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UNIVERSITY OF CALIFORNIA

SANTA CRUZ

**INVESTIGATION OF THE MOLECULAR REGULATION OF PROSTATE
BASAL CELL MULTIPOTENCY**

A dissertation submitted in in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

MOLECULAR, CELLIULAR, AND DEVELOPMENTAL BIOLOGY

by

Corrigan Andrew Horton

September 2022

The Dissertation of Corrigan A. Horton is
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ABSTRACT

Investigation of the Molecular Regulation of Prostate Basal Cell Multipotency

Corrigan Andrew Horton

The goal of my thesis was to discover how basal cell multipotency is regulated in the adult, homeostatic prostate. During development, basal stem cells actively differentiate to produce mature basal, luminal and neuroendocrine cells. Some basal cells retain this ability in adulthood, however the stem cell activity is heavily downregulated. Most of this thesis focuses on understanding cellular mechanisms that are responsible for modulation of basal stem cell activity. Chapter 2 examines a potential inhibitory signal emanating from luminal cell contact. Chapter 3 follows by investigating how the canonical Wnt and androgen receptor (AR) signaling pathways interact to induce basal stem cell activity. The 4th chapter takes a brief look into the internal programming of adult basal stem cells. A better understanding of the regulatory processes that govern stem cell activity is necessary in order to understand the pathogenesis of malignancies that arise from the alterations of these processes. To this end, the final chapter of this thesis uses mouse models of prostate adenocarcinoma to investigate the effect of stromal AR activity on cancer progression.

Dedication

I would like to dedicate this work to my late Grandfather, Joseph Fenick (19-2021)

Your passion for life shines through your loved ones

Acknowledgements

I have so many people to thank for allowing this Ph.D. experience to be a one of fulfillment and growth. This achievement truly would not have been possible if it weren't for the support and collaboration of family, friends, colleagues, and mentors. I will attempt to recognize the many contributors in this section. Appologies if I miss you here.

I want to begin with the acknowledgement of the support and guidance of my mentor, Zhu Wang. I first became interested in Zhu's research when he gave a guest lecture in Amy Ralston's developmental biology course here at UCSC in 2015. I was in my last year of undergrad and had aspirations of becoming a bonafide biologist. I remember his description of the lab's *in vivo* research utilizing the Cre/lox system to perform lineage tracing. I immediately became excited about the potential of this relatively novel technique in its utilization to uncover cells of origin for aggressive cancer. My grandfather had recently been diagnosed with prostate cancer, so I felt an obligation to use my degree to help in the effort to combat this horrible disease.

Once graduated, I was on the search for a job that would allow me to not only remain in Santa Cruz, CA, but also act as a catalyst for my career as a cancer biologist. I serendipitously came across a posting for a junior specialist position in Zhu's lab and knew I had to act fast to land this position. Fortunately, he saw promise in this recent, green grad and decided to bring me into the lab despite my relative lack of experience and skills. I am forever grateful for his leap of faith to trust me to help

establish his lab, as well as his patience while I became more comfortable with basic molecular and cellular biology techniques.

After a little over a year of learning as a junior specialist, I decided to inquire about entering the Ph.D. program as a student in his lab. Again, he showed faith in my abilities and accepted me as a direct admit for the program. Since then, his guidance and passion for science has jettisoned me into becoming the biologist that I am today.

In the early days, I shared the lab with only Zhu and a brilliant postdoc, Qing Xie. Qing directly mentored me in countless molecular biology techniques and showed great patience in doing so. He did all of this while managing the lab and producing publications of the highest quality. To this day, I still do not know how he juggled all of these tasks with such effectiveness. Since moving to role in the lab with fewer stresses, he has continued to help instill confidence in my presentation skills and graciously offered his expertise in experimental design. I can say with confidence that I would not be in this position if it were not for Qing.

So many members of the lab have contributed to my success in countless ways. Chuan Yu and Yueli Liu joined as postdoctoral researchers in 2016 and helped teach me the nuances of being a successful scientist. Chuan has been my go-to person for advise on molecular biology experiments and Lily was integral in helping me understand the intricacies of organoid culture techniques. Ofir Stefanson joined the

lab as a master's student in 2017 and immediately contributed to my projects with his insightful input in lab meetings and his knowledge of molecular cloning.

I cannot mention the contributions of the lab without discussing the undergraduate researchers that I have had the honor of mentoring. In particular, Alexander Zee, Daniel Nguyen, and Jonathan Green have been indispensable in assisting me in the maintenance of numerous mouse lines throughout the years. They all mastered the art of genotyping and tail snipping while managing a busy class schedule. Daniel even found time to develop his own senior year research project within the lab and has since gone on to medical school. Jonathan recently joined the lab as I was preparing my dissertation and quickly picked up the mouse husbandry/PCR skills necessary to take an enormous amount of pressure off of my shoulders. He is now a junior specialist in the lab and shows great promise as not only a biologist, but also a future photographer.

I would like to thank the MCD community for cultivating a wonderfully supportive environment in which to learn and grow. My cohorts were always so helpful and friendly in times of not only need, but also celebration. I would like to give a special thanks to: Gary Mantalas for guiding me through the single cell RNA sequencing protocols, Eric Martin for being patient while I constantly pestered him about technical details of my experiments, and Jeremiah Tsypsin for collaborating with me to nail the Cut and Run experiments. Thesis committee members Lindsay Hinck and Bin Chen provided serious professional support and guidance throughout the later

years of the program. Bari Nazario and Ben Abrams helped me immensely with flow cytometry and microscopy, respectively.

Finally, I would like to thank my family and friends for all of the love and support I received throughout the process. To my parents, Fred and Katheryn Horton, thank you for being such amazing life-guides and friends. Your unrelenting effort to communicate with and always support loved ones is inspirational. You two are the primary reason I have been able to reach many of my dreams thus far in life. Alison, we may have been adversaries much of the time growing up, but we have always been able remain genuine around each other – which means a lot to me. Your and Jared’s ambition, geniality, and love of adventure is contagious and motivating. To Amanda, my partner, thank you so much for being patient throughout this process. You have been my biggest supporter through the highs and lows of the last few months. PhD Power Couple!!

To the many friends that have helped shape my development and have been integral in keeping my sanity through grad school and COVID, thank you! Whether it was losing our cool in a city co-ed softball game that means nothing, throwing frisbees at chains at dela, exploring and camping in the Sierra’s, or riding bikes through upper campus redwoods like speeders on Endor, those were the dang-ol’ times.

Chapter 1. Introduction

Prostate structure and cellular lineage hierarchy

The mouse prostate provides a suitable model for investigating human prostate development, homeostasis, and oncogenesis. Though different in macrostructure, the two homologous organs share the same epithelial microstructure and cell types, as well as cell surface markers (Bonkhoff et al., 1994, Shappell et al., 2004) (Fig 1.1). The prostate arises from the endodermal derived urogenital sinus (UGS), which undergoes epithelial budding at approximately at 17.5 days post coitus (dpc) in the mouse. Once budding is initiated and prostate fate is determined, the urogenital sinus epithelium (UGE) buds into the surrounding urogenital sinus mesenchyme (UGM), initiating branching morphogenesis to form a system of ducts composed of epithelial tubules. This process is dependent on paracrine signaling to the UGE from the UGM, where androgen receptor (AR) activity is necessary to mediate epithelial outgrowth (Toivanen and Shen, 2017). In the adolescent and fully developed mouse, the prostate is divided into 3 lobe pairs: the dorsolateral prostate (DLP), the anterior prostate (AP), and the ventral prostate (VP) (Fig 1.1B). The epithelium within these lobes is primarily comprised of triangular shaped, cytokeratin 5 (CK5), CK14 and P63 expressing basal cells that reside near the basement membrane and columnar secretory luminal cells on the apical side of the epithelium that express CK18, AR, as well as high levels of the prostate specific transcription factor Nkx3.1. Intermediate cells that express both basal and luminal markers, as well as other markers such as CK19, are present within the basal layer (De Marzo et al.,

1998; Wang et al., 2001; Xue et al., 1998) It remains unclear whether intermediate cells represent a functionally distinct cell type. Rare neuroendocrine cells also reside within the epithelium. However, their function remains relatively unknown and they will not be a main focus of this thesis. The mature stromal compartment, which is primarily derived from the UGM, surrounds the epithelium and consists of smooth muscle actin (SMA) and myosin heavy chain 11 (MYH11) expressing smooth muscle cells, as well as fibroblasts, endothelial cells and a collagen rich extracellular matrix (figure 1.1C). Continuous reciprocal signaling between the stromal and epithelial compartments propagates glandular development and differentiation during prostate organogenesis and maintains adult organ homeostasis (Toivenen and Shen, 2017).

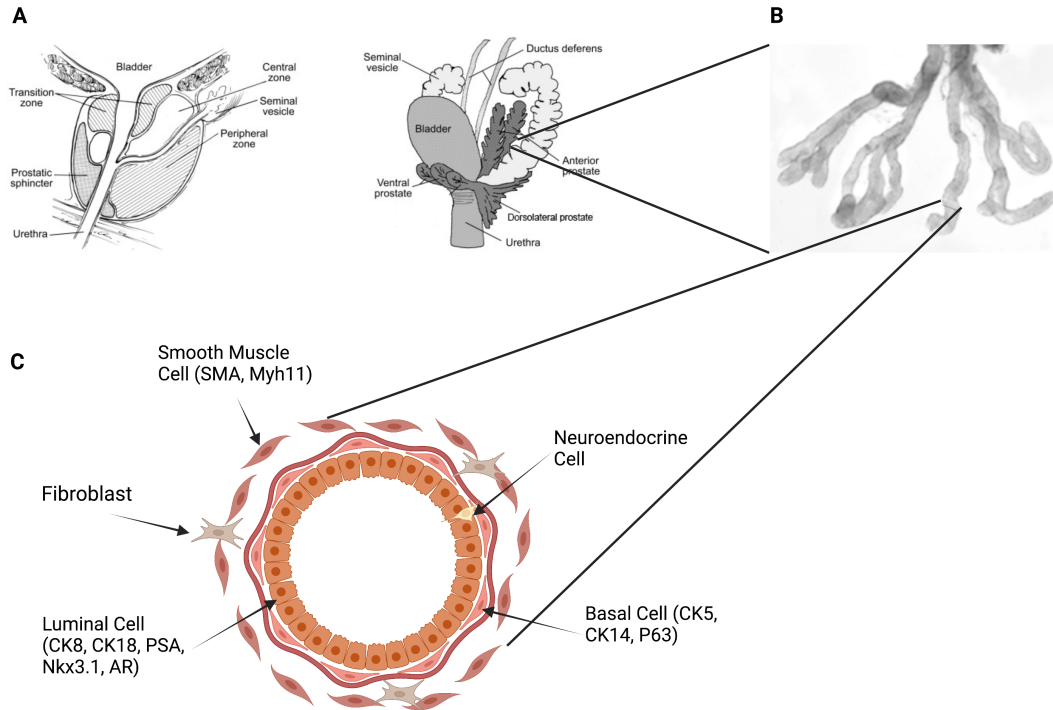


Figure 1.1. Prostate Morphology and Differentiated Cell Types. A.) Schematics of the adult human and mouse prostate gland. Key structures and regions of the prostate are indicated. Adapted from Toivanen and Shen, 2017. B.) Each prostate lobe is composed of multiple secretory epithelial tubules C.) Crossection of these tubules reveals that the epithelium is comprised of two main cell types: Basal cells lining the outer surface that express cell surface markers cytokeratin 5 and cytokeratin 14, and secretory luminal cells lining the apical surface that express cell markers CK8, CK18, PSA, Nkx3.1, and AR. The epithelium is surrounded by a stromal layer that consists of smooth muscle cells and fibroblasts.

Prostate development is driven by basal progenitor cells that give rise to the mature prostate epithelium. Early tissue reconstitution assays demonstrated that when isolated mouse prostate epithelial cells are recombined with developing rat UGM cells, basal cells but not luminal cells can give rise to all three mature cell types found in the adult prostate (basal, luminal and neuroendocrine) (Buger et al., 2005; Xin et al., 2005). More recently, lineage-tracing analyses using inducible Cre drivers under control of CK5, CK14, or CK8 promoters during postnatal epithelial growth reveal that while both basal and luminal cells contain unipotent progenitors, only basal cells give rise to all three mature cells types during development (Ousset *et al.*, 2012). Additionally, adult lineage tracing experiments suggest that some rare adult basal cells retain this multipotency and differentiate into mature luminal cells that lack CK5 expression (Wang et al., 2013). Importantly, lineage tracing experiments performed in this same study found that Pten-deficient adult basal cells can act as the cell of origin for luminal phenotype prostate cancer. Since human primary prostate cancer almost always takes on a luminal phenotype characterized by apparent disorganization and an absence of basal cells (Humphrey, 2011), this lineage tracing data suggest that basal to luminal differentiation is a preliminary and necessary step in basal cell

derived carcinoma. In corroboration with this evidence, it was found that CD49f^{hi} human basal prostate cells with lentivirus mediated overexpression of C-MYC and activated AKT1 can lead to aggressive carcinoma in xenograft models (Goldstein et al., 2010; Park *et al.*, 2016). Taken together, this evidence suggests a lineage hierarchy in which basal cells serve as multipotent progenitor cells during epithelial development, and rare basal cells retain differentiation capabilities in adulthood and are susceptible to oncogenic transformation (figure 1.2). What mechanisms regulate this cellular lineage relationship? Detailed studies of stem cell niche sites in other organs have recently led the field to examine the roles the surrounding environment plays in determining the plasticity of basal progenitor cells.

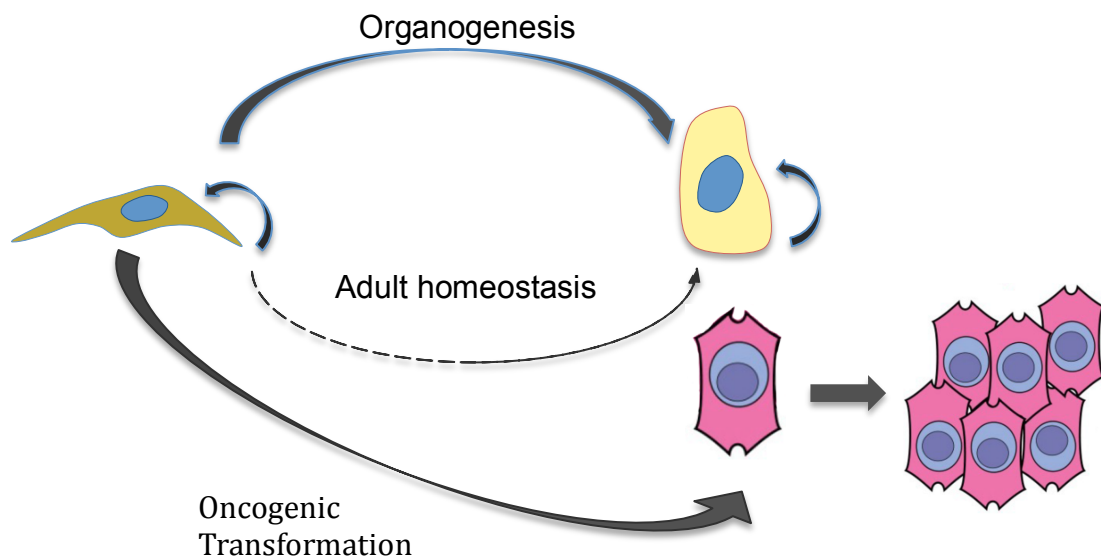


Figure 1.2: Basal Cell Plasticity Basal to luminal differentiation occurs readily and is a hallmark of organogenesis, but is heavily down regulated in adult homeostasis. This differentiation has also been shown to be the necessary first step in the formation of basal derived prostate cancer.

Stem cell niche

Stem cell niches are composed of stem cells as well as a diverse population of neighboring differentiated cell types that secrete and organize an extracellular matrix and other factors that propagate unique intrinsic properties of the resident stem cells (Fuch, Tumber et al. 2004; Morrison and Spradling 2008). Niche sites within many mammalian organs that harbor tissue resident stem cells have been extensively studied and are very well characterized. For example, germline stem cells lie within the basal cell layer of the seminiferous tubules (Cinalli, Rangan et al. 2008); hair epithelial stem cells reside within the bulge of hair follicles (Tumber, Guasch et al. 2004); Alveolar type II (AT2) cells sit in the distal tips of the adult lung alveolar epithelium (Wu and Tang 2021); Lgr5 expressing intestine stem cells reside within the intestinal crypts (Sato et al. 2010); and neural stem cells are in the lateral ventricle subventricular zone of the central nervous system (Doetsch 2003; Zhao, Deng et al. 2008). Examples of mammalian cell niches are shown in table 1.1 below:

Stem Cell	Location	Supporting Cells	Major Signals	Stem cells per niche
HSC	Endosteal, perivascular	osteoblasts, osteoclasts, mesenchymal progenitors, reticular cells	CXCL12; SCF; Tpo; SHH; Ang1	1
Satelite Muscle Cell	Under basal lamina on myofiber	myofiber?	Wnt; Notch; HGF; CXCL12	1
Central Nervous System SVZ stem cell	Subventricular Zone	endothelial; ependymal?	SHH; Notch; Wnt; TGF α ; FGF; VEGF	Many

Intestinal Epithelium	Base of Crypt	fibroblasts?, hematopoietic cells?	Wnt; Notch; BMP	4-6
Hair Follicle Bulge	Bulge	vascular?	Wnt; BMP; TGF β	Many
Interfollicular Epidermis	Basal Layer	dermis	Wnt; Notch	?
Spermatogonial	Basal layer, seminiferous tubules	Leydig, Sertoli, vascular	BMP4; BMP8b; SCF; FGF; GDNF	?

Table 1.1. Summary of well-characterized mouse stem cell niches (Morrison and Spradling, 2008)

The prostate epithelium provides a wonderful model to investigate novel mechanisms that may underlie stem cell niche sites. Relative to other epithelial organs such as the intestine and skin, the prostate is quiescent and therefore its basal stem cells need to be plastic and able to substantially modify their activity level based on input from the surrounding environment. Additionally, due to its relatively simple structure and well-characterized cell populations, as well as the availability of well established ex vivo and in vitro models of organ development, cell recombinant experiments can be performed to determine essential components of the basal stem cell niche. Early tissue reconstitution assays showed that paracrine signaling from embryonic urogenital mesenchyme (UGM) is needed to induce urogenital sinus epithelium (UGE) development from isolated adult basal cells (Kurita *et al.*, 2004). When these tissue recombination experiments are performed, isolated mesenchymal and epithelial components from the same or different tissues and/or donors are combined and implanted under the renal capsule of immunodeficient hosts. UGM or UGE are unable to grow and differentiate on their own in renal grafts, but when they

are grafted together, prostate tissue results. Interestingly, when bladder epithelium is recombined with UGM prostate tissue is also generated, showing that prostate specification is largely conferred by the UGM (Donjacour and Cunha, 1993). This finding indicates a positive regulatory effect of the UGM – specifically on mediating basal to luminal differentiation and specifying prostate epithelial cell fate. As the epithelium begins to expand and reach adult homeostasis, the rate of basal to luminal differentiation slows almost to a complete halt (Wang et al., 2012). Do specific cells within the adult stem cell niche exist that are responsible for this inhibition? What are the mechanisms by which these cells mediate their inhibitory effect? Are there specific basal cells that are primed via their transcriptional programming to be more responsive to external stimuli? What are the stimulatory factors of this stem cell niche and how are they differentially regulated in adulthood compared to development? Chapter two of this thesis will investigate the inhibitory constituents of the niche sites, while chapter three will discuss potential stimulatory stromal signals and how they may be modulated in adult homeostasis to regulate basal stem cell multipotency.

Andromedin Model

Androgen receptor signaling is the most commonly studied pathway in prostate development due to its central role in inducing sexual dimorphism. The androgen receptor is stimulated via the circulating hormone testosterone. Testosterone is produced by the testis and adrenal gland starting at 9 weeks of gestation in humans (Siiteri and Wilson, 1974) and 13-14 dpc in mice (Pointis *et al.*, 1979). Testosterone then reaches the UGS via the bloodstream and is converted to its more potent

metabolite dihydrotestosterone (DHT) by 5α -reductase (Berman *et al.* 1995). DHT then binds to AR, which dimerizes, translocates into the nucleus, complexes with coactivator proteins and general transcription factors and finally binds to androgen response elements (AREs) located in promoter and enhancer regions to activate or repress the transcription of target genes (Fig 1.3).

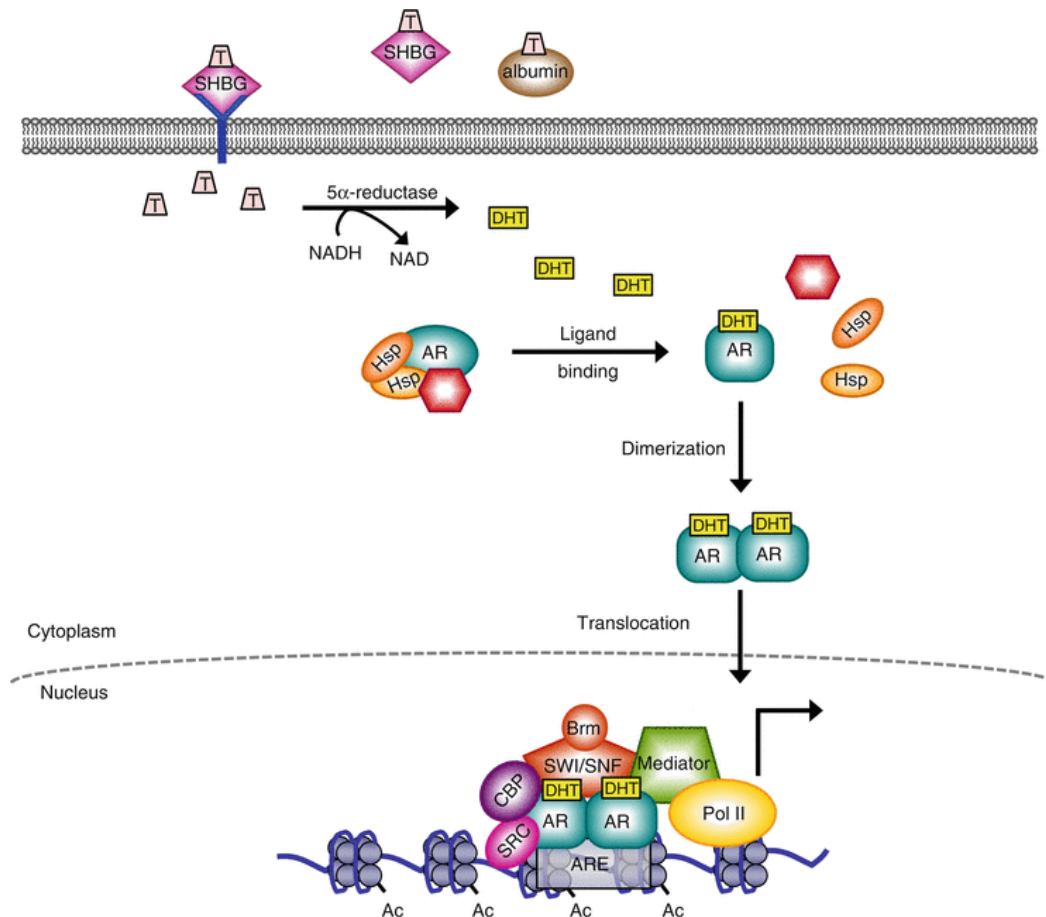


Figure 1.3. AR Pathway AR transcriptional activation. Testosterone (T) circulates in the blood bound to steroid hormone-binding globulin (SHBG) or albumin and enters the cells through diffusion or through a SHBG receptor at the plasma membrane. In some cells, T is converted to the more active metabolite dihydrotestosterone (DHT) through the action of 5α -reductase. Upon ligand binding, AR undergoes a conformational change followed by dissociation from the multi-subunit chaperone complex. It then forms dimers and translocates to the nucleus where it binds androgen response elements (AREs) of target genes and recruits coregulators (some examples of which are shown) and basal transcription machinery, resulting in transcriptional

regulation. HSP, heat shock protein; CBP, CREB binding protein; SRC, steroid receptor coactivator; BRM, Brahma; SWI/SNF, a chromatin modifying complex (Kirli *et al.*, 2012).

The role of AR in the UGM and UGE has been extensively studied using tissue recombination experiments and conditional knockout mouse models. These experiments have shown that AR is necessary in the UGM for prostate induction but dispensable in the UGE for duct formation. However, subsequent AR expression in the epithelium is required for the expression of prostatic secretory proteins and for the differentiation of the surrounding mesenchyme (Cunha *et al.*, 1992; Cunha and Young, 1991; Donjacour and Cunha, 1993; Hayward *et al.*, 1998). To sum, androgen receptor signaling in the UGM induces the UGE to bud into the surrounding mesenchyme and determines prostate cell fate, while AR signaling in the epithelium is required to mediate epithelial cell function as well as determine stromal cell fate.

This leads to two models as to how androgens mediate prostate epithelial development and function: The andromedin model and the smooth muscle model (Toivanen and Shen, 2017) (fig 1.4). In the andromedin model, AR signaling in the UGM results in the upregulation of the intermediary signaling factors (andromedins) that in turn act directly on the UGE to induce prostate formation. In contrast, the smooth muscle model proposes that AR signaling indirectly affects epithelial development by regulating the differentiation of smooth muscle, which forms a barrier to block further epithelial budding and outgrowth (Tovanian and Shen, 2017). Though these models of development differ their explanation of how AR signaling mediates development, they both rely on secondary signaling coming from the

surrounding stromal cells to induce prostate epithelial formation. This thesis uses these developmental models as a basis to investigate how basal stem cell activity is regulated in adult homeostasis.

To date, the field has identified many signaling pathways that are believed to be activated through secretions from cells within the stromal layer and influence prostate development and homeostasis. Three of the most well studied and interesting pathways that are good candidates for further investigation are Fibroblast Growth Factor (FGF) signaling (Finch *et al.*, 1995; Sugimura *et al.*, 1996; Thomson and Cunha, 1999), Bone Morphogenic Growth Factor (BMP) signaling (Lamm *et al.*, 2001; Omori *et al.*, 2014), and the canonical Wnt/ β -catenin signaling pathway. Due to the canonical Wnt pathway's known role in basal cell activity in other epithelial tissues (Veltri *et al.*, 2018; Haas *et al.*, 2019; Hou *et al.*, 2019), how this pathway regulates prostate basal stem cells in adult homeostasis is a main focus of this thesis.

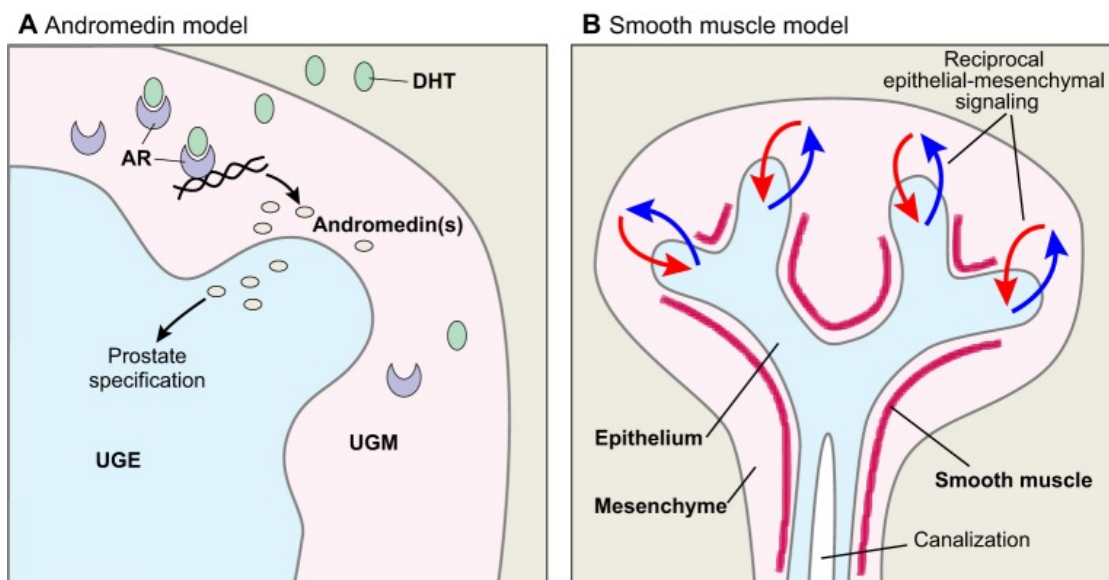


Figure 1.4: Models of prostate bud induction. (A) In the andromedin model, androgen signaling through androgen receptor (AR) expressed in the urogenital mesenchyme (UGM) results in the production of one or more signaling factors (andromedins) that directly induce prostate budding in the adjacent urogenital epithelium (UGE). (B) In the smooth muscle model, signals that promote epithelial budding occur constitutively, and androgen signaling within the mesenchyme indirectly controls this process. In this model, androgen signaling is proposed to regulate the differentiation of smooth muscle around the emerging prostate ducts, thereby inhibiting further epithelial budding in regions where smooth muscle has already formed. DHT, dihydrotestosterone. (Toivanen and Shen, 2017).

Canonical Wnt/ β -catenin pathway

The canonical Wnt/ β -catenin signaling pathway is of significant interest to the field for many reasons. Importantly, Wnt signaling components are heavily involved in prostate development (Mehta *et al.*, 2011) and canonical Wnt ligands are necessary for normal tubule formation within mouse prostate explants (de julio *et al.*, 2013). In addition, canonical Wnt signaling perturbations are implicated in multiple human epithelial cancers including breast and colorectal carcinomas (Polakis, 2012).

Recently, aberrations within the canonical Wnt effectors, β -catenin (positive effector) and APC (negative effector), as well as fusions within *Rspo2*, the gene that codes for the Wnt agonist, R-spondin, have been observed in metastatic, castration resistant human prostate cancer (hCRPC) (Robinson *et al.*, 2015). Given the involvement of canonical Wnt signaling in murine prostate development and advanced hCRPC, as well as the prevalence of basal to luminal differentiation in development and basal derived prostate cancer, it is logical to hypothesize that canonical Wnt is involved in regulation of basal cell multipotency.

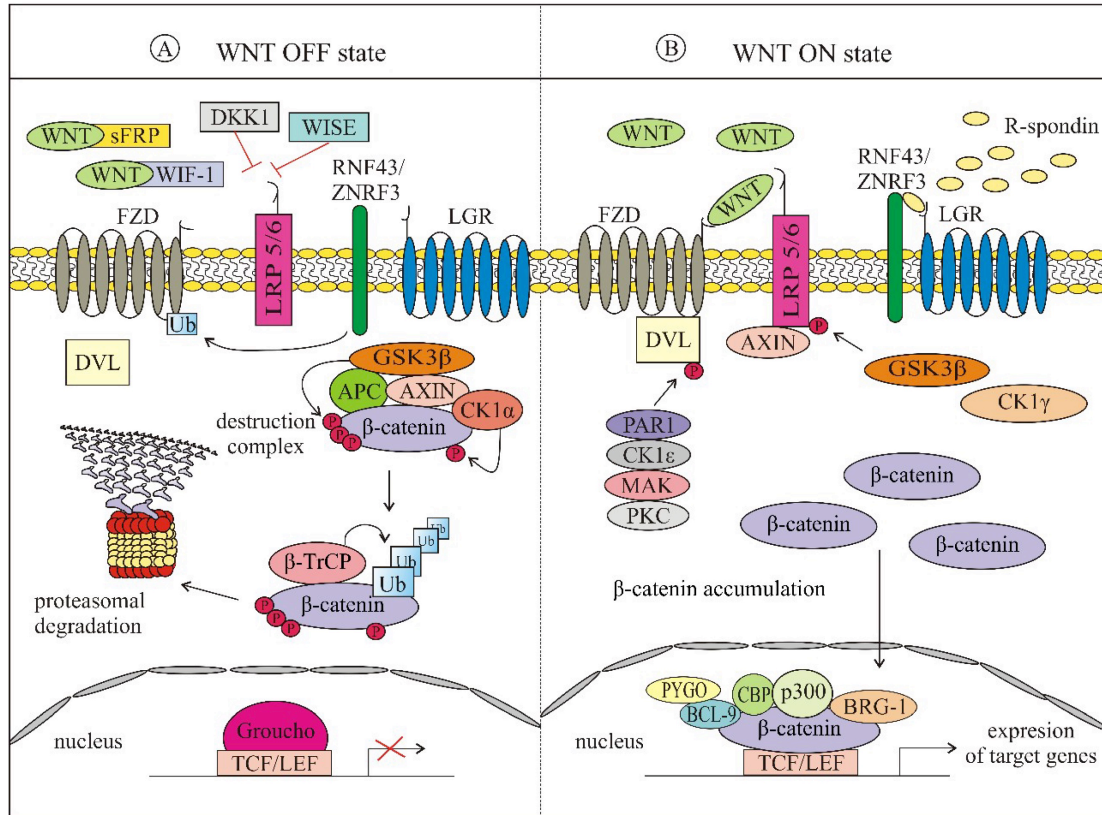


Figure 1.5. Simplified scheme of canonical Wnt signaling pathway. (A) In the absence of WNT ligands (WNT OFF state), β -catenin is phosphorylated by a destruction complex consisting of AXIN, APC, GSK3 β and CK1 α to be further ubiquitinated for proteasomal degradation. In the absence of R-spondins, E3 ubiquitin ligases RNF43/ZNRF3 target FZD for lysosomal degradation; (B) binding of WNT ligands to FZD receptors and LRP co-receptors activates WNT-signaling (WNT ON state). AXIN is associated with LRP5/6, whereas DVL is recruited to FZD, which results in dissociation of the destructive complex. β -catenin is accumulated and stabilized in the cytosol, and then unphosphorylated β -catenin is translocated to the nucleus to activate the expression of WNT target genes. (Gajos-Michniewicz and Czyz, 2020).

Multiple studies have highlighted the necessity of canonical Wnt signaling in prostate bud development and tissue differentiation (Schaeffer et al., 2008; Mehta et al., 2001; Simons et al., 2012). In the canonical Wnt pathway, secreted Wnt ligands bind to Frizzled/LRP5/6 receptor complexes, resulting in a signaling cascade that

leads to the nuclear translocation of β -catenin. Nuclear β -catenin then binds to the transcription factors of the T-cell factor/Lymphoid enhancer factor (TCF/LEF) family to initiate transcription of downstream target genes associated with embryogenesis, such as siamois and twin, and oncogenesis, such as C-Myc and CyclinD1 (Komiya and Habas, 2008; Reya and Clevers, 2005; Clevers, 2006). In 2013, Kruithof-de Julio et al. used a TCF/Lef:H2B-GFP (T/L-GFP) transgene reporter construct to determine spatial distribution of canonical Wnt signaling activity in the developing mouse prostate. The T/L-GFP construct consists of 6 TCF/LEF DNA binding sites, an hsp68 promoter that promotes expression of H2B-GFP (Fig 1.6A), leading to nuclear GFP expression in cells with active canonical Wnt signaling. This allows for visualization of Wnt responsive cells at a single cell resolution. The 2013 study found that before epithelial induction (E15.5-E17.5), Wnt signaling was active in the urogenital sinus mesenchyme (UGM) and absent in the urogenital sinus epithelium (UGE). As the UGE begins to produce buds and become more dynamic, Wnt signaling activity slowly shifts from the UGM to the elongating bud tips of the UGE. By P9, T/L-GFP signal becomes sparse in the UGM but remains active in some, but not all, cells of the epithelial bud tips, suggesting a heterogeneous population of cells within the distal developing UGE. We obtained this transgenic mouse and I was able to determine the levels of Wnt activity in the basal, luminal, and stromal layer of transgenic mice at P20 and P50. Preliminary results show high GFP signal in basal cells at P20 with sporadic GFP⁺ luminal cells. In P50 mice, when the prostate is fully mature and enters a state of homeostasis, GFP⁺; CK5⁺ basal cells are absent while CK18⁺; GFP⁺

cells are numerous (Fig 1.6B). This evidence supports the data from the 2013 study by Kruithof-de Julio and colleagues mentioned above, suggesting that Wnt may be involved in basal stem cell functions during prostate organogenesis. The lack of active Wnt signaling in basal cells during adult homeostasis coincides with a decrease in basal to luminal differentiation, which supports the hypothesis that downregulation of Wnt activities is important for restricting basal stem cell multipotency in the adult prostate. A primary focus of this dissertation research is to test this hypothesis using inducible transgenic mouse models and investigate how Wnt signaling is influenced by other signals such as the AR signaling pathway in the adult, homeostatic prostate.

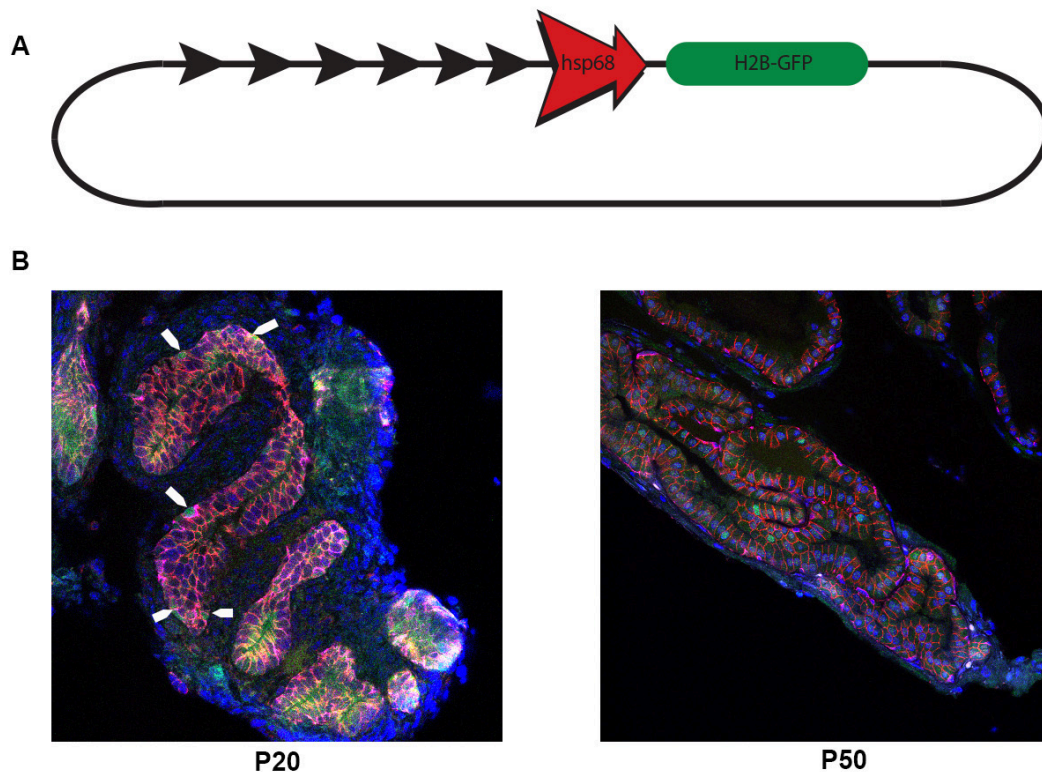


Figure 1.6. Wnt signaling activity in development and adult homeostasis A.) Schematic of the TCF/LEF-GFP reporter construct. Black arrows represent TCF/LEF binding sites. B.) Immunofluorescent images showing TCF/LEF-GFP expression in

prostates of mice at postnatal day 20 and postnatal day 50. White arrows point to GFP+ basal cells.

β -catenin/AR Crosstalk

Both Wnt and androgen receptor signaling pathways are important for regulating prostate development. Recent long-term lineage tracing studies in our lab have shown that cell autonomous AR activity within basal cells is necessary for basal to luminal cell differentiation (Xie *et al.*, 2016). Since signaling crosstalk is crucial for regulating adult stem cells in an organ, we wondered if the canonical Wnt and AR signaling pathways interact in the adult prostate basal cells to regulate their stem cell activities.

Due to the prevalence of Wnt signaling aberrations seen in castration resistant prostate cancer (CRPC) (Yokoyama *et al.*, 2014), multiple studies in the early to mid-2000s investigated interactions between the AR and β -catenin in different CaP cell lines (Table 1.2). To summarize, there is consensus that β -catenin and AR bind to each other via well conserved binding motifs (Song *et al.*, 2003). Binding activity is heavily increased upon activation of AR by addition of DHT. The binding activity leads to a significant increase in nuclear translocation of both transcription factors (very likely in a protein complex). The result of this translocation seems to lead to differing effects on the transcriptome depending on the cell type. Neuronal cell lines exhibit a decrease in AR driven transcription (Powlowski *et al.*, 2002) while prostate cell lines (both PaC cell lines and non PaC cell lines) show a significant increase in AR driven transcription that coincides with increased levels of β -catenin/AR complexes (Yang *et al.*, 2002; Truica *et al.*, 2000; Masiello *et al.*, 2004). Importantly,

there is good consensus from experiments performed in multiple cell lines that this protein complex has an inhibitory effect on expression of Wnt target genes when DHT is present (Powlowski et al., 2002; Chesire and Isaacs, 2002; Song et al., 2003; Schweizer et al., 2008). Interestingly, under low DHT conditions, overexpression of AR leads to enhanced Wnt signaling. This effect is inhibited upon addition of physiological levels of DHT, suggesting that DHT-bound (active) AR is necessary to bind to β -catenin in a manner that inhibits Wnt signaling (Schweizer et al., 2008). Song et al. provided a possible explanation for the inhibitory effect of DHT as they saw that it was possible to reverse this inhibition if excess TCF-4 was added, suggesting that active AR may be competing with TCF/Lef to recruit β -catenin, therefor sequestering it away from its endogenous canonical Wnt binding sites. In support of this hypothesis, Mulholland et al. showed direct evidence that adding DHT to LNCaP cells led to decreased binding between β -catenin and Tcf.

Taken together, these studies support a model in which the AR pathway and the canonical Wnt pathway are intrinsically tied together and share a long evolutionary history of interaction. While the primary goal of these investigations was to gain insight into the mechanisms that underlie castration resistant prostate cancer (CRPC), there is clear evidence to suggest that interaction between these pathways exists outside of the cancer state. Chapter 3 of this thesis delves into this idea and aims to uncover how this interaction may affect basal stem cell activities in the homeostatic prostate.

Publication	Cell Lines Used	Results
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Yang et al., 2002	•LNCaP •CV-1	•Specific interaction in two-hybrid assay and LNCaP cells with addition of DHT •Increase of nuclear translocation of β-catenin upon DHT addition •Increase in minimal AR promoter driven luciferase expression upon addition of β-catenin transcript
Truica et al., 2000	•LNCaP	•β-catenin significantly enhanced androgen-stimulated transcriptional activation by the AR •β-Catenin increased AR transcriptional activation by androstenedione and estradiol and diminished the antagonism of bicalutamide •Coimmunoprecipitation of β-catenin with AR from LNCaP prostate cancer cells showed that the two molecules are present in the same complex. •The amount of β-catenin in complex with AR was increased by androgen.
Powlowski et al., 2002	•GT1-7 GnRH neuronal cells •COS-7 cells •NLT Cells	•Addition of DHT leads to binding, nuclear translocation AR/β-catenin complex •Carboxyl terminus of AR is required to translocate β-catenin. •Addition of DHT reduced expression of Wnt luciferase reporter driven by TCF/Lef promoter •β-catenin/TCF-4 reduced expression of AR luciferase reporter driven by MMTV promoter
Chesire and Isaacs, 2002	•CaP cells: CWR22-Rv1, LAPC-4, DU145 •non CaP cells: HEK-293, TSU, SW480, HCT-116	•β-catenin/TCF-related transcription (CRT), as measured by activation of a synthetic promoter and that of cyclin D1, is inhibited by androgen treatment •CRT suppression depends on transactivation-competent AR in the nucleus, but not on indirect effects stemming from AR target gene expression •Expressed an AR mutant lacking its DNA-binding domain (DBD). This receptor could not orchestrate ligand-dependent CRT repression. •TCF/LEF over-expression counteracts androgen-induced suppression of CRT
Song et al., 2003	•CV-1 Cells	•β-catenin bound to androgen receptor only in the presence of ligand agonists, not antagonists •β-Catenin binding to the androgen receptor LBD was independent of and cooperative with the androgen receptor NTD and the p160 coactivator TIF2, both of which bind to the activation function 2 (AF-2) region of the androgen receptor •Excess androgen receptor could interfere with WNT signaling and excess TCF-4 inhibited the interaction of β-catenin and androgen receptor

Mulholland et al., 2003	•SW480 cells	•Cells transiently transfected with an AR expression construct, treated with DHT and immunoprecipitated for Tcf showed less associated beta-catenin when compared to Tcf precipitates from untreated cells. •In vitro binding assays, with increasing amounts of AR(S35), resulted in decreased Tcf(S35) association with immunoprecipitated recombinant beta-catenin-HIS
Masiello et al., 2004	•LNCaP Cells	•β-catenin coactivated a bicalutamideresponsive mutant AR (W741C) isolated from a bicalutamide-stimulated LNCaP subline •Androgen-stimulated prostate-specific antigen gene expression in LNCaP cells could be modulated by β-catenin, and endogenous c-myc expression was repressed by DHT
Schweizer et al., 2008	•PC3 Cells •CWR22Rv1 Cells •LNCaP cells	•Transient transfections with the AR and multiple components of the Wnt signaling pathway demonstrate that the AR overexpression can potentiate the transcriptional activities of Wnt/β-Catenin signaling. •The presence of physiological levels of androgen or other AR agonists inhibits these effects. •Chromatin immunoprecipitation assays showed that Wnt3A can recruit the AR to the promoter regions of Myc and Cyclin D1.
Samaniego et al., 2015	•MEF Cells •HeLa Cells	•FKBP52 promotes β-catenin interaction with AR and is required for β-catenin co-activation of AR activity in prostate cancer cells

Table 1.2 Review of the literature on Wnt/AR crosstalk

Internal Transcriptome of Basal Cells

Perhaps the rare basal to luminal differentiation events in adulthood are mediated by a small percentage of basal stem cells that retain stem cell capabilities. Intrinsic differences in cells are mostly due to differential transcriptional programming. This programming allows different types of cells to have variable responses to multiple extrinsic signals. Is it possible to detect these rare basal stem cells? Is there a specific set of genes or transcription factors that are responsible for

specifying these cells? Chapter 4 uses single cell RNA sequencing to interrogate these questions.

Stromal AR and luminal cells

The final chapter of this thesis investigates the effect of stromal AR signaling in prostate adult homeostasis. We focus on the role of stromal AR in cancer progression using various mouse prostate cancer models in combination with inducible deletion of stromal cell AR. Again, we take advantage of single cell RNA sequencing to understand how loss of stromal AR affects the aggressiveness of CaP.

Chapter 2. Luminal-contact-inhibition of epithelial basal cell plasticity in prostate organogenesis and homeostasis

[This has been adapted from publication, **Luminal-contact-inhibition of epithelial basal cell plasticity in prostate organogenesis and homeostasis** (Horton et al. 2018)]

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Abstract

Prostate epithelial basal cells are highly plastic in their luminal differentiation capability. Basal stem cells actively produce luminal cells during organogenesis, but become restricted in the adult prostate unless receiving oncogenic or inflammatory stimuli. Given that the number of luminal cells increases relative to basal cells through development and that equilibrium is reached in the adulthood, we hypothesize that a negative-feedback mechanism exists to inhibit basal-to-luminal differentiation. We provide evidence supporting this hypothesis by comparing murine prostatic growth in a tissue reconstitution assay with cell recombinants of different basal-to-luminal ratios. Additionally, in organoid culture, hybrid organoids derived from adjacent basal and luminal cells showed reduced basal stem cell activities, suggesting contact inhibition. Importantly, removal of adult luminal cells in vivo via either an inducible Cre/loxP-Dre/rox dual-lineage-tracing system or orthotopic trypsin injection led to robust reactivation of basal stem cell activities, which acts independent of androgen. These data illustrate the prostate organ as a distinctive paradigm where cell contact from differentiated daughter cells restricts adult stem cell multipotency to maintain the steady-state epithelial architecture.

Introduction

The cell lineage relationship and cell fate transitions in the prostate gland have been under intense investigation recently, because the knowledge is crucial for understanding both prostate development and cancer progression (Davies et al., 2018; Le Magnen et al., 2018; Toivanen and Shen, 2017). The stratified prostate epithelium is comprised of a basal cell layer, a layer of secretory luminal cells, and rare interspersed neuroendocrine cells. In both human and mouse prostate, basal and luminal cells are distinguishable by their different morphologies and molecular marker profiles. Basal cells are triangular or flat-shaped, residing near the basement membrane and expressing marker cytokeratin (CK) 5. In contrast, luminal cells are column-like, residing at the apical side of the epithelium and expressing marker CK18, as well as high levels of the transcription factor Nkx3.1 (Toivanen and Shen, 2017; Xie and Wang, 2017).

Strong evidence supports basal cells behaving as stem cells to generate luminal cells in prostate development. First, epithelial cells in the budding prostate initially show a CK5+CK18+ intermediate cell phenotype before a luminal-specific layer is specified (Toivanen and Shen, 2017; Wang et al., 2001). In this process, basal cells display both symmetrical and asymmetrical divisions leading to different cell fates, while luminal cells only exhibit symmetrical divisions (Wang et al., 2014). Second, basal cells consistently perform better than luminal cells in assays mimicking prostate organogenesis, including the prostate sphere and organoid culture (Chua et al., 2014; Goldstein et al., 2008; Karthaus et al., 2014; Lawson et al., 2007), and the

renal graft-based tissue reconstitution assay (Goldstein et al., 2010; Wang and Shen, 2011; Xin et al., 2003). Finally, mouse genetic lineage-tracing analyses using basal-specific Cre drivers demonstrate that neonatal basal cells efficiently generate luminal cells during postnatal development in vivo (Ousset et al., 2012; Pignon et al., 2013; Wuidart et al., 2016). Recently, sporadic mitochondrial DNA mutations were used to trace human prostate tissues and the data also supported the existence of multipotent basal stem cells (Moad et al., 2017).

Interestingly, basal stem cell functions are highly plastic. Tracing of adult basal cells showed that they are mostly lineage restricted, as basal-to-luminal differentiation is very rare in the mature organ (Choi et al., 2012; Wang et al., 2013). Basal cell plasticity is further demonstrated by their enhanced luminal differentiation under oncogenic (Choi et al., 2012; Lu et al., 2013; Wang et al., 2013) and inflammatory conditions (Kwon et al., 2013). We recently showed that the cell-autonomous androgen receptor is required for basal-to-luminal cell differentiation (Xie et al., 2017), but the mechanism of basal cell plasticity remains poorly understood. Several cues led us to hypothesize that differentiated luminal cells negatively regulate basal stem cell multipotency. First, as more luminal cells are produced, the frequency of basal-to-luminal differentiation decreases through development. Second, purified basal cells appeared to have higher sphere-forming efficiency compared to their counterparts within an unsorted total cell population (Wang et al., 2013). Third, luminal cell anoikis resulting from E-Cadherin loss can lead to an increase of basal cell proliferation, although basal-to-luminal

differentiation has not been definitively shown by lineage tracing (Toivanen et al., 2016). Here, we tested the hypothesis in prostate development using organoid and tissue reconstitution assays, and in the adult prostate by lineage tracing. Our results support a model in which direct basal–luminal cell contact is an essential negative regulator of prostate basal cell bipotentiality.

Results

Luminal cells inhibit prostatic growth from basal cells in tissue reconstitution assay

To test whether there is causality between the increasing number of luminal cells and decrease of basal cell plasticity during prostate development, we mixed fluorescence-labeled luminal and basal cells at different ratios, and analyzed the growth of prostatic tissues using the tissue reconstitution assay (Fig. 2.1A). As we have done previously (Xie et al., 2017), total basal cells were obtained by flow-sorting of YFP⁺ cells from *CK5-CreER^{T2}; R26R-CAG-YFP/+* mice that were tamoxifen-induced at 8 weeks of age (Fig. S2.1A). To isolate luminal cells, we flow-sorted RFP⁺ cells from tamoxifen-induced *CK18-CreER^{T2}; Ai9/+* mice (Madisen et al., 2010; Van Keymeulen et al., 2009) (Fig. S2.1B), in which luminal cells were specifically marked by tdTomato upon induction (Fig. S2.1C). We then mixed the two sorted cell populations at basal-to-luminal ratios of 1:0, 1:0.2, 1:1, and 1:5, to mimic the epithelial cell composition at various developmental stages from prostate budding to adulthood. The mixed cells were recombined with rat urogenital sinus mesenchyme (UGSM) cells and grafted under the renal capsule of nude mice. Since the renal grafting assay is not conducive to prostatic tissue growth from luminal cells

(Lukacs et al., 2010; Xin et al., 2003), we fixed the basal cell number at 5000 in each cell recombinant, so that the influence of luminal cell number on basal cell activities could be compared. Mixed basal and luminal cells organized into small tubules within 7 days of growth (Fig. 2.1B,C). TUNEL staining revealed that most basal cells were not apoptotic in the grafts, while ~40% of luminal cells already showed positive signals by 1 day of growth, and luminal apoptosis persisted at 7 days (Fig. 2.1B,C; Fig. S2.1D). These data suggest that grafted luminal cells were continuously being eliminated due to unfavorable assay conditions. After 2 months, most of the grown grafts were YFP⁺, and RFP⁺ cells were not found (Fig. 2.1D,F), confirming their basal cell origin. On the other hand, small dots of YFP⁺ signals could be found in grafts that failed to grow (Fig. 2.1E,F). We then tested whether the grafted luminal cells, while alive, had any effect on basal-derived prostatic tissues by measuring the sizes of the YFP⁺ tubules 2 months after grafting. We found a trend of higher luminal fraction correlating with smaller prostatic size (Fig. 2.1F,G). Notably, the 1:5 group contained significantly smaller grafts than the 1:0 group ($P=0.016$ by the Mann–Whitney U -test), as many more grafts failed to grow (Fig. 2.1G). The median sizes in the 1:0.2 and 1:1 groups were also smaller than the pure basal group, although the differences did not reach significance. These data support that luminal cells negatively affect basal cell plasticity during prostate organogenesis. Since YFP⁺ basal cells represent the total basal population (Xie et al., 2017), we next tested whether the effect can be observed by using the Trop2⁺ basal cells (Fig. 2.1H), a stem cell population enriched for robust renal graft activity (Goldstein et al., 2008). Indeed,

adding luminal cells significantly suppressed tubule growth from Trop2⁺ basal cells (Fig. 2.1I), confirming they directly inhibit basal stem cells in organogenesis.

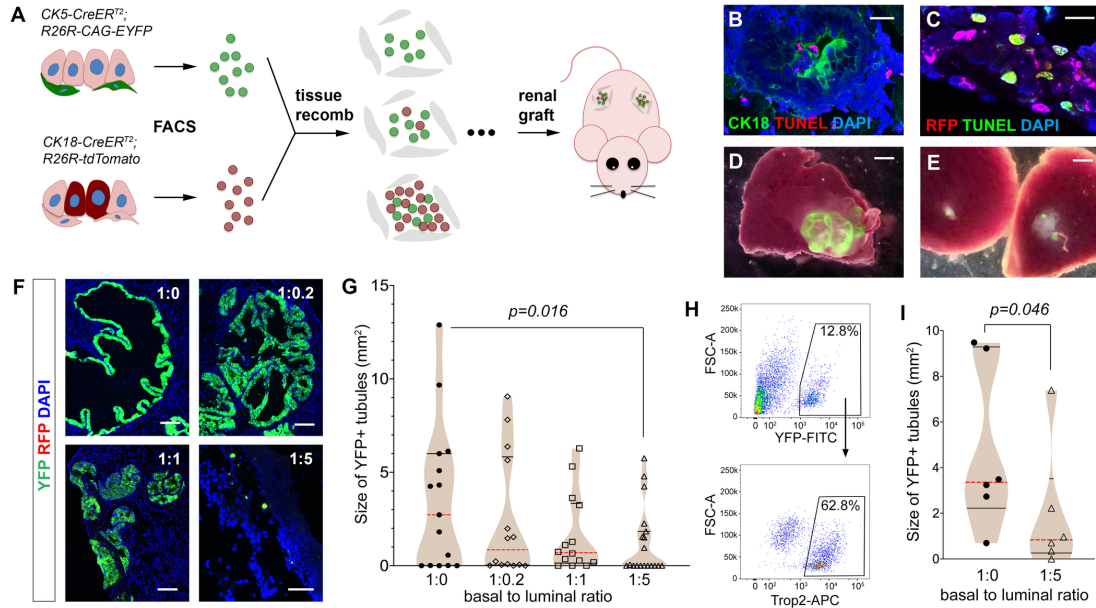


Figure 2.1 Analyzing the effects of mixing basal and luminal cells in prostate tissue reconstitution assay.(A) Diagram of renal grafting experiments with different ratios of basal and luminal cells. (B,C) IF and TUNEL staining of day 7 renal graft showing apoptotic signals in a fraction of luminal cells marked by CK18⁺ (B) or RFP⁺ (C). Scale bars: 20 μ m. (D,E) YFP and white field overlay dissection images showing a graft of the 1:0.2 ratio (D) and grafts of the 1:5 ratio (E). Scale bars: 1 mm. (F) Representative images showing direct visualization for YFP and RFP signals in sectioned grafts. Scale bars: 100 μ m. (G) Violin plot comparing YFP⁺ tubule sizes for all the grafts with different basal-to-luminal ratios by Mann–Whitney *U*-test. Black lines show quartiles and red dashed lines show medians. (H) FACS gating for sorting Trop2⁺ basal cells. (I) Violin plot comparing sizes of YFP⁺ tubules derived from Trop2⁺ basal cells in the cell mixture experiment by Mann–Whitney *U*-test.

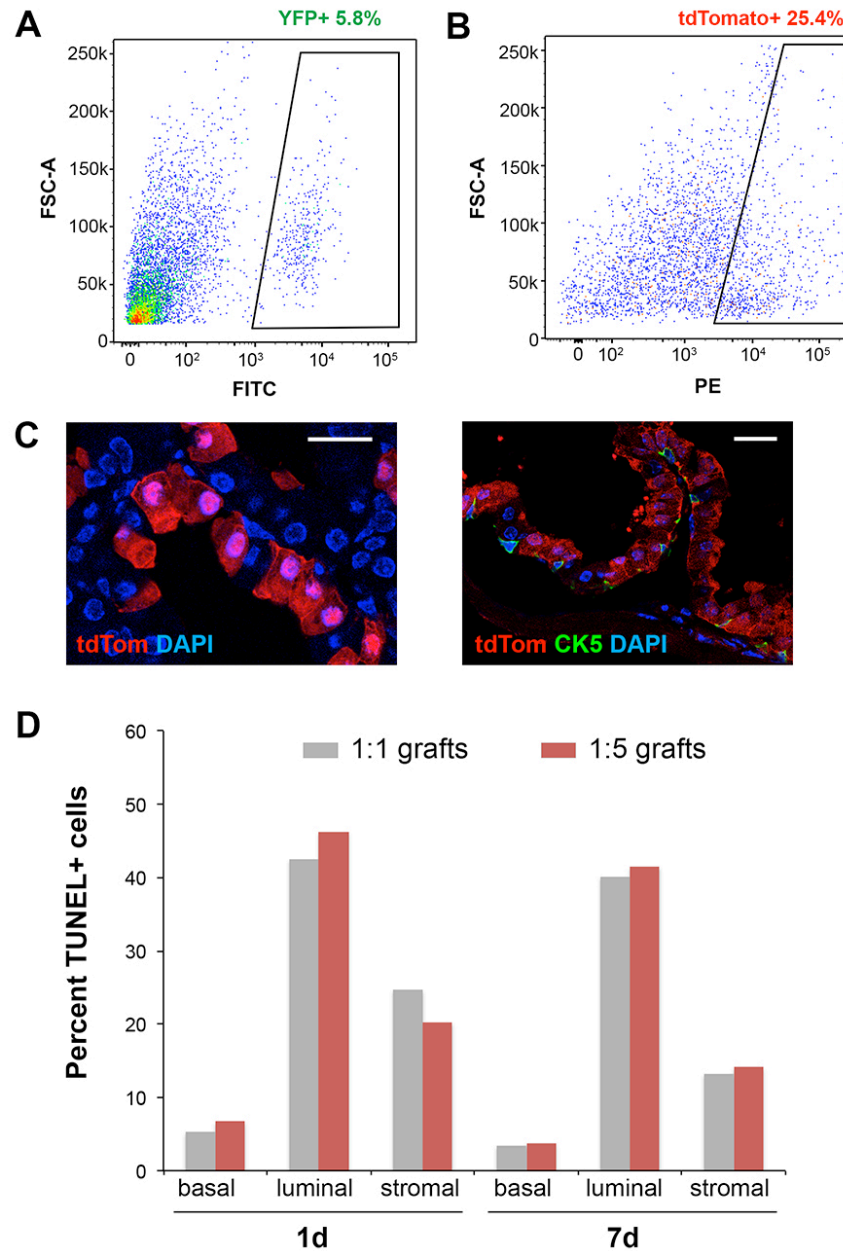


Figure S2.1. Sorting basal and luminal cells for renal grafting. (A, B) FACS gating for YFP+ basal cells (A) and tdTomato+ luminal cells (B) from induced CK5-CreERT2; R26R-CAG-YFP/+ mice and CK18-CreERT2; Ai9/+ mice, respectively. (C) Direct visualization of tdTomato and IF staining with CK5 antibody in CK18-CreERT2; Ai9/+ mice showing specific marking of luminal cells after induction. Scale bars, 20µm. (D) Quantitation of apoptotic cells per cell type for all the 1:1 grafts and 1:5 grafts at 1d and 7d post renal grafting.

Luminal-basal cell contact suppresses basal cell bipotentiality in organoids

The luminal inhibitory effect could be due to luminal-secreted paracrine signals. In the case of renal graft assay, apoptotic luminal cells might send death signals. Alternatively, luminal cells may inhibit basal cells through direct cell contact. To distinguish between these two possibilities, we resorted to prostate organoid culture (Drost et al., 2016; Karthaus et al., 2014) for cell mixing experiments. This assay models prostate organogenesis *in vitro*, but it offers high resolution of prostatic tubule growth from individual cells and, unlike the renal graft assay, is conducive to luminal cell growth (Chua et al., 2014; Karthaus et al., 2014). YFP⁺ basal cells and RFP⁺ luminal cells were sorted and plated in 1:0 (pure basal), 1:1, and 1:5 ratios under organoid culture conditions. Basal cell number was fixed at 5000 in each of the four wells of each ratio (Fig. 2.2A). Eight days after culture, YFP⁺ basal-derived organoids were present in all the wells, while the 1:1 and 1:5 wells also contained RFP⁺ luminal-derived organoids as well as hybrid organoids that comprised intermingled YFP⁺ and RFP⁺ cells (Fig. 2.2B). The numbers of each type of organoids were quantified for each ratio, and adding luminal cells did not affect the formation efficiency of basal-derived organoids (Fig. 2.2C). As expected, CK5 and CK18 staining revealed that extensive basal-to-luminal cell differentiation occurred in pure basal-derived organoids (Fig. 2.2D), and vice versa in pure luminal-derived organoids (Fig. 2.2E). No neuroendocrine cells were detected. In contrast, the hybrid organoids likely developed from coordinated actions of single basal and luminal cells plated in close proximity, as they usually contained YFP⁺ cells on the outer layer

surrounding inner RFP⁺ cells (Fig. 2.2F). Interestingly, those YFP⁺ cells remained CK5⁺ and the RFP⁺ cells remained CK18⁺ (Fig. 2.2F), indicating cell lineage conversion is inhibited when the two cell types are in close contact. Furthermore, when we compared the sizes of YFP⁺ regions between basal-derived organoids and hybrid organoids, we found that those of the hybrid organoids were significantly smaller ($P < 0.001$ by the Mann–Whitney *U*-test) (Fig. 2.2G), indicating that basal stem cells on average underwent fewer rounds of cell division in the hybrids.

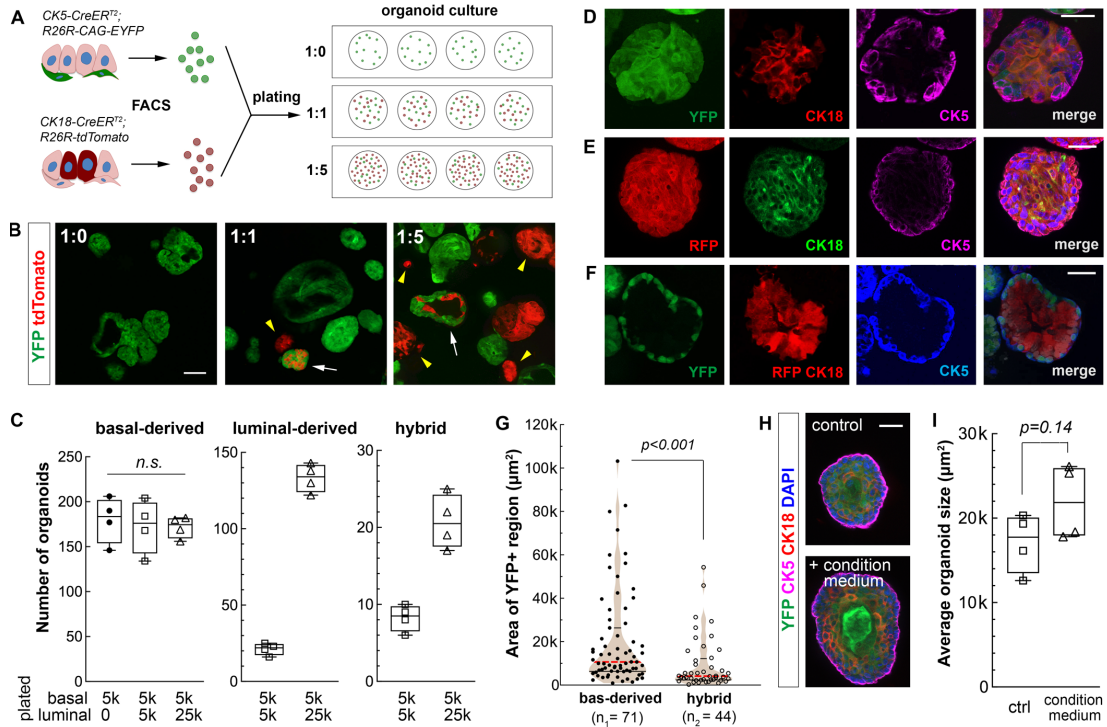


Figure 2.2. Effects of co-culturing luminal cells on basal stem cell activities in prostate organoid assay. (A) Diagram of organoid culture experiments plated with different ratios of basal and luminal cells. (B) Direct visualization of YFP and RFP signals in organoids under different ratio conditions. White arrows, hybrid organoids. Yellow arrowheads, luminal-derived organoids. Scale bar: 100 μ m. (C) Box plot comparing organoid numbers for each type per well ($N=4$) under different basal-luminal mixture conditions. n.s., not significant by Student's *t*-test. (D–F) IF images showing basal-to-luminal differentiation in a basal-derived organoid (D), luminal-to-basal differentiation in a luminal-derived organoid (E), and cell lineage restriction in

a hybrid organoid (F). Scale bars: 50 μm . (G) Violin plot comparing YFP⁺ areas between basal-derived organoids and hybrid organoids by Mann–Whitney *U*-test. Black lines show quartiles and red dashed lines show medians. (H) Representative IF images showing basal-derived organoid cultured with standard medium or luminal-conditioned medium. Scale bar: 50 μm . (I) Box plot comparing average size of basal-derived organoid per well ($N=4$) with and without luminal-conditioned medium by Student's *t*-test.

To directly test whether luminal cells secrete any inhibitory factors, we cultured pure RFP⁺ luminal cells in the organoid assay for 4 days, and transferred the condition medium to pure basal organoid culture. Compared to controls using standard medium, adding the luminal condition medium actually slightly increased the size and luminal differentiation (Fig. 2.2H,I), suggesting luminal inhibitory effects are not mediated in a paracrine fashion. Finally, to test whether basal–basal cell contact has any inhibitory effect, we obtained unmarked average basal cells by sorting from wild-type mice using cell surface markers Lin[−]Sca-1⁺CD49f^{hi} (Lawson et al., 2007; Lukacs et al., 2010) (Fig. S2.2A). We then mixed YFP⁺ basal cells with these unmarked basal cells in 1:5 ratio in the organoid assay (Fig. S2.2B). In the resulting green-white basal hybrid organoids, we frequently observed basal-to-luminal differentiation by CK5 and CK18 staining (Fig. S2.2C). Moreover, the sizes of YFP⁺ regions in those hybrids were not significantly different from pure YFP⁺ organoids (Fig. S2.2D). Taken together, our organoid results support that basal stem cell activities are inhibited by direct luminal cell contact.

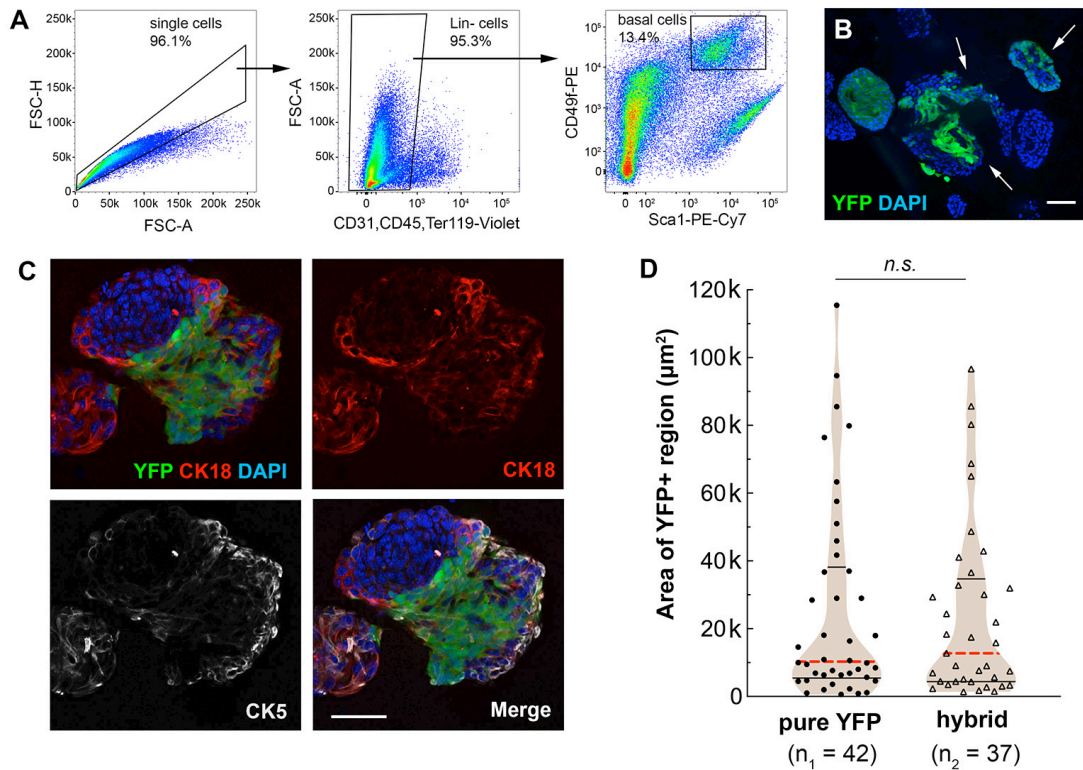


Figure S2.2. Organoid assay with mixture of YFP+ basal cells and unmarked basal cells. (A) FACS gating for sorting average basal cells based on Lin-Sca-1+Cd49fhi. (B) Direct visualization of YFP in organoids derived from green and white basal cell mixture. Arrows point to green-white basal hybrid organoids. Scale bar, 50 μm . (C) IF staining showing YFP+ basal cells can differentiate into luminal cells in the hybrid organoids. Scale bar, 50 μm . (D) Violin plot comparing area of YFP+ regions between pure-YFP+- basal-derived organoids and hybrid organoids. Black lines, quartiles. Red dashed lines, medians. n.s., not significant by Mann-Whitney U Test.

Increased basal cell proliferation upon luminal cell ablation in the adult prostate

After demonstrating an inhibitory role of luminal cells on basal stem cell activities in prostate organogenesis, we next tested whether ablating luminal cells in the adult epithelium reactivates basal cell multipotency. To selectively induce luminal cell death, we used the *Nkx3.1^{CreERT2/+}* driver (Wang et al., 2009) to activate the expression of the cytotoxic protein diphtheria toxin A-chain (DTA) from the *Rosa26*

locus (Wu et al., 2006). *Nkx3.1^{CreERT2/+}; R26R^{DTA/+}* mice were tamoxifen-induced for 4 consecutive days at 2 months of age, and analyzed 1 day, 7 days, 14 days and 28 days later (Fig. 2.3A). Mice without tamoxifen induction were used as negative controls. One day after induction, we performed TUNEL and CK5 staining, and found that 34.2% of luminal cells, but not basal cells were apoptotic (Fig. 2.3B). Disruption of the luminal layer was also visualized by H&E staining (Fig. 2.3C). Staining with CK5 and Ki67 antibodies revealed significantly increased cell proliferation in both the basal and luminal layers (Fig. 2.3D,E), which was absent in control mice. Notably, in many foci that presumably had undergone severe luminal cell loss, multiple CK5⁺ cells were stacked into continuous sheath of cells, some of which turned round-shaped and showed a CK5⁺CK18⁺ intermediate cell phenotype (Fig. 2.3F). The proportion of CK5⁺CK18⁺ intermediate cells among basal cells was 7.8%, significantly higher than the 1.7% in the control (Fig. 2.3G). We observed similar but more modest phenotypes 7 days post induction. At this stage, stacked basal cells were still present, and were more proliferative than normal, albeit with a reduced rate compared to 1 day (Fig. 2.3D,E). By 14 days post induction, the prostate displayed normal morphology (Fig. 2.3C), and the proliferation rates for both basal and luminal cells had decreased back to normal and remained so at 28 days post induction (Fig. 2.3D,E). The transient burst of basal and luminal cell proliferation upon luminal cell death was also confirmed by performing the BrdU incorporation assays in the first and fourth weeks after tamoxifen induction (Fig. 2.3A,H). Notably, increased proliferation was not due to inflammation-induced mechanisms (Kwon et

al., 2016, 2013), since leukocyte or macrophage levels in the tissue was not elevated 1 day after induction (Fig. S2.3A,B), and treating the mice with aspirin alongside tamoxifen did not suppress proliferation (Fig. S2.3C,D). Taken together, our results show that, both basal and luminal cells can promptly respond, via over-proliferation, to the epithelium damage caused by luminal cell death, and that the bulk of the repair process takes place within a week. The increase of CK5⁺CK18⁺ intermediate cells at the apical side of the epithelium support that basal stem cell activities were reactivated to enhance basal-to-luminal differentiation in the repair process, although the possibilities that preexisting intermediate cells underwent expansion or the surviving luminal cells dedifferentiated into an intermediate phenotype cannot be ruled out.

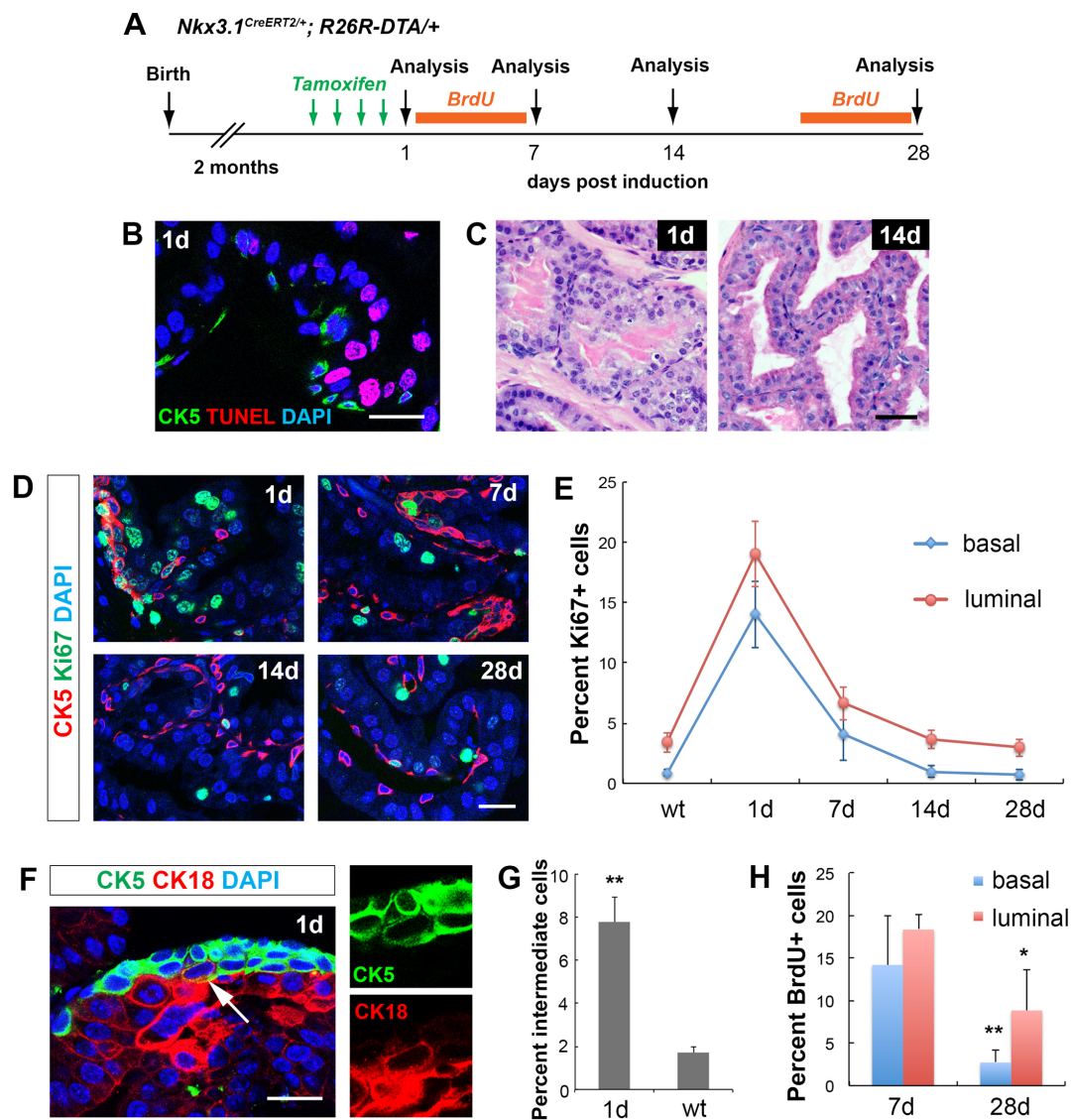


Figure 2.3. Cell dynamics during the epithelial repair process upon luminal cell ablation. (A) Timeline of experiments of luminal cell-specific ablation. (B) IF and TUNEL staining 1 day after tamoxifen induction. Scale bar: 20 μ m. (C) H&E images showing 1 day and 14 days prostate epithelium. Scale bar: 50 μ m. (D) IF images showing proliferating cells in basal and luminal cells at different time points of tissue repair. Scale bar: 20 μ m. (E) Quantitation of Ki67 index in the basal and luminal cell populations at different time points of tissue repair. (F) IF image showing basal cell over-proliferation and CK5⁺CK18⁺ intermediate cells (arrow) at 1 day. Scale bar: 20 μ m. (G) Comparison of intermediate cell percentage between 1 day and wild-type control. (H) Quantitation of basal and luminal cell proliferation by BrdU incorporation assay during tissue repair. Error bars in E,G,H correspond to one s.d. $N=3$ animals per time point. ** $P<0.001$; * $P<0.01$ by two-tailed Student's t -test.

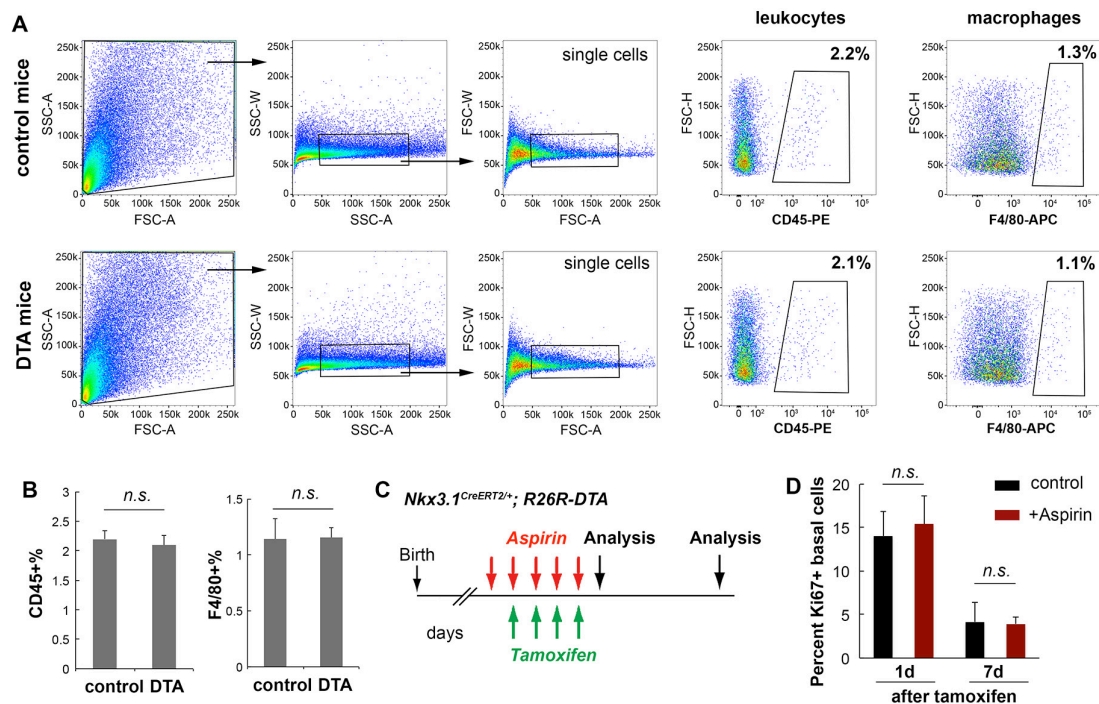


Figure S2.3. Testing potential influence of inflammation response on basal cell proliferation. (A) FACS gating for sorting CD45+ leukocytes and F4/80+ macrophages from control un-induced mice and *Nkx3.1CreERT2/+; R26RDTA/+* mice one day after induction. (B) Quantitation of percentage of leukocytes and macrophages among prostate cells in control and induced *Nkx3.1CreERT2/+; R26RDTA/+* mice. N=3 biological replicates. n.s., not significant by student's t-test. (C) Timeline of aspirin treatment experiment. (D) Quantitation of percentage of proliferating basal cells showing aspirin treatment had no effect both 1d and 7d after induction. N=3 biological replicates per group. n.s., not significant by student's t-test.

Dre-rox lineage-tracing of basal cells demonstrates their reactivated multipotency

To definitively test whether basal-to-luminal cell differentiation occurs upon luminal cell death, we performed basal cell lineage tracing in this context. Since *Cre-loxP* was used for luminal DTA expression, to trace basal cells in the same tissue, we adopted the *Dre-rox* system (Fig. 2.4A), in which the Dre recombinase can bind to *rox* sites and mediate genomic recombination (Sauer and McDermott, 2004). Dre fused to a progesterone receptor (PR) ligand binding domain was shown to respond to

the drug RU486 and could turn on reporter expression in mouse and zebrafish embryos without overlapping activities with Cre (Anastassiadis et al., 2009; Park and Leach, 2013). For marking of adult basal cells, we built a *CK5-DrePR* construct (Fig. S2.4A), and obtained seven transgenic founder lines upon pronuclear microinjection. The lines were then crossed to the *R26-rox-stop-rox-LacZ* reporter mice (Anastassiadis et al., 2009) to test their efficacy. *CK5-DrePR; R26-rox-stop-rox-LacZ* mice (denoted Bas^{LacZ}) were administered with RU486 for 5 consecutive days and analyzed 1 week later. IF staining showed variable degree of basal cell expression and luminal cell leakage for all the lines (Fig. S2.4B). The best line #21 had robust basal cell DrePR activity with 28.8% of basal cells ($n=418/1453$, three animals analyzed) labeled by LacZ (Fig. 2.4B), and relatively low luminal cell leakage with 2.1% of luminal cells ($n=50/2384$, three animals analyzed) also labeled (Fig. 2.4C). This line was chosen for all subsequent experiments.

Next, we generated *Nkx3.1^{CreERT2/+}; CK5-DrePR; R26R-DTA/rox-stop-rox-LacZ* mice (denoted Lum^{DTA}; Bas^{LacZ}) and performed RU486 induction at 8 weeks of age (Fig. 2.4D). One week later, these mice had the same phenotypes as the Bas^{LacZ} mice and showed no signs of luminal cell apoptosis, confirming the non-overlapping activities of the Cre and Dre systems. The labeling ratios for basal and luminal cells were 31.7% and 1.9%, respectively, consistent with the numbers in the Bas^{LacZ} mice (Fig. 2.4E). We tamoxifen-induced the Lum^{DTA}; Bas^{LacZ} mice 2 weeks after RU486 treatment, and analyzed the prostate 2 weeks and 9 weeks later (Fig. 2.4D). At 2 weeks post tamoxifen induction (13-weeks-old), we observed that

the ratio of LacZ⁺ basal cells remained unchanged (Fig. 2.4E), indicating LacZ labeling of basal cells was representative. However, the ratio of LacZ⁺ luminal cells over all luminal cells significantly increased to 8.7% ($n=314/3598$, three animals analyzed, $P=0.0018$ by two-tailed Student's t -test) (Fig. 2.4E). No neuroendocrine cells were marked. We concluded that the increase of luminal cell labeling was due to basal-to-luminal differentiation rather than an expansion of the existing leaked luminal cells, because the LacZ⁺ luminal cells were not more proliferative than the unlabeled ones as shown by Ki67 staining (Fig. S2.4C,D), and because the ratio of labeled luminal cells did not further increase in homeostasis as measured at 9 weeks post tamoxifen induction (20-weeks-old) (Fig. 2.4E). After epithelial repair, clusters of adjacent cells that comprised both LacZ⁺ basal and luminal cells were present (Fig. 2.4F), representing clones of reactivated basal stem cells. Importantly, when we performed these experiments using the *Nkx3.1^{CreERT2/+}; CK5-DrePR; R26-rox-stop-rox-LacZ* control mice (denoted Lum^{WT}; Bas^{LacZ}), no increase of LacZ⁺ luminal cell ratios was observed at either 2 weeks or 9 weeks post tamoxifen induction (Fig. 2.4E), further supporting that ablation of luminal cells triggers the bipotent differentiation of basal cells.

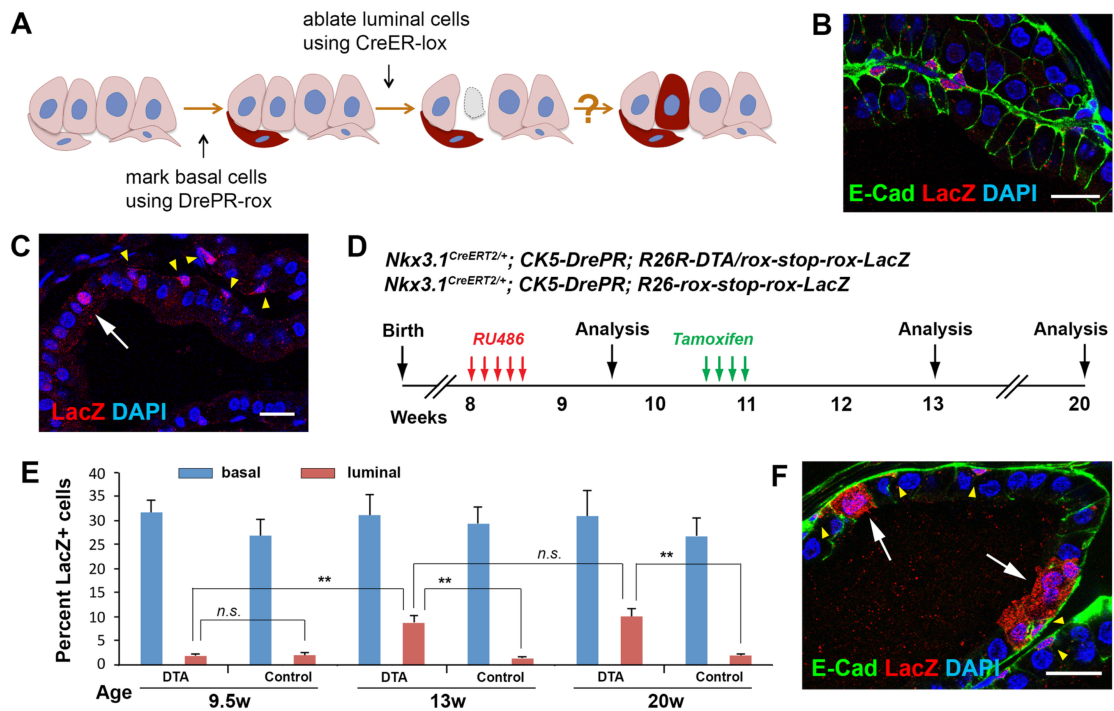


Figure 2.4. Lineage-tracing of basal cells upon luminal cell DTA ablation. (A) Diagram of using the dual-lineage system to trace basal cells. (B) IF showing basal cell labeling upon RU486 treatment of Bas^{LacZ} mice. (C) IF showing a leaked luminal cell (white arrow) that was labeled together with basal cells (yellow arrowheads). (D) Timeline of lineage tracing experiments in Lum^{DTA}; Bas^{LacZ} and Lum^{WT}; Bas^{LacZ} mice. (E) Quantitation of percentages of LacZ⁺ cells in basal and luminal cells at different time points in Lum^{DTA}; Bas^{LacZ} (DTA) and Lum^{WT}; Bas^{LacZ} (control) mice. *N*=3 animals per time point in each cohort. Error bar, one s.d. ***P*<0.001 by two-tailed Student's *t*-test. (F) IF showing LacZ⁺ cell clusters that contained differentiated luminal cells (white arrows). Yellow arrowheads point to LacZ⁺ basal cells in isolation or within the clusters. Scale bars in B,C,F correspond to 20 μ m.

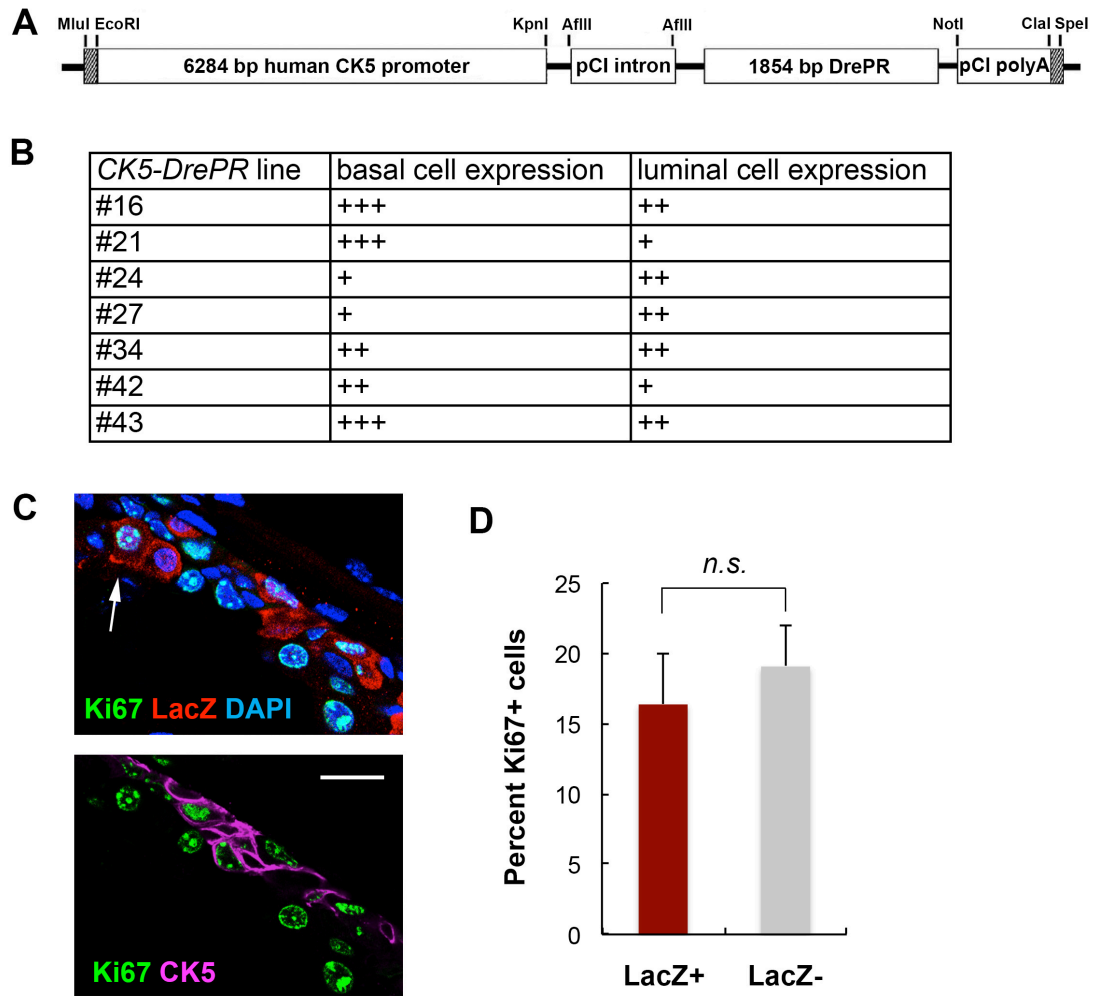


Figure S2.4 Generation and characterization of the Dre-rox lineage tracing system. (A) Schematic diagram of the CK5-DrePR construct for generation of transgenic mice. (B) Table summary of expression levels in prostate basal and luminal cells for all the CK5-DrePR founder lines. “+++”, strong and high-frequency expression; “+”, sporadic expression. (C) Representative IF image showing presence of Ki67+ cells in both LacZ+ (arrow) and LacZ- luminal cells 1d after tamoxifen induction in LumDTA; BasLacZ mice. Scale bar, 20 μ m. (D) Quantitation of Ki67 index between the LacZ+ and LacZ- luminal cells. N=3 animals per group. n.s., not significant by student’s t-test.

Basal cell reactivation upon trypsin injection into intact or regressed prostate

Besides the DTA-ablation context, we also developed a method to chemically remove luminal cells from the prostate epithelium and test basal cell behaviors using *CK5-CreER^{T2}* lineage tracing, which is strictly basal-specific (Wang et al., 2013). Seven days after inducing *CK5-CreER^{T2}; R26R-CAG-YFP* mice, we orthotopically injected 30 μ l 0.05% trypsin into the prostate (see Materials and Methods), and analyzed prostate morphology 8 h, 1 day, 2 days, 1 week and 2 weeks later (Fig. 2.5A). We optimized the trypsin dosage so that it could induce significant luminal cell anoikis in localized regions while leaving the basal layer mostly intact (Fig. 2.5B). Significant luminal cell anoikis was already observed at 8 h post injection and persisted for the first 2 days. Robust cell proliferation was observed at 1 day and 2 days after injection, but was decreased by 1 week, by which time the majority of the epithelium had been repaired (Fig. 2.5B,C). Importantly, lineage-marked $YFP^{+}CK18^{+}$ luminal cells, which were non-existent before trypsin injection, were readily found in clusters 2 weeks after injection (Fig. 2.5D,E). Therefore, luminal layer damage induced by multiple approaches can stimulate basal-to-luminal cell differentiation for tissue repair.

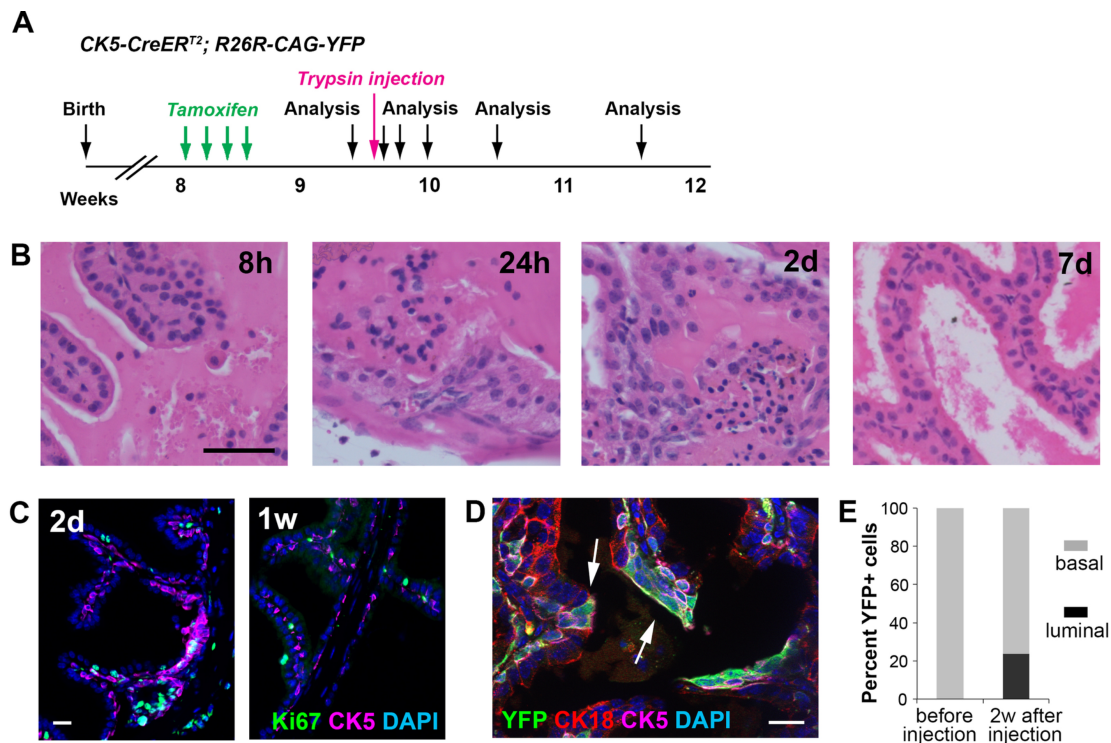


Figure 2.5. Lineage-tracing of basal cells upon trypsin-induced luminal cell anoikis. (A) Timeline of lineage tracing experiment under the trypsin injection condition. (B) H&E images showing luminal cell anoikis and basal cell over-proliferation in the epithelium at early time points after trypsin injection and relatively normal epithelium by 1 week post injection. (C) IF showing proliferating cells 2 days and 1 week after trypsin injection. (D) IF showing YFP⁺ luminal cells (arrows) and repaired epithelium 2 weeks after trypsin injection. (E) Quantitation of basal and luminal proportions of YFP⁺ cells before and 2 weeks after trypsin injection. Scale bars in B correspond to 50 μ m, and in C,D to 20 μ m.

Finally, we tested whether basal cell reactivation upon luminal layer damage is dependent on androgen, since previous grafting studies showed that androgen could promote basal cell proliferation through stromal paracrine signals (Gao et al., 2001; Hayward et al., 1992). We found that in mice that had undergone castration immediately followed by trypsin injection (Fig. 2.6A), large numbers of CK5⁺CK18⁺ intermediate cells emerged during the first week of epithelium repair (Fig. 2.6B). Increased proliferation in basal and luminal cells was also observed

(Fig. 2.6C,D) in similar dynamics to that of hormonally-intact prostates in Fig. 2.3. In addition, we also performed analysis 2 days after trypsin injection in mice that had undergone prostate regression for 1 week (Fig. 2.6E), and still observed similar phenotypes (Fig. 2.6F,G). Therefore, the reactivation of basal cell multipotency upon luminal cell death involves an intrinsic mechanism regulating the prostate epithelial integrity, which is androgen-independent.

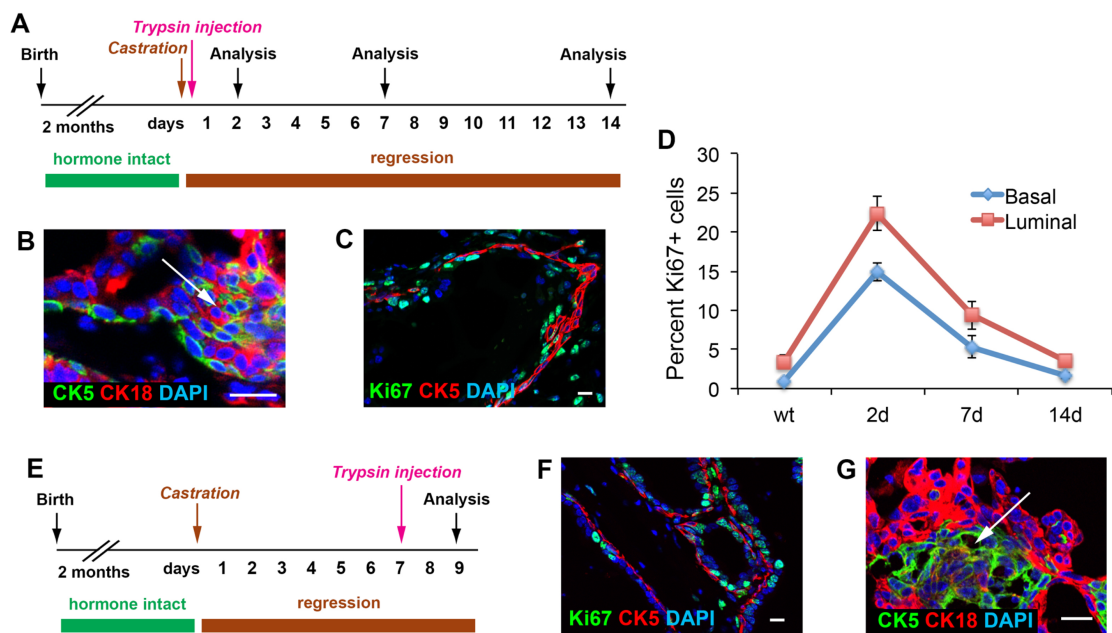


Figure 2.6. Analysis of basal cells after trypsin injection in androgen-deprived prostate. (A) Timeline of experiment of simultaneous trypsin injection and castration. (B,C) IF images showing emergence of intermediate cells (B, arrow) and high proliferation rate in both basal and luminal layers (C) 2 days after trypsin injection. (D) Quantitation of Ki67 index in the basal and luminal cell populations at different time points of tissue repair. N=3 animals per time point. Error bars correspond to one s.d. (E) Timeline of experiment of trypsin injection 1 week after mice castration. (F,G) IF images showing high proliferation rate in both basal and luminal layers (F) and emergence of intermediate cells (G, arrow) 2 days after trypsin injection. Scale bars in B,C,F,G correspond to 20 μ m.

DISCUSSION

Stem cell plasticity is a prominent feature in multiple mammalian organs, since tissue stem cells need to promptly respond to tissue injury while resisting uncontrolled proliferation and differentiation. Both cell-intrinsic transcription factors and external niche signals regulate the decision-making of a tissue stem cell (Blanpain and Fuchs, 2014; Ge and Fuchs, 2018; Wells and Watt, 2018). Unlike some exemplary tissues for studying stem cell plasticity such as skin and small intestine, the mature prostate epithelium is mostly quiescent, and has a relatively simple and rigid architecture of two layers of cells. This may necessitate luminal cells to serve as an important negative niche factor regulating basal stem cell plasticity, counteracting positive stromal signals such as the androgen and Wnt pathways that promote basal cell stemness (Julio et al., 2013; Simons et al., 2012; Xie et al., 2017). This study, using multiple *in vivo* approaches, is the first to definitively show that removal of luminal cells from the adult prostate epithelium induces basal cell multipotency. Our organoid data show that adding luminal cells could directly inhibit basal cell bipotentiality through luminal-basal cell contact. Therefore, the prostate epithelium represents a distinctive paradigm where differentiated daughter cells restrict, rather than support, the multipotency of a tissue stem cell. The mechanisms regulating basal cell reactivation upon luminal cell ablation are likely to be multifaceted. For example, releasing mechanical tension has been shown to promote cell division through Piezo1-dependent pathway (Gudipaty et al., 2017). This might explain the over-proliferation phenotype seen after luminal ablation. It could also explain the different

basal cell behaviors between postnatal and adult stages, since young basal cells, particularly those at the budding tip and branching regions, conceivably receive more mechanical stretch and less luminal contact. Alternatively, inhibition might also work through modulation of extracellular matrix and actin cytoskeleton in basal cells to influence their fate decisions, as studies in other tissues have shown that mechanical activation of YAP activity can promote cell stemness (Panciera et al., 2016; Totaro et al., 2017; Yui et al., 2018). Another possibility is that luminal cell contact potentiates TGF- β signaling in basal cells, as TGF- β /SMAD activation has been associated with basal stem cell quiescence in diverse epithelial tissues including prostate (Mou et al., 2016; Valdez et al., 2012). Future research will test these and other possible mechanisms. It will also be interesting to see whether such heterotypic cell–cell interactions occur in other quiescent epithelial tissues as well.

Our findings could have important implications for prostate cancer. It is well established that oncogenic stimuli such as Pten loss in basal cells promote basal stem cell hyper-activation and luminal differentiation (Choi et al., 2012; Wang et al., 2013). How PI3K pathway activation eventually overrides luminal-contact-inhibition awaits further investigation. Harnessing this information may prove to be a promising therapeutic means for intervening with prostate cancer progression, since luminal differentiation from Pten-deleted basal cells is a slow process (Wang et al., 2013), implying a tug of war between the two forces. On the other hand, treatment that kills a fraction of cancer cells might create vacuum for otherwise more dormant cancer cells to be reactivated, if such negative regulation is inherited and preserved in a

tumor. Recent example in colon cancer showed that plasticity enabled tumor maintenance despite Lgr5⁺ cancer cell ablation (de Sousa e Melo et al., 2017). It remains to be seen whether analogous mechanism exists in prostate cancer.

MATERIALS AND METHODS

Mouse lines and generation of CK5-DrePR

The *CK5-CreER^{T2}*, *CK18-CreER^{T2}*, *Nkx3.1^{CreERT2/+}*, *R26R-CAG-YFP*, *Ai9*, *R26R^{DTA/+}*, and *R26-rox-stop-rox-LacZ* mouse lines were described previously, or obtained from JAX. The *CK5-DrePR* transgenic line was generated by pronuclear injection of fertilized eggs at Cyagen Biosciences with the *CK5-DrePR* construct, which incorporates a 6.3-kb CK5 promoter (GRCh37/hg19 chr12:52,914,147-52,920,430), a chimeric intron, the DrePR fusion gene sequence (Anastassiadis et al., 2009), and polyA sequence. The *CK5-DrePR* construct was built at GenScript using the pUC57 vector. Animals were maintained in C57BL/6N or mixed background. Genotyping was performed by PCR using tail genomic DNA, with primer sequences listed in Table S2.1. All animal experiments received approval from the Institutional Animal Care and Use Committee at UCSC.

Tamoxifen and RU486 induction and aspirin treatment

For tamoxifen induction, mice were administered 9 mg per 40 g body weight tamoxifen (Sigma-Aldrich) suspended in corn oil by oral gavage once daily for 4 consecutive days. For RU486 treatment, mice were administered 1.6 mg per 40 g body weight RU486 (VWR) suspended in corn oil by oral gavage once daily for 5 consecutive days. For aspirin treatment, O-Acetylsalicylic acid (Thermo Fisher

Scientific, Cat No. AC158180500) was dissolved in equimolar NaHCO₃ solution, and administered through oral gavage at 20 mg once daily. Treatment started 1 day before tamoxifen induction, and lasted 5 consecutive days.

Prostate orthotopic trypsin injection

For luminal cell detachment from the prostate epithelium, trypsin solution was orthotopically injected into anesthetized mice. Briefly, a horizontal incision was made at the abdomen above the bladder and the seminal vesicles with their attached anterior prostate lobes were exposed. 30 µl 0.05% trypsin was then slowly injected into the prostate lobes using syringe (Hamilton 7656-01) and 33-gauge 0.5 inch Hamilton needle (Cat No. 89221-012) with the assistance of anatomical lens, before the incision was sutured.

BrdU incorporation assay

BrdU (Sigma-Aldrich) was dissolved in PBS (10 mg/ml) and administered by intraperitoneal injection twice daily (0.1 ml per dose) for 7 consecutive days during tissue repair to label proliferating cells.

Prostate dissociation and flow cytometry

To isolate prostate basal and luminal cells, prostate tissues were dissected and minced to small clumps, followed by enzymatic dissociation with 0.2% collagenase I (Invitrogen) in DMEM media with 5% FBS for 3 h at 37°C. Tissues were digested with 0.25% Trypsin-EDTA (StemCell Technologies) for 1 h at 4°C, passed through 21- to 26-gauge syringes and filtered through a 40-µm cell strainer to obtain single-cell suspensions. Dissociated prostate cells were suspended in Hanks' Balanced Salt

Solution Modified/2% FBS. ROCK inhibitor Y-27632 (StemCell Technologies) was added at 10 μ M throughout the whole process to inhibit luminal cell death. Lineage-marked basal and luminal cells were sorted based on YFP and tdTomato positivity, respectively. Antibodies used for sorting of Trop2⁺ basal cells and Lin⁻Sca-1⁺CD49f^{hi} basal cells, leukocytes, and macrophages are listed in Table S2. Sorting was performed on a BD FACS Aria II instrument in the Flow Cytometry Shared Facility of UCSC.

Tissue reconstitution/renal graft assay

Flow-sorted basal and luminal cells were mixed at different ratios and then mixed with 2.5×10^5 dissociated urogenital sinus mesenchyme (UGM) cells from embryonic day 18.0 rat embryos. UGM cells were obtained from dissected urogenital sinus treated for 30 min in 1% trypsin, followed by mechanical dissociation and treatment with 0.1% collagenase B (Roche) for 30 min at 37°C, and washing in PBS. Pelleted cell mixtures were resuspended in 10 μ l of 9:1 collagen/setting buffer [10 $\times$ Earle's Balanced Salt Solution (Life Technologies), 0.2 M NaHCO₃ and 50 mM NaOH], and gelatinized at 37°C for 20 min. Tissue recombinants were cultured in DMEM/10% FBS supplemented with 10^{-7} M DHT overnight, followed by transplantation under the kidney capsules of immunodeficient NCRNU-M sp/sp nude mice (Taconic Biosciences). Grafts were collected after 2 months of growth and imaged under a Nikon SMZ-1000 stereomicroscope with fluorescence and charge-coupled device digital camera. YFP⁺ graft areas were quantified using ImageJ.

Prostate organoid culture

Flow-sorted basal and luminal cells were washed with advanced DMEM/F12 (Life Technologies), and resuspended in 10 μ l advanced DMEM/F12 and 30 μ l Matrigel per well in the Nunc Lab-Tek II CC2 Chamber Slide System (Thermo Fisher Scientific). Chamber slide was put upside down in the 37°C cell culture incubator for 15 min to let the matrigel solidify. Mouse prostate organoid culture medium was prepared according a previous protocol (Drost et al., 2016). Briefly, the following components were added to advanced DMEM/F12 medium, B27 (50 $\times$ diluted), HEPES 1 M (100 $\times$ diluted), GlutaMAX (100 $\times$ diluted), Penicillin-streptomycin (100 $\times$ diluted), N-acetylcysteine (1.25 mM), EGF (50 ng/ml), A83-01 (200 nM), Noggin (100 ng/ml), R-spondin 1 (500 ng/ml), DHT (1 nM), Y-27632 dihydrochloride (10 μ M). Organoid culture medium was pre-warmed before adding to the wells. The medium was changed with fresh standard medium or paralleled luminal condition medium every 3 days. Organoids were fixed in 4% PFA for 20 min at room temperature before immunofluorescence staining. *In situ* organoid images were taken using the Keyence microscope in the UCSC Microscopy Shared Facility. Organoid sizes were quantified using ImageJ.

Histology and immunofluorescence staining

H&E staining was performed using standard protocols as previously described (Xie et al., 2017), and visualized using a Zeiss Axio Imager. Immunofluorescence staining was performed using 6 μ m cryosections or on organoids *in situ*. Samples were incubated with 10% normal goat serum (NGS) and primary antibodies diluted in 10% NGS overnight at 4°C. Samples were then incubated with secondary antibodies

(diluted 1:500 in PBST) labeled with Alexa Fluor 488, 555, or 647 (Invitrogen/Molecular Probes). Slides were mounted with VectaShield mounting medium with DAPI (Vector Labs), and images were taken on a Leica TCS SP5 spectral confocal microscope in the UCSC Microscopy Shared Facility. All primary antibodies and dilutions used are listed in Table S2.2.

TUNEL assay

TUNEL assay was performed using the In Situ Cell Death Detection Kit, TMR Red or Fluorescein (Sigma-Aldrich) according to the manufacturer's instructions with modifications. Briefly, slides were incubated in freshly prepared permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice, washed with PBS twice, and then incubated with TUNEL reaction mixture (1:20 terminal deoxynucleotidyl transferase solution diluted in label solution) for 40 min at 37°C in a dark humidified chamber. Immunofluorescence staining with CK5 or CK18 was performed immediately afterwards as described above. Signals were detected using the Leica TCS SP5 spectral confocal microscope.

Lineage analysis and statistics

For lineage-tracing analysis, cell numbers were counted manually using confocal $\times 40$ photomicrographs across tissue sections. Basal cells were identified based on the oval or triangular shape of the cells, their positions at the basement of the epithelium, and positive CK5 staining. Luminal cells were determined based on the columnar shape of the cells, their positions at the apical side of the epithelium, and positive CK18 staining. Statistical analyses were performed using the Mann–Whitney *U*-test, or two-

tailed Student's *t*-test as appropriate. At least three animals for each experiment or genotype were analyzed. No statistical methods were used to pre-determine sample size, and animals were not randomized. Investigators were blinded to the animal IDs when analyzing phenotypes. The variances were similar between the groups that were being statistically compared.

Acknowledgements

We thank Sarah Carner and Dr Michael Shen (Columbia) for training C.H. on renal graft procedures. We appreciate the technical assistance from the UCSC FACS and imaging core facilities.

Table S2.1. Primers for mouse genotyping.

Allele		Primer sequence
<i>CreER^{T2}</i>	forward	5'-CAGATGGCGCGCAACACC-3'
	reverse	5'-GCGCGGTCTGGCAGTAAAAAC-3'
	reverse	5'-GTCATCTTCACTTAGCCATTGG-3'
<i>R26R-CAG-YFP</i>	wild-type forward	5'-AAGGGAGCTGCAGTGGAGTA-3'
	wild-type reverse	5'-CCGAAAATCTGTGGGAAGTC-3'
	mutated forward	5'-ACATGGTCCTGCTGGAGTTC-3'
	mutated reverse	5'-GGCATTAAAGCAGCGTATCC-3'
<i>Nkx3.1</i>	wild-type forward	5'-CTCCGCTACCCTAAGCATCC-3'
	wild-type reverse	5'-GACACTGTCATATTACTTGGACC-3'
<i>Ai9</i>	mutated forward	5'-CTGTCCTGTACGGCATGG-3'
	mutated reverse	5'-GGCATTAAAGCAGCGTATCC-3'
<i>R26R-DTA</i>	forward	5'-AAAGTCGCTCTGAGTTGTTAT-3'
	wild-type reverse	5'-GGAGCGGGAGAAATGGATATG-3'
	mutated reverse	5'-GCGAAGAGTTTGTCTCAACC-3'
<i>R26-rox-stop-rox-LacZ</i>	forward	5'-TGGAAATGTTACCAAGGAACT-3'
	wild-type reverse	5'-GCTTTAAAGAAAGCCACAG-3'
	mutated reverse	5'-TGACAGGAGATCCTGCCCCGGCACT-3'
<i>DrePR</i>	forward	5'-CATTCACAGCTCGACAGCTC-3'
	reverse	5'-TGAAGTGGGAGTGGACACCT-3'

Table S2.2. Antibodies used in this study

Antibodies for flow cytometry

Antibody	Supplier	Dilution
Sca-1-PE-Cy7	Biolegend clone E13-161.7 #122513	1:500
CD49f-PE	eBiosciences clone eBioGoH3 #12-0495	1:300
Ter119-eFluor450	eBiosciences clone Ter-119 #48-5921	1:250
CD31-eFluor450	eBiosciences clone 390 #48-0311	1:250
CD45-eFluor450	eBiosciences clone 30-F11 #48-0451	1:250
CD45-PE	eBiosciences clone 30-F11 #12-0451-82	1:250
Trop2- Biotinylated	R&D Systems #BAF1122	1:140
APC Streptavidin	BD #554067	1:140
F4/80-APC	Biolegend #123115	1:250

Primary antibodies used for immunofluorescence staining

Antigen	Supplier	Ig type	Dilution
BrdU	Serotec #MCA2060	rat IgG2a	1:500
CK5	Covance #PRB-160P	rabbit IgG	1:1000
CK18	Abcam #ab668	mouse IgG1	1:100
E-Cadherin	BD Biosciences #610181	mouse IgG2a	1:500
Ki67	DakoCytomation #M7249	rat IgG2a	1:600
LacZ	Fisher #PA1-21477	rabbit IgG	1:2000
Synaptophysin	Zymed #18-0130	rabbit IgG	1:500
YFP	Abcam #13970	chick IgY	1:2000

Chapter 3: Modulation of the canonical Wnt activity by androgen signaling in prostate epithelial basal stem cells

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Abstract

Both the canonical Wnt signaling and androgen signaling are important factors regulating prostate organogenesis. How these two pathways crosstalk to regulate prostate stem cell functions remain unclear. Here, we show that while canonical Wnt activity is required for prostate basal stem cell multipotency in vivo, ectopic Wnt activity does not promote basal-to-luminal cell differentiation. We provide evidence that androgen signaling may keep Wnt activity in check. In prostate organoid culture from basal cells, dihydrotestosterone (DHT) antagonizes R-spondin-stimulated organoid growth in a concentration-dependent manner. Molecular analyses of organoids under different treatment conditions showed that androgen signaling downregulated the expressions of a Wnt reporter as well as many Wnt target genes. Pathway analysis and gene set enrichment analysis of organoid RNA-seq data also revealed the canonical Wnt signaling as a key pathway distinguishing organoids treated with or without DHT. Notably, DHT treatment enhanced AR and β -catenin binding in the nuclei of prostate organoids, providing possible mechanistic clues. Our results reveal a critical role of AR signaling in modulating canonical Wnt activity in prostate basal cells to regulate their multipotency

INTRODUCTION

As mentioned in throughout this thesis, during prostate organogenesis, basal cells behave as stem cells to generate luminal cells and rare neuroendocrine cells (Ousset et al., 2012). Although basal stem cell multipotency become restricted in the mature prostate (Choi et al., 2012; Wang et al., 2013), isolated adult basal cells can be reactivated to generate prostatic tissues in the renal-grafting assay (Wang et al., 2014; Xin et al., 2003), and are more efficient in generating prostate organoids in cultures compared to luminal cells (Chua et al., 2014; Karthaus et al., 2014). The organoid culture medium contains R-spondin, an agonist of the canonical Wnt pathway (Binnerts et al., 2007; Carmon et al., 2011; de Lau et al., 2011), demonstrating a positive role of Wnt signaling for prostate organoid formation and expansion (Karthaus et al., 2014). On the other hand, conditional knockout of β -catenin in adult prostate basal cells appeared to negatively affect their luminal differentiation, as revealed by lineage tracing during a 6-week period (Lu and Chen, 2015). Recently, our lab performed long-term basal cell lineage tracing with AR conditional knockout, and demonstrated that AR within basal cells is required for basal-to-luminal cell differentiation in vivo (Xie et al., 2017). Despite these findings, how Wnt and androgen signaling pathways cooperate to promote basal cell multipotency is unclear. In this study, we show that ectopic Wnt activity leads to prostate basal cell over-proliferation, but not luminal differentiation, and that androgen signaling can down-regulate Wnt activity in basal cells, possibly through binding with β -catenin to interfere with target gene transcription.

Results and Discussion

Intermediate Wnt activity is essential for prostate basal-to-luminal cell differentiation

Although a previous lineage-tracing study suggested a role of canonical Wnt activity in promoting basal-to-luminal differentiation (Lu and Chen, 2015), the longterm effect of β -catenin deletion in basal cells is unknown. To this end, we utilized the *CK5-CreER^{T2}; R26R-CAG-YFP/+* reporter for basal cell lineage tracing, for which we showed previously marks almost all prostate basal cells (Xie et al., 2017). We tamoxifen-induced *CK5-CreER^{T2}; Ctnnb1^{f/f}; R26R-CAG-YFP/+* adult male mice (termed *Basb^{cat-/-}*) and *CK5-CreER^{T2}; R26R-CAG-YFP/+* controls (termed *Bas^{WT}*), and performed long-term lineage tracing under homeostasis (Fig. 3.1A). Since β -catenin is a structural protein expressed in luminal cells contacting the basal layer, its loss in individual basal cells could not be easily distinguished by immunofluorescence (IF) staining (Fig. 3.2A). Nevertheless, efficient β -catenin deletion driven by *CK5-CreER^{T2}* was confirmed by flow-sorting of YFP⁺ prostate basal cells (Fig. 3.2B) for qRT-PCR analysis (Fig. 3.2C), and inferred by observing hair loss through time (Fig. 3.2D) (Kishimoto et al., 2000). Six months after induction, basal-to-luminal cell differentiation was significantly reduced in the *Basb^{cat-/-}* group compared to *Bas^{WT}* (Fig. 3.1B, 3.1C), while basal cell proliferation rates were unchanged as measured by Ki67 staining and BrdU incorporation assay (Fig. 3.1D, 3.1E). These results are consistent with the previous 6-week tracing data in the Lu and Chen study. However, contrary to their finding in the tumor models (Lu and Chen, 2015), we found that β catenin deletion did not rescue the progression of

basal-derived Pten-null tumors, as *CK5-CreER^{T2}; ctnnb1^{fl/fl}; Pten^{fl/fl}; R26R-CAG-YFP/+* tumors (*Bas^{bcat-/-Pten-/-}*) were morphologically indistinguishable from those of *CK5-CreER^{T2}; Pten^{fl/fl}; R26R-CAGYFP/+* (*Bas^{Pten-/-}*) (Fig. 3.1F). The double knockout tumors still contain cribriform pattern and luminal differentiation (Fig. 3.1G), despite their lack of β -catenin expression (Fig. 3.1H). The cause of the discrepancy is unclear. We conclude that Pten loss can override the requirement of β -catenin in promoting basal-to-luminal cell differentiation.

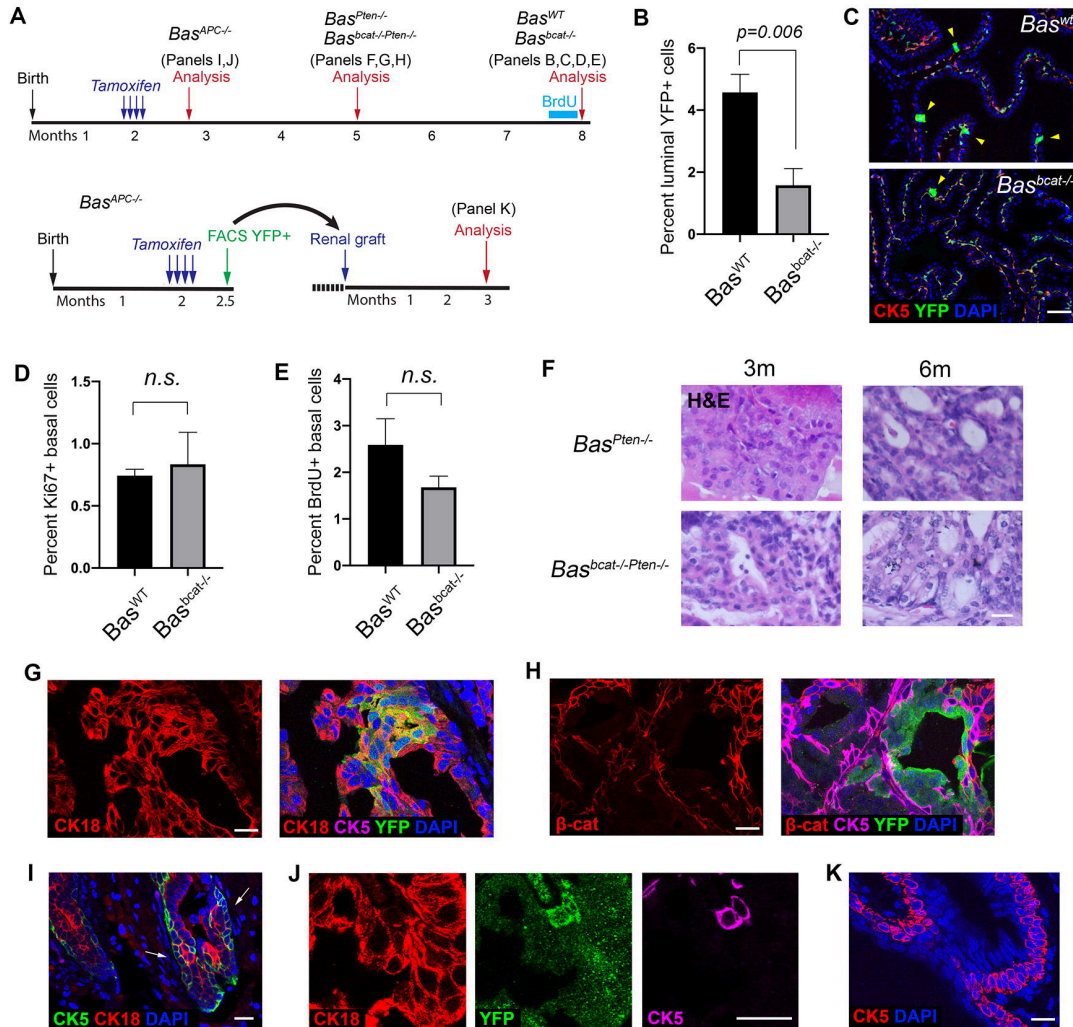


Figure 3.1. Lineage tracing analysis of basal cells of different levels of Wnt activity. (A) Timeline of experiments for lineage tracing and renal grafting of Wnt-mutant-related basal cells. (B) Percentage of YFP⁺ luminal cells decreased significantly in *Bas^{bcat-/-}* mice (N=5) compared to *Bas^{WT}* mice (N=4) at 6 months post induction by student's t-test. (C) Representative IF images of YFP⁺ luminal cells (arrowheads) in the *Bas^{WT}* and *Bas^{bcat-/-}* prostate 6 months post tamoxifen induction. (D, E) Bar graphs showing proliferation rates in *Bas^{WT}* and *Bas^{bcat-/-}* basal cells by Ki67 (D) and BrdU (E). n.s., not significant by student's t-test. (F) H&E staining of tissue sections from *Bas^{Pten-/-}* and *Bas^{Pten-/-;Bcat-/-}* mice at 3 months and 6 months post tamoxifen induction. (G, H) IF image showing increased basal-to-luminal differentiation (G) and loss of β catenin (H) in *Bas^{Pten-/-;Bcat-/-}* tumor regions at 3 months post induction. (I) Squamous basal cell phenotype (arrows) seen in *Bas^{APC-/-}* mice at 2 weeks post induction. (J) IF staining showing the strict basal cell identity of the squamous-like cells. (K) IF showing *Bas^{APC-/-}* basal cell over-proliferation 3 months after renal grafting. Scale bars in C correspond to 50 μ m, and in F-K to 20 μ m. Error bars correspond to one s.d.

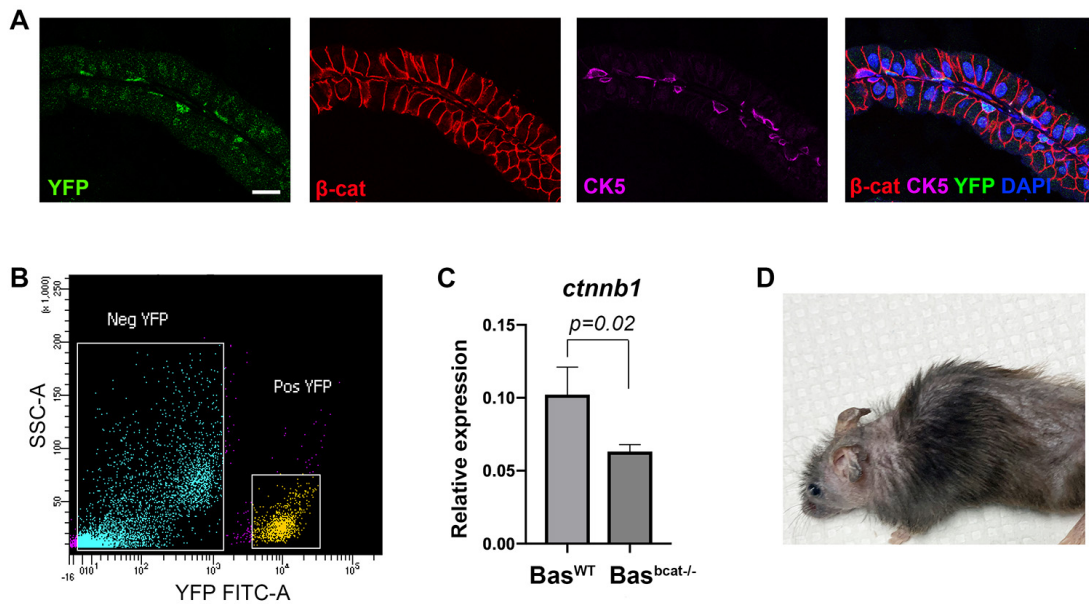


Figure 3.2 Analysis of *Basbcat-/-* basal cells. (A) IF images showing β -catenin expression pattern in *Bas^{bcat-/-}* mice. Scale bar, 20 μ m. (B) FACS plot of sorting YFP⁺ *Bas^{bcat-/-}* basal cells. (C) qRT-PCR showing significantly reduced β -catenin expression in *Bas^{bcat-/-}* basal cells compared to wildtype basal cells by student's t-test. Gene expression levels were normalized to β -actin expression. (D) A mouse losing hair due to β -catenin deletion by the *CK5-CreER^{T2}* driver expression in skin basal cells.

DHT suppresses prostate basal stem cell activities in organoid assay

Given the pivotal role of androgen signaling in prostate development, we next tested how adding DHT affects basal stem cells. To do this, we used a defined organoid culture protocol (Drost et al., 2016). Wildtype basal cells were isolated from induced *Bas*^{WT} mice, and were cultured under six conditions: (1) “basement medium” (BM condition) containing all the factors as described in the protocol except for R-spondin and dihydrotestosterone (DHT), (2) BM plus R-spondin (R condition), the basement medium was supplemented with DHT at two concentrations (3) 10^{-7} M and (4) 10^{-9} M as the DHT-9 and DHT-7 conditions. In another two conditions, DHT was also added together with R-spondin ((5) RD-9 and (6) RD-7 conditions) to test the combinatory effects of activating Wnt and androgen signaling pathways on prostate organoid growth. Representative images of organoids growing for 7 days under these conditions are shown in Fig. 3.3A. Surprisingly, our data consistently showed that DHT suppressed basal stem cell activities in organoid culture in a concentration-dependent manner. When organoid size and structural complexity as measured by the number of branching were quantified, DHT-9 condition led to significantly reduced growth and branching morphogenesis compared to BM alone, and DHT-7 condition further exacerbated such phenotypes as the organoids were significantly stunted in growth (Fig. 3.3A-C). Adding R-spondin was able to rescue these phenotypes to some extent, but DHT could clearly antagonize the growth-promoting effects of R-spondin since organoids under the RD-9 condition were significantly smaller and contained less branching than those under the R condition (Fig. 3.3B, 3.3C). These

data imply that Wnt and androgen signaling pathways have opposite effects on prostate basal stem cell activities and that their combined effects may be subtractive.

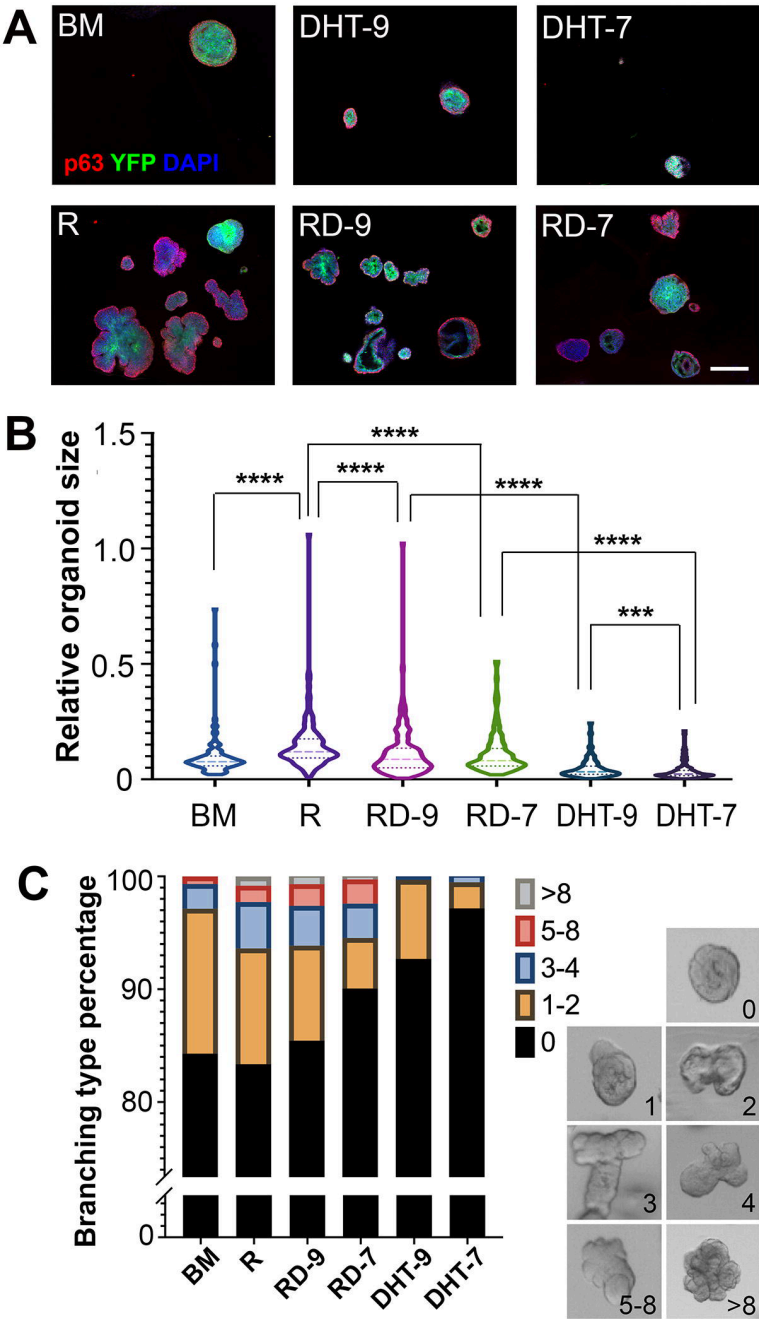


Figure 3.3. Analysis of basal-derived organoids under combined R-spondin and DHT treatment. (A) Representative IF images showing organoid morphology after 7 days under different R-spondin and DHT conditions. Scale bar, 200 μ m. (B) Violin plot comparing relative organoid sizes under different treatments by Mann-Whitney Utest. **** $p < 0.0001$, *** $p < 0.001$. (C) Quantification of organoid branching under different R-spondin and DHT conditions. Examples of different numbers of branching are shown on the right.

DHT decreases Wnt signaling activity in prostate organoids

Based on the results above, we hypothesized that DHT inhibits basal organoid growth through modulation of the Wnt signaling pathway. To test it, we isolated basal cells from the *TCF/Lef-H2B.GFP* reporter mice, in which GFP signal can serve as a readout for canonical Wnt signaling activity (Ferrer-Vaquer et al., 2010). We then cultured these cells as organoids using 6 medium conditions: BM, R, RD-9, RD-7, DHT-9, and DHT-7. After 7 days of culture, we found that the GFP signals were usually present in the center of the organoids, and were most prominent in the R group (Fig. 3.4A). When we quantified the percentages of GFP area size of the total organoid size, we found the trend as follows: $R > BM > RD-9 > RD-7 > DHT-9 > DHT-7$, and adding DHT significantly reduced GFP area even in the presence of R-spondin (Fig. 4B). Expression reduction upon DHT treatment was also observed for several Wnt target genes *Axin2*, *Lef1*, *Ccnd2*, *Cd44*, and *Myc*, as quantified by qRT-PCR (Fig. 3.4C), confirming that DHT decreases Wnt signaling activity.

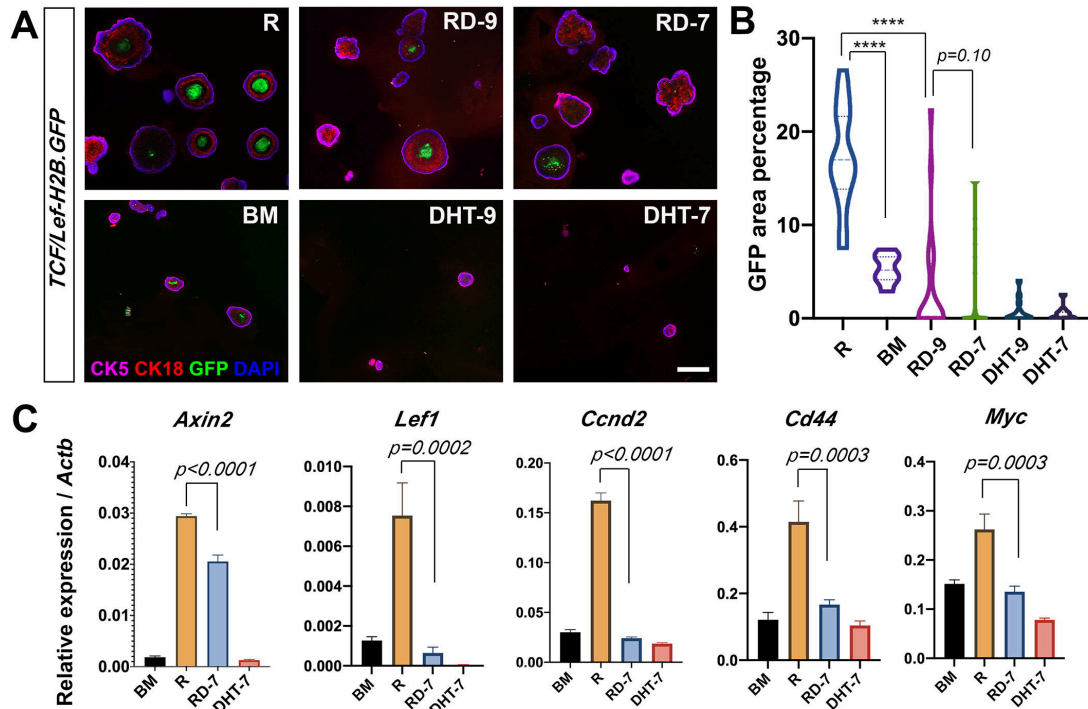


Figure 3.4. DHT decreases Wnt signaling activities in organoids.

(A) Representative IF images showing TCF/Lef-H2B.GFP reporter signals in basallerived organoids cultured under different conditions. Scale bar, 200 μ m. (B) Violin plot showing GFP area percentages for different treatment conditions. **** $P<0.0001$ by using Mann-Whitney U-test. (C) Quantitative real-time PCR analysis of Wnt target genes under different treatments. Gene expression levels were normalized to β -actin expression. P values were analyzed by unpaired student's t-test. Error bars correspond to one s.d.

To further explore the mechanism, we isolated mRNAs from BM, R, and RD-7 organoids and performed RNA-sequencing. Principal component analysis (PCA) and unsupervised hierarchical clustering analysis demonstrated the consistency of the samples within each group and the distinct transcriptomes of the RD-7 group from the BM and R groups (Fig. 3.5A, 3.5B). Gene expression differential analysis revealed

the numbers of upregulated and downregulated genes between each groups as shown in the volcano plots (Fig. 3.6A). Many genes, including Wnt targets, were upregulated in R compared to BM, but were downregulated in RD-7 compared to R (Fig. 3.6B). David GO analysis (Huang da et al., 2009) of differentially expressed gene set between R and RD-7 groups identified key pathways involved, among which were cell cycle regulation and Wnt signaling pathway (Fig. 3.5C), supporting our model that the inhibitory effects of androgen on basal organoids were mediated through modulation of the canonical Wnt activity. In further support of this, gene set enrichment analysis (GSEA) (Subramanian et al., 2005) showed that Wnt signaling pathway genes were highly enriched in the genes that were downregulated in the RD7 group compared to the R group (Fig. 3.5D). Similarly, genes of the GO term Organ Morphogenesis were also highly enriched in the downregulated RD-7 genes, while two gene signatures that were previously shown to be upregulated by androgen (Mulholland et al., 2011; Schaeffer et al., 2008) were highly enriched in the upregulated RD-7 genes (Fig. 3.5D).

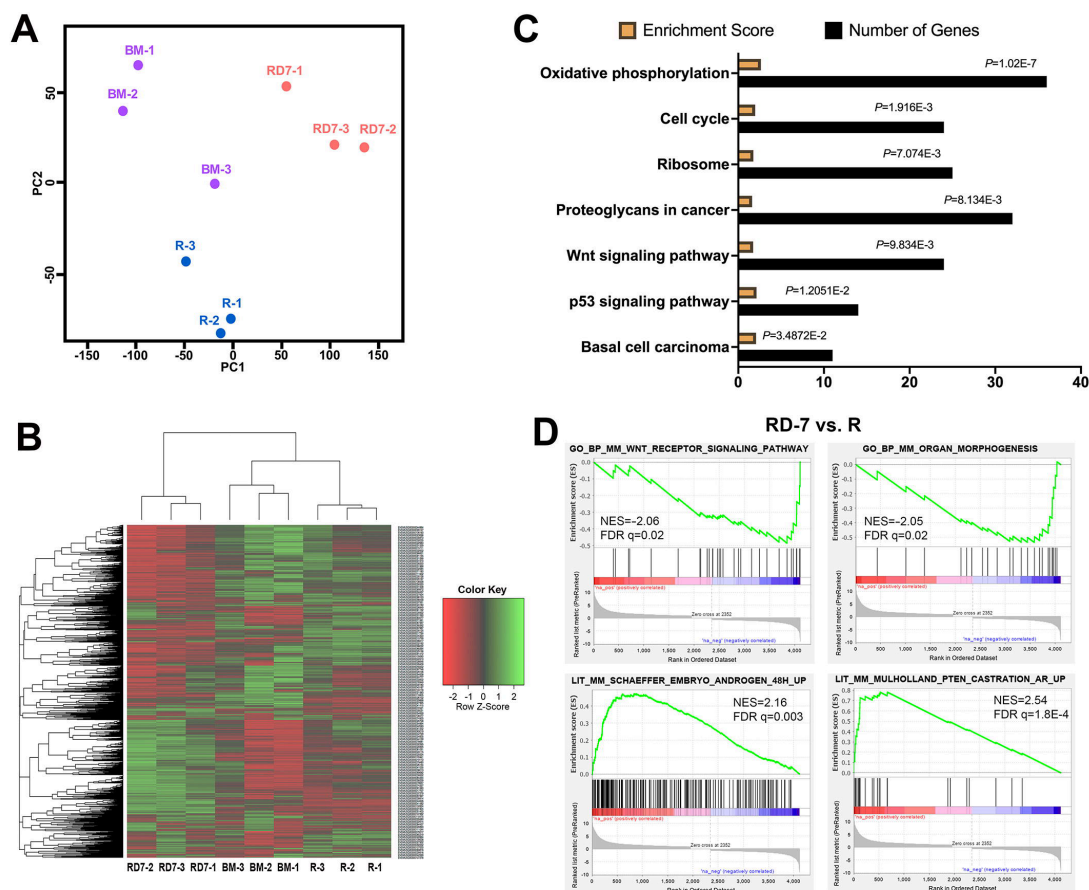


Figure 3.5. RNA-seq analysis of basal-derived organoids under different treatment conditions. (A) Scatter-plot of the two main components from a principle component analysis of the BM (purple), R (blue), and RD-7 (orange) samples. N=3 in each group. (B) Unsupervised hierarchical clustering analysis showing distinct transcriptomes of RD-7 from BM and R. (C) David GO analysis showing the enriched pathways in the comparison of R vs. RD-7, with the number of genes in each pathway. (D) GSEA comparison of RD-7 vs. R showing Wnt pathway genes and organ morphogenesis genes are enriched in the downregulated genes in the RD-7 group, and androgen responsive genes enriched in the upregulated genes in the RD-7 group.

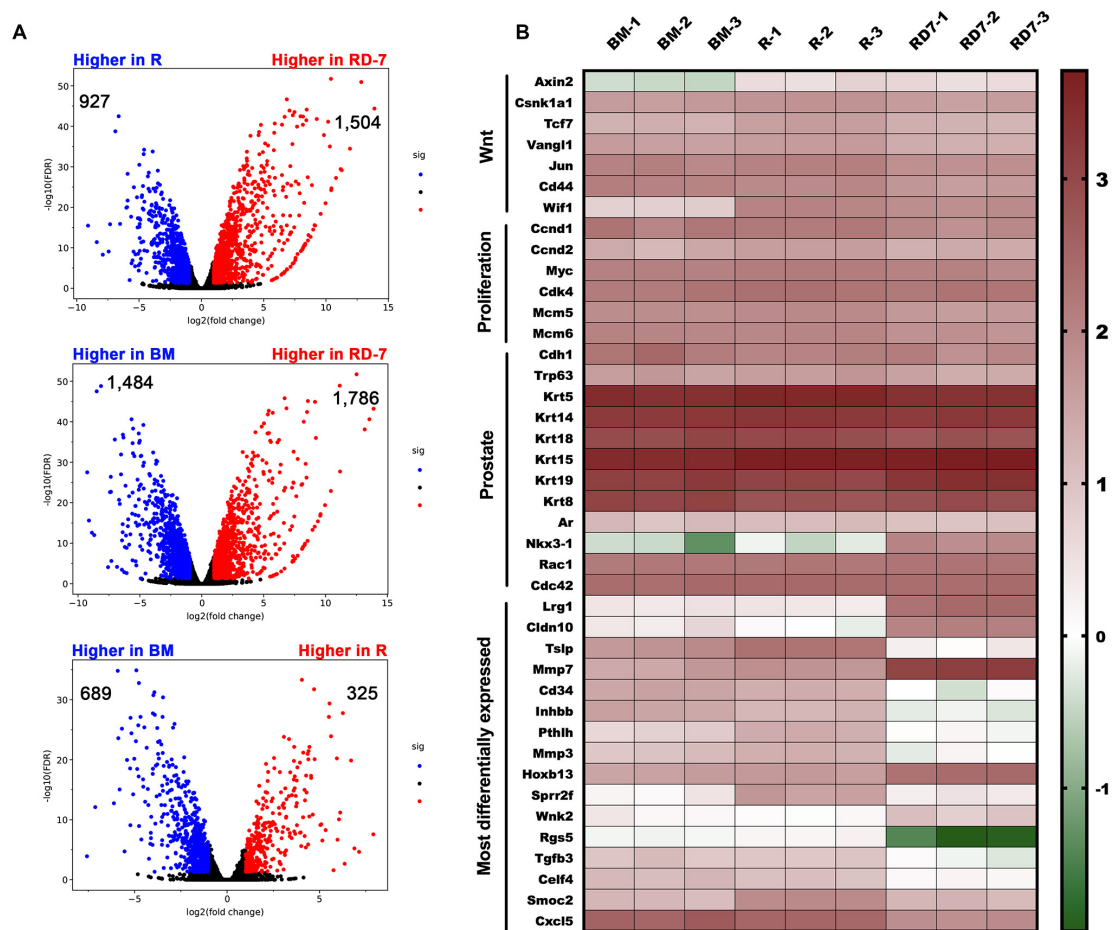


Figure 3.6. Comparison of organoid RNA-seq data of different treatments.
 (A) Volcano plots showing the numbers of upregulated and downregulated genes between R vs. BM, RD-7 vs. R, and BM vs. RD-7 (FDR<0.05 and fold change >2).
 (B) Heatmap showing expression levels of selected genes in different samples.

Increased AR and β -catenin binding in organoid cells upon R-spondin and DHT

Treatment

Previous studies in prostate cancer cell lines have shown that AR can compete with TCF for β -catenin binding (Song et al., 2003), and the binding of AR and β catenin can enhance AR signaling output (Amir et al., 2003; Mulholland et al., 2002; Truica et al., 2000). How this binding affects Wnt signaling activity is unclear. We hypothesize that DHT inhibition of Wnt activity in prostate organoids may be

mediated through AR binding to β -catenin in the nucleus to hamper the normal function of β -catenin as a Wnt signaling transcription factor. To visualize their interactions in organoids, we performed Duolink Proximity Ligation Assay (PLA) (Fredriksson et al., 2002; Soderberg et al., 2008), in which antibodies recognizing both AR and β -catenin in close proximity can yield an amplified signal dot in situ. We cultured prostate organoids for 7 days and performed quality check experiments to confirm that PLA signals can only be detected when both AR and β -catenin antibodies were applied (Fig. 3.7A). Interestingly, when the organoids were cultured under BM, R, RD-9, and RD-7 conditions, we observed significantly more AR and β catenin protein interactions per nucleus or per cell under the RD treatment conditions (Fig. 3.7B, 3.7C), and the interactions increased with the concentration of DHT (Fig. 3.7C).

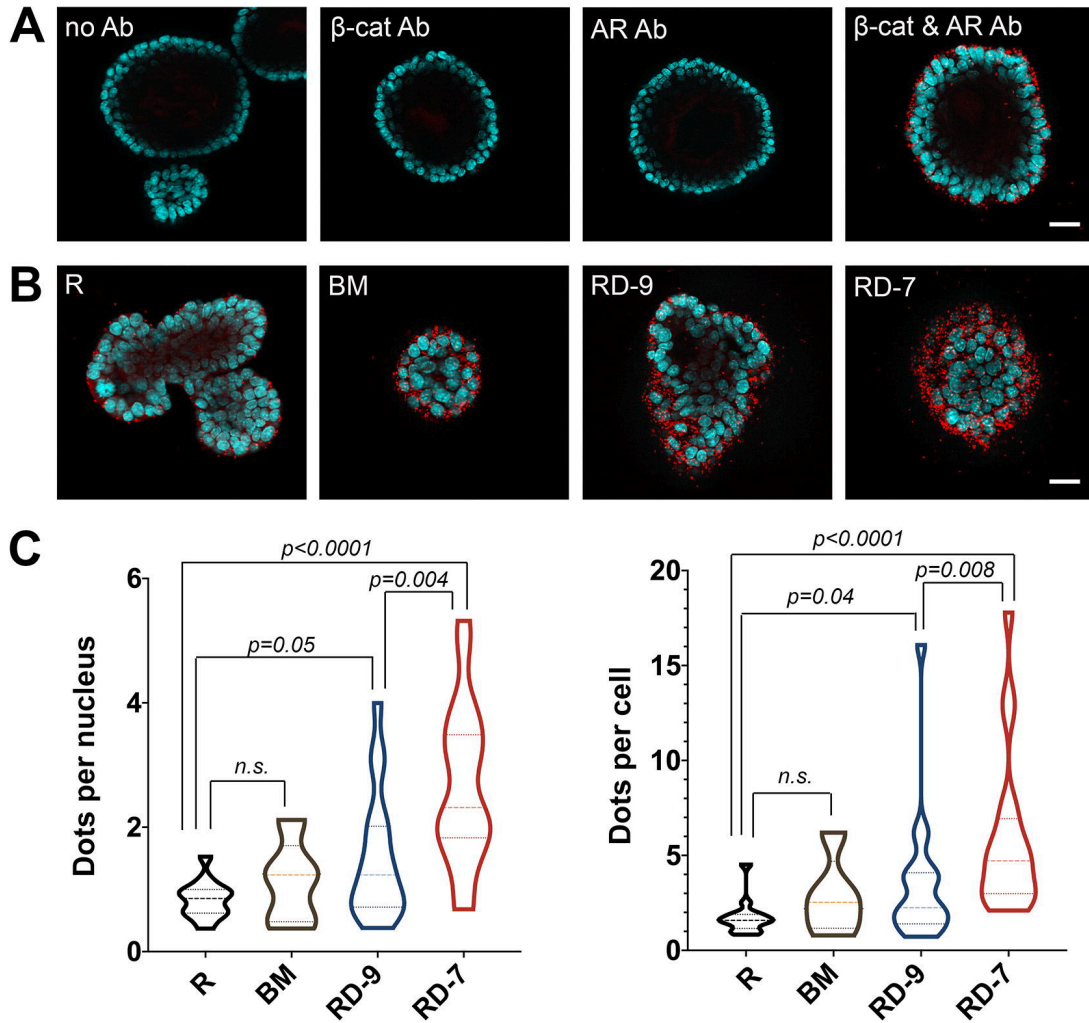


Figure 3.7. Duolink PLA of AR and β -catenin binding in organoids.

(A) Representative IF images showing Duolink PLA signals (red dots) only in the presence of both β -catenin and AR antibodies. (B) Representative IF images showing Duolink PLA signals under R, BM, RD-9 and RD-7 conditions. Cyan represents nuclear DAPI staining. Scale bars, 50 μ m. (C) Violin plots showing quantitation of dots per nucleus (left) and dots per cell (right) in each organoid under different treatment conditions. P values were analyzed by Mann-Whitney U-test.

Addition of DHT sequesters β -catenin away from canonical Wnt target genes

In order to investigate how adding DHT affects AR and β -catenin binding across the genome, we next performed the CUT&RUN assay (Skene et al., 2018) under the R and RD-7 conditions using AR and β -catenin antibodies previously verified for ChIP-seq (Table 3.2). We grew organoids for 5 days in R and RD-7 condition and then isolated and dissociated them into single cells before performing CUT&RUN. Samples were then sequenced at a depth of ~30M reads per sample with a Q30>92.1%. We then compared the peak differences between 4 groups: R_Bcat (R condition with β -catenin antibody), RD_Bcat (RD-7 condition with β -catenin antibody), R_AR (R condition with AR antibody), and RD_AR (RD-7 condition with AR antibody) (Fig 3.7A). Adding DHT greatly shifted the binding patterns for both AR and β -catenin. MEME motif analysis confirmed that an AR binding motif is significantly enriched in the 12745 AR peaks that are present in RD_AR but not R_AR (Fig 3.8B), suggesting DHT promotes AR binding to the androgen-responsive genes. Notably, AR and β -catenin co-binding peaks increased from 516 under R condition to 963 under RD condition, suggesting that adding DHT indeed promoted their co-binding.

We next decided to compare β -catenin binding under R and RD conditions to defined AR peaks across the genome. We first examined R_AR and RD_AR peak sets. Both heat maps show more β -catenin binding in RD condition compared to R (Fig 3.8C), consistent with our hypothesis. We then interrogated peaks that are present in R_AR but not present in RD_AR, and named it OldAR. We found that

there was no difference in the β -catenin binding signals in this peak set between R and RD conditions (Fig 3.8D), which is expected, given that OldAR peak set consists of AR binding sites that are not regulated by DHT but promoted by R-spondin. The final peak set we investigated consisted of all of the peaks that were present in RD_AR but not in R_AR, which we termed NewAR. These are the AR binding sites that are under the regulation of DHT. In these sites, there is clearly more co-binding of β -catenin in the RD condition compared to the R condition (Fig 3.8E). This data confirms that DHT treatment in prostate basal cells promotes the co-binding of AR and β -catenin. To test the hypothesis that DHT bound AR can recruit β -catenin away from its endogenous binding sites to novel binding sites, we decided to run gene ontology (GO) analysis on different β -catenin peak sets (fig 3.8F). First we looked into β -catenin peaks in R (Peak set C) condition where, expectedly, we found the Wnt signaling pathway and stem cell differentiation to be heavily enriched. We then investigated the new β -catenin peaks that were generated by the addition of DHT (peak set A) and saw that negative regulation of the canonical Wnt pathway was enriched. When we examined the peaks within this set that colocalized with AR peaks in RD condition (peak set B) the same negative regulation of Wnt was present. Importantly, this negative regulation in peak set B showed a greater enrichment score, lower P-value, and a lower number of genes enriched. This suggests that the β -catenin/AR protein complex selectively binds to promoter regions that have a high impact on negative regulation of the canonical Wnt pathway.

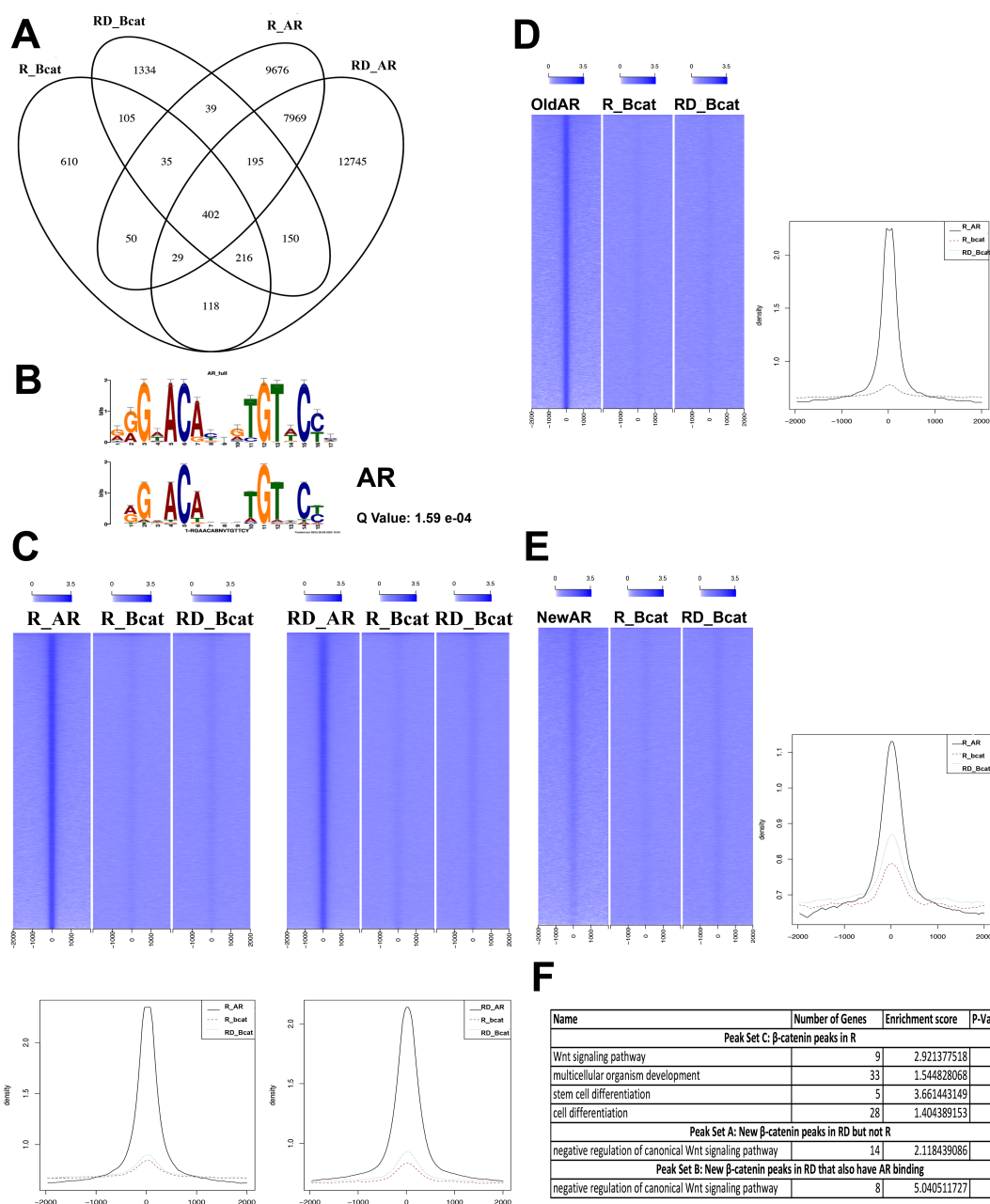


Figure 3.8. Addition of DHT sequesters b-catenin away from canonical Wnt target genes. (A.) Ven diagram showing the colocalization of peaks called from CUT&RUN with β -catenin (Bcat) and AR antibodies in different culture conditions (R and RD). (B.) Consensus motif enrichment for the 12,745 new unique AR peaks created upon the addition of DHT. (C.) Heat maps using all peaks called in R_AR and RD_AR on the Y-axis showing the overlap of β -catenin binding in R and AR conditions. Graphical representation of the heat map densities shown below (Black =

peaks in R_AR or RD_AR; Red = peaks in R_Bcat that colocalize; Green = peaks in RD_Bcat that colocalize). (D.) Heat map using peaks called in R_AR but not RD_AR (OldAR) as the Y-axis showing overlap of β -catenin binding in R and AR conditions. Graphical representation shown below (Black = OldAR; Red = peaks in R_Bcat that colocalize; Green = peaks in RD_Bcat that colocalize) (E.) Heat map using peaks called in RD_AR but not R_AR (NewAR) as the Y-axis showing overlap of β -catenin binding in R and AR conditions. Graphical representation shown below (Black = NewAR; Red = peaks in R_Bcat that colocalize; Green = peaks in RD_Bcat that colocalize) (F.) Table showing pathways found to be enriched in the GO analysis in peak sets A, B, and C.

DISCUSSION

While previous studies performed with cancer cell lines have shown that active Androgen Receptor can inhibit canonical Wnt signaling through interactions with β -catenin, it remains unclear how this interaction affects prostate development and adult homeostasis. Previously we have shown that cell autonomous AR is necessary for basal cell multipotency in the adult prostate (Xie et al., 2016) and here, through longterm in-vivo lineage tracing of basal cells coupled with β -catenin deletion, we confirm the findings of previous studies that suggest a role of canonical Wnt/ β -catenin activity in promoting basal cell multipotency. Interestingly, ectopic Wnt signaling in basal cells does not promote basal-to-luminal differentiation, but rather causes basal cell overproliferation and leads to a squamous tumor phenotype. We hypothesized that an intermediate level of Wnt/ β -catenin is required to induce basal to luminal differentiation, and that AR may be responsible for achieving this intermediate level through modulation of endogenous β -catenin transcriptional activity.

To test this hypothesis, we made use of the prostate organoid culture assay, which promotes basal stem cell activity and allowed us to control the levels of

Wnt and AR activity during growth. Our findings indicate that Wnt activity indeed promotes organoid growth and that growth and Wnt activity levels are stunted by the addition of DHT. In support of previous studies in prostate cancer cell lines, addition of DHT to prostate culture medium increases nuclear colocalization of β -catenin and AR in a dose-dependent manner. CUT&RUN data suggests that AR regulates Wnt signaling by recruiting β -catenin away from endogenous Wnt target sites to new regulatory sites that are controlled by DHT. Overall, adding DHT created 345 new β -catenin binding sites that have AR co-binding and 1334 new unique β -catenin binding sites that were not present under R condition. While it was expected to see new co-binding sites upon DHT addition, we were surprised to see so many new unique β -catenin binding sites. More detailed analysis will need to be performed in order to determine where β -catenin is relocating and how addition of DHT mediates this relocation. Nevertheless, gene ontology analysis of peak set B does indeed support the hypothesis that β -catenin/AR complex regulates genes in a way that inhibits canonical Wnt signaling.

Taken as a whole, our data suggest a model where AR and Wnt/ β -catenin signaling cooperate to determine basal cell activity. Under low Wnt and high AR signaling activity, β -catenin is recruited away from its normal binding sites by activated AR, and recruited to ectopic AR sites that inhibit Wnt signaling activity. In high Wnt and low AR conditions, β -catenin can bind to promoter regions of its normal target genes that promote stem like activities (Fig 3.9). It seems that a careful balance of Wnt and AR activity is necessary to facilitate basal to luminal

differentiation. Future experiments utilizing AR-overexpressing mouse models should further validate the androgen and Wnt pathway interactions in the intact prostate in vivo.

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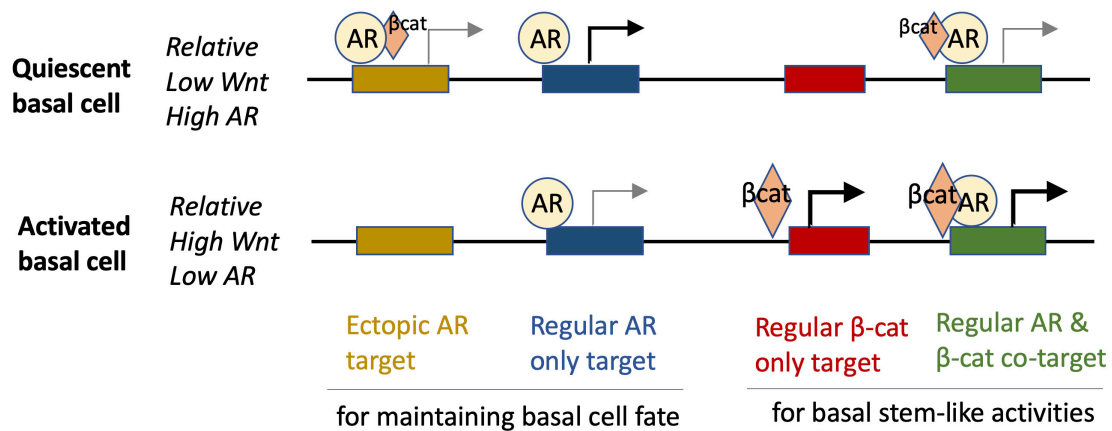


Figure 3.9. How differing levels of canonical Wnt and AR activity affect basal cell state

Table 3.1. Antibodies used in this study

Antibodies for flow cytometry

Antibody	Supplier	Dilution
Sca-1-PE-Cy7	Biolegend clone E13-161.7 #122513	1:500
CD49f-PE	eBiosciences clone eBioGoH3 #12-0495	1:300
Ter119-eFluor450	eBiosciences clone Ter119 #48-5921	1:250
CD31-eFluor450	eBiosciences clone 390 #48-0311	1:250
CD45-eFluor450	CD45-eFluor450eBiosciences clone 30-	1:250

	F11 #48-0451	
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Primary antibodies used for immunofluorescence staining, duolink PLA, and CUT & RUN

Antigen		Supplier	Ig type	Dilution
BrdU		Serotec #MCA2060	Rat IgG2a	1:500
Ki67		DakoCytomation #M7249	Rat IgG2a	1:600
YFP		Abcam #13970	Chick IgY	1:2000
CK5		Covance #PRB-160P	Rabbit IgG	1:1000
CK18		Abcam #ab668	Mouse IgG1	1:200
p63		GeneTex #GTX102425	Rabbit IgG	1:1000
AR		Sigma #A9853	Rabbit IgG	1:500
β -catenin		BD Biosciences #610153	Mouse IgG1	1:500 for Duolink 1:1000 for IF
AR		Cell Signaling (D13A1)	Rabbit IgG	1:100
Active β -catenin	Abcam (ab108341)	Rabbit IgG	1:100	

Table 3.2 Primer sequences used for qPCR

Genes	Primer sequence	
Axin2	forward	5'-CAGAGGGACAGGAACCACTC-3'
	reverse	5'-TGGACACTTGCCAGTTTCTT-3'
Lef1	forward	5'-ACGACAAGGCCAGAGAACA-3'
	reverse	5'-GTCGCTGTTCATATTGGGCA-3'
Ccnd2	forward	5'-AAGGACCGGTGCGAGTCA-3'
	reverse	5'-GGGAGTGCTTCCCTTACCTC-3'
Cd44	forward	5'-TCGATTGAATGTAACCTGCCG-3'
	reverse	5'-CAGTCCGGGAGATACTGTAGC-3'
Myc	forward	5'-CCCTAGTGCTGCATGAGGA-3'
	reverse	5'-TGCCTCTTCTCCACAGACAC-3'
Ctnnb1	forward	5'-CATCCCACTGGCCTCTGATA-3'
	reverse	5'-TCGTGGAATAGCACCCCTGTT-3'
Actb	forward	5'-CGCCACCAGTTCGCCATGGA-3'
	reverse	5'-TACAGCCCCGGGAGCATCGT-3'

METHODS

Mouse strains and genotyping

The *CK5-CreER^{T2}*, *Pten^{fllox}*, and *R26R-CAG-YFP* lines were used previously (Xie et al., 2017). The *Ctnnb1^{fllox}* (Brault et al., 2001) and *APC^{fllox}* (Cheung et al., 2010) lines were obtained from JAX. The *TCF/Lef-H2B.GFP* line (Ferrer-Vaquer et al., 2010) was obtained from Dr. Hadjantonakis. Animals were maintained in C57BL/6N or mixed background. Genotyping was performed by PCR using tail genomic DNA, with the following primer sequences: *CreER^{T2}* allele: 5'-CAGATGGCGCGCAACACC-3' and 5'-GCGCGGTCTGGCAGTAAAAAC-3'; *TCF/Lef-H2B.GFP* allele: 5'-ACAACAAGCGCTCGACCATCAC-3' and 5'-AGTCGATGCCCTTCAGCTCGAT-3'; *Ctnnb1^{fllox}* allele: 5'-ACTGCCTTTGTTCTCTTCCCTTCTG-3' and 5'-CAGCCAAGGAGAGCAGGTGAGG-3'; *APC^{fllox}* allele: 5'-GAGAAACCCTGTCTCGAAAAA-3' and 5'-

AGTGCTGTTTCTATGAGTCAAC-3'; *R26R-CAG-YFP* allele: 5'-

AAGGGAGCTGCAGTGGAGTA-3' (wild-type forward), 5'-

CCGAAAATCTGTGGGAAGTC-3' (wild-type reverse), 5'-

ACATGGTCCTGCTGGAGTTC-3' (mutated forward), 5'-

GGCATTAAAGCAGCGTATCC-3' (mutated reverse).

Mouse procedures

For tamoxifen induction, mice were administered 9 mg per 40 g body weight tamoxifen (Sigma) suspended in corn oil by oral gavage once daily for 4 consecutive days. For BrdU incorporation assay, BrdU (Sigma) was dissolved in PBS (10 mg/ml) and administered by intraperitoneal injection twice daily (0.1 ml per dose) for 11 consecutive days during homeostasis to label proliferating cells. All animal experiments received approval from the Institutional Animal Care and Use Committee at UCSC.

Tissue collection and flow cytometry

Mouse prostate tissues were dissected and fixed in 4% paraformaldehyde for subsequent cryo-embedding in OCT compound (Sakura), or fixed in 10% formalin followed by paraffin embedding. For flow cytometry, prostate tissues were dissected and minced to small clumps, followed by enzymatic dissociation with 0.2% Collagenase/Hyaluronidase (StemCell Technologies) in DMEM/F12 media with 5% FBS for 3 h at 37°C. Tissues were digested with 0.25% Trypsin-EDTA (StemCell Technologies) for 1 h at 4°C, passed through 21- to 26-gauge syringes and filtered through a 40-mm cell strainer to obtain single-cell suspensions. Dissociated prostate

cells were suspended in Hanks' Balanced Salt Solution Modified/2% FBS. Dead cells were excluded by propidium iodide staining. Lineage-marked basal cells were sorted based on YFP positivity. Antibodies (Table 3.2) were used for sorting Lin⁻Sca-1⁺ CD49f^{hi} basal cells from *TCF/Lef-H2B.GFP* mice. Cell sorting was performed on a BD FACS Aria II instrument in the Flow Cytometry Shared Facility of UCSC.

Renal grafting assay

Flow-sorted *APC^{fl/fl}* YFP⁺ basal cells were mixed with 2.5×10^5 dissociated urogenital sinus mesenchyme (UGM) cells from embryonic day 18.0 rat embryos. UGM cells were obtained from dissected urogenital sinus treated for 30 min in 1% trypsin, followed by mechanical dissociation and treatment with 0.1% collagenase B (Roche) for 30 min at 37°C, and washing in PBS. Pelleted cell mixtures were resuspended in 10 µl of 1:9 collagen/setting buffer (10× Earle's Balanced Salt Solution (Life Technologies), 0.2 M NaHCO₃ and 50 mM NaOH), and gelatinized at 37°C for 20 min. Tissue recombinants were cultured in DMEM/10% FBS supplemented with 10^{-7} M DHT overnight, followed by transplantation under the kidney capsules of immunodeficient NCRNU-M sp/sp nude mice (Taconic Biosciences). Grafts were collected after 3 months of growth and embedded in OCT.

Prostate organoids culture

Flow-sorted basal cells were washed with advanced DMEM/ F12 (Life Technologies), and resuspended in 10 µl advanced DMEM/F12 and 30 µl Matrigel per well in the Nunc Lab-Tek II CC2 Chamber Slide System (Thermo Fisher Scientific). Chamber slide was put upside down in the 37°C cell culture

incubator for 15 min to let the matrigel solidify. Mouse prostate organoid culture medium was prepared according to a previous protocol (Drost et al., 2016). Briefly, the following components were added to advanced DMEM/F12 medium: B27 (50× diluted), HEPES 1 M (100× diluted), GlutaMAX (100× diluted), Penicillin-streptomycin (100× diluted), N-acetylcysteine (1.25 mM), EGF (50 ng/ml), A83-01 (200 nM), Noggin (100 ng/ml), Y-27632 dihydrochloride (10 μ M) as basement medium (BM). Rspodin1 (500 ng/ml) and different concentrations of DHT (10^{-7} M, 10^{-9} M) were added alone or in combinations to BM as different treatments. Dkk-1 (GenScript) was added to BM at final concentration of 100 ng/ml. Organoid culture medium was prewarmed before adding to the wells. The medium was changed every 3 days. Organoids were fixed in 4% PFA for 20 min at room temperature before immunofluorescence staining. In situ organoid images were taken using the Keyence microscope, and immunofluorescence images were taken using Zeiss AxioImager microscope in the UCSC Microscopy Shared Facility. Organoid sizes and GFP area percentage were quantified using ImageJ.

Histology and immunofluorescence staining

H&E staining was performed using standard protocols as previously described, and visualized using a Zeiss AxioImager. Immunofluorescence staining was performed using 6 μ m cryosections or on organoids in situ. Samples were incubated with 10% normal goat serum (NGS) and primary antibodies diluted in 10% NGS overnight at 4°C. Samples were then incubated with secondary

antibodies (diluted 1:500 in PBST) labeled with Alexa Fluor 488, 555, or 647 (Invitrogen/Molecular Probes). Slides were mounted with VectaShield mounting medium with DAPI (Vector Labs), and images were taken on a Leica TCS SP5 spectral confocal microscope in the UCSC Microscopy Shared Facility. All primary antibodies and dilutions used are listed in Table 3.2.

Quantitative real-time PCR

Cultured organoids in matrigel were scraped from the incubation chamber with spoon to exclude fibroblasts contamination. Organoids mRNA was isolated using the RNeasy Micro Kit (Qiagen). RNA was reverse transcribed and amplified into cDNA using SuperScrip III kit (Life Technology). Quantitative real-time PCR was carried out using Power SYBR Green PCR Master Mix (Life Technology) in the ViiA 7 Real-Time PCR instrument. Expression values were obtained using the $\Delta\Delta CT$ method and normalized to β -actin (Actb) expression; average values are shown as mean $\pm$ s.d. Primer sequences are provided in Table 3.3.

Duolink proximity ligation assay

Organoids were fixed in 4% PFA for 20 min at room temperature before performing Duolink PLA. Assay was performed as per the kit instruction (DUO92101, Sigma). The antibodies used were AR (Sigma A9853, Rabbit, 1:500 dilution) and β -catenin (BD Bioscience 610153, mouse, 1:500 dilution). Images were taken using the Zeiss AxioImager microscope in the UCSC Microscopy Shared Facility.

Lineage Analysis and Statistics

For lineage-tracing analysis, cell numbers were counted manually using confocal $\times 40$ photomicrographs across tissue sections. Basal cells were determined based on positive CK5 staining and location at the basement membrane. Luminal cells were determined based on positive CK18 staining, the columnar shape, and location at the apical side of the epithelium. Statistical analyses for lineage tracing and organoids experiments were performed using the two-sided student's t-test or Mann-Whitney U test as appropriate. At least three biological replicates for each experiment or genotype were analyzed. The variances were similar between the groups that were being statistically compared.

Organoid bulk RNA-seq

Organoid mRNA was isolated using the RNeasy Micro Kit (Qiagen). RNA was reverse transcribed and amplified into cDNA using the Takara SmartSeq kit at the UC Berkeley QB3 Genomics Center, where library construction and sequencing were performed. The 2x150 bp paired-end sequencing was performed on the NovaSeq 6000 platform. `bc` and `bcl2fastq` was used for converting BCL to fastq format, coupled with adaptor trimming. Sequencing reads were then mapped to mouse genome (mm10) using the STAR package. Mapped sequencing reads were assigned to genes using 'featurecounts' function of Rsubread package (version 1.30.7). Expression of genes was measured by calculating fragments per kilobase of exon model per million mapped reads (FPKM value) using edgeR package (version 3.24.1) with default settings.

Principal components analysis and clustering analysis

Genes with extremely low or high expression (the mean FPKM in all samples < 0.3 or > 6000) were filtered out to decrease data noise and potential outliers.

The logtransformation was performed on the data. PCA was then performed on the data with 'prcomp' function in R v3.5.0 with the parameter `scale. = TRUE`.

The gene hierarchical clustering was done by using 'heatmap.2' function of gplots package (version 3.0.1 in R v3.5.0). Here, the Spearman correlation distance was calculated and the complete linkage clustering algorithm was chosen.

Gene expression and pathway analyses

Differential expression was estimated using the empirical Bayes methods (edgeR v3.24.1 in R v3.5.0) to obtain false discovery rate (FDR) and fold change. The differentially expressed genes (FDR < 0.05 , and fold change > 2) were extracted and fed to the DAVID website for the enriched pathway analysis.

Gene set enrichment analysis

The significantly differentially expressed genes (FDR < 0.05) were ranked by their log-transformed fold change value. Gene Set Enrichment Analysis (GSEA) was conducted using GSEA software (Version 4.0.3). The pre-ranked gene list and MousePath_All_gmt-Format.gmt or MousePath_GO_gmt.gmt gene set (both were downloaded from <http://ge-lab.org/gskb/>) were used for running the tool "Run GSEA Preranked" with default parameters.

CUT & RUN

Basal derived organoids of each culture condition were harvested 5 days after initial plating and enzymatically dissociated into single cells by incubation in pre-warmed TrypLE (Invitrogen) at 37°C for 20 minutes with disruption by pipetting every 7 minutes. CUT&RUN was performed based on the protocol by Skene et al., 2018 with some modifications described. Dissociated cells were spun down at 600g and resuspended in ice cold Wash Buffer (20mM HEPES pH 7.5, 150mM NaCl, 0.5mM Spermidine, 1X EDTA-free protease inhibitor, Roche, 04693159001). Three washes were completed. Cells were then quantified and bound to Concanavalin-A beads (Cell Signaling, 93569). 500,000 cells were used per replicate and each replicate bound to 10µL Concanavalin-A beads. Cell bound to beads were incubated with appropriate antibody at a concentration of 1:100, in 150µL antibody buffer (Wash Buffer with 2mM EDTA, 0.03% Digitonin, MilliporeSigma, 300410) at 4°C overnight with shaking at 400rpm. Beads were then washed twice with DIG-Wash Buffer (Wash Buffer with 0.03% Digitonin), and incubated with protein A/G-MNase (EpiCypher, 15-1016) for 1 hour at 4°C. Beads were washed twice and then Protein A/G-MNase was activated by adding Ca^{2+} to a final concentration of 1mM and incubating at 0°C for 1 hour in a heat block immersed in an ice water slurry. The reaction was stopped by adding 100µL 2X STOP Buffer (170mM NaCl, 20mM EGTA, 0.05%, 50µg/mL RNase A, 20µg/mL Glycogen, 250pg *E. coli* Spike-in DNA, EpiCypher 18-1401). Samples were then incubated at 37°C for 30 minutes, and supernatant was transferred to fresh tubes. Phenol chloroform isoamyl alcohol extraction was performed to purify

CUT&RUN DNA which was resuspended in 30 μ L 1mM Tris-HCl pH 8.0, 0.1mM EDTA.

CUT&RUN Library Prep and Sequencing

We used the NEBNext Ultra II DNA Library Prep Kit with some modifications to the protocol. For the adapter ligation step, the NEBNext Adaptor for Illumina was diluted 1:50, to 0.3 μ M. The adaptor ligated DNA was then cleaned up with 2.1X AMPure XP beads before proceeding. PCR was performed with NEBNext Multiplex Oligos for Illumina (E7600S) with the following thermocycler conditions: Initial denaturation 98°C, 30s, denaturation 98°C, 10s, annealing and extension 65°C, 12s, repeat denaturing and annealing and extension 14 times, final extension 65°C, 5 minutes. 0.5X AMPure beads were added to the reaction, mixed, incubated for 10 minutes, and samples were placed on a magnet stand. Supernatant was transferred to fresh tubes and then cleaned up with 2X AMPure beads. DNA concentrations were determined using Bioanalyzer High Sensitivity DNA kit (5067-4626). Samples were pooled such that equimolar amounts of each sample were present. Samples were sequenced on the NextSeq345 system at the genomics center at UC Davis at a depth of ~30M reads per sample.

CUT&RUN Data Processing

The sequencing data (fastq files) was assessed by FastQC (v0.11.8) for quality control and then were mapped with bowtie2 (v2.4.1) aligner to the mm10 mouse reference genome in very-sensitive-local mode with -I 10, -X 700. The resulting SAM files were converted to BAM files with samtools (v1.9) sorted with GATK (v4.1.9)

SortSam. The peaks were called using MACS2 (v2.2.7.1) from the alignment results with `-p 0.001`, `-keep-dup all`, `-SPMR True` and the resulting signals were converted into bigwig files. The downstream analyses were performed using R ChIPpeakAnno package (v3.28.1). Specifically, `findOverlapsOfPeaks()` was used to find the overlapping peaks between/among different peak sets. To annotate the peaks to known genomic features, annotation data containing only the known genes was first build using *TxDb.Mmusculus.UCSC.mm10.knownGene*, then the peaks were annotated using `annotatePeakInBatch()`. The genes near the peaks were uploaded to DAVID (<https://david.ncifcrf.gov>) to obtain a list of enriched terms. The fasta sequences corresponding to the peaks were extracted using bedtools(v2.30.0) and then MEME-ChIP (<https://meme-suite.org/meme/tools/meme-chip>) performed comprehensive motif analysis on these sequences to discover motifs in these peaks.

Chapter 4: Intrinsic transcriptome of prostate epithelial basal cells impacts their potential for stem cell activities

Corrigan Horton

ABSTRACT

Rare basal to luminal differentiation events in the adult homeostatic prostate may be mediated by a small population of adult basal stem cells. These cells would likely exhibit a unique transcriptome compared to normal adult basal cells. Here, using an unbiased approach, I attempt to identify potential adult basal stem cells based on differences in transcriptomes among the total basal population. Single cell sequencing reveals that the basal cell layer within the adult prostate is mostly homogenous, however two subpopulations can be identified that differentially express transcription factors of interest. Based on shRNA knockdown assays in organoid cultures, one of these transcription factors, *Fosl1*, may be a factor that plays a role in defining basal stem cells.

INTRODUCTION

The rarity of stem like activity in the adult prostate epithelium compared to the developing epithelium could be due not only to differing levels of signaling activity, but also a result of a dwindling pool of stem like cells within the adult epithelium. Intrinsic transcriptional differences between parent basal stem cells and their daughter cells can lead to differing responses to external stimuli. A good example of this type of heterogeneity within a cell population is that of the Lgr5 expressing intestinal stem cells (Barker, van Es *et al.*, 2007) where the canonical Wnt/ β -catenin target gene Lgr5 is expressed exclusively by columnar cells at the crypt base of the intestinal epithelium. Lineage tracing using a Cre knock-in allele of Lgr5 showed that Lgr5-positive crypt base columnar cells self-renew *in vivo* while also giving rise to all intestinal epithelial lineages. This finding paved the way for powerful functional studies on these intestinal stem cells.

Similar attempts have been made to identify unique markers of rare adult prostate stem cells. Although luminal cells are lineage restricted in the homeostatic adult prostate, rare castration resistant Nkx3.1 expressing (CARN) luminal cells have been found to have bipotentiality upon epithelial regeneration (Wang *et al.*, 2009). Interestingly, these CARN cells uniquely retain Nkx3.1 expression upon castration. This suggests an intrinsic difference within the cistrome/transcriptome between these cells and the general population of adult luminal cells. Due to the relatively high stem cell activity of adult basal cells compared to luminal cells, significant effort has been

made to identify potent basal progenitor cells that may possess unique transcriptomes such as the CARN luminal cells.

The use of flow cytometry has allowed researchers to isolate and test the stem cell capabilities of specific populations of basal cells. For example, Sca-1⁺CD49f^{high} basal cells isolated from dissociated adult mouse prostate showed sphere-forming ability and could readily reconstitute prostate tissue following recombination with embryonic urogenital mesenchyme in renal grafts (Lawson *et al.*, 2007). Similarly, Trop2⁺CD49f^{high} basal cells have been shown to produce luminal, basal, and neuroendocrine cells in tissue reconstitution assays (Goldstein *et al.*, 2008). Additionally, markers such as integrin α 2 β 1 (Collins *et al.*, 2001) and CD 133 (Richardson *et al.*, 2004) were used to identify candidate human prostate basal stem cell populations. Presently, it is unknown whether the basal cells isolated in these studies represent unique subsets of basal cells with intrinsic progenitor properties or if their progenitor properties seen in these ex vivo assays are common amongst most or all adult prostate basal cells. Here, I use single cell RNA sequencing in an attempt to find a basal cell population that may have unique transcriptional programming that distinguishes itself from the general basal population.

RESULTS

Transcription factors Fosl1 and Klf2 are differentially regulated between two distinct basal cell populations

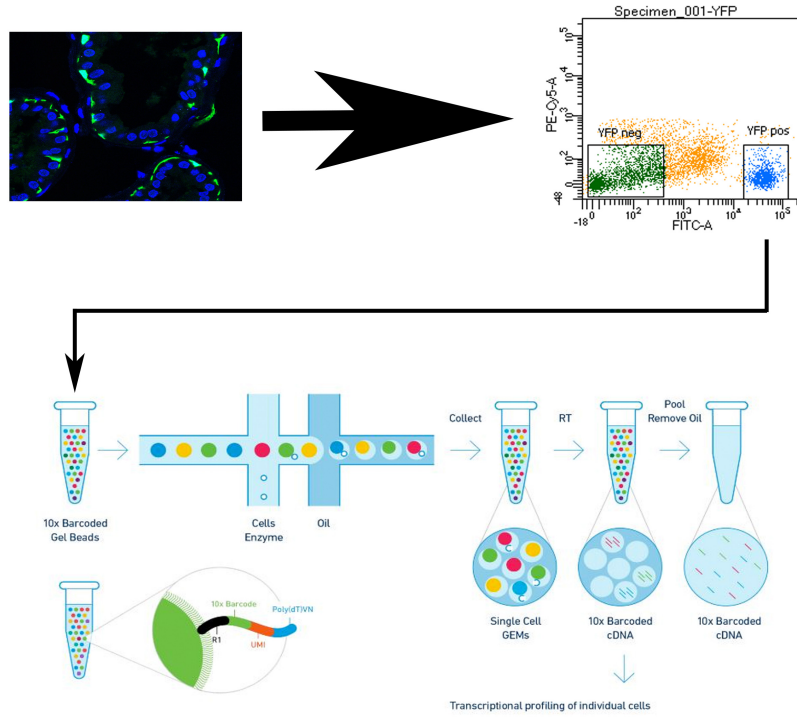
To determine the transcriptional complexity of the basal cell layer I isolated cytokeratin 5 (CK5) expressing basal cells at 2.5 months of age and performed single-

cell RNA sequencing using the 10X genomics Drop-seq platform (Fig. 4.1A). As we have done previously (Horton *et al.*, 2019), total basal cells were obtained by flow-sorting of YFP⁺ cells from *CK5-CreER^{T2}; R26R-CAG-YFP/+* mice that were tamoxifen-induced at 8 weeks of age (Fig. 4.1A). 5,031 out of 10,000 targeted basal cells passed quality control and ~1,900 genes were detected per cell. Based on known marker expression, I determined cell-type identities in the t-distributed stochastic neighbor embedding (t-SNE) plots. I identified the cluster at the bottom left of the t-SNE plot as luminal a luminal population based on the expression of luminal markers *Pbsn*, *Krt8*, *Krt18*, and *Nkx3.1*. A small number of stromal cells that express markers *Acta2* and *Col1a1* can also be seen at the bottom right of the plot. Both of these populations are likely a result of contamination from the sorting process and were therefor filtered during downstream analysis.

As can be seen from the t-SNE plot, the basal population is relatively homogenous in terms of the individual cell transcriptomes. However, when I set the K-means parameter to 2, we were able to see two somewhat distinct clusters that differentially express some genes of interest termed Basal 1 and Basal 2 (Figure 4.1A). I hypothesized that one of the clusters may be more stem-like than the other and decided to dig further into the differentially regulated genes in these two populations. After performing significant feature analysis on the 10X Loupe Browser, two lists were generated showing the upregulated genes in each population (Table 4.1). Two genes of interest were found at the top of each list: *fosl1* and *klf2*. Fosl1 (aka Fra-1) is a co-transcription factor and a member of the Fos family that binds with

members of the Jun family and form the transcription factor Activating Protein-1 (AP-1). AP-1 is known to be involved in cellular proliferation and differentiation (Angel and Karin, 1991) and Fosl1 has been shown to enhance growth and metastasis in human prostate cancer tissue and cell lines (Luo et al., 2018). Krupel like factor 2, or Klf2, which is a member of the krupel like factor family of zinc finger transcription factors which have been implicated in a variety of biological properties including proliferation, differentiation, development, and apoptosis (Pearson et al., 2008).

A



B

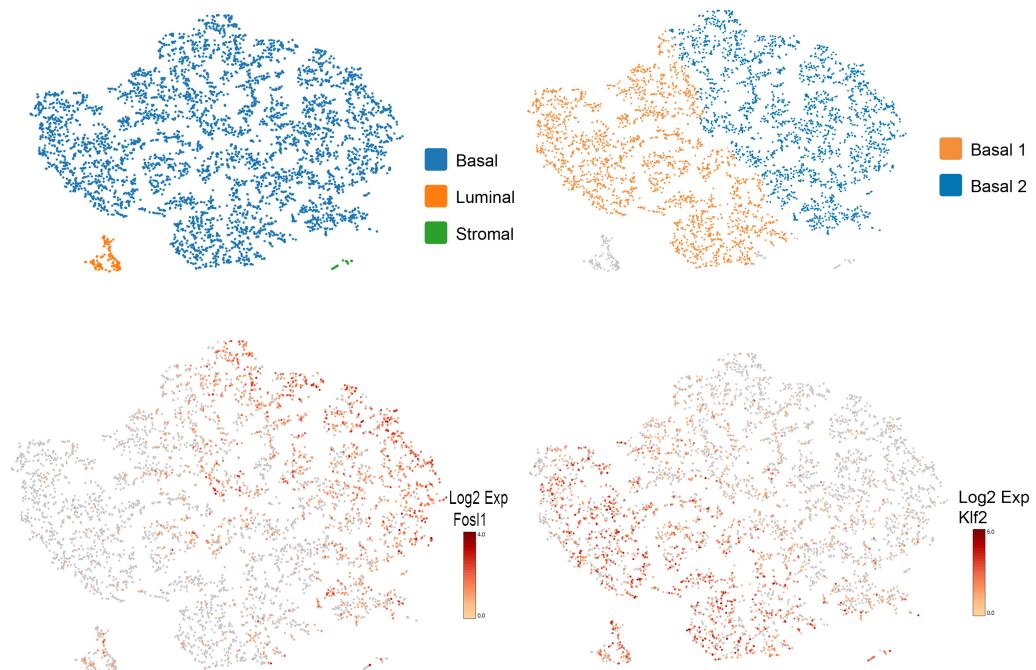


Figure 4.1 Transcription factors Fosl1 and Klf2 are differentially regulated between two distinct basal cell populations. A.) Overview of the method of isolating basal cells from *CK5-CreER^{T2}; R26R-CAG-YFP/+* mice to run single-cell RNA sequencing on the basal layer. B.) t-SNE plot showing cell types listed on the right. Basal population split into two clusters. C.) Differential regulation of *fosl1* (left) and *klf2* (right).

Knock down of Fosl1 decreases basal cell stem cell capabilities

In order to investigate the role these genes play in basal cell multipotency, I turned to prostate organoid culture. I adapted an organoid lentivirus transfection protocol from Karthaus et al., 2014 in order to silence *Fosl1* and *Klf2* using shRNA constructs (Figure 4.2A). In brief, I FACS isolated Sca-1⁺CD49f^{high} basal cells from 2.5 month old wild type mice and cultured them for 7 days to grow into mature organoids using well a established protocol (Drost et al., 2016). I then harvested the organoids, dissociated them into single cells and then infected them with a lentivirus containing a vector with our shRNA construct of interest and an mCherry reporter. I then replated the infected cells to allow them to develop into organoids once again. After two days, I performed a puromycin selection to kill any organoids that were derived from cells not containing the shRNA. 7 days after infection I saw both a greater than 2-fold reduction in *Fosl1* expression (Figure 4.2D) and a significant decrease in the size of the organoids carrying the *Fosl1* shRNA construct compared to scramble control (Figure 4.2B, C) while no size difference was seen in the *Klf2* shRNA containing organoids (Figure 4.3A, B). As a positive control, I infected organoids with the same *Pten* shRNA that was used in Karthaus et al. and similarly saw increased branching and disorganization (Figure 4.3A). Based on these results, I

concluded that Fos11 expression affects the organoid growth potential of prostate basal cells.

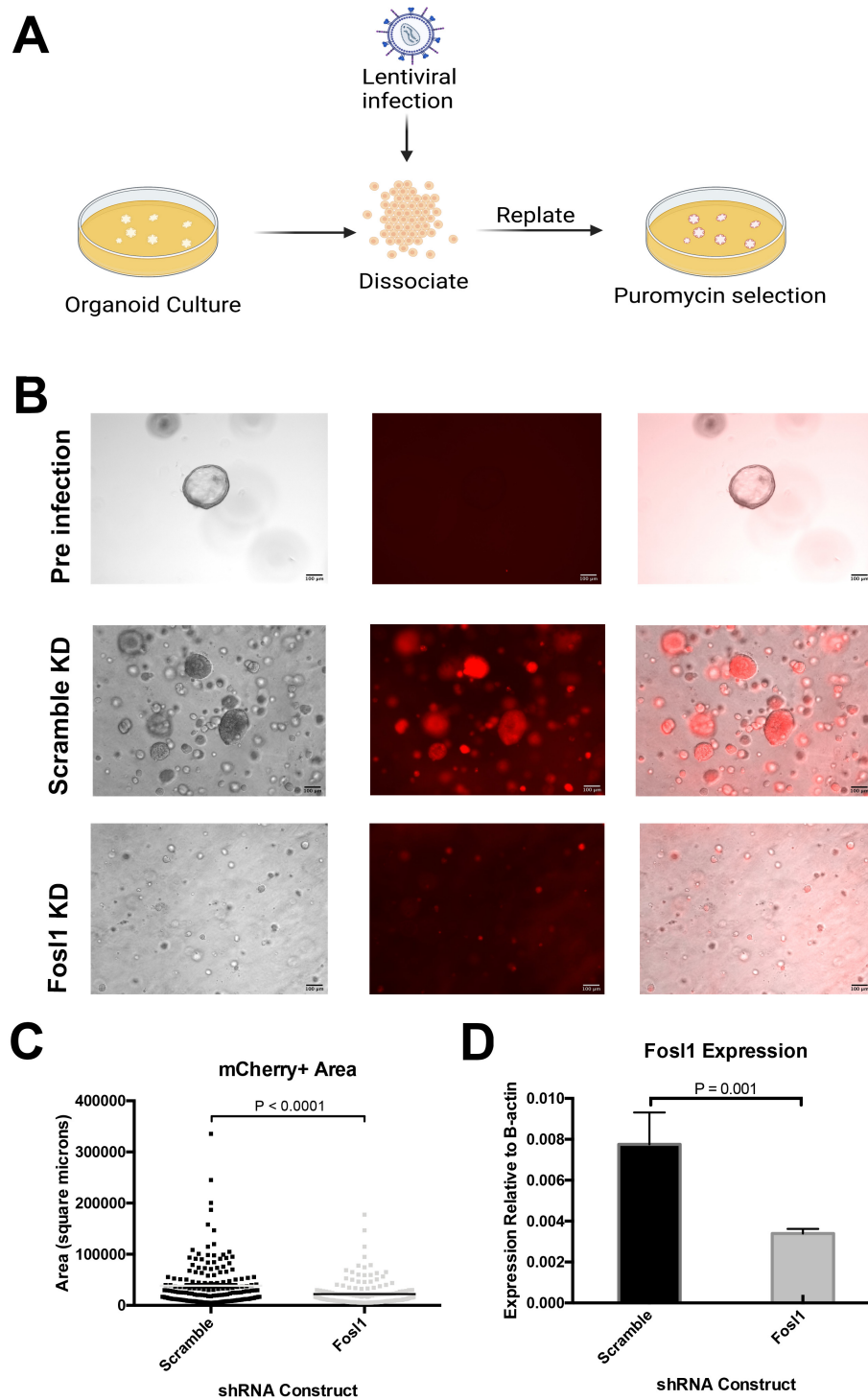


Figure 4.2 Knock down of Fosl1 decreases basal cell stem cell capabilities. A.) Strategy for infecting organoids with lentivirus containing shRNA constructs. B.) Images of full organoids from before infection (top), 7 days post infection scramble shRNA (middle), and 7 days post infection of fosl1 shRNA (bottom). mCherry seen in the red wavelength. Scale bar = 100um. C.) Quantification of organoid size in the scramble and fosl1 knockdowns. D.) Quantitative PCR of fosl1 expression in both groups.

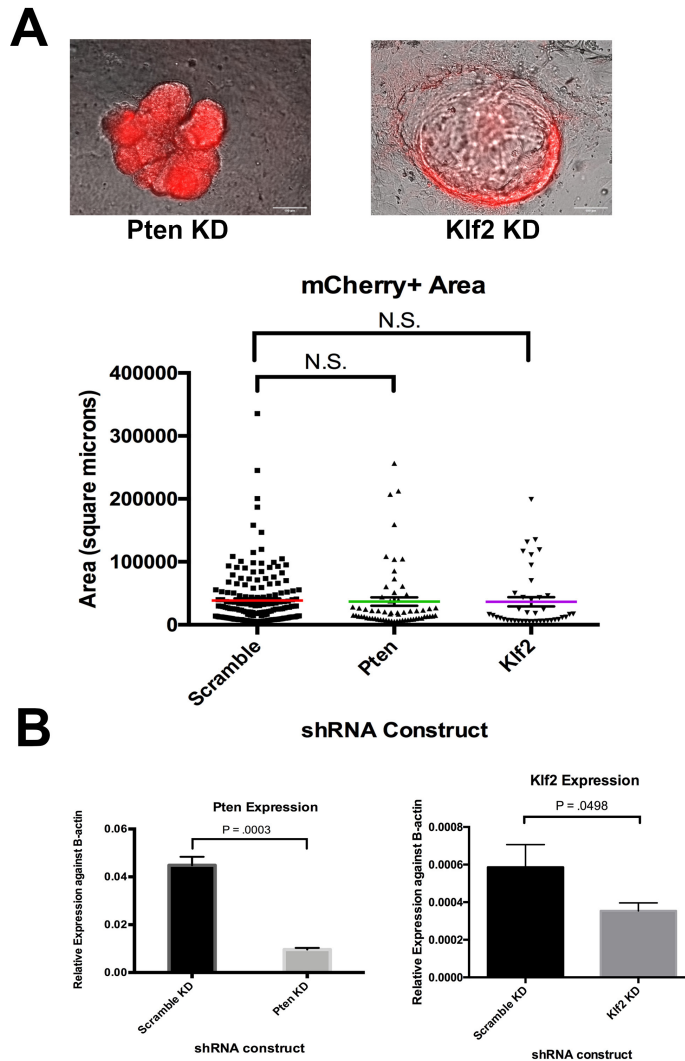


Figure 4.3 Efficiency of shRNA knockdown. A.) Representative images of Pten and Klf2 knock down and quantification of organoid size. scale bar = 100um. Sized compared to scramble control are not significantly different. B.) Quantitative PCR showing relative expression of Klf2 and Pten.

DISCUSSION

Stem cells often display inherent differences in transcriptional and cistrome landscape that leads to differential expression of genes associated with cell proliferation and differentiation compared to the differentiated cells in the resident organ tissue. In the adult prostate, many lineage tracing studies have shown that rare basal to luminal differentiation events occur during adult homeostasis. There is speculation in the field that these differentiation events may be restricted to a subpopulation of basal cells that retain stem like capabilities in adulthood. Here we show through single cell RNA sequencing of the total adult basal layer that such a population is not readily apparent; the basal cell population as a whole is very homogenous at a transcriptional level. While drop-seq is a powerful scRNAseq method that allows for the analysis of up to 10,000 single cells, it has limitations. Drop-seq has relatively low sensitivity compared to methods like smart-seq and SCRB-seq, which leads to lower resolution in the readouts. It may be that drop-seq is simply not sensitive enough to detect rare adult basal stem cells.

Although I did not find an obvious cell population to investigate from the tSNE plot, I was able to split the total basal population into two clusters that showed differential regulation of a couple of interesting genes of interest. After knocking down these genes in basal derived organoid cultures, we showed that *Fosl1* is an important factor that may help define basal stem cells. It is possible that no single cell marker can definitively distinguish adult basal stem cells from regular mature basal

cells. Further studies should be conducted to explore other potential cell markers that may help narrow down the search for adult basal stem cells.

Table 4.1. Quantitative Real-time PCR primers

Klf2	F: ACCAAGAGCTCGCACCTAAA
	R: GTGGCACTGAAAGGGTCTGT
Fosl1	F: CAGCCCAGAGGAGGAAGAG
	R: TCCTCCAATTTGTCGGTCTC
Pten	F: TGGGGAAGTAAGGACCAGAG
	R: GGCAGACCACAACTGAGGA

METHODS

Experimental models and subject details

Adult male mice (7–60 weeks old) were used in this study in accordance with protocols approved by the Institutional Animal Care and Use Committee at UCSC. The mice were housed in an animal facility with a regular 12-h light/dark cycle with ad libitum access to food and water. The *CK5-CreER^{T2}* and *R26R-CAG-EYFP* (Madisen et al., 2010) lines that were used were described previously. All animals used were maintained in C57BL/6N or mixed background, including the *Hi-Myc* allele (originally FVB background), which has been continuously backcrossed to C57BL/6N mice in the lab for over 15 generations. Genotyping was performed by PCR using tail genomic DNA, with the primer sequences listed in the key resources table S2.1.

Tamoxifen induction

Mice were administered 9 mg per 40 g body weight tamoxifen (Sigma) suspended in corn oil by oral gavage once daily for 4 consecutive days.

Tissue collection

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

Immunofluorescence staining

Immunofluorescence staining was performed using 6 μ m cryo sections. Slides were mounted with VectaShield mounting medium with DAPI (Vector Labs), and images were taken on a Leica TCS SP5 spectral confocal microscope in the UCSC Microscopy Shared Facility. YFP protein was directly visualized.

Prostate dissociation and flow cytometry

To isolate prostate basal and luminal cells, prostate tissues were dissected and minced to small clumps, followed by enzymatic dissociation with 0.2% collagenase I (Invitrogen) in DMEM media with 5% FBS for 3 h at 37°C. Tissues were digested with 0.25% Trypsin-EDTA (StemCell Technologies) for 1 h at 4°C, passed through 21- to 26-gauge syringes and filtered through a 40- μ m cell strainer to obtain single-cell suspensions. Dissociated prostate cells were suspended in Hanks' Balanced Salt Solution Modified/2% FBS. ROCK inhibitor Y-27632 (StemCell Technologies) was added at 10 μ M throughout the whole process to inhibit luminal cell death. Lineage-marked basal cells were sorted based on YFP. Sorting was performed on a BD FACS Aria II instrument in the Flow Cytometry Shared Facility of UCSC.

Single cell RNA-seq

Approximately 16,000 dissociated cells were obtained from the prostate

sample. Library preparation was performed using Chromium Single Cell 3' Solution with v3.1 chemistry following the manufacturer's protocol (10x Genomics). The library purity, size, and quantity were validated by capillary electrophoresis using 2100 Bioanalyzer (Agilent Technologies). The libraries were sequenced at the UC Berkeley Genome Center with a HiSeq4000 instrument (Illumina) to a depth of ~75k reads per cell.

Single cell data pre-processing and analyses

Raw sequencing data were processed using the 10x Genomics Cell Ranger pipeline (version 5.0.1) to generate FASTQ files and aligned to the mm10 genome (version 2020-A) to generate gene expression counts and quality control metrics. tSNE plots were then generated using the Cell Ranger pipeline.

Prostate organoid culture

Flow-sorted basal and luminal cells were washed with advanced DMEM/F12 (Life Technologies), and resuspended in 10 μ l advanced DMEM/F12 and 30 μ l Matrigel per well in the Nunc Lab-Tek II CC2 Chamber Slide System (Thermo Fisher Scientific). Chamber slide was put upside down in the 37°C cell culture incubator for 15 min to let the matrigel solidify. Mouse prostate organoid culture medium was prepared according a previous protocol (Drost et al., 2016). Briefly, the following components were added to advanced DMEM/F12 medium, B27 (50 $\times$ diluted), HEPES 1 M (100 $\times$ diluted), GlutaMAX (100 $\times$ diluted), Penicillin-streptomycin (100 $\times$ diluted), N-acetylcysteine (1.25 mM), EGF (50 ng/ml), A83-01 (200 nM), Noggin (100 ng/ml), R-spondin 1 (500 ng/ml), DHT (1 nM), Y-27632

dihydrochloride (10 μ M). Organoid culture medium was pre-warmed before adding to the wells. The medium was changed with fresh standard medium or paralleled luminal condition medium every 3 days. *In situ* organoid images were taken using the Zeiss Live Cell Microscope in the UCSC Microscopy Shared Facility. Organoid sizes were quantified using ImageJ.

Lentiviral Infection of Organoids

Lentiviral infections were performed as described previously (Koo et al., 2012) using pSicoR-Ef1a-mCh-Puro (addgene) targeting *Fosl1* (TGGATGGTGCAGCCTCATTTTC), *Klf2* (CCTAAACAACGTGTTGGACTT), *Pten* (GAGTTCTTCCACAAACAGAA), or control Scramble (CCTAAGGTTAAGTCGCCCTCG).

Quantitative real-time PCR

Cultured organoids in matrigel were scraped from the incubation chamber with spoon to exclude fibroblasts contamination. Organoids mRNA was isolated using the RNeasy Micro Kit (Qiagen). RNA was reverse transcribed and amplified into cDNA using SuperScrip III kit (Life Technology). Quantitative real-time PCR was carried out using Power SYBR Green PCR Master Mix (Life Technology) in the ViiA 7 Real-Time PCR instrument. Expression values were obtained using the $\Delta\Delta$ CT method and normalized to β -actin (*Actb*) expression; average values are shown as mean $\pm$ s.d. Primer sequences are provided in Table 4.1.

Chapter 5: Stromal AR inhibits prostate tumor progression by restraining secretory luminal epithelial cells

[This has been adapted from publication, **Stromal AR inhibits prostate tumor progression by restraining secretory luminal epithelial cells**. Liu et. al, 2022]

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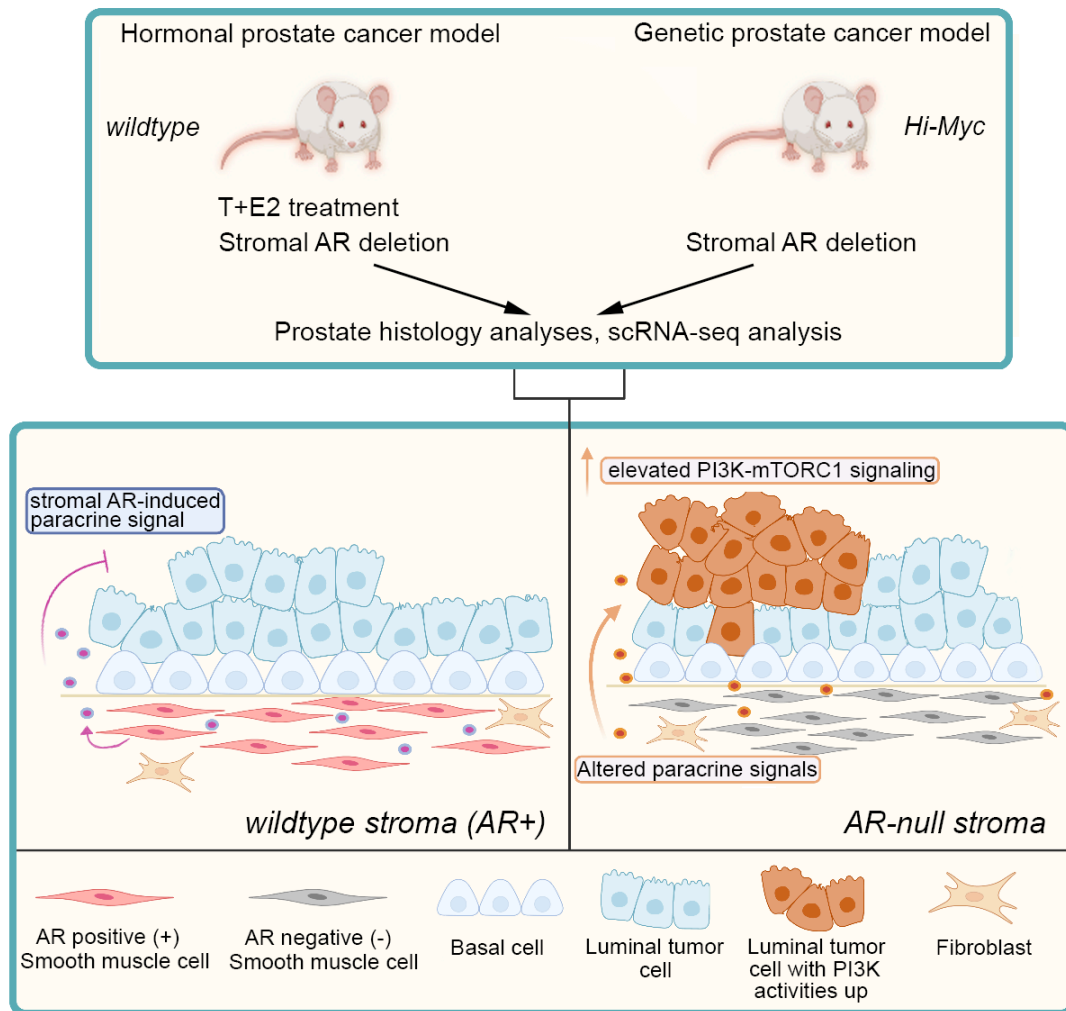
⁴These authors contributed equally

⁵Lead contact

ABSTRACT

Androgen receptor (AR) is expressed in both the prostate epithelium and the prostate stroma and plays diverse roles in prostate physiology. Although low expression of stromal AR is clinically associated with advanced cancer stage and worse outcome, whether stromal AR inhibits or promotes prostate cancer progression remains controversial. Here, we specifically delete *AR* in smooth muscle cells of the adult mouse prostate under two tumorigenic conditions, namely, the *Hi-Myc* genetic model and the T + E2 hormonal carcinogenesis model. Histology analyses show that stromal AR deletion exacerbates tumor progression phenotypes in both models. Furthermore, single-cell analyses of the tumor samples reveal that secretory luminal cells are the cell population particularly affected by stromal AR deletion, as they transition to a cellular state of potentiated PI3K-mTORC1 activities. Our results suggest that stromal AR normally inhibits prostate cancer progression by restraining secretory luminal cells and imply possible unintended negative effects of androgen deprivation therapy.

GRAPHICAL ABSTRACT



INTRODUCTION

Most prostate cancers (PCas) are adenocarcinomas that originate from the epithelial cells of the gland (Shen and Abate-Shen, 2010). Both basal and luminal cells, the two major cell types of the prostate epithelium, can serve as cells of origin for PCa (Choi et al., 2012; Wang et al., 2013). On the other hand, the stromal compartment, which is composed of smooth muscle cells (SMCs),

fibroblasts, endothelial cells, immune cells, and neurons, provides the crucial microenvironment for prostate development and cancer progression. Classic tissue recombination experiments have demonstrated the essential role of stromal-epithelial interactions in prostate organogenesis (Cunha et al., 1987; Hayward, 2002). In PCa, the altered stromal microenvironment, named reactive stroma, can promote cancer progression (Tuxhorn et al., 2001). For example, cancer-associated fibroblasts (CAFs) send paracrine signals, including growth factors and interleukins, to promote epithelial transformation (Sasaki et al., 2017).

Androgen is one of the key signals regulating prostate development and cancer (Murashima et al., 2014; Watson et al., 2015). The androgen receptor (AR) is expressed in both the prostate epithelium and the prostate stroma. In the epithelium, recent lineage-tracing analyses showed that ARs in basal and luminal cells play distinct roles in maintaining adult prostate homeostasis (Xie et al., 2017; Chua et al., 2018). In the stroma, classic experiments showed that AR-deficient urogenital sinus mesenchyme (UGSM) was unable to induce prostate budding when recombined with wild-type epithelium, suggesting that the stromal AR is essential for prostate organogenesis (Cunha and Lung, 1978). Consistent with this finding, genetic deletion of stromal AR in the developing mouse prostate inhibited epithelial cell growth and the differentiation of prostatic ducts and glandular acini (Welsh et al., 2011; Yu et al., 2011, 2012; Lai et al., 2012b; Lee et al., 2021).

How stromal cells and stromal AR affect PCa has been controversial (Singh et al., 2014; Wen et al., 2015; Leach and Buchanan, 2017). Early studies using epithelial-stromal cell co-culture conflicted regarding the role of stromal cells in epithelial cancer cell growth, with some favoring a negative role (Konig et al., 1987; Degeorges et al., 1996; Kooistra et al., 1997) and others a positive one (Kabalin et al., 1989; Lang et al., 2000; Castellon et al., 2005). Clinically, lower expression of stromal AR is associated with advanced PCa and worse patient outcome (Olapade-Olaopa et al., 1999; Henshall et al., 2001; Ricciardelli et al., 2005; Wikstrom et al., 2009; Leach et al., 2015). Paradoxically, AR-deficient mouse stroma was less capable of promoting tumor formation than wild-type stroma in epithelial co-culture assays and tumor cell renal grafts (Lai et al., 2012b; Ricke et al., 2012; Yu et al., 2012). In genetic mouse models, a study comparing AR deletion in the epithelium versus the entire organ in the TRAMP PCa model suggested a tumor-promoting role for stromal AR (Niu et al., 2008). Similarly, stromal AR deletion by FSP1-Cre and Tgln-Cre in the Pten-/+ cancer model was reported to diminish prostatic intraepithelial neoplasia (PIN) development (Lai et al., 2012a). In contrast, Welsh and colleagues used SMH-Cre to ablate AR in SMCs and found stromal AR to play a tumor-suppressing role in a hormonal carcinogenesis model (Welsh et al., 2011).

A possible cause of the discrepancy may be the different approaches used and their associated caveats. Renal grafting assay, and stromal-epithelial co-culture in particular, may not faithfully recapitulate actual cancer progression

due to a lack of orthotopic microenvironment. On the other hand, previous studies of conditional stromal AR knockout utilized Cre lines that were not inducible, potentially complicating data interpretation by having AR loss during development. To date, no stromal AR ablation experiment has been rigorously performed in adult prostate carcinogenesis in vivo. Here, we used an inducible SMC-specific CreER line to delete AR at the adult stage in two distinct PCa mouse models. We show that stromal AR deletion accelerated PCa progression for both, and such effect mostly worked through secretory luminal cells by altering their molecular program to a PI3K-activated state.

RESULTS

Stromal AR maintains normal prostate epithelial homeostasis and morphology

To delete AR in the adult prostate stroma, we obtained the *Myh11-CreER^{T2}* transgenic mouse, in which the inducible Cre is driven by the promoter of the gene smooth muscle myosin heavy polypeptide 11 (Wirth et al., 2008). To test its specificity, we generated *Myh11-CreER^{T2}; R26R-CAG-EYFP/+* (denoted *wt*) mice, induced them with tamoxifen at 7 weeks of age, and analyzed the prostate 2 weeks later (Figure 5.1A). Immunofluorescence (IF) staining showed that YFP⁺ cells were in the stromal layer and did not express basal marker CK5 or luminal marker CK18 (Figure 5.1B). Instead, staining with a smooth muscle actin (SMA) antibody showed that over 99% of the SMCs were labeled by YFP (Figure 5.1C). The vast majority of the YFP⁺ cells were AR positive, with slightly varying

proportions in different lobes (anterior lobe [AP] 92.7%; ventral lobe [VP] 88.4%; dorsal-lateral lobe [DLP] 83.3%) (Figures 5.1D and 5.1F). We then tested the efficiency of stromal AR deletion by tamoxifen induction of *Myh11-CreER^{T2}; AR^{fllox/Y}; R26R-CAG-EYFP/+* (denoted *str^{AR-}*) mice at 7 weeks of age (Figure 5.1A). Two weeks later, the proportions of AR⁺ cells in the YFP⁺ population (SMCs) significantly decreased for all the lobes (AP 16.3%; VP 13.6%; DLP 13.0%) (Figures 5.1E and 5.1F). In contrast, epithelial AR expression was not affected (Figure 5.1E). Therefore, we achieved AR deletion in SMCs with high efficiency and specificity.

We then characterized the long-term effects of stromal AR deletion in the *str^{AR-}* mice by analyzing the prostate phenotypes 9 months after tamoxifen induction (11 months of age) (Figure 5.1A). Prostate weight was reduced in the *str^{AR-}* mice compared with age-matched *wt* mice for all lobes (Figure 5.1G), consistent with the notion that stromal AR mediates androgen-dependent prostate epithelial growth (Yu et al., 2011). Size reduction was also observed for seminal vesicles and testes, since AR was deleted in SMCs of other urogenital organs as well (Figure S5.1). However, no difference in overall body weight or reproductive capability was noticed between *str^{AR-}* and *wt* mice. The proportions of AR⁺ SMCs in the prostate remained constant from 2 weeks to 9 months after induction (Figure 5.1F), and BrdU incorporation assay at 9 months after induction showed that AR⁺ and AR⁻ SMCs proliferated at similar rates (Figure 5.1H). No significant differences in luminal and basal cell proliferation were

detected between *str*^{AR-} and *wt* mice at this stage, either (Figure S5.2). Notably, hematoxylin and eosin (H&E) staining showed that *str*^{AR-} prostate at 9 month after induction contained smaller lumens compared to *wt* and displayed hypertrophy and foci of epithelial hyperplasia (Figure 5.1I), suggesting that stromal AR regulates the normal morphology and epithelial cell integrity in the adult prostate.

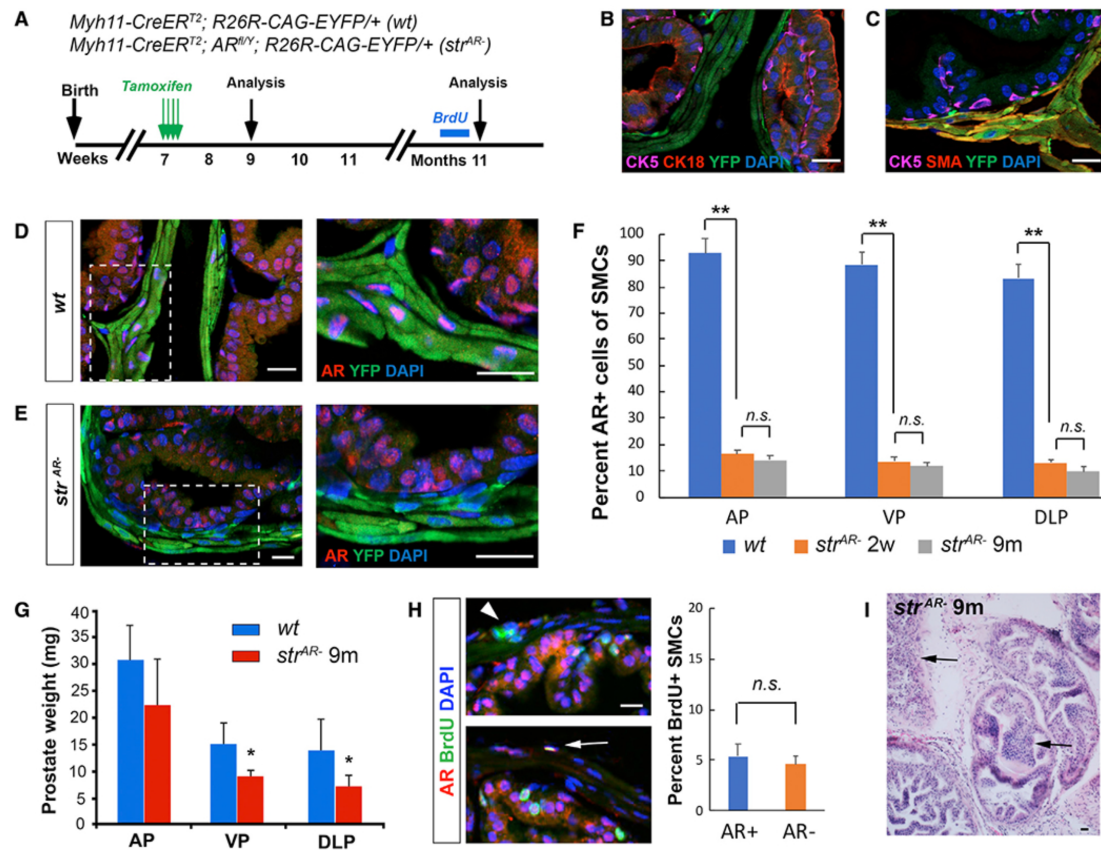


Figure 5.1. AR deletion in SMCs of the normal adult prostate

(A) Timeline of experiments for the *wt* and *str*^{AR-} mice. (B and C) IF staining showing YFP-marked cells present only in the stroma (B) and positive for SMA (C). (D and E) IF staining showing loss of AR expression in YFP⁺ cells from *wt* (D) to *str*^{AR-} (E). Zoom-in views of the boxed areas are shown on the right. (F) Quantitation of the proportions of AR⁺ SMCs in different prostate lobes before and after AR

deletion through time. (G) Quantitation of prostate lobe weights in *wt* and *str^{AR-}* mice 9 months after induction. (H) Representative IF staining of BrdU (left) and quantitation of the proportions of BrdU⁺ cells in AR⁺ and AR⁻ SMCs (right). Arrowhead points to an AR⁻BrdU⁺ SMC and arrow to an AR⁺BrdU⁺ SMC. (I) H&E staining showing prostate morphology of *str^{AR-}* mice 9 months after induction. Arrows point to regions of hyperplasia. **p* < 0.05 and ***p* < 0.001 by Student's *t* test. Scale bars, 20 μ m.

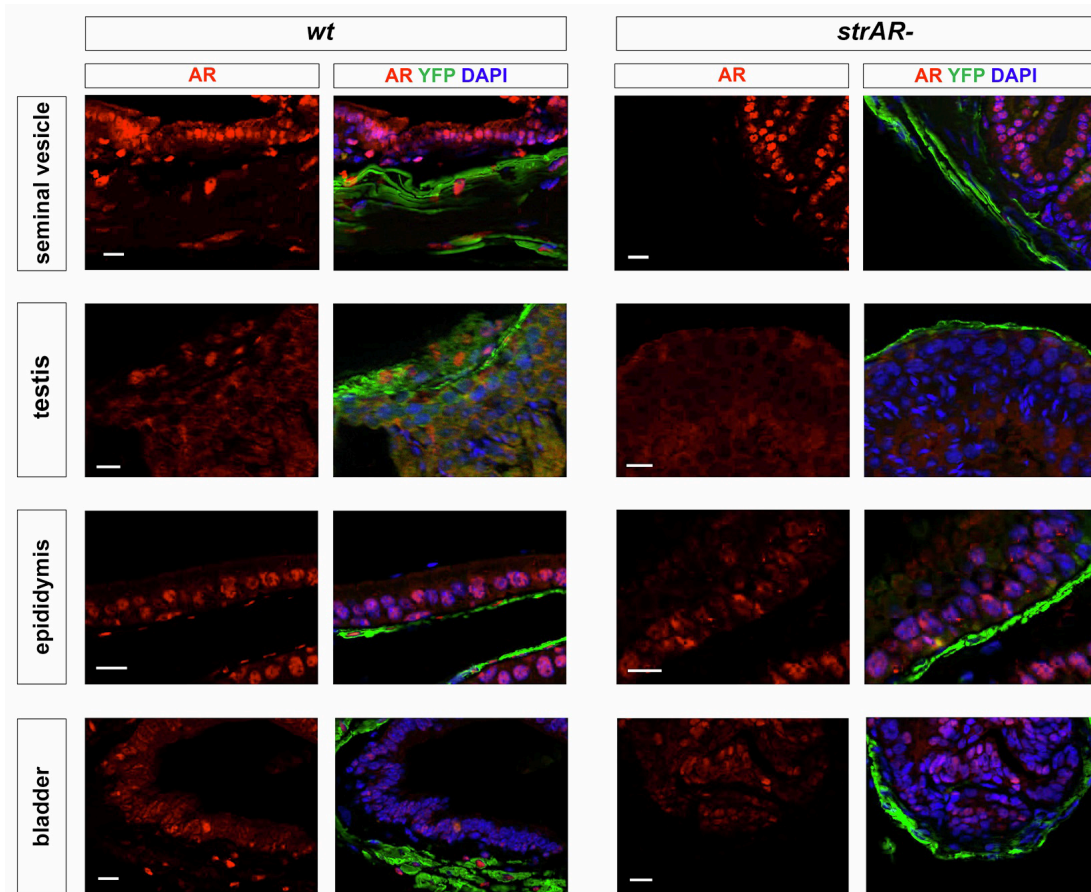


Figure S5.1, *Myh11-CreER^{T2}* deletes stromal AR in other tissues. Related to Figure 1. IF staining showing that AR (red) was deleted in SMCs (green) of the seminal vesicle, testis, epididymis, and bladder in *str^{AR-}* mice. Scale bar, 20 μ m.

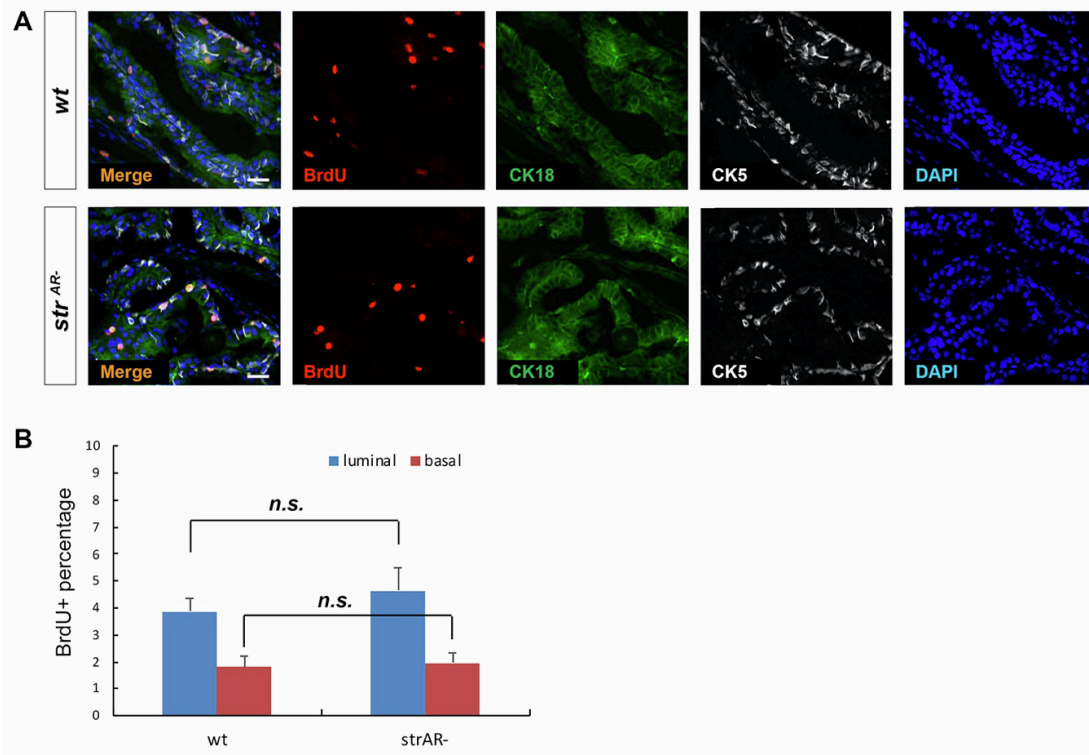


Figure S5.2. BrdU assay comparing epithelial cell proliferation between *strAR*⁻ and wt prostate at 11 months of age. Related to Figure 1. (A) Representative IF images with each individual channel. CK18 and CK5 mark luminal and basal cells, respectively. (B) Quantitation of the proportions of BrdU⁺ cells in luminal or basal cells of each sample. n.s., not significant by student t-test.

Loss of stromal AR enhances Hi-Myc tumor progression

To test the role of stromal AR in PCa progression, we first utilized the *ARR2/probasin-Myc (Hi-Myc)* model (Ellwood-Yen et al., 2003), in which Myc overexpression mimics one of the frequent oncogenic events in human PCa (Fraser et al., 2017). *Myh11-CreERT2; R26R-CAG-EYFP/+; Hi-Myc* (denoted *Myc*) and *Myh11-CreERT2; AR^{lox/Y}; R26R-CAG-EYFP/+; Hi-Myc* (denoted *strAR*⁻ *Myc*) male mice were generated, induced with tamoxifen at 7 weeks of age, and analyzed at different time points later (Figure 5.2A). One month after induction, IF staining

confirmed that AR was absent in over 90% of SMCs in the *str^{AR-} Myc* group (Figure 5.2B). H&E staining at 3 and 9 months post induction revealed frequent hyperplasia and PIN lesions in both groups. However, the *str^{AR-} Myc* group contained more high-grade PINs compared with the *Myc* group, and often displayed dilated ducts, cribriform patterns, and inflammation (Figure 5.2C and Table S5.1). To rigorously compare the histology between the two groups, we quantified tumor grades by evaluating each sample and assigning a histology score using a predefined formula in a double-blind setting (see methods). Our results (Figure 5.2D and Table S5.1) showed that stromal AR deletion promoted PCa progression from the *Hi-Myc* model. Indeed, BrdU incorporation assay showed that luminal cells were more proliferative in the *str^{AR-} Myc* group compared with the *Myc* group (Figures 5.2E and 5.2F), whereas no significant difference was found for basal or stromal cells between the two groups (Figure 5.2F). These data demonstrate that AR in the prostate SMCs plays a tumor-suppressing role in the *Hi-Myc* model.

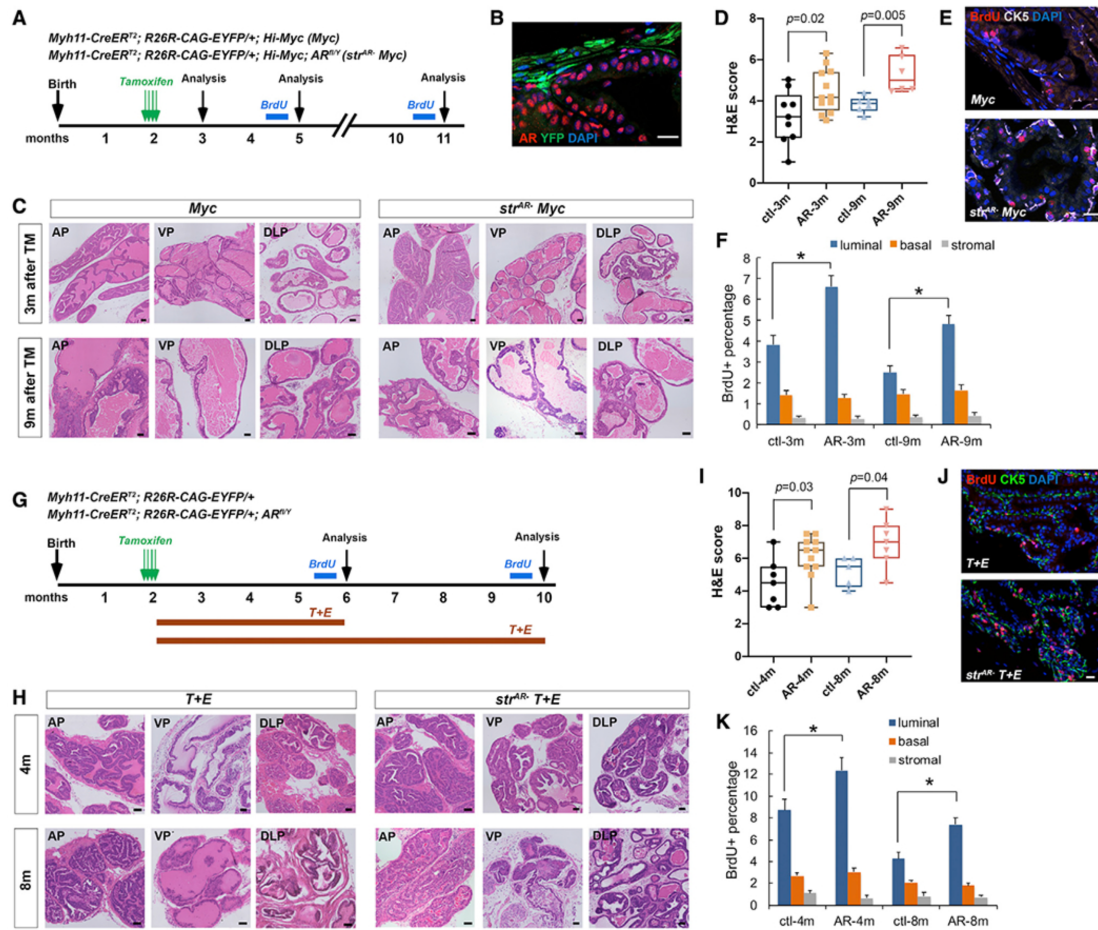


Figure 5.2. Stromal AR loss promotes tumor progression in the Hi-Myc and T + E models (A) Timeline of experiments for the *Myc* and *str^{AR-} Myc* mice. (B) IF showing efficient stromal AR deletion in *str^{AR-} Myc* mice. (C) Representative H&E images of different prostate lobes of *Myc* and *str^{AR-} Myc* mice 3 and 9 months post induction. (D) Quantitation of H&E scores. (E) Representative IF staining of BrdU in *Myc* and *str^{AR-} Myc* mice 9 months post induction. (F) Quantitation of the proportions of BrdU⁺ cells in the basal, luminal, and stromal layers of *Myc* and *str^{AR-} Myc* mice 3 and 9 months post induction. (G) Timeline of experiments for the *T + E* and *str^{AR-} T + E* mice. (H) Representative H&E images of different prostate lobes of *T + E* and *str^{AR-} T + E* mice 4 and 8 months post treatment. (I) Quantitation of H&E scores. (J) Representative IF staining of BrdU in *T + E* and *str^{AR-} T + E* mice 8 months post treatment. (K) Quantitation of the proportions of BrdU⁺ cells in the basal, luminal, and stromal layers of *T + E* and *str^{AR-} T + E* mice 4 and 8 months post treatment. Each dot in (D) and (I) represents a mouse in the group. * $p < 0.05$ by Student's t test. Scale bars in (B), (E), and (J), 20 μ m, and in (C) and (H), 100 μ m.

Mouse ID	Experiment	Score	Histology
425	ctl-3m	5.04	PIN1, dilated ducts, papillary and cribriform structures
426	ctl-3m	4.74	hyperplasia
427	ctl-3m	3.82	hyperplasia
2370	ctl-3m	2.25	overall normal, hyperplasia
2806	ctl-3m	3.22	overall normal, hyperplasia
2878	ctl-3m	3.81	hyperplasia
9018	ctl-3m	1.03	overall normal, some autolysis
9074	ctl-3m	2.14	overall normal, hyperplasia
9076	ctl-3m	2.86	overall normal, hyperplasia
401	AR-3m	3.28	hyperplasia, inflammation
402	AR-3m	5.42	PIN1
1494	AR-3m	4.15	hyperplasia
1496	AR-3m	4.73	hyperplasia, focal PIN1
2919	AR-3m	5.87	PIN2, atypia
2923	AR-3m	4.20	hyperplasia
2975	AR-3m	5.35	PIN3, dilated ducts, atypia in cribriform area
9136	AR-3m	3.06	overall normal, hyperplasia
9165	AR-3m	3.41	hyperplasia
9166	AR-3m	3.93	hyperplasia
9167	AR-3m	6.31	focal PIN3, atypia, cribriform, columnar cells, pleomorphism
9168	AR-3m	3.90	hyperplasia
1101	ctl-9m	4.01	PIN1
2369	ctl-9m	3.97	hyperplasia
2371	ctl-9m	3.77	hyperplasia
2372	ctl-9m	4.39	focal PIN1
2460	ctl-9m	3.22	hyperplasia
2519	ctl-9m	3.61	hyperplasia
1103	AR-9m	4.46	PIN1, focal proliferation to focal cribriform, mild atypia
2337	AR-9m	6.14	PIN3, dilated ducts, proliferation, mild atypia, periductal inflammation
2368	AR-9m	5.43	PIN1, focal thick bridges of epithelium across glands, hyperplasia, marked atypia, cribriform
9045	AR-9m	6.57	PIN2
9046	AR-9m	4.58	focal PIN1, proliferation to cribriform, columnar cells
9047	AR-9m	4.57	PIN1, focal proliferation, mild atypia

Table S5.1 *Hi-Myc* Model H&E scores and histology. Related to figure 2.

Stromal AR inhibits PCa progression during hormonal carcinogenesis

Given that the *Hi-Myc* model relies on the probasin promoter, which is turned on at the neonatal stage, we next set out to test whether our conclusion could be extended to other PCa models where tumor initiates strictly at the adult stage. In rodents, combined treatment of testosterone and estradiol-17 β (named the T + E2 model) has been used to mimic the hormonal change in aging men (Ricke et al., 2008; Wang et al., 2014), whose relative increase in estrogen and decrease in androgen levels are associated with increased PCa incidence (Wilson, 1980). We therefore induced *Myh11-CreER^{T2} ; R26R-EYFP/+* and *Myh11-CreER^{T2} ; AR^{f/y} ; R26R-EYFP/+* mice with tamoxifen at 7 weeks of age, implanted T and E2 tubes subcutaneously to induce tumor initiation (denoted *T + E* and *str^{AR-} T + E*, respectively), and analyzed prostate histology 4 and 8 months later (Figure 5.2G). ELISA confirmed that serum estrogen levels were significantly enhanced after T + E2 treatment for both groups of mice (Figure S5.3). We found that, compared with the *T + E* group, the *str^{AR-} T + E* group contained more prominent high-grade PIN lesions, with some regions displaying cribriform patterns and epithelial cells filling into the lumen (Figure 5.2H and Table S5.2). Double-blind quantification of the histology scores showed that *str^{AR-} T + E* tumors were significantly more aggressive than the *T + E* tumors (Figure 5.2I and Table S5.2), in agreement with the previous report using *SMH-Cre* (Welsh et al., 2011). BrdU incorporation assay again revealed higher proliferation of luminal cells, but not basal and stromal cells, in the *str^{AR-} T + E* group compared with the *T + E* group (Figures 5.2J and 5.2K). Taken together, we conclude that stromal AR deletion

induced more aggressive PCa phenotypes in both the genetic and the hormonal carcinogenesis models.

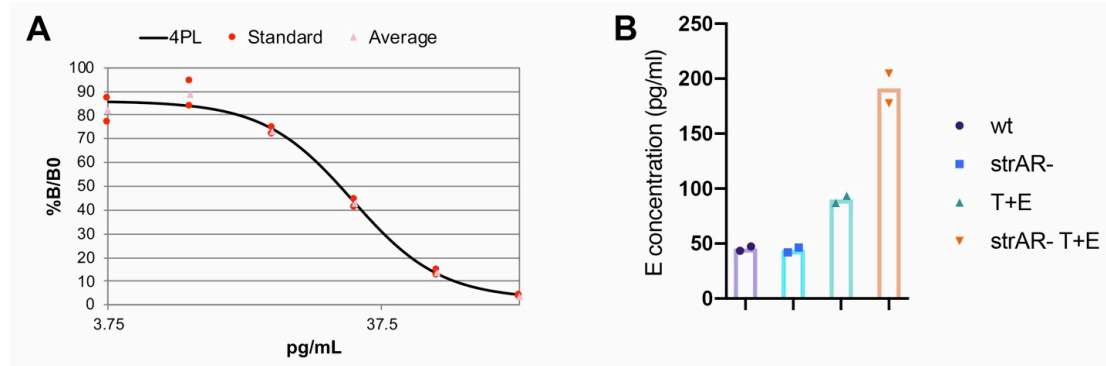


Figure S5.3. ELISA measurement of serum estrogen levels after T+E treatment. Related to Figure 2. (A) Standard curve created by the Arbor Assays Estradiol Serum EIA kit. (B) Quantitation of serum estradiol levels in age-matched *wt*, *strAR⁻*, and *T+E* and *strAR⁻ T+E* mice 8 months after tube implantation. N=2 biological replicates in each group.

Mouse ID	Experiment	Score	Histology
293	ctl-4m	7.0	PIN1
330	ctl-4m	5.0	hyperplasia
1198	ctl-4m	5.5	hyperplasia
2736	ctl-4m	3.5	overall normal, hyperplasia
9384	ctl-4m	3.0	overall normal, hyperplasia
9437	ctl-4m	3.0	overall normal, hyperplasia
9625	ctl-4m	4.5	hyperplasia
1092	AR-4m	5.5	hyperplasia
1123	AR-4m	6.5	PIN1, inflammation
1125	AR-4m	7.0	PIN1, inflammation
1134	AR-4m	6.5	PIN1
1135	AR-4m	5.5	hyperplasia, necrosis
1136	AR-4m	7.5	PIN3, epithelial hyperplasia
1201	AR-4m	7.5	focal PIN3, cribriform, columnar and atypical cells
1203	AR-4m	6.0	hyperplasia, focal PIN1
9446	AR-4m	3.0	overall normal, hyperplasia
9612	AR-4m	5.0	hyperplasia
9615	AR-4m	7.0	hyperplasia, focal PIN1
251	ctl-8m	4.0	overall normal, hyperplasia
1283	ctl-8m	4.5	hyperplasia
1483	ctl-8m	5.5	hyperplasia
1485	ctl-8m	6.0	hyperplasia, focal PIN1
5436	ctl-8m	6.0	PIN1
451	AR-8m	6.5	PIN1, epithelial hyperplasia
579	AR-8m	9.0	PIN3, epithelial filling gland, gland enlargement
1087	AR-8m	6.0	focal PIN3, hyperplasia, gland filled with epithelial cells
1279	AR-8m	4.5	hyperplasia, focal PIN1, inflammation
5419	AR-8m	7.0	PIN1, epithelial hyperplasia, gland enlargement
5421	AR-8m	7.5	PIN2, epithelial hyperplasia
5423	AR-8m	8.0	PIN2

Table S5.2. T+E2 model H&E scores and histology. Related to Figure 2.

Loss of stromal AR primarily affects secretory luminal tumor cells

To understand how stromal AR deletion promotes PCa progression, we next performed single-cell RNA sequencing (scRNA-seq) for the 8-month *T + E* and *str^{AR-} T + E* tumors by 10× Genomics Drop-seq. We profiled 3,373 *T + E* and 2,689 *str^{AR-} T + E* prostate cells that passed quality control, and ~1,500 genes were detected per cell. Based on known marker expression, we assigned cell-type identities in the t-distributed stochastic neighbor embedding (t-SNE) plots (Figure 5.3A), including basal cells, secretory luminal cells, the recently identified proximal luminal cells (Karthaus et al., 2020; Kwon et al., 2020), SMCs, fibroblasts, leukocytes, endothelial cells, erythrocytes, and seminal vesicle cell contaminants. Notably, in both samples, secretory luminal cells (identified by markers *Krt8*, *Krt18*, *Nkx3.1*, and *Pbsn*) contained multiple cell clusters, indicating that they became highly heterogeneous during tumor progression. The two samples were then integrated with single-cell variational inference (scVI) tools (Gayoso et al., 2021) and re-clustered (Figure 5.3B). Differentially expressed genes in each cluster are listed in Table S5.3. This analysis showed that the two samples were similar on a global level, as most clusters aligned well between them, except for the secretory luminal cell clusters (clusters 1, 3, 4, and 6) (Figure 5.3C). Specifically, in uniform manifold approximation and projection (UMAP), clusters 1 and 3 mostly belong to the *T + E* sample, whereas clusters 4 and 6 belong to the *str^{AR-} T + E* sample (Figures 5.3B and 5.3C). We hypothesized that these four secretory luminal cell clusters were responsible for the

phenotypic differences observed between the two groups of mice, and therefore focused on them for further analyses.

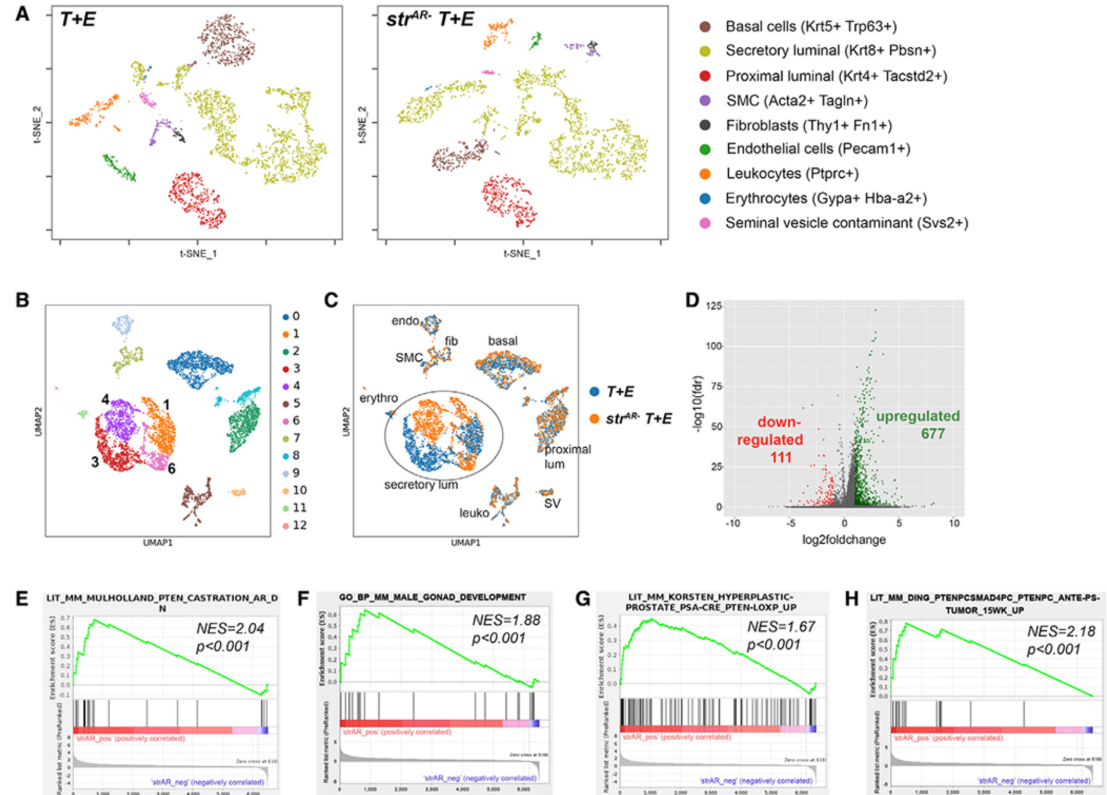


Figure 5.3. scRNA-seq analysis of *T + E* and *Str^{AR}- T + E* prostate (A) t-SNE plots showing various cell populations (identifiable by markers listed on the right) in the *T + E* and *Str^{AR}- T + E* prostate. (B) UMAP showing color-coded cell clusters of integrated samples using scVI tools. (C) UMAP showing alignment of cell types between the *T + E* and the *Str^{AR}- T + E* samples. (D) Volcano plot showing up- and downregulated genes in *Str^{AR}- T + E* versus *T + E*. (E–H) GSEA comparing differentially expressed genes in secretory luminal cells of *Str^{AR}- T + E* versus *T + E* against GO pathways and published gene signatures.

Gene expression differential analysis comparing all secretory luminal cells (clusters 1, 3, 4, and 6) between the two samples showed that 677 genes were upregulated and 111 downregulated in *Str^{AR}- T + E* relative to *T + E* (false discovery rate [FDR] < 0.05 and log fold change > 1 or < -1) (Figure 5.3D, Tables

S5.4 and S5.5). Gene set enrichment analysis (GSEA) (Subramanian et al., 2005) showed that genes overexpressed in the *str*^{AR-} *T + E* sample were enriched in a previously published AR-repressed-gene signature obtained via mouse castration (Mulholland et al., 2011) (Figure 5.3E), indicating that stromal AR deletion has effects similar to those of castration. GSEA further showed that genes overexpressed in *str*^{AR-} *T + E* were enriched in the Gene Ontology (GO) male gonad development (Figure 5.3F), in genes upregulated in a *PSA-Cre*; *Pten*^{f/f} model relative to wild type (Korsten et al., 2016) (Figure 5.3G), as well as genes upregulated in a *Smad4-Pten*-null model relative to *Pten*-null alone (Ding et al., 2011) (Figure 5.3H). GSEA of downregulated genes in the *str*^{AR-} *T + E* sample showed that the reverse trend was also true (Figure S5.4). These data demonstrate that the *str*^{AR-} *T + E* tumor is molecularly more aggressive than the age-matched *T + E* tumor.

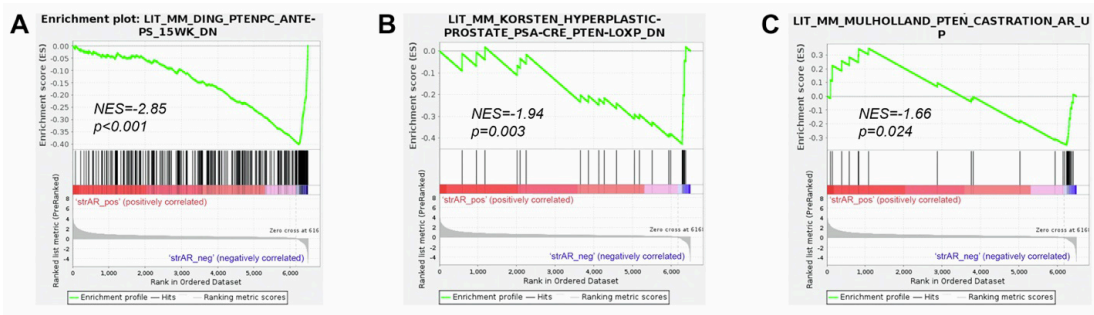


Figure S5.4. GSEA comparing differentially expressed genes in secretory luminal cells of *str*^{AR-} *T+E* vs *T+E* prostate. Related to Figure 3. (A-C) Genes downregulated in *str*^{AR-} *T+E* vs *T+E* are enriched in two independent published gene signatures of downregulated genes of *Pten*-null tumors vs. wt (A, B) and a published gene signature of AR-responsive genes (C).

Subpopulation of luminal tumor cells shows enhanced PI3K-mTORC1 activity

We next examined the heterogeneity within the secretory luminal cells. Clusters 3 and 4 corresponded to the subpopulation of secretory luminal cells with lower luminal marker expression than clusters 1 and 6 in the $T + E$ and $str^{AR-} T + E$ samples, respectively (Figures 5.4A and S5.5). They also appeared to represent more advanced-stage tumor cells compared with clusters 1 and 6. For example, GSEA showed that genes downregulated in cluster 6 relative to 4 were highly enriched in the KEGG pathway chemical carcinogenesis (Figure 5.4B). Furthermore, the progression of these luminal tumor cells was similar between the $str^{AR-} T + E$ and the $T + E$ samples, since GSEA showed that upregulated genes in cluster 4 versus 6 were significantly enriched in the top 300 upregulated genes in cluster 3 versus 1 (Figure 5.4C). When we compared the advanced-stage tumor cells between the two samples (cluster 4 in $str^{AR-} T + E$ versus cluster 3 in $T + E$), we found that genes downregulated in cluster 4 versus 3 were enriched in two published signatures of genes downregulated in *Pten*-null tumors versus wild-type tissues (Ding et al., 2011; Korsten et al., 2016) (Figures 5.4D and 5.4E). These data suggest that the advanced-stage luminal tumor cells in the $str^{AR-} T + E$ prostate were more aggressive than their counterparts in the $T + E$ prostate and that stromal AR deletion elicited a tumor progression program resembling PI3K pathway activation in the secretory luminal cells. Consistent with this notion, we found that, after excluding cells below a minimal detection threshold, *Pten* expression in clusters 3 and 4 was lower than in clusters 1 and 6

(Figures 5.4F and 5.4G). Between clusters 3 and 4, cluster 3 also contained more cells with relatively high *Pten* expression (Figure 5.4G), suggesting that the loss of stromal AR may downregulate Pten in luminal tumor cells to accelerate their progression.

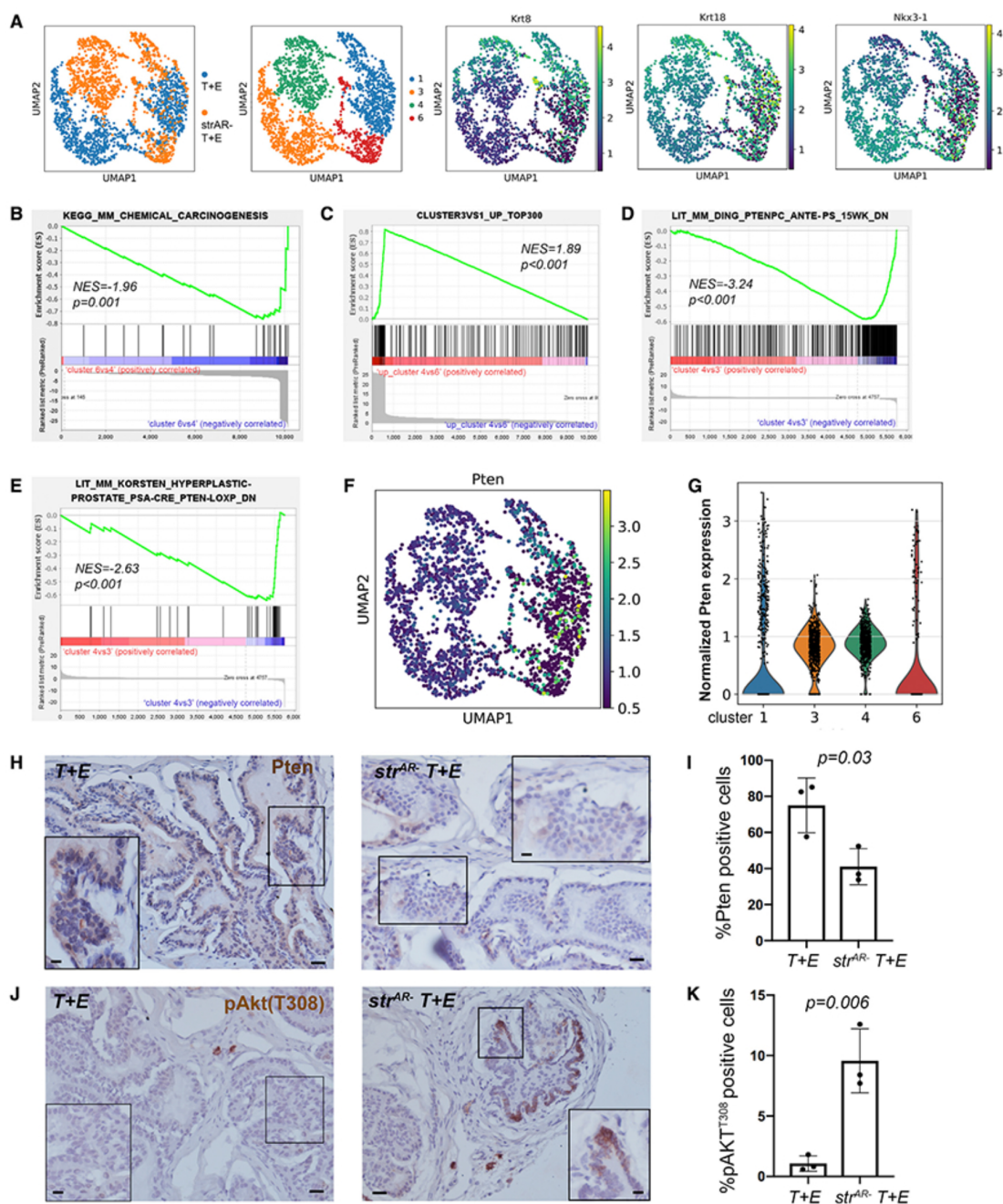


Figure 5.4. Luminal tumor cell heterogeneity in the $T + E$ and $str^{AR-} T + E$ prostate (A) UMAP plots showing the expression of luminal marker genes in secretory luminal clusters 1, 3, 4, and 6. (B and C) GSEA showing that upregulated genes in cluster 4 versus 6 are enriched in KEGG pathway chemical carcinogenesis (B) and in the top 300 upregulated genes in cluster 3 versus 1

(C). (D and E) GSEA showing that downregulated genes in cluster 4 versus 3 are enriched in two downregulated gene signatures of *Pten*-null tumors. (F and G) UMAP (F) and violin plots (G) showing *Pten* expression in clusters 1, 3, 4, and 6. (H and J) Representative IHC images showing *Pten* (H) and pAkt-T308 (J) staining in *T + E* (left) and *str^{AR-} T + E* (right) tissues. Scale bars, 50 μ m. Scale bars in insets, 10 μ m. (I and K) Quantitation of percentages of *Pten*-positive (I) and pAkt-T308-positive (K) epithelial cells. *n* = 3 animals per group. The *p* values were calculated by Student's *t* test.

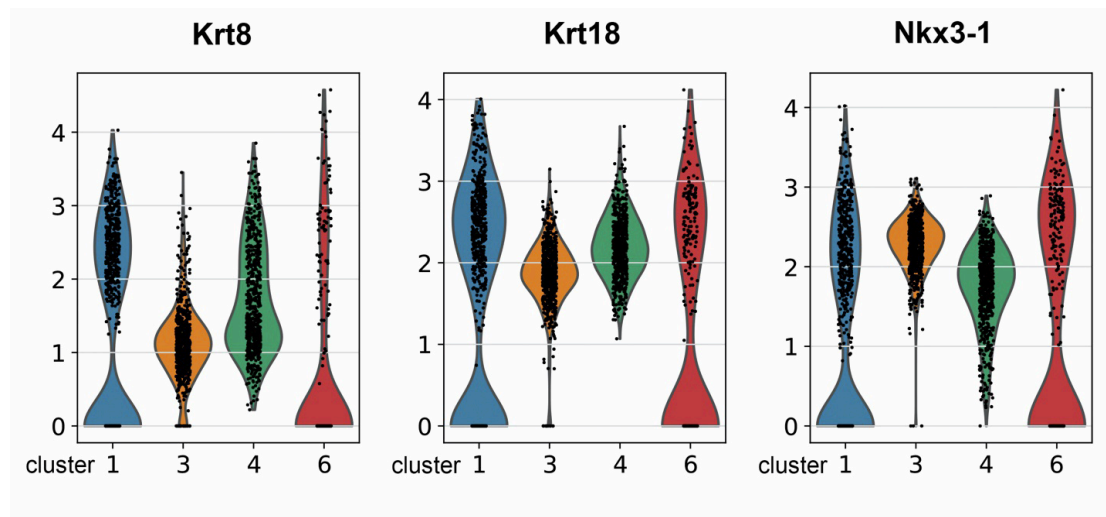


Figure S5.5. Violin plots showing expression of luminal marker genes Krt8, Krt18, and Nkx3.1 in clusters 1, 3, 4, and 6. Related to Figure 4. Clusters 1 and 6 include cells that failed to pass the minimal detection threshold at the base, but generally have cells with higher luminal marker expression levels than clusters 3 and 4.

To further validate this finding, we performed immunohistochemistry (IHC) to detect *Pten* in *T + E* and *str^{AR-} T + E* tissues (*n* = 3 animals each). While the vast majority of the *T + E* epithelial cells showed positive signals, *Pten* staining was significantly decreased in many PIN foci of the *str^{AR-} T + E* epithelium (Figures 5.4H and 5.4I). To evaluate the PI3K signaling downstream effectors, we performed IHC to detect phospho-Akt Ser473 (pAkt-S473), an mTORC2 target, as well as mTORC1

signaling components phospho-Akt Thr308 (pAkt-T308), ribosomal protein S6 phosphorylated at Ser235/236 (pRPS6), and 4e-binding protein 1 phosphorylated at Thr37/46 (p4E-BP1) (Pearson et al., 2018). We did not detect pAkt-S473-positive epithelial cells in either group (Figure S5.6A), suggesting no activation of the mTORC2 pathway. However, clusters of pAkt-T308-positive epithelial cells were present in all the samples, and its percentage significantly increased in the *str*^{AR-} *T* + *E* tissues compared with *T* + *E* (Figures 5.4J and 5.4K). Moreover, *str*^{AR-} *T* + *E* epithelium contained significantly higher percentages of p4E-BP1-positive (Figures S5.6B and S5.6C) and pRPS6-positive cells (Figures S5.6D and S5.6E). Taken together, these results are consistent with the scRNA-seq finding, and indicate that stromal AR loss stimulated the PI3K-mTORC1 signaling activity in a subpopulation of luminal tumor cells.

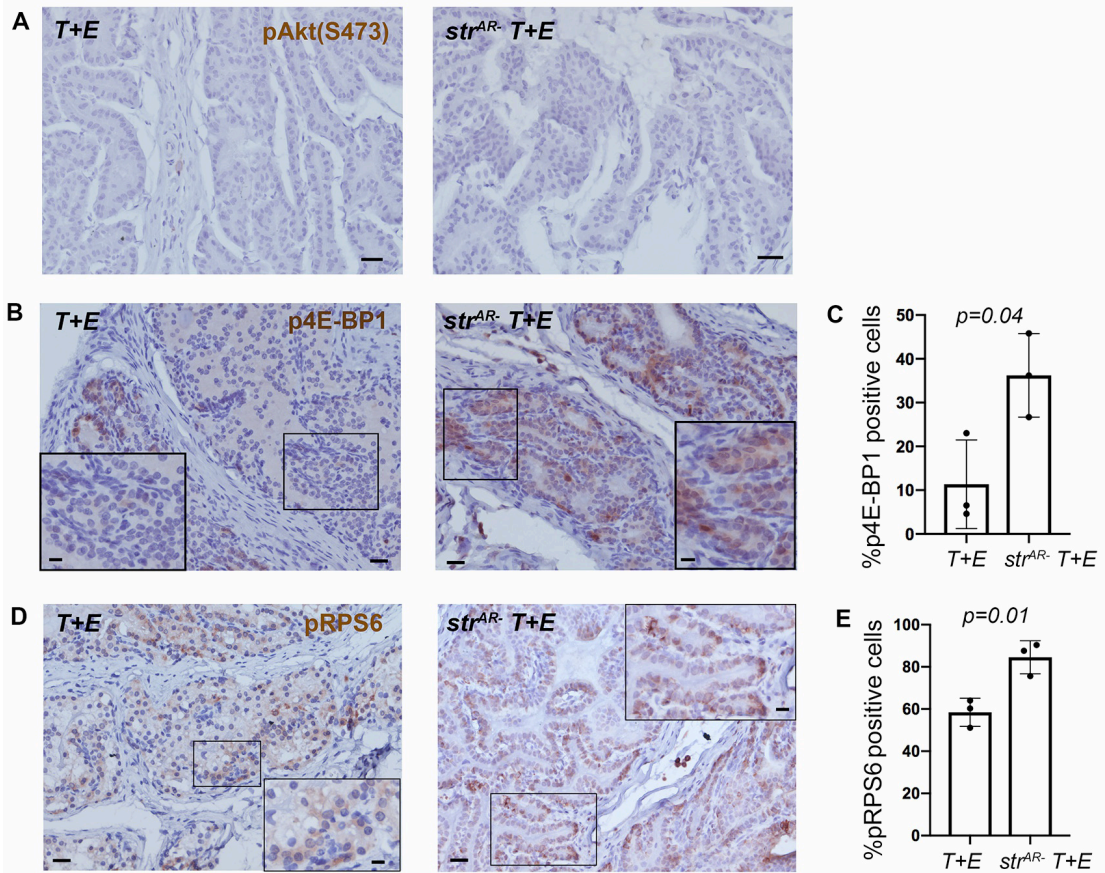


Figure S5.6. Higher PI3K-mTORC1 activity in *str^{AR-} T+E* tumors. Related to Figure 4. (A,B,D) Representative IHC images showing pAkt(S473) (A), p4E-BP1 (B), and pRPS6(S235/236) (D) staining in *T+E* (left) and *str^{AR-} T+E* (right) tissues. Scale bars, 50 μ m. Scale bars in inset, 10 μ m. (C,E) Quantitation of percentages of p4E-BP1 positive cells (C) and pRPS6(S235/236) positive cells (E) in the tumor epithelium. N=3 animals per group. P values calculated by student's t-test.

*Enhanced PI3K activity in a luminal subpopulation of the *str^{AR-} Myc* tumor*

Since the T + E model tends to capture an earlier window of tumorigenesis, while the *Hi-Myc* model offers a wider spectrum of pathological stages, we next asked whether similar mechanisms could also explain the more aggressive phenotypes in the *str^{AR-} Myc* tumors. To this end, we performed scRNA-seq comparing *Myc* and *str^{AR-} Myc* tumors that had progressed for 12 months, with 1,749

cells and 1,713 cells profiled at $\sim 1,200$ genes per cell, respectively. Integration of the two samples and re-clustering by scVI showed that, while most of the cell clusters aligned well between *Myc* and *str^{AR-} Myc* samples, differences again existed in the secretory luminal cell populations (identified by Pbsn⁺ Krt8⁺) (Figures 5.5A and 5.5B). We assigned cell types to different clusters based on marker expression (Figure 5.5C and Table S5.6) and noticed that various stromal cell populations, including leukocytes, were more prominently represented compared with the previous single-cell analysis of the T + E model. Importantly, we found that, while cluster 1 contained secretory luminal cells that overlapped between the two samples, clusters 6 and 11 contained secretory luminal cells specific to *str^{AR-} Myc* (Figures 5.5A and 5.5C). GSEA showed that genes upregulated in cluster 6 relative to cluster 1 were highly enriched in two aforementioned upregulated gene signatures of *Pten*-null prostate tumors (Ding et al., 2011; Korsten et al., 2016) (Figures 5.5D and 5.5E). Conversely, genes downregulated in cluster 6 versus 1 were significantly enriched in the downregulated *Pten*-null gene signature (Ding et al., 2011) (Figure 5.5F). These data suggest that, similar to the T + E model, *str^{AR-} Myc* tumors also harbored more aggressive luminal tumor cells with enhanced PI3K activity. In further support, scRNA-seq data showed lower *Pten* expression in cluster 6 versus cluster 1 (Figure 5.5G) and IHC showed significantly higher proportions of pAkt-T308-positive epithelial cells in the *str^{AR-} Myc* tumors compared with the *Myc* tumors (Figures 5.5H and 5.5I). On the other hand, no *Pten*-related gene signature was significantly enriched in a GSEA comparison between clusters 11 and 1. Instead, genes

upregulated in cluster 11 versus 1 were highly enriched in a gene signature induced by interferon γ (IFN γ) (Jehl et al., 2012) (Figure 5.5J), as well as several upregulated gene signatures of lipopolysaccharide (LPS) treatment (Shell et al., 2005; Kitamura et al., 2008; Bonow et al., 2009) (Figures S5.7A–S5.7C). Given the high frequency of inflammatory foci in *str*^{AR-} *Myc* tumors (Table S5.1), cluster 11 likely represented luminal cells receiving immune cell signals in an inflammatory microenvironment. Overall, upregulation of PI3K activity in luminal tumor cells appears to be a shared feature between the T + E and the *Hi-Myc* models after stromal AR deletion.

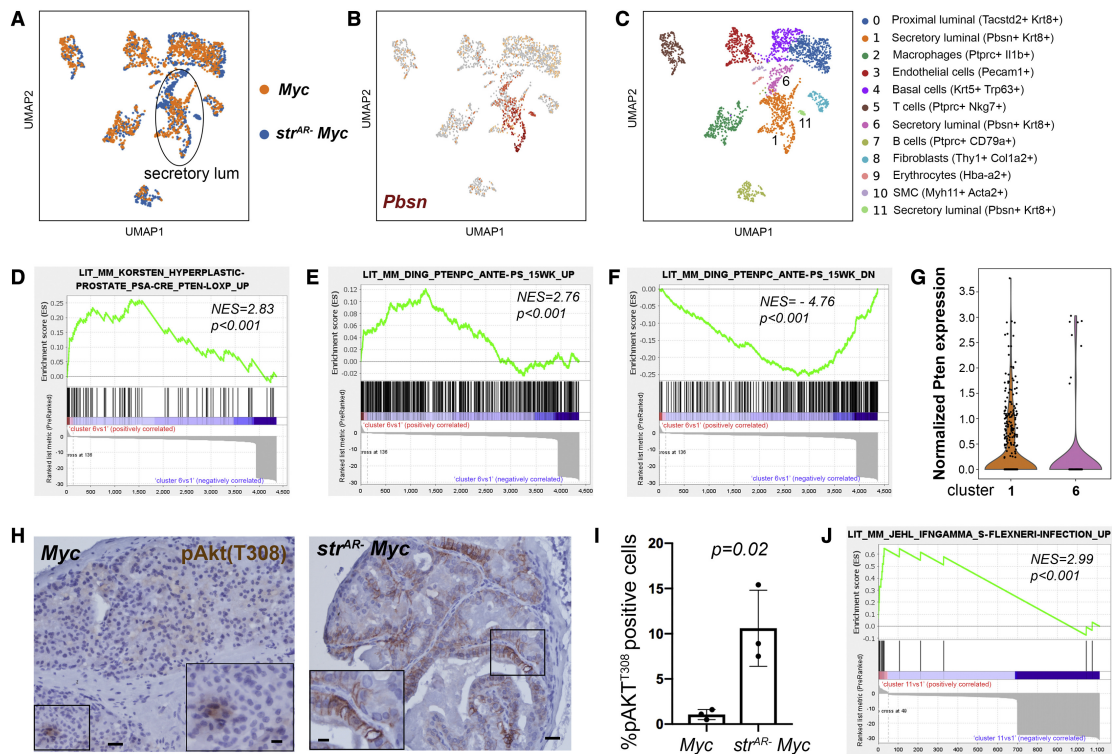


Figure 5.5. Single-cell analysis comparing *Myc* and *str*^{AR-} *Myc* prostate (A) UMAP showing alignment of cell types between the *Myc* and the *str*^{AR-} *Myc* prostate. (B) Identification of secretory luminal clusters based on *Pbsn* expression. (C) UMAP showing color-coded cell clusters of integrated samples using scVI tools and assignment of cell types based on marker expression. (D–F) GSEA comparing differentially expressed genes in cluster 6 versus 1 against published *Pten*-null tumor

gene signatures. (G) Violin plot showing *Pten* expression in clusters 1 and 6. (H) Representative IHC images showing pAkt-T308 staining in *Myc* (left) and *str^{AR-}Myc* (right) tissues. Scale bars, 50 μ m. Scale bars in insets, 10 μ m. (I) Quantitation of percentages of pAkt-T308-positive epithelial cells. n = 3 animals per group. The p value was calculated by Student's t test. (J) GSEA showing enrichment of upregulated genes in cluster 11 versus 1 in a published IFN γ -induced gene signature.

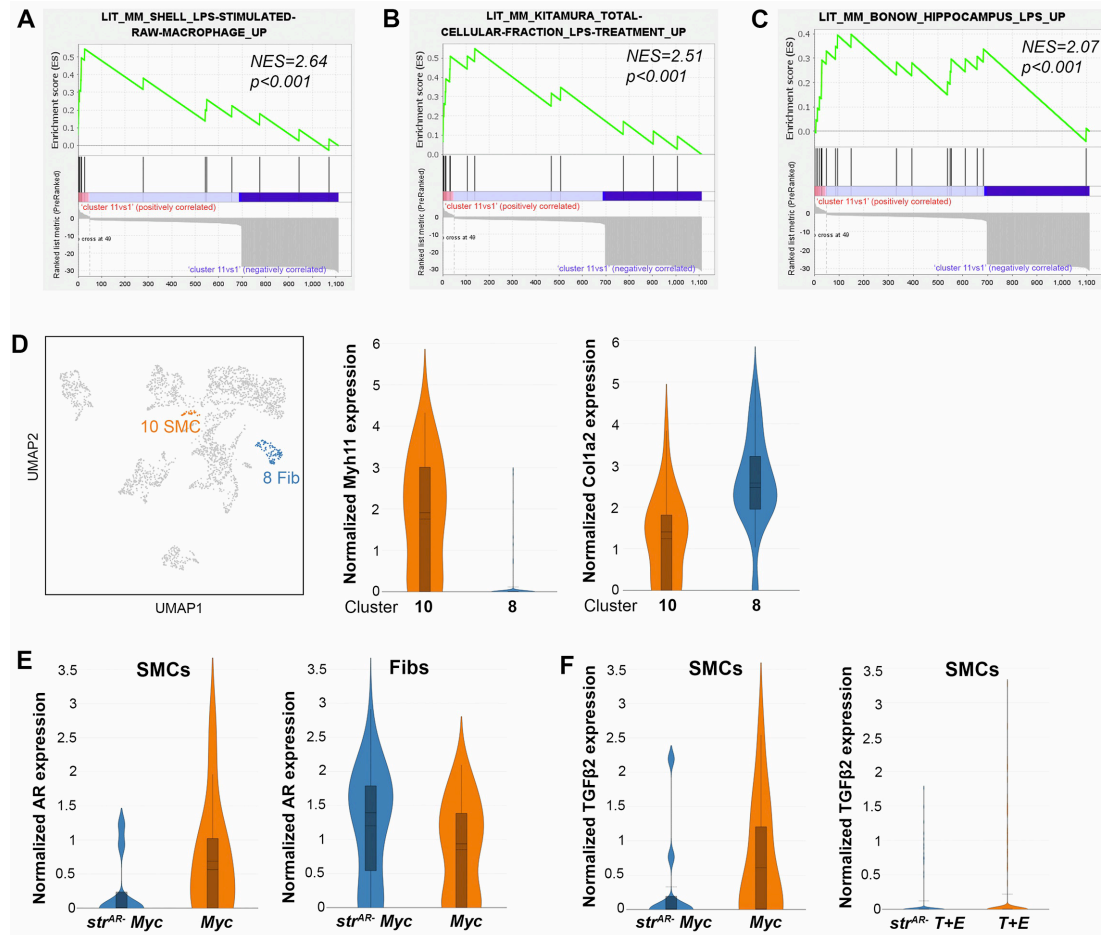


Figure S5.7. GSEA analyses of clusters 11 and 1 and marker expressions in SMCs and fibroblasts. Related to Figure 5. (A-C) GSEA showing enrichment of upregulated genes in cluster 11 vs cluster 1 in three published LPS-induced gene signatures. (D) Violin plots showing expression of *Myh11* (middle panel) and *Col1a2* (right panel) in clusters 10 and 8 of the UMAP (left panel). (E) Violin plots comparing *AR* expression in SMCs (left) and fibroblasts (right) between the *Myc* and *str^{AR-}Myc* samples. (F) Violin plots comparing *TGF β 2* expression in SMCs between the *Myc* and *str^{AR-}Myc* samples (left) and between the *T+E* and *str^{AR-}T+E* samples (right).

DISCUSSION

The role of stromal AR in PCa has been controversial. Experiments recombining AR-deficient mouse stromal cells with transformed prostate epithelial cells in renal grafts suggested that stromal AR promotes PCa progression (Ricke et al., 2012). However, the renal grafting assay mimics prostate organogenesis (Wang and Shen, 2011; Wang et al., 2014), and therefore the requirement of stromal AR for epithelial cell growth in prostate development (Cunha and Lung, 1978; Yu et al., 2011) may have contributed to the outcomes of those studies. Using genetic approaches, AR has been deleted in the stroma, epithelium, or whole prostate via different Cre drivers, and the findings regarding the role of the stromal AR were still contradictory (Niu et al., 2008; Welsh et al., 2011; Lai et al., 2012a). One possibility underlying the discrepancy is the timing of AR deletion, since various Cre drivers may be turned on at different developmental stages. The inducible *Myh11-CreER^{T2}* driver used here ensures stromal AR deletion in the adult prostate. Our results obtained from both genetic and chemical-induced models pointed to a role for stromal ARs in inhibiting PCa progression, explaining the clinical observation that lower stromal AR expression is associated with advanced PCa stages and worse outcomes (Olapade-Olaopa et al., 1999; Henshall et al., 2001; Ricciardelli et al., 2005; Wikstrom et al., 2009; Leach et al., 2015).

SMCs and fibroblasts are the two major stromal cell types in the prostate. In scRNA-seq analysis of the T + E model, SMCs and fibroblasts were clustered together (Figure 3C) and shared marker gene *Myh11*, *Acta2*, and *Tagln* expression to

variable degrees, consistent with a recent study (Kwon et al., 2019). Presumably, AR could also have been deleted in some fibroblasts due to their low *Myh11* expression. Therefore, our conclusion drawn from SMC AR deletion is likely extendable to the whole stroma. For the *Hi-Myc* model, although graph-based clustering assigned SMCs and fibroblasts into one cluster, we could distinguish them into clusters 10 and 8 in Figure 5C, respectively, based on their UMAP spatial distance and distinct expression levels of *Myh11* and *Col1a2* (Figure S5.7D). In this analysis, the difference in AR expression was evident between *Myc* and *str^{AR-} Myc* SMCs, but hardly detectable between *Myc* and *str^{AR-} Myc* fibroblasts (Figure S5.7E), suggesting that accelerated tumor progression was mainly due to AR loss in SMCs. Future research deleting AR using a fibroblast-specific CreER driver should help assess the relative contribution of these two stromal cell types.

We note that AR deletion in SMCs resulted in subtle changes in the SMCs themselves, as reflected by no difference in SMC proliferation as well as the overall alignment of *str^{AR-}* and control SMCs in the UMAP. Such cell overlap suggests that the changes in SMCs were masked by the greater differences among different cell types on the global level. Indeed, secretory luminal cells appeared to be the cell population most affected by stromal AR deletion under tumorigenic conditions, suggesting that *AR*-null SMCs modulate paracrine signals to promote luminal tumor cell progression. A spectrum of growth factors and cytokines has been implicated as under the regulation of stromal ARs in the prostate (Konig et al., 1987; Li et al., 2008; Wen et al., 2015). We observed the expression of factors such as IGF1, HGF,

VEGFb, and FGF2 in both SMCs and fibroblasts in scRNA-seq. However, changes in their expression after AR deletion may not be discernable due to low data resolution. Nevertheless, TGFβ2 appeared to be an interesting candidate, since it was downregulated in the *str*^{AR-} SMCs of both tumor models (Figure S5.7F), and TGFβ signaling was shown to play a cytostatic role in normal prostate epithelial cells (Salm et al., 2005; Valdez et al., 2012) and serves as a barrier to PCa progression (Ding et al., 2011). We caution that our scRNA-seq samples were acquired from progressed tumors, and the stromal cells may have changed since the onset of AR deletion. Future research with an SMC-enriched single-cell analysis at different time points may clarify key paracrine signals.

The finding that stromal AR deletion has distinct impacts on normal and malignant prostate luminal cells is intriguing. One possibility is that the oncogenic stimuli altered the properties of SMCs. Another model we speculate is that stromal AR deletion may result in changes in multiple paracrine signals with opposing roles. In the normal prostate, the tumor-promoting signals may not be sufficient to overcome the growth-inhibitory signals, resulting in overall reduced prostate size but sporadic foci of hyperplasia as seen in Figure 5.11. Under tumorigenic conditions, the oncogenic stimuli may cooperate with the tumor-promoting signals to become the dominant force. Regardless of the cause, the combinatory effects appear to potentiate PI3K-mTORC1 activity in at least a subset of secretory luminal cells, as revealed by our bioinformatics and IHC data. This mechanism is conserved in the two PCa models we tested, but additional mechanisms may exist. For example, we discovered

a cluster of *str*^{AR-} *Myc*-specific luminal cells under the influence of an inflammatory microenvironment, and tumor cells are known to be capable of taking advantage of cytokine signals such as IFN γ to acquire immune evasion and/or high invasiveness (Jorgovanovic et al., 2020).

Our findings could have important implications for PCa treatment. Androgen deprivation therapy (ADT) remains a widely used method for treating advanced PCa. However, despite initial tumor shrinkage after ADT, virtually all patients relapse. Multiple molecular alteration mechanisms have been discovered to explain the castration-resistant PCa, including AR amplification and hypersensitivity, gain-of-function mutations in the AR gene, AR coactivator/corepressor mutations, and intratumoral androgen production (Chandrasekar et al., 2015). These discoveries highlight the importance of continued AR signaling within cancer cells, which are of epithelial origin. Our study suggests that stromal AR signaling plays an opposite role in PCa progression, at least in the early stages. If such mechanism persists in late-stage tumors, ADT may thereby have unintended negative effects in promoting epithelial cancer cell progression through decreased stromal AR signaling. If so, therapies that target epithelial ARs locally or enhance stromal AR downstream signals during ADT may achieve more favorable clinical outcomes.

Limitations of the study

We report the antitumor effects of stromal AR signaling using two distinct mouse PCa models. While our findings suggest elevation of the PI3K signaling pathway in secretory luminal cells as the key mechanism, other mechanisms cannot be ruled out,

especially under different oncogenic conditions. More models are needed to assess the conservation of such mechanism. Due to the limited scope of the scRNA-seq experiments, we were not able to pinpoint the key paracrine factors under stromal AR regulation, and ARs in SMCs and fibroblasts could have different roles. Finally, although low stromal AR is associated with poorer clinical outcome, findings in mouse models may not be fully transferrable to humans, and we do not know whether the mechanism discovered here persists in advanced-stage human PCa.

METHODS

Experimental models and subject details

Adult male mice (7–60 weeks old) were used in this study in accordance with protocols approved by the Institutional Animal Care and Use Committee at UCSC. The mice were housed in an animal facility with a regular 12-h light/dark cycle with ad libitum access to food and water. The AR^{fl^{ox}} (De Gendt et al., 2004), *Hi-Myc* (Ellwood-Yen et al., 2003), and *R26R-CAG-EYFP* (Madisen et al., 2010) lines were used previously. The *Myh11-CreER^{T2}* line (Wirth et al., 2008) was obtained from JAX. All animals used were maintained in C57BL/6N or mixed background, including the *Hi-Myc* allele (originally FVB background), which has been continuously backcrossed to C57BL/6N mice in the lab for over 15 generations. Genotyping was performed by PCR using tail genomic DNA, with the primer sequences listed in the key resources table.

Tamoxifen induction

Mice were administered 9 mg per 40 g body weight tamoxifen (Sigma) suspended in

corn oil by oral gavage once daily for 4 consecutive days.

BrdU incorporation assay

BrdU (Sigma) was dissolved in PBS (10 mg/mL) and administered by intraperitoneal injection twice daily (0.1 mL per dose) for 7 consecutive days to label proliferating cells.

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-17 β (Sigma) were implanted subcutaneously. Mice were treated with hormones for 4 months, and tubes were replenished at that time if the treatment would be extended to 8 months.

Serum estradiol ELISA

Blood sampling was performed by venipuncture from the facial vein and allowed to clot for at least 30 min at 4°C in a BD Microtainer MAP microtube (BD cat #363706). The blood was then centrifuged for 15 min at 2000g, and the serum was collected and stored at –80°C. Serum levels of estradiol were determined using the DetectX serum 17 β -estradiol enzyme immunoassay kit, as per the manufacturer's instructions (Arbor assays). Values for the samples were derived via interpolation using standards provided with the kit.

Tissue collection and dissociation

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

followed by paraffin embedding. For dissociation, prostate tissues were dissected and minced to small clumps, followed by enzymatic dissociation with 0.2% Collagenase/Hyaluronidase (StemCell Technologies) in DMEM/F12 media with 5% FBS for 3 h at 37°C. Tissues were digested with 0.25% Trypsin-EDTA (StemCell Technologies) for 1 h at 4°C, passed through 21- to 26-gauge syringes and filtered through a 40-mm cell strainer to obtain single-cell suspensions. Dissociated prostate cells were suspended in Hanks' Balanced Salt Solution Modified/2% FBS.

Immunofluorescence staining

Immunofluorescence staining was performed using 6 µm cryo sections. Samples were incubated with 10% normal goat serum (NGS) and primary antibodies diluted in 10% NGS overnight at 4°C. Samples were then incubated with secondary antibodies (diluted 1:500 in PBST) labeled with Alexa Fluor 488, 555, or 647 (Invitrogen/Molecular Probes). Slides were mounted with VectaShield mounting medium with DAPI (Vector Labs), and images were taken on a Leica TCS SP5 spectral confocal microscope in the UCSC Microscopy Shared Facility. Primary antibodies and dilutions used are listed in the key resources table.

Tumor histology

For immunohistochemical staining, the procedures followed the instructions of the Vectastain Elite ABC Kit (PK-6101) and Vector NovaRED Substrate Kit (SK-4800). Briefly, 5 µm paraffin sections were deparaffinized and hydrated through xylenes and graded alcohol series, followed by antigen retrieval through boiling in antigen unmasking solution (Vector Labs). Slides were blocked in blocking serum (1.5%

normal serum), and incubated with primary antibodies diluted in blocking serum overnight at 4°C. Biotinylated secondary anti-rabbit antibody from Vectastain Elite ABC Kit was diluted 1:200 in blocking serum. The signal was enhanced using the Vectastain Elite ABC system and visualized with the NovaRed Substrate Kit. The slides were counterstained with Harris Modified Hematoxylin (1:10 diluted in H₂O) and mounted with Clearmount (American MasterTech). Primary antibodies and dilutions used are listed in the key resources table. H&E staining was performed using standard protocols as previously described. Histology slides were visualized using a Zeiss AxioImager.

Single cell RNA-seq

Approximately 16,000 dissociated cells were obtained from each prostate sample. Library preparation was performed using Chromium Single Cell 3' Solution with v3.1 chemistry following the manufacturer's protocol (10x Genomics). The library purity, size, and quantity were validated by capillary electrophoresis using 2100 Bioanalyzer (Agilent Technologies). The libraries were sequenced at the UC Davis Genome Center with a NovaSeq 6000 S4 instrument (Illumina) to a depth of ~225k reads per cell.

Single cell data pre-processing and analyses

Raw sequencing data were processed using the 10x Genomics Cell Ranger pipeline (version 5.0.1) to generate FASTQ files and aligned to the mm10 genome (version 2020-A) to generate gene expression counts and quality control metrics. The count matrices were read into AnnData objects and concatenated using Scanpy

(version 1.7.1) (Wolf et al., 2018). Genes that were not detected in any cells were removed and cells with genes < 200 were removed. The quality of the scRNA-seq dataset was assessed by plotting the numbers of unique molecular identifiers (UMIs), genes, total counts, and mitochondrial gene percentage per cell. The cutoffs for the above metrics were manually determined. Specifically, cells with detected genes > 10,000 or UMI counts > 150,000 or mitochondrial UMI ratio > 15% were excluded. After quality control filtering, the concatenated object was split into objects corresponding to each sample. Cell doublets were removed from the objects using Scrublet (version 0.2.1) (Wolock et al., 2019) with default parameters, while the doublet score thresholds were set by inspecting the simulated doublet score histograms. Then all the objects were concatenated and genes not detected in any cells were filtered again, normalized to 10,000 reads per cell, logarithmized, scaled and centered.

To integrate different samples, top 3000 highly variable genes were selected. Sample batches were registered with scvi-tools (version 0.7.0) (Gayoso et al., 2021), and a deep generative modeling was trained (with `n_latent` set to 30 and `n_layers` set to 2). Then data imputation and integration were carried out to impute dropout events and correct batch effects. The latent variables obtained from the model were stored as annotations of observations (`obsm`). A nearest-neighbor graph using the 30 dimensions of the latent space was calculated using `pp.neighbors()`, followed by clustering using leiden algorithm `tl.leiden()` with a resolution adopting from prior knowledge on prostate cell population composition. Then uniform manifold

approximation and projection (UMAP) dimension reduction was performed for visualizing high-dimensional data.

Scanpy `rank_genes_groups()` and Wilcoxon rank-sum test were used to compute the statistical significance of differential genes expression between any groups and identify marker genes of each cluster.

Gene set enrichment analysis

The significantly differentially expressed genes (FDR <0.01) were ranked by their log-transformed fold change value. Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) was conducted using GSEA software (Version 4.1.0). The pre-ranked gene list and MousePath_All_gmt-Format.gmt or MousePath_GO_gmt.gmt gene set (both were downloaded from <http://ge-lab.org/gskb/>) were used for running the tool “Run GSEA Preranked” with default parameters.

Comparing transition between samples

To investigate the molecular transition from cell cluster 1 to 3 in the $T + E$ sample and cluster 6 to 4 in the $str^{AR-} T + E$ sample, we compared the cluster 3 vs cluster 1 signature with cluster 4 vs cluster 6 signature. Only significant differentially expressed genes between the clusters were kept (FDR<0.01). Then the query signature was defined as a list of top300 positive changed genes ranked by logfoldchange in cluster 3 compared to cluster 1 in sample $T + E$ (higher in 3 than 1). The target signature was defined as a list of genes ranked by logfoldchange in cluster 4 compared to cluster 6 in sample $str^{AR-} T + E$. Statistical significance of the

enrichment between the query signature and the target signature was computed using GSEA.

Quantification and statistical analysis

For tumor H&E evaluation, samples were deidentified and scoring was performed in a double-blinded setting. Each sample was assigned a histology score (highest 10 and lowest 0) using the following formula: score = % area of low/moderate-grade PIN X 5 + % area of high-grade PIN X 10. PIN subtype classification was described previously (Berman-Booty et al., 2012). For BrdU and IHC quantification, the investigators were blinded to the ID/genotype of the mice before performing cell counting. Numbers of positive cells were counted manually across tissue sections.

Statistical analyses for IF and IHC staining images and H&E scores were performed using the two-sided student's t-test. At least four animals for each IF staining quantification, three animals for each IHC staining quantification, and at least five animals per cohort for H&E scoring were used. The variances were similar between the groups that were being statistically compared. Specific details for these statistical tests are listed in figure legends.

Acknowledgments

We thank the Sofie Salama and David Haussler lab for sharing the 10× Genomics Chromium machine and the microscopy core facility at UCSC and DNA Technologies Core at UC Davis for technical support. We also thank Yvonne Gutierrez, Ysabelle-Jeah Habon, and Emily Person for helping with mouse

genotyping and histology analysis. This work was supported by a TRDRP post-doctoral fellowship (Y.L.), a Santa Cruz Cancer Benefit Group award (Z.A.W.), ACS grant 134386-RSG-20-038-01-DDC (Z.A.W.), and NIH grant R01CA271452 (Z.A.W.).

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