral inoculation. CP1 colonizes the mouse prostate after infusion of a very small volume inoculate into the urethra, thus eliminating the confounding inflammation related to urine reflux or distension of prostate ducts. We show that CP1 is capable of inducing inflammation that persists at least one year after a single infusion of bacteria and clearance of the bacteria within 28 days of inoculation [100]. It is not clear if the ability to promote cancer progression is a unique feature of CP1, or if this is a feature of inflammation in general. This will be possible by comparing CP1 induced acceleration of prostate cancer to inflammation induced by other bacteria, or other means of inducing prostatitis.

Inflammatory Microenvironment

Inflammation induced by CP1 recruits a variety of inflammatory cells, especially macrophages and lymphocytes, to the prostate. One component is a strong Th17 response, indicated both by an increase in CD4+ IL-17+ cells and by an increase in IL-17 production during CP1 induced inflammation. This finding is consistent with previous reports of a strong Th17 response to bacteria [95, 109]. However, not all inflammatory models of prostatitis evoke a Th17 response, and the intensity of Th17 response has been shown to be dependent on mouse strain and can vary based on environmental factors [110, 111]. We hypothesize that accelerated cancer progression in CP1 infected mice is, at least in part, due to activation of the IL-17/IL-6/STAT3 pathway, as this pathway is critical for cancer progression cancer in other organs, and IL-17 has been shown to promote prostate cancer progression in mice [95, 112, 113]. Although our investigation focused on immune cell subtypes and cytokine expression during inflammation and cancer, one histologic hallmark of cancer is invasion through the basement membrane. Stromal remodelling and disruption of basement membrane, which occurs during inflammation, may provide a purely mechanical advantage for pre-invasive lesions to progress towards malignancy.

Cytokine Profiles of Inflammation and Cancer

Although we anticipated different cytokine expression profiles for infection and cancer, the distinct profile of coincident inflammation and cancer was surprising. This profile may be due to an increase in intensity of inflammation from the dual stimuli of bacterial infection and cancer, or from synergistic interactions of anti-bacterial and anti-tumour inflammation. Cytokine levels during simultaneous inflammation and cancer were higher than either state alone for 11/20 cytokines

tested. This increase in inflammatory intensity may activate cytokine expression not present in either state alone. For example, IL-17 is significantly increased during inflammation, but concurrent inflammation and cancer caused an 8-fold increase in mean IL-17 expression compared to inflammation alone. IL-6 expression can be induced by IL-17 activity, and the dramatic increase in IL-17 during simultaneous inflammation and cancer may explain the upregulation of IL-6 only in this environment [112]. An alternate explanation for the distinct profiles is that the immune response to infection and cancer initiation are different, and the combination of the two invokes a third distinct immune microenvironment. Inflammation in response to bacterial exposure produces significant tissue damage from reactive oxygen species produced by macrophages and neutrophils. When coupled with immune response to tumour formation, this may result in a different immune phenotype than either process alone.

If confirmed in human studies, these distinct cytokine profiles could provide a useful diagnostic tool to segregate inflammation and cancer. Although it is more difficult to separate the influence of inflammation from cancer on cytokine expression in humans, we see some correlation between our data and published cytokine data in human studies. For example, TNFa and IL-1a were previously found to be upregulated in men with Chronic Prostatitis or Chronic Pelvic Pain Syndrome [114]. We have identified a cytokine profile specific for murine prostate cancer (IL-5, IL-13, CCL2) that may be useful when translated to the human setting. This profile is traditionally associated more with allergic disease than cancer, but IL-13 and CCL2 have been shown to promote prostate cancer cell proliferation and migration [115-117]. Interestingly, the cytokine profiles of

CP1-induced inflammation and cancer were mutually exclusive, i.e. no cytokine was elevated compared to controls in both settings. Although significant immune cell infiltrate is present in Hi-Myc mice with cancer and in mice treated with CP1, this confirms that the microenvironments of the two processes are quite distinct.

Increased Proliferation Despite Decreased Androgen Receptor Expression

Several groups have reported a decrease in AR expression during inflammation [26, 27, 118]. Here, we show inflammation induces increased proliferation in luminal epithelial cells despite diminished AR expression. As this phenotype appears rapidly after the induction of inflammation, it suggests an inherent program for proliferation in luminal cells in an AR diminished setting without evolution of androgen independent clones through mutation. AR has been proposed to function as a growth suppressor in mature luminal cells, and its loss in the context of inflammation may permit the epithelium to respond to proliferative signals from the stroma [119]. The mechanism driving this program is unclear from this study, but understanding this process could uncover mechanisms of other AR independent processes in the prostate, such as castration resistant prostate cancer.

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Statement of author contributions

BWS, PT, AJS, AR, PJH, CGD, DMB, and EMS designed experiments. BWS, ND, TB, and JG carried out experiments and analysed data. PT, AJS, and CGD provided reagents or materials. All authors were involved in writing the paper and had final approval of the submitted and published versions.

List of abbreviations

AR – Androgen receptor

PIA – Proliferative inflammatory atrophy

PIN – Prostatic intraepithelial neoplasia

PSA – Prostate-specific antigen

PMA – Phorbol Myristate Acetate

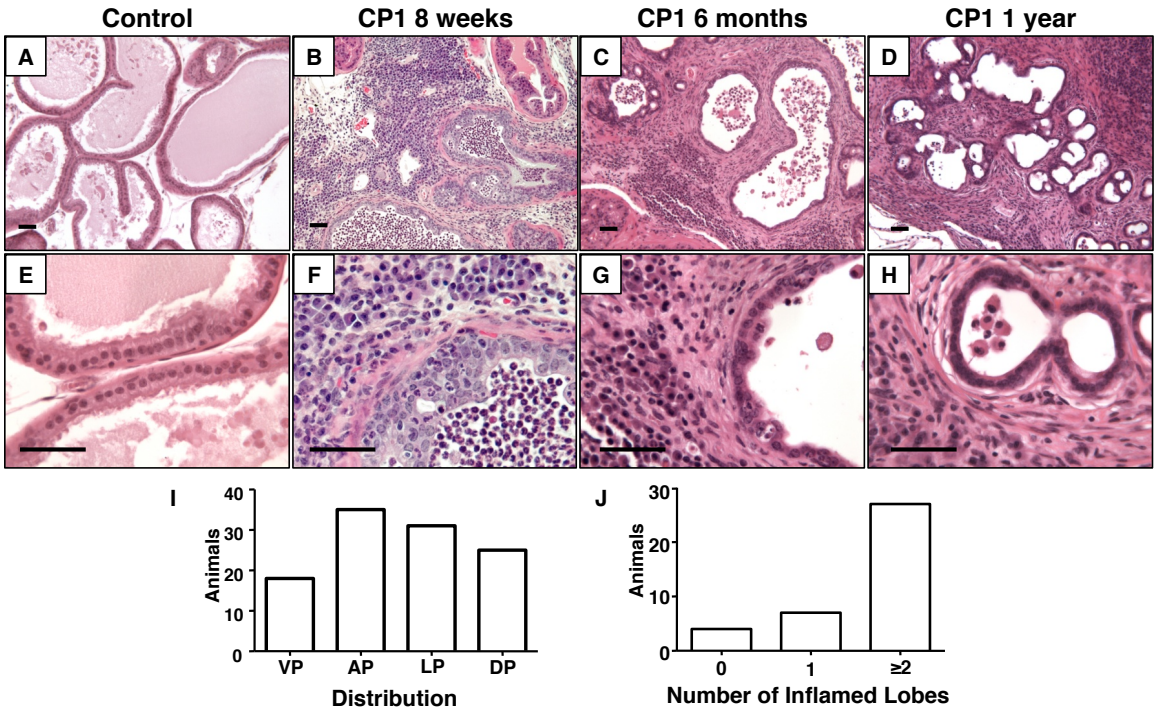

Figure 1: *CP1 induces chronic prostatitis.* Representative images of prostate glands after control saline treatment (A&E), or CP1 induced inflammation at 8 weeks (B&F), 6 months (C&G), and 1 year (D&H) after infection show sustained inflammation. (I) Distribution of inflammation by individual lobe (N=38) DL=dorsal, AP=anterior, LP=lateral, VP=ventral. (J) Total number of inflamed lobes per mouse (N=38). Scale bar = 50 micrometers.

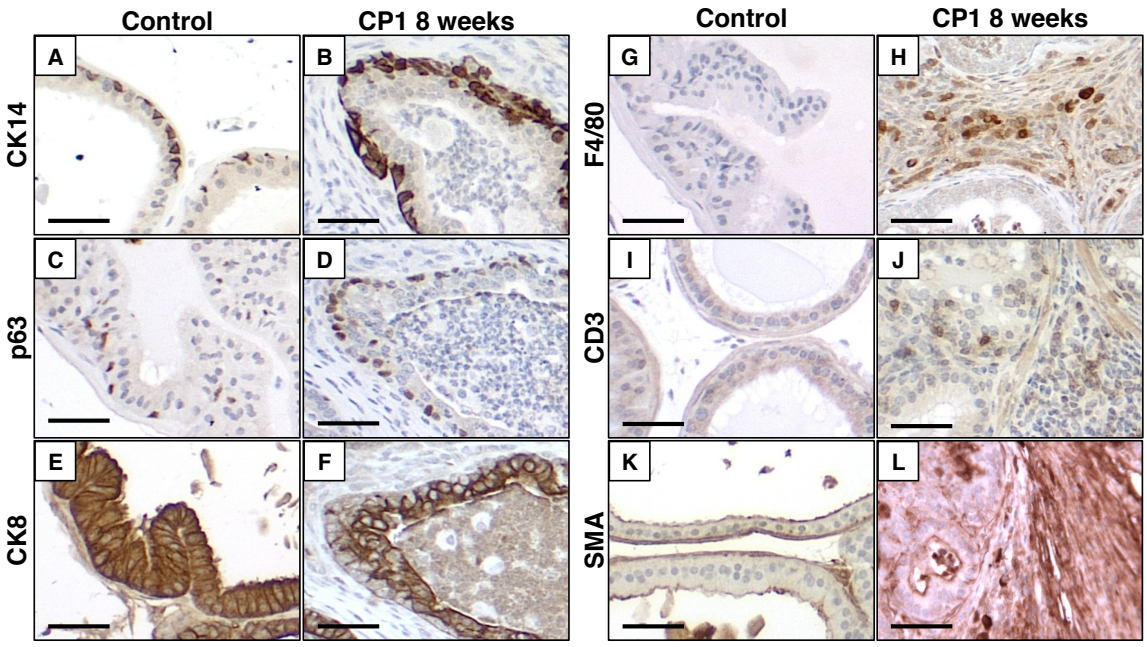

Figure 2: *CP1 infected prostates are hyperplastic and infiltrated by immune cells.* Representative images of immunohistochemistry comparing saline treated controls and CP1 infected prostates 8 weeks after infection demonstrates hyperplasia of basal cells (CK14, A&B; p63 C&D), luminal cells (CK8, E&F), and thickened stroma with disruption of the smooth muscle actin positive layers (SMA, K&L). Inflamed prostates show increased infiltration of macrophages (F4/80, G&H), and T cells (CD3, I&J). Scale bar = 50 micrometers.

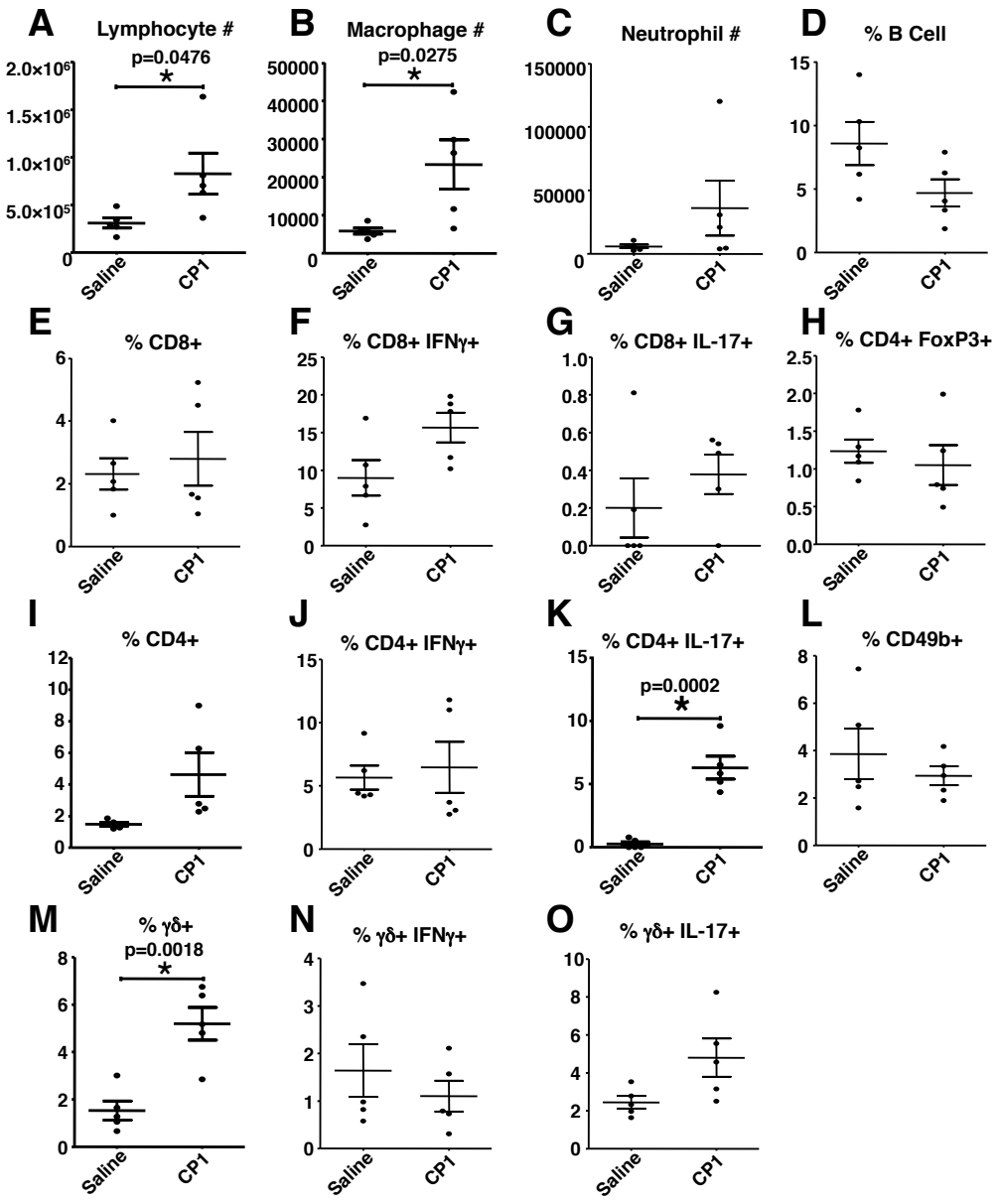

Figure 3: *Prostate inflammatory phenotype.* Analysis of prostate infiltrating immune cells by flow cytometry comparing saline treated controls and CP1 infected wildtype prostates 8 weeks after infection. Significant differences ($P<0.05$) were noted in total lymphocyte number, macrophage number, total CD4+ and gammadelta+ T cells, and CD4+ IL17+ T cells.

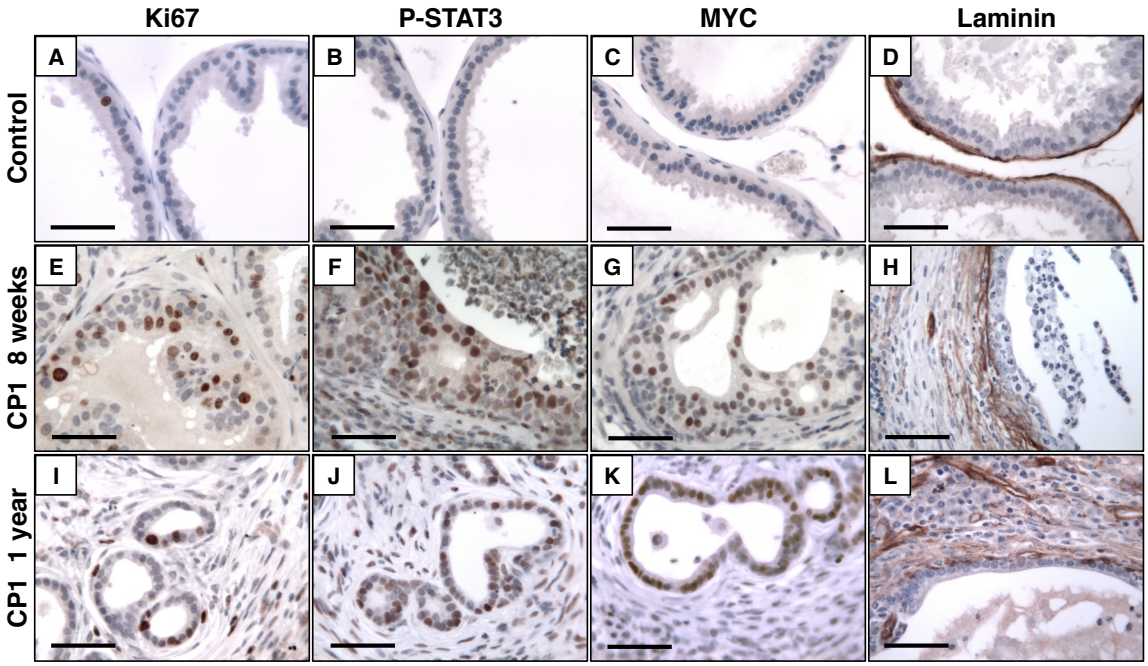

Figure 4: *CP1 inflamed epithelium adopts a proliferative and pro-oncogenic phenotype.* Immunohistochemistry demonstrates increased proliferation (Ki67, A, E, I), phosphorylated-STAT3 (B, F, J) and c-MYC (C, G, K) expression, and disrupted laminin (D, H, L) by CP1 infected mice after 8 weeks (E-H) and 1 year (I-L) compared to saline treated control mice (A-D).

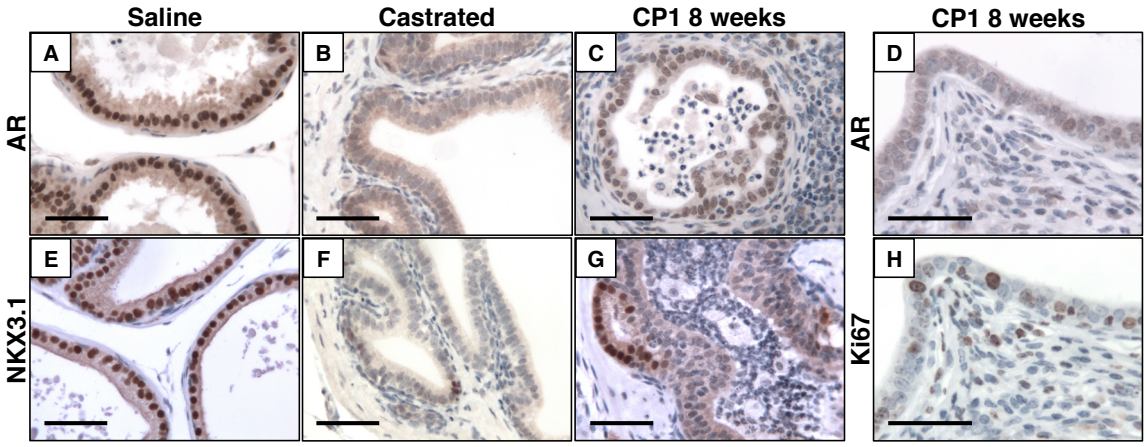

Figure 5: *Diminished AR expression and signaling in inflamed epithelium.* Immunohistochemistry for AR (A-D) and NKX3.1 (E-G), an androgen regulated protein, shows strong nuclear expression in normal glands (A&E), but significantly decreased expression of both proteins two weeks after castration (B&F). Expression of both proteins is decreased to castrate levels in inflamed epithelium 8 weeks post inoculation (C&G). Despite decreased AR expression, inflamed epithelium remains highly proliferative with numerous Ki67 positive nuclei (D&H).

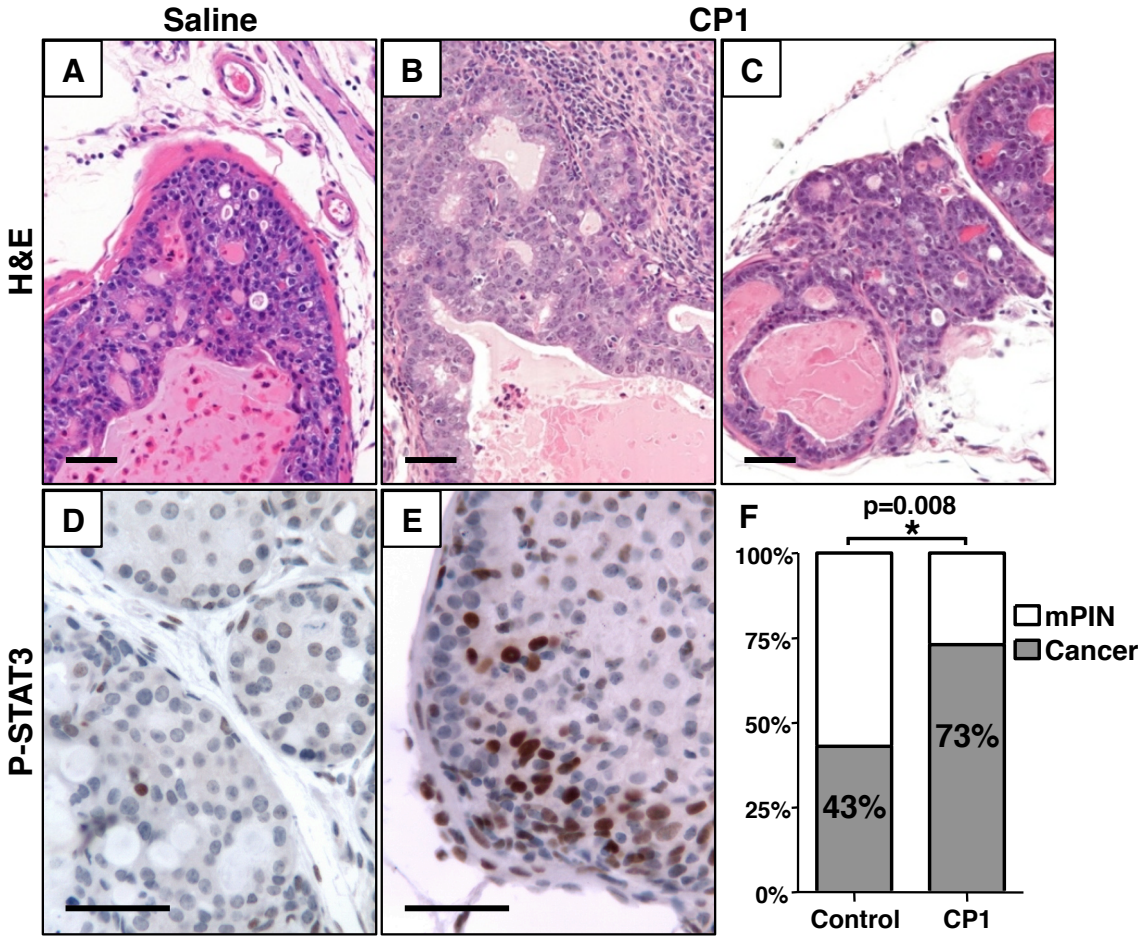

Figure 6: *CP1 infection accelerates cancer progression.* Representative images of prostates from 4.5 month old Hi-Myc mice treated with saline (A&D) or CP1 (B, C, E). Immunohistochemistry for phosphorylated-STAT3 (D&E) shows activation in CP1 treated, but not saline treated controls. At 4.5 months of age (F), significantly more mice have invasive carcinoma in CP1 treated group compared to controls (22/30 vs. 26/60, p=0.008). Scale bar = 50 micrometers.

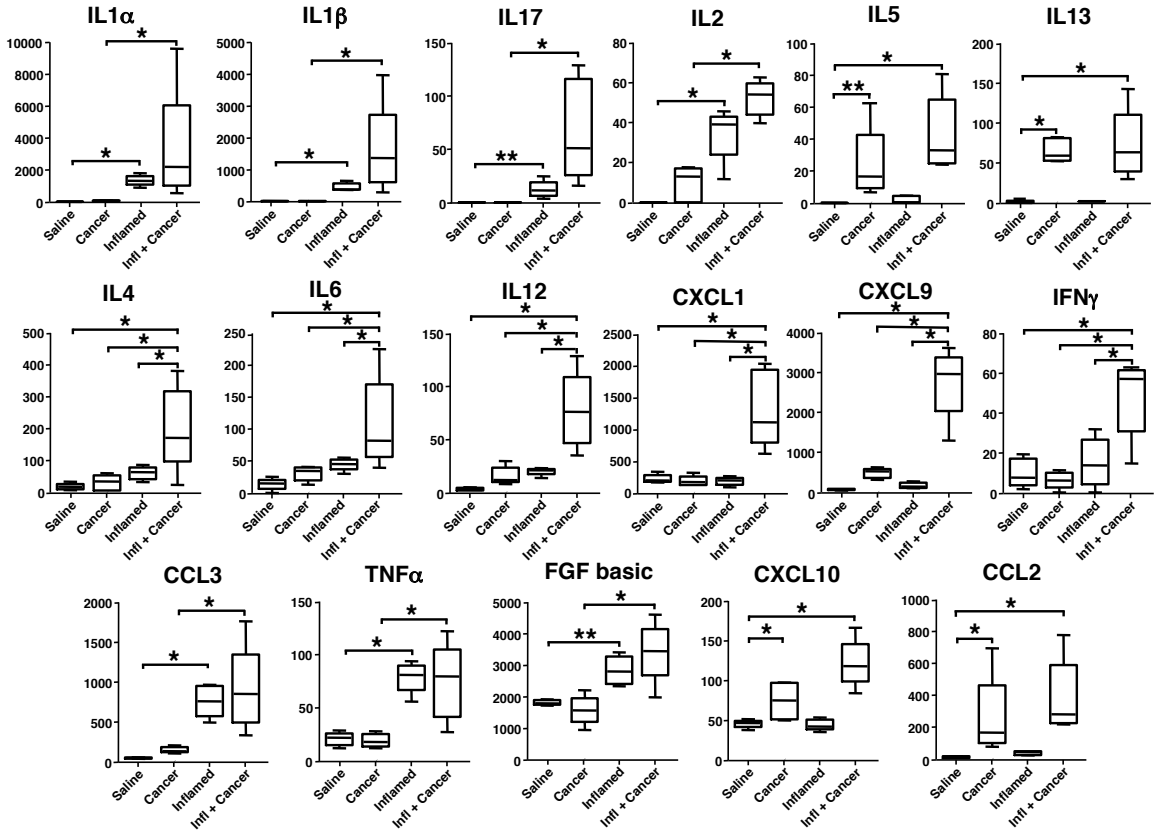

Figure 7: *Cytokine expression during inflammation and cancer.* Multiplex cytokine profiles of whole prostate lysates from 6-month-old (4 months post-inoculation) saline treated wildtype mice (Saline), saline treated Hi-Myc mice (Cancer), CP1 treated wildtype mice (Inflamed), and CP1 treated Hi-Myc Mice (Infl+Cancer). Box plots show median and min/max values of 10 mice for saline treated controls, and 5 mice in other groups, * $p<0.01$, ** $p<0.05$.

Cytokines Associated with Inflammation		Cytokines Associated with Cancer		Only Induced by Concurrent Inflammmation and Cancer
Only Induced by Inflammation	Augmented by Cancer	Only Induced by Cancer	Augmented by Inflammation	
CCL3 FGF TNFα	IL-1α IL-2 IL-1β IL-17	IL-5 IL-13 CCL2	CXCL10	IL-4 CXCL1 IL-6 CXCL9 IL-12 IFNγ

Table 1: *Inflammation and cancer have distinct cytokine profiles*. Cytokines can be grouped according to expression profile. Cytokines associated with inflammation are significantly increased in inflamed prostates, regardless of cancer status. Cytokines associated with cancer are significantly increased in cancer, regardless of inflammation status. Concurrent inflammation and cancer cytokines are significantly increased only when inflammation and cancer are simultaneous.

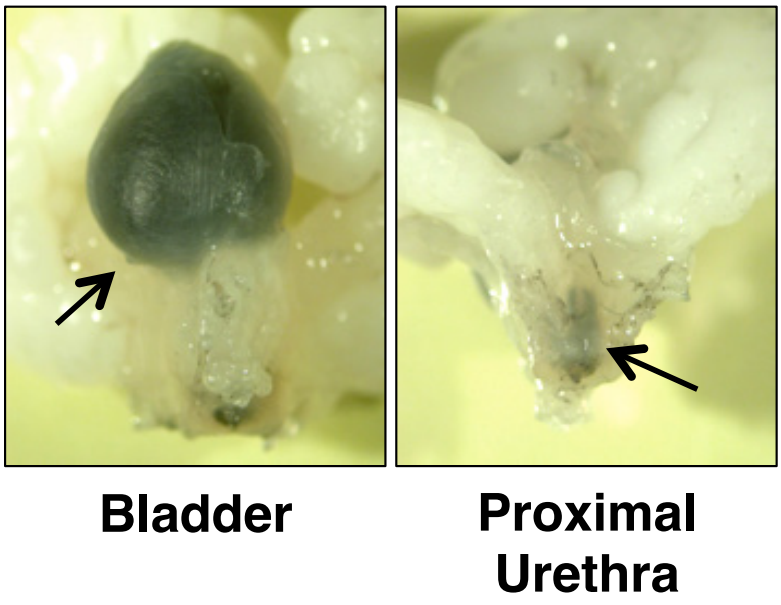

Figure S1: *Catheter placement*. Dye injected into the urethra in an identical manner to CP1 or saline treatment stains the bladder and proximal urethra, but does not infiltrate the prostate gland.

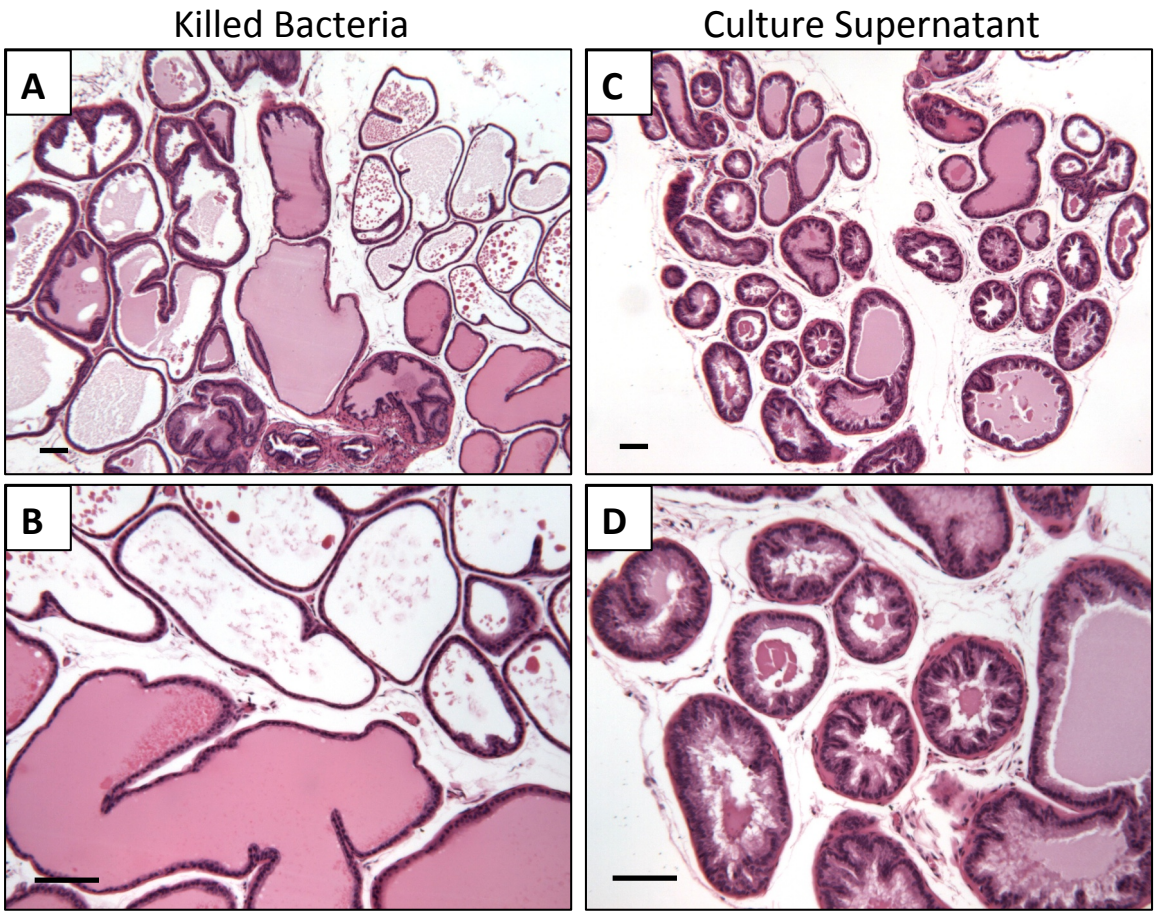

Figure S2: *Live bacteria are required to induce inflammation.* Representative histology of prostate glands two weeks after instillation of heat-killed CP1 culture (A, B) or filtered culture supernatant (C, D) show no inflammatory response. Scale bars = 100 micrometers.

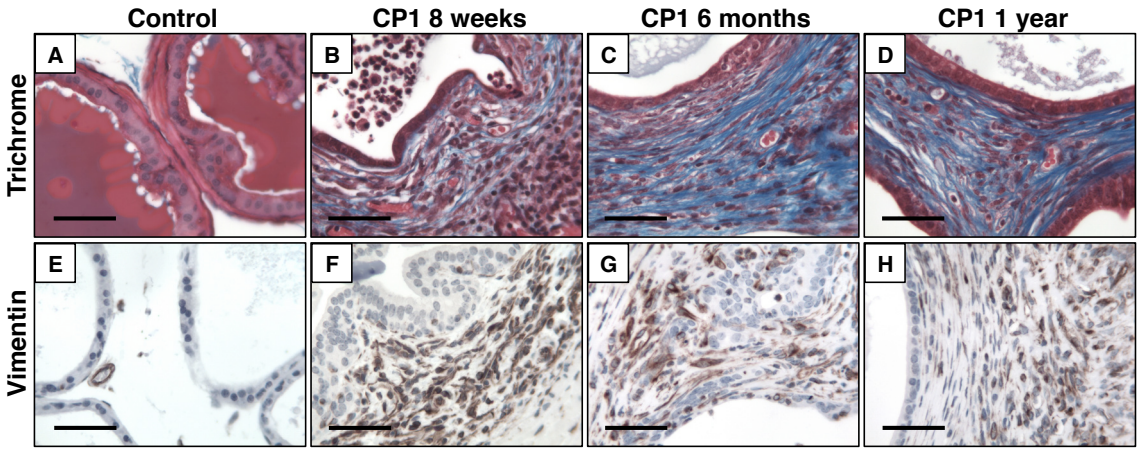

Figure S3: *Inflammation induces reactive stromal hyperplasia.* Masson's trichrome stain (A-D) and immunohistochemistry for vimentin (E-H) show thickened stroma with increasing collagen deposition (blue color) and numerous vimentin-positive stromal cells in inflamed glands at 8 weeks, 6 months, and 1 year after infection compared to saline treated controls.

IV. References

1. Taylor BC, Wilt TJ, Fink HA, Lambert LC, Marshall LM, Hoffman AR, Beer TM, Bauer DC, Zmuda JM, Orwoll ES, and Osteoporotic Fractures in Men Study Research G, *Prevalence, severity, and health correlates of lower urinary tract symptoms among older men: the MrOS study.* Urology, 2006. **68**(4): p. 804-9.
2. Breslow N, Chan CW, Dhom G, Drury RA, Franks LM, Gellei B, Lee YS, Lundberg S, Sparke B, Sternby NH, and Tulinius H, *Latent carcinoma of prostate at autopsy in seven areas. The International Agency for Research on Cancer, Lyons, France.* Int J Cancer, 1977. **20**(5): p. 680-8.
3. Ewing CM, Ray AM, Lange EM, Zuhlke KA, Robbins CM, Tembe WD, Wiley KE, Isaacs SD, Johng D, Wang Y, Bizon C, Yan G, Gielzak M, Partin AW, Shanmugam V, Izatt T, Sinari S, Craig DW, Zheng SL, Walsh PC, Montie JE, Xu J, Carpten JD, Isaacs WB, and Cooney KA, *Germline mutations in HOXB13 and prostate-cancer risk.* N Engl J Med, 2012. **366**(2): p. 141-9.
4. Walsh PC and Wilson JD, *The induction of prostatic hypertrophy in the dog with androstanediol.* J Clin Invest, 1976. **57**(4): p. 1093-7.
5. Teske E, Naan EC, van Dijk EM, Van Garderen E, and Schalken JA, *Canine prostate carcinoma: epidemiological evidence of an increased risk in castrated dogs.* Mol Cell Endocrinol, 2002. **197**(1-2): p. 251-5.
6. McNeal JE, Redwine EA, Freiha FS, and Stamey TA, *Zonal distribution of prostatic adenocarcinoma. Correlation with histologic pattern and direction of spread.* Am J Surg Pathol, 1988. **12**(12): p. 897-906.
7. Suwa T, Nyska A, Haseman JK, Mahler JF, and Maronpot RR, *Spontaneous lesions in control B6C3F1 mice and recommended sectioning of male accessory sex organs.* Toxicol Pathol, 2002. **30**(2): p. 228-34.
8. Wu X, Wu J, Huang J, Powell WC, Zhang J, Matusik RJ, Sangiorgi FO, Maxson RE, Sucov HM, and Roy-Burman P, *Generation of a prostate epithelial cell-specific Cre transgenic mouse model for tissue-specific gene ablation.* Mech Dev, 2001. **101**(1-2): p. 61-9.
9. Placencio VR, Sharif-Afshar AR, Li X, Huang H, Uwamariya C, Neilson EG, Shen MM, Matusik RJ, Hayward SW, and Bhowmick NA, *Stromal transforming growth factor-beta signaling mediates prostatic response to androgen ablation by paracrine Wnt activity.* Cancer Res, 2008. **68**(12): p. 4709-18.
10. Rao V, Heard JC, Ghaffari H, Wali A, Mutton LN, and Bieberich CJ, *A Hoxb13-driven reverse tetracycline transactivator system for conditional gene expression in the prostate.* Prostate, 2012. **72**(10): p. 1045-51.
11. Birbach A, *Use of PB-Cre4 mice for mosaic gene deletion.* PLoS One, 2013. **8**(1): p. e53501.
12. De Marzo AM, Platz EA, Sutcliffe S, Xu J, Gronberg H, Drake CG, Nakai Y, Isaacs WB, and Nelson WG, *Inflammation in prostate carcinogenesis.* Nat Rev Cancer, 2007. **7**(4): p. 256-69.
13. Lundgren R, Holmquist B, Hesselvik M, and Muntzing J, *Treatment of prostatitis in the rat.* Prostate, 1984. **5**(3): p. 277-84.
14. Penna G, Amuchastegui S, Cossetti C, Aquilano F, Mariani R, Giarratana N, De Carli E, Fibbi B, and Adorini L, *Spontaneous and prostatic steroid*

binding protein peptide-induced autoimmune prostatitis in the nonobese diabetic mouse. J Immunol, 2007. **179**(3): p. 1559-67.
15. Coffey DS, *Similarities of prostate and breast cancer: Evolution, diet, and estrogens.* Urology, 2001. **57**(4 Suppl 1): p. 31-8.
16. Harkonen PL and Makela SI, *Role of estrogens in development of prostate cancer.* J Steroid Biochem Mol Biol, 2004. **92**(4): p. 297-305.
17. Naslund MJ, Strandberg JD, and Coffey DS, *The role of androgens and estrogens in the pathogenesis of experimental nonbacterial prostatitis.* J Urol, 1988. **140**(5): p. 1049-53.
18. Sugimura T, Wakabayashi K, Nakagama H, and Nagao M, *Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish.* Cancer Sci, 2004. **95**(4): p. 290-9.
19. Borowsky AD, Dingley KH, Ubick E, Turteltaub KW, Cardiff RD, and Devere-White R, *Inflammation and atrophy precede prostatic neoplasia in a PhIP-induced rat model.* Neoplasia, 2006. **8**(9): p. 708-15.
20. Ponniah S, Arah I, and Alexander RB, *PSA is a candidate self-antigen in autoimmune chronic prostatitis/chronic pelvic pain syndrome.* Prostate, 2000. **44**(1): p. 49-54.
21. Taguchi O, Kojima A, and Nishizuka Y, *Experimental autoimmune prostatitis after neonatal thymectomy in the mouse.* Clin Exp Immunol, 1985. **60**(1): p. 123-9.
22. Fong L, Ruegg CL, Brockstedt D, Engleman EG, and Laus R, *Induction of tissue-specific autoimmune prostatitis with prostatic acid phosphatase immunization: implications for immunotherapy of prostate cancer.* J Immunol, 1997. **159**(7): p. 3113-7.
23. Liu KJ, Chatta GS, Twardzik DR, Vedvick TS, True LD, Spies AG, and Cheever MA, *Identification of rat prostatic steroid-binding protein as a target antigen of experimental autoimmune prostatitis: implications for prostate cancer therapy.* J Immunol, 1997. **159**(1): p. 472-80.
24. Keetch DW, Humphrey P, and Ratliff TL, *Development of a mouse model for nonbacterial prostatitis.* J Urol, 1994. **152**(1): p. 247-50.
25. Lees JR, Charbonneau B, Hayball JD, Diener K, Brown M, Matusik R, Cohen MB, and Ratliff TL, *T-cell recognition of a prostate specific antigen is not sufficient to induce prostate tissue destruction.* Prostate, 2006. **66**(6): p. 578-90.
26. Shinohara DB, Vaghasia AM, Yu SH, Mak TN, Bruggemann H, Nelson WG, De Marzo AM, Yegnasubramanian S, and Sfanos KS, *A mouse model of chronic prostatic inflammation using a human prostate cancer-derived isolate of Propionibacterium acnes.* Prostate, 2013. **73**(9): p. 1007-15.
27. Khalili M, Mutton LN, Gurel B, Hicks JL, De Marzo AM, and Bieberich CJ, *Loss of Nkx3.1 expression in bacterial prostatitis: a potential link between inflammation and neoplasia.* Am J Pathol, 2010. **176**(5): p. 2259-68.
28. Elkahwaji JE, Ott CJ, Janda LM, and Hopkins WJ, *Mouse model for acute bacterial prostatitis in genetically distinct inbred strains.* Urology, 2005. **66**(4): p. 883-7.
29. Kwon OJ, Zhang L, Ittmann MM, and Xin L, *Prostatic inflammation enhances basal-to-luminal differentiation and accelerates initiation of prostate cancer with a basal cell origin.* Proc Natl Acad Sci U S A, 2014. **111**(5): p. E592-600.

30. Sugimura Y, Cunha GR, and Donjacour AA, *Morphogenesis of ductal networks in the mouse prostate.* Biol Reprod, 1986. **34**(5): p. 961-71.
31. Cunha GR, *The role of androgens in the epithelio-mesenchymal interactions involved in prostatic morphogenesis in embryonic mice.* Anat Rec, 1973. **175**(1): p. 87-96.
32. Bhatia-Gaur R, Donjacour AA, Sciavolino PJ, Kim M, Desai N, Young P, Norton CR, Gridley T, Cardiff RD, Cunha GR, Abate-Shen C, and Shen MM, *Roles for Nkx3.1 in prostate development and cancer.* Genes Dev, 1999. **13**(8): p. 966-77.
33. Bieberich CJ, Fujita K, He WW, and Jay G, *Prostate-specific and androgen-dependent expression of a novel homeobox gene.* J Biol Chem, 1996. **271**(50): p. 31779-82.
34. Kim MJ, Bhatia-Gaur R, Banach-Petrosky WA, Desai N, Wang Y, Hayward SW, Cunha GR, Cardiff RD, Shen MM, and Abate-Shen C, *Nkx3.1 mutant mice recapitulate early stages of prostate carcinogenesis.* Cancer Res, 2002. **62**(11): p. 2999-3004.
35. Schaeffer EM, Marchionni L, Huang Z, Simons B, Blackman A, Yu W, Parmigiani G, and Berman DM, *Androgen-induced programs for prostate epithelial growth and invasion arise in embryogenesis and are reactivated in cancer.* Oncogene, 2008. **27**(57): p. 7180-91.
36. Zhang TJ, Hoffman BG, Ruiz de Algara T, and Helgason CD, *SAGE reveals expression of Wnt signalling pathway members during mouse prostate development.* Gene expression patterns : GEP, 2006. **6**(3): p. 310.
37. Blum R, Gupta R, Burger PE, Ontiveros CS, Salm SN, Xiong X, Kamb A, Wesche H, Marshall L, Cutler G, Wang X, Zavadil J, Moscatelli D, and Wilson EL, *Molecular signatures of the primitive prostate stem cell niche reveal novel mesenchymal-epithelial signaling pathways.* PLoS ONE, 2010. **5**(9).
38. Mehta V, Abler LL, Keil KP, Schmitz CT, Joshi PS, and Vezina CM, *Atlas of Wnt and R-spondin gene expression in the developing male mouse lower urogenital tract.* Dev Dyn, 2011. **240**(11): p. 2548-60.
39. Mucenski ML, Wert SE, Nation JM, Loudy DE, Huelsken J, Birchmeier W, Morrisey EE, and Whitsett JA, *beta-Catenin is required for specification of proximal/distal cell fate during lung morphogenesis.* J Biol Chem, 2003. **278**(41): p. 40231-8.
40. Chu EY, Hens J, Andl T, Kairo A, Yamaguchi TP, Brisken C, Glick A, Wysolmerski JJ, and Millar SE, *Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis.* Development, 2004. **131**(19): p. 4819-29.
41. Murtaugh LC, Law AC, Dor Y, and Melton DA, *Beta-catenin is essential for pancreatic acinar but not islet development.* Development, 2005. **132**(21): p. 4663-74.
42. Wells JM, Esni F, Boivin GP, Aronow BJ, Stuart W, Combs C, Sklenka A, Leach SD, and Lowy AM, *Wnt/beta-catenin signaling is required for development of the exocrine pancreas.* BMC Dev Biol, 2007. **7**: p. 4.
43. Yu X, Wang Y, Jiang M, Bierie B, Roy-Burman P, Shen MM, Taketo MM, Wills M, and Matusik RJ, *Activation of beta-Catenin in mouse prostate causes HGPIN and continuous prostate growth after castration.* Prostate, 2009. **69**(3): p. 249-62.

44. Allgeier SH, Lin TM, Vezina CM, Moore RW, Fritz WA, Chiu SY, Zhang C, and Peterson RE, *WNT5A selectively inhibits mouse ventral prostate development.* Dev Biol, 2008. **324**(1): p. 10-7.
45. Huang L, Pu Y, Hu WY, Birch L, Luccio-Camelo D, Yamaguchi T, and Prins GS, *The role of Wnt5a in prostate gland development.* Dev Biol, 2009. **328**(2): p. 188-99.
46. Wang BE, Wang XD, Ernst JA, Polakis P, and Gao WQ, *Regulation of epithelial branching morphogenesis and cancer cell growth of the prostate by Wnt signaling.* PLoS ONE, 2008. **3**(5): p. e2186.
47. Bierie B, Nozawa M, Renou JP, Shillingford JM, Morgan F, Oka T, Taketo MM, Cardiff RD, Miyoshi K, Wagner KU, Robinson GW, and Hennighausen L, *Activation of beta-catenin in prostate epithelium induces hyperplasias and squamous transdifferentiation.* Oncogene, 2003. **22**(25): p. 3875-87.
48. Bruxvoort KJ, Charbonneau HM, Giambernardi TA, Goolsby JC, Qian CN, Zylstra CR, Robinson DR, Roy-Burman P, Shaw AK, Buckner-Berghuis BD, Sigler RE, Resau JH, Sullivan R, Bushman W, and Williams BO, *Inactivation of Apc in the mouse prostate causes prostate carcinoma.* Cancer Res, 2007. **67**(6): p. 2490-6.
49. Li X, Wang Y, Sharif-Afshar A-R, Uwamariya C, Yi A, Ishii K, Hayward SW, Matusik RJ, and Bhowmick NA, *Urothelial transdifferentiation to prostate epithelia is mediated by paracrine TGF-[beta] signaling.* Differentiation, 2009. **77**(1): p. 95-102.
50. Gordon MD and Nusse R, *Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors.* J Biol Chem, 2006. **281**(32): p. 22429-33.
51. Aberle H, Bauer A, Stappert J, Kispert A, and Kemler R, *beta-catenin is a target for the ubiquitin-proteasome pathway.* EMBO J, 1997. **16**(13): p. 3797-804.
52. Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, Kuhl M, Wedlich D, and Birchmeier W, *Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta.* Science, 1998. **280**(5363): p. 596-9.
53. Jho EH, Zhang T, Domon C, Joo CK, Freund JN, and Costantini F, *Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway.* Mol Cell Biol, 2002. **22**(4): p. 1172-83.
54. Ontiveros CS, Salm SN, and Wilson EL, *Axin2 expression identifies progenitor cells in the murine prostate.* Prostate, 2008. **68**(12): p. 1263-72.
55. Yan D, Wiesmann M, Rohan M, Chan V, Jefferson AB, Guo L, Sakamoto D, Caothien RH, Fuller JH, Reinhard C, Garcia PD, Randazzo FM, Escobedo J, Fantl WJ, and Williams LT, *Elevated expression of axin2 and hnkd mRNA provides evidence that Wnt/beta -catenin signaling is activated in human colon tumors.* Proc Natl Acad Sci U S A, 2001. **98**(26): p. 14973-8.
56. Haegel H, Larue L, Ohsugi M, Fedorov L, Herrenknecht K, and Kemler R, *Lack of beta-catenin affects mouse development at gastrulation.* Development, 1995. **121**(11): p. 3529-37.
57. Jin C, McKeehan K, and Wang F, *Transgenic mouse with high Cre recombinase activity in all prostate lobes, seminal vesicle, and ductus deferens.* Prostate, 2003. **57**(2): p. 160-4.

58. Lin Y, Liu G, Zhang Y, Hu YP, Yu K, Lin C, McKeehan K, Xuan JW, Ornitz DM, Shen MM, Greenberg N, McKeehan WL, and Wang F, *Fibroblast growth factor receptor 2 tyrosine kinase is required for prostatic morphogenesis and the acquisition of strict androgen dependency for adult tissue homeostasis.* Development, 2007. **134**(4): p. 723-34.
59. Jokela T and Vainio S, *Conditional tamoxifen Cre induced mutagenesis in the embryonic kidney in organ culture.* Genesis, 2007. **45**(12): p. 757-61.
60. Ghosh S, Lau H, Simons BW, Powell JD, Meyers DJ, De Marzo AM, Berman DM, and Lotan TL, *PI3K/mTOR signaling regulates prostatic branching morphogenesis.* Dev Biol, 2011. **360**(2): p. 329-42.
61. Chen H and Bieberich CJ, *Structural and functional analysis of domains mediating interaction between NKX-3.1 and PDEF.* J Cell Biochem, 2005. **94**(1): p. 168-77.
62. McMullin RP, Mutton LN, and Bieberich CJ, *Hoxb13 regulatory elements mediate transgene expression during prostate organogenesis and carcinogenesis.* Dev Dyn, 2009. **238**(3): p. 664-72.
63. Staack A, Donjacour AA, Brody J, Cunha GR, and Carroll P, *Mouse urogenital development: a practical approach.* Differentiation; research in biological diversity, 2003. **71**(7): p. 402.
64. Livak KJ and Schmittgen TD, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
65. Lasnitzki I and Mizuno T, *Induction of the rat prostate gland by androgens in organ culture.* J Endocrinol, 1977. **74**(1): p. 47-55.
66. Brault V, Moore R, Kutsch S, Ishibashi M, Rowitch DH, McMahon AP, Sommer L, Boussadia O, and Kemler R, *Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development.* Development, 2001. **128**(8): p. 1253-64.
67. Badea TC, Wang Y, and Nathans J, *A noninvasive genetic/pharmacologic strategy for visualizing cell morphology and clonal relationships in the mouse.* J Neurosci, 2003. **23**(6): p. 2314-22.
68. Yu X, Wang Y, DeGraff DJ, Wills ML, and Matusik RJ, *Wnt/beta-catenin activation promotes prostate tumor progression in a mouse model.* Oncogene, 2011. **30**(16): p. 1868-79.
69. Doles JD, Vezina CM, Lipinski RJ, Peterson RE, and Bushman W, *Growth, morphogenesis, and differentiation during mouse prostate development in situ, in renal grafts, and in vitro.* The Prostate, 2005. **65**(4): p. 390.
70. Lepourcelet M, Chen YN, France DS, Wang H, Crews P, Petersen F, Bruseo C, Wood AW, and Shivdasani RA, *Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex.* Cancer Cell, 2004. **5**(1): p. 91-102.
71. Sciavolino PJ, Abrams EW, Yang L, Austenberg LP, Shen MM, and Abate-Shen C, *Tissue-specific expression of murine Nkx3.1 in the male urogenital system.* Dev Dyn, 1997. **209**(1): p. 127-38.
72. Sreenath T, Orosz A, Fujita K, and Bieberich CJ, *Androgen-independent expression of hoxb-13 in the mouse prostate.* Prostate, 1999. **41**(3): p. 203-7.
73. Thomsen MK, Butler CM, Shen MM, and Swain A, *Sox9 is required for prostate development.* Dev Biol, 2008. **316**(2): p. 302-11.

74. Stanfel MN, Moses KA, Carson JA, Zimmer DB, DeMayo F, Schwartz RJ, and Zimmer WE, *Expression of an Nkx3.1-CRE gene using ROSA26 reporter mice.* Genesis, 2006. **44**(11): p. 550-5.
75. Aberle H, Schwartz H, and Kemler R, *Cadherin-catenin complex: protein interactions and their implications for cadherin function.* J Cell Biochem, 1996. **61**(4): p. 514-23.
76. Truica CI, Byers S, and Gelmann EP, *Beta-catenin affects androgen receptor transcriptional activity and ligand specificity.* Cancer research, 2000. **60**(17): p. 4709.
77. Bridgewater D, Cox B, Cain J, Lau A, Athaide V, Gill PS, Kuure S, Sainio K, and Rosenblum ND, *Canonical WNT/beta-catenin signaling is required for ureteric branching.* Dev Biol, 2008. **317**(1): p. 83-94.
78. Prins GS, Birch L, Habermann H, Chang WY, Tebeau C, Putz O, and Bieberich C, *Influence of neonatal estrogens on rat prostate development.* Reprod Fertil Dev, 2001. **13**(4): p. 241-52.
79. Coussens LM and Werb Z, *Inflammation and cancer.* Nature, 2002. **420**(6917): p. 860-7.
80. Palapattu GS, Sutcliffe S, Bastian PJ, Platz EA, De Marzo AM, Isaacs WB, and Nelson WG, *Prostate carcinogenesis and inflammation: emerging insights.* Carcinogenesis, 2005. **26**(7): p. 1170-81.
81. Platz EA and De Marzo AM, *Epidemiology of inflammation and prostate cancer.* J Urol, 2004. **171**(2 Pt 2): p. S36-40.
82. Cheng I, Witte JS, Jacobsen SJ, Haque R, Quinn VP, Quesenberry CP, Caan BJ, and Van Den Eeden SK, *Prostatitis, sexually transmitted diseases, and prostate cancer: the California Men's Health Study.* PLoS One, 2010. **5**(1): p. e8736.
83. Nelson WG, De Marzo AM, DeWeese TL, and Isaacs WB, *The role of inflammation in the pathogenesis of prostate cancer.* J Urol, 2004. **172**(5 Pt 2): p. S6-11; discussion S11-2.
84. Nickel JC, Roehrborn CG, O'Leary MP, Bostwick DG, Somerville MC, and Rittmaster RS, *The relationship between prostate inflammation and lower urinary tract symptoms: examination of baseline data from the REDUCE trial.* Eur Urol, 2008. **54**(6): p. 1379-84.
85. Yoon BI, Kim S, Han DS, Ha US, Lee SJ, Kim HW, Han CH, and Cho YH, *Acute bacterial prostatitis: how to prevent and manage chronic infection?* J Infect Chemother, 2012. **18**(4): p. 444-50.
86. Yanamandra K, Alexeyev O, Zamotin V, Srivastava V, Shchukarev A, Brorsson AC, Tartaglia GG, Vogl T, Kayed R, Wingsle G, Olsson J, Dobson CM, Bergh A, Elgh F, and Morozova-Roche LA, *Amyloid formation by the pro-inflammatory S100A8/A9 proteins in the ageing prostate.* PLoS One, 2009. **4**(5): p. e5562.
87. Hochreiter WW, Duncan JL, and Schaeffer AJ, *Evaluation of the bacterial flora of the prostate using a 16S rRNA gene based polymerase chain reaction.* J Urol, 2000. **163**(1): p. 127-30.
88. Krieger JN, Riley DE, Vesella RL, Miner DC, Ross SO, and Lange PH, *Bacterial dna sequences in prostate tissue from patients with prostate cancer and chronic prostatitis.* J Urol, 2000. **164**(4): p. 1221-8.

89. McDowell KL, Begley LA, Mor-Vaknin N, Markovitz DM, and Macoska JA, *Leukocytic promotion of prostate cellular proliferation.* Prostate, 2010. **70**(4): p. 377-89.
90. Wiseman H and Halliwell B, *Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer.* Biochem J, 1996. **313 (Pt 1)**: p. 17-29.
91. Comito G, Giannoni E, Segura CP, Barcellos-de-Souza P, Raspollini MR, Baroni G, Lanciotti M, Serni S, and Chiarugi P, *Cancer-associated fibroblasts and M2-polarized macrophages synergize during prostate carcinoma progression.* Oncogene, 2013.
92. Fang LY, Izumi K, Lai KP, Liang L, Li L, Miyamoto H, Lin WJ, and Chang C, *Infiltrating Macrophages Promote Prostate Tumorigenesis via Modulating Androgen Receptor-Mediated CCL4-STAT3 Signaling.* Cancer Res, 2013. **73**(18): p. 5633-46.
93. Sfanos KS, Bruno TC, Maris CH, Xu L, Thoburn CJ, DeMarzo AM, Meeker AK, Isaacs WB, and Drake CG, *Phenotypic analysis of prostate-infiltrating lymphocytes reveals TH17 and Treg skewing.* Clin Cancer Res, 2008. **14**(11): p. 3254-61.
94. Vykhovanets EV, Maclennan GT, Vykhovanets OV, and Gupta S, *IL-17 Expression by macrophages is associated with proliferative inflammatory atrophy lesions in prostate cancer patients.* Int J Clin Exp Pathol, 2011. **4**(6): p. 552-65.
95. Wu S, Rhee KJ, Albesiano E, Rabizadeh S, Wu X, Yen HR, Huso DL, Brancati FL, Wick E, McAllister F, Housseau F, Pardoll DM, and Sears CL, *A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses.* Nat Med, 2009. **15**(9): p. 1016-22.
96. Haverkamp JM, Charbonneau B, Crist SA, Meyerholz DK, Cohen MB, Snyder PW, Svensson RU, Henry MD, Wang HH, and Ratliff TL, *An inducible model of abacterial prostatitis induces antigen specific inflammatory and proliferative changes in the murine prostate.* Prostate, 2011. **71**(11): p. 1139-50.
97. Kwon OJ, Zhang L, Ittmann MM, and Xin L, *Prostatic inflammation enhances basal-to-luminal differentiation and accelerates initiation of prostate cancer with a basal cell origin.* Proc Natl Acad Sci U S A, 2013.
98. Boehm BJ, Colopy SA, Jerde TJ, Loftus CJ, and Bushman W, *Acute bacterial inflammation of the mouse prostate.* Prostate, 2012. **72**(3): p. 307-17.
99. Elkahwaji JE, Zhong W, Hopkins WJ, and Bushman W, *Chronic bacterial infection and inflammation incite reactive hyperplasia in a mouse model of chronic prostatitis.* Prostate, 2007. **67**(1): p. 14-21.
100. Rudick CN, Berry RE, Johnson JR, Johnston B, Klumpp DJ, Schaeffer AJ, and Thumbikat P, *Uropathogenic Escherichia coli induces chronic pelvic pain.* Infect Immun, 2011. **79**(2): p. 628-35.
101. Ellwood-Yen K, Graeber TG, Wongvipat J, Iruela-Arispe ML, Zhang J, Matusik R, Thomas GV, and Sawyers CL, *Myc-driven murine prostate cancer shares molecular features with human prostate tumors.* Cancer Cell, 2003. **4**(3): p. 223-38.
102. Thumbikat P, Berry RE, Zhou G, Billips BK, Yaggie RE, Zaichuk T, Sun TT, Schaeffer AJ, and Klumpp DJ, *Bacteria-induced uroplakin signaling mediates bladder response to infection.* PLoS Pathog, 2009. **5**(5): p. e1000415.

103. Ittmann M, Huang J, Radaelli E, Martin P, Signoretti S, Sullivan R, Simons BW, Ward JM, Robinson BD, Chu GC, Loda M, Thomas G, Borowsky A, and Cardiff RD, *Animal models of human prostate cancer: the consensus report of the New York meeting of the Mouse Models of Human Cancers Consortium Prostate Pathology Committee.* Cancer Res, 2013. **73**(9): p. 2718-36.
104. Alex P, Zachos NC, Nguyen T, Gonzales L, Chen TE, Conklin LS, Centola M, and Li X, *Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis.* Inflamm Bowel Dis, 2009. **15**(3): p. 341-52.
105. Blando JM, Carbajal S, Abel E, Beltran L, Conti C, Fischer S, and DiGiovanni J, *Cooperation between Stat3 and Akt signaling leads to prostate tumor development in transgenic mice.* Neoplasia, 2011. **13**(3): p. 254-65.
106. Gurel B, Iwata T, Koh CM, Jenkins RB, Lan F, Van Dang C, Hicks JL, Morgan J, Cornish TC, Sutcliffe S, Isaacs WB, Luo J, and De Marzo AM, *Nuclear MYC protein overexpression is an early alteration in human prostate carcinogenesis.* Mod Pathol, 2008. **21**(9): p. 1156-67.
107. Abdulghani J, Gu L, Dagvadorj A, Lutz J, Leiby B, Bonuccelli G, Lisanti MP, Zellweger T, Alanen K, Mirtti T, Visakorpi T, Bubendorf L, and Nevalainen MT, *Stat3 promotes metastatic progression of prostate cancer.* Am J Pathol, 2008. **172**(6): p. 1717-28.
108. Iwata T, Schultz D, Hicks J, Hubbard GK, Mutton LN, Lotan TL, Bethel C, Lotz MT, Yegnasubramanian S, Nelson WG, Dang CV, Xu M, Anele U, Koh CM, Bieberich CJ, and De Marzo AM, *MYC overexpression induces prostatic intraepithelial neoplasia and loss of Nkx3.1 in mouse luminal epithelial cells.* PLoS ONE, 2010. **5**(2): p. e9427.
109. Osborne LC, Joyce KL, Alenghat T, Sonnenberg GF, Giacomin PR, Du Y, Bergstrom KS, Vallance BA, and Nair MG, *Resistin-like molecule alpha promotes pathogenic Th17 cell responses and bacterial-induced intestinal inflammation.* J Immunol, 2013. **190**(5): p. 2292-300.
110. Ivanov, II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K, and Littman DR, *Induction of intestinal Th17 cells by segmented filamentous bacteria.* Cell, 2009. **139**(3): p. 485-98.
111. Quick ML, Wong L, Mukherjee S, Done JD, Schaeffer AJ, and Thumbikat P, *Th1-Th17 cells contribute to the development of uropathogenic Escherichia coli-induced chronic pelvic pain.* PLoS ONE, 2013. **8**(4): p. e60987.
112. Wang L, Yi T, Kortylewski M, Pardoll DM, Zeng D, and Yu H, *IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway.* J Exp Med, 2009. **206**(7): p. 1457-64.
113. Zhang Q, Liu S, Ge D, Zhang Q, Xue Y, Xiong Z, Abdel-Mageed AB, Myers L, Hill SM, Rowan BG, Sartor O, Melamed J, Chen Z, and You Z, *Interleukin-17 promotes formation and growth of prostate adenocarcinoma in mouse models.* Cancer Res, 2012. **72**(10): p. 2589-99.
114. Nadler RB, Koch AE, Calhoun EA, Campbell PL, Pruden DL, Bennett CL, Yarnold PR, and Schaeffer AJ, *IL-1beta and TNF-alpha in prostatic secretions are indicators in the evaluation of men with chronic prostatitis.* J Urol, 2000. **164**(1): p. 214-8.

115. Loberg RD, Day LL, Harwood J, Ying C, St John LN, Giles R, Neeley CK, and Pienta KJ, *CCL2 is a potent regulator of prostate cancer cell migration and proliferation.* Neoplasia, 2006. **8**(7): p. 578-86.
116. Loberg RD, Ying C, Craig M, Yan L, Snyder LA, and Pienta KJ, *CCL2 as an important mediator of prostate cancer growth in vivo through the regulation of macrophage infiltration.* Neoplasia, 2007. **9**(7): p. 556-62.
117. Maini A, Hillman G, Haas GP, Wang CY, Montecillo E, Hamzavi F, Pontes JE, Leland P, Pastan I, Debinski W, and Puri RK, *Interleukin-13 receptors on human prostate carcinoma cell lines represent a novel target for a chimeric protein composed of IL-13 and a mutated form of Pseudomonas exotoxin.* J Urol, 1997. **158**(3 Pt 1): p. 948-53.
118. Debelec-Butuner B, Alapinar C, Varisli L, Erbaykent-Tepedelen B, Hamid SM, Gonen-Korkmaz C, and Korkmaz KS, *Inflammation-mediated abrogation of androgen signaling: An in vitro model of prostate cell inflammation.* Mol Carcinog, 2012.
119. Litvinov IV, De Marzo AM, and Isaacs JT, *Is the Achilles' heel for prostate cancer therapy a gain of function in androgen receptor signaling?* J Clin Endocrinol Metab, 2003. **88**(7): p. 2972-82.

VI. Curriculum Vitae

Brian W. Simons, DVM

Johns Hopkins University School of Medicine
Department of Molecular and Comparative Pathobiology
Koch Cancer Research Building, Room 532
1550 Orleans Street, Baltimore, MD 21231
simons@jhmi.edu
410-929-4484 (cell)

Education & Training

Postoctoral Fellowship — 2014-present
Johns Hopkins University School of Medicine
Mentors: Edward Schaeffer and Barry Nelkin

Ph.D., Pathobiology — 2014
Johns Hopkins University School of Medicine
Mentor: David Berman

Residency, Veterinary Anatomic Pathology — 2004-2007
Johns Hopkins University School of Medicine

D.V.M. — 2004
Texas A&M University, College Station, TX

B.S., Genetics — 2000
B.A., Philosophy — 2000
Texas A&M University, College Station, TX

Grant Support

Molecular and Clinical Investigations to Reduce the Morbidity of Prostate Cancer
Prostate Cancer Foundation Special Challenge Award
Performance Period: 07/01/13-06/30/18

CDK5 Alters Immune Response to Prostate Cancer
Patrick C. Walsh Prostate Cancer Foundation (PI: Nelkin)
Performance Period: 04/01/12-03/31/15

Designing Polyamide Inhibitors of Twist 1 for Pro-senescence Therapy
Department of Defense CDMRP (W81XWH-13-1-0182, PI: Tran)
Performance Period: 07/01/2013-06/30/2014

Structure-Function Studies of Twist1-induced radioresistance in lung cancer
National Institutes of Healh (R01CA166348, PI: Tran)
Performance Period: 08/01/13-05/31/18

Defining a Role for the Oncogene Beta-Catenin in Prostate Epithelial Growth and Invasion
Department of Defense CDMRP Pre-doctoral Training Award (PC080778, PI: Simons)
Performance Period: 07/01/2009-02/11/2011

Honors and Awards

2014 Poster Award, Honorable Mention, Department of Urology Prostate Cancer Research Day, Johns Hopkins University School of Medicine
2012 First Place Poster Award, 50th Anniversary Year in Review, Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine
2011 Ralph M. Burnett First Place Poster Award, 6th Annual Prostate Research Day, Department of Urology, Johns Hopkins University School of Medicine
2010 3rd place poster, Experimental Disease, American College of Veterinary Pathologists Annual Meeting
2010 Travel award and podium presentation, Society for Basic Urologic Research Annual Meeting
2010 First place award for oral presentation, Pathobiology Graduate Program Annual Retreat, Johns Hopkins University School of Medicine
2010 Award for Excellence in Basic Research, Young Investigator's Day, Department of Pathology, Johns Hopkins University School of Medicine
2010 John L. Willey First Place Poster Award, 5th Annual Prostate Research Day, Department of Urology, Johns Hopkins University School of Medicine
2009 Award for Excellence in Basic Research, Young Investigator's Day, Department of Pathology, Johns Hopkins University School of Medicine
2008 Award for Excellence in Basic Research, Young Investigator's Day, Department of Pathology, Johns Hopkins University School of Medicine
2004 Graduation with honors, College of Veterinary Medicine, Texas A&M University
2000 Graduation with honors, Texas A&M University

Publications

Buscaglia JM, **Simons BW**, Prosser BJ, Ruben DS, Giday SA, Magno P, Clarke JO, Shin EJ, Kalloo AN, Kantsevoy SV, Gabrielson KL, Jagannath SB, Etanercept, a TNF-alpha binding agent, is ineffective in the prevention of post-ERCP pancreatitis in canines. Jop 9, 456-67, 2008a. PMCID18648137
Buscaglia JM, **Simons BW**, Prosser BJ, Ruben DS, Giday SA, Magno P, Clarke JO, Shin EJ, Kalloo AN, Kantsevoy SV, Gabrielson KL, Jagannath SB, Severity of post-ERCP pancreatitis directly proportional to the invasiveness of endoscopic intervention: a pilot study in a canine model. Endoscopy 40, 506-12, 2008b. PMCID18478511
Schaeffer EM, Marchionni L, Huang Z, **Simons B**, Blackman A, Yu W, Parmigiani G, Berman DM, Androgen-induced programs for prostate epithelial growth and invasion arise in embryogenesis and are reactivated in cancer. Oncogene 27, 7180-91, 2008. PMCID18794802

Ohm JE, Mali P, Van Neste L, Berman DM, Liang L, Pandiyan K, Briggs KJ, Zhang W, Argani P, **Simons B**, Yu W, Matsui W, Van Criekinge W, Rassool FV, Zambidis E, Schuebel KE, Cope L, Yen J, Mohammad HP, Cheng L, Baylin SB, Cancer-related epigenome changes associated with reprogramming to induced pluripotent stem cells. Cancer Res 70, 7662-73, 2010. PMCID20841480

Ross AE, Emadi A, Marchionni L, Hurley PJ, **Simons BW**, Schaeffer EM, Vuica-Ross M, Dimeric naphthoquinones, a novel class of compounds with prostate cancer cytotoxicity. BJU Int 108, 447-54, 2010a. PMCID21176082

Ross AE, Marchionni L, Phillips TM, Miller RM, Hurley PJ, **Simons BW**, Salmasi AH, Schaeffer AJ, Gearhart JP, Schaeffer EM, Molecular effects of genistein on male urethral development. J Urol 185, 1894-8, 2010b. PMCID21421236

Zhang W, Zeng X, Briggs KJ, Beaty R, **Simons B**, Chiu Yen RW, Tyler MA, Tsai HC, Ye Y, Gesell GS, Herman JG, Baylin SB, Watkins DN, A potential tumor suppressor role for Hic1 in breast cancer through transcriptional repression of ephrin-A1. Oncogene 29, 2467-76, 2010. PMCID20154726

Boylan NJ, Kim AJ, Suk JS, Adstamongkonkul P, **Simons BW**, Lai SK, Cooper MJ, Hanes J, Enhancement of airway gene transfer by DNA nanoparticles using a pH-responsive block copolymer of polyethylene glycol and poly-L-lysine. Biomaterials 33, 2361-71, 2011. PMCID22182747

Ghosh S, Lau H, **Simons BW**, Powell JD, Meyers DJ, De Marzo AM, Berman DM, Lotan TL, PI3K/mTOR signaling regulates prostatic branching morphogenesis. Dev Biol 360, 329-42, 2011. PMCID22015718

Shabbeer S, Williams SA, **Simons BW**, Herman JG, Carducci MA, Progression of prostate carcinogenesis and dietary methyl donors: temporal dependence. Cancer Prev Res (Phila) 5, 229-39, 2011. PMCID22139053

Huang Z, Hurley PJ, **Simons BW**, Marchionni L, Berman DM, Ross AE, Schaeffer EM, Sox9 is required for prostate development and prostate cancer initiation. Oncotarget 3, 651-63, 2012. PMCID22761195

Hurley PJ, Marchionni L, **Simons BW**, Ross AE, Peskoe SB, Miller RM, Erho N, Vergara IA, Ghadessi M, Huang Z, Gurel B, Park BH, Davicioni E, Jenkins RB, Platz EA, Berman DM, Schaeffer EM, Secreted protein, acidic and rich in cysteine-like 1 (SPARCL1) is down regulated in aggressive prostate cancers and is prognostic for poor clinical outcome. Proc Natl Acad Sci U S A 109, 14977-82, 2012. PMCID22927397

Kahlert U, Maciaczyk D, Doostkam S, Orr BA, **Simons B**, Bogiel T, Reithmeier T, Prinz M, Schubert J, Niedermann G, Brabletz T, Eberhart CG, Nikkhah G, Maciaczyk J, Activation of canonical WNT/beta-catenin signaling enhances in vitro motility of glioblastoma cells by activation of ZEB1 and other activators of epithelial-to-mesenchymal transition. Cancer Lett 2012. PMCID22652173

Kedziorek DA, Hofmann LV, Fu Y, Gilson WD, Cosby KM, Kohl B, Barnett BP, **Simons BW**, Walczak P, Bulte JW, Gabrielson K, Kraitchman DL, X-ray-visible microcapsules containing mesenchymal stem cells improve hind limb perfusion in a rabbit model of peripheral arterial disease. STEM CELLS 30, 1286-96, 2012. PMCID22438076

Simons BW, Hurley PJ, Huang Z, Ross AE, Miller R, Marchionni L, Berman DM, Schaeffer EM, Wnt signaling though beta-catenin is required for prostate lineage specification. Dev Biol 2012. PMCID22960283

Clayton LA, Stamper MA, Whitaker BR, Hadfield CA, **Simons B**, Mankowski JL, Mycobacterium abscessus pneumonia in an Atlantic bottlenose dolphin (Tursiops truncatus). J Zoo Wildl Med 43, 961-5, 2012. PMCID23272373

Carvalho FL, **Simons BW**, Antonarakis ES, Rasheed Z, Douglas N, Villegas D, Matsui W, Berman DM, Tumorigenic potential of circulating prostate tumor cells. Oncotarget 4, 413-21, 2013. PMCID23530114

Ittmann M, Huang J, Radaelli E, Martin P, Signoretti S, Sullivan R, **Simons BW**, Ward JM, Robinson BD, Chu GC, Loda M, Thomas G, Borowsky A, Cardiff RD, Animal Models of Human Prostate Cancer: The Consensus Report of the New York Meeting of the Mouse Models of Human Cancers Consortium Prostate Pathology Committee. Cancer Res 73, 2718-2736, 2013. PMCID23610450

Pascal LE, Ai J, Masoodi KZ, Wang Y, Wang D, Eisermann K, Rigatti LH, O'Malley KJ, Ma HM, Wang X, Dar JA, Parwani AV, **Simons BW**, Ittman MM, Li L, Davies BJ,Wang Z: Development of a Reactive Stroma Associated with Prostatic Intraepithelial Neoplasia in EAF2 Deficient Mice. PLoS One 2013; 8:e79542. PMCID:3832612

Yang M, Yu T, Wang YY, Lai SK, Zeng Q, Miao B, Tang BC, **Simons BW**, Ensign L, Juang CY, Liu G, Chan WY, Mert O, Wood J, Fu J, McMahon MT, Wu TC, Hung CF, Hanes J, Vaginal delivery of paclitaxel via mucus-penetrating nanoparticles suppresses cervical tumor growth. Advanced Healthcare Materials Dec 16 2013. PMID: 24339398

In Press

Saxema P, Haito-Chavez Y, Kord A, Venkata S, Aguila G, Zhang M, Zhang F, Peng J, Gabrielson K, **Simons BW**, Kalloo AN, Khashab MA, A Novel Viscous Dissecting Gel is Safe, Simple and Rapid for Endoscopic Submucosal Dissection: A Prospective Survival Study in a Porcine Model. Endoscopy

Yang M, Yu T, Wang YY, Tang BC, Zeng Q, **Simons BW**, Fu J, Juang CY, Lai S, Wu TC, Hung CF, Hanes J, Intraperitoneal Delivery of Paclitaxel by Poly(ether-anhydride) Microspheres Effectively Suppresses Tumor Growth in a Murine Metastatic Ovarian Cancer Model. Drug Deliver and Translational Research

Abstracts

2014

Simons BW, Miller R, Jabbari J, Ross AE, Hurley PJ, Schaeffer EM, A Murine Model of Prostate Cancer Bone Metastasis In a Syngeneic Immunocompetent Host

- Johns Hopkins Brady Institute Prostate Cancer Research Day

Simons BW, Alme A, Drake CG, Nelkin BD, Cyclin-Dependent Kinase 5 (CDK5) Controls Prostate Cancer Metastasis in Vivo and Alters Tumor Immune Response

- Johns Hopkins Brady Institute Prostate Cancer Research Day

Hurley PJ, **Simons BW**, Hughes RM, Miller R, Kimuara Y, Haffner MC, Esopi D, Yegnasubramanian S, Erho N, Vergara IA, Davicioni E, Faraj S, Netto G,

Schaeffer EM, Sparl1 is an Androgen Regulated Tumor Supressor in the Prostate.

- Johns Hopkins Brady Institute Prostate Cancer Research Day

Hurley PJ, Sundi D, **Simons BW**, Hughes RM, Yan G, Gielzak M, Kasch-Semenza L, Berman DM, Isaacs SD, Isaacs WB, Marchionni L, Ross AE, Schaeffer EM, Germline Variants in the ARPORIN Aspartic Acid Repeat Domain and Adverse Prostate Cancer Outcomes.

- Johns Hopkins Brady Institute Prostate Cancer Research Day

2013

Simons BW, Galindo-Cardiel I, Ward, J, Brayton CF, Berman DM. An optimized method of murine prostate trimming for histologic analysis

- Johns Hopkins Brady Institute Prostate Cancer Research Day

Casey KM, Miller R, Jabbari J, Brayton CF, Hurley PJ, Schaeffer EM, **Simons BW**, Effect of Background Strain on Prostate Cancer Progression in Hi-Myc Mice

- Johns Hopkins Brady Institute Prostate Cancer Research Day

2012

Simons BW, Hurley PJ, Huang Z, Ross AE, Miller R, Marchionni L, Berman DM, Schaeffer EM, Wnt signaling though beta-catenin is required for prostate lineage specification

- Johns Hopkins Brady Institute Prostate Cancer Research Day
- Johns Hopkins Dept of Molecular and Comparative Pathobiology Year in Review

Simons BW, Durham N, Bruno T, Grosso J, Schaeffer AJ, Ross AE, Hurley PJ, Berman DM, Drake CG, Thumbikat P, and Schaeffer EM. Chronic Prostatitis Promotes Prostate Cancer Progression in a Mouse Model

- Johns Hopkins Brady Institute Prostate Cancer Research Day
- Johns Hopkins Dept of Molecular and Comparative Pathobiology Year in Review

Casey KM, Miller R, Jabbari J, Brayton CF, Hurley PJ, Schaeffer EM, **Simons BW**, Effect of Background Strain on Prostate Cancer Progression in Hi-Myc Mice

- Johns Hopkins Brady Institute Prostate Cancer Research Day
- Johns Hopkins Dept of Molecular and Comparative Pathobiology Year in Review

2011

Casey KM, Miller R, Jabbari J, Brayton CF, Hurley PJ, Schaeffer EM, **Simons BW**, Effect of Background Strain on Prostate Cancer Progression in Hi-Myc Mice

- Society for Basic Urologic Research (SBUR) Annual Meeting
- American College of Veterinary Pathologists (ACVP) Annual Meeting

Simons BW, Hurley PJ, Huang Z, Ross AE, Miller R, Marchionni L, Berman DM, Schaeffer EM, Wnt signaling though beta-catenin is required for prostate lineage specification

- Society for Basic Urologic Research (SBUR) Annual Meeting

- Multi-Institutional Prostate Cancer Research Program (SPORE) Meeting

Simons BW, Durham N, Bruno T, Grosso J, Schaeffer AJ, Ross AE, Hurley PJ, Berman DM, Drake CG, Thumbikat P, and Schaeffer EM. Chronic Prostatitis Promotes Prostate Cancer Progression in a Mouse Model
- Multi-Institutional Prostate Cancer Research Program (SPORE) Meeting
- Johns Hopkins Mouse Phenotyping Conference

Simons BW, Galindo-Cardiel I, Brayton CF, An Optimized Method of Murine Prostate Trimming for Histologic Analysis
- Johns Hopkins Mouse Phenotyping Conference

2010

Simons BW, Galindo-Cardiel I, Brayton CF, An Optimized Method of Murine Prostate Trimming for Histologic Analysis
- American Association for the Advancement of Laboratory Animal Science (AALAS) annual meeting
- American College of Veterinary Pathology (ACVP) Annual Meeting

Simons BW, Thumbikat P, Schaeffer EM, A Mouse Model of Chronic Prostatitis Using Human Prostatic Bacterial Isolates
- Society for Basic Urologic Research (SBUR) Annual Meeting
- American Association for the Advancement of Laboratory Animal Science (AALAS) annual meeting
- American College of Veterinary Pathology (ACVP) Annual Meeting
- Johns Hopkins Brady Institute Prostate Cancer Research Day

Simons BW, Schaeffer EM, Marchionni L, Berman DM, Wnt Signaling is Required for Prostate Induction in the Urogenital Sinus
- Department of Defence Prostate Cancer Research Program Meeting (DOD PCRP-IMPACT)
- American College of Veterinary Pathology (ACVP) Annual Meeting
- Society for Developmental Biology Annual Meeting
- Johns Hopkins Brady Institute Prostate Cancer Research Day

Mobley JL, **Simons BW**, Wack A, Bronson E, Montali RJ, Pathologic Features of an Outbreak of Avian Mycobacteriosis in a Captive Population of Waterfowl
- American College of Veterinary Pathology (ACVP) Annual Meeting

2009

Simons BW, Schaeffer EM, Marchionni L, Berman DM, Wnt Signaling is Required for Prostate Induction in the Urogenital Sinus
- Johns Hopkins Dept of Pathology Young Investigator's Day

2008

Simons BW, Brayton CF, Berman DM, New Methods for Assessing Murine Prostate Development

- American College of Veterinary Pathologists (ACVP) Annual Meeting
- John Hopkins Sidney Kimmel Cancer Center Fellows Research Day
- Johns Hopkins Brady Institute Prostate Cancer Research Day
- Johns Hopkins Mouse Phenotyping Conference

2006

Simons BW, Scorpio D, Huso D, Nasal Aspergillosis in a Long-Evans Rat

- American College of Veterinary Pathologists (ACVP) Annual Meeting
- Johns Hopkins Dept of Molecular and Comparative Pathobiology Year in Review

Brayton CF, Li T, Gluckman T, **Simons BW**, Trembley S, Forbes N, Notch Pathway Factors in Cancer and Alzheimer's Disease

- American College of Veterinary Pathologists (ACVP) Annual Meeting

2005

Simons BW, Clayton LA, Stamper MA, Whitaker BR, Hadfield CA, Mankowski JL, Mycobacterium abscessus pneumonia in an Atlantic bottlenose dolphin

- American College of Veterinary Pathologists (ACVP) Annual Meeting

Invited Lectures

Faculty, Workshop on Phenotyping Mouse Models of Lung Disease, The Jackson Laboratory "Histopathology of mouse models of lung disease" September 20-24, 2009.

Guest Lecturer, Basic Research, Department of Defense Center for Prostate Disease Research "A functional requirement for the oncogene beta-catenin in prostate morphogenesis" March 25, 2009.

Manuscript Peer Review

Infection, Genetics, and Evolution (2014)
Life Sciences (2013)
British Journal of Urology International (2012)
Journal of Pathology (2012)
Carcinogenesis (2012)
Current Molecular Medicine (2010)

Teaching Activities (course title; location, dates, role)

Mouse Pathobiology and Phenotyping Short Course, Johns Hopkins School of Medicine, 2010-2013, Laboratory instructor

Toxicologic Pathology; Johns Hopkins School of Public Health, 2006-2013
Guest Lecturer, "Toxicologic pathology of the male reproductive tract"

Comparative Pathobiology and Genetically Engineered Mice, Spring 2010 and 2008, Guest Lecturer, "Comparative Anatomy and Pathology of the Reproductive System"
Pathology For Graduate Students; Johns Hopkins School of Medicine, September 2008, 2009, and 2010, Histology of neoplasia lab instructor
Pathobiology and Disease Mechanisms; Johns Hopkins School of Medicine, Spring 2008, Teaching assistant
Pathology For Graduate Students; Johns Hopkins School of Medicine, Spring 2006 Necropsy Lab, designed and implemented a lab (8 hours contact time) to teach rodent anatomy and tissue trimming for histology
Principles of Animal Pathology; Johns Hopkins School of Medicine, Fall 2005
Guest Lecturer, "Comparative anatomy and pathology of the nervous system"

Mentoring

2012 NIDDK Short-Term Education Program for Underrepresented Persons (STEP-UP) – Supervised the research of an undergraduate student for a two month project in the lab of Dr. Edward Schaeffer (Michael Lopresti, Investigating the differences in chronic bacterial prostatitis caused by CP1 and NU14 strains)
2011 NIDDK Short-Term Education Program for Underrepresented Persons (STEP-UP) – Designed and supervised the research project of an undergraduate student for a two month project in the lab of Dr. David Berman (Anna Miglioretti, Improvement of transduction efficiency in mouse primary prostate cells)
2011 Designed and supervised the research projects of a veterinary pathology extern student for a three month project in comparative mouse pathology (Kerriann Casey, Effect of background strain on prostate cancer progression in Hi-Myc mice)
2011 Designed and supervised the research project of a medical student for a three month project in the lab of Dr. Edward Schaeffer (Spencer Lake, The Role of Wnt Signaling in Human Prostate Cancer Tumor Initiating Cells)
2010 Designed and supervised the research projects of a veterinary pathology extern student for a three month project in comparative mouse pathology (Ivan Galindo-Cardiel, An optimized method of prostate trimming for histologic analysis)
2010 Designed and supervised the research project of a veterinary pathology extern for three months (Jessica Mobley, Pathologic features of an outbreak of avian mycobacteriosis in a captive population of waterfowl)
2010 Designed and supervised the research project of a medical student for a two month project in the lab of Dr. Edward Schaeffer

Credentials and Professional Societies

American College of Veterinary Pathologists, board eligible, anatomic pathology
Maryland veterinary medical and surgical license #5757
Member, C.L. Davis Foundation for the Advancement of Veterinary Pathology

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