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Investigating changes in paracrine signaling and basal cell plasticity during prostatitis and prostate cancer progression

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Table of contents

Contents

Table of contents iii
List of figures iv
List of tables v
Abstract vi
Acknowledgments viii
Chapter 1 1
Chapter 2: How does loss of Wnt/β-catenin signaling affect basal to luminal differentiation during *E. coli*-induced bacterial prostatic inflammation in mice? 12
Introduction 12
Methods 15
Results 18
Discussion 27
Chapter 3: How does stromal AR regulate paracrine signaling from the murine prostate stroma to the epithelium? 30
Introduction 30
Methods 33
Results 37
Discussion 45
Bibliography 49

List of figures

Figure 1: Histological stains from WT mice injected with different concentrations of uropathogenic *E.coli*. 19

Figure 2. Intraurethral UPEC-injection in mice induces prostate inflammation in all lobes. 20

Figure 3: Lineage tracing of prostatic basal cells in *E.coli*-inoculated mice. 22

Figure 4: Loss of β-catenin in $Bas^{Pten-/-bcat-/-}$ tumors. 23

Figure 5: Analysis of β-catenin expression in YFP^{+} luminal cell clusters from $Bas^{bcat-/-}$ mice. 24

Figure 6: Confirmed β-catenin deletion driven by CK5-$CreER^{T2}$. 25

Figure 7: YFP labeling and AR deletion in SMCs. 38

Figure 8: FACS of YFP^{+} stromal cells. 39

Figure 10: Differential expression of significant ($p<0.0001$) secretory proteins in str^{AR-} *T+E* (3m) and str^{AR-} *Hi-Myc* (4.5m) mice. 41

Figure 11: Differential expression of secreted factors in AR-null stromal cells. 42

List of tables

Table 1: Primer sequences used for genotyping of mouse tail genomic DNA. 15

Table 2: Antibodies used for immunofluorescence staining on mouse prostate cryosections. 17

Table 3: Concentration of *E.coli* (cells/ml) used to test the bacterial prostatitis model. 18

Table 4: Details of experimental mice used for basal cell lineage tracing under the bacterial prostatitis condition. 21

Table 5: Primer sequences used for genotyping of mouse tail genomic DNA. 34

Table 6: Sample RNA concentration and RNA integrity number (RIN) from *str*$^{AR-}$ T+E (1.5m & 3m), T+E (1.5m & 3m), *str*$^{AR-}$*Hi-Myc* (4.5m), and *Hi-Myc* (4.5m) mice as measured by the UC Davis Genome center. 40

Investigating changes in paracrine signaling and basal cell plasticity during prostatitis and prostate cancer progression

by

Joosje Bleeker

Abstract

Prostate inflammation is associated with prostate cancer (PCa) development. Basal-to-luminal cell differentiation, a process partly regulated by canonical Wnt signaling, plays a crucial role in PCa initiation. Here, using a basal cell lineage tracing mouse model, I investigated how loss of Wnt/β-catenin signaling in basal cells affects basal-to-luminal differentiation during *E. coli*-induced prostatitis. Results revealed that β-catenin-null basal cells still gave rise to luminal cells, albeit with reduced capacity compared to wild-type cells. Further exploration using single-cell RNA sequencing (scRNA-seq) could unveil distinct subpopulations arising from these basal cells during prostatitis. Future studies should also aim to minimize inflammation variation in bacterial prostatitis models.

Additionally, I examined how knockout of androgen receptor (AR) in stromal cells affected stromal-to-epithelial signaling. Bulk RNA sequencing of WT and AR-KO stromal cells and differential gene expression analysis identified

potential gene candidates for future investigations into the impact of stromal AR deletion on PCa progression. This study provides insights into the intricate cellular and molecular processes underlying PCa initiation and development, shedding light on potential therapeutic targets.

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Chapter 1

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Applications of Vertebrate Models in Studying Prostatitis and Inflammation-Associated Prostatic Diseases

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It has long been postulated that the inflammatory environment favors cell proliferation, and is conducive to diseases such as cancer. In the prostate gland, clinical data implicate important roles of prostatitis in the progression of both benign prostatic hyperplasia (BPH) and prostate cancer (PCa). However, their causal relationships have not been firmly established yet due to unresolved molecular and cellular mechanisms. By accurately mimicking human disease, vertebrate animals provide essential *in vivo* models to address this question. Here, we review the vertebrate prostatitis models that have been developed and discuss how they may reveal possible mechanisms by which prostate inflammation promotes BPH and PCa. Recent studies, particularly those involving genetically engineered mouse models (GEMMs), suggest that such mechanisms are multifaceted, which include epithelium barrier disruption, DNA damage and cell proliferation induced by paracrine signals, and expansion of potential cells of origin for cancer. Future research using rodent prostatitis models should aim to distinguish the etiologies of BPH and PCa, and facilitate the development of novel clinical approaches for prostatic disease prevention.

Keywords: mouse model, prostatitis, prostate cancer, BPH, chronic inflammation

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INTRODUCTION

Prostatitis is the inflammation of the prostate gland, and is characterized by immune cell (lymphocytes, neutrophils, macrophages, basophils, eosinophils) infiltration in the stromal compartment or localized regions surrounding the prostatic epithelial ducts. Often causing pelvic pain and sexual dysfunction, it is the most common urinary tract problem for men under the age of fifty (Collins et al., 1998). In the United States, prostatitis is estimated to account for two million visits to the clinics each year. Prostatitis is also gaining increasing attention because pathological and epidemiological evidence suggest that it is a significant etiologic factor in prostate cancer (PCa) (De Marzo et al., 2007; Sfanos et al., 2018). However, the mechanisms of prostatitis pathogenesis and its contribution to PCa development remain poorly understood. Vertebrate systems, particularly rodent models, provide invaluable tools to address these questions in the *in vivo* setting. By mimicking human prostatitis conditions and symptoms, those models allow for experimentation on various prostatic disease mechanisms and possible treatment options. In this review, we discuss commonly used vertebrate models of prostatitis in the field with a focus on their potential roles in elucidating the etiologic relationship of prostatitis, benign prostatic hyperplasia (BPH), and PCa.

TABLE 1 | Animal models of prostatitis and inflammation-associated BPH and PCa.

Model	References	Species, Strain	Histology
Bacterial prostatitis by intraprostatic injection	Olsson et al. (2012)	Rats, Sprague Dawley	Focal inflammation in dorsal-lateral prostate, diffuse and low inflammation in ventral prostate
	Xiong et al. (2017)	Rats, Sprague Dawley	Moderate to severe inflammation
Bacterial prostatitis by intraurethral inoculation	Boehm et al. (2012)	Mice, C57BL/6J	Significant acute inflammation, highest in anterior and dorsal-lateral prostate lobes
	(Elkahwaji et al., 2005, 2007, 2009)	Mice, BALB/c, C3H/HeJ, C3H/HeOuJ, C57BL/6J	Acute and chronic inflammation, hyperplasia, and PIN lesions
	Lilljebjörn et al. (2020)	Mice, C57BL/6	Mild acute and chronic inflammation
	Rippere-Lampe et al. (2001)	Rats, strain not specified	Moderate to high inflammation
	Shinohara et al. (2013)	Mice, C57BL/6J	Mild to chronic inflammation only in dorsal prostate
	Khalili et al. (2010)	Mice, C3H/HeOuJ	Acute inflammation and epithelial hyperplasia, ventral lobe most affected, lateral lobe least affected
	Kwon et al. (2013)	Mice, *K14-CreER; mTmG* and *K14-CreER; Pten$^{fl/fl}$; mTmG*	Inflammation induced basal to luminal cell differentiation, accelerated tumor initiation
	Le Magnen et al. (2018)	Mice, *Nkx3.1$^{-/-}$* and wildtype C57BL/6	Acute and chronic inflammation as well as hyperplasia, progression to PIN-lesions in *Nkx3.1$^{-/-}$* mice
Spontaneous CPPS	Jackson et al. (2013)	NOD	Inflammation
	Penna et al. (2007a)	Mice, NOD	Chronic inflammation, more severe in aged mice
Hormone-induced CPPS and BPH	Konkol et al. (2019)	Rats, Wistar and Noble	Chronic inflammation, PIN-lesions and adenocarcinoma, Noble rats more susceptible than Wistar
	(J. Li J et al., 2018a)	Rats, Sprague-Dawley, and dogs, Beagle	Epithelial hyperplasia
	(Z. Li et al., 2018b)	Rats, Wistar	Epithelial hyperplasia
	Nicholson et al. (2012)	Mice, C57BL/6 and BALB/c	Increased prostate weight
	Yokota et al. (2004)	Dogs, Beagle	Epithelial hyperplasia
	Zou et al. (2017)	Mice, ICR	Epithelial hyperplasia
	(M. Zhang et al., 2020)	Rats, Sprague-Dawley	Hyperplasia, mild inflammation
	(Y. Li et al., 2019b)	Rats, Sprague-Dawley	Epithelial hyperplasia, mild inflammation
Hormone + castration-induced CPPS	Zang et al. (2021)	Rats, Sprague-Dawley	Chronic inflammation, testosterone increased and estradiol repressed prostate growth
	Jia et al. (2015)	Rats, Sprague-Dawley	Less inflammation but more hyperplasia with increasing testosterone doses
	Kamijo et al. (2001)	Rats, Wistar	Severe inflammation, stromal proliferation and fibrosis
	Tsunemori et al. (2011)	Rats, Wistar	Significant inflammation in ventral prostate lobe
High-fat diet-induced CPPS and PCa	Kwon et al. (2016)	Mice, C57BL/6	Inflammation and PIN-formation
	Shankar et al. (2012)	Mice, C57BL/6	Chronic inflammation
	(H. Xu et al., 2015)	Mice, TRAMP	High-fat diet increased mortality and tumor formation rate in the TRAMP model
EAP by LPS or autoantigen injection	dos Santos Gomes et al. (2017)	Mice, Swiss and C57Bl/6	Inflammation, hyperplasia
	(D. Xu et al., 2019)	Rats, Sprague-Dawley	Inflammation, hyperplasia
	Kim et al. (2013)	Rats, Sprague-Dawley	Inflammation, hyperplasia
	Jackson et al. (2013)	Mice, Balb/c, B10.D2, NOD, SWR, MRL and NZB	Chronic inflammation in Balb/c, minor in SWR, acute inflammation resolved in NZB
	Penna et al. (2007b)	Mice, NOD	Chronic inflammation, slightly more severe immune cell infiltration when injected with MAG instead of just PSBP
	Popovics et al. (2017)	Mice, BALB/c	Chronic inflammation and hyperplasia
	(X. J. Wang et al., 2016)	Rats, Sprague-Dawley	Chronic inflammation and hyperplasia
	(M. Zhang et al., 2020)	Rats, Sprague-Dawley	Severe inflammation, moderate hyperplasia
POET model for CPPS	Burcham et al. (2014)	Mice, POET-3	Chronic inflammation and rare hyperplastic lesions
	Haverkamp et al. (2011)	Mice, POET-3	Severe acute inflammation
	Lees et al. (2006)	Mice, POET-1 and POET-3	Mild to moderate acute inflammation
	(H. H. Wang et al., 2015)	Mice, POET-3	No histological images
CPPS in other GEMMs	Ashok et al. (2019)	*Hoxb13-rtTA; TetO-IL1B* Mice, FVB/N	IL1b overexpression, acute and chronic inflammation, epithelial proliferation, fibrosis
	Liu et al. (2017)	*Pb-IL6* transgenic mice, C57BL/6	IL6 overexpression, infiltrating inflammatory cells, PIN-lesions and adenocarcinoma
	Pascal et al. (2021a)	*PSA-CreER; Cdh1$^{fl/fl}$* mice, C57BL/6J	Deletion of E-cadherin, inflammation, hyperplasia and fibrosis in all lobes
	(B. Zhang et al., 2016)	*K8-CreER; AR$^{fl/Y}$* mice	Luminal AR deletion, up-regulation of inflammatory cytokines and down-regulation of tight-junction proteins
	Hou et al. (2009)	*Aire-KO* Mice, B6 and NOD Lt/J backgrounds	Moderate to severe inflammation

Rodent Models for Different Types of Prostatitis

Clinically, prostatitis can be divided into four types: acute bacterial inflammation, chronic bacterial inflammation, abacterial prostatitis or chronic pelvic pain syndrome (CPPS), and asymptomatic prostatic chronic inflammation (Vykhovanets et al., 2007; Gill and Shoskes, 2016; Liu et al., 2020). Prostatitis pathology differs among the types of inflammation and may be distinguished by immune cell types and their localization in different regions of the prostate (Sfanos et al., 2018). For example, acute inflammation usually features neutrophil infiltration, whereas chronic inflammation is mostly characterized by lymphocytes and macrophages (Sfanos et al., 2018; Ashok et al., 2019). Type IV or asymptomatic inflammation, due to its lack of symptoms in patients, can only be diagnosed based on increased leukocytes in biopsy samples taken after a prostate-specific antigen (PSA) test in prostate cancer screens (Porcaro et al., 2015). As a result, animal models of asymptomatic prostatitis are rare and difficult to define. In contrast, various methods, including bacterial infection, hormone treatment, immunization, stress, and diet manipulation, have been used to study acute and chronic bacterial prostatitis as well as CPPS in rodent models (Vykhovanets et al., 2007) (summarized in **Table 1**).

Bacterial infection is frequently used to study acute and chronic bacterial inflammation and is induced either by direct injection of uropathogenic bacteria into the prostate lobes of rodents (Olsson et al., 2012; Xiong et al., 2017) or by inoculation via an intraurethral catheter (Rippere-Lampe et al., 2001; Elkahwaji et al., 2005; Elkahwaji et al., 2007; Elkahwaji et al., 2009; Khalili et al., 2010; Boehm et al., 2012; Shinohara et al., 2013; Le Magnen et al., 2018; Lilljebjörn et al., 2020). Different rodent species and strains have been used, including Wistar and Sprague-Dawley rats and C57BL/6 and C3H/HeJ mice. While some of the infected rodents recover spontaneously, many will develop chronic inflammation following initial acute inflammation response (Vykhovanets et al., 2007). Commonly used bacterial strains for infection include various uropathogenic *Escherichia coli* strains (Rippere-Lampe et al., 2001; Elkahwaji et al., 2005; Elkahwaji et al., 2007; Elkahwaji et al., 2009; Boehm et al., 2012; Lilljebjörn et al., 2020), as well as other species such as *Propionibacterium acnes* (Olsson et al., 2012; Shinohara et al., 2013). Although *P. acnes* infection might take longer to induce inflammation compared to *E. coli,* both can induce acute and chronic inflammation, with lesions featured by higher cell proliferation and diminished Nkx3.1 and androgen receptor (AR) expression (Shinohara et al., 2013). Clinically, chronic bacterial prostatitis is often developed from acute bacterial prostate inflammation. Therefore, these infection models are highly relevant as they mimic disease etiology.

In contrast to bacterial inflammation, the direct cause of abacterial prostatitis/CPPS remains unclear (Liu et al., 2020; Tsunemori and Sugimoto, 2021). Possible disease mechanisms include physical and chemical damage by urine reflux, sexually transmitted pathogens, diet, hormone imbalances, and autoimmunity (De Nunzio et al., 2011). Consequently, a wide range of animal models has been developed to explore the many potential causes of CPPS. Notably, certain rodents such as Wister, Lewis and Copenhagen rats, develop abacterial chronic prostatitis spontaneously as they age (Lundgren et al., 1984; Sharma et al., 1992; Keith et al., 2001). In men, aging is associated with increased prevalence of CPPS and a decline of the serum testosterone to estradiol ratio (T-to-E2 ratio) (Harman et al., 2001; Bernoulli et al., 2008). One proposed mechanism is that a decreased T-to-E2 ratio disrupts the balance between the immunosuppressive effect of testosterone and the pro-inflammatory effect mediated by estrogen (Cutolo et al., 2002). To mimic this, hormone-induced animal models of CPPS are often based on decreasing the T-to-E2 ratio, either by administration of estradiol or a combination of estradiol and testosterone (Kamijo et al., 2001; Tsunemori et al., 2011; Jia et al., 2015; Konkol et al., 2019; Zang et al., 2021). In other approaches, a high fat diet (HFD) has been shown to induce chronic inflammation in rodents (Shankar et al., 2012; Shankar et al., 2015; Xu et al., 2015; Kwon et al., 2016). HFD-induced oxidative stress and NF-κB and Stat3 signaling activation may play important roles in this process (Shankar et al., 2012; Shankar et al., 2015), but the mechanisms by which HFD promotes chronic inflammation remain to be fully elucidated.

One of the great advantages of using mouse models is the capability of genetically manipulating gene expression *in vivo*. Several genetically engineered mouse models (GEMMs) have been reported to be able to induce chronic inflammation. These include prostate-specific knockout of the gene encoding AR or E-cadherin (Zhang et al., 2016; Pascal et al., 2021b), which increases prostate epithelial barrier permeability. Genetically modified mice are particularly useful for modeling immune-related chronic prostate inflammation, whose phenotypes are commonly referred to as experimental autoimmune prostatitis (EAP). For example, an inherent lack of immunity can cause chronic prostatitis in aging NOD mice, a strain prone to developing organ-specific autoimmune disease (Kikutani and Makino, 1992). In these models, the autoimmune origin is evident by a T-cell response to prostate autoantigens and characterized by $CD4^+$ T-cell intraprostatic infiltration (Penna et al., 2007a; Jackson et al., 2013). Other genetic models include overexpression of the pro-inflammatory cytokines IL-1β or IL-6 in transgenic mice (Liu et al., 2017; Ashok et al., 2019), and the *Aire*-deficient mouse model, in which knockout of the important immune regulator *Aire* led to development of chronic prostatitis (Hou et al., 2009).

Notably, EAP can also be triggered by injection of lipopolysaccharide (LPS), a component of the Gram-negative bacterial cell wall, which stimulates the release of pro-inflammatory cytokines to induce chronic inflammation (Kim et al., 2013; dos Santos Gomes et al., 2017; Xu et al., 2019). Other EAP models induce inflammation by injecting a combination of autoantigens with an adjuvant. These autoantigens are prostate specific, such as male accessory gland extract (Jackson et al., 2013) and prostate tissue homogenate (Wang et al., 2016; Popovics et al., 2017). However, as homogenized tissue contains multiple antigens, this makes some of these immunological models unfit to study T-cell/antigen specific interactions. Furthermore, many

models use endogenous T-cell pools that have had previous antigen exposure, further limiting specificity of the T-cell response (Lees et al., 2006). Consequently, the prostate ovalbumin-expressing transgenic (POET) mouse model was developed as an antigen-specific autoimmune model of both acute and chronic prostate inflammation (Lees et al., 2006; Haverkamp et al., 2011; Burcham et al., 2014; Wang et al., 2015). Using the ARR_2PB promoter, POET mice express high levels of membrane-bound ovalbumin in the different lobes of the prostate (Lees et al., 2006; Haverkamp et al., 2011). Using adoptive transfer of transgenic T-cells that recognize ovalbumin, the POET model circumvents general tolerance mechanisms and provides the opportunity to monitor a specific T-cell population during both chronic and acute prostate inflammation.

Vertebrate Models That Involve Inflammation and Benign Prostatic Hyperplasia

Benign prostatic hyperplasia (BPH) is a condition in which hyperplasia of the stromal and glandular prostatic cells causes prostate enlargement (Nickel, 2008). Clinically, BPH is characterized by lower urinary tract symptoms (LUTS) such as voiding, storage and post-micturition symptoms, and can be associated with bladder outlet obstruction (BOO) (Roehrborn, 2005; Chughtai et al., 2011). Some BOO animal models involve mechanical obstruction of the urethra by sutures or ligatures, thus directly affecting urine outflow (Austin et al., 2004; Kanno et al., 2016). However, the relevance of these models to BPH-induced BOO is unclear due to the invasiveness of the procedure and the fact that BPH is a disease that develops over a long period of time. Rather, animal models that recapitulate age-related spontaneous BPH development are desired. In contrast to spontaneous prostatitis models, only macaques, chimpanzees, and dogs are known to naturally develop BPH, with dogs being the most commonly used animal model for BPH (Sun et al., 2017; Zhang et al., 2021). Interestingly, since age-related change in hormone ratios is thought to contribute to BPH development, rodent models of BPH have been developed by castration and administration of testosterone and/or estrogen (Yokota et al., 2004; Nicholson et al., 2012; Zou et al., 2017; Li J. et al., 2018; Li Z. et al., 2018; Li Y. et al., 2019; Zhang et al., 2020), similar to the hormone-induced CPPS rodent models (Kamijo et al., 2001; Tsunemori et al., 2011; Jia et al., 2015; Konkol et al., 2019). Indeed, many aforementioned chronic prostatitis models also show BPH phenotypes. For example, high-fat diet as well as LPS and *E. coli* injection can induce both inflammation and BPH phenotypes in rodents (Elkahwaji et al., 2007; Escobar et al., 2009; Shankar et al., 2012; Kim et al., 2013; Kwon et al., 2016; dos Santos Gomes et al., 2017; Li Y. et al., 2019; Xu et al., 2019), and the EAP model used to induce chronic prostatitis can induce BPH in rats (Wang et al., 2016; Zhang et al., 2020). This overlap between animal models for prostatitis and BPH is reflected in clinical findings: biopsies taken from patients with BPH often show immune cell infiltration and markers of inflammation (Taoka et al., 2004; Penna et al., 2007b; Nickel, 2008; Robert et al., 2009; Taoka and Kakehi, 2017). Similarly, several studies found bacterial and viral strains in BPH specimens, suggesting that bacterial inflammation may play a role in BPH development (Nickel et al., 1999; Chughtai et al., 2011).

Mechanistically, it has been postulated that chronic inflammation can create a microenvironment that induces wound healing repair processes, leading to the activation of proliferative pathways and hence prostate hyperplasia (Taoka et al., 2004; Fibbi et al., 2010). For example, inflammation-induced leakage of the epithelial barrier could lead to an influx of luminal-secreted autoantigens into the stromal compartment and subsequently produce an autoimmune response (Chughtai et al., 2011; Li F. et al., 2019; Pascal et al., 2021b). Indeed, PSA has been detected in stroma surrounding BPH nodules from patients (O'Malley et al., 2014), and a large scale analysis of BPH patient tissues revealed that high serum PSA values were associated with inflammation (Gandaglia et al., 2013). One possible mechanism of epithelial barrier leakage may be through down-regulation of E-cadherin, an important regulator of the epithelial barrier and tissue homeostasis. E-cadherin expression is often found to be lower in BPH tissues (Kim et al., 2013; Li F. et al., 2019; Xu et al., 2019; Pascal et al., 2021a), and conditional knockout of E-cadherin in the mouse prostate causes loss of epithelial barrier function, inflammation and hyperplasia (Pascal et al., 2021b). Additionally, conditional overexpression of the pro-inflammatory cytokine interleukin-6 (IL-6) down-regulates E-cadherin (Liu et al., 2017), suggesting that a positive feedback loop between inflammation and epithelial barrier disruption may be present to promote BPH. Similarly, a recent study showed that attenuation of luminal epithelial AR signaling can induce prostate inflammation and impair epithelial cell tight junctions, while inflammation can suppress AR expression (Zhang et al., 2016). Such a positive feedback loop may also be involved in sustaining chronic inflammation during BPH progression. Despite these progresses, whether inflammation directly causes BPH or is an associated factor during BPH progression remains unclear. Further research is needed to clarify the relationship between chronic prostatitis and BPH.

Rodent Models for Studying the Relationship Between Prostatitis and Prostate Cancer

Prostate cancer (PCa) is the second leading cause of cancer-related morbidity and mortality in American men. The etiologic link between prostatitis and PCa has long been suggested (De Marzo et al., 2007; Sfanos et al., 2018). For example, in human prostatectomy specimens, lesions characterized by proliferating epithelial cells and activated inflammatory cells (named proliferative inflammatory atrophy, PIA) are often adjacent to areas of prostatic intraepithelial neoplasia (PIN) (De Marzo et al., 1999). Recently, inflammation in benign tissues identified in the Prostate *Cancer* Prevention Trial was positively associated with later development of PCa (Platz et al., 2017), strongly suggesting

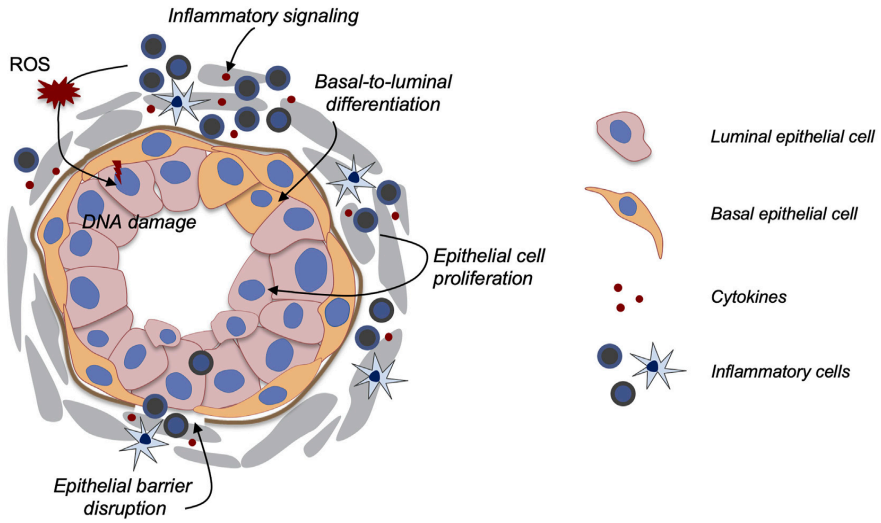

FIGURE 1 | Model of how prostatitis promotes PCa. Multiple possible mechanisms have been proposed as illustrated in the diagram. Secreted cytokines may promote epithelial cell proliferation, basal-to-luminal cell differentiation, and epithelial barrier disruption. Inflammation-induced ROS production and *Nkx3.1* down-regulation could also enhance DNA damage.

that chronic prostatitis is a precursor of PIN and PCa. To date, however, the mechanisms linking prostatitis and PCa development remain unclear. Uncovering these mechanisms should aid PCa prevention and early intervention. Below, we discuss three major possible avenues of how prostatitis may facilitate PCa progression (**Figure 1**) with a focus on applications of mouse models: 1) enhanced secretion of cytokines and growth factors to promote epithelial cell proliferation, 2) inflammation-induced epithelial cell DNA mutations, and 3) increasing basal-to-luminal differentiation to enlarge the pool of cells of origin for PCa.

Enhanced Secretion of Cytokines and Growth Factors to Activate Epithelial Cell Proliferation

The mechanisms by which inflammation contributes to PCa development are multifaceted. One of the more direct ways may be through activating epithelial cell proliferation via paracrine signals from the inflammatory stroma. The normal prostate mostly contains quiescent cells, while cell proliferation is necessary for tissue wound healing. Interestingly, the reactive stroma observed in BPH and PCa undergoes changes resembling a wound healing response (Tuxhorn et al., 2001; Schauer and Rowley, 2011). Infiltration of inflammatory cells, increased growth factor availability, angiogenesis, and extracellular matrix remodeling are among the major features of such a pro-tumor microenvironment. The infiltrating inflammatory cells can produce a wide range of cytokines such as tumor necrosis factor (TNF) and interleukins (ILs), which can induce further secretion of growth factors to promote epithelial cell proliferation (Giri and Ittmann, 2001; Steiner et al., 2002; Sokol and Luster, 2015). For example, an *in vitro* study showed that in prostate epithelial cells, cytokines secreted by macrophages could activate ERK and Akt, two protein kinases that promote cell proliferation and survival (Dang and Liou, 2018). Furthermore, GEMMs offer great models to study the effects of inflammatory signaling on the prostate *in vivo*. In particular, overexpression of human IL-6 in the mouse prostate showed development of chronic inflammation and progressive neoplasia, with PIN lesions and prostate adenocarcinoma observed later (Liu et al., 2017). Moreover, in the genetic mouse prostatitis model where interleukin 1β (IL-1β) is overexpressed, increased expression of downstream cytokines were observed, along with formation of PIA-like lesions and high expression of the proliferation marker Ki67 (Ashok et al., 2019). As discussed previously regarding inflammation and BPH, these genetic mouse models suggest the involvement of a positive feedback loop between inflammation and epithelial barrier disruption to promote cell proliferation, as evidenced by the down-regulation of E-cadherin in the IL-6 overexpression model (Liu et al., 2017). However, it is important to note that cancer development requires more than just cell proliferation. Additional inflammation-induced mechanisms must be in play to explain the phenotypic differences between BPH and PCa.

Inflammation-Induced Oxidative Stress and Loss of Nkx3.1 can Induce DNA Damage

Studies across many organ types have suggested that inflammation can increase genomic instability (Colotta et al.,

2009; Grivennikov et al., 2010). One proposed mechanism is the release of reactive oxygen species (ROS) by infiltrating inflammatory cells. ROS, such as superoxide, nitric oxide and hydrogen peroxide, are highly reactive oxygen-containing molecules that are produced during natural metabolic processes (Ihsan et al., 2018). Excess ROS production can result in an imbalance between ROS and antioxidants, leading to insufficient ROS degradation. This state of oxidative stress can cause oxidative damage in DNA, RNA, proteins, and lipids (Olinski et al., 2002; Lugrin et al., 2014; Ihsan et al., 2018). Notably, although much focus is placed on DNA damage, proteins and lipids are also important targets for oxidative attack, as modification of these molecules can increase the risk of mutagenesis (Reuter et al., 2010; Murata, 2018). Continuous exposure to inflammation and concurrent immune cell infiltration can lead to increased levels of ROS (Xia and Zweier, 1997; Eiserich et al., 1998), which can lead to genetic mutations and instability (Weitzman and Stossel, 1981; Weitzman and Gordon, 1990). It is hypothesized that in PIA lesions, where inflammatory injury stimulates epithelial cell proliferation, ROS released by infiltrating inflammatory cells can increase formation of PIN-lesions and carcinoma (Wiseman and Halliwell, 1996; Xia and Zweier, 1997; De Marzo et al., 1999). Using animal models, a mechanistic link between oxidative stress and inflammation has been established in mice susceptible to colon inflammation, in which knockout of *Gpx1* and *Gpx2*, two genes that encode antioxidant enzymes, results in a high incidence of tumors in the intestinal epithelium (Chu et al., 2004). However, similar models in the PCa context are currently lacking. To functionally test the role of ROS in promoting inflammation-induced PCa, it will be very informative to genetically perturb the ROS production pathway in mice or combine ROS production perturbation with other oncogenic pathways to assess the effect on PCa development.

Genetic mouse model studies also suggested that another possible mechanism of inflammation-induced epithelial DNA damage could be related to *Nkx3.1* down-regulation. Nkx3.1, besides serving as a transcription factor in prostate development, is also a tumor suppressor (Bhatia-Gaur et al., 1999; Kim et al., 2002). Its tumor suppressing functions can at least be partially attributed to its role in preventing DNA damage (Bowen and Gelmann, 2010; Bowen et al., 2013; Debelec-Butuner et al., 2015). PIN formation in *Nkx3.1*$^{-/-}$ mice was reported to be associated with deregulation of prooxidant and antioxidant enzymes, as well as oxidative damage in DNA (Ouyang et al., 2005). Notably, acute bacterial prostatitis in mice leads to down-regulation of Nkx3.1 (Khalili et al., 2010; Shinohara et al., 2013), and lower Nkx3.1 expression was also observed in the genetic prostatitis model of IL-1 overexpression (Ashok et al., 2019). Moreover, inducing prostate inflammation in *Nkx3.1*$^{-/-}$ mice accelerates PCa initiation (Le Magnen et al., 2018). These findings suggest that there may be a positive feedback or inflammatory storm mechanism at play. In such a model, inflammatory cytokines such as TNF-α and IL-1β could stimulate Nkx3.1 down-regulation (Markowski et al., 2008; Debelec-Butuner et al., 2014), which in turn would increase susceptibility to oxidative stress and further DNA damage (Ouyang et al., 2005). Such a combined environment of inflammatory signaling, oxidative stress, and high epithelial proliferation, could give rise to PIN and PCa (De Marzo et al., 1999).

Inflammation-Induced Basal-To-Luminal Differentiation Expands Cells of Origin for PCa

Cell of origin for PCa has been implicated as a link between prostatitis and PCa. A cell of origin is defined as a normal tissue cell that can give rise to a tumor after its oncogenic transformation (Blanpain, 2013; Lee and Shen, 2015). Tissue stem cells, due to their self-renewal and multipotent capabilities, can serve as potent cells of origin for cancer. In an earlier colon cancer study, inflammation induces tissue stem cell expansion, potentially enlarging the cellular pool for oncogenic transformation (Umar et al., 2009). In the prostate, lineage-tracing studies in mice have shown that epithelial basal cells are the stem cells that can generate luminal cells during prostate organogenesis (Ousset et al., 2012). However, basal stem cell activities become restricted in the mature prostate as basal and luminal cells are mostly two self-sustained lineages at adulthood and basal-to-luminal cell differentiation is rare (Choi et al., 2012; Wang et al., 2013). Importantly, basal-to-luminal differentiation appears to be an important step towards PCa initiation. In mouse lineage-tracing models, loss of the tumor suppressor gene *Pten* in basal cells promoted basal-to-luminal differentiation, and the resulting tumor had a luminal phenotype (Choi et al., 2012; Wang et al., 2013), resembling the predominant luminal feature in human PCa (Shen and Abate-Shen, 2010). In fact, loss of the basal cell layer is often considered a hallmark of PCa (Humphrey, 2007; Grisanzio and Signoretti, 2008). We previously showed that luminal cells are the favored cell type of origin for PCa (Wang et al., 2014). Therefore, by enhancing basal cell plasticity and basal-to-luminal differentiation, the cellular pool for oncogenic transformation is enlarged, potentially facilitating PCa development.

In light of this, it is particularly interesting to note that basal-to-luminal differentiation was reported to be enhanced in two prostatitis mouse models. When mice were either inoculated with uropathogenic *E. coli* (UPEC) or fed with HFD, basal cells rapidly proliferated and produced luminal cells (Kwon et al., 2013; Kwon et al., 2016). Both treatments also accelerated PCa initiation in the basal-specific *Pten*-knockout model (*K14-Pten*) (Kwon et al., 2013; Kwon et al., 2016). PCa developed relatively slowly in basal-specific *Pten*-knockout models since it takes time for *Pten* deletion to drive basal cells towards transformed luminal cells (Choi et al., 2012; Wang et al., 2013). *K14-Pten* mice treated with UPEC or HFD showed accelerated disease progression, indicating that faster basal-to-luminal differentiation due to inflammation-induced signals facilitated PCa development. In the future, identifying those signals that promotes basal-to-luminal differentiation should be beneficial for delaying PCa progression in patients with chronic prostatitis.

CONCLUSION AND DISCUSSION

Numerous rodent models have been developed to study the different types of clinically defined prostatitis. While bacterial prostatitis models have recapitulated many aspects of the acute and chronic bacterial inflammation observed in humans, it remains challenging to pinpoint the most relevant model for CPPS, since the molecular pathways responsible for abacterial chronic prostatitis are not yet fully understood. The etiology of chronic prostate inflammation can vary among individuals, and different CPPS models, including hormone, high fat, autoimmune, and GEMMs may capture different important aspects of CPPS development. These animal models have been playing crucial roles in our efforts to elucidate the relationship between prostatitis and other prostatic diseases such as BPH and PCa. The association of prostatitis to these diseases is well documented in clinical studies. In recent years, applications of GEMM prostatitis models have revealed possible mechanisms by which inflammation causes BPH and PCa. Among those mechanisms, disruption of the epithelial barrier and the ensuing auto feedback loop of enhanced inflammation appear to be a common theme. Nonetheless, inflammation may contribute to PCa development in many other ways, such as oxidative stress-induced DNA damage, down-regulation of the tumor suppressor Nkx3.1, and expansion of luminal epithelial cells as cells of origin. Future research utilizing rodent models will continue to shed light on the mechanistic, causal links between chronic prostate inflammation and progressive prostatic diseases, and should distinguish the etiology between BPH and PCa. Such insights will be invaluable for prostatic disease prevention and early intervention.

AUTHOR CONTRIBUTIONS

JB and ZAW wrote the manuscript.

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Chapter 2: How does loss of Wnt/β-catenin signaling affect basal to luminal differentiation during *E. coli*-induced bacterial prostatic inflammation in mice?

Introduction

Prostate inflammation, or prostatitis, is the inflammation of the prostate gland. It is characterized by immune cell (lymphocytes, neutrophils, macrophages, basophils, eosinophils) infiltration, localized primarily to the stromal compartment that surrounds the prostatic epithelial ducts. Pathological and epidemiological evidence suggest that prostatitis is a significant etiologic factor in prostate cancer (PCa) (De Marzo et al., 2007; Sfanos et al., 2018). For example, human prostatectomy specimens often show lesions characterized by proliferating epithelial cells and activated inflammatory cells (named proliferative inflammatory atrophy, PIA) adjacent to areas of prostatic intraepithelial neoplasia (PIN), a precursor to PCa (De Marzo et al., 1999). Similarly, the Prostate *Cancer* Prevention Trial identified that inflammation in benign tissues was positively associated with later development of PCa (Platz et al., 2017). However, the mechanisms by which prostatitis can contribute to PCa development remain poorly understood.

Basal and luminal cells are the two major cell types lining the prostate epithelium. Basal cells behave as stem cells to generate luminal cells and rare neuroendocrine cells during organogenesis (Ousset et al., 2012) but become restricted in the mature prostate (Choi et al., 2012; Z. A. Wang et al., 2013). However, isolated adult basal cells can regain plasticity in both *in vitro* and *in vivo* cell regeneration assays (Goldstein et al., 2008, 2010) and can generate prostate organoids more efficiently compared to luminal cells (Karthaus et al., 2014). Basal cell plasticity, particularly basal-to-luminal differentiation, appears to be an important step toward PCa initiation. In genetic mouse models of prostate carcinogenesis, oncogenic transformation of basal cells has been shown to give rise to adenocarcinomas with a luminal phenotype (Choi et al., 2012; Stoyanova et al., 2013; Z. A. Wang et al., 2014), which resembles the predominantly luminal feature of human PCa (Shen & Abate-Shen, 2010). Consistent with this, luminal cells have been suggested as the favored cell type of origin (Z. A. Wang et al., 2014), giving rise to luminal tumors after oncogenic transformation (Blanpain, 2013; Lee & Shen, 2015). Therefore, enhanced basal-to-luminal differentiation could enlarge the luminal cell pool susceptible to oncogenic transformation, thus potentially facilitating PCa development.

Notably, prostatitis induced by intraurethral uropathogenic *E.coli* (UPEC) injection or a high-fat diet (HFD) has previously been shown to increase

basal-to-luminal differentiation in mice (Kwon et al., 2014, 2016), potentially as a result of inflammation-induced changes in the surrounding stroma (Goldstein & Witte, 2013; Kwon et al., 2014). UPEC injection also accelerated PCa initiation in a basal-specific *Pten*-knockout model (*K14-Pten*), indicating that increased basal-to-luminal differentiation due to inflammation-induced signaling may facilitate PCa development (Kwon et al., 2014).

In the prostate, paracrine signaling from the surrounding stroma plays a vital role in regulating adult epithelial cell plasticity (Berry et al., 2008; A. Y. Liu et al., 2011). Specifically, the canonical Wnt signaling pathway is a significant factor in the self-renewal and differentiation of stem cells (Clevers et al., 2014; Holland et al., 2013; Wei et al., 2019). Consistent with this, lineage tracing studies in mice demonstrate that loss of Wnt signaling in adult prostate basal cells decreased basal-to-luminal differentiation (Horton et al., 2023; Lu & Chen, 2015). These results, combined with Kwon's group's findings on increased basal cell plasticity during prostatitis (Kwon et al., 2014, 2016), raise the question: does loss of canonical Wnt signaling in basal cells reduce basal-to-luminal differentiation during prostate inflammation? To investigate this question, I employed a basal lineage tracing (*CK5-CreER*^{T2}) and β-catenin knockout model (*Ctnnb1*$^{fl/fl}$) in concert with a mouse model of UPEC-induced prostatitis.

Methods

Mouse strains and genotyping

The *CK5-CreER^{T2}* (Rock et al., 2009), *R26R-CAG-YFP* (Madisen et al., 2009), and *Ctnnb1^{flox}* line (Brault et al., 2001) lines were used previously (Y. Liu et al., 2020; Q. Xie et al., 2017). Animals were maintained in C57BL/6N or mixed background. Genotyping was performed by PCR using tail genomic DNA with the following primer sequences:

Primer	**Sequence**
CK5-CreER^{T2} forward	5'-CAGATGGCGCGGCAACACC-3'
CK5-CreER^{T2} reverse	5'-GCGCGGTCTGGCAGTAAAAAC-3'
Ctnnb1^{flox} forward	5'-ACTGCCTTTGTTCTCTTCCCTTCTG-3'
Ctnnb1^{flox} reverse	5'-CAGCCAAGGAGAGCAGGTGAGG-3'
R26R-CAG-YFP wt forward	5'-AAGGGAGCTGCAGTGGAGTA-3'
R26R-CAG-YFP wt reverse	5'-CCGAAAATCTGTGGGAAGTC-3'
R26R-CAG-YFP mutated forward	5'-ACATGGTCCTGCTGGAGTTC-3'
R26R-CAG-YFP mutated reverse	5'- GGCATTAAAGCAGCGTATCC-3'

Table 1: Primer sequences used for genotyping of mouse tail genomic DNA.

Mouse procedures

Mice were administered 9 mg per 40 g body weight tamoxifen (Sigma) suspended in corn oil by oral gavage once daily for four consecutive days. 2 weeks after the last tamoxifen induction, C57BL/6 mice were put under anesthesia (2.5% Isoflurane, MWI Veterinary Supply Company) and intraurethrally inoculated with 200 µl of sterile uropathogenic *E. coli* 1677 (4.6 x 10^7 cells/ml) using sterile polyethylene tubing (BD Intramedic™ PE Tubing, 0.011in). All animal experiments described in this study were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at UCSC.

Tissue collection

Mouse prostate tissues were dissected in 1xPBS and fixed in 4% paraformaldehyde for subsequent cryo-embedding in OCT compound (Sakura), or fixed in 10% formalin followed by paraffin embedding.

Histology and immunofluorescence staining

H&E staining was performed using standard protocols on 5 µm paraffin sections, and slides were imaged using a Zeiss Axio Imager in the UCSC Microscopy Shared Facility. Immunofluorescence staining was performed using 4 µm cryosections. Samples were incubated with 10% normal goat serum (NGS) blocking buffer at room temperature for 1 hour and primary antibodies diluted in 5% NGS overnight at 4°C. Samples were then incubated

with secondary antibodies (diluted 1:600 in 5% NGS) labeled with Alexa Fluor 488, 555, or 647 (Invitrogen/Molecular Probes) for 1 hour at room temperature. Slides were mounted with VectaShield mounting medium with DAPI (Vector Labs) and imaged on a Leica TCS SP5 spectral confocal microscope in the UCSC Microscopy Shared Facility. Cell counting was performed via Image J Software.

Antibody	**Supplier**
rabbit anti-CK5 (1:1000)	BioLegend, #PRB-160P
mouse anti-β-catenin (1:1000)	BD Biosciences, #610153
chicken anti-YFP (1:1000)	Abcam, #ab13970
mouse anti-CK18 (1:200)	Abcam, #ab668

Table 2: Antibodies used for immunofluorescence staining on mouse prostate cryosections.

Results

Establishing the bacterial prostatitis model

Intraurethral injection of bacteria, typically uropathogenic strains of *E. coli*, is a widely used mouse model to investigate acute and chronic bacterial inflammation of the prostate (Boehm et al., 2012; Elkahwaji et al., 2005, 2007; Lilljebjörn et al., 2020; Rippere-Lampe et al., 2001). However, due to the various usage of species, strains, and concentrations in previous studies, we opted to first establish an accurate inflammatory response by performing intraurethral injection of 6 different concentrations of uropathogenic *E.coli* (UPEC) 1677 into 12 WT mice (Table 3).

Mouse number	Dilutions of *E. coli* (OD_{600} = 0.92)	Cells/ml
1899	1x	$4.6 * 10^8$
1930		
1898	2x	$2.3 * 10^8$
1929		
1897	5x	$9.2 * 10^7$
1928		
1896	10x	$4.6 * 10^7$
1927		
1895	20x	$2.3 * 10^7$
1926		
1894	50x	$9.2 * 10^6$
1900		

Table 3: Concentration of E.coli (cells/ml) used to test the bacterial prostatitis model.

Histological images confirmed inflammatory phenotypes in all mice, indicated by infiltrating leukocytes in the stroma and areas of disorganized tissue and hyperplasia (Figure 1).

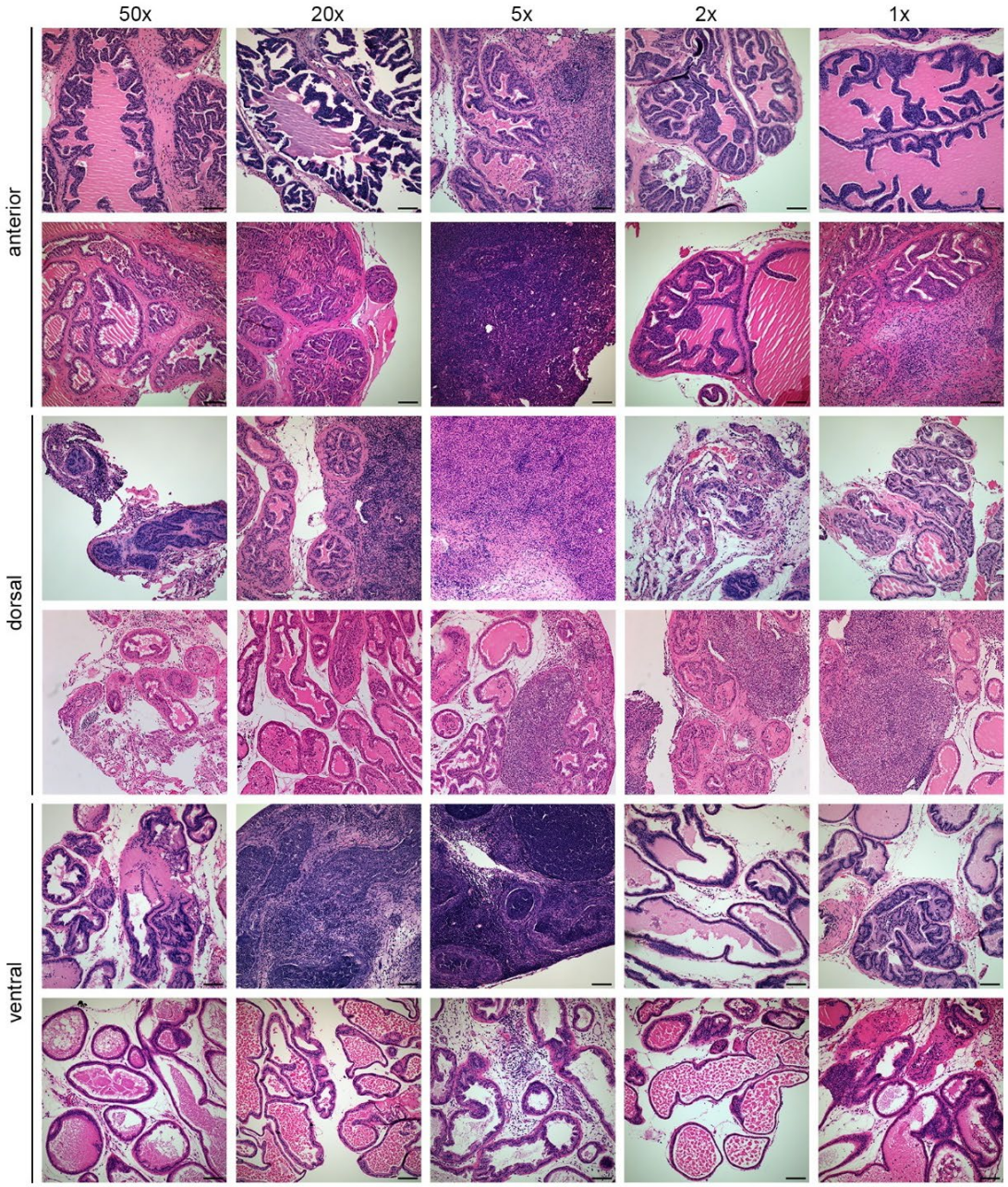

Figure 1: Histological stains from WT mice injected with different concentrations of uropathogenic E.coli. Scale bars: 200µm.

Variation in severity of inflammation between duplicate mice was observed (Figure 1), possibly due to inherent mouse-to-mouse differences (Chisolm et al., 2019), or as a result of injection efficiency. Additionally, the ventral and dorsal lobes appear to be more greatly affected by UPEC injection than the anterior lobes, most likely because of their proximity to the site of infection (Figure 2A). Based on the observed histological phenotypes, the 10x dilution was chosen as the injection concentration for subsequent basal cell lineage tracing experiments.

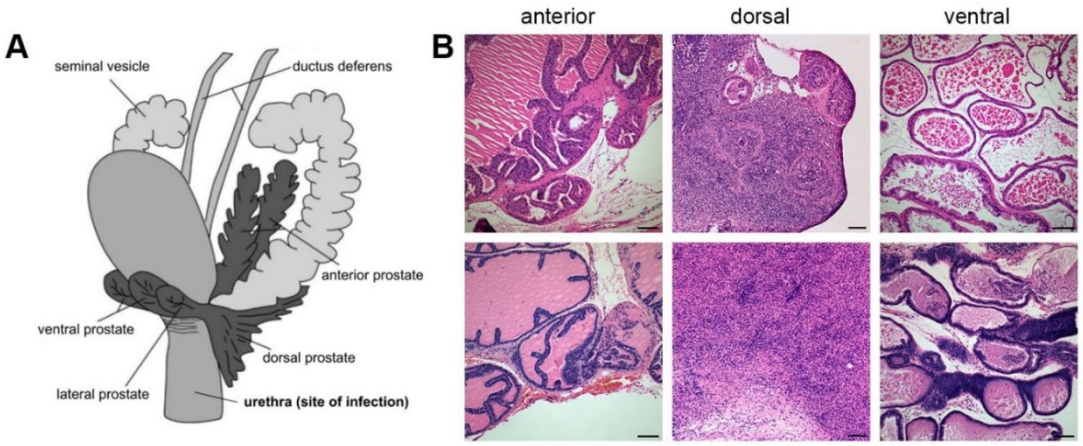

Figure 2. Intraurethral UPEC-injection in mice induces prostate inflammation in all lobes. (A) Anatomy of the mouse prostate and site of UPEC injection. Adjusted from Bhatia-Gaur et al., 1999. (B) Prostate H&E stains from UPEC-injected (10x) WT mice. Scale bar = 200μm

Basal cell lineage tracing

To assess the effect of disrupted Wnt signaling on basal-to-luminal differentiation, I utilized a CK5-CreER^{T2}; R26R-CAG-YFP/+ reporter for basal cell lineage tracing, which has previously been shown to mark almost all prostate basal cells (Q. Xie et al., 2017). I tamoxifen-induced CK5-CreER^{T2};

Ctnnb1$^{fl/fl}$; R26R-CAG-YFP/+ adult male mice (denoted *Bas$^{bcat-/-}$*) and CK5-CreER^{T2}; R26R-CAG-YFP/+ control mice (denoted *Bas^{WT},* Table 4).

Experimental group	n	mouse #	age at dissection (days)
Bas$^{bcat-/-}$	1	9416	161
	1	9493	164
	1	9629	143
	2	1671, 1674	99
Bas^{WT}	1	9943	108
	1	1107	170
	1	9014	204
	1	227	140

Table 4: Details of experimental mice used for basal cell lineage tracing under the bacterial prostatitis condition.

After two weeks, the mice were inoculated with *E.coli* 1677 transurethrally before collecting the prostate four weeks later (Figure 3A).

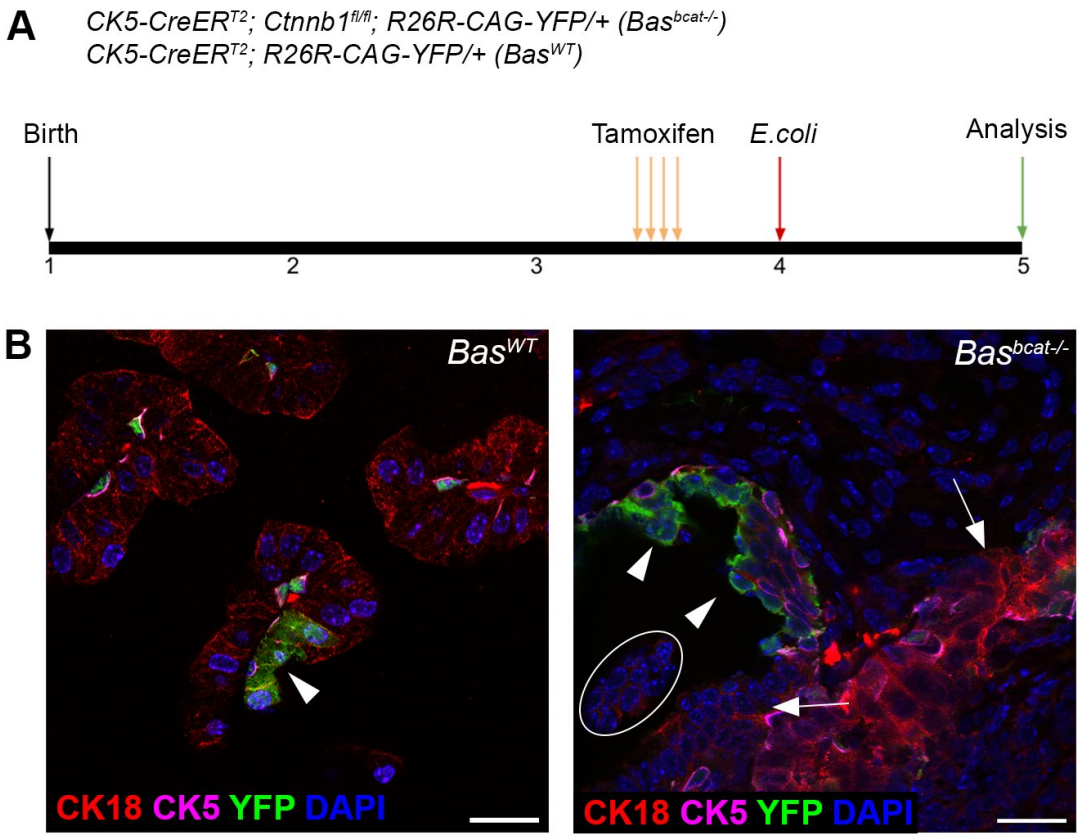

Figure 3: Lineage tracing of prostatic basal cells in E.coli-inoculated mice. (A) Timeline of lineage tracing experiments. (B) IF images of disorganized epithelium and hyperplasia (arrows), immune cell infiltration (circle), and YFP$^+$ luminal cells (arrowheads) in the Bas^{WT} and Bas$^{bcat-/-}$ ventral prostate. Scale bar = 20μm.

Typically, the differentiation rates between basal and luminal cells are determined by quantifying all YFP$^+$ cells in a tissue section. Nevertheless, this method has proven unreliable here due to significant variation in the inflammatory phenotypes among different mice and sections of the same tissue. Some mice showed, specifically in the ventral and dorsal lobes, evident inflammation-induced changes to the epithelium (Figure 3B). Similarly, parts of the epithelium exhibited areas containing bright clusters of YFP$^+$ luminal cells (Figure 3B), resulting in skewed quantification data.

However, instead of using quantification of YFP$^+$ luminal cells to assess how β-catenin knockout affects basal-to-luminal differentiation rates, perhaps β-catenin immunofluorescence staining of these luminal YFP$^+$ clusters in *Bas$^{bcat-/-}$* mice can give more conclusive evidence as to what extent β-catenin-negative basal cells are giving rise to β-catenin-negative luminal cells. Because β-catenin is a key structural protein in the luminal cell membrane, it can be challenging to visualize loss of β-catenin in individual luminal YFP$^+$ cells via IF staining. Therefore, due to less contact with WT luminal cells, clusters of luminal YFP$^+$ cells can provide much clearer IF staining results (Figure 4).

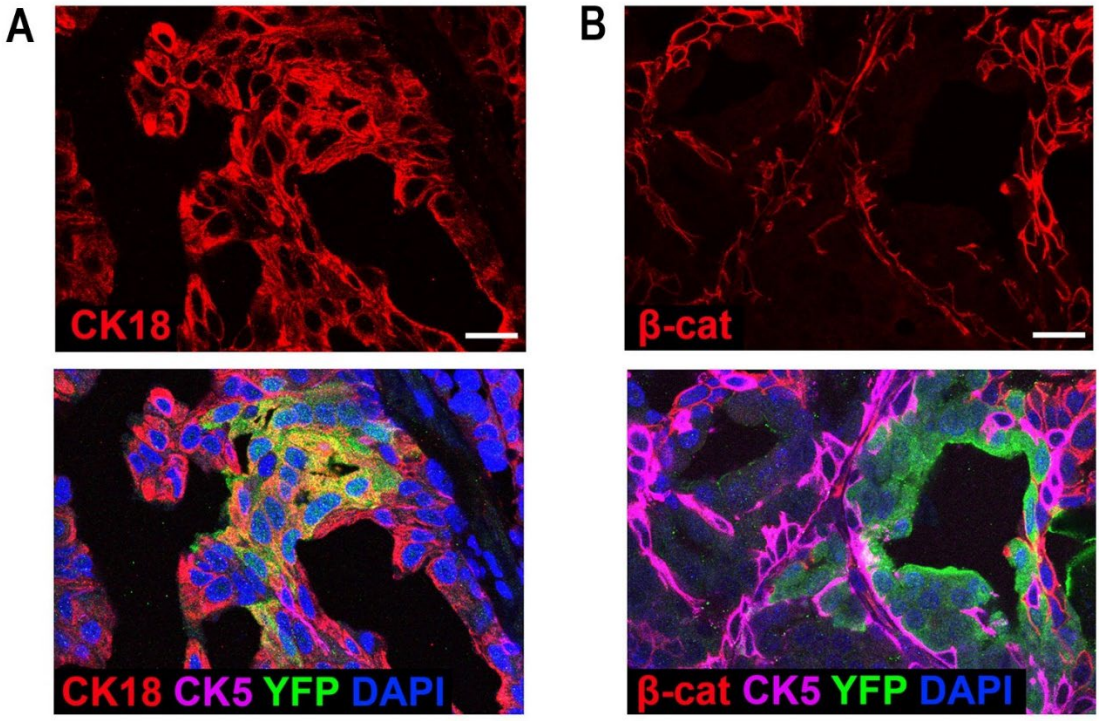

Figure 4: Loss of β-catenin in Bas$^{Pten-/-bcat-/-}$ tumors. (A) IF images showing increased basal-to-luminal differentiation and (B) loss of β-catenin in tumors from CK5-CreER^{T2}; ctnnb1$^{fl/fl}$; Pten$^{fl/fl}$; R26R-CAG-YFP/+ mice 3 months post tamoxifen induction. Scale bar = 20 μm. Adapted from Horton et al., 2023.

Immunofluorescence staining of *Bas*$^{bcat-/-}$ YFP^{+} luminal cell clusters demonstrated that only ~4% of YFP^{+} luminal cells stained negative for β-catenin, while ~96% were β-catenin-positive. (Figure 5).

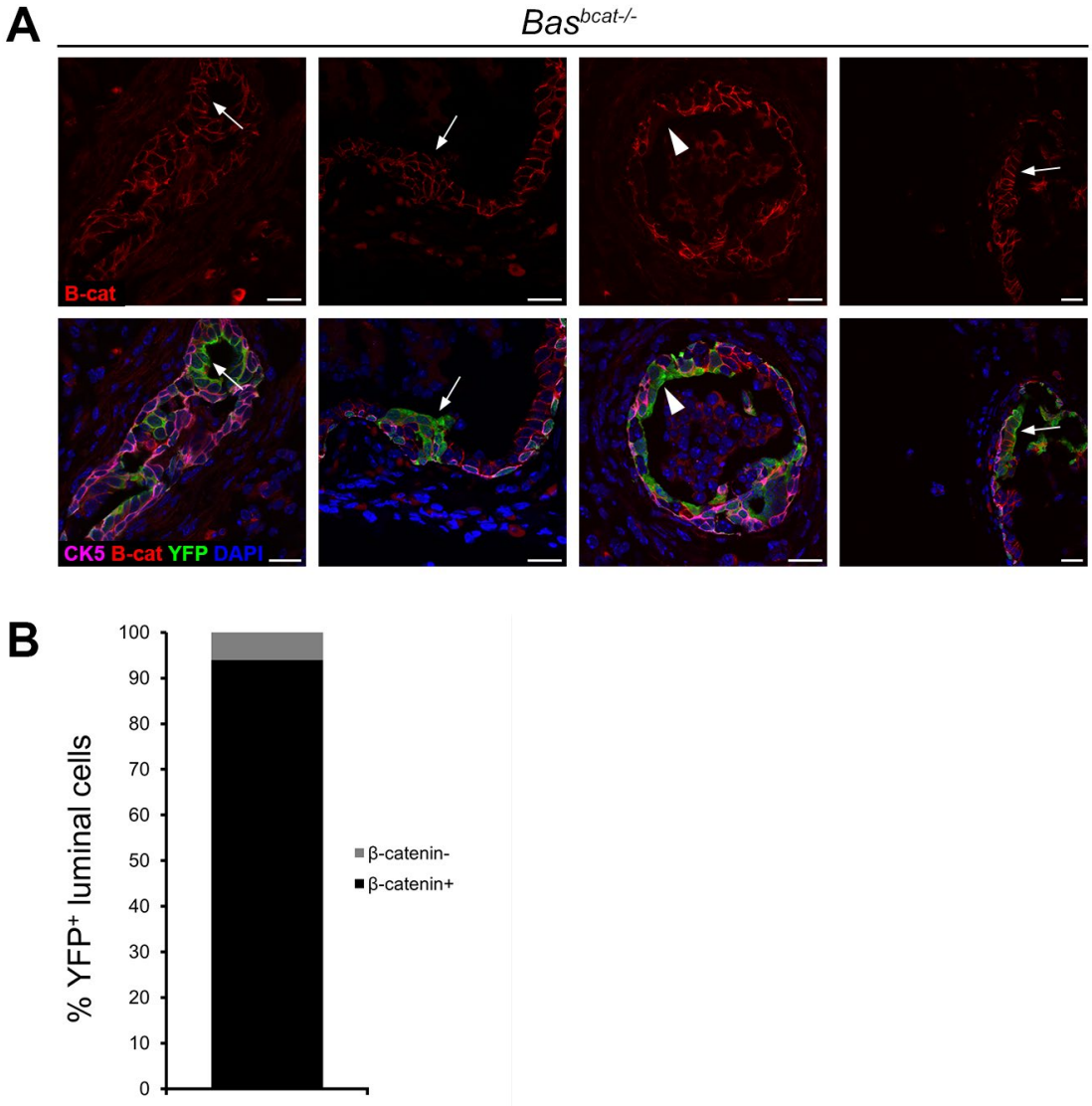

Figure 5: Analysis of β-catenin expression in YFP^{+} luminal cell clusters from Bas$^{bcat-/-}$ mice. (A) Immunofluorescence images from Bas$^{bcat-/-}$ mice (n=4). Most clusters stain positive for β-catenin (arrows), although a small subset of cells stain negative (arrowhead). Scale bar = 20μm. (B) Quantification of β-catenin expression in YFP^{+} luminal cells from Bas$^{bcat-/-}$ mice (n=4).

Although previous qRT-PCR analysis conducted on FACS-sorted basal cells

from CK5-CreER^{T2}; R26R-CAG-YFP/+ mice demonstrated a reasonably effective elimination of β-catenin (Figure 6), further confirmed by β-catenin-negative luminal tumors in *Bas$^{Pten-/-bcat-/-}$* mice (Figure 4), the data obtained here indicates that the majority of YFP^{+} luminal cells originated from basal cells that escaped β-catenin deletion.

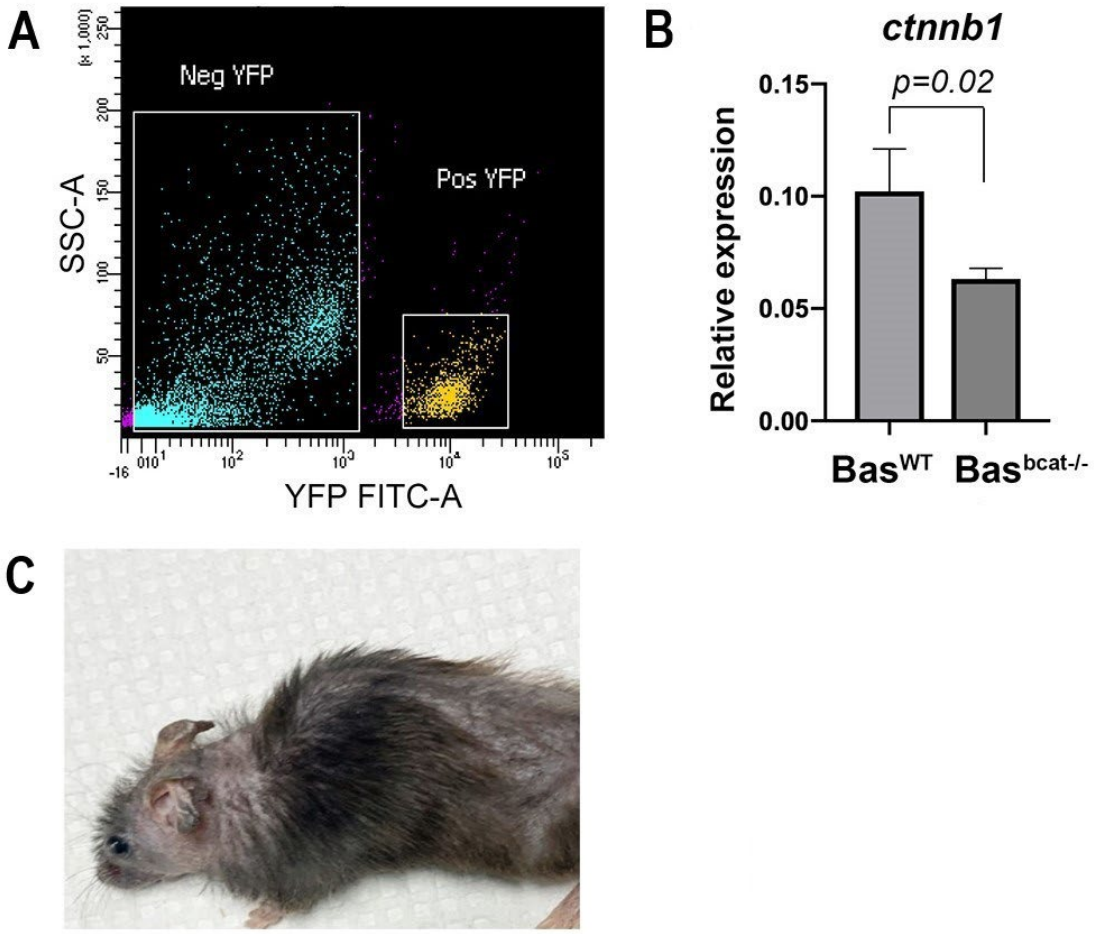

Figure 6: Confirmed β-catenin deletion driven by CK5-CreER^{T2}. YFP-labeled cells were flow sorted (A) and analyzed by qRT-PCR (B). CK5-CreER^{T2}; Ctnnb1$^{fl/fl}$; R26R-CAG-YFP/+ mice also showed hair loss over time. Adapted from Horton et al., 2023.

Nonetheless, I observed that some β-catenin-negative basal cells still possess the capability to undergo basal-to-luminal differentiation, albeit

potentially at a diminished rate. Consequently, I anticipate that the quantification of basal-to-luminal differentiation would exhibit a reduced rate in the *Bas*$^{bcat-/-}$ group compared to *Bas*^{WT} mice, which is consistent with our lab's previous finding that *Bas*$^{bcat-/-}$ mice had reduced basal-to-luminal differentiation rates compared to *Bas*^{WT} mice (Horton et al., 2023).

Discussion

Despite the epidemiological link between PCa and inflammation (De Marzo et al., 2007; Sfanos et al., 2018), the specific mechanism(s) by which prostatitis contributes to tumor development are not yet fully understood. However, considering that PCa often displays a luminal phenotype (Shen & Abate-Shen, 2010), which aligns with a preferred luminal cell of origin for PCa (Z. A. Wang et al., 2014), one possible mechanism could involve inflammation-induced alterations in the plasticity of basal cells. Using basal lineage tracing, Kwon's group has shown that prostatitis, induced either by UPEC-injection or a high-fat diet, increases basal-to-luminal differentiation in mice and accelerates PCa progression in the *Pten*$^{-/-}$ mouse model (Kwon et al., 2014, 2016). Here, using a similar basal lineage tracing method, I investigated the effect of disrupted Wnt signaling on basal-to-luminal differentiation in a mouse model of bacterial prostatitis.

Despite the successful induction of inflammation, the presence of significant variation among mice and different regions within each prostate lobe posed challenges in accurately quantifying basal-to-luminal differentiation. To address this issue, a potential strategy would be to visualize immune cell infiltration through immunofluorescence staining of CD45. This approach would enable a more comprehensive assessment and control of inflammation severity. Additionally, conducting combined staining of CK5, YFP, and CD45

and subsequently analyzing whether increased presence of YFP^{+} luminal cell clusters coincides with higher levels of immune cell infiltration could provide valuable insight into whether inflammation affects basal cell plasticity.

In order to further alleviate this observed high inflammation variability, direct injection into the prostate as previously performed in Sprague Dawley rats (Olsson et al., 2012; Xiong et al., 2017), could be considered as an alternative to intraurethral injection of bacteria. Additionally, the bacterial concentration was established based on its OD_{600} value, which does not distinguish between alive and dead bacteria. Instead, a colony forming assay could be used to determine the injection concentration, as colony forming units (cfu) more accurately describe the number of viable cells in a bacterial culture.

Analysis of tamoxifen-induced and UPEC-injected *Bas*^{WT} and *Bas*$^{bcat-/-}$ mice prostates revealed regions containing many YFP^{+} luminal cells. Staining of these clusters in *Bas*$^{bcat-/-}$ mice showed that only a small minority (~4%) of YFP^{+} luminal cells were actually derived from β-catenin-negative basal cells, suggesting that basal cells that evaded β-catenin deletion are responsible for most of the basal-to-luminal differentiation in the *Bas*$^{bcat-/-}$ mice, since we know that β-catenin deletion in the CK5-CreER^{T2}; Ctnnb1$^{fl/fl}$; R26R-CAG-YFP/+ mouse line is not 100% efficient (Figure 6). These findings indicate that while canonical Wnt activity in basal cells is not indispensable (as evidenced

by the presence of β-catenin-negative luminal YFP$^+$ clones), it plays a significant role in driving basal-to-luminal differentiation during prostatitis. However, to enhance the accuracy of future IF-staining analyses of β-catenin expression in YFP$^+$ luminal clones, it could be beneficial to include staining for the non-phosphorylated, active form of β-catenin. This approach would provide a more precise representation of β-catenin knockout by avoiding the staining of adjacent cell membranes that express β-catenin as a structural protein.

A proposed subsequent experimental approach entails conducting single-cell RNA sequencing (sc-RNAseq) on whole prostates obtained from tamoxifen-induced and UPEC-injected *Bas*^{WT} and *Bas*$^{bcat-/-}$ mice, as well as tamoxifen-induced R26R-CAG-YFP/+ control mice. The comparative analysis between the control and *Bas*^{WT} groups can provide insights into the impact of inflammation on the transcriptome and composition of the epithelial and stromal compartments. Simultaneously, comparing *Bas*^{WT} with *Bas*$^{bcat-/-}$ will yield a comprehensive understanding of how specifically the loss of β-catenin influences the composition and transcriptome of the epithelium and stroma. Additionally, this analysis will enable the identification of any distinct epithelial subpopulations arising from basal cells during prostatitis.

Chapter 3: How does stromal AR regulate paracrine signaling from the murine prostate stroma to the epithelium?

Introduction

The majority of prostate tumors are adenocarcinomas that originate from the gland's epithelial cells (Shen & Abate-Shen, 2010). However, the surrounding stromal compartment, comprising smooth muscle cells (SMCs), fibroblasts, endothelial cells, immune cells, and neurons, plays a crucial role in the prostate's development (Cunha et al., 1987; Hayward & Cunha, 2000) and cancer progression (Barron & Rowley, 2012; Josson et al., 2010; Levesque & Nelson, 2018; Tuxhorn et al., 2001) by providing the necessary microenvironment. Androgen receptor (AR) signaling in the prostate epithelium and stroma plays a key role in regulating prostate development and cancer (Berry et al., 2008; Dai et al., 2017; Watson et al., 2015).

Clinically, decreased expression of stromal AR has been linked to advanced PCa and poor patient outcomes (Henshall et al., 2001; Leach et al., 2015; Ricciardelli et al., 2005; Wikström et al., 2009). In line with this, deletion of AR in the epithelium compared to the whole prostate suggested stromal AR has a tumor-promoting role in the TRAMP PCa mouse model (Niu et al., 2008).

However, in epithelial co-culture assays and tumor cell renal grafts, the stroma of AR-deficient mice was paradoxically found to be less effective in promoting tumor formation than that of wild-type mice (Lai, Yamashita, Vitkus, et al., 2012; Ricke et al., 2012; Yu et al., 2012). Similarly, a study using *SMH-Cre* to knock out AR in smooth muscle cells found that in a hormonal carcinogenesis model, stromal AR plays a role in suppressing PCa (Welsh et al., 2011). Likewise, in the $Pten^{-/+}$ cancer model, deletion of stromal AR through *FSP1-Cre* and *Tgln-Cre* was found to reduce the development of PIN (Lai, Yamashita, Huang, et al., 2012).

These contradictory findings could potentially be attributed to the use of non-inducible Cre, which may not eliminate the impact of AR loss during development, as well as the use of different Cre-drivers. For this reason, Liu and colleagues of our lab employed inducible *Myh11*-driven Cre to delete AR in smooth muscle cells (SMCs). Their findings revealed that the loss of stromal AR accelerated cancer progression in a hormonal (T+E2) and genetic (*Hi-Myc*) mouse model of PCa (Y. Liu et al., 2022). Furthermore, their results indicated that the deletion of stromal AR primarily impacted secretory luminal tumor cells, as demonstrated by the development of luminal cell clusters with enriched inflammatory signaling markers compared to mice with WT stroma (Y. Liu et al., 2022). The rise of such populations in mice with AR-deficient stroma could perhaps be mediated by the alterations in paracrine signaling in

AR-null stromal cells, as a spectrum of growth factors and cytokines has been implicated as under the regulation of stromal ARs in the prostate (König et al., 1987; Li et al., 2008; Wen et al., 2015). However, how loss of stromal AR affects specific stromal-to-epithelial signaling pathways during different stages of prostate carcinogenesis remains unclear. In an effort to elucidate these pathways, I used bulk RNA sequencing to investigate changes in gene expression in AR-null and wild-type stromal cells isolated from mice at different time points during the development of PCa, induced either hormonally via testosterone and estradiol-17β (T+E2) or genetically via *Myc*-overexpression (*Hi-Myc*).

Methods

Mouse strains and genotyping

The *Myh11-CreER^{T2}* (Wirth et al., 2008), *R26R-CAG-YFP* (Madisen et al., 2009), *AR^{flox}* (De Gendt et al., 2004), and *Hi-Myc* (Ellwood-Yen et al., 2003) lines were used previously (Y. Liu et al., 2022). Animals were maintained in C57BL/6N or mixed background. Genotyping was performed by PCR using tail genomic DNA, with the following primer sequences:

Primer	**Sequence**
Myh11-CreER^{T2} forward	5'-TGACCCCATCTCTTCACTCC-3'
Myh11-CreER^{T2} reverse	5'-AGTCCCTCACATCCTCAGGTT-3'
Hi-Myc forward	5'-AAACATGATGACTACCAAGCTTGGC-3'
Hi-Myc reverse	5'-ATGATAGCATCTTGTTCTTAGTCTTTTT CTTAATAGGG-3'
AR^{flox} forward	5'-GTTGATACCTTAACCTCTGC-3'
AR^{flox} reverse	5'-CTTCAGCGGCTCTTTTGAAG-3'
R26R-CAG-YFP wt forward	5'-AAGGGAGCTGCAGTGGAGTA-3'
R26R-CAG-YFP wt reverse	5'-CCGAAAATCTGTGGGAAGTC-3'

R26R-CAG-YFP mutated forward	5’-ACATGGTCCTGCTGGAGTTC-3'
R26R-CAG-YFP mutated reverse	5’-GGCATTAAAGCAGCGTATCC-3'

Table 5: Primer sequences used for genotyping of mouse tail genomic DNA.

Mouse procedures

Mice were administered 9 mg per 40 g body weight tamoxifen (Sigma) suspended in corn oil by oral gavage once daily for four consecutive days. For T+E2 treatment, a 1.0 cm Silastic capsule (No. 602–305 Silastic tubing; 1.54 mm inside diameter, 3.18 mm outside diameter; Dow-Corning #2415569) filled with testosterone (Sigma) and a 0.4 cm Silastic capsule filled with estradiol-17β (Sigma) were implanted subcutaneously. Mice were treated with hormones for 1.5 or 3 months.

All animal experiments described in this study were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at UCSC.

Tissue collection, dissociation, flow cytometry, and RNA isolation

Mouse prostate tissues were dissected in 1xPBS and minced into small clumps, followed by enzymatic dissociation with 1% Collagenase/Hyaluronidase (StemCell Technologies) in DMEM/F12 media

with 5% FBS in a vertical Eppendorf tube rotator for 1 hour at 37°C. Tissues were digested with 0.25% Trypsin-EDTA (StemCell Technologies) in a rotator for 10 min at 37°C, followed by 10 min at room temperature. Homogenates were mixed with Hanks' Balanced Salt Solution (HBSS)/2% FBS. Pellet was further digested with 900 µl 5mg/ml Dispase (StemCell Technologies) and 100 µl 1 mg/ml DnaseI (StemCell Technologies), then suspended in 4 ml HBSS/2% FBS and filtered through a 40-mm cell strainer to obtain single-cell suspensions.

Dissociated prostate cells were suspended in HBSS/2% FBS/1% EDTA, and YFP^+ cells were sorted on a BD FACS Aria II instrument in the Flow Cytometry Shared Facility of UCSC. Total RNA from FACS-purified stromal cells was isolated using the RNeasy Micro Kit (Qiagen). Samples were measured via Nanodrop and frozen at -80°C until shipped on dry ice to the UC Davis Genome Center for library prep and bulk-RNA 3' Tag Sequencing on the Illumina HiSeq 4000.

Differential Gene Expression Analysis (DGEA)

TagSeq data were trimmed using TrimGalore, version 0.6.7 (Babraham Institute, 2019) and UMI deduplication was performed using UMI-tools, version 1.1.2 (Smith et al., 2017). Reads were aligned to GRCm39 using STAR, version 2.7.10a (Dobin et al., 2013).

Differential expression analyses were conducted using limma-voom (Ritchie et al., 2015), limma version 3.54.2, edgeR version 3.40.2. DE analyses were conducted in R version 4.2.2 (2022-10-31) (R Core Team, 2022). The model used in limma included experimental group and RNA extraction batch.

Results

To our goal of studying the effect of stromal AR deletion on stromal-to-epithelial signaling, the mouse lines *Myh11- CreER^{T2}; R26R-CAG-EYFP/+; Hi-Myc* (denoted as *Myc*) and *Myh11-CreER^{T2}; AR$^{flox/Y}$; R26R-CAG-EYFP/+; Hi-Myc* (denoted *str^{AR-} Myc*) were generated. The use of *Myh11-CreER^{T2}*, in which the inducible Cre is driven by the promoter of the gene smooth muscle myosin heavy polypeptide 11 (Ellwood-Yen et al., 2003), allows for very efficient and specific YFP labeling and AR knockout in stromal SMCs (Figure 7A, Y. Liu et al., 2022). Additionally, the *ARR2/probasin-Myc* (*Hi-Myc*) model (Ellwood-Yen et al., 2003), provides an excellent model of human PCa (Fraser et al., 2017). The *Hi-Myc* model utilizes the probasin promoter, which becomes active during the neonatal stage. However, it is crucial to consider models of PCa in which tumors only initiate during the adult stage. In line with this, a combined treatment of testosterone and estradiol-17b (named the T+E2 model) has been employed in rodents to mimic the relative increase in estrogen and decrease in androgen levels in aging men. I induced Myh11-CreER^{T2}; R26R-EYFP/+ (denoted T+E) and Myh11-Cre ER^{T2}; AR$^{fl/Y}$; R26R-EYFP/+ (denoted *str^{AR-}*T+E) mice at 7 weeks of age, implanted T and E2 tubes subcutaneously to initiate tumor growth 2 weeks post-tamoxifen and analyzed them at 1.5m and 3m post-hormone treatment (Figure 7B). *Myc* and *str^{AR-} Myc* mice were identically induced with tamoxifen at 7 weeks old, but then analyzed at 4.5 and 6 months of age (Figure 7A).

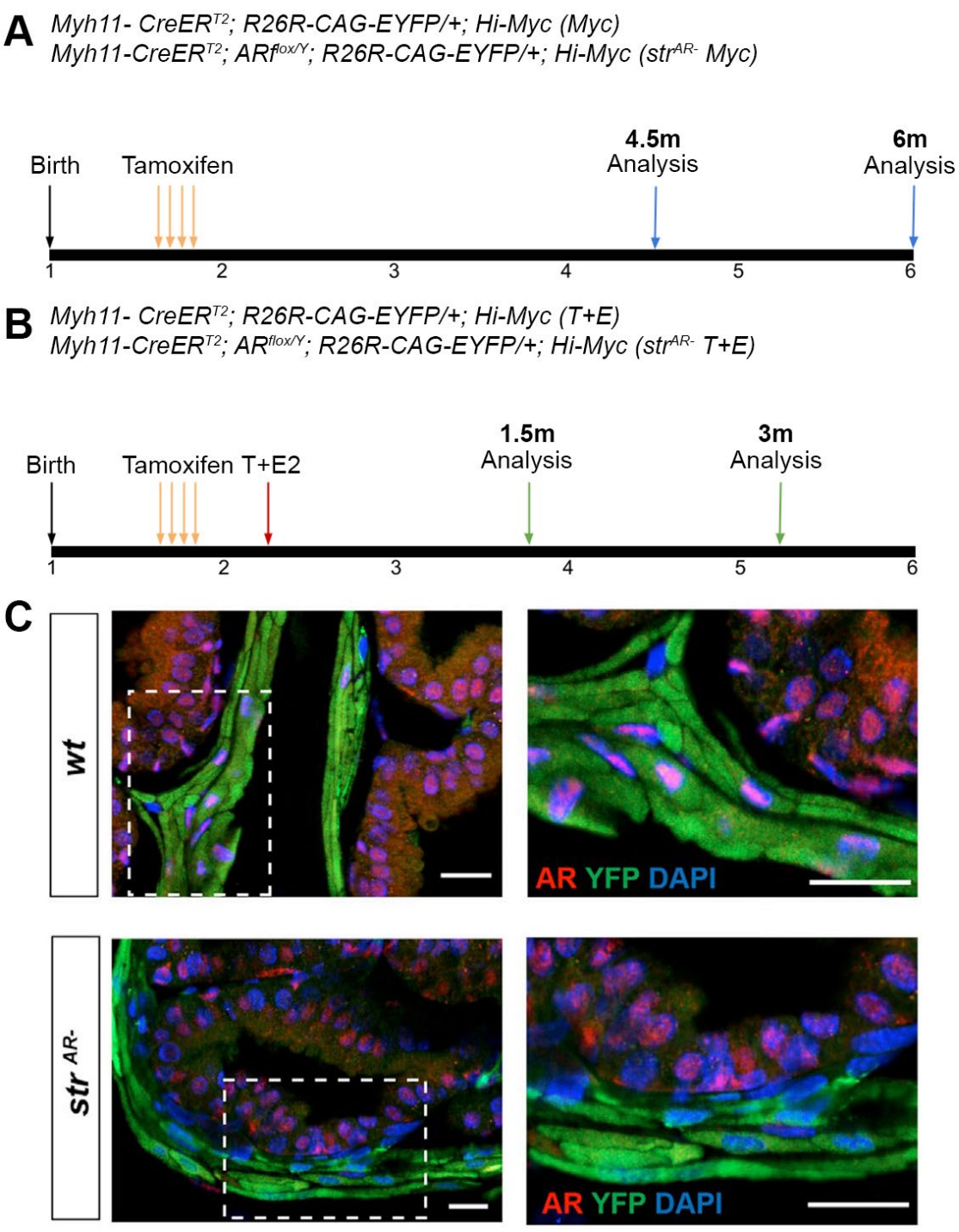

Figure 7: YFP labeling and AR deletion in SMCs. (A) Timeline of experiments for T+E and str^{AR-} T+E mice. (B) Timeline of experiments for Myc, and str^{AR-} Myc mice. (C) IF staining showing loss of AR expression in YFP^{+} cells from Myh11-CreER^{T2}; R26R-CAG-EYFP/+ (wt) and Myh11-CreER^{T2}; AR$^{fl/Y}$; R26R-CAG-EYFP/+ (str^{AR-}) mice. Images from Y. Liu et al., 2022.

The objective of the analysis is to examine and contrast gene expression in AR-null and WT stromal cells, with the goal to offer valuable understanding regarding the impact of stromal AR on stromal-to-epithelial signaling during tumor progression in different models of prostate cancer (T+E2 and *Hi-Myc*), as well as at various stages of prostate cancer development (multiple time points for analysis).

A

Experimental group	mouse #	#sorted YFP⁺ cells
T+E (1.5m)	9447	57607
	9448	56543
	9451	43669
str^{AR-} T+E (3m)	9195	51403
	9197	109911
	1181	30779
T+E (3m)	1183	15768
	1184	18004
	9140	70083
	9141	70072
str^{AR-} *Hi-Myc* (4.5m)	1164	67904
	1163	39154
	1177	56973

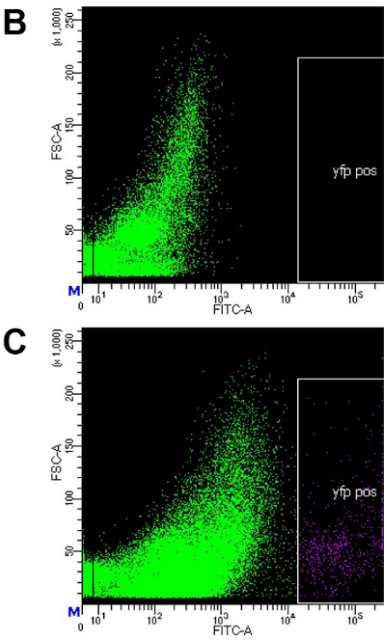

Figure 8: FACS of YFP⁺ stromal cells. (A) Number of sorted YFP⁺ cells per mouse. (B) FACS-plot of WT control mouse. (C) FACS-plot of mouse #9448.

To initiate analysis, prostates were collected, digested and dissociated in single-cell suspensions. Stromal cells were flow-sorted based on YFP expression (Figure 8), after which RNA was extracted and shipped to the UC Davis Genome Center for bulk-RNA 3' Tag Sequencing.

Unfortunately, not all 6-month *Hi-Myc* samples could be collected due to unexpected complications with the FACS machine. Additionally, *str*$^{AR-}$ T+E (1.5m) and *Hi-Myc* (4.5m) samples were excluded from sequencing due to low RNA concentrations (Table 6).

Experimental group	mouse #	[RNA] in ng/µl	RIN
str^{AR-} T+E (1.5m)	9431	0.032	1
	9432	0.019	1
	9434	0.02	1
T+E (1.5m)	9447	8.5	5.1
	9448	9.32	4.7
	9451	5.98	4.2
str^{AR-} T+E (3m)	9195	2.12	6.7
	9197	2.4	6.8
	1181	0.458	7.6
T+E (3m)	1183	0.332	7.2
	1184	0.982	6.9
	9140	0.689	2.5
	9141	3.44	3.4
Hi-Myc (4.5m)	9161	0.201	4.1
	9162	0.093	3.8
	9163	0.086	1
str^{AR-} *Hi-Myc* (4.5m)	1164	1.02	5
	1163	0.653	7.7
	1177	0.519	7

Table 6: Sample RNA concentration and RNA integrity number (RIN) from str^{AR-} T+E (1.5m & 3m), T+E (1.5m & 3m), str^{AR-}Hi-Myc (4.5m), and Hi-Myc (4.5m) mice as measured by the UC Davis Genome center.

In order to investigate the impact of stromal AR loss on stromal-to-epithelial signaling, raw read counts were used as input for edgeR analysis to derive a catalog of genes that displayed significant differential expression. After narrowing down this extensive list to one comprising 159 genes of high

significance (p<0.0001), a subset of 32 genes encoding secretory proteins were identified (Figure 10).

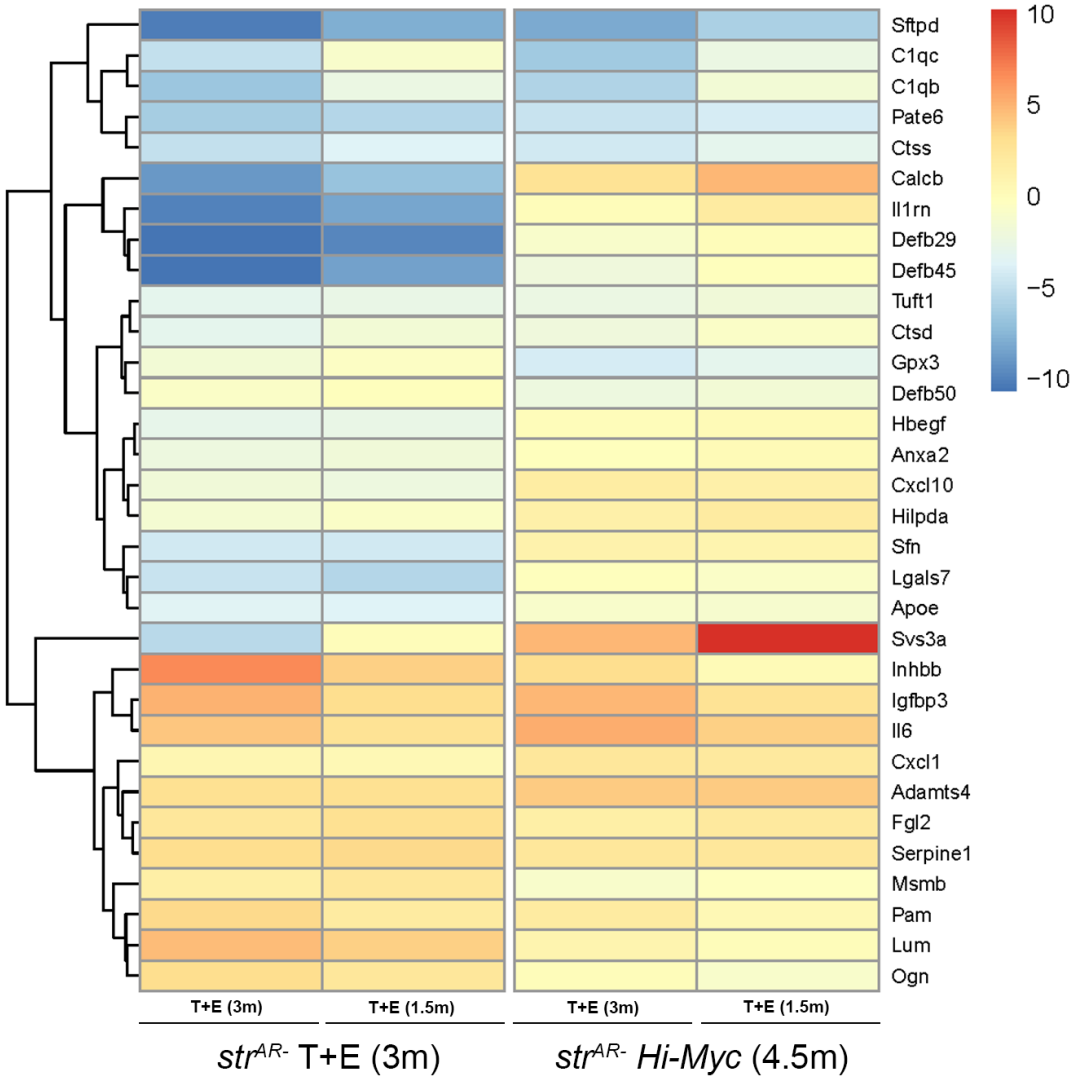

Figure 9: Differential expression of significant (p<0.0001) secretory proteins in str^{AR-} T+E (3m) and str^{AR-} Hi-Myc (4.5m) mice. Colors represent negative fold change (blue) to positive fold change (red). Fold changes in str^{AR-} mice are relative to either 3m or 1.5m T+E control groups, as labeled under each column.

While there is a slight decrease in the gene expression of these secreted factors when comparing *str*$^{AR-}$ mice to T+E (3m) or T+E (1.5m), overall expression patterns do not exhibit significant changes with further cancer progression in the T+E model (Figure 10). However, in *str*$^{AR-}$ T+E (3m) mice, strongly downregulated genes such as *Il1rn* and *Calcb* are observed to be upregulated in *str*$^{AR-}$ *Hi-Myc* (4.5m) mice (Figure 10). These changes could possibly result from differences between each PCa model or could be attributed to variations in PCa progression at different time points of analysis.

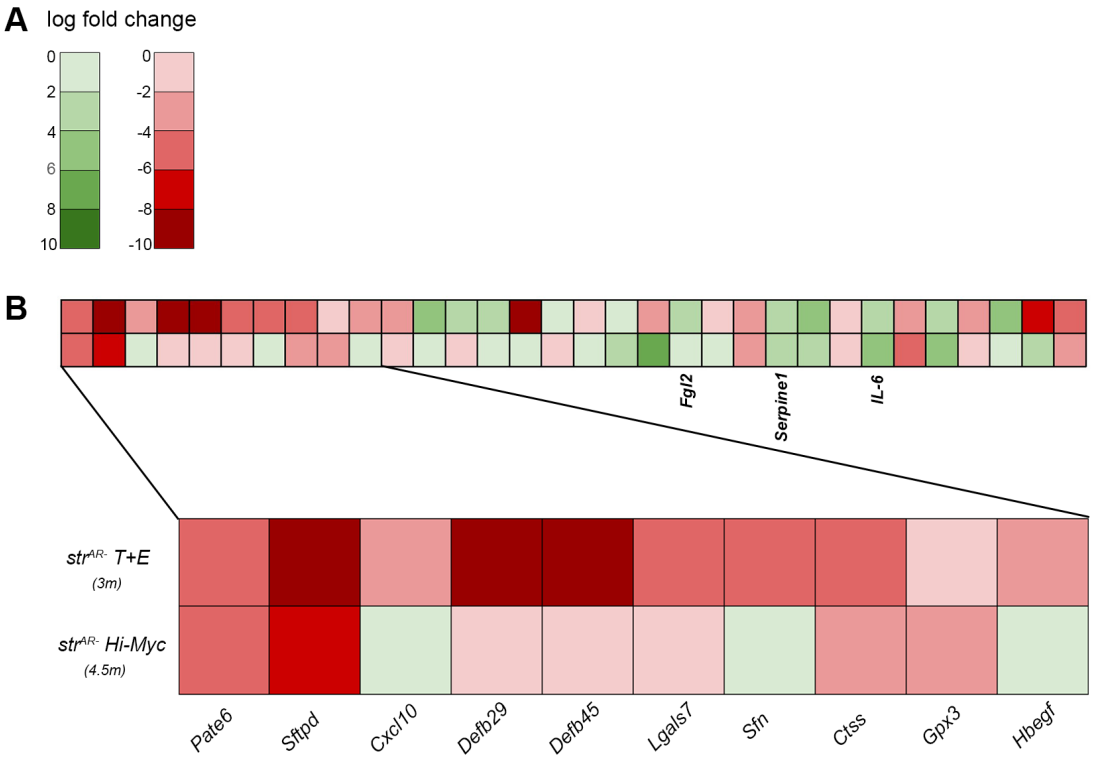

Figure 10: Differential expression of secreted factors in AR-null stromal cells. (A) Legend for log fold change. (B) Average log fold change enlarged for the top 10 most significant secreted factors in stromal cells from str^{AR-} *T+E (3m) and str*$^{AR-}$ *Hi-Myc (4.5m) mice when compared to T+E (1.5m) and T+E (3m). Candidate genes Fgl2, Serpine1, and IL-6 are shown as well.*

Figure 11 presents the logarithmic fold change values for the top 10 secreted proteins exhibiting remarkable significance, with the gene *prostate and testis expressed 6* (*Pate6*) emerging as the most prominently significant among them. However, as a previous study utilizing scRNA-seq on whole prostates identified a distinct luminal subpopulation exhibiting enhanced PI3K-mTORC1 activity in *str^{AR-} Hi-Myc* and *str^{AR-}* T+E mice compared to *Hi-Myc* and T+E controls (Y. Liu et al., 2022), it is prudent to explore secreted factors within the context of the PI3K-mTORC1 signaling axis. A promising candidate is *fibrinogen-like protein 2* (*Fgl2*), which has been demonstrated in orthotopic mouse glioblastoma models to induce self-renewal and tumorigenicity of glioma cells by recruiting macrophages into the tumor microenvironment and stimulating their secretion of CXCL7 via the PI3K pathway (Yan et al., 2021). Correspondingly, *Fgl2* knockout in mice was found to suppress cerebral PI3K pathway activation compared to wild-type mice (Huang et al., 2023), aligning with my data indicating upregulated *Fgl2* expression in *str^{AR-}* mice (Figure 10). An additional potential candidate worthy of consideration is *Serpine1,* also known as *plasminogen activator inhibitor 1* (*PAI-1*). Previous studies in fibrosarcoma and endothelial cells has elucidated its role in modulating cell growth and proliferation through the PI3K-Akt pathway (Balsara et al., 2006; Rømer et al., 2008). Furthermore, fibrosarcoma tumor growth was notably suppressed in *PAI-1$^{-/-}$* mice compared to WT mice (Gutierrez et al., 2000). Additionally, *Adamst4*, a gene that is also upregulated in *str^{AR-}* mice (Figure

10), is highly correlated with increased *Serpine1* expression in colon cancer patients (Y. Wang et al.)

Lastly, a compelling candidate gene is *interleukin-6* (*IL-6*), as existing evidence suggests that androgen inhibits *IL-6*-mediated PI3K activation (S. Xie et al., 2004), which is consistent with my data showing upregulation of *IL-6* in str^{AR-} mice (Figure 10). Moreover, elevated levels of *IL-6* have been detected in PCa cell lines, as well as clinical specimens from PCa patients and sera of patients with advanced, therapy-resistant PCa (Culig, 2014).

Taken together, my analyses identified 3 highly significant gene candidates that were upregulated in the str^{AR-} mice when compared to T+E controls. The increased expression of these genes in str^{AR-} mice is consistent with previously published findings on the role of these genes in promoting tumorigenicity in various cell types and regulating PI3K signaling. Further studies into these candidate genes can help identify how loss of stromal AR may accelerate PCa development by modulating stromal-to-epithelial signaling.

Discussion

PCa is primarily derived from the epithelial cells of the prostate gland, but the surrounding stromal compartment, consisting of various cell types, plays a critical role in prostate development and cancer progression. Androgen receptor (AR) signaling in both the epithelium and stroma is known to regulate prostate development and cancer. A recent study from our lab showed an inhibitory role of stromal AR in prostate carcinogenesis in the T+E2 and *Hi-Myc* mouse models (Y. Liu et al., 2022). In addition, scRNA-seq analysis revealed that deletion of stromal AR primarily affected secretory luminal tumor cells, potentially mediated by the modulation of paracrine signaling in AR-null SMCs. However, the scRNA-seq samples were obtained from advanced tumors (8 months in T+E mice, 12 months in *Hi-Myc* mice), which posed challenges in discerning alterations in stromal cells at various time points following onset of AR deletion. Likewise, scRNA-seq data alone does not offer comprehensive insights into genes that are regulated by stromal AR. To address these challenges and gain a deeper understanding of how loss of stromal AR may alter signaling between the stroma and the epithelium during prostate carcinogenesis, I performed bulk RNA Seq on AR-null and wild-type stromal cells isolated from mice at different time points during PCa development.

From the comprehensive dataset obtained, a subset of 32 highly significant genes encoding secretory proteins was identified. Notably, the expression patterns of these secreted factors did not exhibit significant changes with further cancer progression in the T+E model. Nevertheless, in the AR-null mice, certain genes such as *Il1rn* and *Calcb* that were strongly downregulated in the T+E model were found to be upregulated in the *Hi-Myc* model. These differences could be attributed to variations in the PCa models or the timing of analysis at different stages of cancer progression in each model.
However, the primary objective of this experiment is to identify stromal-secreted proteins that are strongly regulated by AR. To achieve this, my analyses primarily focused on identifying genes that exhibited consistent expression patterns in both *str*$^{AR-}$ *Hi-Myc* and *str*$^{AR-}$ T+E mice, indicating a robust regulatory effect of AR deletion. As a result, any variations in the timing of analysis are not anticipated to significantly impact the findings outlined in this study.

Building upon our lab's previous study where we observed a distinct luminal subpopulation influenced by enhanced PI3K-mTORC1 activity in the prostates of *str*$^{AR-}$ *Hi-Myc* and *str*$^{AR-}$ T+E mice (Y. Liu et al., 2022), I aimed to further identify gene candidates that produce secretory factors involved in the activation of the PI3K pathway. Analyses identified *fibrinogen-like protein 2* (*Fgl2*), *plasminogen activator inhibitor 1* (*PAI-1*), and *interleukin-6* (*IL-6*) as

potential candidate genes due to their implication in modulating the PI3K pathway.

Subsequent investigations could be directed towards evaluating the functional roles of these identified candidate genes *in vitro* using an epithelial-smooth muscle cell (SMC) prostate organoid co-culture system (Janecki et al., 1987)). This approach could involve assessing the impact of stromal cell knockout of the candidate ligands on the expression of genes associated with the PI3K pathway, employing quantitative real-time PCR (qRT-PCR) analyses. Furthermore, *in vivo* experimentation could entail renal grafting of candidate gene-deleted SMCs with mouse or patient-derived PCa cells. Additionally, SMC conditional knockout (KO) of the identified ligands could be employed to investigate their effects on tumor development and progression in mouse models of PCa. These multifaceted experiments would provide valuable insights into the functional significance of the candidate genes and their potential roles in PCa pathogenesis.

It is important to note that stromal AR may influence the development of prostate cancer (PCa) through an additional mechanism that is not addressed in this study, namely by modulating epithelial cell plasticity. Although previous studies have explored the impact of both stromal AR and epithelial AR knockout on morphological and transcriptional alterations in the prostate epithelium, a comprehensive investigation examining how stromal AR loss

affects epithelial cell differentiation patterns has yet to be conducted. To address this gap in knowledge, a combined Cre-loxP and Dre-rox system could be employed, enabling targeted knockout of AR in stromal cells while simultaneously conducting lineage tracing in basal or luminal cells. This approach would elucidate the specific effects of stromal AR signaling on epithelial cell plasticity, both in adult homeostasis and in the context of PCa development.

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