RESIDENT AND RECRUITED STROMA CONTRIBUTE TO CASTRATE
RESISTANT PROSTATE CANCER

By

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ABSTRACT

Progression to castrate resistant prostate cancer (CRPC) is associated with high morbidity. Disease recurrence and progression is influenced by the tumor microenvironment. Resident and recruited stroma contribute to cancer progression through paracrine signaling. A conditional stromal TGF-β type II receptor knockout mouse model (Tgfbr2^{fspKO}) was characterized to be a model for understanding CRPC progression. We demonstrated the resident stromal fibroblast responsiveness to TGF-β mediated paracrine canonical Wnt signaling in the adjacent prostate epithelia in castrate resistance. Regrowth of the prostate was associated with recruitment of stromal cells from the bone marrow and a contributor to CRPC. We determined that a population of bone marrow derived cells, particularly the mesenchymal stem cells (MSCs), were recruited and fused to prostatic ductal epithelia. These MSCs were found to be a source of Wnt ligands that could contribute to castrate resistant prostatic epithelia. In summary, the tumor microenvironment composed of both resident and recruited stromal cells mediate CRPC through paracrine signaling.

Dedicated to my loving and supportive family.

Especially to my loving grandmother, "Grandma Bully" Elvira Gomez Placencio,

who always encouraged me to pursue my dreams. She passed away during my

Ph.D. studies, but her memory remains in my heart.

De Colores

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of exciting findings, kept me moving forward, and helped me to look on the bright side of things when I was disappointed.

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PREFACE

Of all the ailments that affect our society, cancer is among those that bring the most fear. Perhaps this is because it affects everyone on the planet either directly or indirectly. Cancer is commonly described as an accumulation of diseases in which cells within the body grow uncontrollably. This definition seems elusive and rightly so as we have just begun to grasp the complexities in an effort to treat and prevent its inception. There are many types and sub-classifications of cancer, which continue to expand as we are able to distinguish the differences among these. This may seem daunting when the number one correlate with cancer is aging and our society continues to have an increase in the elderly population. Despite this, there have been many advances toward therapies to treat cancer due to the ongoing research efforts. Although a cure to treat all cancers may not be possible, it is hopeful that multiple treatments will be developed in the future to treat each individual cancer.

This thesis focuses on understanding castrate resistant prostate cancer (CRPC). The majority of men with prostate cancer can be treated by surgical resection or radiation therapy if they are diagnosed early. Unfortunately, the development of CRPC is inevitable for some unlucky individuals. Within this thesis we focus on the stroma as it contributes to CRPC. As seen in our studies, knowledge obtained from the initial studies on the resident stroma served as a basis for understanding the contribution of the recruited stroma in the prostate. We show in the first group of studies through various transgenic mouse models,

the importance of resident stromal TGF-β signaling and how this contributes to CRPC. Based on our Tgfbr2$^{\text{fspKO}}$ mouse model we showed how the loss of stromal TGF-β signaling led to enhanced stromal Wnt production. Through paracrine signaling these Wnt ligands caused transformation of the epithelia and castrate resistance. SFRP-2 treatment to inhibit Wnt signaling restored the castrate response and slowed CRPC progression. In the second group of studies we focus on recruited stroma and how this also led to the progression of CRPC. BMDCs were recruited and incorporated into the prostate epithelia during regrowth. MSCs were responsible for this incorporation through fusion. In CRPC, recruited MSCs were shown to enhance Wnt signaling in CRPC tumor epithelia. Overexpression of SFRP-2 in MSCs recruited to the tumors suppressed the enhanced Wnt signaling and caused tumor associated apoptosis and necrosis. Together, these studies highlight the importance of understanding how components of the tumor microenvironment enhance cancer progression, with a focus on the resident and recruited stroma.

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AR	Androgen receptor
BCL2	B-cell lymphoma 2
BMDC	Bone marrow derived cell
BPH	Benign prostatic hyperplasia
Ca^{++}	Calcium
CCL5	Chemokine (C-C motif) ligand 5
CRPC	Castrate resistant prostate cancer
CXCR4	CXC chemokine receptor 4
DHT	5α-dihydrotestosterone
DKK	Dickkopf
DN	Dominant negative
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-regulated kinase
GSK3-β	Glycogen synthase kinase-3β
HER-2/neu	Human epidermal growth factor receptor 2
HSC	Hematopoietic stem cell
IFN	Interferon
IGF	Insulin-like growth factor
JNK	c-Jun N-terminal kinase

KGF	Keratinocyte growth factor
LEF	Lymphoid enhancer
LDL	Low-density lipoprotein
LRP	Low-density lipoprotein receptor-related protein
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
NSAIDs	Non-steroidal anti-inflammatory drugs
PCP	Planar cell polarity
PI3K	Phosphoinositide 3-kinase
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
SDF-1	Stromal cell-derived factor-1
shRNA	Small hairpin RNA
SV40	Simian virus 40
Smad	Mothers against decapentaplegic homolog
SFRP-2	Secreted frizzled related protein-2
Tag	Simian virus 40 large T antigen
TCF	T-cell factor
Tfm	Testicular-feminized mice
TGF-β	Transforming growth factor beta
TβRII	TGF-β type II receptor protein
Tgfbr2	TGF-β type II receptor gene

Tgfbr2$^{floxE2/floxE2}$	TGF-β type II receptor exon 2 floxed mouse
Tgfbr2^{fspKO}	TGF-β type II receptor conditional fibroblastic knockout mouse
Tgfbr2$^{NKX3.1KO}$	TGF-β type II receptor conditional prostate epithelial knockout mouse
Tgfbr2$^{floxE2/floxE2}$/TOPGal	β-catenin/TCF reporter mice expressing β-galactosidase crossed with Tgfbr2$^{floxE2/floxE2}$ mice
Tgfbr2^{fspKO}/TOPGal	β-catenin/TCF reporter mice expressing β-galactosidase crossed with Tgfbr2^{fspKO} mice
TST	Testosterone
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UGE	Urogenital epithelium
UGM	Urogenital mesenchyme
UGS	Urogenital Sinus
VEGF-R1	Vascular endothelial growth factor receptor 1
WIF-1	Wnt inhibitory factor-1
WT	Wild-type

CHAPTER I

INTRODUCTION

Prostate Anatomy and Function

The prostate is a male accessory reproductive organ located just below the bladder and in front of the rectum (Figure 1). While it is not an essential organ, it greatly enhances male fertility by producing a zinc, citric acid, fructose, and prostate-specific antigen (PSA) containing fluid to further liquefy the semen (consisting of sperm and seminal fluid) as it is ejaculated through the urethra. The urethra traverses the prostate and continues through the penis. In humans the prostate is divided into three anatomic regions (the central zone, the transitional zone, and the peripheral zone), which differentially correspond to prostate disease. The central zone is not commonly associated with prostatic disease. The transitional zone is the primary site that develops benign prostatic hyperplasia (BPH) with many symptoms including weak or interrupted urine flow as the prostate can grow inward narrowing the urethra. Development of BPH is estimated to occur as early as age 30 (Verhamme, Dieleman et al. 2002). Most men will develop BPH if they live long enough; however there does not seem to be a connection between BPH and prostate cancer development. The peripheral zone is the site of most prostate cancers, and symptoms are often not apparent until late stages of cancer development. For this reason, digital rectal exams are
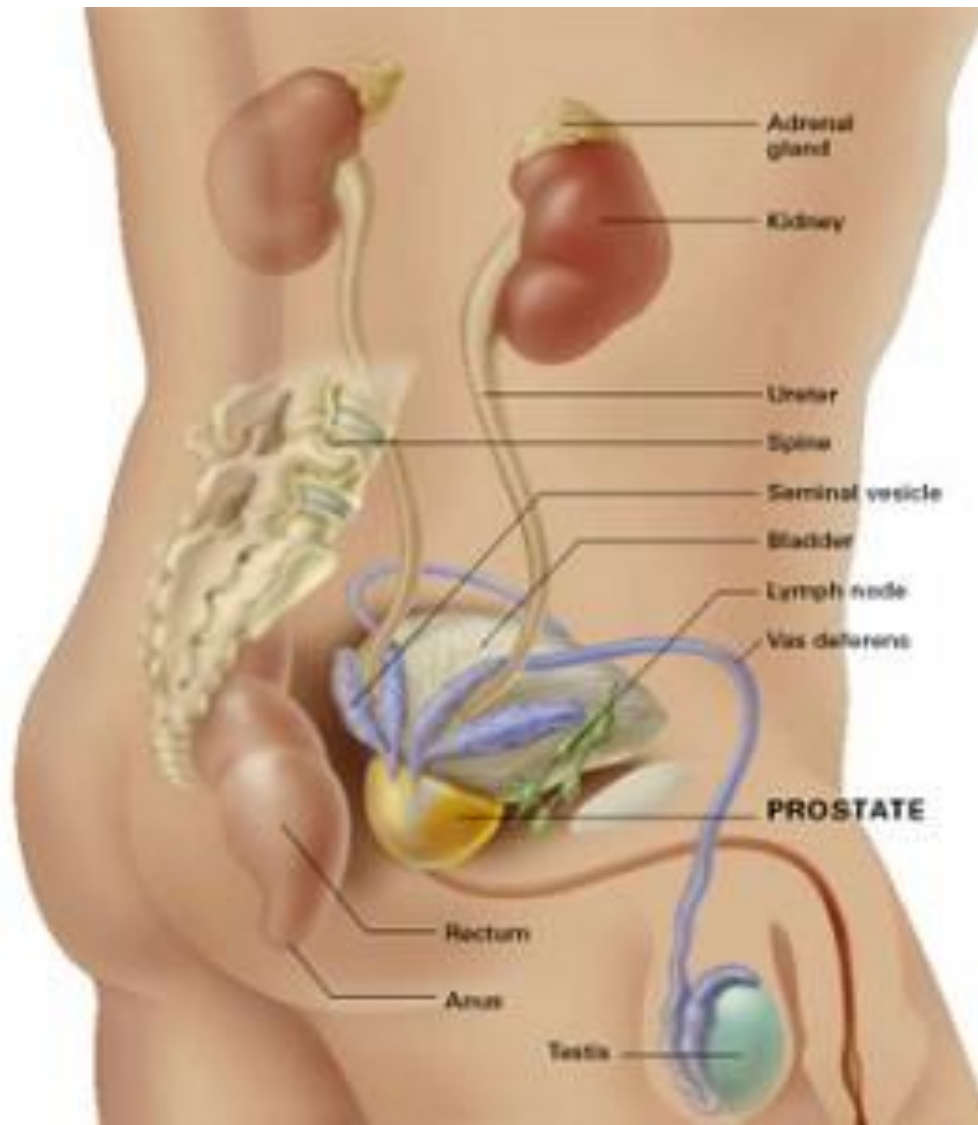
Figure 1. Prostate location in the male body. The human prostate is shown centrally located in yellow. The bladder is on top of the prostate (beige) and the ureter runs from the prostate through the penis (brown). (Image taken from www.pillsforall.com).

useful as the cancer may be identified by abnormalities such as nodules or hardened tissue within the organ.

In murine models, the prostate is divided into lobes as opposed to zones. There is no definitive correlation between the lobes in mice with the zones of the human prostate. Although the gross anatomical structure is different, the secretory function and thus secretory glands of humans and mice share many similarities. Transgenic mouse models, therefore, serve as the gold standard for mechanistic understanding of human prostate cancer development and progression.

Prostate Glandular Structure

The secretory glandular structure of the prostate is composed of organized epithelia surrounded by a basement membrane and fibromuscular stroma (Figure 2) (Cunha, Hayward et al. 2002). Prostatic homeostasis requires paracrine signals from the fibromuscular stroma to the epithelia and vice versa to maintain growth quiescence. These studies will focus on prostate cancer paracrine signaling, which consists of one cell type communicating with another cell type (ex: stromal- epithelial signaling). There are many parallels to prostate development. Human and mouse fibromuscular stroma is made up of a heterogeneous mixture of mainly smooth muscle cells and some fibroblasts and myofibroblasts. The human prostate stromal compartment, however, has a greater ratio of stroma to epithelia compared to mice. Developmental parallels
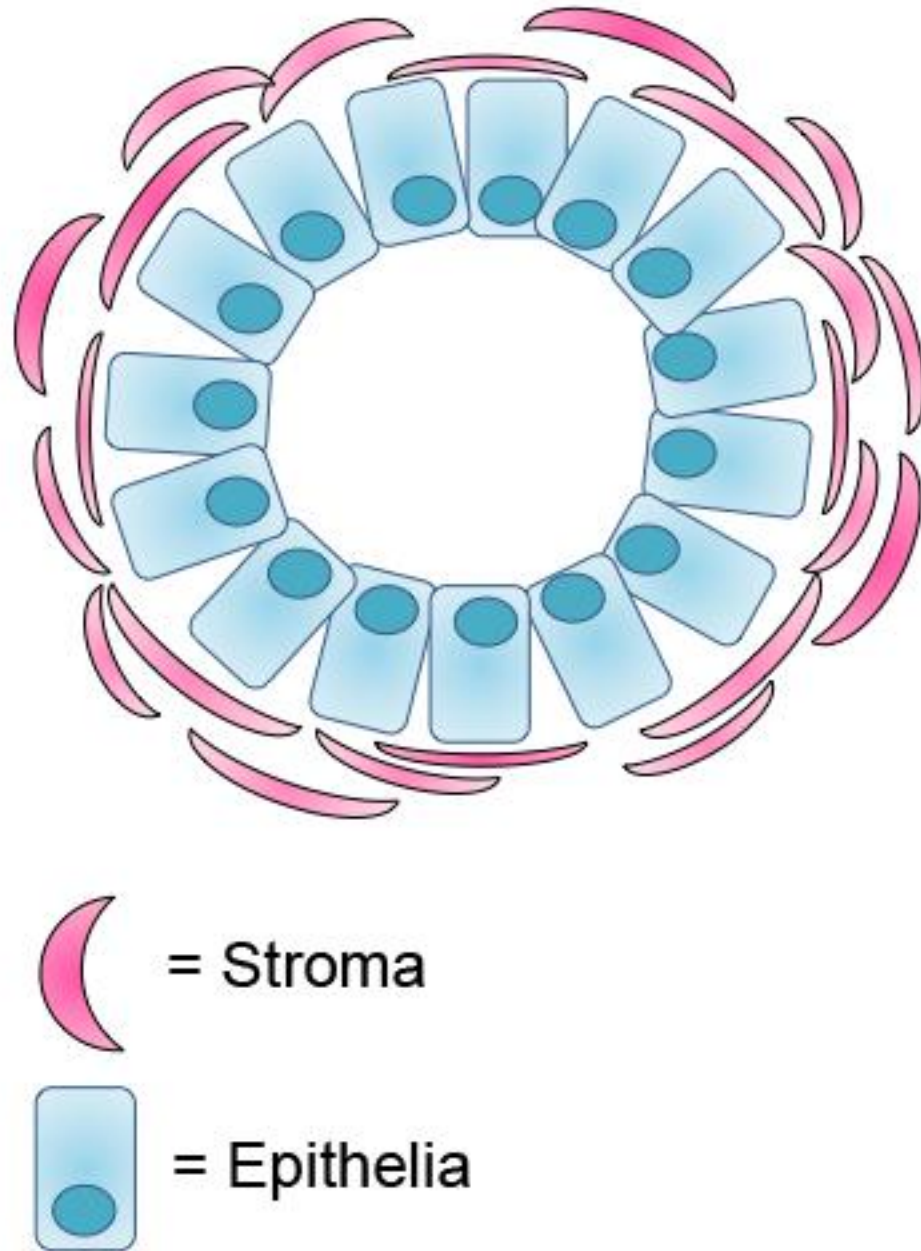
Figure 2. Prostate glandular structure. The epithelial cells (blue rounded rectangles) compose the glandular structure. The stroma (pink half moons) surround the epithelial glands. Not shown is the basement membrane between the epithelial and stromal layers. Prostatic fluids are secreted into the lumen (empty space in the center of the gland).

will be discussed where appropriate, although there are far too many to discuss in depth. An overview of paracrine signaling in prostate development can be reviewed in the many articles by the pioneering father of this field, Gerald Cunha (Cunha, Ricke et al. 2004; Cunha 2008). Proper epithelial differentiation is necessary for the production of prostatic fluid to enhance sperm survival in the female reproductive tract. This alkaline prostatic fluid serves to counteract the acidic environment exiting the male urethra and within the vagina producing a desirable pH supporting sperm survival and subsequent fertilization. Stromal-epithelial interactions play a role in prostate development, tissue homeostasis, and later in life contribute to prostate tumorigenesis as discussed within the following topics (Bhowmick and Moses 2005).

Androgen Dependence of the Prostate

The prostate is an androgen dependent organ, thus androgens regulate proper prostate growth, development, and maintenance (Cunha, Hayward et al. 2002; Nantermet, Xu et al. 2004; Sakko, Ricciardelli et al. 2007). The prostate develops from an ambisexual organ termed the urogential sinus (UGS). Paracrine signals from the surrounding connective tissue termed the urogenital mesenchyme (UGM) respond to testicular androgens to induce prostatic structures. In the absence of sufficient androgens, female sex organs will be generated by default. The UGS will then expand into cords of urogenital epithelium (UGE) invading into the surrounding UGM to produce prostatic buds.

These budding structures elongate and undergo branching morphogenesis with hollowing of the epithelial structure to produce prostatic ducts. In humans these ducts organize into a solid compact gland divided into zones. Whereas in mice, these ducts organize into individual lobes.

Early studies using testicular-feminized (Tfm) mice showed the necessity of androgen receptor (AR) expression in both the stroma and epithelia for complete maturation of the prostate during development (He, Kumar et al. 1991). In tissue recombinants resulting from epithelia lacking AR combined with wild-type (WT) stroma expressing AR, the prostate ducts that formed did not fully mature and lacked prostate secretions indicative of a mature and functional prostate. In addition, tissue recombinants that combined stroma lacking AR and WT epithelia resulted in vagina-like tissues, which showed that stromal AR was necessary to instruct prostatic duct formation by the epithelia. These studies stressed the importance of androgen signaling in prostate development.

More recent studies have shown how androgens regulate many pathways thought to be involved not only in development, but also in cancer. In one study, primary cultured rat epithelial cells were treated with the synthetic androgen, R1881, and analyzed for genes regulated by androgens (Asirvatham, Schmidt et al. 2006). They found that androgen regulated genes included those associated with differentiation, proliferation, and apoptosis. Interestingly, they also found genes associated with inflammatory and immune response pathways including the interleukin gene, IL-6. IL-6 is often associated with castrate resistant prostate cancer (Dossus, Kaaks et al. 2010). Another study looked at the role of stromal

androgens on normal and malignant prostate epithelial growth. When using the stroma from Tfm mice, which lack AR expression in the stroma, normal adult prostate epithelial cells failed to grow (Gao, Arnold et al. 2001). When malignant prostatic epithelial cells- including human PC-82, human LNCaP, human LAPC-4, and rat R3327G cells- were used combined with Tfm stroma these epithelial cells grew in response to androgens. This suggests that normal prostate epithelial cells require stromal paracrine androgen signaling for growth; however malignant epithelia developed the ability to respond to autocrine androgen signaling instead. Further information on altered androgen signaling is discussed within the mechanisms for castrate resistant prostate cancer development.

Prostate Cancer Statistics, Diagnosis, and Current Therapies

Among those cancers that have had great strides towards its understanding, prostate cancer is still the number one diagnosed cancer and the second in cancer deaths among aging American men (Ho, Boileau et al. 2004; Arap, Trepel et al. 2008; ACS 2010). When diagnosed early, the five year survival rate is nearly 100%. Diagnosis at late stages of progression, however, drops the five year survival rate to a mere 34%. It was estimated that in 2010 there would be 217,730 cases diagnosed, and 32,050 deaths from prostate cancer in the United States (NCI 2010).

Cancer detection and diagnosis have improved dramatically over the last few decades (Arap, Trepel et al. 2008). One of the greatest advances in the field

of prostate cancer has been the introduction of blood test screening for PSA. Low circulating levels of PSA, on average less than 4 ng/mL, are thought to be normal and anything higher is carefully monitored (ACS 2010). A significant increase over basal levels is also a sign of cancer. This screening test is often used in conjunction with the digital rectal exam for tactile detection of cancer. Potential tumors are verified with prostate ultrasound and biopsy. Upon diagnosis, there are many treatment options and the course of action is determined by the physician in accordance with individual patient circumstances.

Prostate cancer is a slow growing cancer and takes many years to develop (Figure 3) (Abate-Shen and Shen 2000). As depicted, prostate cancer progresses from normal epithelium to prostatic intraepithelial neoplasia (PIN), invasive carcinoma, and finally metastasis. Early stage prostate cancer is usually androgen dependent. At this stage the cancer can be effectively treated through a prostatectomy or radiation therapy. If surgery or radiation is not a treatment option, then androgen ablation therapy may be used. Drugs to block androgen signaling impede growth of the prostate and the resulting cancer. The prostate regresses as a result and cancer progression is halted. For older men diagnosed with prostate cancer, it may not be pertinent to treat it aggressively with surgical methods or radiation therapy. Active surveillance is often used if a man is too old to risk surgery or the potential side effects from therapy outweigh the benefits of treatment. For younger men, prostate cancer is a greater concern since there is a greater likelihood for the development of aggressive disease, which will ultimately lead to the development of bone metastatic disease. Prostate cancer
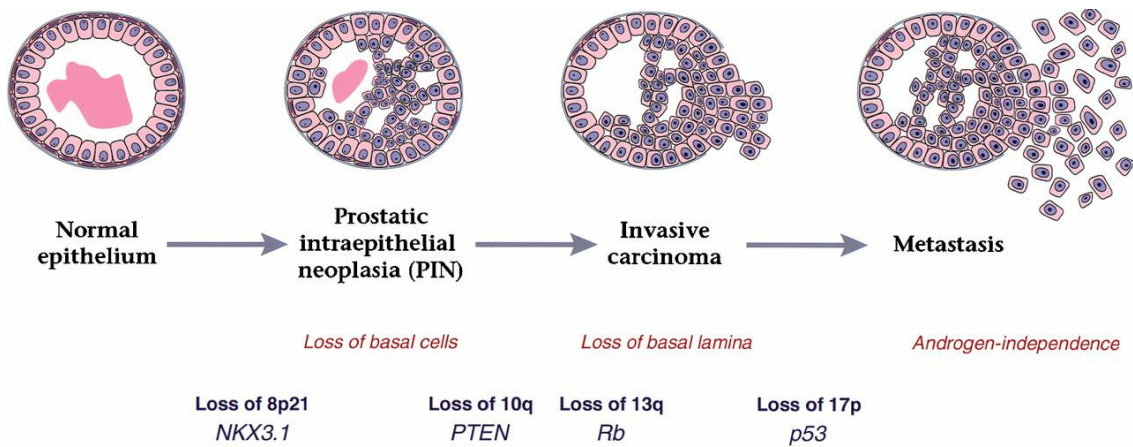
Figure 3. Pathway for human prostate cancer progression. The stages of progression are shown. Loss of glandular architecture and changes in androgen status associated with each stage are indicated in red. Loss of specific chromosome region and tumor suppressor genes associated with progression are shown in dark blue. Image taken from (Abate-Shen and Shen 2000).

preferentially metastasizes to the bone (Mundy 2002; Pienta, Abate-Shen et al. 2008). Currently there are no curative therapies for castrate resistant prostate cancer or metastatic disease. Palliative therapies are the only available options for castrate resistant disease.

Castrate Resistant Prostate Cancer

The development of castrate resistant prostate cancer (CRPC) is accompanied by regrowth of the cancer in addition to metastatic dissemination. A significant proportion of the morbidity associated with prostate cancer occurs at this late stage of progression. CRPC can arise from multiple mechanisms. The prevailing hypothesis suggests that CRPC arises from the primary tumor through selective pressure to enable the more aggressive cancer cells to survive in diminished or depleted androgen levels. The mechanisms thought to lead to the development of CRPC can be classified into five main categories according to Feldman and Feldman: 1) hypersensitive AR, 2) promiscuous AR, 3) outlaw AR, 4) bypass AR, and 5) lurker cells (Figure 4) (Feldman and Feldman 2001).

1) Hypersensitive AR is when the cancer cells adapt by being able to respond to very low levels of androgens. Amplification of the androgen receptor will better allow the cancer cell to sense and respond to the low amounts of androgen available (van der Poel 2005). These cells that are able to respond to the low androgen concentration will be selected for with their ability to survive in
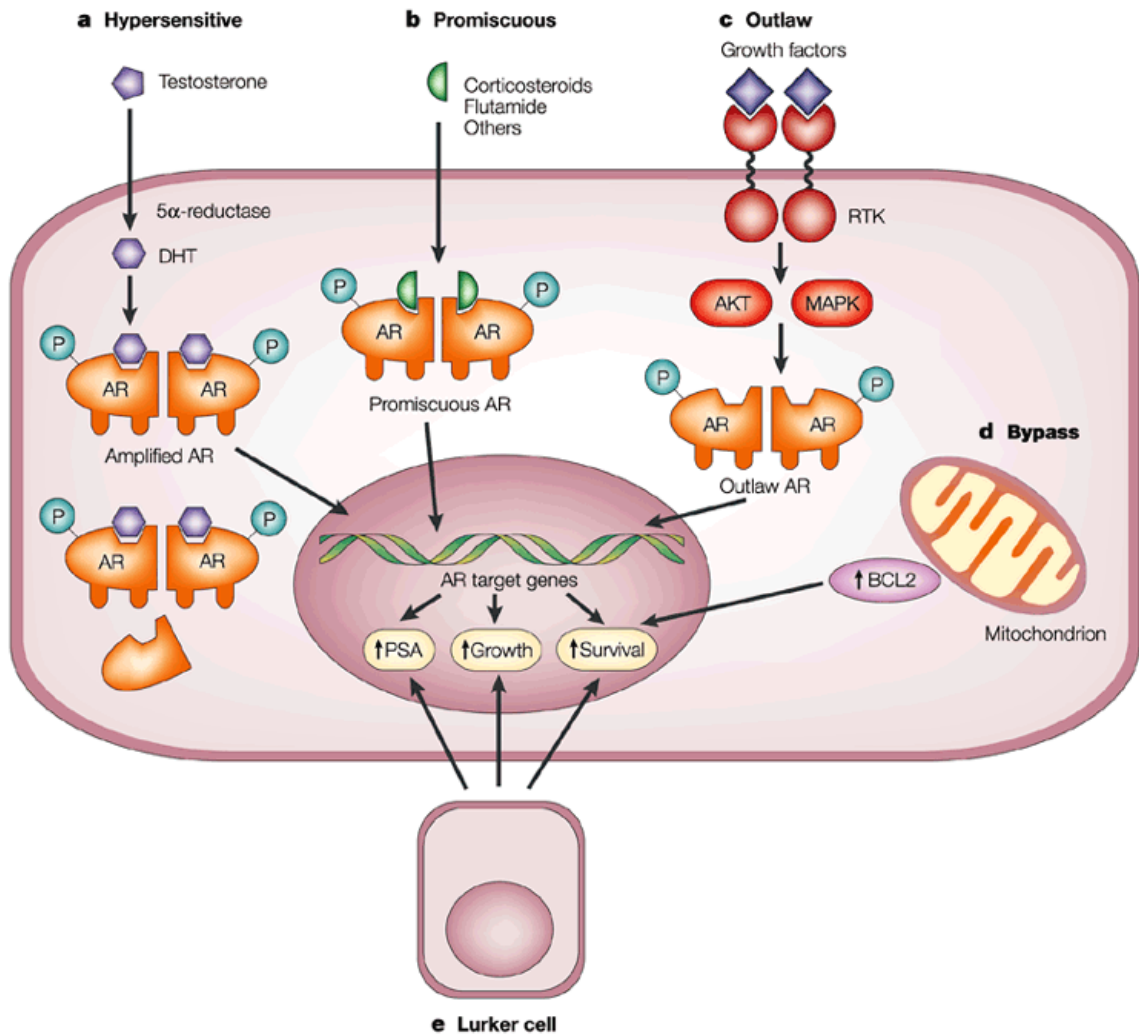
Figure 4. Five possible pathways to androgen independence. a). Hypersensitive pathway. More AR is produced, or has enhanced sensitivity (not shown) to compensate for low levels of androgen or more testosterone is converted to DHT by 5α-reductase. b). Promiscuous pathway. The specificity of AR is broadened so it can be activated by non-androgenic molecules normally present in the circulation. c). Outlaw pathway. Receptor tyrosine kinases (RTKs) are activated and AR is phosphorylated by AKT/PKB or the MAPK pathway to produce a ligand-independent AR. d). Bypass pathway. Parallel survival pathways such as BCL2 obviate the need for AR or its ligand. e). Lurker cell pathway. Castrate resistant cancer cells that are present all the time in the prostate- possibly epithelial stem cells- might be selected for by therapy. Figure taken from (Feldman and Feldman 2001).

the depleted levels. In vitro studies have shown that patient tumor cells with AR amplification could withstand androgen ablation longer than the cells devoid of amplification (Koivisto, Kononen et al. 1997).

Hypersensitivity can be achieved at the transcriptional level. The amount of 5α-dihydrotestosterone (DHT) needed to stimulate growth in castrate resistant cells was four orders of magnitude lower than that necessary for stimulation in androgen-dependent cells (Gregory, Johnson et al. 2001). This study showed that in models classified as castrate resistant, there can still be proliferation resulting from AR transcriptional activity in low androgen concentrations.

In addition, hypersensitivity can be a result of increased local production of androgens. This local increase is a compensatory mechanism to make up for the lower levels of total circulating androgens. Intracrine steroidogenesis allowed tumors and metastases to survive in low circulating androgen levels (Montgomery, Mostaghel et al. 2008). This may be due to enhanced 5α-reductase activity, which converts the circulating testosterone (TST) into DHT within the prostate. This would explain the discrepancy observed that after androgen ablation therapy total serum testosterone levels decrease by 95%, however, DHT concentration in the prostate has a reduction by only 60% (Makridakis, Ross et al. 1997). Also of interest is the correlation of high 5α-reductase activity, notably higher in African American men compared to Caucasian men, with increased prostate cancer incidence (Ruijter, van de Kaa et al. 1999). This was found to be due to a 5α-reductase polymorphism, V89L, within the enzyme resulting in higher enzymatic activity.

2) The second main category for the development of CRPC is androgen receptor promiscuity. This is when non-androgen or androgen antagonists are able to bind the androgen receptor to activate signaling. This may be a result of AR mutations. In various studies, it has been shown that the frequency of AR mutations was higher following androgen ablation therapy compared to the primary tumor before therapy (Taplin, Bubley et al. 1995; Tilley, Buchanan et al. 1996; Marcelli, Ittmann et al. 2000). LNCaP cells were found to have a missense mutation in the AR at amino acid 868, T868A, in the ligand binding domain (Veldscholte, Berrevoets et al. 1992). This allowed other steroid hormones and the androgen antagonist flutamide to bind in its place and activate signaling. Other studies have also found missense mutations, including T877A, in the AR ligand binding domain that seem to have a selective advantage in metastatic growth (Gaddipati, McLeod et al. 1994). 25% of patient tumor samples were shown to have the T877A mutation suggesting that it is common in CRPC. Five out of 16 patients treated with flutamide were found to have bone marrow metastases with AR T877A mutations (Taplin, Bubley et al. 1999). This suggested that the treatment selected for cells that could grow in response to flutamide. Patients not treated with flutamide were shown to have other mutations not associated with flutamide treatment.

Co-regulators can also alter receptor promiscuity. Steroid hormone receptors work with co-activators and co-repressors to activate or repress transcription (Shang, Myers et al. 2002; Litvinov, De Marzo et al. 2003; Heitzer and DeFranco 2006; Cano, Godoy et al. 2007; Lee, Kim et al. 2008). It has been

shown that over-expression of AR co-activators, SRC1 and TIF2, can combine with ARs harboring ligand binding domain mutations and enhance the misguided signaling (Gregory, He et al. 2001). It was suggested that most patients with CRPC have an over-expression of co-activators to allow signaling in low androgen levels. Another study showed that lentiviral-AR transduced PC3 cells resulted in growth inhibition (Litvinov, Antony et al. 2006). This was accompanied by increased p21 and p27 expression upon synthetic androgen R1881 treatment. This study emphasized that PC3 cells retain the co-regulators required for the tumor suppressor ability of AR, but without a functional AR this suppression is lost.

3) The third process for the development of CRPC is the outlaw pathway. Outlaw receptors are steroid hormone receptors that can be activated by ligand-independent mechanisms. It has been shown that growth factors including IGF-1, KGF, and EGF can activate the AR (Culig, Hobisch et al. 1994). In LNCaP cells, IGF-1 was shown to activate AR signaling greater than the other growth factors inducing a five-fold increase in PSA secretion. Furthermore, over-expression of these growth factors in prostate cancer suggests that this contributes to the development of CRPC. It has also been shown that HER-2/neu is frequently over-expressed in CRPC (Craft, Shostak et al. 1999). Androgen-dependent cell lines can also be converted to the castrate resistant phenotype by over-expressing HER-2/neu. This causes activation of AR-dependent genes in the absence of DHT. Furthermore, when cells were treated with the AR antagonist

bicalutamide, there was no alteration in AR signaling resulting from HER-2/neu activation showing that this took place independent of the ligand binding domain. It has been shown that HER-2/neu can activate the PI3K/AKT pathway and thereby lead to the phosphorylation of AR (Zhou, Hu et al. 2000). In addition, dominant-negative AKT blocked the activation of AR mediated through HER-2/neu (Wen, Hu et al. 2000). Studies have also shown the importance of GSK-3β and PTEN in CRPC when targeting therapies to PI3K/AKT signaling (Mulholland, Dedhar et al. 2006). The loss of PTEN correlates with activation of AKT, which can then lead to phospho-inhibition of GSK-3β and subsequent inactivation of apoptotic factors. PTEN was suggested as a "gatekeeper" of progressive prostate cancer. The frequency of mutations and deletions of PTEN in prostate cancer was found to be 60% (Vlietstra, van Alewijk et al. 1998). These studies emphasized that alterations in many signaling pathways can ultimately lead to the development of CRPC.

4) The fourth mechanism thought to lead to CRPC is the bypass pathway. Androgen signaling activation causes cancer cell proliferation. However, there are alternative ways to cause cancer cell proliferation and growth without AR. BCL2, which blocks apoptosis, has been shown to be expressed in CRPC cells. It is not normally expressed in benign prostate epithelial cells. One study indicated that tumors grown in castrated mice selected for cells expressing BCL2 to bypass apoptotic signals (Liu, Corey et al. 1996). Further support comes from another model using LNCaP xenografts with antisense oligonucleotides that

delayed the progression to CRPC tumors (Gleave, Tolcher et al. 1999). These models showed how BCL2 is able to overcome the normal signals for apoptosis and allow carcinoma cells to sustain growth. In human CRPC tumors, BCL-2 was over-expressed in 62% of locally invasive prostate cancers and 100% of metastases after androgen ablation therapy (Colombel, Symmans et al. 1993).

5) The fifth route that may lead to CRPC is the lurker cell pathway. Prostate epithelial stem cells are thought to be androgen-independent. When prostate stem cells become cancer stem cells, they are not affected by androgen ablation and will continue to proliferate. These cancer stem cells give rise to androgen-dependent daughter cells comprising the bulk of primary tumors, which would be eliminated with androgen ablation therapy. Androgen ablation therapy would act as a selective pressure to allow the androgen-independent cancer stem cells to remain, and this leads to CRPC recurrence (Isaacs 1999). A study indicated that androgen-independent cells are present in the prostate at a frequency of 1 per every 10^5 -10^6 androgen-dependent cells (Craft, Chhor et al. 1999). Prostate cancers are known to be heterogeneous. Similarly, androgen status within the cancer cells of primary tumors is also heterogeneous. As most advanced prostate cancers are comprised of clonal outgrowths of castrate resistant cells, it is likely that these more aggressive cells resulted from selective pressure.

Collectively these five mechanisms demonstrate varied approaches a cancer cell may adapt to survive in response to androgen ablation. Undoubtedly, new mechanisms to develop CRPC will be discovered as our understanding increases. It is also likely that combinations of these mechanisms will be used to enable enhanced survival in a single cancer cell. These mechanisms to evade androgen ablation therapy suggest the need for multimodality therapeutic approaches that can target both castrate dependent and resistant cells. These five mechanisms are similar with respect to evading androgen ablation, but to understand the unique differences will help in tailoring therapies to target specific subsets of cancer cells. Each patient has a unique tumor profile, thus efficient therapy must be tailored to these differences.

Prostatic Stroma

The stroma is commonly thought to be a layer of supportive connective tissue cells. Prostate stroma is composed of both resident stromal cell types such as smooth muscle, fibroblasts, myofibroblasts, fat and vascular cells, as well as recruited stromal cell types that include immune cells and other bone marrow derived cells (Li, Fan et al. 2007). Paracrine signaling from the stroma to the epithelial cells of the prostate is necessary for maintenance of prostate homeostasis (Gerdes, Dang et al. 1996; Kurita, Wang et al. 2001; Jackson, Franco et al. 2008). Many studies have shown the importance of the stroma in preventing or supporting cancer initiation and progression (Cunha, Hayward et al.

2002; Tuxhorn, Ayala et al. 2002; Chung, Baseman et al. 2005; Zechmann, Woenne et al. 2007; Sung, Hsieh et al. 2008). In contrast, tumor-associated stroma plays a role in local cancer growth, invasion, and metastasis.

Within the tumor microenvironment, the stroma has a vital role in cancer progression. Studies have shown that tumor cells and their associated stroma co-evolve (Li, Fan et al. 2007; Polyak, Haviv et al. 2009). It was thought for many years that cancer is initiated only in the epithelia. However, studies have since demonstrated that the stroma can transform adjacent epithelia through paracrine signaling (Bhowmick, Chytil et al. 2004; Kuperwasser, Chavarria et al. 2004). Growth factors and chemokines produced by stromal cells enhance epithelial growth and transformation. Cancer cells have the ability to undergo morphologic transitions influenced by their microenvironment. Epithelial to mesenchymal transition (EMT) as well as osteomimicry is thought to enhance the ability of carcinoma cells to evade the primary tumor (Thiery 2002). EMT provides cells enhanced motility and invasive properties to escape the primary tumor and enter circulation (Hay 1995; Condeelis and Segall 2003). Osteomimicry by the cancer cells, characterized by expression of genes generally restricted to cells within the bone, is thought to result from interaction with the resident prostate stroma in preparation for its dissemination to survive in the bone (Koeneman, Yeung et al. 1999). This shows that the stroma can be a powerful mediator of both tumor initiation and tumor progression.

Metastasis of cancer cells is influenced by the stroma. Stromal cells enhance metastatic events through paracrine signaling to carcinoma cells. A

study showed that tumor cells recruited hematopoietic bone marrow-derived cells in a VEGF-R1 dependent mechanism (Kaplan, Riba et al. 2005). These recruited stromal cells were responsible for creating a pre-metastatic niche for tumor cells. Importantly, this showed that expression of VEGF-R1 is responsible for bone marrow-derived cell recruitment to primary tumors as well as determining organ-specific tumor metastases. Moreover, the metastatic potential of LNCaP cells was enhanced in a 3-D co-culture system with prostate stromal cells and in subcutaneous xenografts (Thalmann, Anezinis et al. 1994; Thalmann, Rhee et al. 2009). Another study demonstrated the ability of prostate cancer cells to metastasize to bone using the SDF-1/CXCR4 signaling pathway (Taichman, Cooper et al. 2002). Cancer cells were shown to express varying levels of CXCR4 and migrate in response to SDF-1, which is normally produced by osteoblasts and endothelial cells. In addition, SDF-1 treatment enhanced cancer cell adhesion to endothelial cell lines and osteosarcomas.

Therapies to target the stroma in addition to the cancer epithelial cells will be more efficacious. An effective study that targeted the stroma used non-steroidal anti-inflammatory drugs (NSAIDs) to decrease inflammation associated with the cancer microenvironment (Sandler, Halabi et al. 2003). Decreased inflammation associated with the cancer will decrease the amount of cells recruited to the tumor microenvironment. In particular, tumor-associated macrophages and neutrophils have been shown to contribute to tumor progression by contributing growth factors aiding vasculogenesis (Serafini, De Santo et al. 2004; Yang, DeBusk et al. 2004). A study on castrate resistant

prostate cancer demonstrated that androgen ablation resulted in the infiltration of leukocytes, including B cells, with IKK-β activation (Ammirante, Luo et al. 2010). This led to enhanced chemokine production that activates IKK-α and Stat3 in prostate cancer cells. It has also been shown that nuclear cytokine-activated IKK-α controls prostate cancer metastasis through the tumor suppressor, Maspin (Luo, Tan et al. 2007). In addition, reduced Maspin expression in prostate epithelial cells correlates with decreased infiltration of RANKL-expression inflammatory cells. These stromal components contribute to tumor progression. Effective therapies will need to target multiple cell types to eradicate existing tumors as well as prevent recurrence or formation of new tumors (Loeffler, Kruger et al. 2006).

Transforming Growth Factor-β

TGF-β signaling is a master regulatory pathway that functions in almost every cell. TGF-β signaling is important for many processes including maintenance of slow cycling cell turn-over, regulating differentiation, maintaining the stem cell niche, immune surveillance, extracellular matrix formation, cellular morphogenesis, migration, bone remodeling, fibrosis, angiogenesis, and wound healing (Massague 1990; Border and Ruoslahti 1992; Sporn and Roberts 1992; Wahl 1992; Kulkarni, Huh et al. 1993; Salm, Burger et al. 2005).The activation of this pathway begins when the TGF-β1 ligand binds the TGF-β type II receptor (TβRII), which subsequently recruits the TGF-β type I receptor (Figure 5)
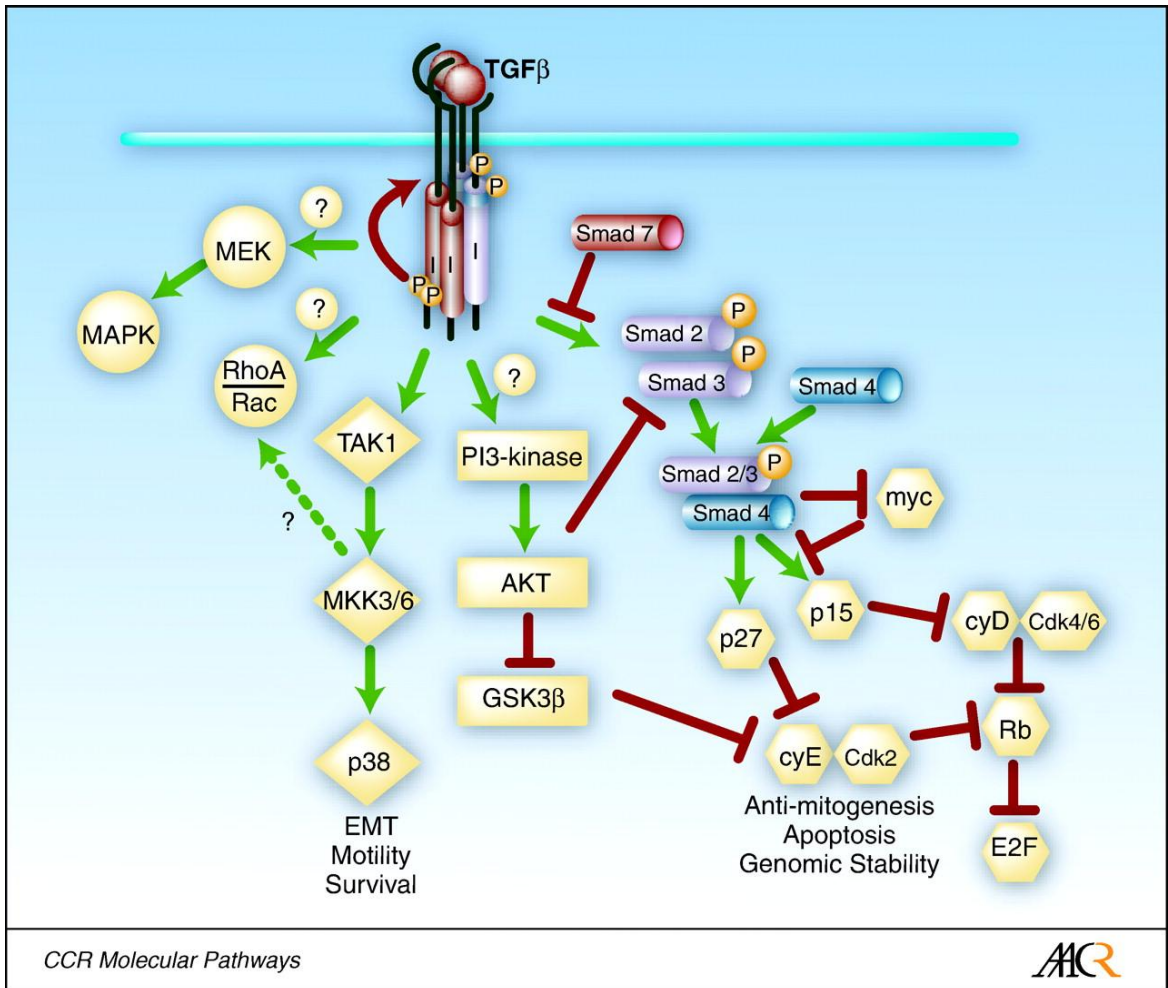
Figure 5. Signaling pathways induced by TGF-β receptors. The ligand-activated type I and II receptor complex recruits and phosphorylates the receptor-specific Smad2/3 which then recruit Smad4. Transcriptional responses induced by the Smad complex have been demonstrated to be associated with ligand-induced inhibition of cell proliferation, apoptosis, and genomic stability. The molecular mechanisms by which TGFβ activates non-canonical Smad pathways such as PI3K/AKT, Rho/Rac GTPases, and Ras/MAPK are less clear. Figure taken from (Biswas, Criswell et al. 2006).

(Biswas, Criswell et al. 2006). This causes phosphorylation of the receptor Smad proteins, Smad2 and Smad3, which can then bind Smad4. These Smad complexes then translocate to the nucleus and activate the canonical TGF-β signaling pathway. TGF-β can also activate non-canonical pathways involving ERK, p38, and c-Jun N-terminal kinase (JNK). Activation of various genes at the transcriptional level occurs in a cell-specific manner through interaction of these factors with other transcription factors and co-factors at the level of the genomic DNA (Derynck and Zhang 2003; Elliott and Blobe 2005).

Inflammation is regulated by TGF-β signaling. Mice lacking TGF-β1 resulted in extensive autoimmune inflammation affecting many organs (Shull, Ormsby et al. 1992; Kulkarni, Huh et al. 1993). Similarly, conditional knockout mice with the TβRII gene disrupted after induction with interferon-$\alpha\beta$ or polyI:polyC resulted in a lethal inflammatory disease (Leveen, Larsson et al. 2002). Bone marrow transplantation studies using these mice further confirmed that the lethal inflammation was a result of aberrant TGF-β signaling within cells of the hematopoietic lineage. Other cells of the immune system require proper TGF-β signaling demonstrated by mouse conditional knockout mice. The loss of Smad4 signaling in T cells resulted in gastrointestinal cancer (Kim, Li et al. 2006). In addition, reduced TGF-β signaling in T lymphocytes led to expansion of CD8+ memory T-cells (Lucas, McNeil et al. 2004). This led to the formation of leukemia/lymphoma. TGF-β signaling is crucial for proper immune function.

TGF-β signaling has been implicated in the development and progression of many cancers (Letterio 2005; Massague 2008). Its importance in biological

processes makes it one of the most frequently altered pathways in human cancer (Akhurst 2004; Xu and Pasche 2007). Among those studied, TGF-β signaling has been shown to be altered in prostate, breast, colon, leukemia, lymphoma, lung, pancreatic, and skin cancers (Bottinger, Jakubczak et al. 1997; Akhurst and Balmain 1999; Holloway, Davis et al. 2003; Tu, Thomas et al. 2003; Wolfraim, Fernandez et al. 2004; Cheng, Bhowmick et al. 2005; Kim, Li et al. 2006; Chen, Ghosh et al. 2007).

In the prostate, TGF-β plays a role with other signaling pathways to maintain growth and differentiation. AR was shown to down-regulate TGF-β1 expression in LNCaP cells (Chipuk, Cornelius et al. 2002). Specifically, AR binds Smad3, but not Smad2 nor Smad4. When Smad3 is bound to AR, binding to Smad-binding elements is repressed to inhibit transcription. In prostate smooth muscle cells, TGF-β1 (but not TGF-β2) blocked androgen-stimulated proliferation and altered AR distribution from the nucleus to the cytoplasm (Gerdes, Dang et al. 1998; Gerdes, Larsen et al. 2004). The crosstalk between androgen and TGF-β signaling affects differentiation of prostate stromal cells.

TGF-β signaling is known to regulate prostate cancer initiation and progression. There is a loss of epithelial and stromal TβRII in human prostate cancer compared to benign prostate tissues (Guo, Jacobs et al. 1997; Kim, Ahn et al. 1998; Li, Placencio et al. 2008). Another study looking at human prostate cancer samples and cell lines found lower mRNA and protein expression of TβRII compared to BPH samples (Zhao, Shiina et al. 2005). Low mRNA and protein expression also correlated with increased CpG methylation of the TβRII promoter

at the Sp1 binding site -140. Interestingly, treatment of prostate cancer cell lines with 5-aza-2'-deoxycytidine restored TβRII expression in PC3 cells. Using a transgenic mouse with a dominant negative TβRII mutant driven by the ventral prostate-specific C3(1) promoter, loss of TGF-β signaling caused accumulation of ventral prostate epithelial cells and a decrease in apoptosis compared to controls (Kundu, Kim et al. 2000). Another mouse model with a dominant negative TβRII mutant in the epithelia in combination with SV40 transformation led to increased metastasis (Tu, Thomas et al. 2003). Conditional loss of stromal Tgfbr2 in Tgfbr2[fspKO] mice caused transformation of the adjacent epithelia that led to the formation of prostatic intraepithelial neoplasia (PIN) lesions (Bhowmick, Chytil et al. 2004). In long-term studies, this also led to the development of adenocarcinoma (Bhowmick, Chytil et al. 2004; Li, Placencio et al. 2008). This showed the importance of paracrine signaling from the fibromuscular resident stroma to the epithelia. Further studies presented in this thesis characterized the mechanism of action within this Tgfbr2[fspKO] mouse model.

Wnts

One of the signaling pathways important in transferring signals from the resident stroma to the epithelia is the Wnt signaling pathway. Wnt signaling has been shown to be crucial to developmental processes and contributes to cancer progression (Polakis 2000; Giles, van Es et al. 2003; Verras and Sun 2006). Wnt signaling begins at the receptor (Figure 6) (Hall, Kang et al. 2006). The primary
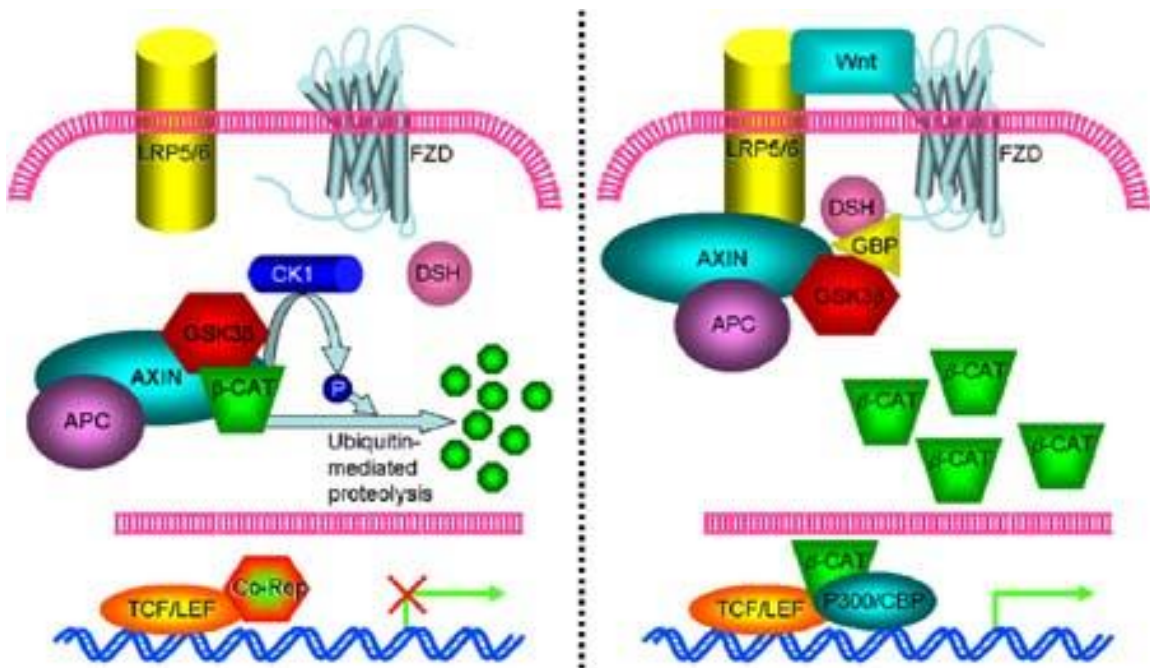
Figure 6. The canonical Wnt signaling pathway. In the absence of Wnt signals (shown in the left side of the diagram) cytoplasmic β-catenin is sequestered by a complex of Axin, APC, and glycogen synthase kinase 3β (GSK3β). Phosphorylation of GSK3β leads to ubiquitination and the subsequent degradation of β-catenin. In the presence of Wnt signals (shown in the right side of the diagram), Wnt proteins bind at the cell membrane to a co-receptor consisting of Frizzled (FZD) and low-density lipoprotein receptor-related protein (LRP). This union results in the hyperphosphorylation of disheveled (DSH), which blocks the activity of GSK3β. Inhibition of GSK activity results in the stabilization and accumulation of β-catenin. Upon translocation to the nucleus β-catenin serves as a co-factor for the TCF family of transcription factors. Histone acetylases, p300, and CBP act as co-activators of β-catenin activity. Figure taken from (Hall, Kang et al. 2006).

receptors for Wnt signaling are the seven-transmembrane Frizzled proteins. These proteins interact with a single transmembrane LDL receptor-related protein 5/6 (LRP5/6), which serve as co-receptors (He, Semenov et al. 2004). This receptor complex binds the Wnt ligands from the extracellular matrix. Individual Wnt ligands activate the canonical or non-canonical signaling pathways. The most studied is the canonical pathway, which stabilizes β-catenin leading to accumulation in the nucleus. β-catenin forms a transcription complex with members of the TCF/LEF family, which then bind DNA to activate transcription of target genes (Eastman and Grosschedl 1999). Non-canonical pathways include the planar cell polarity (PCP) pathway or the Ca^{++} pathway, however their involvement in prostate cancer is not well established.

Interactions between canonical Wnt and androgen signaling implicate their cooperation in prostate cancer progression. Studies demonstrate that β-catenin acts as an AR co-activator (Mulholland, Cheng et al. 2002). Wnt-3a conditioned medium enhanced AR-mediated transcription of PSA with and without androgens in LNCaP cells (Shibamoto, Higano et al. 1998; Verras, Brown et al. 2004). Alternatively, knockdown of AR with shRNA or bicalutamide treatment reduced AR-mediated transcription by Wnt-3a conditioned medium. This suggests that Wnt-3a directly acts on AR. Furthermore, Wnt-3a was shown to enhance cell proliferation and colony formation in LNCaP cells.

Direct binding of β-catenin with AR was shown to enhance AR transcriptional activity in LNCaP cells (Truica, Byers et al. 2000). This has been confirmed in numerous other studies including yeast two-hybrid systems, *in vitro*

and *in vivo* protein binding assays (Mulholland, Cheng et al. 2002; Pawlowski, Ertel et al. 2002; Yang, Li et al. 2002). Androgens enhanced this interaction. More specifically, this interaction occurred within the ligand binding domain of AR. Interestingly, β-catenin was shown to have greater binding affinity to AR compared to other hormone receptors including estrogen receptor, progesterone receptor, and the glucocorticoid receptor. AR enhances the nuclear translocation of β-catenin in response to androgens, suggesting its role to enhance Wnt signaling in the prostate. It has also been shown that reduced E-cadherin enhanced AR-mediated transcription by increasing β-catenin levels in both the cytoplasm and nucleus (Sasaki, Lin et al. 2000). Reduced expression of E-cadherin was observed in advanced and poorly differentiated prostate cancer (Umbas, Schalken et al. 1992; Richmond, Karayiannakis et al. 1997; Luo, Lubaroff et al. 1999). Of interest is the low frequency of β-catenin mutations in prostate cancer with one study showing only 5% of patient samples having a mutation in the serine or threonine residues in the NH2-terminus of β-catenin (Voeller, Truica et al. 1998; Chesire, Ewing et al. 2000; Gerstein, Almeida et al. 2002). This is the region responsible for the interaction with AR (Yang, Li et al. 2002). Analysis of castrate resistant prostate cancer tissues showed an increase in β-catenin mutations in approximately 20-40% of patient samples (Chesire, Ewing et al. 2002; de la Taille, Rubin et al. 2003). The low frequency of β-catenin mutations suggests that more studies are needed to understand how other signaling pathways can activate Wnt signaling in prostate cancer progression. Collectively, Wnt signaling plays an important role in prostate cancer progression

in cooperation with androgen signaling and likely other major regulatory pathways.

Wnt Inhibitors

There are known inhibitors to the Wnt signaling pathway. These include secreted frizzled-related proteins (SFRPs), Wnt inhibitory factor-1 (Wif-1), and Dickkopf (Dkk) (Figure 7) (Truica, Byers et al. 2000; Jones and Jomary 2002; Kawano and Kypta 2003 ; Hall, Kang et al. 2006). SFRPs inhibit Wnt signaling by binding Wnt ligands or frizzled receptors, Dkk binds to LRP receptors to block LRP-Wnt interactions, and Wif-1 binds Wnt ligands to inhibit binding to receptors. SFRPs and Wif-1 inhibit canonical and non-canonical Wnt-mediated signaling (Hsieh, Kodjabachian et al. 1999). Dkk only inhibits canonical Wnt signaling (Zorn 2001). They are expressed in distinct and overlapping patterns depending on the cell and tissue (Leimeister, Bach et al. 1998; Terry, Magan et al. 2000). They are often expressed in a complementary pattern to individual Wnt ligands (Jaspard, Couffinhal et al. 2000). In the prostate, Wnt-3a is often associated with cancer progression (Verras, Brown et al. 2004). SFRP-2 has been shown to inhibit Wnt-3a mediated signaling (Galli, Barnes et al. 2006). Understanding the role of Wnt inhibitors may advance treatment options for prostate cancer.
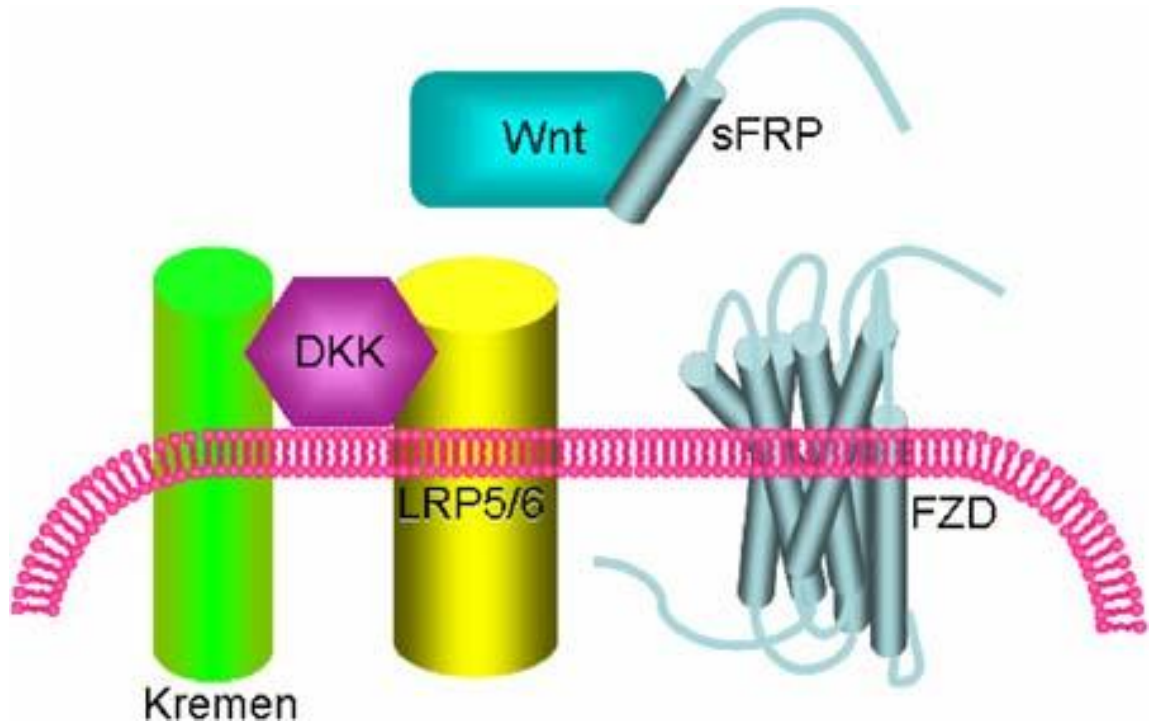
Figure 7. Endogenous inhibitors of Wnt signaling. Soluble Frizzled related proteins (SFRPs) can sequester Wnts so that they cannot bind to cell surface Frizzled (FZD) receptors. Kremen facilitates the binding of Dickkopf (DKK) to the Low-density lipoprotein receptor-related protein (LRP), which results in blocking Wnt from binding LRP. Wif-1 (not shown) is bound to the cell membrane, but acts similar to SFRP by sequestering Wnt ligands inhibiting their binding to receptors. Figure taken from (Hall, Kang et al. 2006).

Bone Marrow Derived Cells

Bone marrow derived cells (BMDCs) have been shown to play a role in cancer progression (Avital, Moreira et al. 2007; Guest, Ilic et al. 2009). They are composed of many cell types. The two main progenitor cell types are the hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). HSCs are known to differentiate into various cell types including endothelial cells and monocytes. MSCs are known to differentiate into many other cell types including pericytes and fibroblasts (Roorda, ter Elst et al. 2009). BMDCs have been shown to home to various tissues under basal conditions (Krause, Theise et al. 2001). However, when inflammation is present in the tissues recruitment of BMDCs is enhanced (Li, Stoicov et al. 2006). BMDCs are important for tissue repair to enhance subsequent cell and tissue growth through paracrine signaling or angiogenic responses. They are also important for removing dead cells or debri to restore homeostasis (Roorda, ter Elst et al. 2009).

Alternatively, BMDCs play a role in tumor progression and it is thought that the capacity for self-renewal, longevity, chemoresistance, and susceptibility to mutations make these potential cancer stem cells (Liu, Chen et al. 2006; Avital, Moreira et al. 2007). BMDCs are known to be recruited to the stroma. Tumor stromal cells have been shown to be recruited from progenitor cells in the bone marrow (Lyden, Hattori et al. 2001; Rajantie, Ilmonen et al. 2004; De Palma, Venneri et al. 2005; Peters, Diaz et al. 2005; Sangai, Ishii et al. 2005).These bone marrow-derived progenitor cells are mobilized into the

circulation, migrate to tumors, and incorporate into the tumor microenvironment to enhance growth (Asahara, Murohara et al. 1997; Hattori, Dias et al. 2001; Yamaguchi, Kusano et al. 2003; Kawada, Fujita et al. 2004; Ramasamy, Lam et al. 2007). The exact role of BMDCs is variable depending on the tissue they are recruited to.

Inflammation in a wound healing response or cancer is known to recruit BMDCs (Alison, Lim et al. 2009). One example is the gastrointestinal tract. Chronic inflammation is associated with cancer development (Spaeth, Klopp et al. 2008). Inflammation serves to recruit BMDCs from the circulation. These cells can then incorporate into the associated stroma and contribute by enhancing the production of cytokines and induce blood vessel growth (Roorda, ter Elst et al. 2009). In this milieu, inflammatory cells, including those derived from the bone marrow, produce highly reactive oxygen and nitrogen species that can interact with cellular DNA to induce mutations (Li, Stoicov et al. 2006). The normal cell response to DNA mutations may be to undergo apoptosis. However, in a chronic inflammatory environment the chances to bypass apoptosis are favorable and growth is allowed to continue (Li, Stoicov et al. 2006). These mutated cells continue to divide accumulating further genetic defects leading to the development of cancer cells. In a model of gastric cancer induced with helicobacter, BMDCs were recruited in this model of chronic inflammation leading to cancer development (Houghton, Stoicov et al. 2004). These BMDCs incorporated into the prostate epithelial cells through fusion and played a role in the progression through metaplasia, dysplasia, and eventually intraepithelial

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cancer. BMDCs secrete cytokines and growth factors to promote blood vessel formation and support stem cell niches to enable cancer development in the affected tissue. Other studies have shown that BMDCs are recruited to and incorporate into resulting epithelial tissues found in lung, liver, skin, heart, and gastrointestinal tract (Krause, Theise et al. 2001; Kawada, Fujita et al. 2004; Li, Stoicov et al. 2006). Few studies have examined the recruitment of BMDCS in benign and malignant prostate in normal and cancer tissues (Palapattu, Meeker et al. 2006; Luo, Tan et al. 2007; Ammirante, Luo et al. 2010). These studies suggest multiple BMDC types could contribute to cancer progression, including macrophage and B cells. Further studies are needed to understand how BMDCs play a role in prostate cancer progression, in particular the progression to castrate resistant disease.

Mesenchymal Stem Cells

One sub-population of BMDCs include MSCs. They are classified as a non-hematopoietic adult stem cell thought to have properties of a mesenchymal lineage. They can be isolated from multiple tissues, but are most commonly isolated from the bone marrow. Like most other stromal cell types, they are heterogeneous in nature and thus their characterization is not well defined. They are able to differentiate into a multitude of cell lineages including osteocytes, chondrocytes, adipocytes, stromal cells, and muscle cells. MSCs are routinely isolated and expanded in culture (Phinney, Kopen et al. 1999; Short, Brouard et

al. 2001). They are characterized as an adherent cell population expressing, though disputable, stem cell markers including CD44 and stromal cell markers CD90 and CD105 while lacking hematopoietic markers such as CD34 and CD45. Studies have shown their ability to home to sites of tissue or organ damage, inflammatory microenvironments, and cancer (Spaeth, Klopp et al. 2008). These recruited MSCs have been shown to promote tumor growth through expression of growth factors, enhance tumor vessel growth, and create tumor stem cell niches (Roorda, ter Elst et al. 2009).

Therapeutic uses with MSCs are of high interest. Pre-clinical studies have begun to explore the possibilities of MSCs for therapeutic use. These have been used to reduce graft-versus-host disease, tissue repair of cerebral injury, bone fractures, myocardial ischemia/infarction and muscular dystrophy (Niedzwiedzki, Dabrowski et al. 1993; Li, Chen et al. 2001; Kawada, Fujita et al. 2004; Dezawa, Ishikawa et al. 2005). They have been shown to home preferentially to tumor stroma as opposed to normal organ stroma in mouse studies (Roni, Habeler et al. 2003; Karnoub, Dash et al. 2007). Their migration is induced by growth factors or cytokines secreted from the tumor microenvironment. MSCs possess chemotactic properties similar to inflammatory cells that enable their recruitment to sites of inflammation or tissue remodeling. MSCs express a multitude of growth factor, chemokine/ cytokine, adhesion and innate immune surveillance receptors (Table 1) (Spaeth, Klopp et al. 2008). These receptors are also expressed on inflammatory cells commonly found in tumor microenvironments. This suggests that MSCs home and become recruited to the tumor

Table 1. Cell surface markers and receptors associated with cell migration that are known to be expressed on MSCs. The known ligands for each receptor are listed in the third column. The various cell types expressing the same receptors are listed in the fourth column. Table taken from (Spaeth, Klopp et al. 2008).

	Cell surface receptors found on MSC	Ligands	Present on other cell types
Growth hormone receptors	EGFR (ErbB)	EGF	DC, neutrophil
	HGFR (c-met)	HGF	Leukocytes, macrophages
	IGF1R	IGF1	Leukocytes, HSC
	PDGFR (Ra-b)	PDGFa/b	HSC
	VEGFR1	VEGF	HSC, monocytes, neutrophils
	VEGFR2	VEGF	HSC
	FGFR2	FGF2	HSC, leukocytes
	Tie-2	Ang-1	HSC, leukocytes
Chemokine/cytokine receptors	CCR1	CCL3, CCL5, CCL7, CCL13, CCL14, CCL15, CCL16, CCL23	monocyte, T cell, DC
	CCR2	CCL2, CCL7, CCL8, CCL13, CCL16	Monocyte, T cell, DC
	CCR3	CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL16, CCL24, CCL26, CCL28	T cell, DC
	CCR4	CCL17, CCL22	T cell, macrophage, DC
	CCR5	CCL3, CCL4, CCL5, CCL8, CCL11, CCL14, CCL16	Monocyte, T cell, DC, HSC
	CCR6	CCL20	T cell, B cell, DC
	CCR7	CCL19, CCL21	T cell, DC
	CCR8	CCL1	monocyte, T cell, DC
	CCR9	CCL25	T cell
	CCR10	CCL27, CCL28	T cell
	CXCR1	CXCL6, CXCL7, CXCL8	Neutrophil, monocyte
	CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8	Neutrophil, monocyte
	CXCR3-A/B	CXCL4, CXCL9, CXCL10, CXCL11	T cell, microvascular cells
	CXCR4	CXCL12	T cell, B cell, monocyte, macrophage, DC
	CXCR5	CXCL13	B cell, Th cells, HSC
	CXCR6	CXCL16	CD8 T cells, NK cells, CD4 T cells
	CX3CR1	CX3CL1	Macrophage
	XCR1	XCL1, XCL2	T cell, NK cell
Adhesion molecules	VCAM-1 (VLA-4)	$\beta1$ integrin/$\alpha4$ integrin	Leukocytes
	ICAM-1/3	LFA-1	Leukocytes, DC
	ALCAM	CD6	Leukocytes
	Endoglin (CD105)	TGFβ1/3	Leukocytes, HSC
	TLR1	Lipopeptides	Leukocytes
Innate immune surveillance	TLR2	Peptidoglycans, lipopeptides	Monocytes, DC
	TLR3	dsRNA	DC
	TLR4	LPS	Monocytes, DC
	TLR5	ECM molecules	Monocytes
	TLR6	Peptidoglycans	Epithelium

microenvironment. Studies have also shown that MSCs can be used as vehicles for targeted delivery in tumor cells. One study forced expression of IFN-β in MSCs (Studeny, Marini et al. 2002). Upon homing to tumor cells, the IFN-β produced by the MSCs caused inhibition of growth. Another study showed that preconditioning MSCs with IGF-1 increased the expression of CXCR4 on the cell surface of MSCs allowing them enhanced migration to SDF-1 (Li, Yu et al. 2007). Using this information, future trials can use preconditioned MSCs to express a plethora of receptors to more efficiently home to certain tumor microenvironments versus others. Forced over-expression of receptors in MSCs can also be a valuable tool for more specific expression. Targeting MSCs for delivery of anticancer drugs and treatments to the tumor microenvironment holds promise with a further understanding of these complex cells.

Summary and Dissertation Goals

Our current understanding of castrate resistant prostate cancer is in its early stages. The Nobel Prize discovery that prostate cancer was responsive to castration was realized 69 years ago by Huggins and Hogdes (Huggins and Hodges 1972). Since then the PSA blood screening test and multiple therapeutics have been developed to treat the primary tumor. It has become clear in recent years that current androgen ablation therapy, although effective initially, results in subsequently enhancing the aggressiveness and castrate resistance of the recurrent cancer. The goal of this thesis was to enhance our

understanding of CRPC. These studies focused on understanding the role of the tumor microenvironment on prostate cancer progression. Paracrine signaling from the resident and recruited stroma to the epithelia is discussed in the following chapters.

Stromal TGF-β signaling mediates prostatic response to androgen ablation by paracrine Wnt activity

These studies were based on our previously developed conditional stromal TGF-β type II receptor knockout mouse model (Tgfbr2^{fspKO}). Within this thesis work, prostates were characterized as castrate resistant providing a novel transgenic model to understand CRPC. We demonstrated the involvement of paracrine TGF-β, androgen, and Wnt signaling in castrate resistance. These studies are based on understanding paracrine signaling originating in the resident prostate stroma during prostate cancer progression.

Bone marrow derived mesenchymal stem cells incorporate into the prostate during regrowth

Regrowth of the prostate with respect to recruitment of stromal cells was studied to understand this aspect of CRPC. We began by examining the collective population of BMDCs recruited to the prostate during regrowth. We determined that a small population of these total BMDCs, MSCs, were not only recruited, but also fused to prostatic ductal epithelia. These MSCs were shown to enhance Wnt signaling within castrate resistant tumor epithelia. This study

serves as a foundation for future studies on the role of recruited MSCs in the prostate cancer microenvironment.

CHAPTER II

STROMAL TGF-β SIGNALING MEDIATES PROSTATIC RESPONSE TO ANDROGEN ABLATION BY PARACRINE WNT ACTIVITY

Introduction

Prostate cancer continues to be a major cause of death in aging men (Jemal, Tiwari et al. 2004). The prostate is an androgen-dependent organ. As a result, treatment for prostate cancer includes the inhibition of androgens. Regardless of the initial positive response to androgen ablation, the cancer frequently overcomes its dependence on androgens and results in a drug-resistant cancer with few options for treatment. Although androgen ablation therapy is intended to target the prostate epithelia, the influence of the prostatic stroma on androgen responsiveness of the adjacent epithelia is likely to be critical in the long-term effectiveness of treatment.

Mature and differentiated prostate tissue is formed and maintained by effects on androgen receptors within the stromal compartment (Cunha and Chung 1981; Cunha and Donjacour 1989). The concept of mesenchymal cells relaying androgen sensitivity to the epithelia through paracrine interactions is supported by tissue recombination experiments. Prostate epithelia expressing a non-functional androgen receptor were equally sensitive to androgen ablation, carried out by castration of host mice, when compared to the control tissue recombinations generated with wild type mesenchyme and wild type epithelia (Donjacour and Cunha 1993; Kurita, Wang et al. 2001). Yet mesenchymal cells

with a non-functional androgen receptor did not even support prostate epithelial development (Cunha and Lung 1978). This suggested that the prostatic stroma plays an instructive role in glandular development and potentially influences responsiveness to androgen ablation in prostate cancer progression (Kurita, Wang et al. 2001). As a basis to understanding androgen independent prostate cancer, we investigated the mechanism for prostate epithelial survival in the absence of androgens.

Following androgen ablation the prostate undergoes apoptotic regression. In mouse models, reintroduction of androgens following androgen ablation results in prostate regrowth originating from the proximal ducts. This observation indicates that the proximal ducts are inherently refractory to androgen ablation (Lee, Sensibar et al. 1990; Rouleau, Leger et al. 1990; Prins, Cooke et al. 1992). Canonical Wnt signaling is known to play a role in cell survival in many tissues (Mount, Muzylak et al. 2006; Terry, Yang et al. 2006)Verras, 2006 #120}. Thus, we wanted to investigate a potential role for Wnt signaling in the survival of proximal ductal tissue upon castration. Another growth factor, TGF-β, is thought to support prostatic apoptosis as its expression coincides with androgen ablation in benign and cancer tissues (Kyprianou and Isaacs 1989; Hsing, Kadomatsu et al. 1996; Brodin, ten Dijke et al. 1999). TGF-β binds the TGF-β type II receptor (Tgfbr2) at the cell surface to phosphorylate the TGF-β type I receptor and activate cytoplasmic proteins, predominantly Smad2 and Smad3 (Massague and Gomis 2006). Both cooperative and antagonistic interactions of Wnt, androgen, and TGF-β signaling pathways occur in the prostatic epithelia (Hayes, Zarnegar

et al. 2001; Kang, Lin et al. 2001; Chipuk, Cornelius et al. 2002; Yang, Chen et al. 2006). However, the role of Wnt and TGF-β signaling on androgen dependence of the prostate is unknown.

We tested the hypothesis that paracrine Wnt signaling regulates stromal-epithelial interactions in response to androgen ablation in a TGF-β-dependent manner using mouse and allografting models. Canonical Wnt signaling involves the activation of cognate frizzled receptors at the cell surface that mediate β-catenin accumulation leading to transcriptional activity in the nucleus (Widelitz 2005). Here we report that canonical Wnt activity is present in the prostatic epithelia of the proximal ducts of the prostate following androgen ablation. Previously, we generated a Tgfbr2 conditional fibroblast knockout mouse through FSP-1 Cre-mediated recombination of Tgfbr2 exon 2 (Tgfbr2^{fspKO}), which resulted in the development of pre-neoplastic lesions (prostatic intraepithelial neoplasia) by five to seven weeks of age (Bhowmick, Chytil et al. 2004). To further study the role of TGF-β on the prostate epithelia following androgen ablation, here we developed a conditional epithelial Tgfbr2 knockout by crossing NKX3.1-Cre mice with Tgfbr2$^{floxE2/floxE2}$ mice (Chytil, Magnuson et al. 2002), termed Tgfbr2$^{NKX3.1KO}$. Another valuable tool was the Tg(Fos-lacZ)34Efu/J mouse model, termed TOPGal, enabled the visualization of canonical Wnt signaling activity (DasGupta and Fuchs 1999). Interestingly, the prostates of Tgfbr2^{fspKO} mice had constitutive Wnt signaling throughout the prostate and were resistant to androgen ablation induced regression. The 12T7f LADY mouse model served as a transformed epithelial counterpart, expressing SV40 Large T antigen in the prostatic epithelia

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(Kasper, Sheppard et al. 1998). Tissue recombination allografting of the Tgfbr2^{fspKO} prostatic stroma was able to convert the androgen-dependent 12T7f LADY prostatic epithelia to become refractile to androgen ablation. Together, the data provides a mechanism for androgen independent survival of the prostate epithelia.

Materials and Methods

Transgenic Mice

Tgfbr2$^{floxE2/floxE2}$ (Shappell, Thomas et al. 2004) and Tgfbr2^{fspKO} mice of C57BL/6 background were generated as previously described (Bhowmick, Chytil et al. 2004). The NKX3.1-Cre mice, also in the C57BL/6 background, were crossed with Tgfbr2$^{floxE2/floxE2}$ mice to generate the Tgfbr2$^{NKX3.1KO}$ mouse model. The Tgfbr2$^{NKX3.1KO}$ mice were further crossed with Rosa26 mice to enable visualization of cells undergoing Cre-mediated recombination. TOPGal mice (DasGupta and Fuchs 1999) were purchased from Jackson Laboratories and crossed with Tgfbr2$^{floxE2/floxE2}$ and the Tgfbr2$^{floxE2/wt}$-Cre^{FSP} mice to generate the Tgfbr2$^{floxE2/floxE2}$/TOPGal and Tgfbr2^{fspKO}/TOPGal mouse models. Prostatic epithelial organoids were generated from 15 week-old 12T7f LADY mice, at which time they developed high-grade hyperplastic lesions and foci of adenocarcinoma (Kasper, Sheppard et al. 1998; Tuxhorn, Ayala et al. 2002). All mice were genotyped from ear punch biopsies. NKX3.1-Cre mice were

genotyped using the same Cre primers as the Tgfbr2^{fspKO} mice (Bhowmick, Chytil et al. 2004). A single approximately 200bp band indicated Cre expression. All other mouse genotyping was performed as previously reported (Kasper, Sheppard et al. 1998; DasGupta and Fuchs 1999; Shappell, Thomas et al. 2004). Harlan Sprague Dawley (Indianapolis, IN) SCID CB17/ICR hsd and C57BL/6 mice were used for tissue recombination and tissue rescue allografting techniques, respectively. All animal procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Cell Culture

Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} mouse primary prostate stromal cell cultures were generated from prostates of 6-8 week old mice, as described previously (Roberts, Sporn et al. 1986). Cultures grown between passage ten and thirty were used for experiments. LNCaP cells were purchased and grown as recommended by ATCC.

Conditioned media experiments and ^{3}H-thymidine Incorporation

Conditioned stromal media was generated by plating 750,000 Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} cells or for control 3,000,000 LNCaP cells on a 100mm dish similar to previously published reports (Bhowmick, Ghiassi et al. 2001). Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} stromal cells were transduced with either GFP (control) or secreted

frizzled related protein-2 (SFRP-2) adenovirus at 10^5-virus particles/mL for 24 hours prior to replacing standard stromal cell culture media with the lower serum media to allow virus production in stromal cells. The stromal cells were incubated for 72 hours in the stromal cell media containing 10^{-8} M testosterone or if indicated, bicalutamide (10^{-5} M). The stromal conditioned media was transferred to 15 mL conical tubes and stored in -80°C at least 24 hours up to three weeks. The stromal conditioned media was thawed, and plated over LNCaP cells (10,000 cells per well) in 24 well plates. The conditioned media was replaced after 72 hours of incubation with fresh conditioned media. In select conditions, fresh bicalutamide was also included as part of the conditioned media. Following 120 hours of incubation of LNCaP cells with stromal conditioned media, ^{3}H-thymidine incorporation assays were performed. Three hours prior to assaying for proliferation, cells were given 2 µCi ^{3}H-thymidine (PerkinElmer, Waltham, MA) in serum free RPMI per well. Cells were washed in 1 mL 10% TCA for 10 min, three times then lysed with 300 µl 200 mM NaOH for 30 min. The cell lysates (100 µl) were measured for ^{3}H-thymidine activity using a scintillation counter (Hayward, Haughney et al. 1998). All treatment conditions were performed in triplicate.

Immunohistochemistry

Tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned for histological analysis. GFP (1:1000, Santa Cruz, CA), Ki67 (1:3000,

Vector Laboratories), and SV40 (1:1000,Calbiochem, SanDiego, CA) immunohistochemistry was performed by using antigen retrieval with antigen unmasking solution (Vector Laboratories, Burlngame, CA) diluted 1:100. Following primary antibody incubation overnight, Dako Cytomation Universal or Rabbit kits were used for the secondary antibody and development with DAB. TUNEL staining was performed using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA). Double immunohistochemical staining was performed for TUNEL followed by staining for ß-galactosidase (1:5000, Abcam, Cambridge MA) developed with TrueBlue Peroxidase Substrate (KPL, Gaithersburg, MD). Immunohistochemical staining was systematically quantitated by taking a ratio of positively stained cells per epithelial nuclei per field (200x). Statistical significance was determined by two tailed Student's t test.

Immunofluorescence

50,000 cells were treated with 5 ng/ mL TGF-β (Cell Sciences, Canton, MA) on glass coverslips for 6 hours. The cells were fixed with 4% paraformaldehyde for 10 min. at 4°C. Smad2 localization was visualized using primary (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) and secondary anti-goat Alexa Fluor 594 antibody (1:500, Invitrogen. Carlsbad, CA) on a Nikon epifluorescence microscope.

β-galactosidase tissue staining

Whole mouse prostates were dissected and fixed in 4% PFA for one to two hours, washed in PBS, incubated with X-gal for 3 hr at 30ºC, washed in PBS, and fixed in 4% PFA overnight. Processed paraffin embedded tissues were sectioned at 8 μm and counterstained with Nuclear Fast Red (Electron Microscopy Sciences, Hatfield, PA).

Tissue recombination allografting

The tissue recombinations were performed as previously described (Strutz, Okada et al. 1995). Epithelial organoids were derived by digesting prostates of 6-12 week old wild type C57BL/6 mice or 15 week- old LADY 12T7f mice in 675 units/mL collagenase with 0.04% DNAse type I at 37ºC for 40 min as previously described (Strutz, Okada et al. 1995). The organoids were washed and pipetted into 50 μl collagen with stromal cells from $Tgfbr2^{floxE2/floxE2}$ or $Tgfbr2^{fspKO}$ mouse prostates and allowed to incubate overnight at $37^{o}C$. The tissue recombinants were then allografted under the renal capsule of syngenic C57BL/6 for 5 to 7 weeks or SCID mice for 6 weeks. Castration of host mice was performed 3-7 days prior to sacrifice as indicated.

RNA purification and RT-PCR

RNA from cell lysates was purified using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's directions. RT-PCR was performed for 32 cycles with the following primer sets: Wnt-2 forward 5'GTTTGCCCGTGCCTTTGTAGATG reverse 5'CCGGGTGACGTGGATGTG, Wnt-3a forward 5'TCTGCAGGAACTACGTGGAGATCA reverse 5'TCCCGAGAGACCATTCCTCCAAAT, Wnt-5a forward 5'TCGCCATGAAGAAGCCCATTGGAA reverse 5'TGTCCTTGAGAAAGTCCTGCCAGT, Wnt-9a forward 5'ACTGCTTTCCTCTACGCCATCTCT reverse 5'TTTGCAAGTGGTTTCCACTCCAGC, Wnt-11 forward 5'CTGACATGCGCTGGAACTGCTC reverse 5'AGGCCCGGGCGATGGTGT, and 18S forward 5' CAAGAACGAAAGTCGGAGGTTC reverse 5'GGACATCTAAGGGCATCACAG.

Results

<u>Stromal TGF-β responsiveness enables prostatic regression
after androgen depletion</u>

Androgen ablation causes prostate regression with a transient elevation of TGF-β expression (Kyprianou and Isaacs 1989). However, the role of TGF-β on the prostate following androgen ablation is not clear. We first developed a conditional Tgfbr2 knockout targeted to the epithelia by crossing NKX3.1-Cre

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mice with Tgfbr2$^{floxE2/floxE2}$ mice, termed Tgfbr2$^{NKX3.1KO}$ (conditional epithelial Tgfbr2 knockout). Further crossing the Tgfbr2$^{NKX3.1KO}$ mice into the Rosa26 line enabled the immunohistochemical localization of β-galactosidase expression associated with Cre-mediated recombination in the prostatic epithelia (Figure 8A). Since the Tgfbr2$^{NKX3.1KO}$ mice died perinatally, the prostates of these and Tgfbr2$^{floxE2/floxE2}$ mice were rescued to the renal capsule of syngenic C57BL/6 male hosts. Hematoxylin and eosin (H&E) staining revealed little difference in the ductal structures of the Tgfbr2$^{NKX3.1KO}$ prostates compared to Tgfbr2$^{floxE2/floxE2}$ controls (Figure 8B). However, the Tgfbr2$^{NKX3.1KO}$ prostates had significantly higher epithelial turnover as demonstrated by the apoptotic TUNEL staining (Figure 8B, lower panels; statistical graph shown in Figure 9) compared to Tgfbr2$^{floxE2/floxE2}$ allografts. Following castration there was a further elevation of epithelial TUNEL staining in the Tgfbr2$^{NKX3.1KO}$ prostates as observed in Tgfbr2$^{floxE2/floxE2}$ allografts. Taken together, there was no significant difference in apoptotic response observed between Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2$^{NKX3.1KO}$ prostates following castration compared to their intact controls, respectively.

As epithelial TGF-β signaling did not seem to affect androgen responsiveness, the role of stromal TGF-β signaling was studied using the Tgfbr2^{fspKO} mouse model (conditional stromal Tgfbr2 knockout). The castration of Tgfbr2$^{floxE2/floxE2}$ mice resulted in 42% and 55% decrease in the total prostate wet weights, after seven and fourteen days, respectively (Figure 10A). In contrast, there was a negligible decrease in Tgfbr2^{fspKO} prostates wet weight seven days following castration, with approximately 20% regression by fourteen days.
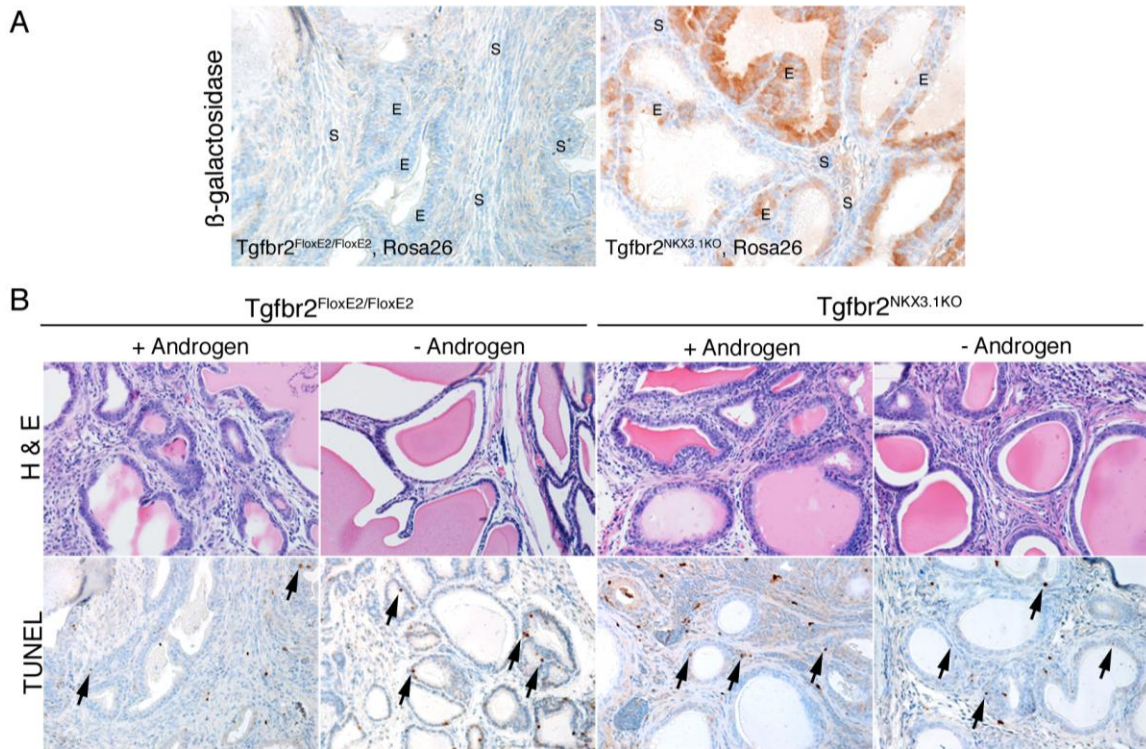
Figure 8. Conditional knockout of *Tgfbr2* in the prostate epithelia (Tgfbr2[NKX3.1KO]) did not significantly affect the response to androgen ablation compared to control Tgfbr2[floxE2/floxE2] prostate allografts. (A) Immunohistochemistry for β-galactosidase expression (brown) in tissue rescued Tgfbr2[NKX3.1KO]/Rosa26 prostates indicates Cre-recombination in the prostatic epithelia compared to control, Tgfbr2[floxE2/floxE2]/Rosa26 prostates with no detectible staining. Sections were counterstained with hematoxylin (blue). The epithelial (E) and stromal (S) compartments are indicated. (B) (Upper Panels) H&E staining of Tgfbr2[NKX3.1KO] and Tgfbr2[floxE2/floxE2] tissue rescue allografts suggest similar prostatic development (n=4). (Lower Panels) TUNEL staining (brown) indicates differential apoptosis of the prostatic epithelia of Tgfbr2[NKX3.1KO] and Tgfbr2[floxE2/floxE2] allografts in hosts that were not castrated (+Androgen) compared to hosts that were castrated (-Androgen). Increased TUNEL positive epithelia after castration indicates responsiveness to androgen ablation. The increase in TUNEL positive epithelia in intact compared to castrated Tgfbr2[floxE2/floxE2] prostates was significant (P value = 0.0379). The increase in TUNEL positive epithelia in intact compared to castrated Tgfbr2[NKX3.1KO] prostates was significant (P value = 0.0056). Student's t test evaluation determined P value < 0.05 is significant. Figure taken from (Placencio, Sharif-Afshar et al. 2008).

Corresponds to Figure 1: Percent Positive Epithelial TUNEL staining of Tgfbr2$^{NKX3.1KO}$ Prostates

P-Value	Comparison
0.0379	Tgfbr2$^{floxE2/floxE2}$ (+Androgen) vs Tgfbr2$^{floxE2/floxE2}$ (-Androgen)
0.0058	Tgfbr2$^{floxE2/floxE2}$ (+ Androgen) vs Tgfbr2$^{NKX3.1KO}$ (+ Androgen)
0.0056	Tgfbr2$^{NKX3.1KO}$ (+ Androgen) vs Tgfbr2$^{NKX3.1KO}$ (- Androgen)

Corresponds to Figure 2: Percent Positive Epithelial Staining in Tgfbr2$^{FloxE2/FloxE2}$ and Tgfbr2^{fspKO} Prostates

P-Value	Comparison
0.0152	TUNEL: Tgfbr2$^{floxE2/floxE2}$ vs Tgfbr2^{fspKO}
0.0003	Ki67: Tgfbr2$^{floxE2/floxE2}$ vs Tgfbr2^{fspKO}

Corresponds to Figure 3: Percent Positive Epithelial Staining in Allografts of Tgfbr2$^{FloxE2/FloxE2}$ or Tgfbr2^{fspKO} Stroma with Wt Epithelial Organoids

P-Value	Comparison
0.0249	TUNEL: Tgfbr2$^{floxE2/floxE2}$ vs Tgfbr2^{fspKO}
0.0030	Ki67: Tgfbr2$^{floxE2/floxE2}$ vs Tgfbr2^{fspKO}

Corresponds to Figure 6: Percent Positive Epithelial TUNEL Staining in Tissue Rescues treated with GFP or SFRP-2 Adenovirus

P-Value	Comparison
0.0296	GFP-Tgfbr2$^{floxE2/floxE2}$ vs GFP-Tgfbr2^{fspKO}
0.2940	SFRP-2-Tgfbr2$^{floxE2/floxE2}$ vs SFRP-2-Tgfbr2^{fspKO}
0.2819	GFP-Tgfbr2$^{floxE2/floxE2}$ vs SFRP-2-Tgfbr2$^{floxE2/floxE2}$
0.0373	GFP-Tgfbr2^{fspKO} vs SFRP-2-Tgfbr2^{fspKO}

Corresponds to Figure 6: Percent Positive Epithelial TUNEL Staining in Allografts Using GFP-Tgfbr2^{fspKO} Stroma and LADY TAg Organoids Treated with GFP or SFRP-2

P-Value	Comparison
0.0472	GFP-Tgfbr2^{fspKO} -stroma vs GFP-Tgfbr2^{fspKO} -stroma

Figure 9. Quantitation for figures with TUNEL and Ki67 staining of the epithelia in Chapter II. Bar graphs show the average percent positive epithelial staining of TUNEL or Ki67 staining. The graph title indicates the corresponding figure. The tables of p-values designate a description of the compared values. P-values <0.05 indicate significance according to Student's t test analysis.
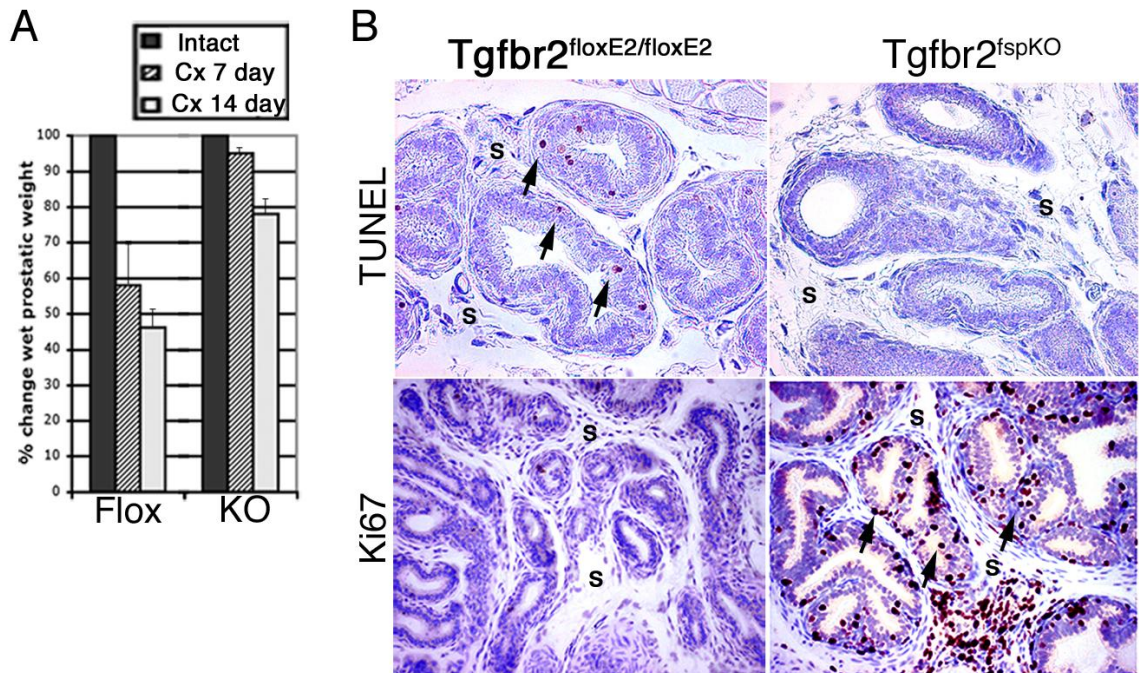
Figure 10. Tgfbr2^{fspKO} prostates lose androgen responsiveness after androgen ablation. (A) Percentage change in total wet prostate weight is shown seven and fourteen days following castration (Cx). Average Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} total wet prostate weights are shown as a percentage of the respective total prostate weight from intact Tgfbr2$^{floxE2/floxE2}$ (Flox) and Tgfbr2^{fspKO} (KO) mice (n=5). (B) Three days following castration, DL prostate lobes of Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} mice were subjected to TUNEL staining (brown) indicating apoptotic cells and Ki67 staining (brown) indicating proliferative cells. Hematoxylin (blue) was used as a nuclear counter stain. The stromal (S) compartment is indicated and black arrows indicate positively stained epithelial cells. Prostates were dissected from 5-7 week old male mice. Dorsolateral lobes are shown and these effects are consistent with all other lobes (n=8). The increase in TUNEL positive Tgfbr2^{fspKO} epithelia after ca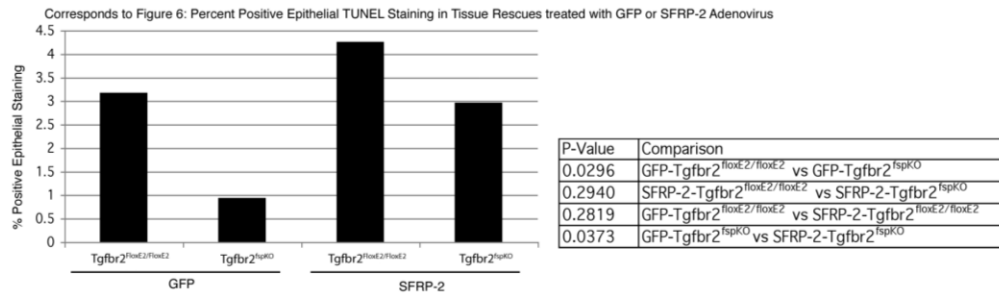stration was significantly greater than Tgfbr2$^{floxE2/floxE2}$ epithelia (P value = 0.0152). The level of Ki67 positive Tgfbr2^{fspKO} epithelia after castration was significantly greater than Tgfbr2$^{floxE2/floxE2}$ epithelia (P value = 0.0003). Figure taken from (Placencio, Sharif-Afshar et al. 2008).

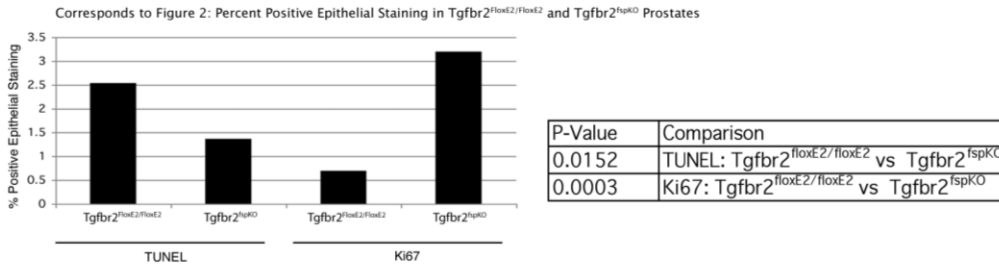Histologic analysis revealed that seven days following castration the Tgfbr2$^{floxE2/floxE2}$ prostates, with otherwise low cellular turnover, had an expected increase in TUNEL-positive apoptotic cells in both the epithelial and stromal compartments and little evidence of proliferation observed by Ki67 staining (Figure 10B). Prior to castration, Tgfbr2^{fspKO} mice exhibited slightly higher rates of apoptosis than Tgfbr2$^{floxE2/floxE2}$ mice (1.4-fold), accompanied by significant proliferation consistent with the described PIN phenotype (Bhowmick, Chytil et al. 2004). Seven days following castration, prostates from Tgfbr2^{fspKO} mice had little apparent epithelial apoptosis, yet remained highly proliferative based on TUNEL and Ki67 staining, respectively. The apparent elevated proliferation of the Tgfbr2^{fspKO} stroma coincided with greater stromal expansion compared to Tgfbr2$^{floxE2/floxE2}$ prostates. Together, these data suggested that normal prostate epithelial regression occurs in response to stromal TGF-β signals.

Before further studying the androgen independence of the prostates in Tgfbr2^{fspKO} mice, we needed to establish that the epithelial response resulted from the prostatic stroma and not other systemic factors in these mice. The FSP-1 Cre promoter targets a subset of fibroblasts throughout the body, including prostatic fibroblasts (Hayward 2002; Bhowmick, Chytil et al. 2004). We verified the loss of TGF-β responsiveness in Tgfbr2^{fspKO} cultured stromal cells by immunolocalization of Smad2 (Figure 11A). A tissue recombination allograft approach was then utilized to identify the role of the prostatic stromal cells in the phenotype observed. The tissue recombination technique consisted of combining cultured stromal cells derived from Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} prostates with

Tgfbr2^FloxE2/FloxE2   Tgfbr2^fspKO

A

Smad2 Hoechst   Smad2 Hoechst

B

+ Androgen

Kd   Kd

- Androgen

Kd   Kd

C

TUNEL

Kd

D

Ki67

Kd   Kd
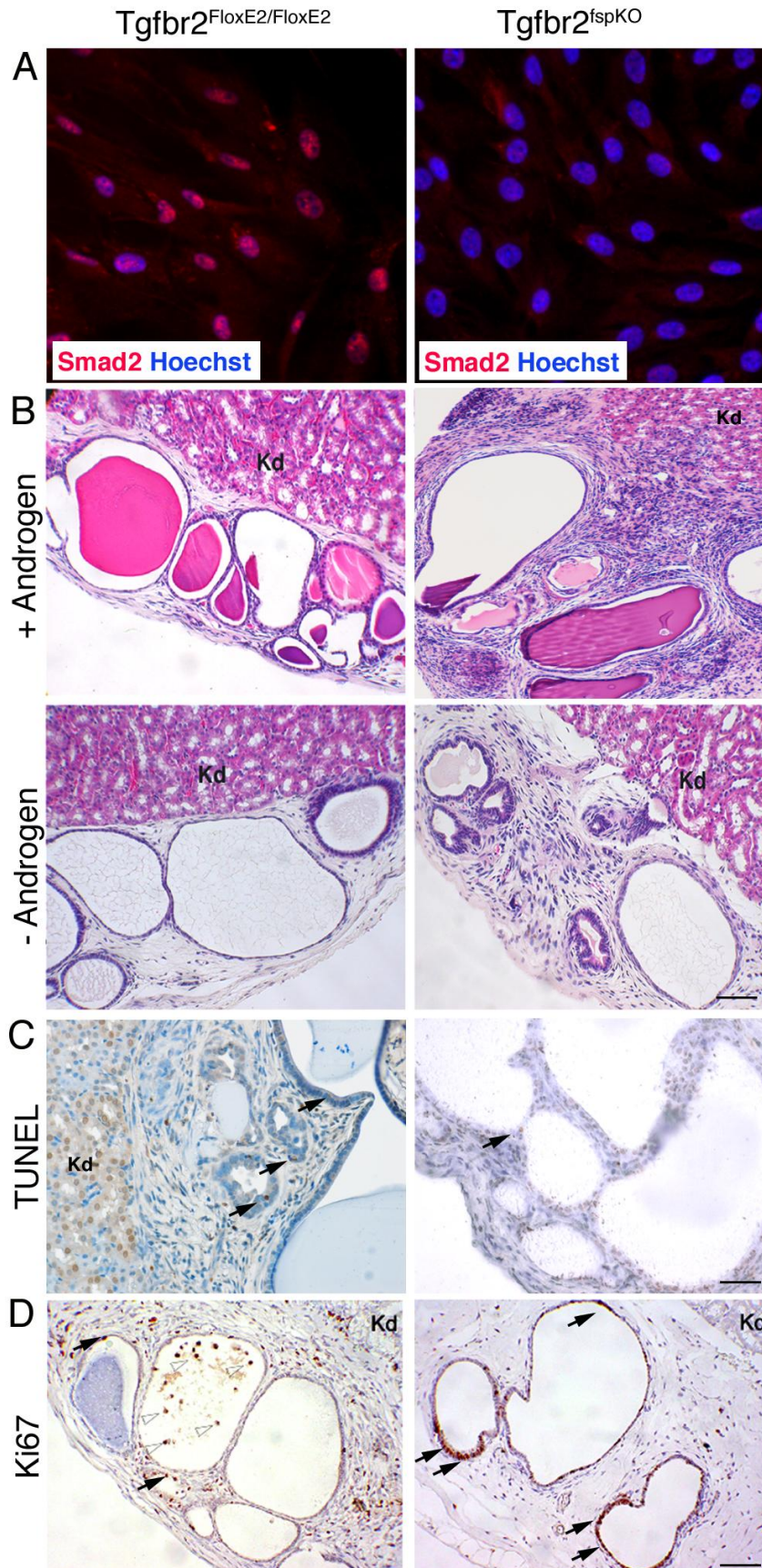
Figure 11. Loss of TGF-β responsiveness in prostatic stromal cells causes the prostate to become refractory to androgen ablation. (A) To assess the competency of Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} prostatic stromal cells for TGF-β signaling Smad2 was localized by Alexa Fluor 594 (red) immunofluorescence staining following TGF-β treatment. Hoechst nuclear counter stain was used (blue). Colocalization of Smad2 with Hoechst indicates active TGF-β signaling, whereas disparate staining indicates a lack of TGF-β signaling. (B) H&E staining of prostatic glandular structures in tissue recombination grafts of Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} stromal cells with epithelial organoids were allografted for six weeks in intact (2 upper panels) syngenic host male mice (+ Androgen) (n=8) or castrated (2 lower panels) for the last week of grafting in syngenic host male mice (- Androgen) (n=8). "Kd" indicates kidney tissue. The black bar indicates 100uM. (C) TUNEL staining (indicated with black arrows) in Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} tissue recombination grafts from castrated mice is shown (n=4). The level of TUNEL positive Tgfbr2^{fspKO}–associated epithelia after castration was significantly lower than Tgfbr2$^{floxE2/floxE2}$–associated epithelia (P value = 0.0249). The black bar indicates 50 uM. (D) Ki67 staining (black arrows) indicate proliferative cells of Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} allografts from castrated mice (n=4). Open arrowheads indicate background staining of likely dead cells and "Kd" indicates kidney tissue. The level of Ki67 positive Tgfbr2^{fspKO}–associated epithelia after castration was significantly greater than Tgfbr2$^{floxE2/floxE2}$– associated epithelia (P value = 0.0030). The black bar indicates 50 uM. Figure taken from (Placencio, Sharif-Afshar et al. 2008).

epithelial organoids isolated by digesting mature wild type C57BL/6 mouse prostates. The tissue recombinants were allografted to the sub-renal capsules of syngeneic male mice (Voeller, Truica et al. 1998). Five weeks following grafting, the Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} stromal cells organized themselves around the epithelial organoids to form prostatic glands (Figure 11B). Seven days following castration of the hosts there was a 6-fold increase in TUNEL-positive epithelia in the Tgfbr2$^{floxE2/floxE2}$ stroma-associated glands (Figure 11C). Note, in comparison, the Tgfbr2^{fspKO} stroma-associated glands did not show a significant increase in TUNEL-positive epithelia. Ki67 staining indicated minimal proliferation in recombinants associated with Tgfbr2$^{floxE2/floxE2}$ stroma following castration (Figure 11D). In contrast, the recombinants associated with Tgfbr2^{fspKO} stroma had greater epithelial proliferation following castration. Collectively, castration of the host mice resulted in regression of the allografted tissue recombinants associated with Tgfbr2$^{floxE2/floxE2}$, but not Tgfbr2^{fspKO} stromal cells. These observations further supported that the androgen ablation refractile phenotype of our Tgfbr2^{fspKO} mice resulted from interactions within the prostate microenvironment.

Proximal ductal epithelial activation of Wnt signaling following androgen ablation mediates stromal-epithelial crosstalk

The next step was to determine how TGF-β signaling within the stroma was responsible for the observed regression in the Tgfbr2$^{floxE2/floxE2}$, or lack thereof in the Tgfbr2^{fspKO}, mouse prostates. The proximal ducts of the prostate

remain viable as the distal ducts regress in the absence of androgen signaling. Up-regulation of Wnt ligands and activating mutations of β-catenin in prostate cancer epithelium is a potential mechanism for androgen refractory prostatic epithelial proliferation (Truica, Byers et al. 2000; Chen, Shukeir et al. 2004; Galli, Barnes et al. 2006). To address the potential of a Wnt signaling mechanism to support the androgen independent prostate survival phenotype observed, we developed a Tgfbr2^{fspKO}/TOPGal mouse model (conditional stromal Tgfbr2 knockout with a canonical Wnt signaling reporter). Intact and castrated male Tgfbr2$^{floxE2/floxE2}$/TOPGal and Tgfbr2^{fspKO}/TOPGal prostates were subjected to whole mount β-galactosidase staining to visualize canonical Wnt activity. In whole mount staining of prostates from intact Tgfbr2$^{floxE2/floxE2}$/TOPGal mice, β-galactosidase expression was not detected (Figure 12A). However, three days after castration β-galactosidase activity was detected exclusively in the proximal ducts of the prostate indicating activated β-catenin signaling. This was further supported by elevated immuno-localization for β-galactosidase expression in the proximal glands of Tgfbr2$^{floxE2/floxE2}$/TOPGal prostates following castration (Figure 13). By the seventh day following castration, little β-galactosidase activity was detected in Tgfbr2$^{floxE2/floxE2}$/TOPGal prostates (data not shown). In contrast to that observed in Tgfbr2$^{floxE2/floxE2}$/TOPGal prostates, Figure 12A showed that the Tgfbr2^{fspKO}/TOPGal prostates had β-galactosidase expression in the entire gland before castration with further elevated expression following castration. In both Tgfbr2$^{floxE2/floxE2}$/TOPGal and Tgfbr2^{fspKO}/TOPGal prostates, only the epithelial compartment showed strong positive β-galactosidase activity (Figure 12B, C).
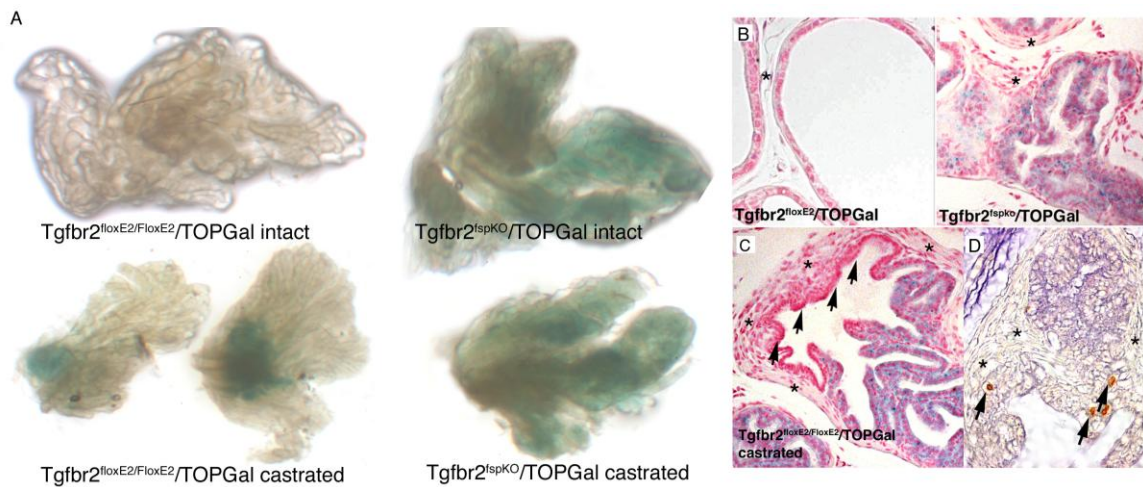
Figure 12. Inhibiting TGF-β signaling in the prostatic stroma results in constitutive Wnt signaling throughout the prostatic epithelia associated with survival following androgen ablation. Six week old TOPGal mice were stained for β-galactosidase activity and anterior and dorsolateral prostate lobes from intact and three day castrated mice were analyzed. (A) Tgfbr2floxE2/floxE2/TOPGal and Tgfbr2fspKO/TOPGal prostates from intact and three days following castration were stained for β-galactosidase activity (blue) and imaged as whole mounts (n=8) to show areas of canonical Wnt signaling activity. (B) Paraffin sections of the β-galactosidase stained intact Tgfbr2floxE2/floxE2/TOPGal and Tgfbr2fspKO/TOPGal distal prostates were counter stained with Nuclear Fast Red. Asterisks indicate the stromal compartment. (Prostates from intact and castrated Tgfbr2fspKO/TOPGal mice were similar. Only the intact Tgfbr2fspKO/TOPGal tissue section is shown.) (C) Three days after castration, paraffin sections of the β-galactosidase stained Tgfbr2floxE2/floxE2/TOPGal prostate was counter stained with Nuclear Fast Red. Tissue section shows the distal prostate as it is regressing toward the proximal area. Asterisks indicate the stromal compartment. (D) Following castration of control Tgfbr2floxE2/floxE2/TOPGal mice, the β-galactosidase activity stained prostate sections were co-stained for TUNEL (brown) indicated with black arrows and β-galactosidase expression (blue) (n=4). Tissue section shows the distal prostate as it is regressing toward the proximal area. Asterisks indicate the stromal compartment and black arrows indicate the epithelia with no β-galactosidase activity following castration (n = 8). Figure taken from (Placencio, Sharif-Afshar et al. 2008).
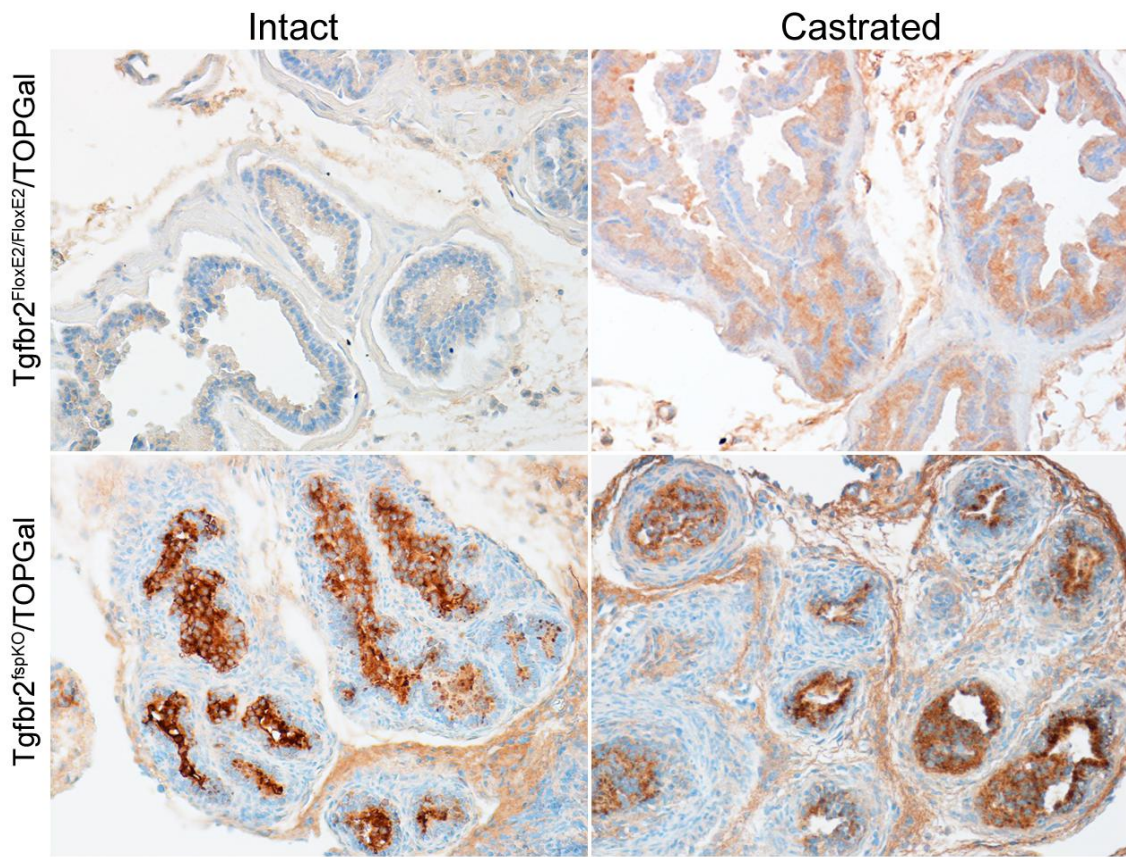
Figure 13. Wnt signal activity in the prostatic proximal ducts. Tgfbr2^floxE2/floxE2^/TOPGal and Tgfbr2^fspKO^/TOPGal prostates were sectioned and stained for β-galactosidase using immunohistochemistry. Note, the increase in β-galactosidase staining in the proximal ducts after castration in both the Tgfbr2^floxE2/floxE2^/TOPGal and Tgfbr2^fspKO^/TOPGal glandular epithelia. Scale bare indicates 25 μM. Figure taken from (Placencio, Sharif-Afshar et al. 2008).

Co-staining for TUNEL and β-galactosidase in Tgfbr2$^{floxE2/floxE2}$/TOPGal flox prostate glands, four days following castration, illustrated that the epithelia expressing β-galactosidase did not overlap with epithelia undergoing apoptosis (Figure 12D). This finding suggested that the epithelia in the proximal glands supported canonical Wnt activity during normal prostatic regression following androgen ablation. In addition, the hormone refractory Tgfbr2^{fspKO} prostates supported constitutive paracrine canonical Wnt signaling. The coincident localization of canonical Wnt signaling in Tgfbr2$^{floxE2/floxE2}$/TOPGal prostates and the reported region surviving androgen ablation supported the possibility of a causal relationship between the two events.

To determine if the observed epithelial Wnt activity could be due to stromal regulation of Wnt ligand production, Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} cultured prostatic stromal cells were treated with an androgen receptor antagonist, bicalutamide. The expression of Wnt ligands was measured following androgen ablation over a time course of five days by semi-quantitative RT-PCR. There was a transient increase in Wnt-2, Wnt-3a and Wnt-5a one and three days following bicalutamide treatment of Tgfbr2$^{floxE2/floxE2}$ stromal cells, with a decrease in expression by five days (Figure 14A). A similar trend was seen with Tgfbr2^{fspKO} stromal cells, but with elevated basal expression for Wnt-2, Wnt-3a and Wnt-5a. Wnt-9a and Wnt-11 expression were unchanged in both stromal cell types under the same conditions. When Tgfbr2$^{floxE2/floxE2}$ stromal cells were treated with bicalutamide and TGF-β there was a lack of Wnt expression compared to treatment with bicalutamide alone (data not shown). Thus, inhibiting either the
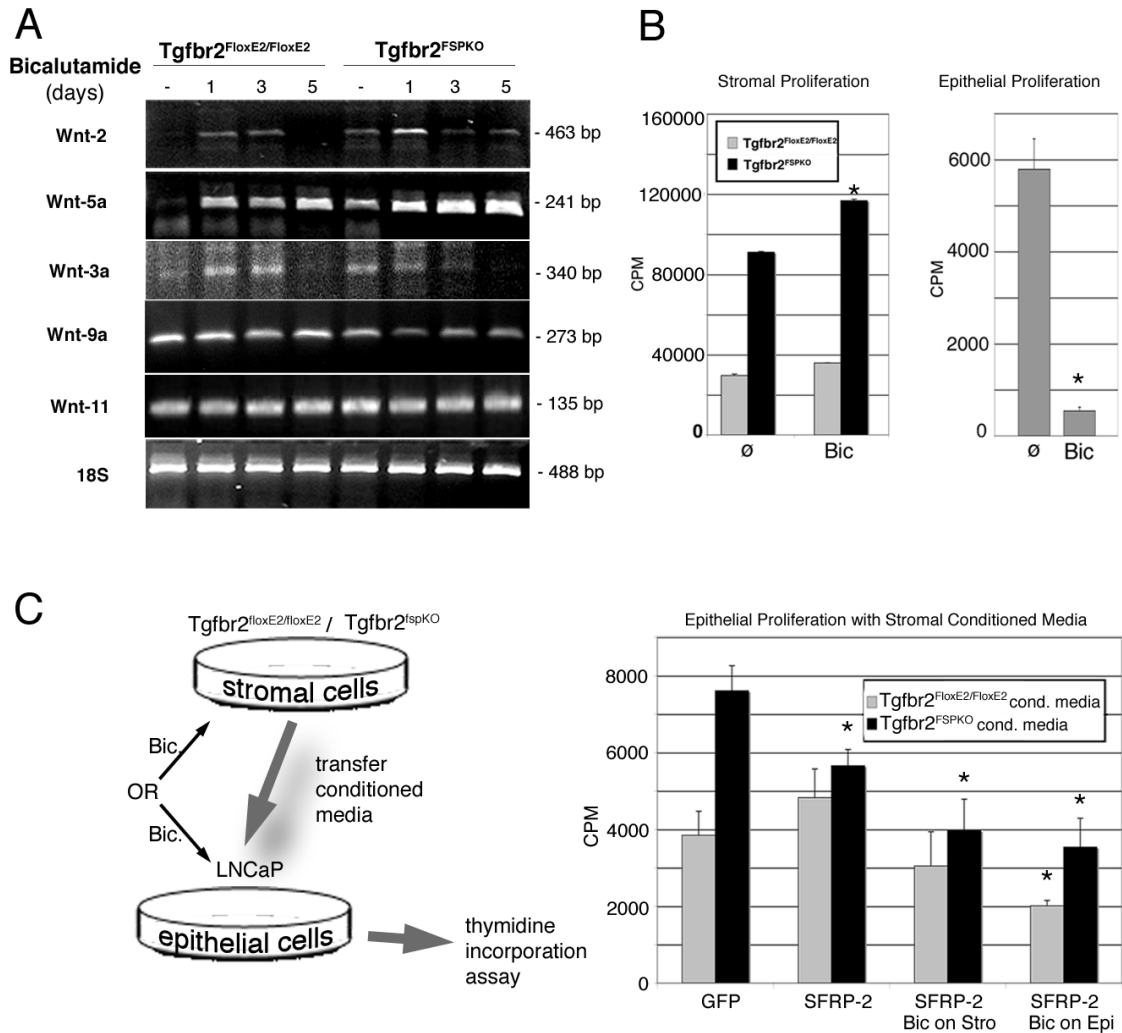
Figure 14. Wnt signaling induced by androgen ablation of stromal cells affects epithelial proliferation. (A) Tgfbr2[floxE2/floxE2] and Tgfbr2[fspKO] stromal cells were treated with bicalutamide over five days to analyze Wnt gene expression by semi-quantitative RT-PCR. The expression of 18S ribosomal RNA was used as a loading control. The results shown are representative of three or more sets of RNA used to test each Wnt gene. (B) ^{3}H-thymidine incorporation assays were performed on Tgfbr2[floxE2/floxE2] and Tgfbr2[fspKO] stromal cells transduced with GFP-adenovirus and grown either in media containing testosterone or bicalutamide (Bic) in the absence of testosterone. ^{3}H-thymidine incorporation assays were performed on LNCaP epithelial cells also treated with or without bicalutamide for five days. Each bar represents the average of three or more replicates and error bars indicate standard deviation. Asterisks indicate significance compared to the respective control. GFP- Tgfbr2[floxE2/floxE2] stromal cells were not significantly affected by bicalutamide treatment compared to control GFP- Tgfbr2[floxE2/floxE2] stromal cells (P value = 0.0580). GFP-Tgfbr2[fspKO] stromal cells had a significant increase in proliferation with bicalutamide treatment compared to control GFP-Tgfbr2[fspKO]stromal cells (P value = 0.0016). LNCaP epithelial cells treated with

bicalutamide (Bic) proliferated significantly faster than untreated LNCaP cells (P value = 0.0025). Data is representative of three or more individual experiments. (C) On the left, a cartoon of the generation of conditioned media is shown. Conditioned media was collected from Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} stromal cells and used to treat the target LNCaP epithelial cells. To the right, bar graphs show the results of these conditioned media experiments. Androgen responsive proliferation of LNCaP cells was measured in response to a 120 hour treatment with conditioned stromal cell media from Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} cells by ^{3}H-thymidine incorporation assays. Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} stromal cells were transduced either with GFP adenovirus as a control or Wnt antagonist SFRP-2 adenovirus prior to starting collection of conditioned media. 'Bic on Stro' indicates that bicalutamide was added to stromal cell cultures during the generation of conditioned media. 'Bic on Epi' indicates that bicalutamide was added to epithelial LNCaP cells simultaneously with stromal conditioned media treatment. Each bar represents the average of three or more replicates and error bars indicate standard deviation. Compared to the GFP control, P values for SFRP-2- Tgfbr2$^{floxE2/floxE2}$ conditions are 0.207, 0.339, and 0.011, and for SFRP-2-Tgfbr2^{fspKO} conditions 0.002, 0.005, and 0.004, respectively. Each asterisk indicates statistical significance between the control and knockout data points for a given condition with a P value < 0.05 according to a Student's t test evaluation. Figure taken from (Placencio, Sharif-Afshar et al. 2008).

androgen or the TGF-β signaling pathway could induce Wnt-2, Wnt-3a, and Wnt-5a gene expression in prostatic stromal cells.

To determine the role of paracrine Wnt signaling on the epithelial response to androgen ablation, the proliferation of prostatic epithelial cells was measured in the presence of conditioned media collected from either Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} stromal cell cultures. As seen in Figure 14B, bicalutamide treatment had little proliferative effect on either stromal cell type grown in culture, as measured by ^{3}H-thymidine incorporation assays. As anticipated, the Tgfbr2^{fspKO} stromal cells were notably more proliferative than the Tgfbr2$^{floxE2/floxE2}$ stromal cells. Bicalutamide treatment decreased the proliferation of the androgen responsive prostate cancer line, LNCaP epithelial cells, as expected. Therefore, LNCaP cells were subsequently used as the target epithelia to assess proliferative responsiveness to conditioned stromal media treatment and androgen ablation. Control green fluorescent protein (GFP)-transduced Tgfbr2$^{floxE2/floxE2}$-conditioned media had lower proliferative response on LNCaP cells compared to the enhanced proliferation using the GFP-transduced Tgfbr2^{fspKO}-conditioned media (Figure 14C and Figure 15). Wnt expression by the stroma was antagonized through adenoviral expression of SFRP-2 to stromal cell cultures. The SFRP-2 transduced Tgfbr2^{fspKO}-conditioned media significantly decreased proliferation of LNCaP cells, compared to GFP-transduced Tgfbr2^{fspKO}-conditioned media. Bicalutamide treatment to the Tgfbr2^{fspKO} stroma during generation of conditioned media (Bic on Stro) further decreased LNCaP proliferation, demonstrating the paracrine effect of androgen ablation on epithelial

LNCaP Epithelial Proliferation with GFP-Stromal Conditioned Media

GFP–Tgfbr2$^{floxE2/FloxE2}$ cond. media
GFP–Tgfbr2^{fspKO} cond. media
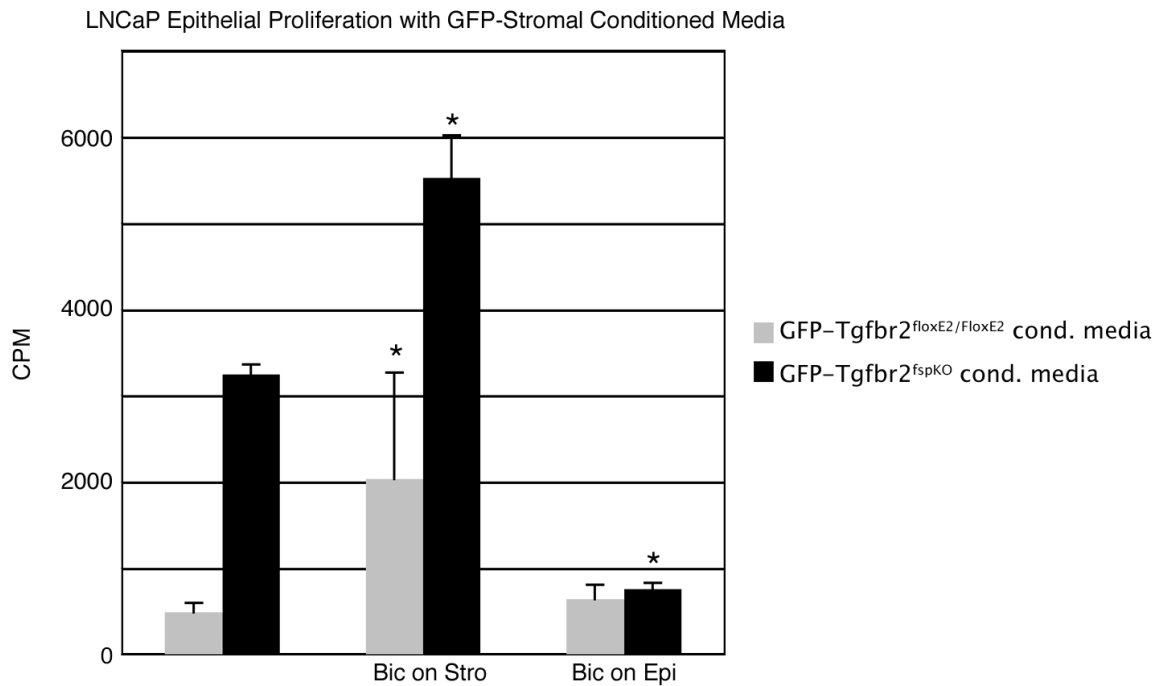
Figure 15. Bicalutamide treatment directly to prostatic stromal cells leads to a paracrine mediated increase in epithelial proliferation whereas bicalutamide treatment to the epithelia directly causes a decrease in proliferation. ^{3}H-thymidine incorporation assays were performed on LNCaP epithelial cells also treated with or without bicalutamide for five days. Each bar represents the average of three or more replicates and error bars indicate standard deviation. Asterisks indicate significance compared to the respective control. GFP-Tgfbr2$^{floxE2/floxE2}$ and GFP-Tgfbr2^{fspKO} stromal cells treated with bicalutamide generated 'Bic on Stro' conditioned media (see figure 14) Figure taken from (Placencio, Sharif-Afshar et al. 2008).

cells. However, most dramatic was the direct effect of androgen ablation on the epithelia with SFRP-2 transduced Tgfbr2^{fspKO}-conditioned stromal media (Bic on Epi), which resulted in a 50% decrease in LNCaP proliferation compared to the GFP-transduced Tgfbr2^{fspKO}-conditioned stromal media control. This suggested that Wnt ligands expressed by both Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} stromal cells support epithelial survival in response to androgen antagonism.

Next, the role of TGF-β on Wnt expression during androgen ablation in mice was determined. Previously, SFRP-2 has been shown to inhibit Wnt-3a, and Wnt-5a in vivo (Hou, Tan et al. 2004; Liu, Kirschenbaum et al. 2007). Based on SFRP-2 mediated inhibition of prostatic epithelial cell proliferation in vitro, we hypothesized that it may restore androgen dependence to the Tgfbr2^{fspKO} prostate. The fragile condition of Tgfbr2^{fspKO} mice did not allow expression of SFRP-2 in these mice directly. Therefore, we transduced control GFP or SFRP-2 adenovirus in intact Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} prostate lobes and subsequently allografted the tissues under the renal capsules of SCID mice in tissue rescue experiments. GFP staining confirmed successful transduction of the allografted tissues (Figure 16). Three days following allografting the host mice were either left intact or castrated for an additional three days. There were no observable differences in apoptotic rate resulting from the expression of GFP or SFRP-2 on Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} rescued prostates in the intact host mice, based on TUNEL staining (Figure 17). Following castration of the host mice, the Tgfbr2$^{floxE2/floxE2}$ prostates had the expected elevation of TUNEL staining in both GFP and SFRP-2 transduced prostates (Figure 18 A, B). The

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Tissue Rescue Allografts Showing GFP Staining

GFP SFRP-2
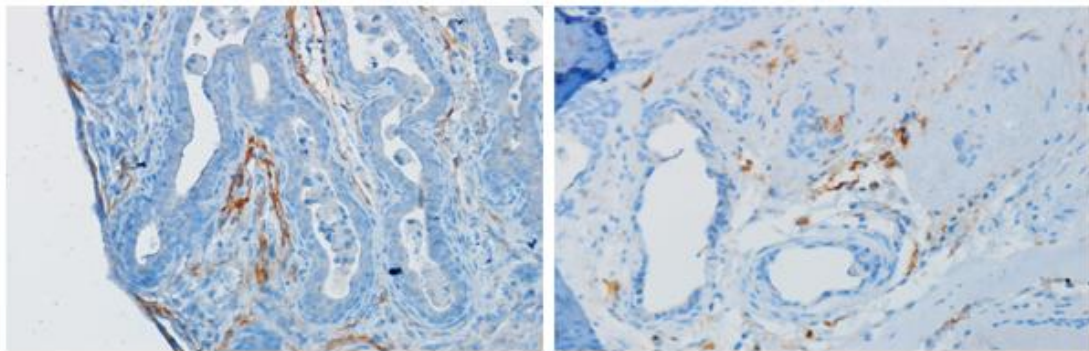
Figure 16. GFP staining indicates successful adenovirus allograft transduction. GFP immunohistochemistry staining is positive in both GFP and SFRP2 tissue rescue allografts (see Figure 18). Figure taken from (Placencio, Sharif-Afshar et al. 2008).

Intact Tissue Rescue Allografts
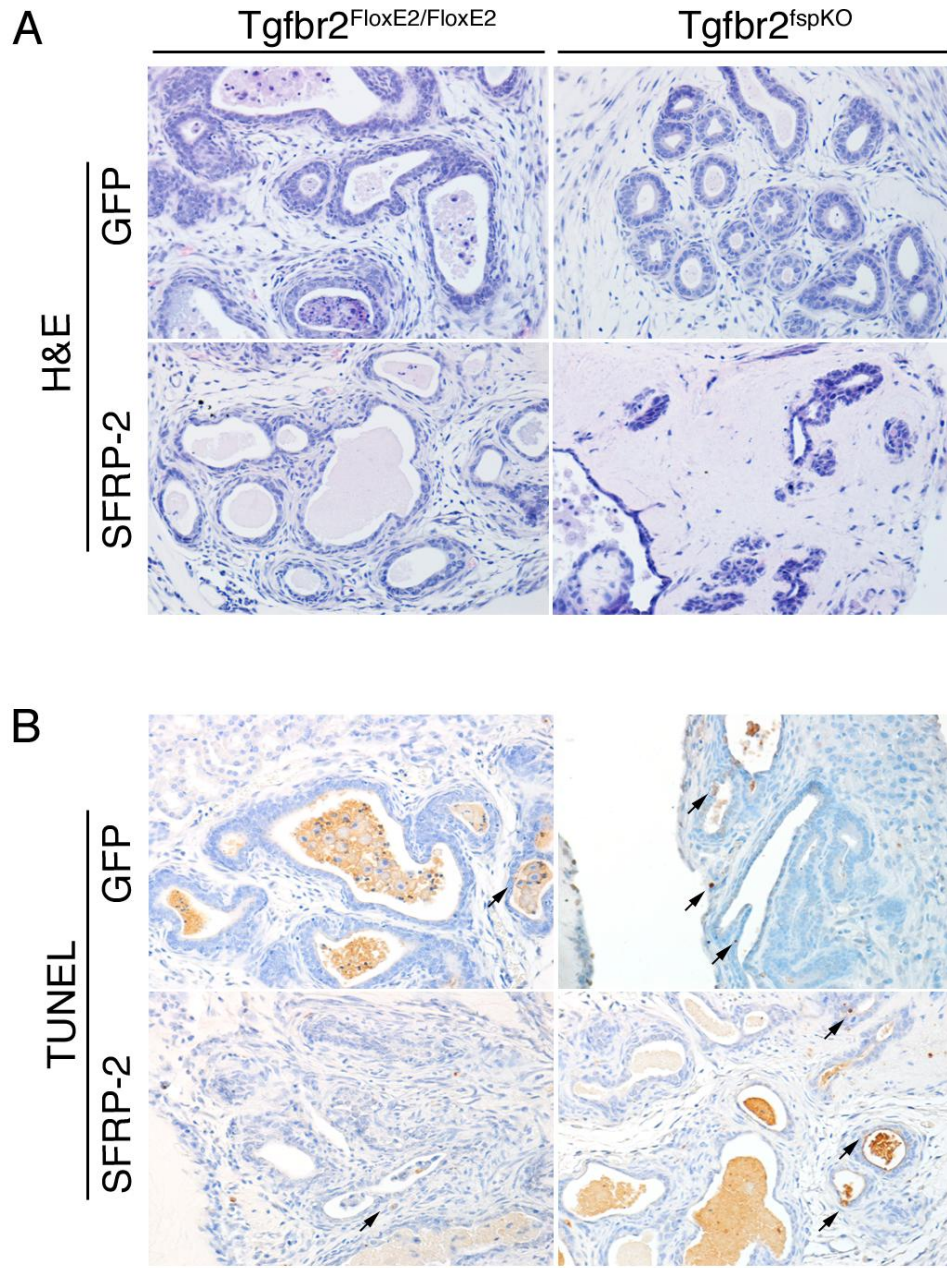
Figure 17. Inhibition of Wnt signaling does not alter prostate phenotypes of intact Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} mice. (A) Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} mature mouse prostates were transduced with GFP or SFRP2 adenovirus and allografted in the renal capsules of male SCID mice. Tissues were harvested on day six (n=12) and subjected to H&E staining. (B) Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} mature mouse prostates were transduced with GFP or SFRP2 adenovirus and allografted in the renal capsules of male SCID mice. Tissues were harvested on day six (n=12) and subjected to TUNEL staining for apoptotic cells (brown). Scale bar indicates 25µM. Figure taken from (Placencio, Sharif-Afshar et al. 2008).
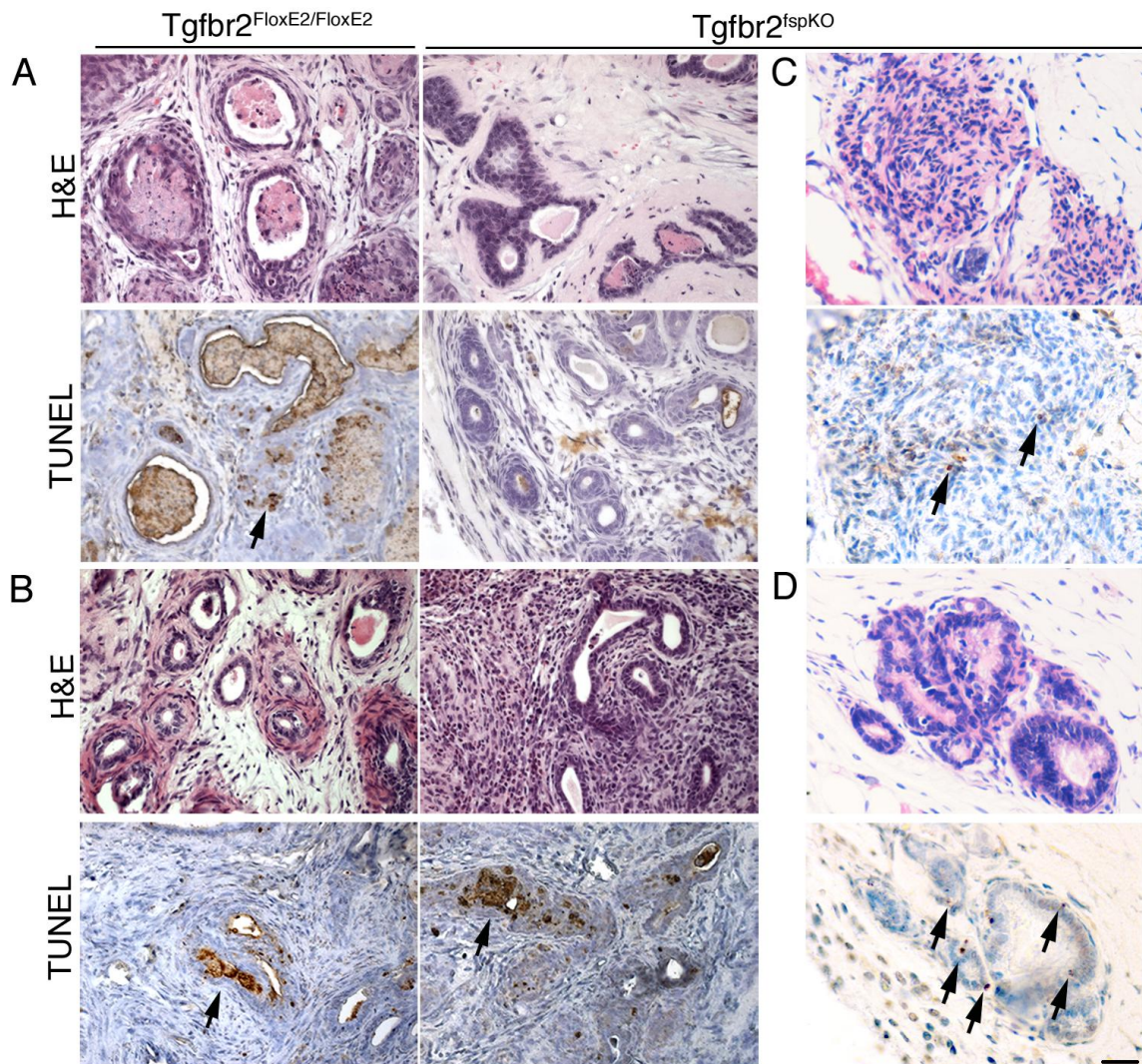
Figure 18. Inhibition of Wnt signaling restores Tgfbr2^{fspKO} prostate responsiveness to androgen ablation. (A) Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} mature mouse prostates were transduced with GFP adenovirus and allografted in the renal capsules of male SCID mice. Host mice were castrated for three days. Tissues were harvested on day six (n=12) and subjected to H&E staining (upper panels) as well as TUNEL staining (lower panels) for apoptotic cells (brown). (B) Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} mature mouse prostates were transduced with SFRP-2 adenovirus and allografted in the renal capsules of male SCID mice. Host mice were castrated for three days. Tissues were harvested on day six (n=12) and subjected to H&E staining (upper panels) as well as TUNEL staining (lower panels) for apoptotic cells (brown). Percent positive epithelial TUNEL positive staining was not statistically different between GFP-Tgfbr2$^{floxE2/floxE2}$ and SFRP-2-Tgfbr2$^{floxE2/floxE2}$ allografts (P value = 0.2819). Percent positive epithelial TUNEL positive staining in SFRP-2- Tgfbr2^{fspKO} allografts was statistically greater than GFP-Tgfbr2^{fspKO} allografts (P value = 0.0373). (C) Tissue recombinations of 12T7f LADY epithelial organoids and Tgfbr2^{fspKO} prostatic stromal cells were

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allografted in SCID mice for six weeks. The host mice were given GFP adenovirus throughout the grafting period. Host mice were castrated seven days prior to harvesting the prostatic grafts. Tissue recombinants were harvested at week six (n=4) and subjected to H&E staining (upper panel) as well as TUNEL staining (lower panel) for apoptotic cells (brown). (D) Tissue recombinations of 12T7f LADY epithelial organoids and Tgfbr2^{fspKO} prostatic stromal cells were allografted in SCID mice for six weeks. The host mice were given SFRP-2 adenovirus throughout the grafting period. Host mice were castrated seven days prior to harvesting the prostatic grafts. Tissue recombinants were harvested at week six (n=4) and subjected to H&E staining (upper panel) as well as TUNEL staining (lower panel) for apoptotic cells (brown). Percent positive epithelial TUNEL positive staining in tissue recombinations of 12T7f LADY epithelial organoids and SFRP-2-Tgfbr2^{fspKO} allografts was statistically greater than those in tissue recombinations of 12T7f LADY epithelial organoids and GFP-Tgfbr2^{fspKO} allografts (P value = 0.0472). Scale bar indicates 25 μm. Figure taken from (Placencio, Sharif-Afshar et al. 2008).

expression of GFP with Tgfbr2$^{\text{fspKO}}$ prostates recapitulated the original observation of refractivity to androgen ablation. TUNEL-positive epithelia in GFP-Tgfbr2$^{\text{floxE2/floxE2}}$ prostates, three days following castration, were significantly elevated compared to GFP-Tgfbr2$^{\text{fspKO}}$ prostates. Note, in comparison, the expression of SFRP-2 resulted in a three-fold increase in the apoptosis of Tgfbr2$^{\text{fspKO}}$ prostates following castration similar to Tgfbr2$^{\text{floxE2/floxE2}}$ prostates. The restoration of prostate regression in SFRP-2-Tgfbr2$^{\text{fspKO}}$ prostates following castration indicated cooperation among the TGF-β, Wnt, and androgen signaling pathways.

Stromal TGF-β signaling allows prostatic ductal regression after androgen ablation in prostate cancer

Next, the role of stromal TGF-β signaling in a prostate cancer model system was examined. Our objective was to determine if the paracrine instruction from the stroma to the epithelia observed in the development of androgen refractory PIN lesions continued to have an androgen refractory influence in the progression from PIN to adenocarcinoma. This was achieved by allografting tissue recombinants consisting of Tgfbr2$^{\text{floxE2/floxE2}}$ or Tgfbr2$^{\text{fspKO}}$ stromal cells with prostate epithelial organoids from a prostate cancer mouse model expressing the SV40 Large T Antigen in the epithelia, 12T7f LADY (Kasper, Sheppard et al. 1998). Host male SCID mice were treated weekly with either SFRP-2 or GFP control adenovirus. Five weeks following allografting, half of the host mice were castrated. All grafts were analyzed six weeks following grafting. The GFP-

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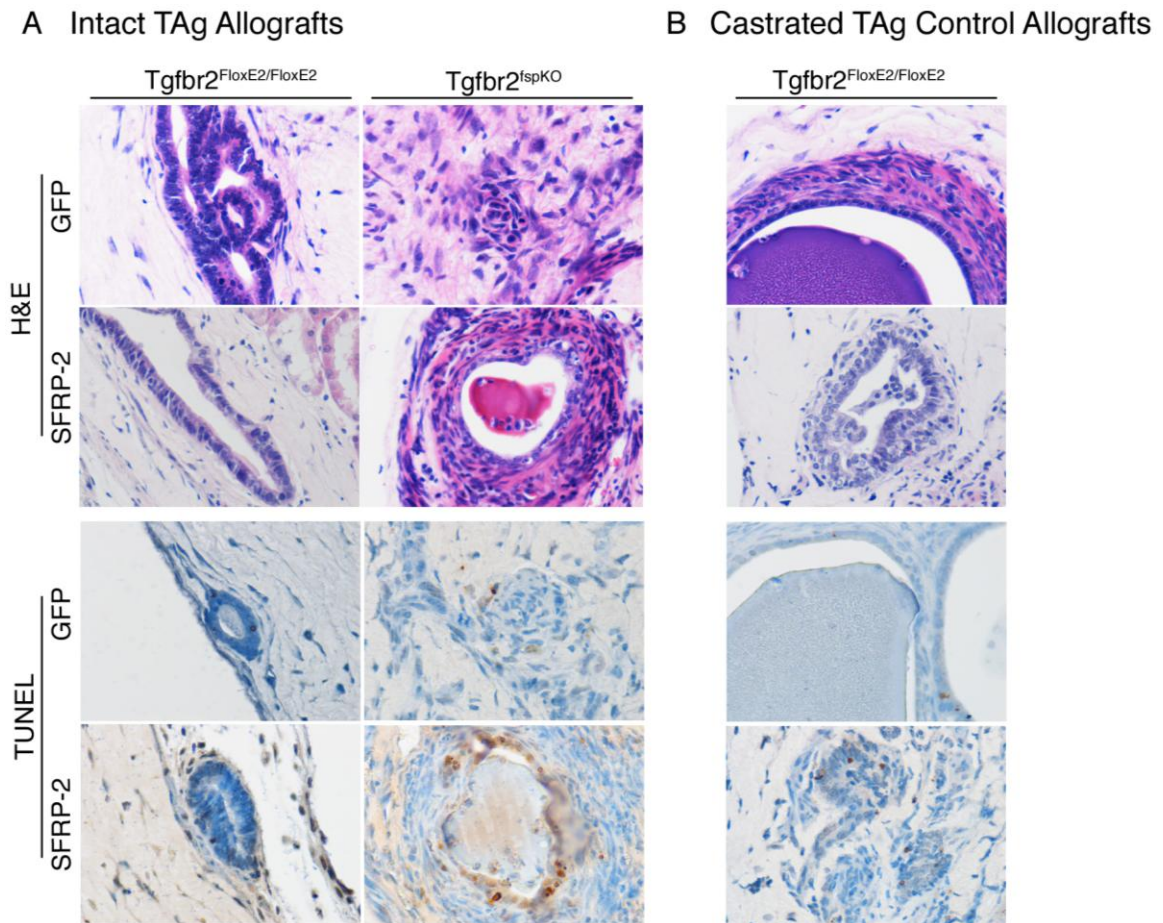
Figure 19. Inhibition of Wnt signaling in Tgfbr2$^{floxE2/floxE2}$- and Tgfbr2^{fspKO}-stroma allografts with LADY Tag organoids. (A) Tissue recombinations of 12T7f LADY epithelial organoids and Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} prostatic stromal cells were allografted in SCID mice for six weeks. The host mice were given GFP adenovirus or SFRP2 throughout the grafting period. Tissue recombinants were harvested at week six (n=4) and subjected to H&E staining (upper panels) as well as TUNEL staining (lower panels) for apoptotic cells (brown). (B) Tissue recombinations of 12T7f LADY epithelial organoids and Tgfbr2^{fspKO} prostatic stromal cells were allografted in SCID mice for six weeks. The host mice were given GFP or SFRP2 adenovirus throughout the grafting period. Tissue recombinants were harvested at week six (n=4) and subjected to H&Estaining (upper panels) as well as TUNEL staining (lower panels) for appropriate cells (brown). Figure taken from (Placencio, Sharif-Afshar et al. 2008).
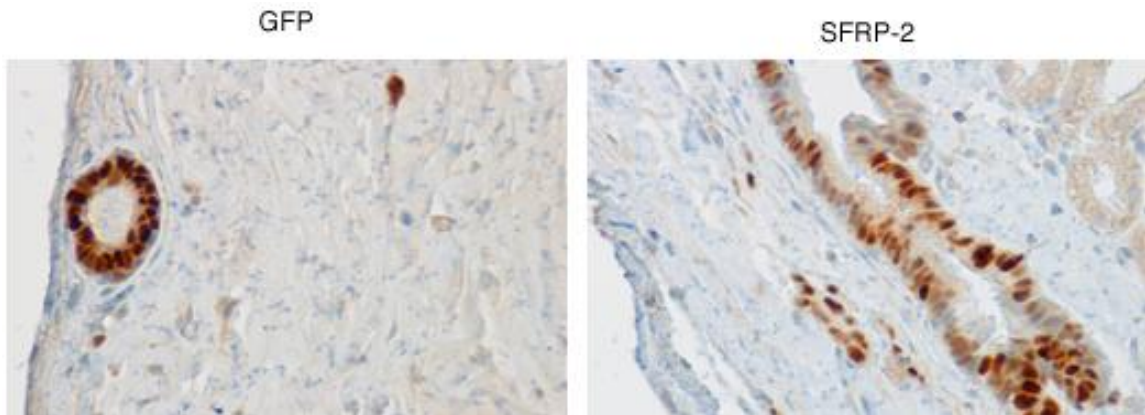
Figure 20. SV40 positive epithelia within tissue recombination allografts.
SV40 T antigen staining localizes LADY epithelia within the tissue recombination allografts using Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} prostatic stromal cells. Shown are representative prostate glands from GFP or SFRP2-Tgfbr2$^{floxE2/floxE2}$ allografts. Tgfbr2^{fspKO} allografts had similar epithelial staining (data not shown). Figure taken from (Placencio, Sharif-Afshar et al. 2008).

Tgfbr2$^{floxE2/floxE2}$ stroma grafts (with LADY organoids) formed prostatic ductal structures with minimal hyperplasia (Figure 19A, 19B, and 20). Following castration, the GFP- Tgfbr2$^{floxE2/floxE2}$ stroma-associated grafts had elevated TUNEL-positive apoptotic epithelia. In contrast, GFP-Tgfbr2^{fspKO} stroma-associated grafts developed adenocarcinoma (Figure 18C, upper panel). The GFP-Tgfbr2^{fspKO} stroma-associated grafts had low levels of apoptosis (Figure 18C, lower panel). SFRP-2-Tgfbr2$^{floxE2/floxE2}$ and GFP-Tgfbr2$^{floxE2/floxE2}$ stroma-associated grafts were similar in regard to apoptotic response to androgen ablation. Notably, the SFRP-2-Tgfbr2^{fspKO} stroma-associated grafts had elevated levels of TUNEL-positive epithelia following castration, typical of normal prostate regression (Figure 18D lower panel). We also found that the SFRP-2-Tgfbr2^{fspKO} stroma-associated grafts appeared more differentiated than analogous GFP-Tgfbr2^{fspKO} stroma-associated grafts. Thus, stromal androgen and TGF-β signaling cooperates with epithelial Wnt signaling to regulate responsiveness to androgen ablation in benign, pre-neoplastic, and adenocarcinoma of the prostate. These results are summarized (Figure 21).

Discussion

Stromal responsiveness to TGF-β allows androgen sensitivity in the prostate epithelia through paracrine Wnt signaling. The direct role of TGF-β signaling on the prostate epithelia following castration was minimal based on histologic and apoptotic differences between the Tgfbr2$^{NKX3.1KO}$ and

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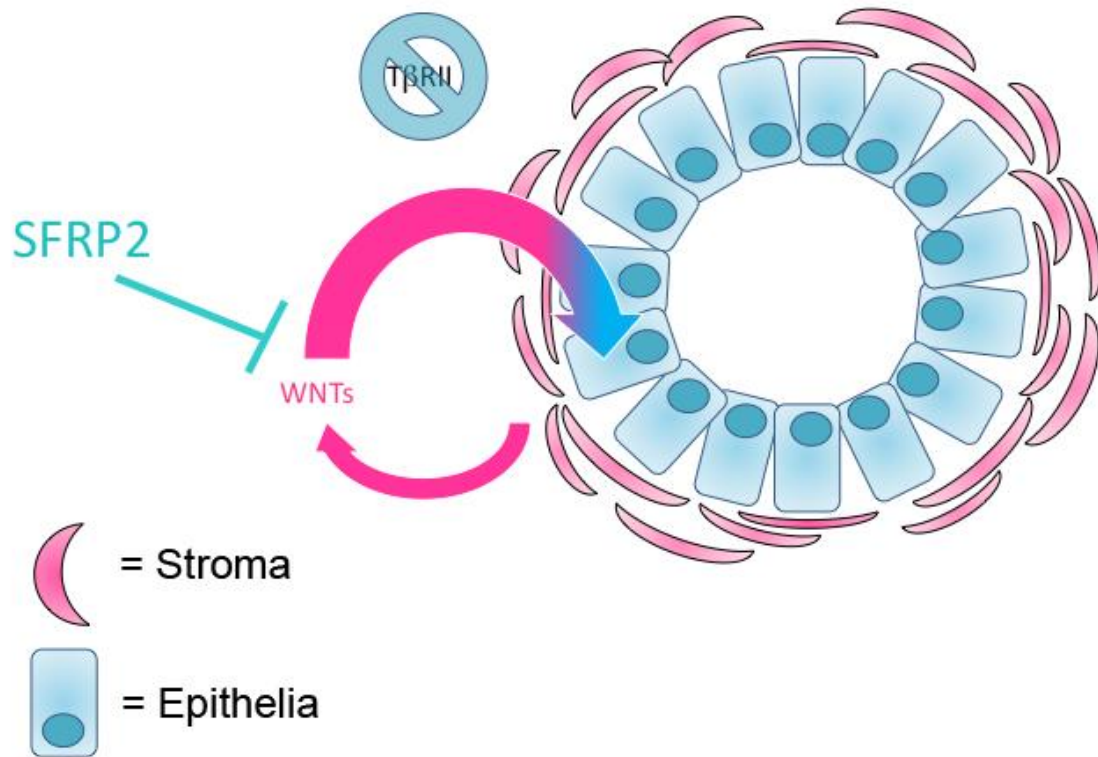
Figure 21. Summary diagram depicting that the loss of stromal TGF-β signaling influences the development of CRPC. A representative prostate gland is depicted. The epithelia (blue rounded rectangles) comprise the prostate ducts, which are surrounded by the stroma (pink half moons). The diagram depicts that the loss of the TGF-β type II receptor in the stroma leads to production of Wnt ligands (pink arrow). Through paracrine signaling, these Wnts in the stroma signal to the epithelia (pink to blue gradient arrow) resulting in castrate resistance. Inhibition of these Wnts with SFRP2 restored the castrate responsiveness of the epithelia. Summarized from (Placencio, Sharif-Afshar et al. 2008).

Tgfbr2$^{floxE2/floxE2}$ prostates. It is possible that compensatory signaling by Activin and its cognate receptors may provide Smad protein activity similar to TGF-β. However, the conditional knockout of the TGF-β type II receptor in fibroblasts, in the Tgfbr2^{fspKO} mice and tissue recombinants, indicated the importance of TGF-β responsiveness in the stromal compartment following castration. Further crossing of the Tgfbr2^{fspKO} mice to the TOPGal reporter gene mouse model demonstrated the regulatory role of stromal TGF-β signaling on Wnt signaling in epithelial cells refractory to androgen ablation. The Tgfbr2^{fspKO} stromal cells themselves acquired a more proliferative phenotype, presumably due to the loss of the growth inhibitory TGF-β signaling, and promoted nearby epithelia to increase their rate of proliferation and overcome hormonal dependence. The mechanisms behind the observed phenomena highlight a stromal derived paracrine Wnt signaling axis that is triggered upon androgen ablation. The stark proximal ductal localization of canonical Wnt signaling activity in control prostates following castration indicated duplicity of responses to androgen ablation, one of survival and another for cell death. Thus, the data indicate that the role for TGF-β signaling following androgen ablation is to suppress Wnt signals in much of the distal prostate to enable regression.

The stem cell niche is regulated by TGF-β and Wnt signaling. Prostate regression-regeneration cycles surpassing 30 cycles in a single mouse suggest the presence of prostate epithelial stem cells (Isaacs 1987). Prostate stem cells are not well defined, but studies have focused on identifying their location within the basal cells in the proximal ducts (De Marzo, Nelson et al. 1998; Zhou,

Flesken-Nikitin et al. 2007). Our studies also indirectly suggest the presence of a prostate stem cell niche in the proximal ductal epithelia (Figure 12). The lack of β-gal staining in Tgfbr2$^{floxE2/floxE2}$ prostates compared to widespread expression in Tgfbr2^{fspKO}/TOPGal prostates indicates that stromal TGF-β signaling regulates the spatial localization of canonical Wnt signaling. Of greater significance in relation to the stem cell niche is revealed after castration. Three days following castration Tgfbr2$^{floxE2/floxE2}$/TOPGal prostates exhibit transient β-gal expression in the proximal ductal epithelia compared to widespread expression in Tgfbr2^{fspKO}/TOPGal prostates. Based on known information, these studies reveal the cooperation of TGF-β, androgen, and Wnt signaling in regulating the prostate stem cell niche. Characteristics to suggest that the proximal epithelial ducts contain a stem cell population include having a high in vitro proliferative potential, are slow cycling, and can reconstitute highly branched glandular ductal structures in collagen (Reya, Morrison et al. 2001; Tsujimura, Koikawa et al. 2002). Using NRP-152 rat epithelial cells in tissue recombination studies, both basal and luminal epithelial cells were shown to be derived from a common progenitor (Hayward, Haughney et al. 1999). These studies suggested it was due to cells within the basal population that were capable of differentiating into both cell types. The proximal ducts are known to be surrounded by a thick layer of smooth muscle cells rich in TGF-β1 (Nemeth, Sensibar et al. 1997). This TGF-β gradient is thought to help maintain the stem cell niche by inhibiting proliferation. Furthermore, expression of a dominant negative Tgfbr2 in the prostate caused increased proliferation of the proximal ductal epithelia (Kundu, Kim et al.

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2000).The protective effects within the stem cell niche by TGF-β was shown in a castration study. TGF-β receptor levels transiently increased following castration, with a peak at four days and diminished by 14 days (Kyprianou and Isaacs 1988). Prostate regression is greatest between three to seven days following castration and lessens by 14 days and thereafter. However, even after full regression at two months the prostate does not ever diminish completely, but remains as a small bud of proximal ductal epithelia thought to encompass the stem cells. This suggests that TGF-β was essential to maintain the stem cell niche with an increased need during regression. A gene profiling study revealed TGF-β signaling in maintaining stem cells with mouse embryonic and adult prostates (Blum, Gupta et al. 2009). In addition, embryonic stem cells relied on canonical Wnt signaling associated with proliferation and self-renewal, whereas adult stem cells used the non-canonical Wnt/Ca+ signaling pathway to maintain quiescence. Another study suggested that Wnt signaling regulates self-renewal of prostate cancer cells with stem cell characteristics independent of androgen receptor activity (Bisson and Prowse 2009). In particular, Wnt-3a increased self-renewal and prostasphere size in LNCaP and C4-2B cells. These studies in combination with our Tgfbr2$^{floxE2/floxE2}$/TOPGal and Tgfbr2$^{floxE2/floxE2}$/TOPGal studies indicate TGF-β as a master regulator in inhibiting widespread canonical Wnt signaling under normal conditions. Following androgen ablation, TGF-β transiently increases, triggering a transient increase in canonical Wnt signaling within the proximal ducts to maintain the stem cell niche during regression. This

emphasizes the potential for Wnt inhibitors to treat CRPC in combination with currently used androgen ablation regimens.

Previous studies have shown that Wnt genes or proteins in the Wnt signaling pathway are up-regulated or mutated in castrate resistant prostate cancers (Terry, Yang et al. 2006; Verras and Sun 2006). Castration provided a transient elevation of canonical Wnt signaling in Tgfbr2$^{floxE2/floxE2}$ prostates (Figure 12). In parallel, Tgfbr2$^{floxE2/floxE2}$ prostatic stromal cells transiently express specific Wnt genes in response to an androgen antagonist, bicalutamide (Figure 14). In contrast, Tgfbr2^{fspKO} stromal cells had elevated basal expression of Wnt-2, Wnt-3a, and Wnt-5a. Recently, preosteoblasts were reported to induce Wnt signaling as a result of androgen stimulation, and through paracrine signaling the preosteoblasts subsequently increased the proliferation of prostate cancer cells in a coculture system *in vitro* (Kim, Zelner et al. 1996). As preosteoblasts and prostatic stromal cells are both of mesenchymal origin, together with the results presented, this suggests a directional regulation of paracrine Wnt signaling by androgens. LNCaP cells, used in our co-culture studies, are derived from a human prostate cancer lymph node metastatic lesion that lack a functional TGF-β type II receptor and have a functional, yet mutant, androgen receptor (Yardy and Brewster 2005). The LNCaP cells, analogous to the Tgfbr2$^{NKX3.1KO}$ prostates, enabled us to show that androgen responsiveness mediated by the stroma is not dependent on epithelial TGF-β signaling. This supports earlier developmental studies in the prostate. The prostate stroma, but not the epithelia was shown to dictate androgen response in the prostate (Cunha and Chung 1981).

Antagonizing Wnt signaling in the absence of androgen ablation in Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} prostates did not induce apoptosis (Figure 18). Thus, although TGF-β and androgen signaling converge in the stroma to affect canonical Wnt signaling they act through distinct pathways.

We demonstrated that activated canonical Wnt signaling helps to limit prostatic regression. It is known that Wnt ligands bind Frizzled receptors on the epithelial surface and transmit signals through the canonical pathway that activates β-catenin/TCF in the nucleus (Sugimura, Cunha et al. 1986). Activating mutations in β-catenin have been identified to affect androgen receptor transcriptional activity and ligand specificity (Truica, Byers et al. 2000; Chen, Shukeir et al. 2004). Based on the specific TCF/β-catenin activity in the proximal prostatic ducts of the castrated Tgfbr2$^{floxE2/floxE2}$/TOPGal mice, the mechanism for the survival of the proximal ducts may be through an initial activation of epithelial canonical Wnt signaling. Comparing the Tgfbr2$^{floxE2/floxE2}$/TOPGal and Tgfbr2^{fspKO}/TOPGal mice it was evident that TGF-β signaling is important to regulate the spatial localization of Wnt signaling to enable regression of the distal ducts in a temporal coordination with androgen signaling. TGF-$\beta 1$ is distributed in a gradient throughout the prostate stroma, with the highest concentration residing at the proximal ducts (Nemeth, Sensibar et al. 1997). This is consistent with studies showing androgen-induced regeneration occurring at the distal tips of the prostate (Polakis 2000). One mechanism by which the localization of Wnt signaling is limited could be due to secreted Wnt inhibitors (e.g. SFRP-2, DKK-1) in the distal glands. Long term androgen ablation responses were not studied, as

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the focus of the study was to determine the role of the immediate upregulation of TGF-β following androgen ablation. However, as the prostate continues to regress after day seven, Wnt signaling apparently is replaced by another mechanism to prevent continued regression of the proximal ducts. The latter events and maintenance of the regressed prostate is through a separate mechanism that is more dependent on the availability of androgens since some prostate regression was observed in Tgfbr2^{fspKO} mice 14 days following castration (Figure 10).

Together, the upregulation of TGF-β expression in the prostate, coincident with androgen ablation, would support the regression of the prostate by antagonizing stromal Wnt expression brought on from an androgen regulated mechanism. In normal proximal prostatic tissue the epithelial Frizzled receptor is activated resulting in canonical, and possibly non-canonical, Wnt signaling which may contribute to the phenotypes seen in our mouse models. However, based on previous reports that canonical Wnt signaling supports survival and proliferation (Wright, Douglas et al. 1999; Widelitz 2005), this is the most likely mechanism that maintains the viability of proximal prostate tissues in the context of androgen ablation. The entire prostate does not involute in the absence of androgens, since subsequent replacement of testosterone results in the regeneration of prostatic tissue (Knudson, Hethcote et al. 1975). Intriguingly, in other mammals such as deer, antler regeneration is reported to demonstrate a site-specific Wnt signaling activation during regeneration (Mount, Muzylak et al. 2006). The fact that activating mutations of β-catenin are found in castrate resistant prostate

cancers (Voeller, Truica et al. 1998; Truica, Byers et al. 2000) suggests that cancer cells can hijack the same Wnt signaling pathways used to support proximal prostate survival to aid in tumor survival. Understanding paracrine interactions of TGF-β, androgen, and Wnt signaling in regulating prostate regression may be linked to its regeneration following androgen supplementation.

Stromal-epithelial interactions have proven to be important in embryonic development and tumorigenesis. Based on the Knudson multi-hit hypothesis of tumor development we attempted to further the progression of the PIN lesions associated with LADY 12T7f epithelia, expressing the large T antigen, by recombining them with Tgfbr2^{fspKO} prostatic stromal cells, as the second mutagenic hit (Knudson, Hethcote et al. 1975). Figure 6 illustrated that the Wnt signaling associated with the Tgfbr2^{fspKO} cells not only enabled the PIN lesions to progress to adenocarcinoma, but also enabled the epithelia to become resistant to androgen ablation. Inhibition of Wnt signaling with SFRP-2 appeared to restore the androgen sensitivity and decrease the tumorigenicity of the resulting tumors. Future prostate cancer therapies would most likely benefit by not only antagonizing the traditional androgen signaling pathway, but also inhibiting Wnt signaling. This would allow therapies to target both the epithelial and stromal compartments as well as androgen dependent and independent tumor cells.

Our studies concentrated on canonical stromal TGF-β signaling. We showed that the loss of stromal TβRII led to transformation of the epithelia as well as castrate resistance. Studies have shown that the downstream Smad proteins can activate different pathways depending on the transcriptional

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complexes they form with binding DNA and activating target genes. It would be interesting to determine if Smad2, Smad3, or Smad4 played similar or different roles with respect to pathways influencing transformation or castrate resistance. It has been shown that AR can bind Smad3 directly, but not Smad2 (Chipuk, Cornelius et al. 2002). In addition, Smad4 controls the ability of Smad3 to inhibit AR-mediated gene transcription (Nishi, Oya et al. 1996). This suggests that Smad3 and Smad4 would have a greater role in androgen regulated events including castrate resistance compared to Smad2. In a breast cancer model using the MMTV-Neu mouse, activated Smad2 correlated with loss of the TβRII receptor (Landis, Seachrist et al. 2005). In prostate cancer, the loss of TβRII is found in the majority of patient samples suggesting that Smad2 may play a greater role in prostate transformation. In our Tgfbr2^{fspKO} mouse model it is not possible to delineate the contribution of individual Smad proteins to transformation or castrate resistance. Further understanding would make targeting castrate resistance in late stage prostate cancer versus transformation as a preventative or early stage treatment possible.

CHAPTER III

BONE MARROW DERIVED MESENCHYMAL STEM CELLS INCORPORATE INTO THE PROSTATE DURING REGROWTH

Introduction

Prostate cancer mortality continues to rise as the aging population expands. After surgical intervention, radiation, and androgen ablation therapy, recurrence is androgen-independent and is termed castrate resistant prostate cancer (CRPC). Patients with CRPC are primarily given palliative care since conventional chemotherapeutics do little to delay mortality from the disease. Prostate growth and regrowth has been the subject of stem cell studies, but not with respect to recruited cell types (Tsujimura, Koikawa et al. 2002; Wang, Kruithof-de Julio et al. 2009). We used transgenic mouse models to help understand the involvement of recruited cells in prostate regrowth.

Prostatic epithelial Wnt signaling was identified in transgenic mouse models of CRPC. One such model we developed had a conditional stromal knockout of the TGF-β type II receptor, termed Tgfbr2^{fspKO}, (Bhowmick, Chytil et al. 2004). FSP1-Cre enabled targeted recombination in mesenchymal-derived cells, including fibroblasts (Iwano, Plieth et al. 2002; Bhowmick, Chytil et al. 2004). The prostatic stroma of Tgfbr2^{fspKO} mice independently contributed to transformation of the adjacent epithelia as well as castrate resistance. The mechanism for the castrate resistant phenotype was associated with stromal fibroblastic expression of Wnt ligands, that in turn activated canonical Wnt

signaling in the epithelia (Li, Placencio et al. 2008; Placencio, Sharif-Afshar et al. 2008). Adenoviral transduction of the Wnt signaling antagonist, secreted frizzled related protein-2 (SFRP2) restored castrate responsiveness in the Tgfbr2^{fspKO} mice (Placencio, Sharif-Afshar et al. 2008). However, systemic SFRP2-adenovirus treatment in Tgfbr2^{fspKO} mice was associated with morbidity. Another model with prostatic epithelial expression of constitutively activated β-catenin developed HGPIN and sustained growth following castration (Yu, Wang et al. 2009). Additionally, human CRPC C4-2B cells reportedly have elevated autocrine Wnt signaling compared to their androgen dependent parent cell line, LNCaP (Bisson and Prowse 2009).

CRPC, tissue remodeling, and cancer progression are generally associated with the recruitment of bone marrow derived cells (Liu, Chen et al. 2006; Houghton, Morozov et al. 2007; Ammirante, Luo et al. 2010). In a mouse model of damaged liver, during regeneration the recruited BMDCs contributed to the new liver epithelia (Vassilopoulos, Wang et al. 2003). Similar BMDC incorporation occurs in gastrointestinal epithelia associated with elevated proliferation and inflammation (Houghton, Stoicov et al. 2004; Davies, Powell et al. 2009). A specialized type of BMDCs, mesenchymal stem cells (MSCs), are recruited to sites of inflammation and proliferation including wound healing and cancer (Ichim, Alexandrescu et al. 2010). Multipotent MSCs can differentiate into multiple cell types including osteoblasts, chondrocytes, adipocytes, and fibroblasts (Bianco, Robey et al. 2008). In various models of cancer, MSCs potentiate disease progression. In a model of breast cancer, MSCs promoted

metastasis (Karnoub, Dash et al. 2007). In a model of prostate cancer metastasis, manipulating MSCs to deliver the urokinase-type plasminogen antagonist amino-terminal fragment by co-injection with PC3 cells in the bone decreased tumor angiogenesis and osteolytic activity (Fritz, Noel et al. 2008). MSCs home to tumors as a result of cytokine and chemokine expression in the tumor microenvironment. The efficacy of MSCs as a therapeutic tool has been tested through the targeted delivery of exogenously expressed soluble factors (Fritz and Jorgensen 2008; Reinders, Fibbe et al. 2010). For example, patients with heart damage have received MSCs that home to damaged and necrotic tissue for wound repair (Song, Song et al. 2010). We rationalized that MSC therapy may benefit patients with CRPC in a similar manner.

We examined BMDC recruitment to the prostate during regrowth. Co-expression of prostate markers with BMDCs suggested that these recruited cells were also incorporated into the prostate epithelia. We further identified MSCs fusing with prostatic epithelia. Using an orthotopic C4-2B xenograft model system, we found that recruited MSCs could further contribute to tumor progression by enhanced Wnt signaling. The overexpression of SFRP2 by MSCs homed to the tumors and restored tumor responsiveness to castration.

Materials and Methods

Ethics Statement

The Vanderbilt Institutional Animal Care and Use Committee approved all animal procedures (M/06/236).

Mice and Fetal Liver Transplants

Harlan C57BL/6 wild type mice were purchased (Harlan, Indianapolis, IN). Chicken beta actin-GFP mice were purchased from Jackson Laboratories and GFP expression was confirmed by visualization with UV light. Tgfbr2$^{floxE2/floxE2}$ (Chytil, Magnuson et al. 2002) and Tgfbr2^{fspKO} (Bhowmick, Chytil et al. 2004) mice with C57BL/6 background were generated as previously described. Fsp-Cre+/Rosa26 mice enabled β-gal visualization of cells after Cre-mediated recombination. All mice were genotyped from ear punch biopsies as previously reported (Kasper, Sheppard et al. 1998; DasGupta and Fuchs 1999; Chytil, Magnuson et al. 2002). Fetal liver transplants (FLTs) were performed as previously described (Everhart, Han et al. 2005). Briefly, E14 embryonic livers from GFP+ mice were made into single cell suspensions for tail vein injections (2×10^6 cells per mouse) into lethally irradiated recipient male mice using a [^{137}Cs] gamma source by giving a split dose of 1200 rads. The host mice were either intact or castrated four weeks prior to the FLT procedure. To minimize infection

from a diminished immune system, mice were kept on acidified antibiotic water for two weeks before and four weeks after FLT. Four weeks after FLT, prostate regrowth was monitored following subcutaneous implantations of testosterone pellets.

Laser Capture Microdissection and RNA Isolation for Microarray

Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} mouse prostates were dissected and frozen in liquid nitrogen. Cryosections of the prostates were used for laser capture microdissection of the stroma. RNA was isolated using a µMACS mRNA isolation kit following the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). Samples were lysed in SuperAmp Lysis Buffer and sent for processing and microarray analysis. Miltenyi Biotec amplified the RNA, produced cDNA, and hybridized to Agilent whole genome oligo microarrays. Fluorescent signals were detected and Agilent Feature Extraction Software was used to read and process the microarray image files. Gene lists were given as normalized Cy5/Cy3-fold changes. The microarray data sets were submitted to the NCBI GEO database accession number GSE22130. The selected table included fold change values averaged from three data sets per genotype, each having a fold change >2 and p-value <0.01.

MSC generation and verification

Bone marrow derived mesenchymal stem cells were derived as previously described (Phinney, Kopen et al. 1999; Spagnoli, Longobardi et al. 2005; Anumanthan, Makari et al. 2008). Bone marrow was flushed from mice femurs and tibias aged eight to twelve weeks. Red blood cells were lysed and the remaining bone marrow cells plated in MSC expansion media (Phinney, Kopen et al. 1999; Spagnoli, Longobardi et al. 2005). Adherent MSCs were selected and expanded for ten days. Cells were then trypsinized and plated for differentiation assays or used for tail vein injections into host mice (10^6 cells injected per mouse).

Osteogenic differentiation of MSCs was performed as previously described (Phinney, Kopen et al. 1999; Alfaro, Pagni et al. 2008). MSCs were switched to osteogenic inducing media for three weeks, consisting of high glucose DMEM supplemented with 10% fetal bovine serum, 100 µM dexamethasone, 0.1 mM ascorbic acid, and 10nM β-glycerophosphate. Osteogenic differentiation was confirmed by alkaline phosphatase staining according to the manufacturer's directions (Sigma-Aldrich, St. Louis, MO)

Chondrogenic differentiation of MSCs was performed as previously described (Phinney, Kopen et al. 1999; Alfaro, Pagni et al. 2008). MSCs were switched to chondrogenic inducing media for three weeks, consisting of high glucose DMEM supplemented with 10% fetal bovine serum, 100µM dexamethasone, and 0.1 µg/mL TGF-β. Chondrogenic differentiation was

confirmed by alcian blue (pH 1.0) staining. Cells were fixed for 20 min at RT in 10% formalin, washed with PBS, stained with alcian blue for 20 min and washed with PBS.

Adipogenic differentiation of MSCs was performed as previously described (Phinney, Kopen et al. 1999; Alfaro, Pagni et al. 2008). MSCs were switched to adipogenic inducing media for three weeks, consisting of high glucose DMEM supplemented with 10% fetal bovine serum, 10μM dexamethasone, 10μg/ mL insulin, and 100μg/mL IBMX. Adipogenic differentiation was confirmed by Oil Red-O staining.

Immunohistochemistry and Immunofluorescence

Tissues were fixed with 4% paraformaldehyde or 10% formalin, embedded in paraffin, and sectioned for histological analysis. GFP (1:1000, Santa Cruz, CA) and phosphorylated-histone H3 (1:500, Upstate, Temecula, CA) immunohistochemistry was performed using antigen retrieval with antigen unmasking solution (Vector Laboratories, Burlngame, CA) diluted 1:100. Following primary antibody incubation overnight, Dako Cytomation Universal or Rabbit kits were used for the secondary antibody and development with DAB. TUNEL staining was performed using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, Temecula, CA) as directed. Immunohistochemical staining was quantitated by taking a ratio of positively stained cells per field

(400x) MetaMorph 7.6 software was used to help quantitate the immunohistochemical staining. Statistical significance was determined by two tailed Student's *t* test.

Tissues were fixed with 4% paraformaldehyde or 10% formalin, embedded in paraffin, and sectioned for histological analysis. Antigen retrieval with antigen unmasking solution (Vector Laboratories, Burlngame, CA) diluted 1:100 was performed. Staining was performed for GFP (1:100, Santa Cruz, CA), β-galactosidase (1:750, Abcam, Cambridge MA), Cytokeratin 8 (1:100, University of Iowa Hybridoma Bank, Iowa City, IA), p63 (1:50, Calbiochem, Gibbstown, NJ), androgen receptor (1:50, Santa Cruz, CA), and CD45 (1:50, BD Biosciences, San Jos, CA). Secondary anti-mouse or anti-rabbit Alexa Fluor 594 (red), Alexa Fluor 546 (orange-red) or Alexa Fluor 488 (green) were used as indicated (1:500, Invitrogen, Carlsbad, CA). TO-PRO-3 iodide was used for confocal nuclear counterstaining (Invitrogen, Eugene, OR). DAPI mounting media was used for widefield immunofluorescence staining (Vector Laboratories, Burlngame, CA). Widefield images were taken on a Nikon epifluorescence microscope and a Leica DM IRB inverted microscope. Z-series slices were taken on a Zeiss LSM510 META inverted confocal microscope.

Statistical Analysis

Oneway ANOVA was performed to test differences between the experimental and control groups, followed by Dunnett's post-hoc tests when the variances were equal. When variances were unequal, oneway Welch ANOVA allowing for unequal variances was used to test differences between the experimental groups and the intact group, followed by a two-sample t tests with unequal variances.

Results

Castrate resistance mediated by the stroma and prostate regrowth
recruit BMDCs

Previous studies with the Tgfbr2^{fspKO} mouse model showed that the conditional stromal knockout of TGF-β signaling led to CRPC (Placencio, Sharif-Afshar et al. 2008). To fully understand the stromal changes that led to the development of CRPC, we isolated prostate stroma RNA through laser capture microdissection for microarray analysis and compared Tgfbr2^{fspKO} to control Tgfbr2$^{FloxE2/FloxE2}$ mouse stroma. The microarray data has been deposited in the NCBI Gene Expression Omnibus (GEO, GSE22130). This revealed the differential regulation of numerous cytokines and chemokines in Tgfbr2^{fspKO} compared to control Tgfbr2$^{FloxE2/FloxE2}$ mouse stroma (Table 2). Microenvironments rich in chemokine and cytokine signaling recruit many cell types, including BMDCs (Alison, Lim et al. 2009).

Table 2. Selected chemokines altered in Tgfbr2^{fspKO} mouse stroma compared to Tgfbr2$^{FloxE2/FloxE2}$ mouse stroma. Negative values indicate the chemokine was downregulated in the Tgfbr2$^{FloxE2/FloxE2}$ stroma and upregulated in the Tgfbr2^{fspKO} stroma. Positive values indicated the chemokine was upregulated in the Tgfbr2$^{FloxE2/FloxE2}$ stroma and downregulated in the Tgfbr2^{fspKO} stroma. Values are averaged from three independent data sets. Each value had > 2-fold difference and a P-value < 0.01.

<u>Selected List of Chemokines</u>

Primary Sequence Name	Average Fold Change
Ccl12	-3.5079
Ccl19	2.9717
Ccl5	-70.1794
Ccl6	-26.8950
Ccl8	-39.9607
Ccl9	-11.6369
Csf1r	-9.6176
Csf2	162.4770
Cx3cl1	-25.0406
Cxcl10	-25.4580
Cxcl12	77.3219
Cxcl13	-47.1429
Cxcl16	-20.5419
Cxcl9	-49.5591
Epo	40.0280
Ifna12	38.8468
Ifnz	159.8488
Il18	-2.1320
Il18bp	-13.0765
Il1f8	3.3513
Il28	10.2366
Il4	-3.7385
Il6st	-31.2994

BMDCs are recruited to the prostate during cancer development. Since BMDCs are also recruited during tissue remodeling, we wanted to identify their role in prostate regrowth following castration. Previous studies have described the histologic identification of macrophage and monocytes recruited in prostate cancer (Luo, Tan et al. 2007; Ammirante, Luo et al. 2010). To better localize BMDCs during prostate regrowth we generated GFP-bone marrow chimeric wild type mice, which were either intact or castrated. Prostate regrowth was monitored at day zero, three, seven, and 28 days following implantation of exogenous testosterone. Immunohistochemical GFP localization identified BMDCs recruited to the prostate during regrowth in response to castration (Figure 22). Intact prostates had low basal BMDC recruitment (Figure 22A). Castration induced a slight, but statistically significant increase in recruitment (Figure 22B). Three and seven days following testosterone supplementation, prostates reached the highest levels of recruitment (Figure 22C, D). At 28 days following testosterone supplementation, when the prostate was fully regrown, the level of detectible BMDCs had fallen, comparable to basal levels (Figure 22E-F). Of particular interest was the appearance of BMDCs residing within the epithelial compartment (Figure 22F). BMDC recruitment during active prostate remodeling is quantitated in Figure 22G. BMDC incorporation in the epithelial compartment was observed throughout the duration of recruitment from castration to full regrowth. However, the presence of BMDCs at four weeks when the prostates had resumed normal homeostasis suggested that BMDCs directly contributed to the regenerated prostate tissue.
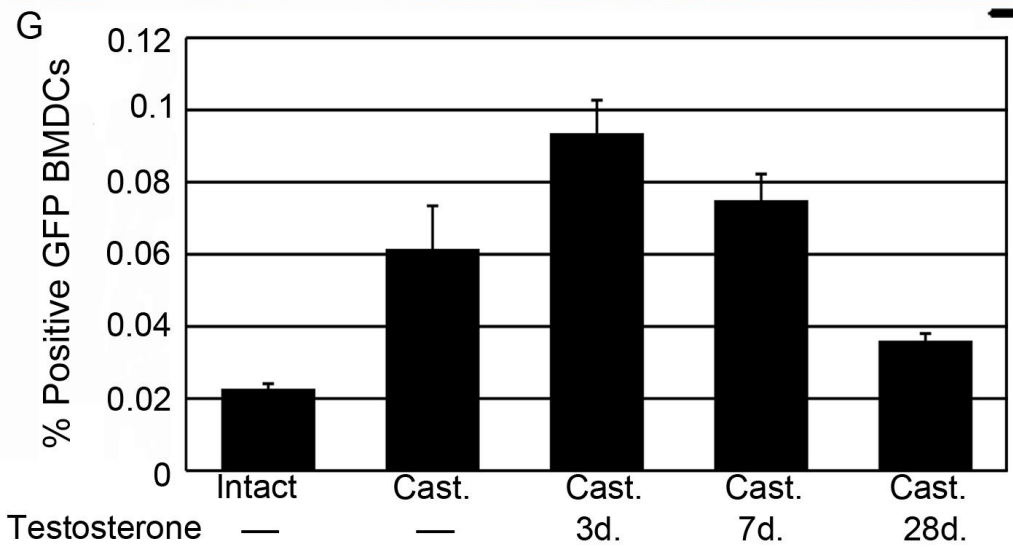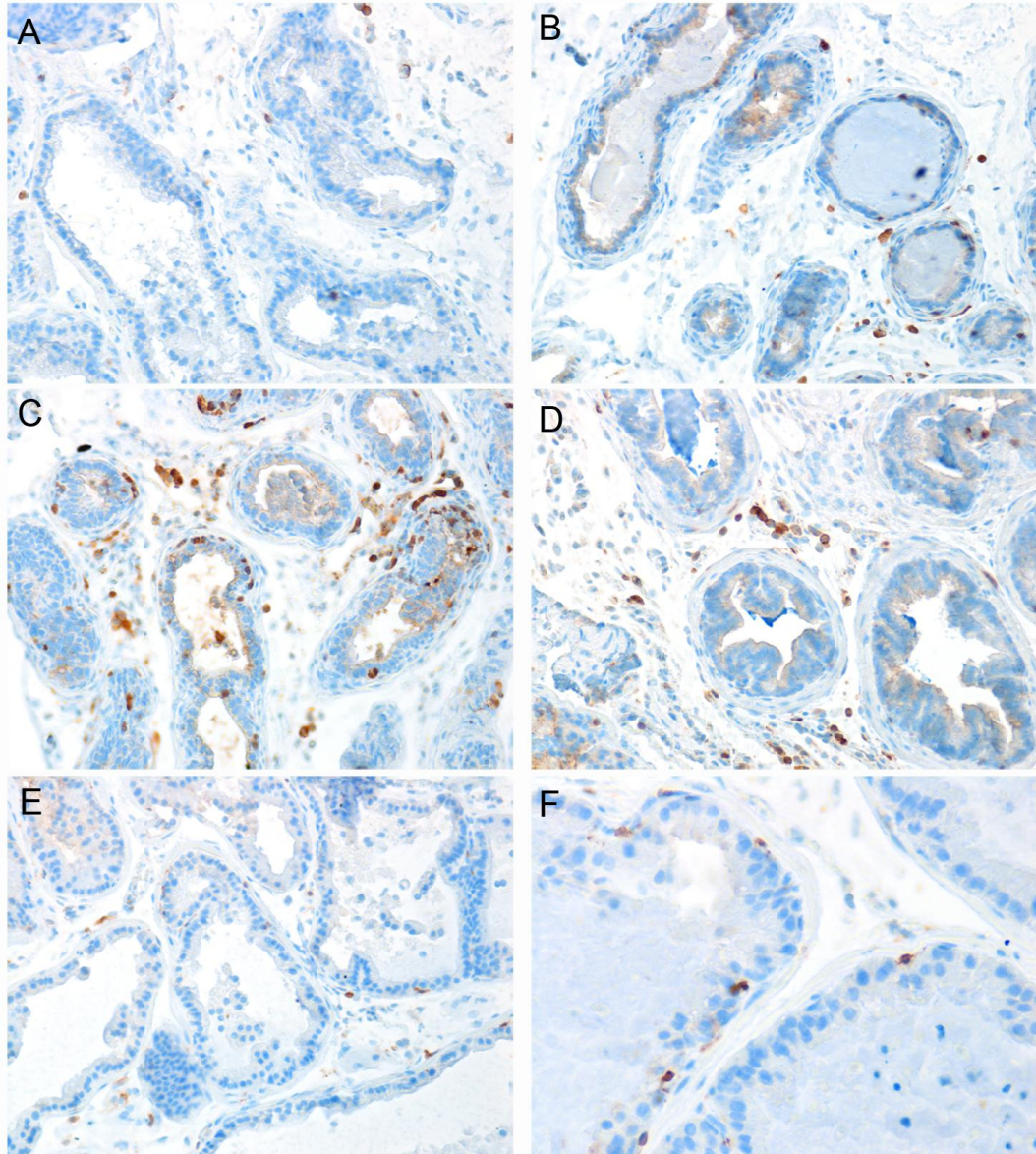
Figure 22. BMDCs were recruited to the prostate during regrowth. Prostates from GFP chimeric mice were stained using GFP immunohistochemistry to show GFP-BMDC (brown) recruitment to the prostate. A) Intact prostates had low basal levels of BMDC recruitment. B) Two months post-castration, prostates show a slight but significant increase in BMDCs (p value = 0.0254). C) Castrated prostates supplemented with exogenous testosterone for three days show the highest levels of BMDC recruitment (p value = 0.0064). D) Castrated prostates supplemented with exogenous testosterone for seven days show high levels of BMDC recruitment (p value = 0.0084). E) Castrated prostates supplemented with exogenous testosterone for 28 days show low levels of BMDC recruitment comparable to basal levels (p value = 0.162). F) Higher magnification of castrated prostates supplemented with exogenous testosterone for 28 days shows BMDC recruitment to epithelial duct cells. Scale bar indicates 50 μm for panels A-E and 25 μm for panel F. G) Bar graph quantifies the percentage of GFP-positive BMDCs recruited to the prostate at various time points during regrowth.

We characterized the BMDCs that appeared to incorporate into the prostate epithelia. GFP expression was immunolocalized with prostate tissue markers (Figure 23). We found that co-expression of GFP with androgen receptor indicated the prostate cell lineage of stromal or epithelial cells (Figure 23A). Furthermore, basal and prostate luminal epithelial cells identified by p63 and cytokeratin 8, respectively, also co-expressed GFP (Figure 23B, C). We also noted limited co-expression of GFP with CD45, indicating hematopoietic lineage cells (Figure 23D). These results indicated that although a large population of BMDCs is recruited to the prostate, comparatively smaller populations of the total BMDC population seem to incorporate into the tissue long term.

MSCs incorporate into the prostate epithelia through fusion

We hypothesized that at least a subset of the incorporated BMDCs were mesenchymal stem cells (MSCs) based on evidence that 1) MSCs are less than one percent of the total bone marrow population of cells, 2) MSCs are reported to home to sites of active tissue remodeling, and 3) MSCs are known to incorporate into regenerated tissues (Vassilopoulos, Wang et al. 2003; Wang, Willenbring et al. 2003). We next determined the recruitment of MSCs to the prostate during regrowth. We generated primary cultured MSCs from adult chicken β-actin-GFP mouse bone marrow (Phinney, Kopen et al. 1999). The MSC trilineage differentiation potential was verified. The MSCs were shown to differentiate into adipocytes, as indicated with oil red-O staining, chondrocytes indicated with alcian blue staining, and osteocytes indicated by alkaline phosphatase staining
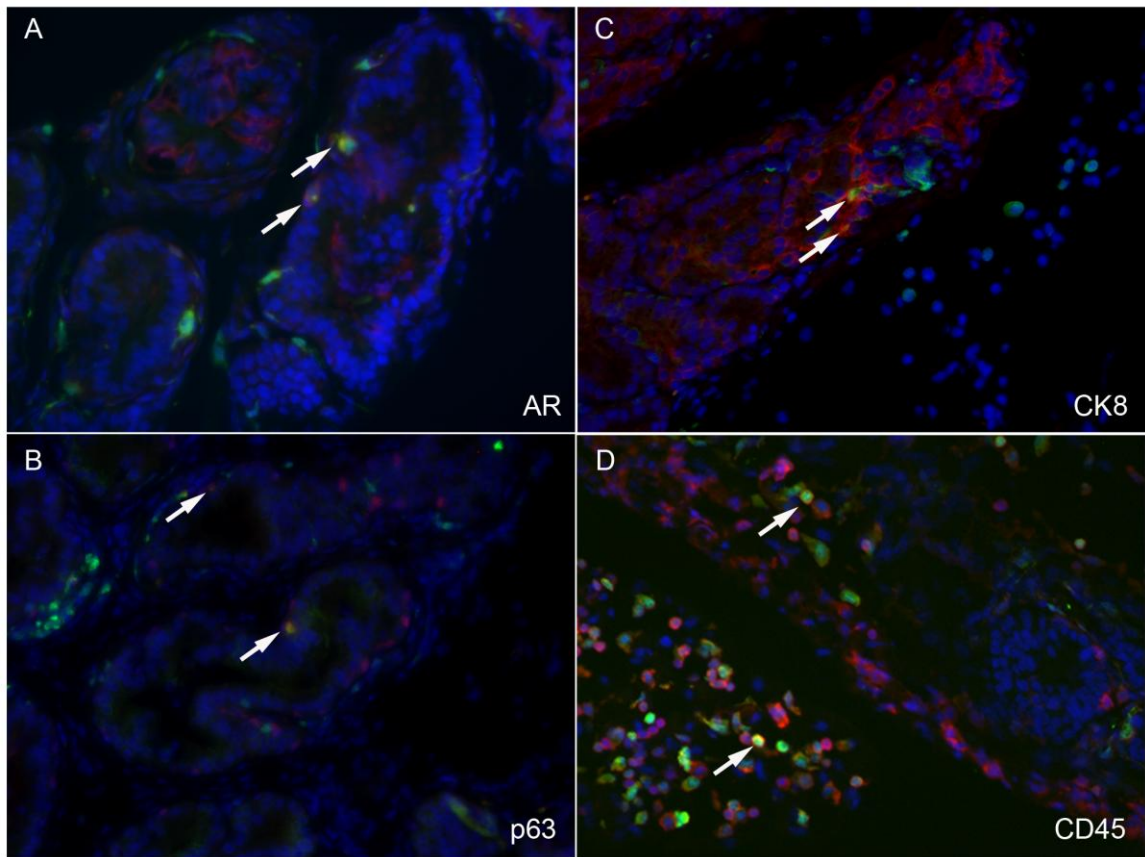
Figure 23. BMDCs incorporated into the prostate during regrowth. Immunofluorescence staining localized GFP-BMDCs in green with prostate markers in red. DAPI staining in blue indicates nuclear DNA. A) GFP-BMDCs co-express the prostate lineage marker, androgen receptor, in red. B) GFP-BMDCs co-express the prostate basal epithelial marker, p63, in red. C) GFP-BMDCs co-express the prostate luminal epithelial marker, cytokeratin 8, in red. D) GFP-BMDCs show minimal co-expression of the hematopoietic marker, CD45, in red.
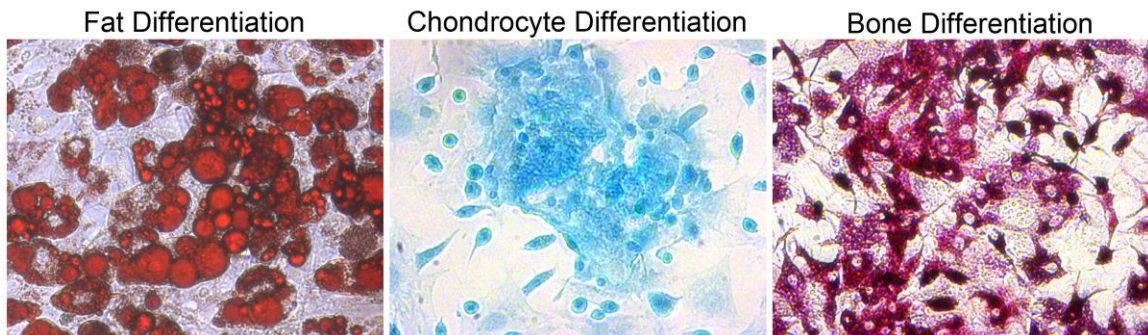
Figure 24. MSCs were functionally verified. Functional assays verified tri-lineage differentiation potential of mesenchymal stem cells after culturing for three weeks. Adipogenic differentiation was verified by staining with Oil Red-O; lipid droplets stained a red color. Chondrogenic differentiation was verified by staining with alcian blue; matrix sulfated proteoglycans stained a turquoise blue color. Osteogenic differentiation was verified by alkaline phosphatase staining; osteogenic cells stained a deep fuschia color.

(Figure 24). The multipotential MSCs were tested for the expression of known chemokine receptors. As expected, the MSCs expressed CCR5, CCR2, and CXCR4. Next, we measured the expression of corresponding chemokines in prostates during regrowth, to determine a mechanism for MSC homing. We isolated RNA from castrated prostates and castrated prostates given testosterone for three days. CCL5 was confirmed to be elevated 2.6-fold during prostate regrowth (ANOVA, $p = 0.0224$) compared to castrated controls. However, CCL2 and SDF-1 were not significantly elevated during regrowth. Thus, the CCR5-CCL5 chemokine signaling axis is a potential mechanism for MSC recruitment during prostate regrowth.

GFP-tagged MSCs were injected into mice to identify MSC recruitment to the prostate. We used intact or castrated host mice that were given exogenous testosterone for zero or three days, correlating with the highest level of BMDC recruitment. We then monitored MSC recruitment by GFP immunohistochemical staining and found a pattern similar to that seen with BMDC recruitment (Figure 25). The GFP-MSCs were recruited to intact prostates at low levels (mean = 7.25 cells per field of view). Castrated mice had elevated MSC recruitment to the epithelial compartment of the prostate (mean = 25.75). Further elevation in MSC recruitment was observed following three days of testosterone supplementation (mean = 38.55). Importantly, examination of other tissues of the same mice (liver, intestine) revealed no detectible GFP-MSCs. Thus, the bone marrow derived MSC population could selectively incorporate into the prostate during regrowth.
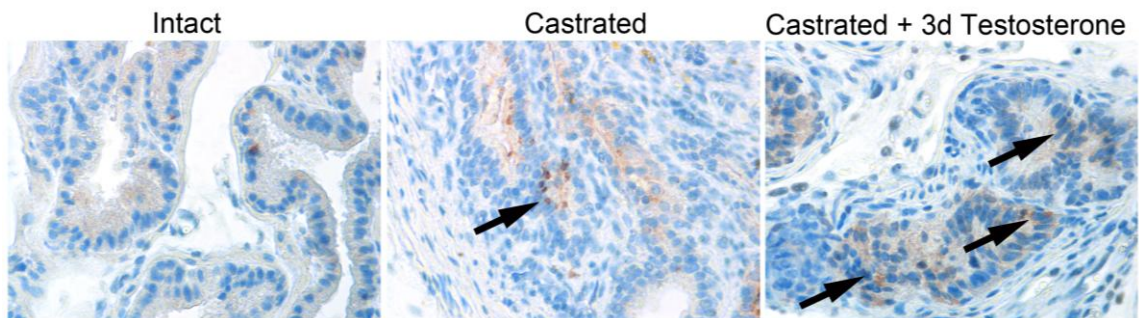
Figure 25. MSCs were recruited to the prostate during regrowth. GFP immunohistochemistry was performed to stain GFP-MSCs (brown) that were recruited to the prostates of mice at the indicated time points during regrowth. Intact prostates had low basal recruitment of MSCs. Castrated prostates had increased MSC recruitment. Castrated prostates supplemented with exogenous testosterone for three days showed the greatest increase in MSC recruitment.

The fusion or transdifferentiation of MSC is reported in other model systems (Lee, Kuo et al. 2004; Dan and Yeoh 2008).

We tested the potential of using MSC cell fusion or differentiation as a mechanism for targeting genetic material to CRPC. We used chicken β-actin-GFP mice as hosts for MSCs obtained from FSP1-Cre/Rosa26 mice. The development of FSP1-Cre/Rosa26 mice enabled MSC lineage tracing through the expression of β-galactosidase. Prostates of castrated mice were visualized by confocal fluorescent microscopy following three days of testosterone supplementation. We identified prostate epithelial cells that co-expressed GFP with β-galactosidase as an indication of fusion of the MSCs to the prostate epithelia (Figure 26). We calculated the percentage of recruited MSCs shown to undergo fusion at 48.9% (n= 5 mice, 20 fields of view).

MSCs are recruited to human CRPC and enhance Wnt signaling in the tumor epithelia

To further explore the role of MSCs in CRPC growth and their potential for targeted therapy, we used the C4-2B human CRPC cell line, well characterized to grow as xenografts in castrated host mice (Thalmann, Anezinis et al. 1994). Based on the documented importance of Wnt signaling in CRPC (Terry, Yang et al. 2006; Verras and Sun 2006), we stably transduced C4-2B cells with a TCF/LEF responsive luciferase reporter lentivirus. This allowed us to monitor canonical Wnt activity within the tumor epithelia. We then transduced MSCs with either GFP adenovirus as a control or SFRP2 adenovirus. We hypothesized that

| Prostate Epithelia | MSC | Nuclei | Merged |

GFP · ß-galactosidase · TO-PRO-3
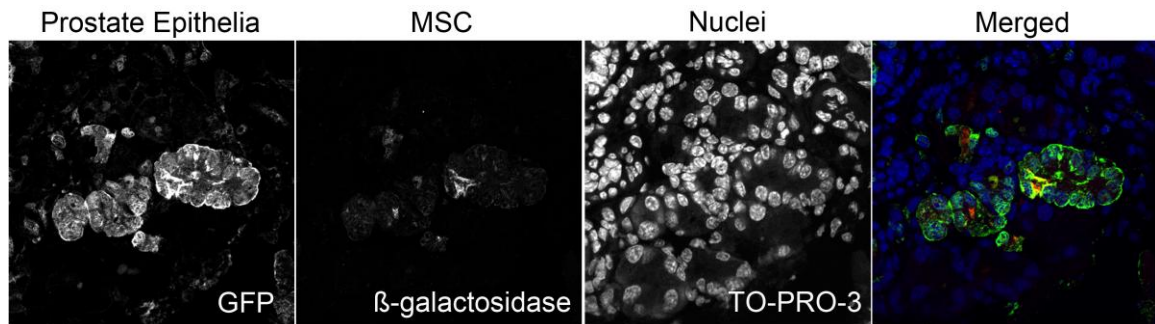
Figure 26. MSCs fused to the prostate during regrowth. Rosa26-expressing MSCs were injected into castrated GFP mice supplemented with exogenous testosterone for three days. Confocal microscopy showed colocalization of β-galactosidase-expressing MSCs with GFP prostate ducts. β-galactosidase-expressing MSCs were stained with red and shown in black and white. GFP-expressing prostate ducts were stained in green and shown in black and white. Nuclear DNA was stained blue with TO-PRO-3 stain and shown in black and white. A merged color panel shows the colocalization of the red MSCs with green prostate epithelia, indicated by the overlay of yellow color and counterstained blue nuclei.

MSCs could home to C4-2B tumors and secrete SFRP2 to inhibit tumor progression. To generate the CRPC model, we orthotopically grafted C4-2B cells into each of the anterior lobes of SCID mouse prostates. The experimental timeline is summarized (Figure 27A). After ten days of tumor growth, the host mice were castrated. Serum testing indicated continued expression of PSA in C4-2B tumor bearing mice following castration, as expected. To test the efficacy of MSCs as a therapeutic tool for CRPC, we subsequently injected host mice with vehicle, GFP-transduced MSCs, or SFRP2-transduced MSCs and harvested the tumors five days after castration.

After harvesting the C4-2B tumors, we measured TCF/LEF-luciferase activity, proliferation, and apoptosis. Analysis of luciferase expression from the lysed tumors revealed that mice given GFP-MSCs showed a 24-fold increase of canonical Wnt activity within the C4-2B tumor epithelia compared to controls (Figure 27B). Tumors from mice injected with SFRP2-MSCs had no difference in canonical Wnt activity compared to controls. This surprising finding suggested that five days following castration, wild type MSCs contribute to CRPC by enhancing Wnt signaling activity. Manipulating the MSCs to secrete SFRP2 caused reduced proliferation and elevated apoptosis associated with increased necrosis within the tumors.

H&E staining indicated focal areas of necrosis in control C4-2B tumors and in mice given GFP-MSCs (Figure 27C, D). The C4-2B tumors in mice given SFRP2-MSCs had comparatively greater areas of necrosis (Figure 27E). Tumor proliferation was analyzed by immunohistochemical localization of
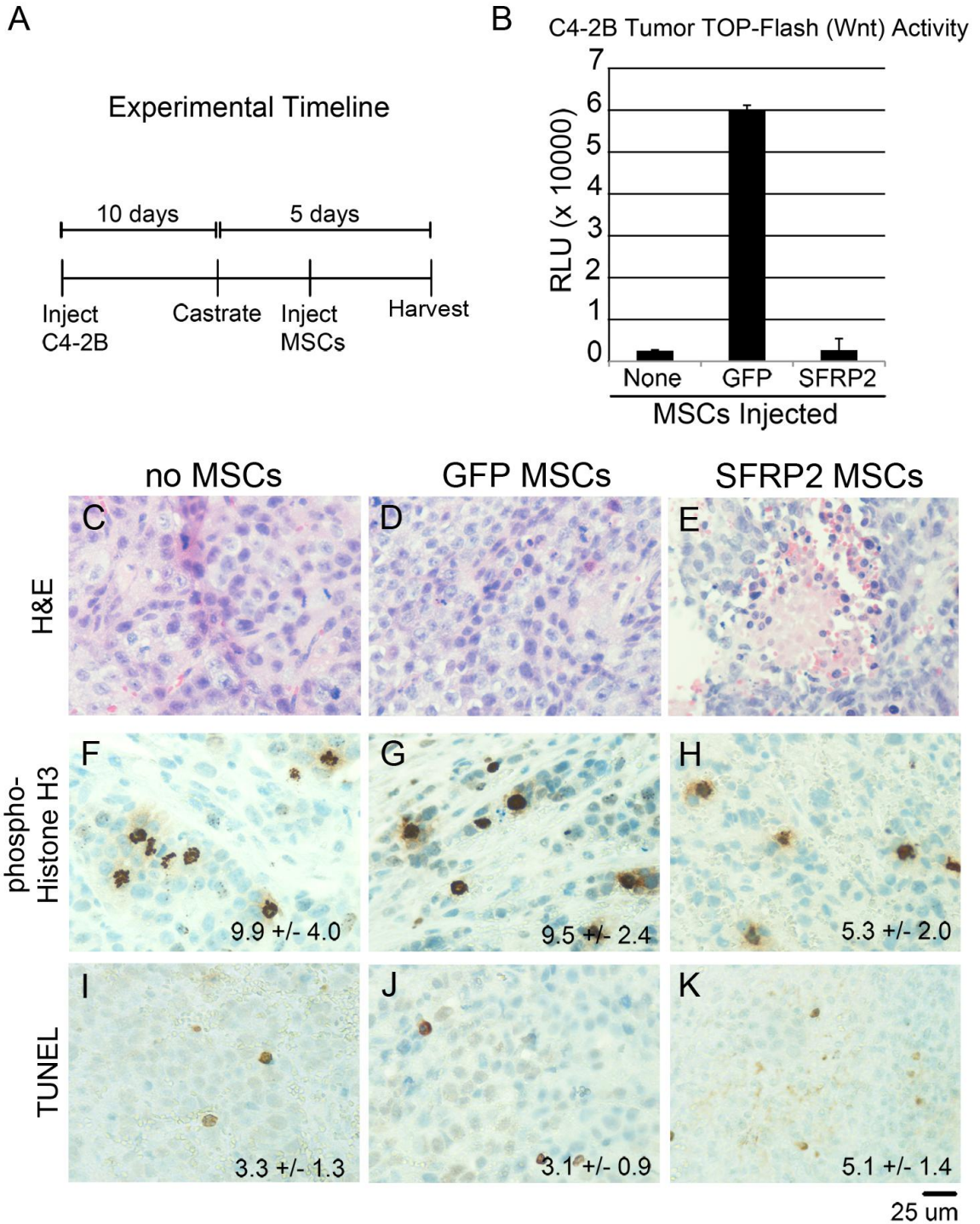
Figure 27. GFP-MSCs recruited to human CRPC xenografts enhanced Wnt signaling. The experimental setup is summarized in the timeline (Figure 27A). C4-2B cells were orthotopically injected into the prostates of SCID mice. After 10 days the mice were castrated and MSCs were given 24hours following castration. The tumors were harvested five days following castration. TOP-Flash luciferase activity was measured in C4-2B tumors (Figure 27B). GFP-MSCs

increased Wnt activity within the tumors by 24-fold compared to control uninjected tumors. SFRP2-MSCs suppressed Wnt activity to basal levels. C4-2B tumors were sectioned and stained histological analysis five days following castration. H&E staining shows some necrosis in C4-2B tumors without MSCs (Figure 27C). Necrosis was also seen with GFP-MSCs (Figure 27D), and increased necrosis was seen with SFRP2-MSCs (Figure 27E). Phosphorylated histone-H3 staining was used to stain tumors for mitotic proliferation. Basal levels of proliferation were seen in tumors without MSCs (Figure 27F). There was no difference in proliferation of tumors with GFP-MSCs (Figure 27G), and proliferation was significantly decreased in tumors with SFRP2-MSCs (p value = 0.0069, Figure 27H). TUNEL staining shows levels of apoptosis. Areas of necrosis were avoided in quantitating TUNEL positive cells. Basal levels of apoptosis were seen in tumors without MSCs (Figure 27I), similar to tumors with GFP-MSCs (Figure 27H), and a significant increase in apoptosis was seen in tumors with SFRP2-MSCs (p value = 0.0066, Figure 27J).

phosphorylated histone-H3, indicating cells undergoing mitotic division (Figure 27F-H). The mitotic rates of control and GFP-MSC-associated tumors were similar (Figure 27D, E). However, the mitotic rate in SFRP2-MSC-associated tumors was significantly lower than in control tumors (p value =0.0069, Figure 27F). We next analyzed apoptosis in these tumors using TUNEL staining (Figure 27G-I). Control and GFP-MSC-associated tumors had similar apoptotic cell numbers (Figure 27G, H). However, SFRP2-MSC tumors had a significant increase in apoptotic cells (p value = 0.0066, Figure 27I). Together, we found MSC recruitment to CRPC xenografts to support Wnt signaling and the targeted antagonism of Wnt signaling by MSCs can potentiate reduced tumor growth.

Discussion

Recruited BMDCs enhanced tumor progression through paracrine Wnt signaling. We found that the highest levels of BMDC recruitment occurred during prostate regrowth at three days. Interestingly, previous reports indicated that the highest level of prostate proliferation also occur three days following testosterone re-introduction, suggesting that peak tissue remodeling corresponds to greatest recruitment of BMDCs. This was to be expected, since BMDCs include inflammatory responsive cells such as leukocytes and macrophages, associated with clearing dead cells. However, this study demonstrated that a sub-population of BMDCs, MSCs, are recruited to potentiate re-growth. MSCs may provide growth factors that aid cancer progression in this complex signaling

microenvironment (Figure 28). We were able to employ MSC to target regrowing prostate tissue and deliver SFRP2, antagonizing Wnt-mediated tumor progression.

Based on the microarray analysis of the Tgfbr2^{fspKO} CRPC mouse model, (Table 2) and xenografted human CRPC C4-2B cells (Figure 27) it is likely that MSCs were recruited during regrowth through the CCL5-CCR5 axis. To broaden our understanding of mechanisms of CRPC, signaling pathway alterations based on LCM-microarray from Tgfbr2^{fspKO} compared to Tgfbr2$^{floxE2/floxE2}$ mouse stroma were analyzed. Interestingly, many more chemokines were upregulated than downregulated in the Tgfbr2^{fspKO} prostate stroma. Of particular interest, CCL5 had a 70-fold increase in Tgfbr2^{fspKO} stroma (Table 2). CCL5 is known to be upregulated in prostate cancer (Vaday, Peehl et al. 2006). It has also been shown to be a potent recruiter of BMDCs, including MSCs (Xu, Bian et al. 2009). A significant alteration of chemokine signaling within the tumor microenvironment was likely to result in BMDC recruitment. The multiple co-morbidities of the Tgfbr2^{fspKO} mice (Bhowmick, Chytil et al. 2004; Li, Placencio et al. 2008; Placencio, Sharif-Afshar et al. 2008; Boomershine, Chamberlain et al. 2009) prevented us from generating chimeric mice using these as the host to track BMDC recruitment to the prostate. Therefore, we looked at recruitment during prostate regrowth in wild type mice as a way to understand this aspect of CRPC.

We demonstrated how the recruited mesenchymal stem cells home to and contribute to CRPC by enhancing Wnt signaling within the tumor epithelia. Using MSCs as a delivery tool for secreted frizzled related protein 2 suppressed the
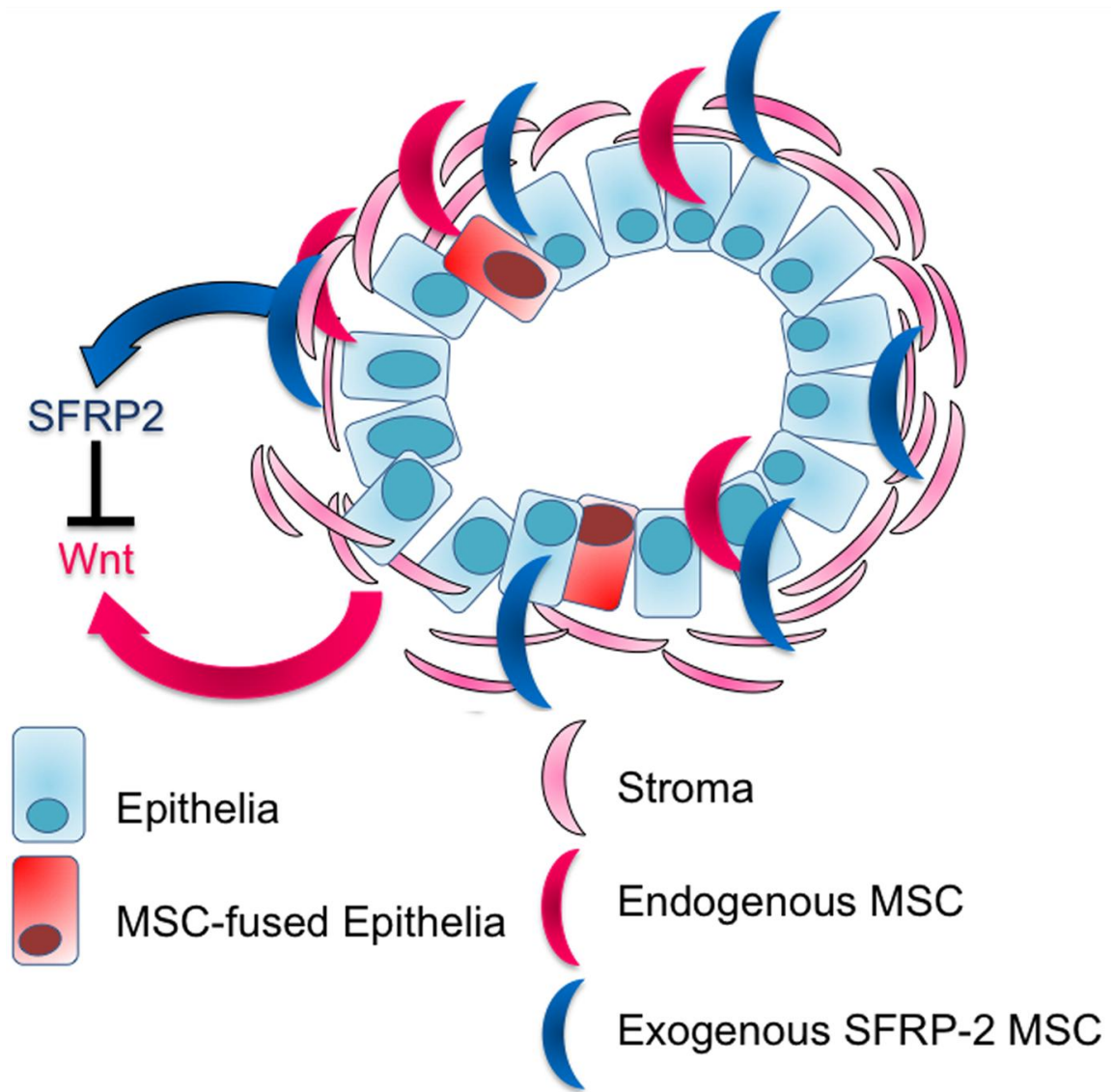
Figure 28. MSCs contribute to CRPC. This diagram represents a transformed prostate gland in CRPC. The rounded turquoise rectangles indicate the epithelial cells with an irregular nuclear size. The stroma is indicated with the pink half moon shapes, which surrounds the ducts and is being invaded by the transformed epithelia. Red and blue half moon shapes indicate recruited MSCs. The red MSCs represent the endogenous MSCs, which we showed enhance Wnt signaling in CRPC. The blue MSCs represent exogenous MSCs that express SFRP-2, antagonizing Wnt signaling in the CRPC.

enhanced Wnt signaling in the tumor epithelia to effectively increase apoptosis. MSCs may be used to deliver other gene therapeutics to enable CRPC regression. Using MSCs as a targeted delivery tool is also a way to avoid the toxicity associated with other less specific therapies. When targeting signaling pathways that have systemic effects, such as Wnt, it is especially necessary to avoid widespread repercussions.

We showed that MSCs incorporate themselves into the prostate epithelia through fusion. It is likely that the total number of recruited MSCs is over-estimated due to the experimental conditions of administering one million MSCs. However, the percentage of recruited MSCs observed to undergo fusion (48.9%), is likely similar to endogenous fusion events, respectively. Other studies have demonstrated how hematopoietic stem cells incorporate into the gastrointestinal epithelia through fusion during inflammation and proliferation (Rizvi, Swain et al. 2006). Prostate injury from vaccinia virus infection can cause inflammation and glandular disruption, resulting in BMDC reconstitution of 4% of the prostate epithelium (Palapattu, Meeker et al. 2006). Other studies have shown BMDCs recruited to and then incorporate into resulting epithelial tissues found in lung, liver, skin, heart, and gastrointestinal tract (Krause, Theise et al. 2001; Kawada, Fujita et al. 2004; Li, Stoicov et al. 2006). Cancer recurrence is linked to cell fusion (Dittmar, Nagler et al. 2009). CRPC cells have properties including increased drug resistance, increased resistance to apoptosis, and enhanced malignancy that are known to result from cells that have undergone cell fusion (Dittmar, Nagler et al. 2009). We did not rule out the possibility of

transdifferentiation into prostate epithelial lineages. The chicken β-actin-GFP mice used in the fusion experiment had focal expression of GFP in the prostate. Therefore, the presence of β-galactosidase expressing MSCs in the absence of GFP could indicate transdifferentiation of the MSCs that incorporated into the prostate epithelia or fusion that we could not visualize due to the lack of GFP expression in those prostate epithelial cells. It is possible that both fusion and transdifferentiation are mechanisms of MSC incorporation into the prostate epithelia. Others have suggested that cancer stem cells may arise from the fusion of BMDCs with cancer cells, giving rise to cells more capable of evading immune control. While our study could not look at every possibility, we demonstrated the involvement of MSCs in the cancer microenvironment and the possibility of using MSCs to deliver therapeutic genes to control prostate cancer.

In the C4-2B xenograft studies, the recruitment of MSCs enhanced Wnt activity within the tumors. However, there was little effect on proliferation, apoptosis, or tumor size in C4-2B xeongrafts in the presence or absence of MSC recruitment. This may be because C4-2B cells express high levels of Wnt ligands endogenously, masking the Wnt expression by the MSCs. This led us to hypothesize that MSCs may have a greater influence on androgen dependent tumors with lower amounts of Wnt that would be more sensitive to additional Wnt produced in the prostate microenvironment. By understanding how these MSC act upon cancer cells and contribute to the tumor microenvironment, we could learn to manipulate these cells to counteract their cancer promoting effects. We had some success in suppressing C4-2B tumor proliferation and promoting

apoptosis with MSC-targeted SFRP2 expression. However, we did not observe a significant reduction in tumor size. Targeting Wnt signaling is just one route for suppressing cancer progression. An approach targeting multiple signaling pathways is likely to be more effective in this late stage of cancer progression to provide a lasting effect on tumor size.

Collectively, these studies demonstrated BMDCs are involved in prostate regrowth and cancer progression. BMDCs, and in particular MSCs, are recruited to tissues undergoing active remodeling, including the cancer microenvironment. Given the innate cancer-homing capabilities of MSCs, it may be possible to use these cells to treat CRPC with therapeutic gene delivery.

CHAPTER IV

CONCLUDING DISCUSSION

Resident and recruited stroma contribute to castrate resistant prostate cancer. The prostate tumor microenvironment, as demonstrated in these studies, is greatly influenced by TGF-β, Wnt, and androgen signaling (Table 3). These studies highlight Wnt signaling as a common pathway in CRPC that is influenced by resident stromal cells or recruited mesenchymal stem cells. The loss of TGF-β signaling in Tgfbr2$^{\text{fspKO}}$ mice resulted in the enhanced production of Wnt ligands in the resident stroma. In addition to increased monocyte, macrophage, and leukocytes recruited there were also likely MSCs being recruited although this was not confirmed in these studies. These MSCs would also add to the Wnt produced in the prostate stromal microenvironment to activate epithelial Wnt signaling resulting in castrate resistance. Androgen ablation would select for castrate resistant epithelia, coupled with the transient elevation of TGF-β and Wnt signaling will perpetuate the wound healing response to attract BMDCs to the tumor microenvironment. This would enhance the chemokine signaling in the tumor microenvironment and cause fusion of MSCs to tumor epithelia resulting in more aggressive cancer and enhanced metastasis. This 'cancer wound' will never 'heal' and Wnt signaling will ensue. The tumor epithelia will have increased proliferation as a result of enhanced Wnt signaling, castrate resistance, and uncontrolled growth. Simultaneously, BMDC recruitment will continue to further

Table 3. Summary of stromal associated events during CRPC. The major regulatory pathway with alterations are in the left column. The effects resulting from the pathways are described in the two columns on the right as they pertain to the resident or recruited stroma. As indicated, there are many overlapping effects between signaling pathways.

Change in Regulatory Pathway	Associated with Resident Stroma	Associated with Recruited Stroma
Loss of TGF-β	• PIN lesions and adenocarcinoma leading to castrate resistance of the epithelia	• Enhanced recruitment of BMDCs • Increased monocytes, macrophage, leukocytes • Likely also increased T cells, B cells, MSCs
Increased Wnt	• Castrate resistance of epithelia by paracrine resident stroma	• Castrate resistance of epithelia by paracrine recruited MSCs
Loss of Androgens (castration)	• Regression of normal epithelia with selection for castrate resistant epithelia • Transient elevation of TGF-β and Wnt signaling • Increased chemokine signaling	• Wound healing response and increased inflammatory/ chemokine signaling to recruit BMDCs
Replacement of Androgens (castration + testosterone) or Adaptation to Low Levels of Androgens	• Regrowth of castrate resistant epithelia • Increased chemokine signaling • More aggressive cancer • Will lead to metastasis	• Regrowth/ Remodeling of the prostate enhances BMDC recruitment • Increased chemokine signaling • More aggressive cancer • Enhances metastasis • Increased MSC recruitment to enhance Wnt signaling

sustain the cancer resulting in a perpetual loop. Modulating Wnt signaling is a target for CRPC therapy as these studies have demonstrated. Further targets will also need to be identified, as our studies have shown the complex crosstalk between signaling pathways in CRPC development and progression.

At the present time, we are unable to adequately study non-canonical Wnt signaling as it is altered by stromal TGF-β and androgen signaling. Bicalutamide transiently elevated non-canonical Wnt-5a expression in Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} stromal cells (Figure 14). Most studies in the prostate look at the canonical Wnt pathway involving β-catenin, since this pathway is more defined. Studying non-canonical Wnt signaling is complicated and not well defined. Therefore, these studies focused on understanding canonical Wnt signaling as multiple tools are available. It will be important to understand how the non-canonical PCP, JNK, or calcium Wnt pathways are affected by androgens. These pathways are likely to affect CRPC. Interestingly, SFRP-2 is known to inhibit canonical Wnt-3a and also non-canonical Wnt-5a (Hou, Tan et al. 2004). These were two of the Wnt genes altered in our Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} stromal cells after bicalutamide treatment. It is likely that effects we saw with SFRP-2 also affected non-canonical Wnt signaling in ways that we were not able to assess. This suggests further crosstalk between non-canonical Wnt and androgen signaling in CRPC. Recent evidence also suggests that non-canonical Wnt signaling is important in MSC differentiation. Specifically, Wnt-5a was shown to oppose Wnt-3a in regulating the MSC differentiation potential (Baksh and Tuan 2007). This is increasingly important as castrate resistant C4-2B human

prostate cancer cells are known to have more stem cell-like properties compared to the androgen-dependent LNCaP cells from which they were derived. The role of canonical and non-canonical Wnt signaling with respect to TGF-β and androgen signaling is important for future CRPC therapy.

We continue to understand the complexities of prostate cancer. Once CRPC develops in this late stage, cancer mortality is primarily due to metastases that have arisen from the primary tumor. We still do not understand how these metastases develop and disseminate. It is likely that metastatic cells have entered circulation since the first development of the primary tumor. Therefore, even before diagnosis, metastases are frequently colonizing the bone and other organs. They may remain dormant for many years, with the event that triggers subsequent growth at distant sites unknown. In many cases, these metastatic cells remain inactive and benign, but those few that are activated to proliferate are problematic.

When thinking of treatments for CRPC, we must keep in mind treatments for castrate resistant bone metastases. It has been shown that MSCs can home to tumors. MSCs home to tumors using a similar mechanism as that used by immune cells. MSCs are thought to possess chemotactic properties similar to inflammatory cells that enable their recruitment to sites of inflammation or tissue remodeling. These properties also allow them to evade immune detection when circulating throughout the body. For this reason, directed therapy with MSCs is likely to cause less systemic effects and allow a more targeted approach to tumors. The bone marrow is the natural home for MSCs, thus directed targeting

to the metastases in the bone marrow would be an option worth exploring. These cells would survive and even thrive in their natural environment, and this would give them an advantage over foreign prostate cancer epithelial cells. It would be extremely important to fully understand how MSCs proliferate and contribute to their microenvironments. For use as a therapeutic, MSCs would need to be engineered in a way to target cancer cells while not enhancing their growth or the tumor microenvironment, yet maintaining the natural balance within the bone microenvironment. As we used in our studies, SFRP-2 over-expression is a candidate to start with, though it is probable that multiple gene targets would be most effective. Wnt signaling is also a major pathway in the bone microenvironment. Thus, it may be more effective to focus on individual Wnt target genes rather than inhibiting all Wnt ligands with SFRP-2. More studies will be necessary, but MSC directed therapy may be effective for bone metastases.

As we continue to study how cancer develops we must understand the role our immune system plays to regulate this process. It is known that in most people there are microscopic cancers. Determining how the cancer is kept dormant and small would be of use to future treatments to allow us to treat cancer. Studies have also used other cell types besides MSCs as therapeutic agents. These include dendritic cells and T cells to target tumor antigens. Foreign pathogen immune response elicits CD4$^+$ and CD8$^+$ T cells. It is known that TGF-β in the tumor microenvironment has immunosuppressive effects. To circumvent this, CD4$^+$ and CD8$^+$ T cells were engineered to express dominant negative TβRII (Gorelik and Flavell 2001). Mouse tumor models showed that

these engineered T cells, by not responding to the immunosuppressive effects of TGF-β in the tumor microenvironment, were able to inhibit and eradicate tumor growth. These studies suggest that it is possible to eradicate cancer with our own immune defense system, albeit with alterations to boost a necessary response. Our immune system is unique to each individual, as unique as each cancer. Thus, it makes sense that we must study cancer from the perspective that a given cancer can behave very differently in individuals with a slight alteration in their genetic makeup. In addition, ageing negatively impacts the ability of the immune system to fight cancer (Gruver, Hudson et al. 2007), thus a major reason that cancer incidence increases with age (Yancik and Ries 2000). As we understand how our immune system keeps our bodies healthy we can use this information to help fight cancer. A greater understanding of our immune system would allow us to target specific tumors.

These studies have demonstrated the importance of understanding the complex tumor microenvironment to effectively treat prostate cancer. As described, CRPC development and progression is not well understood, which is evident in the fact that there are no current therapies for late stages of cancer. The development of CRPC was classified into five main categories: 1) hypersensitive AR, 2) promiscuous AR, 3) outlaw AR, 4) bypass AR, and 5) lurker cells (Figure 4) (Feldman and Feldman 2001). Based on our studies, I propose adding a sixth category: stromal paracrine signaling. Herein, these studies have described how the prostate resident stroma influences epithelial androgen responsiveness and development of CRPC through paracrine Wnt

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signaling. In addition, recruited stroma such as MSCs were shown to increase Wnt signaling in CRPC through paracrine signaling. More studies must be conducted in order to understand all the components- besides TGF-β, androgen, and Wnt signaling- that influence the ever changing tumor microenvironment from the site of the primary cancer to the metastatic site(s). As suggested, when developing effective therapy it is necessary to keep in mind not only the cancerous epithelia, but the resident and recruited stromal cell types that are heterogeneous in nature with respect to innate characteristics and androgen status. The need for multimodality treatment approaches for prostate and other carcinomas is evident.

CHAPTER V

FUTURE DIRECTIONS

These studies using the Tgfbr2^{fspKO} mouse model have expanded the current knowledge on the development and progression of CRPC. However, the limited lifespan of the Tgfbr2^{fspKO} mice (less than two months) coupled with their weak state as they age make long-term studies impossible. Of importance is the need to know if the transformation of epithelial PIN lesions and eventually prostate adenocarcinoma is unique to the FSP-1 expressing fibroblast lineage in which the FSP-1 promoter is driven by Cre. Collagen-1$\alpha2$–Cre-ER mice have a Cre-ER fusion protein driven by the Collagen-1$\alpha2$ promoter that specifically binds tamoxifen. Collagen-1α-2 is expressed ubiquitously in all stromal cells. Rosa/Tgfbr2$^{floxE2/floxE2}$ mice were crossed with collagen-1$\alpha2$–Cre-ER mice to generate Rosa/Tgfbr2^{ColFlox} mice. Treatment of the dam of these mice starting after birth of the pups (on days 1, 3, 5) by intra-peritoneal injection with tamoxifen results in the production of viable and healthy Rosa/Tgfbr2^{ColTKO} mice with detectable Tgfbr2 recombination by PCR. It will be important to characterize these new mice to determine if the knockout of TβRII in Collagen-1α-2 expressing stromal cells is similar to that of FSP-1 expressing stromal cells.

Preliminary studies suggest the phenotypes are similar. The Rosa/Tgfbr2^{ColTKO} mice develop PIN lesions and areas of focal adenocarcinoma as they age. Preliminary castration studies, however, have been difficult with

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older mice aged to 32 weeks. In many mice, the testes for an unknown reason are firmer and appear to have overgrown into the adjacent scrotum tissue. Excision is complicated by the testicles not having a defined shape and not being easily released from the scrotum. Future studies would likely benefit from chemical castration with bicalutamide or another pharmacologic agent to avoid incomplete removal of the testes. Confirmation will further support the importance of the stroma in prostate cancer initiation, progression, and development of CRPC. In addition, pathologic analysis of the testicular tissue may reveal development of testicular hyperplasia, cancer, or some other abnormality of importance resulting from the long-term knockout of stromal TGF-β signaling.

Stem cell/ progenitor cell studies with the Rosa/Tgfbr2^{ColTKO} mice would highlight the importance of the stroma with respect to the stem cell niche. We have analyzed Tgfbr2^{fspKO} mice labeled with long term incorporation of BrdU to track progenitor cells in the prostates. These mice, as the Tgfbr2^{fspKO}/TOPGal studies alluded to, have increased BrdU- positive progenitor stem cells. It would be interesting to analyze the incorporation of BrdU in Rosa/Tgfbr2^{ColTKO} prostates compared to their controls as they develop adenocarcinoma. Even more interesting would be to analyze the BrdU-positive cells in relation to recruited BMDCs or MSCs. Coupled by the comparison of fusion to progenitor stem cells versus non-progenitors in prostate cancer. The Rosa/Tgfbr2^{ColTKO} mice would serve as an ideal host to analyze the prostates at various stages of cancer progression.

It is important to understand the mechanism that leads to loss of TβRII in prostate cancer stroma. Previous studies have suggested methylation as the most likely mechanism (Kim, Im et al. 2000). It is thought that repression of Tgfbr2 is responsible for tumor resistance to TGF-β. Methylation of CpG islands in the promoter region of Tgfbr2 represses transcriptional activity. Other studies have also shown that Tgfbr2 is regulated by distinct ets family members. The ets family consists of potent transcriptional transactivators. Disregulation of the ets family has been shown to lead to loss of TβRII expression (Macleod, Leprince et al. 1992). Microsatellite instability also led to TβRII frameshift mutations in colon cancer (Markowitz, Wang et al. 1995; Samowitz and Slattery 1997). There are many potential mechanisms that can lead to loss of TβRII expression. It is important to determine the mechanism of prostate stromal loss of TβRII.

It is possible that compensatory signaling by Activin and its cognate receptors may provide Smad protein activity similar to TGF-β. Activin is known to be active in fibroblasts. Thus, in the Tgfbr2$^{\text{fspKO}}$ stromal cells it is likely that Activin signaling acts as a compensatory signaling pathway. This may be why many organs and tissues in the Tgfbr2$^{\text{fspKO}}$ mouse do not have an overt phenotype. In Tgfbr2$^{\text{fspKO}}$ mouse prostate tissues, phosphorylated Smad2 and Smad3 immunohistochemistry revealed focal expression (data not shown). The knockout was not in 100% of the prostate fibroblasts, thus some of this signaling was due to the incomplete knockout. It is also likely that this was also due to compensatory Activin signaling also leading to phosphorylated Smad2 and

Smad3 activation. Further studies are needed to verify the involvement of Activin signaling in the Tgfbr2^{fspKO} stromal cells.

The majority of our studies on BMDC and MSC recruitment were performed in wild type mice. It will be important to determine how BMDCs and MSCs are recruited in transgenic mouse models of prostate cancer. With improved imaging techniques, monitoring recruitment from the stages of cancer initiation to development of CRPC will be important. This will provide insight for BMDC therapy to determine when inhibition of recruitment would be the most effective in treating cancer. It is likely that different populations of BMDCs are recruited at different stages of progression. Monitoring individual populations of BMDCs in tumor progression may allow us to determine which populations enhance or inhibit tumor growth. In addition, we must determine what cues trigger their recruitment. If Rosa/Tgfbr2^{ColTKO} mice are confirmed to develop CRPC, BMDC recruitment studies could be performed as they remain healthy into late adulthood. Recruitment of BMDCs to tumors is likely to give insight on designing targeted therapies for CRPC.

CRPC is thought to arise from clonal selection following androgen ablation through various mechanisms previously described. Cancer recurrence is linked to cell fusion (Dittmar, Nagler et al. 2009). CRPC cells have properties including increased drug resistance, increased resistance to apoptosis, and enhanced malignancy that are known to result from cells that have undergone cell fusion. It is not clear which cell types may fuse to give rise to cancer stem cells with resistance to androgen ablation. Fusion has been shown to occur in an effort to

restore degenerated tissue function. The tumor microenvironment on its own, but even more prevalent during cancer therapy, has tissue destruction and remodeling, increased inflammation, and thus enhanced recruitment of various BMDCs. This suggests that androgen ablation regimens to treat prostate cancer will promote recruitment of BMDCs capable of fusion with cancer cells that may actually lead to more aggressive cancer upon recurrence. Fusion of MDA-MB435 breast cancer cells has been shown to occur spontaneously (Duelli and Lazebnik 2003). Evidence of cell fusion in humans also exists. Renal cell carcinoma patients that received bone marrow transplants had donor DNA within their cancer cells suggesting fusion occurred (Chakraborty, Lazova et al. 2004; Yilmaz, Lazova et al. 2005). Increasing evidence for fusion in cancer suggests that BMDC therapy may inevitably result in cell fusion with tumor cells or other cells within the body. It may require extra safeguards that need to be engineered into immune cell therapies such as a self-destruct function. BMDCs or immune cells would need to induce apoptosis once their task was completed or after a given period of time to avoid acquiring mutations.

Concomitant with age, the immune system becomes less effective at fighting off illness and disease. Cancer incidence therefore increases with age. One could envision that as our understanding of the immune system and other processes increases we will develop preventative measures to boost our immune systems as a way to eradicate cancer as it develops. Preventative maintenance for automobiles consists of regular oil changes and tune-ups to keep vehicles running smoothly. Analogous to automobile maintenance, "immune system tune-

ups" could keep the body in better shape to detect and prevent cancer. As discussed previously, dominant negative-TβRII CD4+ and CD8+ T cells were able to eradicate cancer in mice. Using this principle, protective measures may consist of periodically boosting our immune systems by harvesting our own cells, altering them to detect and destroy cancer, and giving them back to elicit a protective immune response to cancer. This concept may be years away from inception as a preventive measure given our limited knowledge. However, clinical trials are currently being investigated to treat tumors and diseases in this manner. At late stage cancer progression when patients have no other alternatives, immune therapy may be desirable even without foresight for long-term side effects. As described in these studies, the use of MSCs as a potential targeted therapy for CRPC shows promise with correct manipulation to inhibit tumor progression. As our knowledge expands, treatment and preventative options increase. The future for cancer treatment holds promise.

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