CANCER BIOLOGY

A ROLE FOR ESTROGEN RECEPTOR AND THE ESTROGEN-REGULATED PROSTEASE CATHEPSIN D IN STROMALLY-DRIVEN PROSTATIC CARCINOGENESIS

FREDDIE LEE PRUITT III

Dissertation under the direction of Professor Simon W. Hayward

Stromal-epithelial interactions are important in both prostate development and cancer. Stromal changes have been shown to be powerful prognostic indicators of prostate cancer progression and of patient death helping to define lethal versus indolent phenotypes. The specific molecular underpinnings of these interactions are incompletely understood. Several molecules found to be aberrantly expressed in cancer associated fibroblasts (CAFs) (including cyclin D1 [CD1], stromal derived factor 1 [SDF-1]) contribute to tumorigenesis and malignant transformation in xenograft experiments. These molecules can be regulated by a number of different factors, but are both putative estrogen regulated genes. In this study, we show that dysregulation of ERα expression in cancer associated stroma results in the differential regulation of estrogen responsive genes that are key factors in enhancing the invasive potential of the epithelial tumor. The cell cycle regulator CD1 and the estrogen receptor are known to interact and can induce estrogenic gene transcription. Cathepsin D

(CathD) is an estrogen regulated aspartic endopeptidase, known to be involved in a number of physiological processes as well as in the regulation of apoptosis. In this study, we highlight CathD as a mediator of cancer associated stromal promotion of prostate tumorigenesis. An examination of human prostate tissue revealed significantly increased stromal staining of CathD in malignant prostate tissue in comparison to benign prostate tissue. Stromal specific overexpression of CathD in benign prostate stromal cells induced malignancy in adjacent epithelium through increased TGFβ signaling and responsive gene expression. The proteolytic function of stromally-derived CathD is dependent on the activity of hydrogen-proton pump activity on the surface of prostate epithelial cell lines. The study presented here indicates that CathD is not only an important mediator of stroma-epithelial cross talk, but also an essential component in promotion of tumorigenesis *in vivo*, and Inhibition of ER signaling in the cancer associated stroma inhibits malignant transformation in the adjacent epithelium.

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That Man

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We did it babe!

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AI – Androgen Insensitive

AR – Androgen Receptor

AS – Androgen Sensitive

bFGF – basic Fibroblastic Growth Factor

BPH – Benign Prostatic Hyperplasia

CAB – Combined Androgen Blockade

CAFs – Cancer Associated Fibroblasts

CathD – Cathepsin D

CD1 – Cyclin D1

ChIP – Chromatin Immunoprecipitation

Col. IVα2 – Collagen Type Four Alpha 2

CR – Castration Resistance

CRPCa – Castration Resistant Prostate Cancer

CSC – Cancer Stem Cells

CT – Computed Tomography

CZ – Central Zone

DES – Diethylstilbestrol

DHT – Dihydrotestosterone

DRE – Digital Rectal Exam

ERE – Estrogen Response Element

EIPA – 5-(N-ethyl-N-isopropyle)-amiloride

ERα - Estrogen Receptor Alpha

ERβ - Estrogen Receptor Beta

ERSPC – European Randomized Study of Screening from Prostate Cancer

GAG - Gylcosaminoglycans

GnRH – Gonadotropin Releasing Hormone

HS – Heperan Sulfate

I^{125} – Iodine 125

IGF-I/ II – Insulin-like Growth Factor 1 and 2

HPC – Hereditary Prostate Cancer

LH – Leutinizing Hormone

LHRH – Leutinizing Hormone Releasing Hormone

MMR – Mismatched Repair

NE – Neuroendocrine

NEPCa – Neuroendocrine Prostate Cancer

PCa – Prostate Cancer

Pd^{103} – Palladium 103

PSA – Prostate Specific Antigen

Pz – Peripheral zone

RALP – Robotically Assisted Laparoscopic Radical Prostatectomy

ROS – Reactive Oxygen Species

SV – Seminal Vesicles

TEM – Trans-Endothelial Migration

TGFβ – Transforming Growth Factor Beta

TNM – Tumor Lymph Node Metastasis

TZ – Transitional Zone

UGE – Urogenital epithelium

UGM – Urogenital Mesenchyme

UGS – Urogenital Sinus

XMRV – Xenotropic Murine Leukemia Virus-related Virus

CHAPTER 1

GENERAL INTRODUCTION

1.1 Cancer development and metastasis

During normal tissue homeostasis, quiescent, terminally differentiated cells are eliminated by apoptosis (Kerr et al., 1972), and replaced by progeny of adult tissue specific stem cells (Ferrari et al., 1998), resulting in the steady state renewability of a particular organ. However, the process by which normal proliferating cells enter quiescence can go awry, and lead to a population of cells characterized by the ability to grow and divide without respect to normal limits (Kerr et al., 1972). The building blocks for organs in the human body are cells and tissues. As an organ develops, the cells which compose its various tissues undergo a predetermined number of proliferative cycles (Hayflick, 1965) along with defined stages of differentiation, until a mature organ is formed. Some organs are capable of self renewal: a select population of cells, called stem cells, will proliferate in a controlled manner in order to replenish the lost cells.

Cancer is a condition in which cells obtain the ability to grow in a poorly controlled or uncontrolled manner. The Greek physician and father of medicine, Hippocrates, observed that the blood vessels that surrounded a tumor mass resembled that of the crab claw within the

constellation of stars termed cancer, thus explaining the ancient origin of this modern clinical term (Karpozilos and Pavlidis, 2004; Miller et al., 1979). The Greek translation for crab is karkinos, which translates in English to carcinoma. A carcinoma is, by definition, any cancer which originates from an epithelial cell. Carcinomas are the most common type of cancer diagnosed in humans. The term “tumor” denotes a neoplasm with growth in region of the normal surrounding tissue (McKinnell, 1998).

Tumors exist in one of two states; benign and malignant. Benign tumors are categorized as being a well-organized, relatively differentiated and rarely cause death. Examples of benign tumors include adenomas and lipomas, and in the prostate the condition benign prostatic hyperplasia (BPH). While untreated BPH could cause death by bladder outlet obstruction putting the patient into urinary retention, this is a secondary consequence of the disease, rather than the aggressive invasive mechanisms utilized by malignant tumors. Unlike benign tumors, the malignant counterparts are less differentiated, and have the ability to kill their hosts (McKinnell, 1998). Cell differentiation is the process by which cells acquire the specialized properties that distinguish different types of cells from each other. In general there is a balance between cell proliferation and differentiation. Perhaps more importantly, malignant tumors posses the ability to invade and destroy the normal surrounding

areas of the diseased organ, as well as other organs. This acquired ability to invade the surrounding tissue allows for malignant cells to disseminate, implant, and colonize into distal organs, a process referred to as metastasis.

Metastasis is an event by which the development of secondary tumor appears in distant organs (Miller et al., 1979). Malignant tumors of different origins can metastasize to a variety of sites throughout the body. Furthermore, specific cancers have the ability to metastasize to preferred sites (McKinnell, 1998). However, the steps involved in the process of metastasis are common to all. As denoted in *Figure 1* the general mechanism for tumor metastasis are as follows: Once a tumor has developed to a size of approximately 2mm (1) the overproduction of proteases by the tumor, allows for degradation of the epithelial basement membrane. These cells also begin to secrete pro-angiogenic factors. (2) Angiogenesis, the formation of new blood vessels from preexisting vessels which is initiated by factors secreted from the developing tumor. The newly formed vessels provide nutrients to the growing tumor, and also provide a route for dissemination. (3) Tumor cell detachment and dissemination. Through differential regulation of adhesion molecules and proteases, tumor cells acquire motility. (4) Tumor cell intravasation into blood vessel (5). Arrest and adhesion of tumor cell at distal site. (6) Extravasation of tumor cell from blood vessel into

the stroma of distal organ. (7) Tumor cell proliferation and establishment of distal organ metastasis (Hanahan and Weinberg, 2000a).

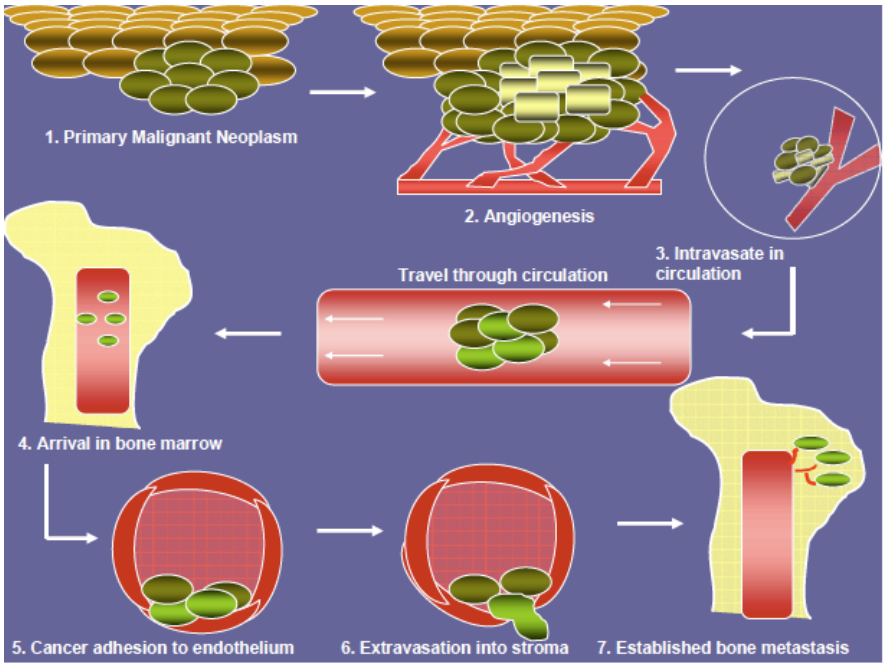

Figure 1.1 Cancer metastasis model. Diagrams of the general events leading to the establishment of metastatic cancer**.** After the development of a primary malignant neoplasm **(1),** angiogenesis occurs that provides the growing tumor with a blood supply and a route for metastasis to occur **(2)**. Tumor cells intravasate into the circulatory system **(3)**, and eventually arrest in a distal organ like the bone **(4)**. To arrest, cancer cells adhere to the endothelium **(5)**, which allows for the extravasation of the cancer cell into the stroma of the organ **(6)**, and eventually a successful metastasis is established **(7)**.

As previously mentioned, different cancers show a predilection for metastasis to specific distal target tissues, an idea explored in the late 19th century by Paget in his classic “seed and soil” hypothesis (Paget, 1889). One common distal organ that metastatic cancer cells colonize is bone. For example, lung, breast, and prostate cancer

(PCa) cells metastasize to bone in a non-random manner (Nicolson, 1993). A key point to remember when considering metastasis is that surgery and radiation therapies are ineffective as curative therapies. The current standard therapy for treatment of secondary metastasis is with the use of cytotoxic chemotherapeutics (Nicolson, 1991). Initial treatments of metastatic cancers with chemotherapeutics individually or in combinations have shown some efficacy in killing the metastatic tumors, unfortunately, such approaches are not curative for most metastatic tumors (Terranova et al., 1986). Metastatic tumors acquire resistance to the chemotherapeutics by mechanisms not fully understood to date. The development of chemoresistant tumors ultimately leads to the death of affected person through the development of secondary metastasis to vital organs such as brain and kidney which leads to organ failure (Kaplan et al., 2006).

1.2 Prostate physiology and development

Prostate Physiology and Function

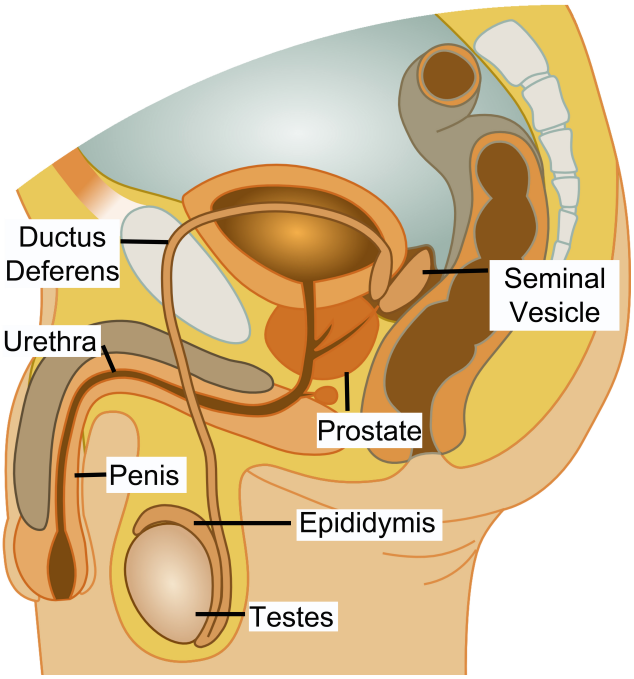

Figure 1.2. The male reproductive system. The sexual reproduction system in men includes the Ductus Deferens, Epididymis, Penis, Prostate, Seminal Vesicle, Testes, and Urethra.
Figure adopted and modified from Van De Graaff, Human Anatomy, Wm. C. Brown: Dubuque, IA, 1988. (Van De Graaff, 1998)

The male reproductive system in humans comprised of the penis, testes, epididymis, ductus deferens, seminal vesicles, and the prostate. The prostate is a walnut sized gland located immediately below the bladder and in front of the rectum. While it is believed that the prostate gland enhances male fertility, the prostate is not required for fertility, and is thus classified as a male accessory reproductive organ (Rui et al., 1986). The human prostate gland has commanded a considerable amount of interest from the medical community. It is the only accessory genital organ in men that undergoes abnormal growth in the later stages of life (Berry et al., 1984). The individual

glands that comprise the prostate are arranged in canalized structures and empty their contents into the urethra. The epithelial glands are surrounded by a dense fibromuscular stroma which is controlled by the sympathetic nervous system (Keast, 1999). Contraction of the fibromuscular stroma during ejaculation injects prostatic fluid into the urethra which traverses the prostate. The prostate produces a solution rich in ions, polyamines and proteins including zinc, and citrate, spermine, cholesterol, prostatic acid phosphatase, and prostatic specific antigen (PSA) (Aumuller et al., 1990). This rich solution contributes approximately 20-30% of the total volume of the ejaculate. The testes contribute approximately 5%, and the remainder of the ejaculate volume is produced by the seminal vesicles (SV). Semenogelin, and fibronectin are among the SV derived proteins that are found in the ejaculate (Lilja and Laurell, 1984). The solution produced by the prostate is not strictly essential for fertilization, however, these elements are thought to optimize conditions for fertilization by enhancing sperm motility, providing protection from the low pH of the vagina, and providing nutrients for the sperm (Frick and Aulitzky, 1991).

Secretions from the SV contribute to coagulation of the ejaculate. Liquefaction of the coagulated ejaculate is the result of the protease PSA, a prostate derived kallikrein serine protease (MacLeod and Wang, 1979).

1.3 Steroid hormone biogenesis

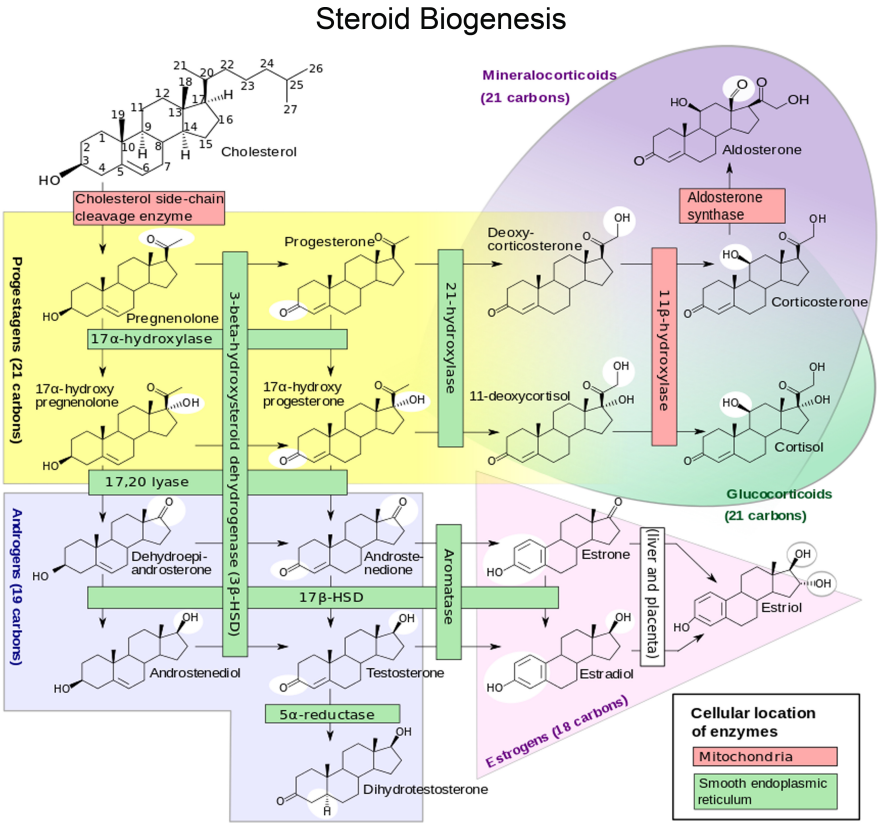

Figure 1.3. Schematic diagram of steroid synthesis from cholesterol. Biosynthetic pathway showing the production Progestagens, Mineralocorticoids, Glucocorticoids, Androgens, and Estrogens from Cholesterol.

Modified figure adopted Boron, Medical Physiology: A Cellular And Molecular Approach; Elsevier/Saunders 2003. (Boron and Boulpaep, 2003)

Steroid biosynthesis is an anabolic metabolic pathway that produces steroids from cholesterol. There are five major classes of steroid hormones in humans; progestagens (21 carbons), mineralocorticoids (21 carbons) glucocorticoids (21 carbons),

androgens (19 carbons), and estrogens (18 carbons). Progesterones serve as precursors to all other human steroids. Cholesterol, produced mainly in the liver, is initially converted into pregnenolone by cytochrome P450 in the mitochondria (Shikita and Hall, 1974).

Pregnenolone is converted to progesterone, and is further reduced to deoxycorticosterone the precursor for mineralocorticoids. Mineralocorticoids are produced in the adrenal cortex and are responsible for maintaining electrolyte and water balance in mammals (Axelrad et al., 1954).

In the production of glucocorticoids, pregnenolone is further reduced to 17α-hydroxy pregnenolone which serves as a precursor for glucocorticoids, and androgens. In the synthesis of glucocorticoids, 17α-hydroxy pregnenolone is converted to 17α-hydroxy progesterone, which can also be produced directly from progesterone by the enzyme 17α-hydroxylase (Nakajin et al., 1984). 17α-hydroxy progesterone is processed to 11-deoxycortisol and then to the glucocorticoid, Cortisol. Cortisol is also produced in the adrenal cortex and functions to regulate gluconeogenesis (Weber et al., 1967).

Androgen production occurs when 17α-hydroxy pregnenolone is converted to dehydroepiandrosterone, the precursor for androstenediol and androstenedione. Testosterone is the primary male sex hormone that is produced almost exclusively by the testis,

and in smaller quantities in humans (but not mice) by the adrenal cortex (Cohn and Mulrow, 1963; Lipsett and Tullner, 1965). Testosterone can be produced directly from androstenediol, or androstenedione. Once in the prostate or other peripheral tissues, testosterone is converted to the more potent androgen dihydrotestosterone (DHT) by the enzyme 5α-reductase (Farnsworth, 1963). 5α-reductase is expressed as two isotypes that are differentially expressed in tissues (Andersson et al., 1991; Andersson and Russell, 1990). Type II 5α-reductase is expressed in the stroma and epithelium of the prostate (Normington and Russell, 1992).

Both testosterone and DHT can activate AR transcriptional activity. Physiologically, testosterone and DHT differ based on their interaction with the AR. Stabilization and function of the AR require prolonged ligand occupancy. Testosterone dissociates from the AR at a rate three times faster than DHT. Higher concentrations of testosterone are required in order to achieve sufficient AR activation to compensate for the low dissociation constant (Zhou, 1995).

Androstenedione and testosterone serve as precursors for the synthesis of estrogens. The enzyme aromatase, also known as cytochrome P450, is responsible for the reduction of androstenedione to estrone. Estrone is further processed to the biologically active estradiol by 17β-hydroxysteroid dehydrogenase. Aromatase also directly reduces testosterone directly into estradiol

(Goto and Fishman, 1977). The aromatase enzyme is expressed by adipose tissue, osteoblasts, skin fibroblasts, and prostate stroma (Bruch et al., 1992; Fujimoto et al., 1986; Mahendroo et al., 1991; Srinivasan et al., 1995).

1.4 Male urogenital development

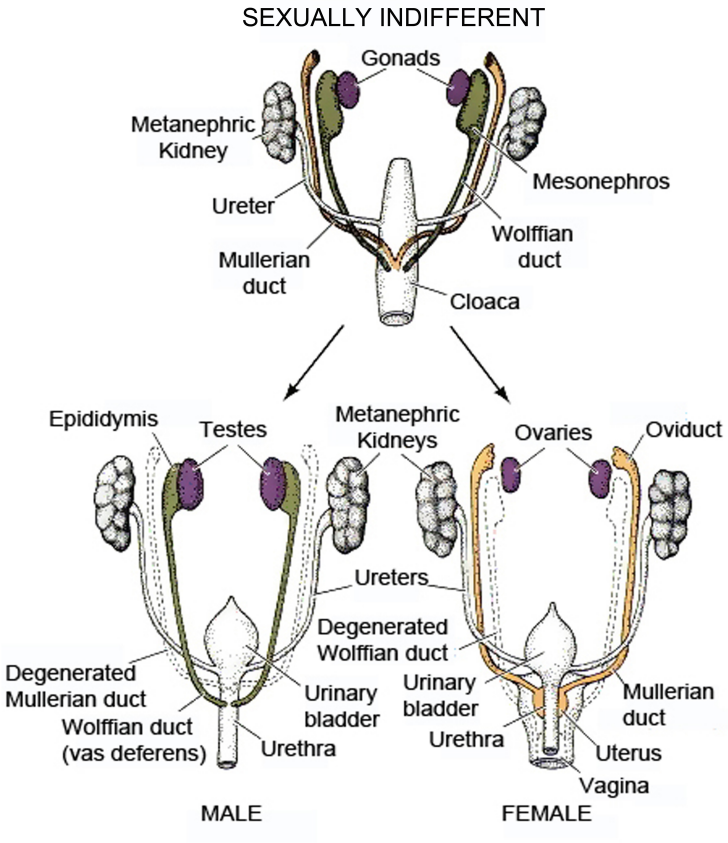

Figure 1.4. Sexual differentiation during embryogenesis. Formation of internal genitalia tract in males (left) and females (right).
Figure adapted from Gilbert SF. *Developmental biology, sixth edition.* Sunderland, MA: Sinauer Associates, 2000.

The genetic sex of an embryo is determined at fertilization, however, mammalian embryos exhibit an ambisexual stage. During this ambisexual stage, the gonads remain undifferentiated, and both sets of gonaducts (Wölffian and Müllerian ducts, also known as mesonephric and paramesonephric ducts respectively) are present (Price, 1947). Masculine morphogenesis occurs between 6-8 weeks of embryogenesis in humans, where the gonads differentiate into testes, and the mesonephric tubules differentiate into the efferent ducts. In an adult

male, the efferent ducts deliver sperm from the testes to the epididymis. The Wölffian ducts differentiate into the epididymis, ductus deferens, and SV while a diverticulum of each duct grows to the developing kidney and forms the ureters. In males the Müllerian ducts degenerate, under the influence of antimüllerian hormone (also known as Müllerian inhibiting substance), except for the tail portion fused with urogenital sinus (UGS) which develops into the prostatic utricle. In female morphogenesis, the Wölffian ducts and mesonephric tubules, with the exception of the section that becomes the ureters, degenerate due to the absence of androgenic stimulation. While the gonads develop into ovaries, and the Müllerian ducts differentiate into the oviducts, uterus, cervix, and the upper portion of the vagina. The UGS give rise to the lower portion of the vagina and forms the urethra. In male embryos, under the influence of androgens the UGS differentiates to become the bulbourethral glands, urethra, and prostate (Zuckerman and Groome, 1940).

Masculine morphogenesis is attributed to the production testicular hormones around fetal week 8. Antimüllerian hormone, a testicular factor secreted specifically by Sertoli cells, acts to suppress Müllerian duct development into female specific structures. However, if antimüllerian hormone production is inhibited, a uterus and fallopian tubes will develop in a genotypic male (Wilson et al., 1981). The default phenotype during sexual morphogenesis is female, unless

both androgens and antimüllerian hormone is secreted. Leydig cells secrete testosterone which acts specifically in Wölffian duct morphogenesis (Siiteri and Wilson, 1974). The differentiation of the UGS is controlled by dihydrotestosterone (DHT), the reduced form of testosterone (Imperato-McGinley et al., 1985).

1.5 Prostate development

The human prostate develops from the direct interactions between the epithelium of the urogenital sinus (UGE) and its surrounding mesenchymal (UGM) (Cunha, 1972a). During the eighth week of fetal development, the production of androgens from Leydig cells of the testis is initiated (Siiteri and Wilson, 1974). The interactions of testicular androgens with the androgen receptors (AR) of the UGM elicit the proliferation and expansion of UGM. Signaling elicited by androgens in the UGM induces prostatic budding of the UGE around fetal week 10, followed by growth and branching morphogenesis, through the production of UGM-derived mitogens, which diffuse into the epithelial compartment (Kellokumpu-Lehtinen, 1985; Kellokumpu-Lehtinen et al., 1980). Signaling from the UGM also initiates epithelial differentiation into secretory luminal and basal epithelial cells. The communication between UGM and UGE is not unidirectional, a secretory/chemical dialogue exists between the

UGM and the UGE. Signaling from the UGE induces differentiation of the UGM into smooth muscle and fibroblast cells (Cunha, 1972c).

Growth and differentiation of the epithelial compartment via the paracrine-acting secretory effects of the UGM is spatially controlled. Spatial signaling has been documented in the development of the rat prostate. The rat prostate consists of four distinct lobes, designated ventral, dorsal, lateral and anterior lobes. The lobes are named based on their locations relative to the urethra. Characterization of the rat prostate has shown the different lobes vary based on morphology and secretory products (Jesik et al., 1982). Heterotypic tissue recombinations of rat UGM and adult rat ductal tissue showed that the fetal UGM can induce prostatic ductal growth and morphogenesis and change the expression of secretory proteins that are not expressed from the original lobe of the prostatic ductal tissue (Hayashi and Cunha, 1991; Timms et al., 1995).

1.6 Zonal prostate anatomy

The prostate is a glandular organ primarily comprised of secretory luminal epithelial cells, which are proximally surrounded by basal epithelial cells. A third cell type, neuroendocrine (NE) cells comprise a minor proportion (approximately 1%) of the glandular component of the prostate (Sun et al., 2009; Vashchenko and Abrahamsson, 2005; Yuan et al., 2007). NE cells are hypothesized to

play important roles in prostate development and function, however, little evidence exists to provide significant insights (Zong and Goldstein, 2012).

In the adult human, the fully developed prostate consists of 3 zones; the peripheral zone, the central zone, and the transitional zone, all of which is surrounded by a dense fibromuscular stroma. The peripheral zone (Pz) encompasses the majority (70-75%) of the adult prostate, and it is the sub-capsular portion of the prostate which surrounds the distal urethra (McNeal, 1968). It is in the Pz where a majority of PCa occur (70%) with greater frequency than any other zone (McNeal et al., 1988b). The central zone (Cz) comprises 20-25% of the adult prostate mass, and surrounds the ejaculatory ducts (McNeal, 1981). Cancer rarely originates in the Cz with a rate of 5-10% of all PCa cases (McNeal et al., 1988b). The transitional zone (Tz) comprises the remaining 5% of the total prostate mass, and it surrounds the urethra. 20-25% of PCa originates in this zone (McNeal et al., 1988b).

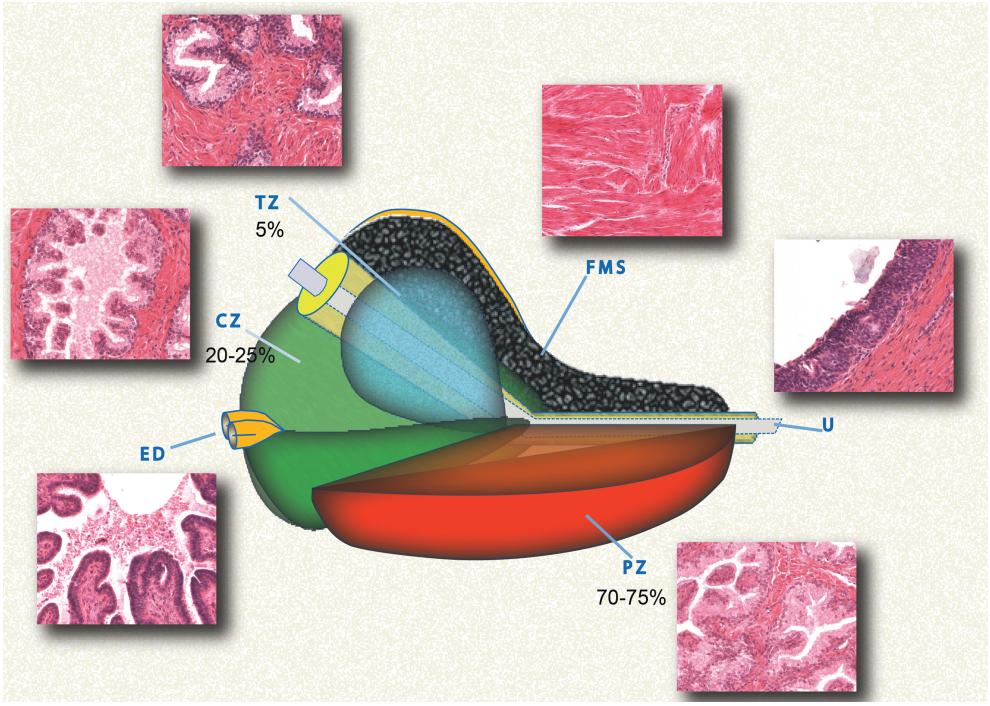

Figure 1.5. Zonal anatomy of the human prostate.
The transition zone (Tz) makes up approximately 5% of the prostate's mass, and surround the urethra (U) located below the neck of the bladder. The ejaculatory ducts (ED) surrounded by the central zone (Cz) which comprises 20-25% of the prostate's mass. The Cz is surrounded by the peripheral zone (PZ) which makes up 70-75% of the prostate. The entire prostate is surrounded by the fibromuscular stroma (FMS).
Figure adopted and modified from Jiang, M *et al*. 2011. (Jiang et al., 2011

1.7 Prostate cancer

Statistics

Worldwide, PCa is the second most commonly diagnosed cancer in men (Siegel et al., 2012) and is the most commonly diagnosed non-cutaneous form of cancer found in American men (Siegel et al., 2012). Over 899,000 men worldwide were diagnosed with PCa in 2008. Of new diagnoses in 2012; 241,740 American men were diagnosed with PCa, resulting in 28,170 deaths (Siegel et al., 2012). Men in western countries have a greater risk of developing PCa, and more specifically, African American men are at the greatest risk (Gronberg, 2003). African American men are about 1.6 times more likely to develop PCa than their Caucasian counterparts, and are twice as likely to die from PCa (Siegel et al., 2012).

When detected early, men diagnosed with PCa have a disease-specific five year survival rate of 100% and a ten year survival rate of 95%. Unfortunately most men that present with urological complications associated with their prostate are diagnosed with an advanced form of PCa. The five year survival rate of men with advanced PCa and distal metastasis is 34% (Siegel et al., 2012).

Development and Progression

PCa development, like most cancers, can be classified in four distinct phases: Initiation, promotion, malignant conversion, and propagation (Boutwell, 1974; Hennings et al., 1983; Rous and Friedewald, 1944; Slaga et al., 1980). Tumor initiation results from a mutation in the DNA. Initiated cells are irreversibly altered and are considered to be “initiated” and at a greater risk for malignant conversion than normal cells (Mottram, 1944). More than 30 years ago, it was proposed by Knudson, that two mutations are necessary for carcinogenesis to occur. The Knudson hypothesis states that a recessive disease could be inherited on a cellular basis with a high penetrance, as long as there was a high probability of somatic mutation in the wild type allele in a susceptible cell (Knudson, 1971). Tumor promotion can be accomplished through the repeated exposure to a non-tumorigenic agent, that stimulates the growth of the tumor. The effects of a single tumor promoter are reversible, but it is repeated exposure to promoting agents that allow for the clonal expansion of an initiated cell into a focus of cells or a small neoplasm (Berenblum and Shubik, 1949). Malignant conversion is described as the transformation of a pre-neoplastic cell into a cell which expresses a malignant phenotype (Holley, 1972). Once transformed to the

malignant state, propagation or progression of the malignant cell is an increase into an aggressive phenotype overtime.

Androgen deprivation therapy for the treatment of advanced PCa allows for the emergence of a castration resistant (CR) disease state and the eventual progression to metastatic disease. One model suggests that the development of a CR state is an adaptation to castration. This work suggests that the change in prostate environment induces the transformation in the prostate cells to a hormone independent state. In contrast to this model it has been argued by Isaacs that the initial PCa is composed of a heterogeneous population of androgen sensitive (AS) and castrate resistant PCa (CRPCa) cells (Isaacs and Coffey, 1981). According to this model, the post-castration progression of PCa to a CR state is inevitable consequence of castration due to selective pressure for cancer cells that are already androgen resistant.

In line with the previous statement about selective pressure for the emergence of an aggressive phenotype, castration therapy also allows for the emergence of NE tumors. Up to 30% of late stage PCas have a predominance of NE differentiation (Abrahamsson and di Sant'Agnese, 1993; di Sant'Agnese, 1992). NEPCa is more aggressive than non-NE advanced PCa. Men diagnosed with NEPCa have a life expectancy of approximately one year. One explanation for the poor prognosis associated with NEPCa is their CR phenotype,

due to lack of AR expression (Abrahamsson et al., 1987; Cohen et al., 1990). A caveat to the clonal selection model for the development of CRPCa is the inability to isolate cells that are already CR from either PCa cell lines or human tumor tissue. An emerging new model for the development of CRPCa is the cancer stem-cell (CSC) hypothesis.

CSC are a minority of tumor cells with ability for self renewal and differentiation (Kasper, 2008; Maitland and Collins, 2005; Reya et al., 2001). This model proposed that the tumor is comprised of a heterogeneous population of cells that exist in a hierarchical lineage relationship with different proliferative potentials. The CSC hypothesis was first described in acute myeloid leukemia in 2005, and has since become the established model (Huntly and Gilliland, 2005). Unlike the clonal selection model for CRPCa, CSC have been identified in clinical PCa specimens based on the expression of panels of markers expressed in stem cell, and have been demonstrated to have self-renewal properties (Collins et al., 2005). It is estimated that CSC comprise approximately 0.1% of the tumor population. Prostate CSCs are thought to be derived from a multi-lineage stem cell, which 1) develops over time from the accumulation of genetic alterations (Liu et al., 2011; Reya et al., 2001; Sell and Pierce, 1994), or 2) result from the arrest of differentiation in stem cell development (Sell, 1993; Wang et al., 2009). However the prostate

CSCs develop, it is the selective pressure from castration therapy which allows for their growth and CR proliferative advantage. Studies have provided evidence that prostate CSC isolated from clinical samples are responsible for PCa initiation, progression to CR and resistance to apoptosis (Bui and Reiter, 1998; Maitland and Collins, 2005).

Initiation of prostate carcinogenesis

When the idea was first proposed, tumor initiation suggested that a chemical carcinogen was the cause for the altered DNA. However, agents for the initiation of human PCa remain to be identified. Several hypotheses have been suggested including obesity, and inflammation leading to the formation of DNA damaging reactive oxygen species (ROS) (Hietanen et al., 1994; Snowdon et al., 1984). In relation to obesity and PCa, findings from the 4 largest patient studies were not conclusive (Moyad, 2002). The current knowledge pertaining to ROS, is insufficient to determine if ROS are a significant cause of PCa (Shan et al., 2011). More recently a γ-retrovirus called xenotropic murine leukemia virus-related virus (XMRV) was reported in human PCa tissues (Fischer et al., 2008; Schlaberg et al., 2009). In general, it is hypothesized that XMRV induces malignant transformation by insertional mutagenesis.

Infection of prostate cells by XMRV resulting in a malignant transformation implies a clonal selection for XMRV positive tumors. However, XMRV DNA is only detected in a minute fraction of prostate tumors cells, this implies that XMRV insertional mutagenesis does not play a causal role in most PCa initiation (Aloia et al., 2010). Aging is the biggest risk factor for the development of PCa. For men over the age of 60, one out of 15 men will develop PCa, compared to one out every 8,500 men under the age of 40 (Siegel et al., 2012). However, studies of prostates from men ranging in ages from 20 to 40 years old have detected histologic foci of PCa (Sakr et al., 1994; Shiraishi et al., 1994; Yatani et al., 1989). These studies suggest that PCa initiation takes place at an early age.

Another risk factor for PCa development is family history. In fact, a positive family history of PCa is reported to increase the risk for cancer development in relatives by 2-fold (Kalish et al., 2000). The mode for PCa inheritance is uncertain, but through linkage analysis a model system can be generated that approximates the mode of inheritance. Genomic analysis of 1,233 families with PCa showed evidence of linkage to five regions; 5q12, 8p21, 15q11, 17q21, and 22q12 (Xu et al., 2003). The first hereditary prostate cancer locus to be identified was mapped to chromosome 1 (1q24-25). This gene was called hereditary prostate cancer (HPC) susceptibility gene, HPC2/ELAC2 (Gronberg et al., 1997). The

common missense mutations in HPC2/ELAC2 are a Ser-Leu change at amino acid 217, and an Ala-Thr change at amino acid 541. It has been show that men who carry the Leu217/Thr541 variants had an increased probability for developing PCa (Rebbeck et al., 2000). BRCA1 and BRCA2 mutations are two highly penetrant genes that predispose individuals to breast cancer development. In PCa, BRCA1 and BRCA2 carriers show a 3-fold and 4-fold increased risk of development (Gayther et al., 2000). Another PCa susceptibility locus mapped to chromosome 17 is the HOXB13 gene. Expression of this HOXB13 variant is associated with a 20-fold higher risk of PCa development (Breyer et al., 2012; Stott-Miller et al., 2013). The evidence presented does make a strong argument for an inherited mutation serving as an initiator in PCa development. However, the genetic influences responsible for the development of PCa remain largely unidentified. Genome-wide association studies simply identify genes capable of affecting the risk for the development of PCa.

Promotion of prostate carcinogenesis

Strong evidence exists that androgens act as a promoter during the development of PCa (Bosland, 1992; Noble, 1977). Tumor promoters are usually non-mutagenic and non-carcinogenic. The mode of action for a promoter is usually to increase the rate of

proliferation of an initiated cell, thus increasing the rate of tumor formation (Bosland, 1992). Within normal prostate epithelial cells, the interaction of androgens with the AR control the rate which epithelial cells proliferate (Evans and Chandler, 1987). Huggins and Hodges in 1941 demonstrated that lowering testosterone levels through castration or through supplementation with diethylstilbestrol (DES) in dogs produced a marked decrease in the size of the prostate gland (Huggins, 1945). Studies in animals treated with testosterone presented an increased incidence of tumor formation in their dorsal prostate, it was concluded the testosterone is a strong promoter of tumorigenesis in rat prostate (Noble, 1977). Thus it could be possible for androgen to be a major contributor towards the promotion of an initiated prostate epithelial cell. Men who are castrated early in life do not develop prostatic disease as they age (Scott, 1953). Androgen depravation by castration induces regression of the prostate by apoptosis, primarily in the epithelium (Kyprianou et al., 1990; Kyprianou and Isaacs, 1988). However, regeneration of the prostate will occur in a castrated being if androgens are restored (Bruchovsky et al., 1975; Sugimura et al., 1986). As previously stated, the mode of action for a promoter is usually to increase the rate of proliferation. The reintroduction of androgens into a castrated host inducing the re-growth of the prostate, supports the hypothesis of androgens acting as a promoter in the development of PCa.

Conversion to malignancy in prostate cancer

The repeated promotion events initiated by androgens allow for the propagation of the initiated cell population (Hayward et al., 2001). Initiated cells in the prostate have a growth advantage which increases the risk for a conversion to malignancy because of the infidelity of the DNA replication machinery (D'Antonio et al., 2009; Loeb and Loeb, 2000). Errors such as base deletions or additions made by DNA polymerase have been identified in every polymerase studied *in vitro*, and have been implicated in the development of human cancer (Buermeyer et al., 1999; Kunkel, 2004; Kunkel and Bebenek, 2000). Mutations have been identified in loci coding for DNA mismatched repair (MMR). Loss of function in MMR leads to the accumulation of errors made by DNA polymerase that are normally repaired. The accumulation of errors in DNA culminates in genomic instability (Loeb and Loeb, 2000). Karyotype analysis of all cancers have demonstrated chromosomal rearrangements and duplications, which are the hallmark of genomic instability. Mutations in the MMR genes MLH1, MSJ2, MSH6, and PMS2 are predominantly associated with colon and endometrial cancers (Vasen et al., 2007), however, studies have shown that mutations in these MMR genes confer a high risk for the development of PCa (Grindedal et al., 2009). Human

PCa is multifocal and heterogeneous in nature as a direct result of genomic instability. Primary tumors contain numerous independent cancer foci that are genetically distinct (Aihara et al., 1994; Bostwick et al., 1998; Macintosh et al., 1998). Chromosomal rearrangements in PCa have been identified in chromosomes 7, 8, 10, 13, 16, 17, and 18 (Chen et al., 2001). From these observations it is believed that chromosomal arrangements may contribute to malignancy and more aggressive prostate cancer phenotype (Suzuki et al., 1998; Taylor et al., 2010).

PCa progression

The progression of cancer is described as the tendency for the cancer cells to acquire a more aggressive phenotype over time (Hanahan and Weinberg, 2000b). The increase in aggressive characteristics is a result of more genomic alterations as described in conversion to malignancy. By the end of the progression phase, the initiated, propagated, and converted prostate epithelial cell has been fully converted into a neoplasm (Thiagalingam, 2006). In general as neoplasms, these cells have evolved mutations which allow for them to escape the physical restraints of the microenvironment, as well as regulatory growth restraints (Hanahan and Weinberg, 2000b).

As clinical treatments for advanced PCa can act to select subpopulations of PCa cells which fail to respond to continued treatment, it can be argued that these treatments eventually act to further progression. For example, the observations made by Huggins and Hodges established androgen ablation therapy as the most effective treatment for men with locally advanced and metastatic PCa to date (Denmeade and Isaacs, 2002). The initial effects from androgen ablation are very positive, with 80-90% of men showing a response (Huggins and Hodges, 1972). The removal of androgens is accomplished by chemical and/or surgical castration (orchiectomy), which results in the rapid regression in androgen dependent prostate tissue. However, the positive effects seen from ablation therapy are only transient, as men that undergo ablation therapy relapse as the cancer develops to a CR disease. CRPCa poses a major clinical obstacle because there is no cure. These CR cells will eventually repopulate the prostate and become more aggressive. Thus castration, while initially very beneficial for the treatment of advanced PCa, can result in disease progression.

The exact mechanism for the development of CR is unknown, however, several molecular mechanisms are under investigation. 1) Amplification of the AR, 2) Gain of function mutations in the AR, 3) De novo synthesis of androgens, 4) Growth factor signaling leading to ligand-independent AR activation, 5) Inflammatory mediators.

1) Amplification of the AR gene.

The gene coding for the AR was reported to be amplified in patients who had failed castration therapy (Koivisto et al., 1997). In a study of 54 prostate cancer patients who had failed therapy, genomic wild-type AR amplification was associated with increased mRNA expression in 28% of the patients analyzed, and concluded the clonal expansion of tumor cells with amplified AR allows androgen dependent growth in the presence of castrate levels of circulating androgens (Koivisto et al., 1997).

2) Gain of function mutations in the AR.

Numerous reports have identified molecular changes in the AR which allow for continued activation of AR signaling pathways in castrate conditions by mutations in ligand binding domain that aid in greater androgen sensitivity (Taplin, 2003), increased AR protein stability (Robzyk et al., 2007), ligand-independent AR activity (Hu et al., 2009), novel responses to other steroid hormones (Zhao et al., 2000), increased recruitment of AR coactivators (Brooke et al., 2007), and splicing variants that produce a constitutively active AR (Dehm et al., 2008).

3) De novo synthesis of androgens

Under androgen deprivation conditions recurrent PCa retains AR expression and AR-regulated protein expression (Edwards et al., 2003; Linja et al., 2001). This lead to the hypothesis that recurrent PCa develops the ability to biosynthesize androgens. Studies of men with recurrent PCa found levels of DHT in tissue were sufficient for AR activation (Titus, 2005). Other studies have found increased expression of genes involved in the conversion of adrenal androgens to testosterone in CRPCa patient tissues (Montgomery et al., 2008) (Stanbrough et al., 2006).

4) Growth factor signaling leading to ligand-independent AR activation.

Transgenic mouse models have been developed that provide a molecular insight into the development of hormone refractory disease during early stages of carcinogenesis. Prostate epithelial cells from the double knockout $Pten^{-/-}$;$Nkx3.1^{-/-}$, which is characterized as developing prostatic intraepithelial neoplasia and progressing to adenocarcinoma, proliferate in the absence of androgens (Gao et al., 2006). This CR proliferation is linked to increased Akt and Erk signaling in the epithelium which opposes the pro-apoptotic stimuli coming from the stroma (Gao et al., 2006).

5) Inflammatory mediators.

Resistance to castration therapy is enhanced by inflammatory mediators. Production of interleukin-1β by macrophages can lead to the repression of the AR co-repressor complex in PCa cell lines. This repression converts AR antagonists into AR agonists (Gong et al., 2006).

Androgen deprivation therapy for the treatment or advanced PCa consequently allows for the emergence of an AI disease state and the eventual progression to metastatic disease. One model suggests that the development of PCa to an AI state is an adaptation to castration (Isaacs and Coffey, 1981). This model suggests that the change in prostate environment induces the transformation in the prostate cells to a hormone independent state. In contrast to this model it is argued by Isaacs that the initial PCa is composed of a heterogeneous population of androgen sensitive (AS) and AI PCa cells (Isaacs and Coffey, 1981). According to this model, the post-castration progression of PCa to an AI state is inevitable consequence of castration due to selective pressure for cancer cells that are already androgen AI.

Screening, Diagnosis, Grading and Staging of Prostate Cancer

Detection of PCa was historically performed by digital rectal exam (DRE). However this would only detect tumors that were in the Pz and large enough to be palpable through the anus (McNeal et al., 1988b). As previously stated, the majority (70-75%) of PCa originate in the Pz, this means the remaining 20-25% of tumors originate in regions not palpable through the rectum (McNeal, 1981). A reliable biomarker/biomarkers of PCa was needed.

The first antigens specific to the prostate were identified in 1960 (Flocks et al., 1962; Flocks et al., 1960). Follow-up studies compared antigenic properties of normal, benign, and malignant prostate tissues identified prostatic acid phosphatase and PSA (Ablin, 1972; Ablin et al., 1970; Ablin et al., 1973). Chemical assays for prostatic acid phosphatase based on activity in citrate and tartrate buffers were developed for commercial use but were problematic (Hudson, 1956; Hudson et al., 1955). The development of immunoassays for PSA detection and the clinical applications for prostate disease was pioneered by the PCa research group at Roswell Park Cancer Institute (Kuriyama et al., 1980; Nadji et al., 1981; Wang et al., 1981; Wang et al., 1979). The first report to show PSA could be detected in serum of men with metastatic PCa was published in 1980 and marked the beginning of the new era of PCa screening by blood testing (Papsidero et al., 1980). The first study to

show that PSA is a tumor marker was performed by Stamey *et al* (Stamey et al., 1987). This study showed serum levels of PSA in patients correlated with advancing stage of PCa, and showed that PSA levels dropped below detectable thresholds after castration (Stamey et al., 1987). This work showed that PSA monitoring could be used to detect a patient's response to therapy. In 1992 the American Cancer Society approved the inclusion of screening PSA in the blood along with DRE for basic check-ups in men over the age of 50, with the acceptable limit for serum PSA of 4.0 ng/ml (Mettlin et al., 1993). PSA screening alone cannot be used as a method for detecting PCa. In the study 473 men screened for PCa using PSA alone, 82 (17.3%) of 473 men had PCa with PSA levels below 4.0 ng/ml (de Koning and Schroder, 1998), indicting that PSA levels are not always elevated in men who have developed PCa.

Recently the use of PSA blood testing in the screening of PCa has come under heavy scrutiny. In the European randomized study of screening for PCa (ERSPC) trial, 182,000 men were randomized to being screened for PCa by DRE and PSA every four years. The study concluded close to 1,400 men needed to be screened, and an additional 48 men with PCa would have to be treated to prevent one PCa associated death. The study also concluded that PSA based screening only reduced PCa associated death rates by 20%, and that PSA screening was associated with over diagnosis of PCa (Schroder

et al., 2009). Based on this study, a U.S. preventative services task force completed an examination on the benefits/ harm of PCa screening. Similar to the Schroder study, the U.S. study concluded there is moderate or high certainty that the service has no net benefit to patients. The findings suggests that PSA screening should not be eliminated, but that screening decisions should be made based on individual patient's risk (e.g. family history, genetic factors) (Schröder, 2011).

At initial onset, PCa is asymptomatic, perhaps due to the fact that the majority of the malignancies arise in the peripheral zone of the prostate distal to the prostatic urethra (McNeal et al., 1988b). Once symptoms present themselves, the disease has often progressed to a locally advanced or metastatic disease (Coley et al., 1997). As the cancer grows in the peripheral zone of the prostate, it begins to infringe on the central prostatic zone adjacent to the urethra. Symptoms associated with advanced PCa include difficulty with initiating urinary flow, interruption or weak stream while urinating, and frequent urge to urinate. If abnormal DRE with a high PSA is detected and/or symptoms associated with PCa are present, a biopsy is performed to examine the differentiation of the prostate gland.

Differentiation of the prostate gland is determined using the Gleason grading system (Gleason and Mellinger, 1974). The Gleason grading system is based entirely on the histologic pattern of

the epithelial cells in hematoxylin and eosin stained prostatic tissue sections. It categorizes tumors into grades 1-5 representing increasingly poor differentiation. The grade of the prostate biopsy is an indication of the likelihood the tumor will spread. Specifically, the method is one of categorization of histologic patterns by the extent of glandular differentiation and the pattern of growth of the tumor compared with the normal glandular structure. The grade for the biopsy determined by the Gleason system is the sum of two scores. The first score represents the predominant histologic pattern seen. The second score represents the second most common histologic pattern. The scores of the Gleason system can be grouped together, a score of 2-4 is well differentiated, 5-7 is moderately differentiated, and 8-10 is poorly differentiated (Gleason and Mellinger, 1974). The Gleason grading system was updated in 2005, because our understanding of PCa has changed since conception of the Gleason grading system in the 1960s. The international society of Urological Pathology concluded that Gleason score 2-4 should rarely be diagnosed on needle biopsy because this is usually inaccurate. There is also a poor correlation with prostatectomy grade, leading clinicians and patients into believing the patient's tumor is indolent. Gleason patterns 3-4, including all cribriform cancers should be graded a 4 because of the rarity of candidates for cribriform Gleason

grade 3, and cribriform pattern 3 cancers almost always occur in association with typical Gleason grade 4 cancer (Epstein, 2010).

Staging is performed to determine how much cancer is present in a person and to determine where the cancer is located. Staging provides information for physicians in order to determine the appropriate course of treatment. Staging is based on 3 factors; 1) The size of the primary tumor, and whether it has spread to nearby areas. 2) presence of lymph node metastasis and 3) presence of distal metastasis. PCa staging is performed using the TNM classification system. T refers to the original tumor. This category describes the size of the tumor and will determine if the tumor has invaded nearby structures. A number is associated with the size and invasiveness of the tumor. This ranges from T_X which means a tumor can not be measured or detected, up to T4. N refers to whether or not the cancer has reached local lymph nodes. The score ranges from N_X being undetectable, to N_3 indicating a lot of lymph node metastasis. The M, in TNM, refers to distal metastasis. The score for M ranges from MX being undetectable, to M1 indicating distal metastasis.

Treatment and Therapy

The aggressiveness of therapies used for the treatment of PCa are directly correlated with the aggressiveness of disease to be treated. For men with clinically localized PCa (i.e. organ confined disease) three treatment options exist, radical prostatectomy (Millin, 1945), external beam radiotherapy (Bagshaw et al., 1965) (Bagshaw et al., 1975) and brachytherapy (Whitmore et al., 1972). The prostate gland is in an intimate location, meaning that is there are neurovascular bundles important for erectile function that traverse the prostate. Early radical prostatectomies damaged these nerves, leaving men with erectile dysfunction. During the 1970s a novel nerve-sparing approach was developed for the surgical treatment of early stage locally confined cancer (Walsh et al., 1983). The nerve-sparing approach is very effective at decreasing disease-specific mortality in patients with PCa, however, this approach is still invasive and associated with significant morbidity and prolonged recovery (Lepor et al., 2001). To overcome the morbidity associated with invasive radical prostatectomy, the robotically assisted laparoscopic radical prostatectomy (RALP) in combination with the da Vinci Surgical System was developed in 2000, and is the current standard for radical prostatectomies (Binder and Kramer, 2001).

External beam radiotherapy is the second treatment option for locally confined PCa. A radiation oncologist delivers a high dose of radiation, which is focused on the cancerous region of the prostate through the use of a three-dimensional computed tomography (CT) imaging (Thoeni et al., 1981). The use of CT imaging allows for the concentrated delivery of the radiation to an exact area, which minimizes the damage to surrounding normal tissue .

Brachytherapy involves the implantation of radioactive “seeds” into the prostate (Whitmore et al., 1972). Iodine 125 (I^{125}) and palladium 103 (Pd^{103}) are two commonly used isotopes used in brachytherapy to deliver a high dose of radiation to the cancerous prostate (Porter et al., 1995). Monitoring of cancer reoccurrence after therapy is done through PSA screening (Stamey et al., 1987). Rising PSA levels are indicative of cancer recurrence, but are not diagnostic. Radiographic imaging with CT or magnetic resonance, or with biopsy of regional lymph nodes are performed to diagnose recurrent or locally metastatic disease (Carroll et al., 2006). TNM results that have determined that a cancer has spread beyond the prostatic fossa, surgery and beam therapy are no longer treatment options. For men that have locally advanced PCa, the “gold standard” therapy is hormone ablation.

Hormone ablation can be accomplished by two mechanisms, used alone or in combination. The first and oldest method of

hormone ablation is bilateral orchiectomy with the use of the oral oestrogen DES (Huggins and Hodges, 1972). Androgen ablation by DES treatment is accomplished by suppressing the release of the gonadotropin luteinizing hormone (LH). LH acts on the testis, via the LH-receptor to initiate the metabolism of cholesterol to testosterone. Castration therapy with DES was effective at lowering serum testosterone levels in men with advanced PCa, however, significant side effects became evident during the 1960s. A study revealed systemic hormonal therapy with DES caused cardiovascular and thromboembolic toxicity (Byar, 1972), and DES treatment did not completely suppress all androgen production (Huggins, 1945). To overcome the significant side effects associated with DES treatment, novel hormonal therapies were developed to block both adrenal androgen production and inhibit androgen interaction with the AR. The second method of hormone ablation is with the use of gonadotropin releasing hormone (GnRH), also called leutinizing hormone releasing hormone (LHRH), agonist. These agonists act to produce transient spikes increases in serum testosterone levels, which eventually lead to the downregulation of the LHRH receptors, decreasing LH release, resulting in inhibition of cholesterol metabolism into testosterone (Schally et al., 2000). For a complete suppression of androgen's stimulating effects on the prostate, a combined androgen blockade (CAB) is utilized.

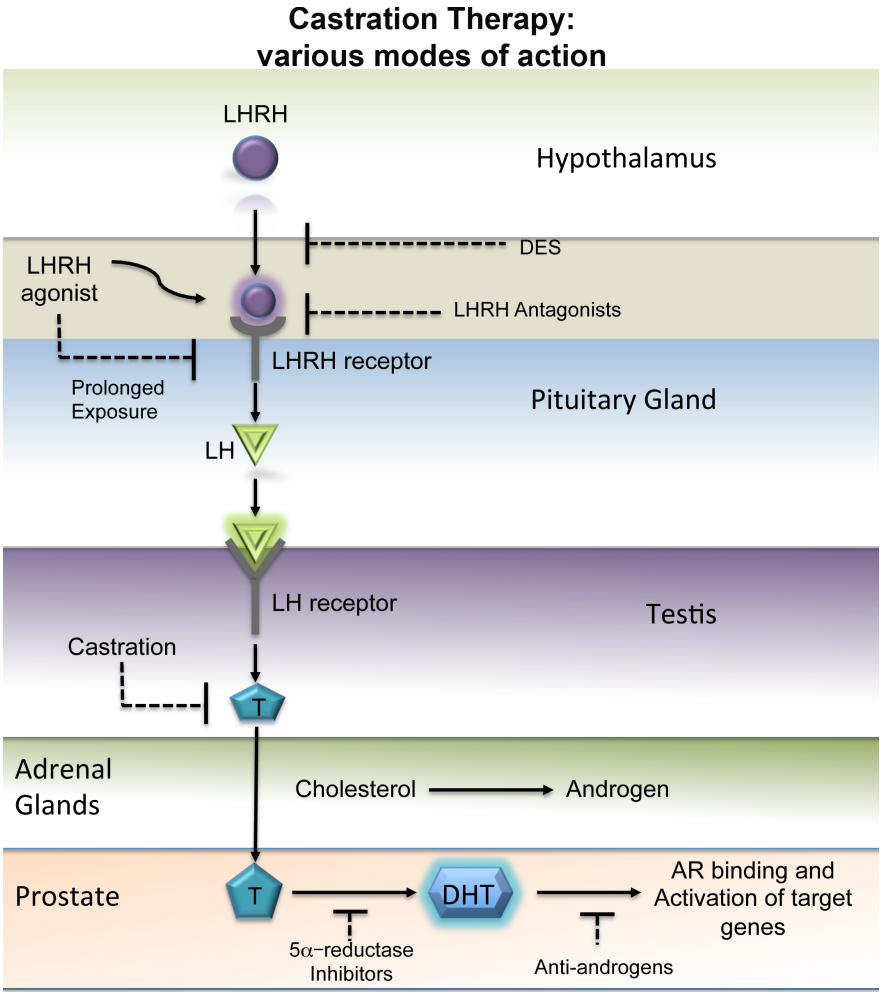

Figure 1.6. Modes of action for castration therapy in men with advanced PCa. Luteinizing hormone (LH) releasing hormone (LHRH) produced in the hypothalamus acts on the pituitary through the binding on LHRH receptor, for the release of LH. LH binding with LH-receptor on the testis signals for the production of testosterone. Testosterone travels to the prostate where it is converted to dihydrotestosterone (DHT).
Modified figure adopted from (Denmeade and Isaacs, 2002).

A CAB uses an agonist of LHRH, such as Zoladex, along with an anti-androgen which competes with androgen binding for the AR (Denmeade and Isaacs, 2002). By doing so, it prevents androgens from stimulating proliferation in androgen dependent cells. The

removal of androgens by castration will induce apoptosis of androgen sensitive prostate cells. Castration by physical or chemical means will reduce tumor size in most men, however, this reduction is short lived as the cancer usually progresses to CR disease.

The progression to CRPCa is an ominous clinical finding since the median survival is approximately one year. A variety of treatment approaches are used for the treatment of CRPCa, all of which include a combination of chemotherapeutics. Chemotherapeutics are cytotoxic agents that induce cell death by damaging DNA and/or disrupting microtubule actions. The standard chemotherapeutic for CRPCa is docetaxel. Docetaxel stabilizes microtubules and provides an increase in mean survival of 9.4 months (range 1.6 to 18.2 months) (Mackler and Pienta, 2005). There is no curative therapy for CRPCa, chemotherapeutics only serve as palliative agents to improved the quality and/or quantity of life. The palliative benefits received from regiments of other chemotherapeutic agents are short lived, resulting in a mean increased survival time of two months due to the development of chemoresistance by the CRPCa (Petrylak, 2007; Petrylak et al., 2004).

1.8 BONE METASTASIS

Men with advance CRPCa almost always develop metastasis to bone, and at the time of death the majority of the tumor burden will be in the bone (Mundy, 2002). Metastasis to bone is a nonrandom occurrence with a reported 90% occurrence in autopsy studies (Bubendorf et al., 2000).

Steps leading up to the formation of bone metastasis are similar to metastasis to any other organ site. The production of matrix remodeling proteases are an example of the aggressive phenotype acquired as the cancer progresses (Bok et al., 2003). The secretion of destructive enzymes by the PCa allow for degradation of extracellular matrix and invasion of the surrounding stroma where blood vessels of normal tissue or blood vessels of the tumor are compromised (D'Andrea et al., 2001). Once in the circulation, the cancer cells are required to evade the normal host immune surveillance, and “dock” and “lock” on the sinusoid bone marrow endothelial cells. Once arrested on the endothelial cells that line the bone marrow cavity, the cancer cells undergo trans-endothelial migration (TEM). TEM is the extravasation of the cancer from the bone marrow cavity into the bone marrow stroma (Miles et al., 2007). Once established in the bone marrow stroma, the PCa initiates the formation of blood vessels from the marrow compartment. After

forming a blood supply, the tumor can continue migrating into the endosteal bone surface. Once at the bone, the metastatic PCa stimulate the activity of the osteoblasts (Chirgwin and Guise, 2006). A number of factors produced by PCa have been identified to stimulate osteoblast activity (Chiao et al., 2000).

The reason(s) why PCa preferentially metastasizes to the bone over other vascularized organs is unknown. The most popular hypothesis is the “seed and soil” theory proposed by Stephen Paget in 1889 (Paget, 1889). According to Paget’s hypothesis, the “Seed” (PCa cell) plants its self in the fertile “Soil” (bone) which is fortified in growth factors including transforming growth factor-beta (TGF-β) (Mundy, 1991), insulin like growth factors (IGF-I/II) (Canalis et al., 1991), and basic fibroblastic growth (bFGF) (Tang et al., 2004). The growth factors present in the bone microenvironment act in a paracrine fashion to stimulate the proliferation of the metastatic PCa cells.

1.9 PROSTATE STROMA AND ROLE IN CARCINOGENESIS

As previously stated the prostate develops from two tissue compartments: (1) the UGE which gives rise to the epithelium and ductal structures, and (2) the UGM which gives rise to the stroma (Cunha, 1972a). Stroma is a collective term to describe the connective tissue which supports the function of the epithelium. The stroma is comprised of numerous cell types including smooth muscle cells, fibroblasts, myofibroblasts, adipocytes, endothelial cells, immune cells, and other bone marrow derived cells (Grossfeld, 1998; Price et al., 1990; Rowley, 1998). Paracrine signaling from cells comprising the stroma provides microenvironmental cues for maintenance of prostate homeostasis (Cunha, 1972b). In the study of PCa, the stroma was originally thought to play a bystander role in the progression of the disease, however, heterotypic recombinations of inductive mesenchyme with PCa cell lines show reduction in growth rate and loss of tumorigenesis (Hayashi and Cunha, 1991).

Prostatic adenocarcinoma originates from the epithelial cells which comprise the ducts of the prostate gland. Consequently the field of PCa research has primarily focused on studying the molecular events of initiation, promotion, and malignant conversion in the epithelium. There is, however, a growing body of evidence showing the non-malignant cells associated with the cancer cells (cancer

associated fibroblasts [CAFs]) experience changes that enhance the malignant potential of epithelial tumor (Grossfeld, 1998). *In vivo* model systems using human cancer associated fibroblasts recombined with an initiated, but non-transformed prostate epithelial cell line BPH-1 produced large tumors that exhibited poorly-differentiated prostatic adenocarcinoma. Control recombinations of BPH-1 cells with normal associated fibroblasts demonstrated minimal growth with benign prostatic structures (Olumi et al., 1999b). These experiments showed that CAFs can induce a transformed phenotype in initiated cells, and stimulate progression of tumorigenesis.

The factors which potentiate the malignant epithelial tumor are mediated, in large part, by paracrine signaling between tumor epithelial cells and neighboring stromal cells (Adam et al., 1994). In addition to receiving signals from malignant epithelial cells, the stromal fibroblasts stimulate tumorigenesis by releasing factors that act on adjacent epithelial tumor cells or exchange enzymes that modify local microenvironment promoting the proliferation and survival of the neoplastic cells (Ao et al., 2007; Basanta et al., 2009; Franco et al., 2011; Pruitt et al., 2013). Angiogenesis is a critical hallmark in the development of metastatic disease (Hanahan and Weinberg, 2000). *In vitro* co-culture experiments using PCa cell lines showed stromal fibroblasts were required for the induction of capillary formation of endothelial cells in matrix (Janvier et al., 1997). Just as

the mesenchyme has been shown to be absolutely required for the development of organs, the cancer associated stroma has been hypothesized to be essential for the development of cancers (Bosman et al., 1993) and specific inhibition of tumor stroma may be a better target for therapeutic intervention rather than targeting the tumor directly (Ronnov-Jessen et al., 1996).

1.10 Dissertation Goals

The tumor stroma has been implicated in the regulation of cell growth, determining metastatic potential, and impacting the outcome of therapy. Evidence shows that the stroma is radically changed during cancer progression and may actually contribute to this progression. Our laboratory has previously published on several molecules found to be aberrantly expressed in cancer associated stroma that induce tumorigenesis and malignant conversion. We have found that the up-regulation of a cell cycle regulator known as cyclin D1 (CD1) in normal prostate fibroblasts mimics the phenotype of ability to induce malignant conversion seen in cancer associated stroma. Genetic analysis of CD1 overexpressing prostate fibroblasts, and of human prostate CAFs identified cathepsin D (CathD), which is a protease known to be associated with the development of aggressive and metastatic breast cancer (Tumminello et al., 1996). Further investigation revealed that CathD is essential for cancer associated stroma to induce a malignant conversion in prostate epithelial cells. The mechanism underlying CathD overexpression in cancer associated stroma, as well as a mechanism explaining how CathD can induce a malignant conversion is unknown.

The first objective was to determine how overexpression of CD1 in the stroma induces the upregulation of the estrogen regulated protease CathD, and to determine how overexpression of CathD in

the stroma can contribute to tumorigenesis in the epithelium. The second objective was to determine if estrogen receptor alpha (ERα) in CAFs differentially regulates estrogen responsive genes that are key factors in enhancing the invasive potential of the epithelial tumor.

The study of stroma in association with cancer is advantageous for the development of novel therapies because stromal cells are more genetically stable and therefore less likely to develop mutations resulting in chemoresistance. The information gained from this study hopefully increases our understanding of the complexity within the tumor microenvironment opening the possibility of providing potential targets for suppressing lethal PCa phenotypes.

CHAPTER 2

Cathepsin D Acts as an Essential Mediator to Promote Malignancy of Benign Prostatic Epithelium.

2.1 Introduction

Historically, the field of cancer biology has primarily been focused on studying the malignant tumor epithelium (Weinberg, 2008). The emergence of the field of tumor microenvironment is providing some much needed insight into how non-malignant cells associated with cancer (cancer associated stroma) can promote or suppress tumorigenesis. The stromal phenotype has been shown to be a powerful prognostic indicator of cancer progression and of patient death underlining the importance of local stromal cells in defining lethal versus indolent phenotypes (Li et al., 2007).

Stromal-epithelial interactions are important in both the development of the prostate, and in prostate cancer (PCa) (Cunha, 1972; Cunha et al., 2002; Olumi et al., 1999). During carcinoma evolution, the stroma cells adjacent to the pre-malignant or malignant epithelium experience phenotypic alterations that have been shown to enhance the invasive potential of the epithelial tumor (Ao et al.,

2007; Grossfeld, 1998; Joesting et al., 2005). These stromal-epithelial interactions are mediated, in large part, by paracrine signaling between epithelial tumor cells and neighboring stromal fibroblasts (Ao et al., 2007). We have previously published on several molecules found to be aberrantly expressed in cancer associated stroma that induce tumorigenesis and malignant conversion (Ao et al., 2007; Franco et al., 2011; He et al., 2007; Joesting et al., 2005; Orr et al., 2011). These intercellular interactions are clearly complex and there are likely a number of molecular routes, which can either promote or suppress tumor-inducing activity. One purpose of pursuing these studies is to start to determine the identity of pathways which are either sufficient or necessary to induce transformation and to examine how such pathways might interact. We showed that the up-regulation of a cell cycle regulator known as CD1 in normal prostate fibroblasts mimics aspects of the phenotype of malignant conversion seen in cancer associated stroma. In addition to receiving signals from malignant epithelial cells, the stromal fibroblasts stimulate tumorigenesis by releasing factors that act on adjacent epithelial tumor cells or exchange enzymes that modify local microenvironment promoting the proliferation and survival of the neoplastic cells (Ao et al., 2007; Bhowmick et al., 2004; Cheng et al., 2005; Joesting et al., 2005).

One mechanism by which modifications to the local tumor

microenvironment are accomplished is via the actions of several different families of proteases produced by either the tumor or the stroma (MacDougall and Matrisian, 1995). These enzyme families include matrix metalloproteases (MMP), cysteine, and serine proteases, which have been shown to play a role in the degradation of the basement matrix, promotion of angiogenesis, and the liberation of growth factors to stimulate tumor cell growth (McCawley and Matrisian, 2001) (Tuxhorn et al., 2001).

CathD is a ubiquitous lysosomal aspartic endoproteinase. CathD, has been shown to be involved in a number of physiological processes, playing a critical role in barrier function, regulation of apoptosis, and epithelial differentiation (Chen et al., 2000; Egberts et al., 2004; Guicciardi et al., 2004). In cancer, CathD is overexpressed and hypersecreted in various malignancies including PCa (Hara et al., 2002; Laurent-Matha, 2005). In breast cancer, CathD expression is associated with a poor prognosis and increased likelihood for the development of metastasis (Rochefort et al., 2000). Experimental evidence has shown CathD can stimulate the proliferation of PCa cell lines (Vetvicka et al., 1997).

There are limited data defining CathD's function in prostate cancer progression. Some studies have concluded that CathD is overexpressed in the epithelium and stroma of PCa, and may promote proliferation (Konno et al., 2001; Vetvicka et al., 1998).

Other studies have concluded that CathD produced by PCa may be inhibiting tumor growth (Morikawa et al., 2000; Tsukuba et al., 2000). In the present study, we highlight CathD as a mediator of cancer associated stromal promotion of prostate tumorigenesis.

2.2 Material and methods

Cells. BPH-1 (a non-tumorigenic human prostatic epithelial cell), and its tumorigenic derivatives BPH^{CAFTD} were isolated from our own stocks (Hayward et al., 1995b; Hayward et al., 2001). CAF cells were isolated from human prostate tumor samples and their activity validated in a tissue recombination model. The technique for the isolation of CAF is described in Olumi et al. (Olumi et al., 1999b) which also describes a bioassay which was used to confirm the tumor-inducing activity of the CAF used in the present study. NPF^{CD1} cells, which we have shown in the past to overexpress CathD, were generated as previously described (He et al., 2007). Benign human prostate stromal cells (BHPrS) were isolated from a prostate surgical sample and immortalized with hTERT as previously described (Franco et al., 2011). Cells were maintained in RPMI 1640 (Gibco, Carlsbad, CA) with 1% antibiotic/antimycotic (Life Technologies, Grand Island, NY) and 5% Cosmic Calf Serum (CCS-HyClone, Logan, Utah).

Generating genetically modified cell lines. The pSuper.Retro-control (PSR- OligoEngine, Seattle, WA) and pSuper.Retro-CD1 shRNA (PSR-CD1sh) were kindly provided by Drs. Rene Bernards and Daniel Peeper from the Netherlands Cancer Institute. The two

plasmids were engineered into CAF by retroviral transduction as previously described (He et al., 2007). Positively transduced cells were selected for resistance to puromycin (5μg/ml) to generate two cell strains (CAF^{scram} and CAF^{CD1sh}) The pSilencer 2.1-CathD1shRNA vector was kindly provided by Dr. Daniel E. Johnson from the University of Pittsburgh Cancer Institute. PSR-cathepsin Dsh was generated by removing the CathD1sh coding sequence from pSilencer 2.1-CathD1shRNA with HindIII and BamH1 and ligated into the PSR construct. The PSR and PSR-CathD sh were engineered into CAF by retroviral infection as described previously (He et al., 2007). The positively transduced cells were selected for resistance to puromycin (5μg/ml) to generate the cell line ($CAF^{CathDsh}$). BPH-1^{NPF}, BPH-1^{CAFTD1}, and BPH-1^{NPFCD1} cells were re-isolated from resulting growths as previously described (He et al., 2007). BHPrS cells were engineered to overexpress CathD by lentiviral transduction (Genecopoeia Inc, Rockville, MD.) Viral supernatant was generated, centrifuged at 3000 rpm for 5 min and passed through a 0.45 μm filter before being frozen at -80°C until used. Polybrene (Sigma-Aldrich, St Louis, MO) was added to the viral suspension at 5 μg/mL to increase the efficiency of the transduction. GFP-expressing cells were selected by fluorescence-activated cell sorting (FACS) to establish the $BHPrS^{CathD}$ and $BHPrS^{EV}$ as an empty vector control.

Western blotting analysis. Cell lysates were prepared and Western blotting was performed as previously described (Williams et al., 2005). Membranes were incubated with mouse primary antibody to PTEN (1;1000, Santa Cruz Biotechnology, Santa Cruz, CA), Cdk2 (1;1000, Santa Cruz), Cdk4 (1;1000, Santa Cruz), Cdk6 (1;1000, Santa Cruz), cyclin E (1:1000, Santa Cruz), CD1 (1:1000, BD Biosciences Pharmingen, San Jose, CA) , b-actin (1:5000, Sigma) or CathD (1:1000, Cell Signaling, Denvers, MA) overnight and washed with PBS-Tween 20 for 1 hour, and incubated with horseradish-Peroxidase linked anti-mouse secondary antibody (Amersham Biosciences, Piscataway, NJ, 1:10,000 dilution) for 1 hour. Bound antibodies were visualized using enhanced chemiluminescence western blotting detection reagents (Amersham Bioscences).

Tissue recombination and xenografting. Rat urogenital mesenchyme (rUGM) was obtained from 18-day embryonic fetuses (plug date denoted as day 0). Urogenital sinuses were dissected from fetuses and separated into epithelial and mesenchymal components by tryptic digestion, as described previously (Hayward et al., 1998). BPH-1 + rUGM, BPH-1 + NPF, BPH-1 + NPF^{CD1}, BPH + CAF, BPH-1+ $CAF^{CyclinD1sh}$, BPH-1 + $CAF^{CathDsh}$, BPH-1 + $BHPrS^{CathD}$, and BPH-1 + $BHPrS^{EV}$ tissue recombinants were made as previously

described (Hayward et al., 1999). $1.0x10^5$ epithelial cells and $2.5x10^5$ stromal cells combined in type I rat tail collagen were used to make the recombinants. After overnight incubation, the tissue recombinants were grafted under the kidney capsule of adult male severe combined immunodeficient (SCID) mice (Harlan, Indianapolis, IN) supplemented with 25 mg testosterone pellets (PCCA, Houston TX). All the experiments were repeated six times. Mice were sacrificed at eight weeks and grafts were harvested, fixed, and paraffin embedded. Graft dimensions were measured using the formula: volume=width x length x depth x $\pi/6$ as described previously (Franco et al., 2011).

Wound healing assays. Confluent monolayers of NPF and NPF^{CD1} cells were grown in 6 well plates. Confluent cell monolayers were wounded by scratching with a pipette tip. Specific points on the wounds were identified and marked. These open areas were then inspected microscopically over time as the cells migrated in and fill the damaged area. Wounds were imaged at 0, 3, 6, and 8 hours post wounding and the cell migration rate into the wound was calculated. Experiments were performed in triplicate.

Outgrowth assay. $1.0x10^5$ NPF, NPF^{CD1}, $NPF^{CD1\text{-}CathD\ control}$ or $NPF^{CD1\text{-}CathDsh}$ were resuspended at 4°C in Matrigel (0.2 ml, 10

mg/ml; Becton and Dickinson), and overlaid to a previously solidified layer of Matrigel in 24-well plates as described previously. The top Matrigel layer was solidified at 37°C for 30 minutes and covered with culture medium containing 10% FCS (0.5 ml).

Conditioned Medium. NPF or NPF^{CD1} were seeded with 5% FCS in RPMI 1640 at a density of $5.0x10^5$ per 75-cm^2 flask, allowed to grow, and attached overnight. Confluent cultures of NPF or NPF^{CD1} were rinsed twice in PBS and incubated for 3 days in RPMI + 0.5% FCS. The medium was collected, centrifuged, passed through a 0.45-μm filter (Millipore), and stored at –80°C for later use. Conditioned medium was thawed and diluted 1:1 with fresh DMEM + 0.5% FCS before use. BPH-1 cells were seeded at $2.0x10^4$ per well in six-well plates in conditioned medium. The cultures were incubated for 3 days and the total number of cells was determined by direct counting in a hemacytometer.

Human Prostate and Prostate Tissue Microarray. Human prostate tissue array (PR806) was obtained from US Biomax, Inc. The array contained duplicates from 30 cases of adenocarcinoma ranging in Gleason scores and 10 cases of normal prostate tissue. Normal human prostate tissue was also obtained from the Vanderbilt University Medical Center Department of Pathology.

Histochemical and Immunohistochemical staining. Masson's trichrome stain was performed as previously described using Diagnostics Accustain Masson trichrome stain kit (Sigma), Bouin's solution (Sigma) and Weigert's Iron Hematoxylin set (Sigma) (Franco et al., 2011). Immunohistochemical staining was performed following a protocol that was described previously (Williams et al., 2005). Tissue slides were then incubated with the primary antibody against CD1 (1:200, BD Biosciences Pharmingen), CathD (1:200, Santa Cruz), p-SMAD2/3 (1:400, Santa Cruz), Col4α2 (1:200, Santa Cruz), The polyclonal rabbit or mouse immunoglobulins/biotinylated anti-mouse secondary antibody (DAKO, Carpentiria, CA) was incubated for 60 min after the slides were washed with PBS buffer for 1 hour. After washing the slides in PBS extensively, slides were incubated in ABC-HRP complex (Vector Laboratories) for 30 minutes. Bound antibodies were then visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride (liquid DAB, DAKO). Slides were then rinsed extensively in tap water, counterstained with hematoxylin, and mounted.

Immunofluorescence. For histological analysis, 5 μm tissue sections were dewaxed, and the antigen was unmasked by heating samples in unmasking solution (Vector Laboratories). Slides were

blocked in 12% BSA in PBS for 30 minutes at room temperature before incubating with primary antibodies against CathD and GFP (1:200, Santa Cruz). After 1 hour of washing in PBS buffer, slides were incubated with secondary antibodies (1:200; AlexaFluor 488 anti-Rabbit IgG and AlexaFluor 594 anti-mouse IgG2a) for 30 minutes at room temperature. Slides were incubated in Hoechst 33258 (4 mg/L) for 5 min. Tissue sections were washed for 30 minutes in PBS, mounted, and visualized.

Quantitative Image Analysis. Immunostainded slides were analyzed using the Ariol SL-50 automated slide scanner (Applied Imaging, San Jose, CA) to quantitate the amount of staining for CathD in the stroma of benign and malignant human prostate tissue sections. Positive staining was calculated by applying 2 thresholds, with one recognizing weaker brown-positive cells, and another recognizing stronger brown-positive cells. The intensity of the stain was calculated by masking out all non-stromal areas from the tissue section and calculating the integrated optical density of brown within the remaining area. This value was divided by the area in pixels of the brown mask to calculate the average intensity of the tissue section.

Statistical Analysis. Data from *in vitro* and *in vivo* are presented as the mean ± standard deviation (SD). The data was analyzed using GraphPad PRISM software (La Jolla, CA). P values less than .05 were considered statistically significant. Quantitated intensity of CathD expression in human prostate samples were compared with analysis of variance followed by post hoc analysis of significant means by Mann Whitney's test was used in comparison of normal to tumor tissue. Post hoc analysis of significant means by Dunn's Multiple Comparison test was used for the comparisons of normal tissue with low grade and high grade malignant prostate tissue. P values less than .05 were considered statistically significant.

2.3 Results

CathD expression is upregulated in prostate clinical samples, and CathD is overexpressed in the stroma of tumorigenic tissue recombinants.

We examined the expression patterns of CathD in human prostate clinical samples using a tissue microarray containing 30 cases of adenocarcinoma, and 10 cases of normal prostate tissue. The tissue microarray contained duplicate cores per case. Quantification of positive CathD staining in stromal regions of prostate tissue showed significantly greater areas of CathD expression in tumor tissue in comparison to normal prostate tissue. When tumor tissue was stratified between low and high grades, a significant difference was only observed in high grade tumors compared with normal tissue with a non-significant elevation of expression in low grade tumors (Figure 2.1B). A similar trend with no significant difference was also observed in comparisons of low and high grade tumors. It was noteworthy that the expression of CathD apparently corresponded to areas, that stained a light red color in the adjacent trichrome-stained sections. This likely indicates the presence of myofibroblastic cells in this area, which would correspond to the source of our experimental CAF.

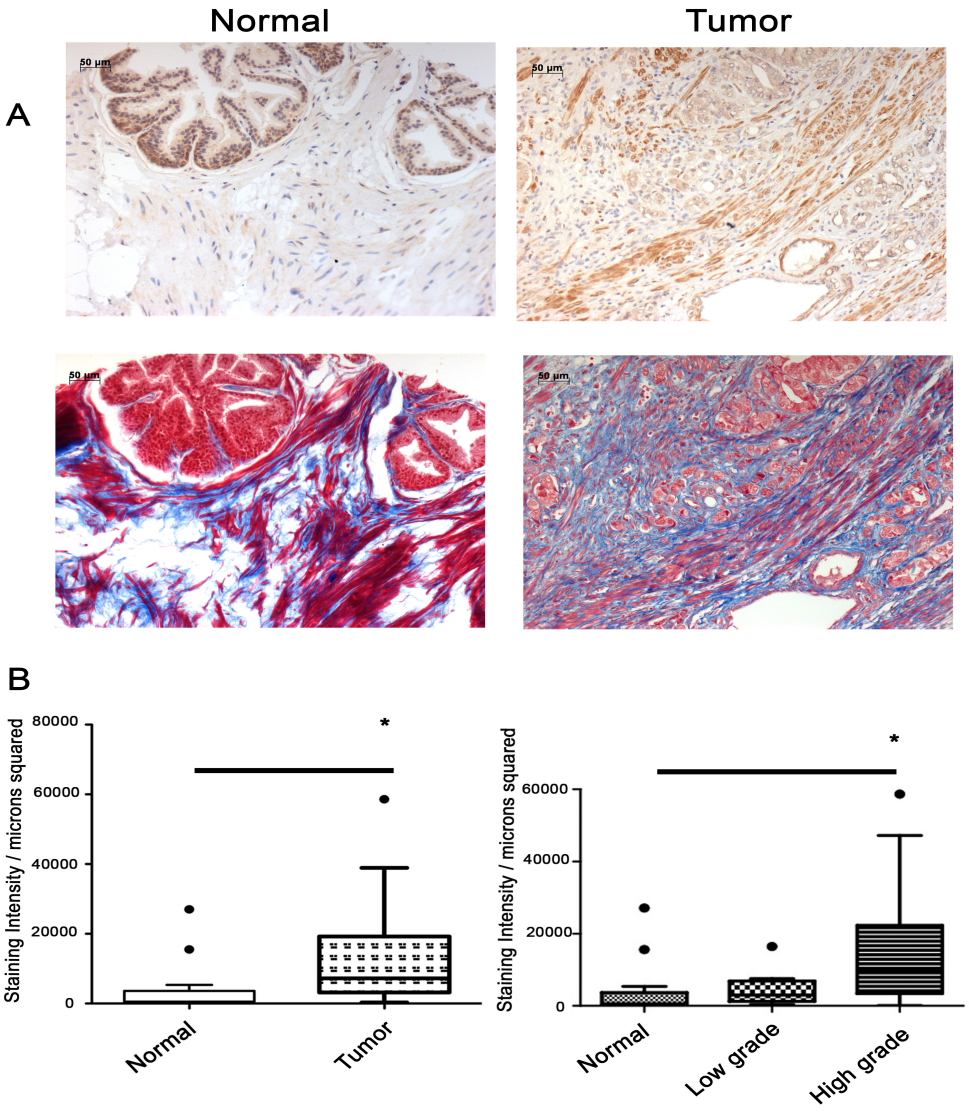

Figure 2.1. Cathepsin D is overexpressed in malignant prostate clinical samples.
(A) Representative images from immunohistochemical (top) analysis of CathD expression in normal (left) n = 18 and tumor (right) n = 30 human prostate tissues. Representative images of Masson's tri-chrome staining (bottom) from normal (left) and tumor (right). Scale bar is equal to 50μm. (B) Quantitation of CathD expression in the prostate comparing normal to malignant tissue. Data are presented as means ±SD.

Examination of CathD expression in tissue recombinations of BPH-1+NPF, BPH-1+rUGM, BPH-1+NPF^{CD1}, and BPH-1+CAF was performed by IHC. The recombinations of BPH-1 + NPF and BPH-1 + UGM isolated after 8 weeks of growth produced small growths overall which displayed solid epithelial cord structures surrounded by a muscular stroma. IHC staining displayed minimal expression of CathD in the stroma with some epithelial expression seen in the BPH-1 + NPF recombinants (Figure 2.2A, 1 and 2.2A, 2). In marked contrast, recombinations of BPH-1 + NPF^{CD1} and BPH-1 + CAF isolated after 8 weeks produced poorly differentiated carcinoma along with areas of squamous metaplasia similar to previously published results (Figure 2.2A, 3 and 2.2A, 4) (He et al., 2007). Recombinations of BPH-1 + NPF^{CD1} and BPH-1 + CAF displayed strong CathD staining in the stroma and epithelium (Figure 2.2A, 3 and 2.2A, 4). These results are consistent with the observations of CathD overexpression in the stroma of human PCa clinical tissues. These data raised the question of whether the upregulation of CathD protein is a passive result of prostatic tumorigenesis or plays an active role as a paracrine mediator required to induce a malignant transformation in the adjacent prostatic epithelium.

Stromal expression of CD1 affects cell cycle regulators in adjacent epithelium.

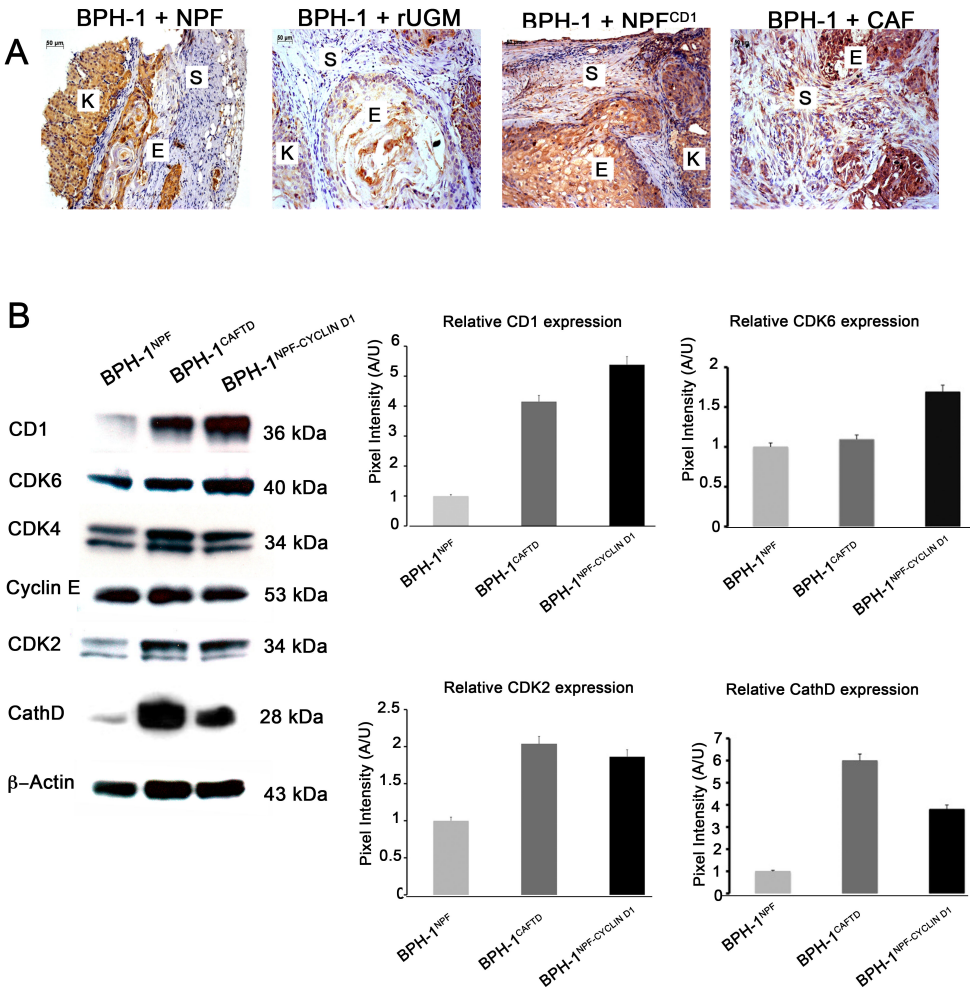

Figure 2.2 Evaluation of Cathepsin D as a paracrine mediator of neoplastic epithelial cell growth in tissue recombinants *in vivo*. (A) Immunohistochemical analysis of CathD expression in recombinations of 1) BPH-1 + NPF, 2) BPH-1 + rUGM, 3) BPH-1 + NPF^{CD1}, 4) BPH-1 + CAF. Scale bar is equal to 50μm (Letters K,S,E, refer to kidney, stroma, and epithelium). (B) Evaluation of densitometric analysis of cell cycle regulators and CathD in $BPH\text{-}1^{NPF}$, $BPH\text{-}1^{CAFTD1}$, $BPH\text{-}1^{NPFCD1}$ cells. Expression of Beta-actin was used for a loading control. Graphical representation of the mean ± SD of band intensities.

Fluorescence-activated cell sorting of BPH-1 cells isolated from the xenografts of BPH-1 + NPF, and BPH-1 + NPF^{CD1} tissue recombinants showed striking differences in cell population distributions. Previously published DNA flow cytometric analysis showed a majority (55%) of BPH-1 cells isolated from the tissue xenografts of BPH-1 + NPF^{CD1} were hyperploid along with another large population of BPH-1 cells (23.1%) that were polyploid (He et al., 2007). In order to gain some insight as to what signaling pathways in BPH-1 cells were being affected by NPF^{CD1} we performed western blot analysis for several proteins involved in cell cycle regulation. Epithelial cells were isolated and cultured from xenografts of BPH-1+NPF, BPH-1+CAF and BPH-1+NPF^{CD1}. The resulting cells were designated BPH-1^{NPF}, BPH-1^{CAFTD} and BPH-$1^{NPF\text{-}CD1}$ respectively. Densitometric analysis of band intensities from western blots revealed that overexpression of CD1 in the local stromal cells increased the expression of the cell cycle related proteins CD1, cyclin dependent kinases-6 (CDK6) and 2 (CDK2), and CathD in BPH-$1^{NPF\text{-}CD1}$ over BPH-1^{NPF} (Figure 2.2B), consistent with increasing proliferative activity in these cells. Similar results were observed in BPH-1^{CAFTD1}. No changes in the expression CDK4, cyclin E were observed.

CathD is a critical component in NPF^{CD1} motility and 3D outgrowth.

We have previously reported the abilities of NPF^{CD1} and CAF to induce tumorigenesis in tissue recombination experiments (He et al., 2007; Olumi et al., 1999a). In order to further investigate mechanisms underlying this malignant transformation we characterized the effects of knocking down CathD expression in NPF^{CD1}. NPF^{CD1} displayed enhanced motility in wound healing assays compared with control NPFs (Figure 2.3A). The enhanced motility displayed by NPF^{CD1} was significantly abrogated when CathD expression was knocked down, with the use of CathD specific shRNA ($NPF^{CD1\text{-}CathDsh}$) (p-value<0.005). These findings demonstrate that CathD plays a role in NPF^{CD1} migration *in vitro*. Western blot analysis was performed to confirm the knockdown of CathD expression in CD1 overexpressing fibroblast (Figure 2.3D). Overexpression of CD1 in NPF results in increased CathD expression as previously published (He et al., 2007). Stable expression of stable hairpin RNA (shRNA) specific for CathD results in 66% knockdown in CathD expression. Expression of non-specific shRNA in NPF^{CD1} does not alter CathD expression.

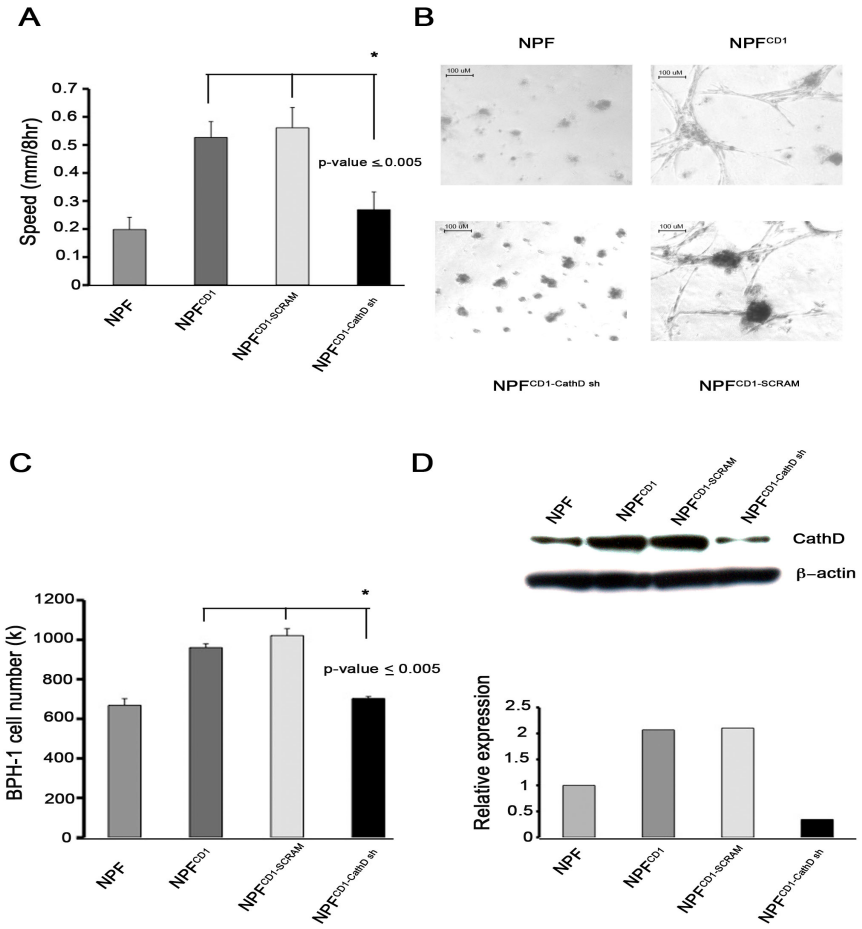

Figure 2.3 Cathepsin D is a critical mediator between BPH-1 cells and NPF^{CD1} *in vitro*. (A) Wound healing assay. Confluent monolayers of NPF, NPF^{CD1}, NPF$^{CD1\text{-}control}$, NPF$^{CD1\text{-}CathD\ sh}$ were scratched with a pipette tip. Bar graphs represent the mean ± SD of rate of wound closure over 8 hour period. Significance determined by ANOVA, p-value ≤ 0.005 n=3. (B) 3D outgrowth assays. NPF cell lines were embedded in matrigel and cultured for 14 days, scale bar = 100μm. Images taken at 10x (3B, b). (C) Evaluation of CathD as a paracrine mediator of growth. BPH-1 cells were treated with conditioned media collected from NPF cell lines for 3 days. Cell numbers were quantitated by direct counting, graphical representation of the mean ± SD of the experiment is shown, significance determined by ANOVA *p-value* ≤ 0.005 n=3. (D) Western blot confirming knockdown of CathD in NPF, NPF^{CD1}, NPF$^{CD1\text{-}control}$, NPF$^{CD1\text{-}CathD\ sh}$ cells (Top). Densitometric analysis of band intensities performed to determine knockdown efficiency (Bottom).

To further characterize the requirement for CathD expression in NPF^{CD1}, we examined fibroblast outgrowth in 3D matrices. As shown in Figure 2.3B, overexpression of CD1 promoted outgrowth of normal prostatic fibroblasts embedded into Matrigel. After 14 days of culture, NPF^{CD1} cells had adopted a stellate morphology and formed invasive colonies with protrusions sprouting into the surrounding matrix (Figure 2.3B,2). In contrast, normal prostatic fibroblasts presented a well-delineated spherical appearance of quiescent and/or dying cells and grew poorly, neither invading nor forming protrusions to the surrounding matrix (Figure 2.3B, 1). $NPF^{CD1\text{-}CathDsh}$ cultured in 3D matrix failed to form invasive colonies that protruded into the surrounding matrix (Figure 2.3B, 3), unlike $NPF^{CD1\text{-}control}$, which retained the ability to form invasive growth feature (Figure 2.3B, 4). These data strongly imply a role for CathD as a factor in promoting the invasive growth of NPF^{CD1} cells *in vitro*.

CathD is a paracrine mediator of neoplastic epithelial cell growth *in vitro*.

To investigate the role of CathD as a paracrine mediator of prostate epithelial cell growth, we generated conditioned media from NPF and NPF^{CD1} cells, and measured BPH-1 cell numbers after growth for three days in the conditioned media. Conditioned medium from NPF^{CD1} increased the proliferation of BPH-1 cells by 1.7-fold, when compared with medium conditioned by parental NPF (Figure 2.3C). The pro-mitogenic effects from NPF^{CD1} conditioned medium were abrogated when CathD expression was knocked down in $NPF^{CD1\text{-}CathDsh}$ (p-value $\leq$ 0.005). These results suggest that a significant component of NPF^{CD1} proliferative influence toward epithelium is mediated through secreted CathD.

CathD is an essential mediator of CAF induced tumorigenicity *in vivo*.

To elucidate the role of CD1 and CathD in CAF's ability to induce tumorigenesis of BPH-1, we took a knockdown expression approach. CAF were engineered to express shRNA vectors specific for either CD1 or CathD. Western blotting was used to assess knockdown efficiency. CD1 expression was knocked down 50% and CathD expression was knocked down 95% in CAF cells (Figure 2.4C). Based on gross morphology it was found that BPH-1+ $CAF^{CyclinD1sh}$ and BPH-1+$CAF^{CathDsh}$ recombinants formed significantly smaller grafts compared with BPH-1+CAF grafts (p-value ≤ 0.05) (Figure 2.4A and C) Histologically, BPH-1+CAF recombinants formed adenosquamous carcinoma as previously described (Olumi et al., 1999a). Knockdown recombinants formed benign, small cords structure with no tumorigenic response (Figure 2.4B). These findings establish CathD to be key mediators in prostate stoma-epithelial interaction in the development of tumorigenesis.

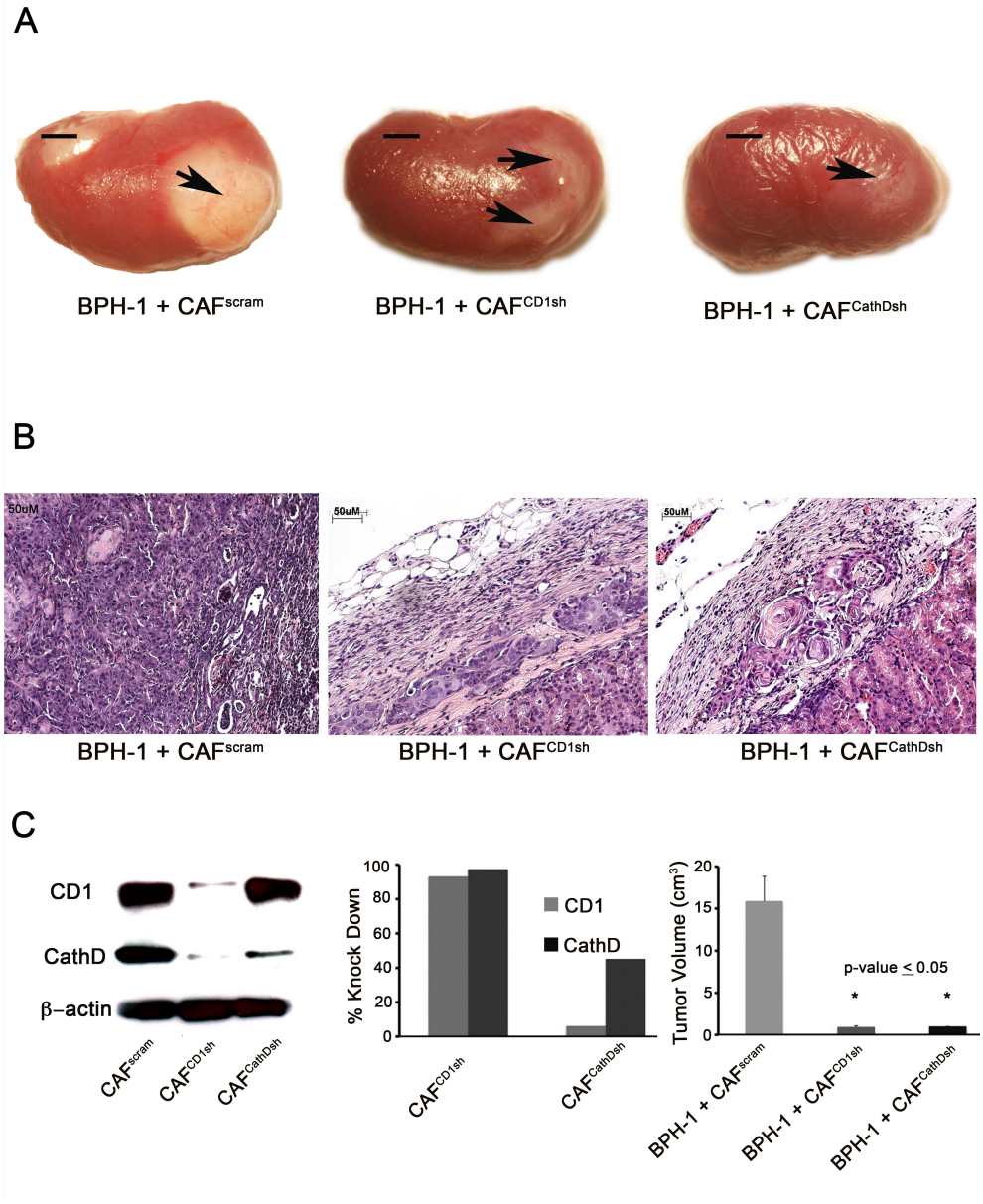

Figure 2.4 Cyclin D1 and Cathepsin D are required for CAF induced tumorigenicity *in vivo*. (A) Gross morphology of 2 month grafts of BPH-1 + CAF^{PSR}, BPH-1 + CAF^{CD1sh}, and BPH-1 + $CAF^{CathDsh}$, scale bar equal to 5cm. (B) H&E staining of BPH-1 + CAF^{PSR}, CAF^{CD1sh}, or $CAF^{CathDsh}$. BPH-1 + CAF^{PSR} recombinants formed adenosquamous carcinoma as previously described. Scale bar equal to 100μm (C) Western blot confirming knockdown of CD1 and CathD in CAFs (left). Knockdown efficiency determined by performing densitometric analysis of western blot represented by bar graph (middle). Quantiation of tumor volume of 2 month grafts of BPH-1 + CAF^{PSR}, BPH-1 + CAF^{CD1sh}, and BPH-1 + $CAF^{CathDsh}$ (right), graphical representation of the mean ± SD of the grafts is shown in n=6. Significance determined by ANOVA, p-value ≤0.05.

To further clarify the role of stromal derived CathD in promoting tumorigenesis we engineered BHPrS, a benign human prostate stromal cell line, to overexpress CathD ($BHPrS^{CathD}$) by lentiviral transduction. In comparison to recombinations of BPH-1 + $BHPrS^{EV}$, BPH-1 + $BHPrS^{CathD}$ recombinants exhibited a malignant transformation. Based on the H&E staining recombinations of BPH-1 + $BHPrS^{EV}$ exhibited thick stromal regions delineating BPH-1 cells from the kidney interface (Figure 2.5A). The opposite was observed in recombinations of BPH-1 + $BHPrS^{CathD}$, where BPH-1 cells are directly adjacent to kidney interface (Figure 2.5D). IHC staining for CathD indicates strong stromal expression of CathD in the recombinations of BPH-1 + $BHPrS^{CathD}$ (Figure 2.5E). IF staining for GFP positive stromal cells (red) and CathD expression (green) show strong stromal specific expression of CathD seen in the yellow overlay (Figure 2.5F).

Masson's trichrome staining was performed on tissue sections from recombinations of BPH-1 + $BHPrS^{EV}$ and BPH-1 + $BHPrS^{CathD}$ (Figure 2.5 G+2.5 J). Heavy aniline blue stains indicated increased deposition of newly synthesized collagen fibrils in the CathD overexpressing recombinations (Figure 2.5J). IHC staining was performed to examine the phosphorylated-SMAD2/3 (p-SMAD2/3), a surrogate reporter of transforming growth factor-beta (TGF-β) activity (Figure 2.5 H + 2.5 K). CathD overexpressing recombinations (Figure

5K) shows increased nuclear p-SMAD2/3 staining. Quantitation of p-SMAD2/3 positive cells indicated a significant difference in the CathD overexpressing recombinations. Type IVα2 collagen (Col.IVα2) is a known TGF-β responsive gene. We performed IHC staining for Col.IVα2 and the CathD overexpressing recombinations (Figure 5L) displayed strong expression for Col.IVα2 in comparison to recombinations with the empty vector construct (Figure 2.5I).

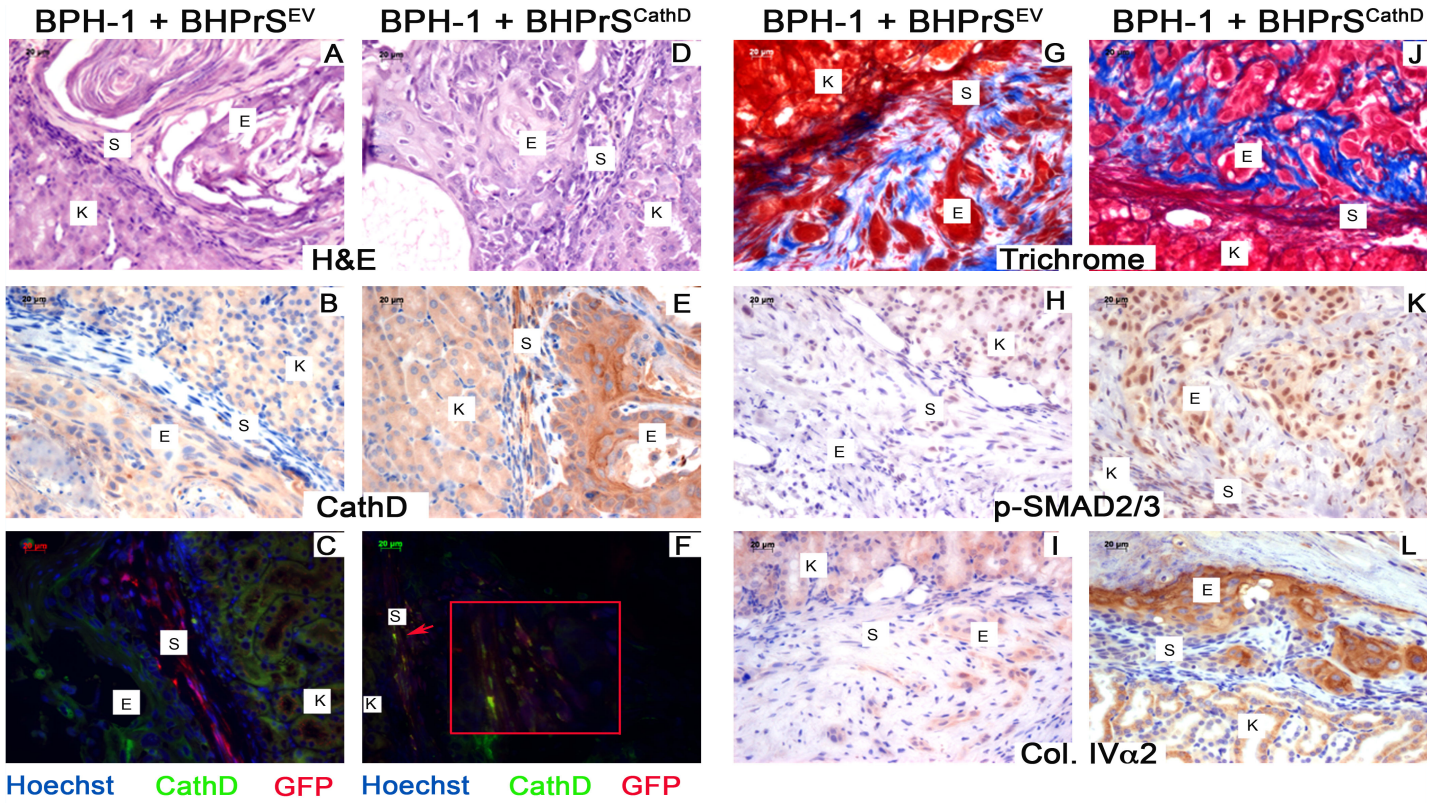

Figure 2.5 Overexpression of Cathepsin D induces a malignant transformation through activation of TGFβ signaling. (A) Characterization of CathD overexpressing grafts. H&E staining (top) of BPH-1 + BHPrS^{EV} and BPH-1 + BHPrS^{CathD}. Recombinations of BPH-1 + BHPrS^{CathD}produced malignant transformations. IHC for CathD (middle) strong expression visible in the stroma of recombinations of BPH-1 + BHPrS^{CathD}. Immunofluorescence for CathD (green) and GFP (red) show co-localization for GFP and CathD (yellow) in recombinations of BPH-1 + BHPrS^{CathD}. (B) Masson's trichrome staining (top) of recombinations of BPH-1 + BHPrS^{EV} and BPH-1 + BHPrS^{CathD}. IHC for p-SMAD2/3 (middle) and Collagen IVα2 (lower) in recombinations of BPH-1 + BHPrS^{EV} and BPH-1 + BHPrS^{CathD}. Scale bar equal to 50μm.

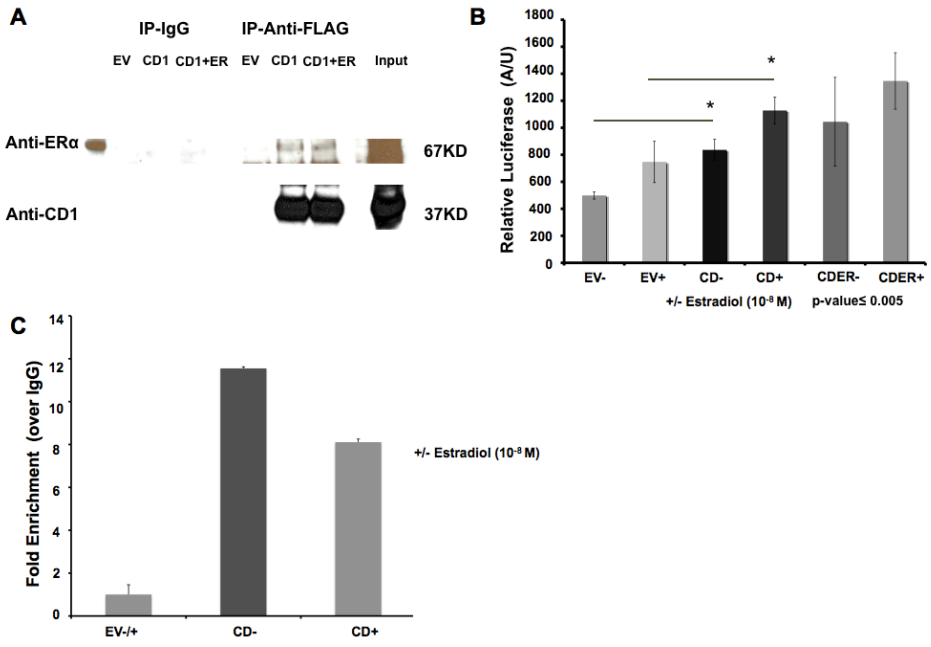

Figure 2.6 CD1 interacts with ERα on the CathD promoter. **(A)** Overexpression of CD1 in BHPrS cells show co-interaction with endogenous ERα. **(B)** CD1 overexpression drives ER transcriptional activity in luciferase experiments. **(C)** CD1 is recruited to the estrogen response elements in the CathD gene.

Recombination experiments showed that NPF^{cyclinD1}+BPH-1 induced tumorigenesis and malignant transformation of the epithelium, a phenotype which is also seen in recombinations with CAF+BPH-1 (He et al., 2007). The shared phenotype between CAF and NPF$^{cyclin\ D1}$ of the ability to induce tumorigenesis and malignant transformation led us to compare gene expression profiles between these two cell types. Expression results identified CathD expression to be upregulated almost 7 fold in both CAF and NPF$^{cyclin\ D1}$ in

comparison to normal primary fibroblasts. This finding led us to question how a cell cycle regulator could affect the transcription of an estrogen regulated protease that is strongly associated with the progression of other metastatic cancers (Benes et al., 2008).

The D-type cyclins are rate limiting and essential for the progression through the G1 phase of the cell cycle (Xiong et al., 1991). Under *in vitro* conditions CD1 and the estrogen receptor alpha (ERα) were shown to interact by co-immunoprecipitation and to stimulate estrogenic gene transcription (Shang et al., 2000). We hypothesized that the overexpression of CD1 in prostate stromal fibroblasts modifies the expression of estrogen regulated genes through an interaction with ERα. In Figure 2.6A, Co-immunoprecipitation experiments were performed to determine if CD1 could interact with ERα in prostate stromal cells. Co-overexpressing BHPrS$^{CD1\text{-}ER\alpha}$ cells demonstrate CD1 and ERα can interact when ectopically expressed. Overexpression of CD1 in BHPrS alone demonstrated interaction with endogenous ER in prostate stromal cells. We next examined if CD1 overexpression modifies ERα transcriptional activity. In Figure 2.6B, luciferase activity assays were performed with the use of estrogen responsive element fused with the luciferase gene. These experiments were performed in presence/absence of β-estradiol. In comparison to the empty vector control BHPrS^{EV}, CD1 overexpression induced

increased ER transcriptional activity in the absence of hormone. This indicates that CD1 can induce the transcriptional activity of the ER without the ligand bound in prostate stromal cells. This finding is in agreement with studies of ER and CD1 in breast cancer (Neuman et al., 1997). Co-overexpression of ERα and CD1 in BHPrS cells induced greater luciferase expression compared to CD1 overexpression alone, both in the presence and absence of hormone. These data show that CD1 can interact with ERα to drive transcriptional activity of the estrogen receptor on non-chromosomal DNA. To determine if the CD1-ERα interaction binds chromosomal DNA we performed chromatin immunoprecipitation (ChIP) experiments with the BHPrS^{CD1} and BHPrS$^{CD1\text{-}ER\alpha}$ cell lines. In figure 2.6C overexpression of CD1 in BHPrS showed greater than 11 fold recruitment over the IgG control of CD1 to the estrogen receptor element (ERE) in the CathD gene in the absence of hormone. In the presence of hormone CD1 recruitment to the ERE in the CathD gene was only increased 8 fold over the IgG control .

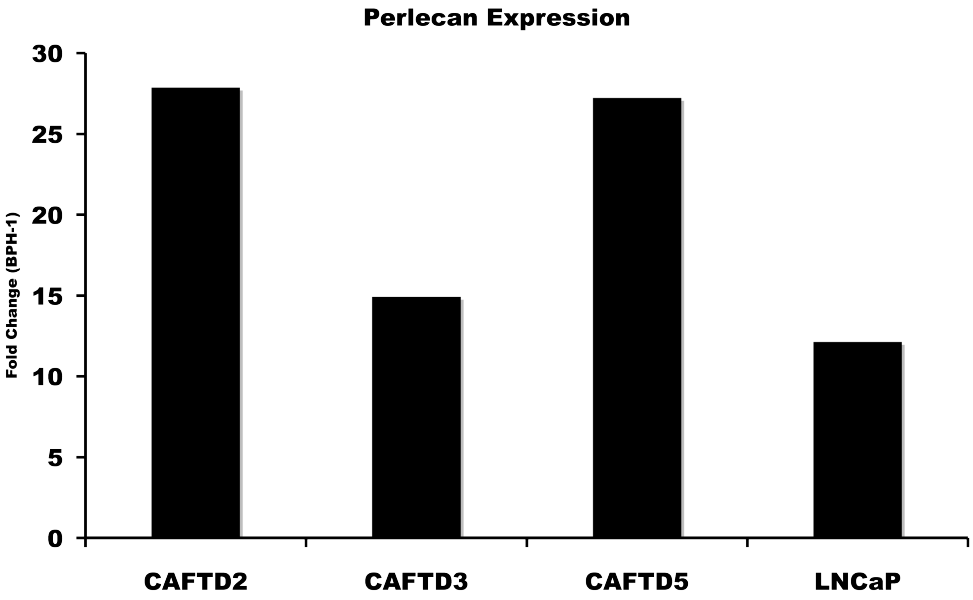

Figure 2.7. Perlecan expression in transformed prostate cell lines. Real-time expression analysis of Perlecan in the transformed derivatives of BPH-1 cells, CAFTD2,3,5. The PCa cell line LNCaP was used as a positive control. Expression is relative to the non-transformed cell line BPH-1.

CathD is a highly decorated glycoprotein that is trafficked to the lysosome through the mannose-6-phosphate receptor, also known as the insulin-like growth factor II receptor (IGFIIR), however no pro-tumorigenic correlations between CathD and the IGFIIR have been identified (Capony et al., 1989; Vetvicka and Vetvickova, 1998; Vetvicka et al., 1997). Experimental evidence suggests that the basic amino acids contained with in the pro-domain of CathD can interact glycosaminoglycans (GAG), leading to activation of the protease (Beckman et al., 2009; Masa et al., 2006). GAGs are large linear polysaccharide structures that can contain sulfate groups. The

presence of these sulfate groups provides a net negative charge to the GAG. Heparin Sulfate (HS) is a common glycosaminoglycan (GAG) that is linked to a protein core to form a proteoglycan. The HS domains on proteoglycans are important for providing docking sites for proteins like growth factors and enzymes (Kirn-Safran et al., 2009). In PCa proteoglycans are overexpressed and are thought to facilitate tumor growth by binding and presenting growth factors to malignant cells.

A functional hallmark of prostate CAFs is their ability to induce tumorigenesis when recombined with BPH-1 cells (Olumi et al., 1999b). When the BPH-1 cells were isolated from malignant tissue recombinations they exhibited a permanent transformed phenotype (Hayward et al., 2001). The resulting series of cell lines established from recombinations of CAF with BPH-1 were designated CAFTD. We questioned whether the expression of the HS proteoglycan Perlecan changed with the conversion of BPH-1 cells to the malignant CAFTDs. In figure 2.7, real-time PCR was performed using primers specific for human Perlecan. The expression of Perlecan in CAFTD-2, -3, and -5 was compared to Perlecan expression in parental BPH-1. In human PCa and PCa cell lines Perlecan in reported to be overexpressed and is necessary for *in vivo* growth of PCa cell lines (Savore et al., 2005). As a control for Perlecan overexpression we used the weakly tumorigenic PCa cell line

LNCaP. In comparison to BPH-1 expression levels, Perlecan expression is upregulated more than 15 fold in all of the CAFTD cell lines examined.

Next we wanted to determine if the conversion of pro-CathD to the active protease is dependent on the presence of glycosaminoglycans (GAGs) on the surface of PCa cells. To accomplish this task we treated a series of PCa cell lines with heparinase to remove heparin sulfate containing proteoglycans from the cell surface prior to the addition of pro-CathD. The heparin sulfate proteoglycans have been previously shown to convert the CathD zymogen to the active state (Lyons et al., 1988). Completion of the CathD activity assay after treatment with heparin lyase (Figure 2.8) did not show any inhibition in the conversion of pro-CathD to the active protease.

We also performed this experiment with 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), a specific inhibitor for Na(+)/H(+) antiporters. It has been shown that human PCa and PCa cell lines have lower extracellular pH due to increased proton pump expression (Steffan et al., 2009). We hypothesized that CathD activity was dependent on the activity of proton pumps present on the surface of PCa cells. PCa cell lines treated with EIPA show drastic decreases in extracellular CathD activity similar to levels when of cell lines treated with the CathD inhibitor pepstatin A. Our results show that

extracellular CathD activity is not dependent on heperan sulfate containing proteoglycans, however is dependent on the extracellular pH.

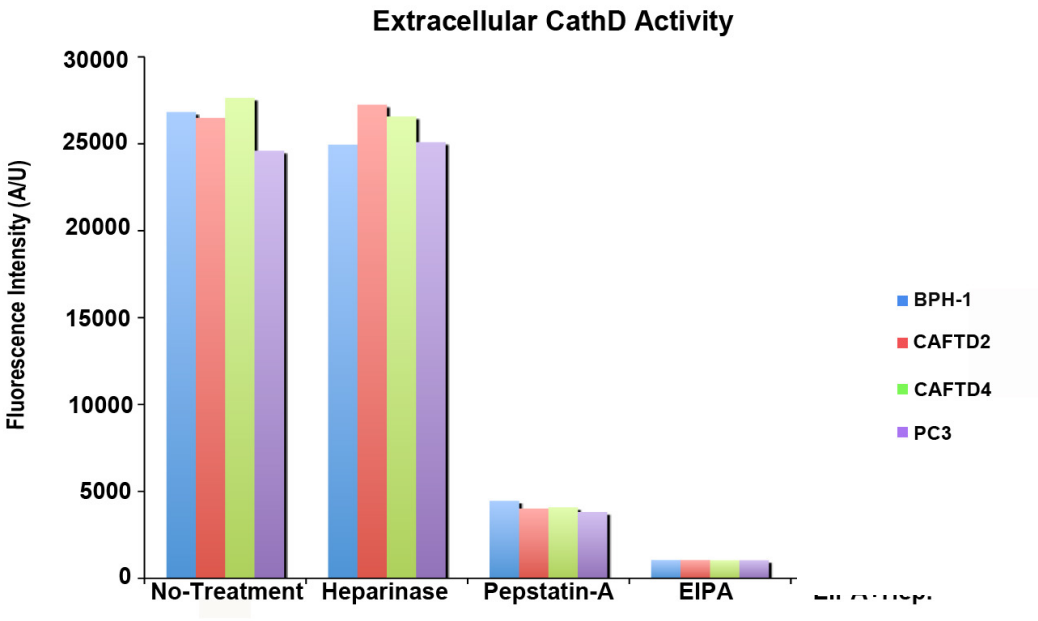

Figure 2.8. Extracellular CathD proteolytic activity is dependent on proton pump activity. Pro-CathD (inactive) was added to monolayer cultures of ■ BPH-1 cells , ■ CAFTD2, ■ CAFTD4, and ■ PC3 cells along with CathD specific substrate. Fluorescence is produced after cleavage of substrate by active CathD. Cell monolayers were pre-treated with heparinase to remove HS-GAGs. Cells were also treated with the CathD inhibitor pepstatin A, or EIPA,an inhibitor of Na+/H+ pumps.

2.4 Discussion

Tumor stroma has been implicated in the regulation of cell growth, determining metastatic potential, and impacting the outcome of therapy. Stromal-epithelial interactions in cancer been have implicated as promoting several malignancies including prostate, breast, colon, and pancreatic cancers (Olumi et al., 1999b; Weinberg, 2008). The stroma is often radically changed around malignant tumors and such changes both predict prognosis and may actually contribute to disease progression (Olumi et al., 1999b; Weinberg, 2008). We have previously examined the role of several molecules found to be aberrantly expressed in cancer associated stroma that induce tumorigenesis and malignant conversion (Ao et al., 2007). We reported that CD1-overexpressing BPH-1 cells are non-tumorigenic in the presence of rUGM in tissue recombination experiments, but in contrast, the overexpression of CD1 in prostate fibroblasts induces a strong tumorigenic response in the non-malignant but genetically initiated BPH-1 cells (He et al., 2007). The tumor-promoting abilities of NPF^{CD1} produce changes very similar to published descriptions of tissue recombinations of CAF with BPH-1 (Olumi et al., 1999b). A comparison of the genetic profiles from CAF and NPF^{CD1} identified CathD as being upregulated 7-fold in comparison to NPF (He et al., 2007). From this finding we hypothesized that CathD may be a mediator of stromal-epithelial interactions contributing to prostate

tumorigenesis.

CathD overexpression in neoplastic cells and neoplasia-associated connective tissue was described as long as 24 years ago, and is reported to play several roles in cancer progression (Glondu et al., 2002; Nomura and Katunuma, 2005; Reid et al., 1989; Weidner et al., 1992). Cathepsins have recently been shown to be upregulated in a pancreatic tumor model and also contribute to invasive breast tumor growth (Nadji et al., 1996; Shen et al., 2004; Tumminello et al., 1996). We previously reported that CathD is upregulated in both NPF^{CD1} cells (which mimic CAF) as well in CAF. Microarray analysis revealed a 7-fold increase in CathD resulting from CD1 expression in NPF. NPF^{CD1} cells display increased motility in comparison to control NPFs in a wound healing assay which was shown to depend upon the expression of CathD (Olumi et al., 1999a). Here we show that the ability of NPF^{CD1} to survive and invade into 3D matrices was also dependent on CathD. The overexpression of CD1 in NPF produced invasive colonies with protrusions sprouting into the surrounding matrix. This invasive growth was inhibited in NPF^{CD1} cells when CathD expression was knocked down. This finding is supported by similar results from Laurent-Matha et al. where CathD was critical for outgrowth of human fibroblast in 3D matrices (Laurent-Matha, 2005). Our findings were consistent with a model in which CD1-induced overexpression of CathD resulting in increased fibroblast motility and

invasion.

An immunohistochemical examination of clinical specimens revealed low levels of expression of CathD in normal prostate stromal tissue. Malignant areas showed prominent stromal expression of CathD, with the significantly greater stromal CathD expression in high grade tumor samples. IHC analysis of CathD expression in tissue recombinations of BPH-1+CAF and NPF^{CD1} also revealed strong stromal staining in comparison to recombinations of BPH-1+NPF and BPH-1+rUGM. CathD expression in human prostate cancer stoma correlates, with shorter survival and recurrence-free periods (Bacac et al., 2006). Our experimental data establishing a link between the overexpression of CD1 with the up-regulation of CathD in prostate CAF, coupled with the similar findings in human disease indicates a strong association between cell cycle regulation and protease expression in prostate tumorigenesis. The cell cycle regulator CD1 and the ERα are known to interact and can induce estrogenic gene transcription (Neuman et al., 1997). This suggests the possibility that the overexpression of CathD in PCa associated stroma is due to the interaction of ERα with CD1.

Further investigation into the role of CathD in the tumor microenvironment showed that CathD expression is necessary for NPF^{CD1} cells to promote epithelial growth under *in vitro* conditions. The pro-mitogenic effect of NPF^{CD1} conditioned medium on BPH-1

cells was inhibited when CathD expression was knocked-down in NPF^{CD1}. This result mirrors published findings showing that CathD is mitogenic to PCa cell lines (Vetvicka et al., 1998). These data do not, of course, imply that CathD is a direct mitogen, merely that its presence results in a mitogenic environment. Given the possibility that this protease may activate latent growth factors associated with extracellular proteoglycans an indirect mechanism is not only possible but likely.

To further pursue an underlying mechanism we engineered BHPrS cells to overexpress CathD and combined these $BHPrS^{CathD}$ cells with BPH-1 and performed renal grafting experiments. Recombinations of BPH-1 cells with BHPrS resulted in benign solid epithelial cords similar to recombinations of NPF with BPH-1 cells (Franco et al., 2011). However, the overexpression of CathD in BHPrS in recombination experiments with BPH-1 cells induced a malignant transformation with invasion into the mouse kidney. This is consistent with our findings with the CathD knockdown approach in experiments with the CAFs. A feature of the prostate tumor microenvironment in human disease is the expansion of myofibroblast like cells with increased deposition of extracellular matrix proteins (Tuxhorn et al., 2001). Masson's trichrome staining of tissue xenografts from the CathD overexpressing stromal cells revealed increased production of collagen in comparison to

recombinations with the EV control stromal cells. These staining patterns were similar to previous publications that pointed towards altered TGF-β signaling. We have previously shown that the overexpression of TGF-β in BHPrS cells resulted in the development of poorly differentiated adenocarcinoma with increased deposition of collagen in tissue recombination experiments (Franco et al., 2011). TGF-β is expressed by most cultured cells in an inactive form due to binding with latent complex, and activation requires the proteolytic degradation of this complex. CathD derived from fibroblast conditioned media has been shown to liberated active TGF-β from the latent complex (Lyons et al., 1988). Investigation of altered TGF-β signaling in our model revealed increased p-SMAD2/3 staining, a surrogate marker for TGF-β response, in the CathD overexpressing recombinations. Examination of Col.IVα2, a direct TGF-β responsive gene, expression in the tissue xenografts revealed increased staining for Col.IVα2. Collectively, the differences in stromal composition observed from trichrome staining can be linked to increased TGF-β signaling and responsive gene expression as a result of stromal derived CathD. The overexpression of CathD in the stroma resulted in a somewhat minor, all though sufficient, malignant transformation of initiated epithelial cells similar to the tumor inductive properties of CAF.

We previously demonstrated that NPF^{CD1} cells and CAF

elicited permanent malignant transformation of BPH-1 cells (Hayward et al., 2001; Olumi et al., 1999a; Phillips et al., 2001) (Olumi et al., 1999b). Data from IHC of clinical tissue showed increased CathD in the stroma adjacent to malignant regions of the prostate. To address the contribution of CathD in CAF-induced tumorigenesis of BPH-1, we engineered CathD knock-down CAF. The ability of CAF to induce tumorigenesis in BPH-1 recombinations was abolished when CathD expression was knocked-down. Similar results were observed when CD1 expression was knocked down in CAF. These data indicated that CathD is not only an important mediator of stroma-epithelial cross talk *in vitro*, but also an essential component in promotion of tumorigenesis *in vivo*, at least in this model.

In summary, the study presented here demonstrates that CathD can play a role as a paracrine mediator contributing to prostate tumorigenesis. We show that the presence of HS containing proteoglycans do not influence the conversion of pro-CathD to the active protease, however, HS containing proteoglycans serve as a reservoir for numerous growth factors, including. TGF-β. We cannot rule out a role for proteoglycans like Perlecan in tumor microenvironment in facilitating the presentation of mitogenic factors to malignant epithelial cells. *In vitro* experiments showed the proteolytic function of extracellular CathD is dependent on the activity of Na+/H+ exchangers on the surface of malignant epithelial cells. It

has yet to be determined if inhibition of these proton pumps inhibits the malignant conversion observed in tissue recombinations with CathD overexpressing stromal cells.

The identification of key players, such as CathD, that participate in the promotion of the tumor microenvironment contributes to our understanding of the molecular mechanisms underlying this process and may prove to be valuable for the development of novel anti-cancer therapies. Current anti-cancer therapies target the malignant epithelial cells, which progressively acquire genetic alterations during the progression of the disease (Ling, 1997; Pérez-Tomás, 2006; Reles et al., 2001). The biggest obstacle facing clinicians treating people with cancer in general is the toxicity of treatments combined with the development of resistance to therapy. The tumor microenvironment has been shown to be more genetically stable and therefore less likely to develop resistance to novel anti-cancer therapeutics (Allinen et al., 2004; Qiu et al., 2008). Since tumor promotion by the microenvironment is a function of many different signaling molecules it should be possible to develop therapeutic strategies which appropriately modify several pathways simultaneously rather than simply attempting to totally block a single signal. This is likely to be both more effective and better tolerated, since the normal biological effects of the molecules concerned will be less affected. Further investigation is needed to explain in detail how

CathD is acting. A better understanding of the complexities of CathD in the tumor microenvironment may provide targets for suppressing lethal PCa phenotypes.

CHAPTER 3

The Role of Stromal Estrogen Receptor Alpha in Hormonal Carcinogenesis

3.1 Introduction

Androgens and the AR have been shown to have an integral role in mediating normal proliferation, differentiation, and maintenance of the prostate (Cunha et al., 1982). However, androgens and the actions of the AR are also generally accepted to play a role in the development and progression of PCa (Cunha et al., 2002; Gao et al., 2001; Gao and Isaacs, 1998; Titus, 2005). Prior to the development of the radical prostatectomy procedure, the only treatment option for men with PCa was physical or hormonal castration to reduce the levels of circulating androgens. The hormonal castration approach, establish in 1941 by Charles Huggins and Clarence Hodges, is still the basis of treatment for men with advanced PCa. The likelihood for developing PCa correlates with a man's increasing age (Crawford, 2003). Past 30 years of age, the circulating levels of androgens in men begin to decrease, a process that continues with age (Harman et al., 2001). Conversely, after the age of 50, serum estrogen levels increase (Vermeulen and Verdonck, 1969). This results in a shift with an increase in the amount of free

estrogen relative to free androgens in older men. The shift in levels of androgens relative to estrogen has been used to support the suggestions that estrogens are involved in the development of PCa.

Animal models of prostate development and carcinogenesis have been used extensively to examine the effects of estrogen on the prostate. Castrated dogs supplemented with estrogen were shown to undergo stromal expansion of the prostate by enhancing the actions of the AR (Moore et al., 1979). Estrogen treatment of castrated dogs also induces alterations in the epithelial histology characterized as squamous metaplasia (Trachtenberg et al., 1980). Developmental models have shown that intermittent exposure to estrogens during neonatal and/or perinatal development periods induces dysplastic changes and increased proliferation in the adult prostates of mice (Ho et al., 2006; Naslund and Coffey, 1986; Pylkkanen et al., 1993). Prolonged exposure to high dose estrogens in combination with androgens in the NBL rat model produced a 100% incidence of PCa, whereas treatment with androgens alone induced PCa with a 40% incidence (Bosland et al., 1995; Noble, 1977). Similar to the experiments with the NBL rat model, tissue recombination experiments using rUGM and the human prostate epithelial cell line BPH-1, grafted in mice supplemented with estrogen and testosterone induced invasive carcinoma in the human epithelial cells (Wang et al., 2001). Recombinations of rUGM with BPH-1 cells in mice

supplemented with testosterone alone produced benign solid branched epithelial cords and ductal structures (Hayward et al., 1998). In these animal models, it appears that estrogen in combination with androgen is required for the maximal carcinogenic response.

The actions of estrogen are mediated through two receptor subtypes; estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ). In the normal prostate, expression of ERα is restricted to stromal cells and the expression of the ERβ is localized to secretory luminal cells, and is also expressed in basal cells (Prins and Korach, 2008). Increased nuclear accumulation of estrogen receptor is seen in the stroma of human BPH samples, suggesting that stromal proliferation is mediated by the actions of ERα (Kozak et al., 1982). The role of ERβ expression in the prostate is somewhat unclear, the prevailing hypothesis is ERβ plays a role in maintaining epithelial differentiation and suppresses epithelial proliferation (Imamov et al., 2004; Weihua et al., 2001). During PCa progression, the expression patterns of the ER subtypes become dysregulated. The expression of ERα is reported to be elevated in the stroma during the progression of PCa (Fixemer et al., 2002). ERβ expression experiences dynamic changes from early stage diseases progressing to the development of metastatic lesions. Most reports conclude the ERβ expression decreases in PIN, and in disease states of low to high Gleason

scores (Fujimura et al., 2001; Horvath et al., 2001; Leav et al., 2001). In metastatic disease, ERβ expression is restored in varying degrees (Fixemer et al., 2002; Lai et al., 2004). This suggests that ERβ may serve to inhibit tumor proliferation, while ERα overexpression may serve to promote tumor progression.

Our laboratory has published extensively on the role of the stroma in carcinogenesis of the prostate. We have previously described several molecules found to be aberrantly expressed in cancer associated fibroblasts (CAFs) including CD1, stromal derived factor-1 (SDF-1), also known as CXCL-12, and CathD that contribute to tumorigenesis and malignant transformation in tissue recombination experiments (Ao et al., 2007; He et al., 2007; Pruitt et al., 2013). All of these molecules can be regulated by a number of different factors, but are putative estrogen regulated genes (Eeckhoute, 2006; Hall, 2003; Klinge, 2001).

Steroid hormone receptor biology has provided a world of insight into the structure and function of transcription factors in regulation of gene transcription and organ development. Studying the structure and function of this class of transcription factors has made ideal targets for therapeutic intervention (Heuson et al., 1975; J C Heuson et al., 1975; Noble, 1977b; Ward, 1973). Tamoxifen is a non-steroidal anti-estrogen, that was first synthesized in 1966, but was not entered into clinical trials for the treatment of breast cancer until

1971 (Cole et al., 1971). Anti-estrogens like tamoxifen have a broad therapeutic spectrum. Tamoxifen has been widely used for the treatment of several neoplastic diseases, and non-neoplastic diseases such as endometriosis or uterine fibroids (Tonietto et al., 1997). Tamoxifen has also been used as preventative therapeutic in women who are at a high-risk for the development of breast cancer, and in the prevention of osteoporosis in postmenopausal women (Fisher et al., 1998; Gotfredsen et al., 1984). Tamoxifen has shown to be very effective in the prevention of post-surgical recurrence of breast cancer (Fowble et al., 1996).

Tamoxifen is a member of a class of drugs, which act as competitors of estrogen for the same binding site on the ER. At the molecular level, tamoxifen serves as an anti-estrogen by occupying the ligand binding domain of the estrogen receptor, also known as the AF-2 domain. This binding induces a conformational change in the ER which differs from usual conformation when estrogen is bound. This altered conformational structure prevents the ER from interacting with co-activators, resulting in the inhibition of the transactivation function of the receptor (Dhingra, 1999; McDonnell, 1999). Tamoxifen is one of the most successfully used ER modulators (Park and Jordan, 2002). However, the numerous benefits tamoxifen provides as an anti-estrogen come with a laundry list of significant side-effects. Since the conception of Tamoxifen 47

years ago, a new class of drugs has been developed to improve upon the antagonistic effects in the treatment of various malignancies without the adverse side effects.

Some of the side effects associated with tamoxifen include the development of endometrial cancer and thromboembolic phenomena (Han and Liehr, 1992; Hemminki et al., 1996; Hendrick and Subramanian, 1980). The concept of an ideal drug that would have tissue selectivity, and ER-agonist or antagonist effects led to the development of selective estrogen receptor modulators (SERMs). Raloxifene is an example of a SERM that has potent anti-ER effects without the negative side effects associated with Tamoxifen.

Tamoxifen is reported to be an effective therapy for several conditions in men. Men diagnosed with coronary artery disease that were treated with tamoxifen were reported to show improved endothelial function along with decreases in several plasma cardiovascular risk factors (Clarke et al., 2001; McCrohon et al., 1997; New et al., 1997). Tamoxifen therapy is has also been shown to have efficacy in the treatment of painful gynaecomastia (Hanavadi et al., 2006; McDERMOTT et al., 1990). In the treatment of several types of cancers in men, tamoxifen has been shown to be tolerated at high doses (Trump et al., 1992). However, a 4-year phase II clinical trial on the use of tamoxifen in men with hormone-refractory metastatic PCa, concluded that high dose tamoxifen therapy does

not warrant any further clinical applications. The investigators also noted that no notable stabilization in PSA increase was observed in men treated with tamoxifen (Bergan et al., 1999). No investigation has been performed to elucidate the reason why men with advanced PCa failed to respond to treatment with tamoxifen. Studies using tamoxifen for the treatment of idiopathic oligozoospermia, reported significant increases in testosterone, and testosterone precursors, along with increases in LH and FSH in men (Hampl et al., 2009). Being purely speculative, increased androgen production by tamoxifen may be a possible explanation for the failed tamoxifen trial in men with metastatic AIPCa.

3.2 Materials and Methods

Cells. BPH-1 cells (a non-tumorigenic human prostatic epithelial cell) were derived from our own stock (Hayward et al., 1995a). CAF cells were isolated as described in Olumi et al. (Olumi et al., 1999b) from human prostate tumor samples and their ability to induce tumors was confirmed. Normal associated fibroblasts (NAF) cells were isolated as previously described from benign human prostate tumor samples. Benign human prostate stromal cells (BHPrS) were isolated from a prostate surgical sample and immortalized with hTERT as previously described (Franco et al., 2011). Cells were maintained in RPMI 1640 (Gibco, Carlsbad CA) with 1% antibiotic/antimycotic (Life Technologies, Grand Island, NY) and 5% Heat-inactivated Charcoal-stripped fetal calf serum (Gibco, Carlsbad, CA).

Generation of genetically modified cell lines. pEGFP-C1-ERα and pEGFP-C1-ERβ (Addgene) vectors were transfected into BHPrS cells to generate $BHPrS^{ER\alpha}$ and $BHPrS^{ER\beta}$. Cells positive for the ERα and ERβ expressing constructs were selected for resistance to G418. BHPrS cells were also engineered to express and pEGFP empty vector ($BHPrS^{EV}$) and selected for resistance to neomycin.

Real-time PCR. Total RNA was isolated from pelleted NAFs, CAFs, and BPH-1 cells using the TRIzol method. The aqueous phase was collected and combined with 3.5 times the volume of buffer RLT from the RNeasy Kit (Qaigen) and 2.5 times the volume of 100% EtOH. Isolation of RNA was performed according the RNeasy protocol from Qiagen. RNA was reverse transcribed using I-script (BioRad), and real-time PCR was performed using human specific primers.

Gene	Primer Sequence
ERα	5' - **GAGGATTCCCGTAGCTCTTC**-FOR 5' - **CCCTTGACCTAGCTTTCTCC**-REV
ERβ	5' - **GGCAGAGGACAGTAAAAGCA**-FOR 5' - **GGACCACACAGCAGAAAGAT**-REV
Aromatase	5' - **CCTCTGAGGTCAAGGAACAC**-FOR 5' - **CAGAGATCCAGACTCGCATG**-REV
GAPDH	5' - **TGCACCACCAACTGCTTAGC**-FOR 5' - **GGCATGGACTGTGGTCATGAG**-REV

TABLE 3.1. Primer sequences for real-time PCR

Western Blot. Cell lysates were prepared and Western blotting was performed as previously described (Pruitt et al., 2013). Membranes were incubated with rabbit primary antibody to Vimentin (Sigma, A2547), α-smooth muscle actin (Sigma, V6630), β-actin (1:5000, Sigma) overnight, washed with PBS-Tween 20 for 1 hour, and incubated with horseradish-peroxidase linked anti-mouse secondary antibody (Amersham Biosciences, Piscataway, NJ, 1:10,000 dilution) for 1 hour. Bound antibodies were visualized using enhanced chemiluminescence western blotting detection reagents (Amersham

Bioscences).

Crystal Violet Growth Assays. Genetically modified BHPrS cell lines were plated sub-confluently at 5,000 cells/well in 24-well tissue culture plates (Corning Inc, Corning, NY), in RPMI media supplemented with 1% charcoal-stripped FCS supplemented with $1.0x10^{-8}$M β-estradiol. Cells were fixed in a solution 11% glutaraldehyde with ddH_2O for 15 min shaking (500 cycles/min). Fixed cells were washed extensively in ddH_2O and allowed to air dry. Crystal violet stain was prepared in a solution of 0.1% crystal violet (w/v) with 200mM Boric Acid (w/v) pH 9. 300µl of crystal violet stain was incubated with adhered cells for 20min at room temperature shaking (500 cycles/min). Excess crystal violet was removed and cells were washed extensively in ddH_2O, and allowed to air dry completely. Bound crystal violet was solubilized in 300 µl of 10% acetic acid for 30min. Quantification of cell growth was determined by measuring absorbance at 570nm using automated plate reader (Bio-Tek Instruments Inc., Winooski, VT) and subtracting value from an acetic acid blank.

Human Prostate Tissue Microarray. Human prostate tissue array (PR806) was obtained from US Biomax, Inc. The array contained 30 cases of adenocarcinoma in duplicate with Gleason scores ranging

from 4-7, 5 cases of adjacent normal tissue (NAT), and 5 cases of normal tissue with duplicate cores per case.

Immunohistochemical Staining. Immunohistochemical staining was performed following a protocol that was described previously (Williams et al., 2005). Tissue slides were then incubated with the primary antibody against ERα (1:200, ABCAM), SV-40 (1:200, Santa Cruz), SDF-1 (1:50 Santa Cruz), CathD (1:200 Santa Cruz). The polyclonal rabbit or mouse immunoglobulins/biotinylated anti-mouse secondary antibody (DAKO, Carpenteria, CA) was incubated for 60 min after the slides were washed with PBS buffer for 1 hour. After washing the slides in PBS extensively, slides were incubated in ABC-HRP complex (Vector Laboratories) for 30 minutes. Bound antibodies were then visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride (liquid DAB, DAKO). Slides were then rinsed extensively in tap water, counterstained with hematoxylin, and mounted.

Immunofluorescent Staining. Cell lines were plated on glass slides in media containing 5% charcoal stripped serum for 24 hours. Slides were treated with 100nm estradiol for 4 hours. prior to fixation in 100% EtOH. Non-specific staining from antibodies was prevented by blocking slides in 15% BSA in PBS for 2 hours. Slides were treated

with the antibodies against ERα (1:100 Santa Cruz) or ERβ (1:100 ABCAM) overnight in blocking buffer. Slides were washed in PBS for 30 minutes prior to the addition of AlexaFluor-488 goat anti-rabbit IgG for 30 min in blocking buffer. After a 30 minute wash in PBS, slides were counterstained with VECTASHIELD mounting media with DAPI (Vector Laboratories, Burlingame, CA).

Quantitative Image Analysis. Immunostained slides were analyzed using the Ariol SL-50 automated slide scanner (Applied Imaging, San Jose, CA). Positive staining was calculated by masking out all non-stromal areas for the tissue cores and applying a threshold for recognizing brown-positive cells. The values are presented as the total number of positive cells in the tissue core punch.

Tissue recombination and Xenografting. BPH-1 + BHPrS^{ERα}, BPH-1 + BHPrS^{ERβ}, BPH-1 + BHPrS^{EV}, BPH-1 + NAF, BPH-1 + CAF, BPH-1 + rUGM tissue recombinants were made as previously described (Hayward et al., 1999). $1.0x10^5$ epithelial cells and $2.5x10^5$ stromal cells combined in type I rat tail collagen were used to make the recombinants. After overnight incubation, the tissue recombinants were grafted under the kidney capsule of castrated adult male severe combined immunodeficient (SCID) mice (Harlan, Indianapolis, IN) supplemented with 25mg testosterone pellets (PCCA, Houston TX),

2.5mg β-Estradiol pellets combined with 22.5mg cholesterol (Sigma), 5mg Tamoxifen pellets combined with 20mg cholesterol (Sigma). Recombinants were performed in replicates of six. Mice were sacrificed at eight weeks and grafts were harvested, fixed, and paraffin embedded. Graft dimensions were measured using the formula: volume=width x length x depth x π/6 as described previously (Pruitt et al., 2013).

Statistical Analysis. Data from the *in vitro* and *in vivo* experiments are presented as the mean ± standard deviation (SD). The data were analyzed using GraphPad PRISM software (La Jolla, CA). Quantitation of ERα expression in human prostate clinical samples were compared with analysis of variance followed by post hoc analysis of significant means by Kruskal-Wallis test in comparison of normal to tumor tissue and NAT. Post hoc analysis of significant means by Dunn's multiple comparison test was used for the comparison ERα expression in normal tissue to NAT and normal tissue to tumor tissue. p-values less than 0.05 were considered statistically significant.

3.3 RESULTS

ERα expression is upregulated in the stroma of prostate clinical samples.

We examined the expression of ERα in human prostate clinical samples using a tissue microarray, which contained 30 cases of adenocarcinoma, 5 cases of normal prostate tissue, and 5 cases of normal prostate tissue adjacent to malignant tissue (NAT). The tissue array contained duplicate cores per case. Quantification of nuclear ERα in stromal regions was significantly greater in tumor prostate tissue in comparison to normal prostate tissue (Figure 3.1). Since we observed increased expression of ERα in the stroma of malignant prostate tissue, we examined the consequences of ERα modulation in stromal cells. To address this question we engineered BHPrS cells to stably overexpress ERα ($BHPrS^{ER\alpha}$). As a control, we also overexpressed ERβ in BHPrS ($BHPrS^{ER\beta}$). Quantitative real-time PCR was performed to confirm overexpression of ERα and ERβ in the BHPrS cell line. Relative to the EV control, there is a 30 fold increased expression of ERα and a 8 fold increase of ERβ expression in the engineered BHPrS cell line (Figure 3.2A). Crystal violet growth curves were performed to determine if the overexpression of the ERs affected cell proliferation. $BHPrS^{ER\alpha}$

proliferate at a significantly greater rate than the empty vector control ($BHPrS^{EV}$) and the $BHPrS^{ERβ}$ (Figure 3.2B) in the presence of estrogen. Western blot analysis was performed to characterize any differences in the expression of filament proteins as a result of ER overexpression. $BHPrS^{EV}$ cells display expression of vimentin, but do not express α-SMA, or calponin. $BHPrS^{ERα}$ and $BHPrS^{ERβ}$ display expression for the filamentous proteins vimentin, α-SMA, and calponin (Figure 3.2C). To show the overexpression of the transgene was functional, we performed Immunofluorescent staining for ERα and ERβ localization with and without the addition of estradiol. Treatment with hormone after 4 hours induces the nuclear accumulation of both ERs. No nuclear localization of ERs was observed under hormone-free conditions (Figure 3.2D).

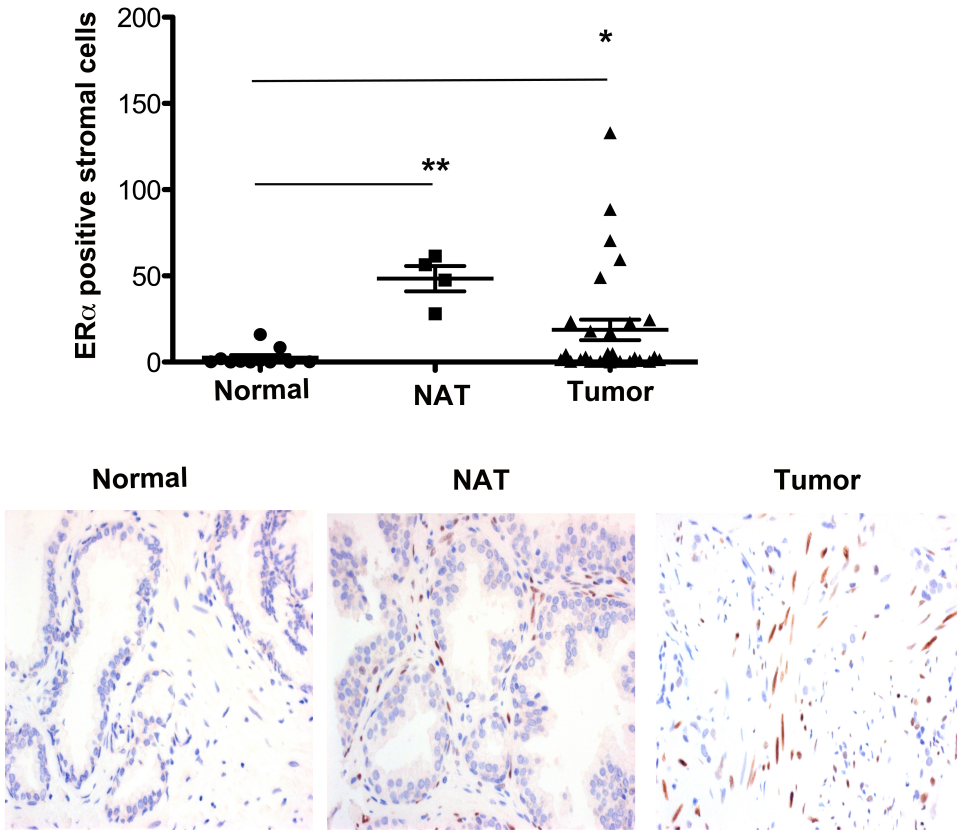

Figure 3.1. ERα is overexpressed in the stroma of malignant clinical prostate tissue. Quantitation of total ERα expressing stromal cells in each prostate core comparing normal to tumor, and normal to NAT. Data are presented as means ±SD, Statistical analysis performed by ANOVA, *p-value≤ 0.05, **p-value≤ 0.0005 **(top)**. Representative images from immunohistochemical analysis of ERα expression in normal (n = 5), NAT (n=5), and tumor (n = 30) human prostate tissues **(bottom)**.

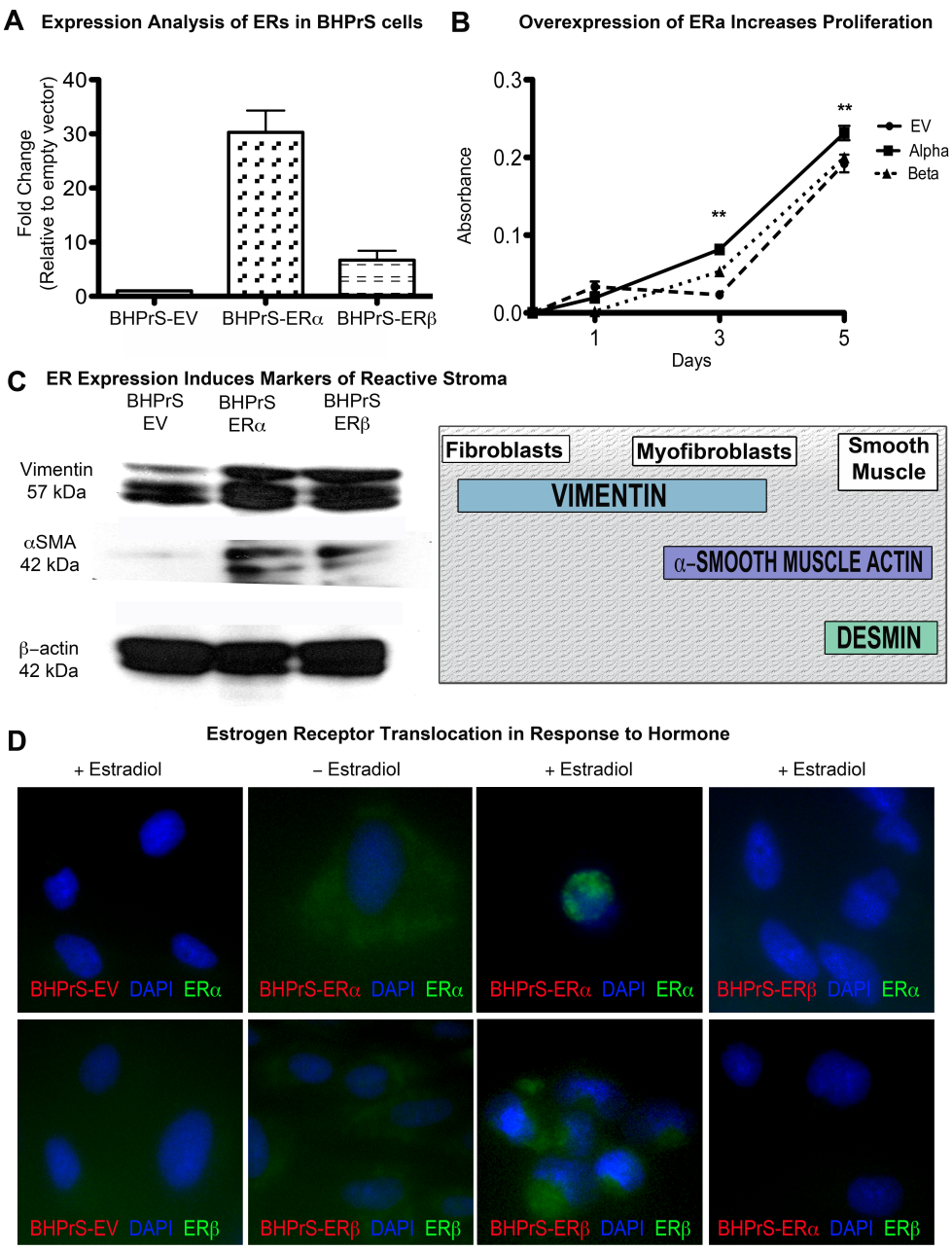

Figure 3.2. Overexpression of ERα increases stromal cell proliferation and induces myofibroblast phenotype. A) Expression analysis confirming overexpression of ERα and ERβ in BHPrS cell line. **B)** Crystal violet growth curve of $BHPrS^{EV}$ (●), $BHPrS^{ER\alpha}$ (■), and $BHPrS^{ER\beta}$ (▲), p-value ≤ 0.05. **C)** Western blot analysis of structural proteins in engineered stromal cells. **D**) Immunofluorescence of ER (green) localization after treatment with Estradiol (100nm) for 4 hours. Nuclei counterstained with Dapi (blue).

Overexpression of ERα in benign human prostate stromal cells induces tumorigenesis.

To further investigate the consequences of ERα in the stroma, we prepared tissue recombinations using the prostate epithelial cell line BPH-1 as a reporter with the BHPrS^{ERα} and BHPrS^{ERβ} stromal lines and grafted under the renal capsule in castrated SCID mice supplemented with a combination of 17β-estradiol and testosterone pellets, testosterone alone, or no-treatment (NT). As a control we also used rat urogenital mesenchyme (rUGM) recombined with BPH-1 cells and grafted under the renal capsule in castrated mice supplemented pelleted testosterone and 17β-estradiol. Recombinations of rUGM under these conditions induces carcinogenesis of the adjacent BPH-1 cells as previously described by Wang et al. 2001 (Wang et al., 2001).

Results of Tissue Recombinations				
Treatments				
	rUGM	EV	ERα	ERβ
NT	No Growth	No Growth	No Growth	No Growth
T	Small Benign Growth	Small Benign Growth	Small Benign Growth	Small Benign Growth
T + E	Largest Malignant Tumors	Small Benign Growth	Large Malignant Tumors	Small Benign Growth
E	Small Growth SQM	Small Growth SQM	Small Growth SQM	Small Growth SQM

Table 3.2. Results of various recombinations of BPH-1 cells with rUGM, $BHPrS^{EV}$(**EV**), $BHPrS^{ERα}$ (**ERα**), and $BHPrS^{ERβ}$ (**ERβ**), supplemented with the different hormones testosterone (**T**), estrogen (**E**), and testosterone + estrogen (**T+E**).

Table 3.2 shows the results from the various recombinations with different hormone treatment groups. After an 8 week engraftment period, recombinations of $BHPrS^{ERα}$ + BHP-1 cells gave rise to significantly larger tumors in comparison to recombinations of BPH-1 with either $BHPrS^{ERβ}$ or $BHPrS^{EV}$. Recombinants composed of rUGM + BPH-1 cells grafted in mice supplemented with the same hormone combination produced significantly larger tumors in comparison to castrated mice (Figure 3.3). Similar experiments were performed in mice supplemented with testosterone and β-estradiol independently. These resulted in smaller growths in comparison to mice supplemented with the combination of hormones. H&E staining of xenografts was performed to examine the histology of the resulting recombinations (Figure 3.4). $BHPrS^{ERβ}$ + BPH-1 produced benign solid epithelial structures reminiscent of the solid cords seen in

developing prostate. Stromal cells were organized around the BPH-1 cells, similar to recombinations with the empty vector control (Figure 3.4, d and h). Overexpression of ERα in stromal cells resulted in very disorganized and aggressive tumors displaying a malignant transformation (Figure 3.4, f) in mice supplemented with the combination of β-estradiol and testosterone. The histology of recombinations with BHPrS^{ERα} resembled the malignant conversion that resulted from recombinations of rUGM + BPH-1 in mice supplemented with β-estradiol and testosterone (Figure 3.4, e),

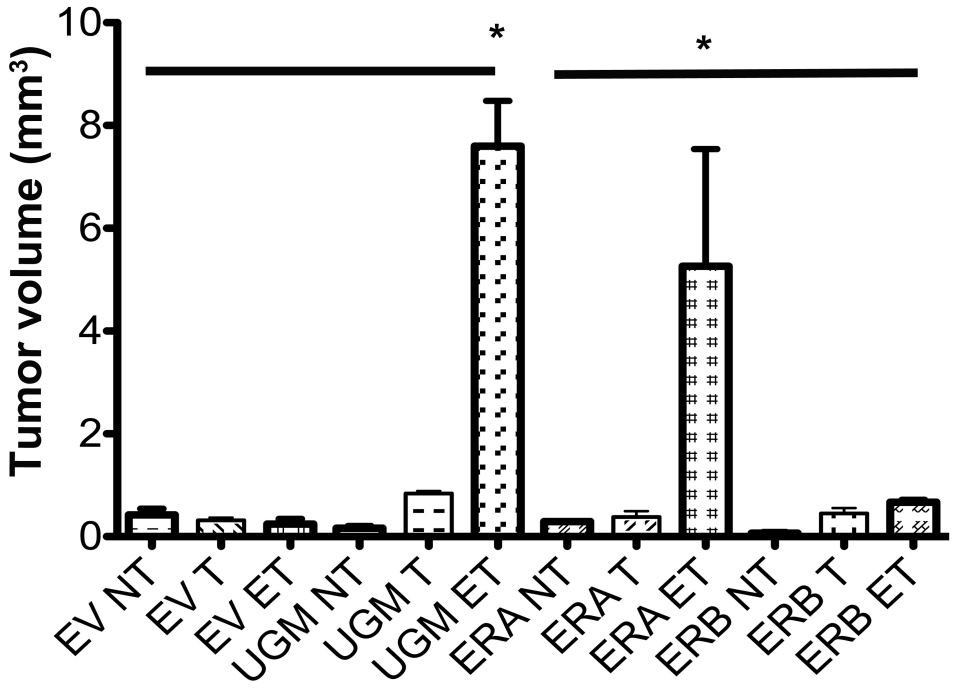

Figure 3.3. Overexpression of ERα in prostate stromal cells induces tumorigenesis *in vivo*. Comparison of tumor volume in recombination of BPH-1 cells with BHPrSEV (EV), BHPrSERα (ERA), BHPrSERβ (ERB), or rUGM. Castrated mice were supplemented with testosterone (T), estrogen and testosterone (ET), or no treatment (NT). Significance determined by ANOVA, p-value ≤0.05.

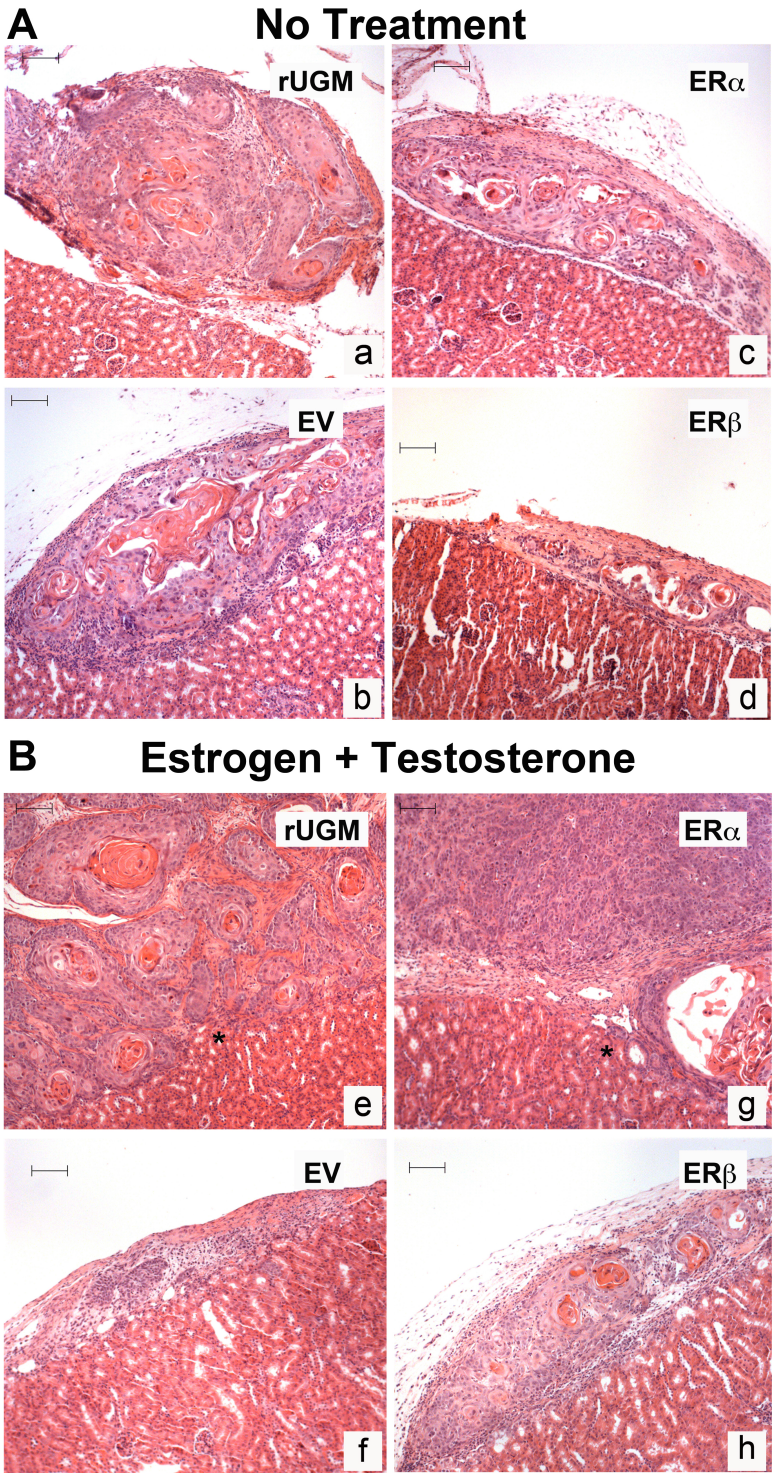

Figure 3.4 Histology of ERα overexpressing recombinations
Resulting recombinations of BPH-1 cells with rUGM (a,e). EV (b,f), ERα (c,g), ERβ (d,h). Castration mice not supplemented with hormones (**A**), and Estrogen and testosterone supplemented mice (**B**). Asterisk denoted areas exhibiting malignant conversation by invasion.

Inhibition of the estrogen receptor abrogates CAFs ability to induce malignant transformation.

Gene expression analysis of ERα and aromatase in primary fibroblasts isolated from benign/normal human prostate tissue (NPF) and malignant prostate tissue (CAFs) showed significantly greater expression in CAFs in comparison to NPFs with a greater than 5 fold difference (Figure 3.5 A, B). Due to our observation of increased ERα expression in CAFs we questioned the role of the ER in the cancer associated stroma's ability to promote transformation in tissue recombination experiments. To test this question, we recombined CAFs or NPFs with BPH-1 cells and grafted under the renal capsule of SCID mice supplemented with either testosterone pellets, or a combination of testosterone and tamoxifen pellets. Historically, recombinations of BPH-1 cells with CAFs in the presence of testosterone produces robust tumorigenesis and conversion to malignancy as previously described Olumi 99 (Olumi et al., 1999b). Recombinations of NPFs with BPH-1 cells in mice supplemented with testosterone produced small growths with benign-appearing structures (Figure 3.6, a). Recombinants of CAFs plus BPH-1 cells grafted in mice supplemented with testosterone resulted in large tumors exhibiting poorly differentiated adenosquamous carcinoma as previously reported (Figure 3.6, c). Mice supplemented with

tamoxifen in combination with testosterone inhibited the ability of the CAFs to induce a malignant transformation in the adjacent epithelium. Based on examination of the histology, when the estrogen receptor was inhibited with tamoxifen, the cancer associated stroma organized around the epithelial cells to produce benign structures resembling recombinations with the NAFs (Figure 3.6, g). IHC for SV-40 large T antigen allows for tracking of the transformed BPH-1 cells in our recombination experiments. IHC staining for SV-40 large T antigen confirms that the epithelial cells are not invading into the kidney of the mouse, supporting our findings of inhibition of malignant transformation when ER is inhibited in the cancer associated stroma (Figure 3.6, h).. A comparison of BPH-1 cell invasion into the kidney in recombinations with CAFs shows a significant decrease in mice treated with tamoxifen (Figure 3.5C).

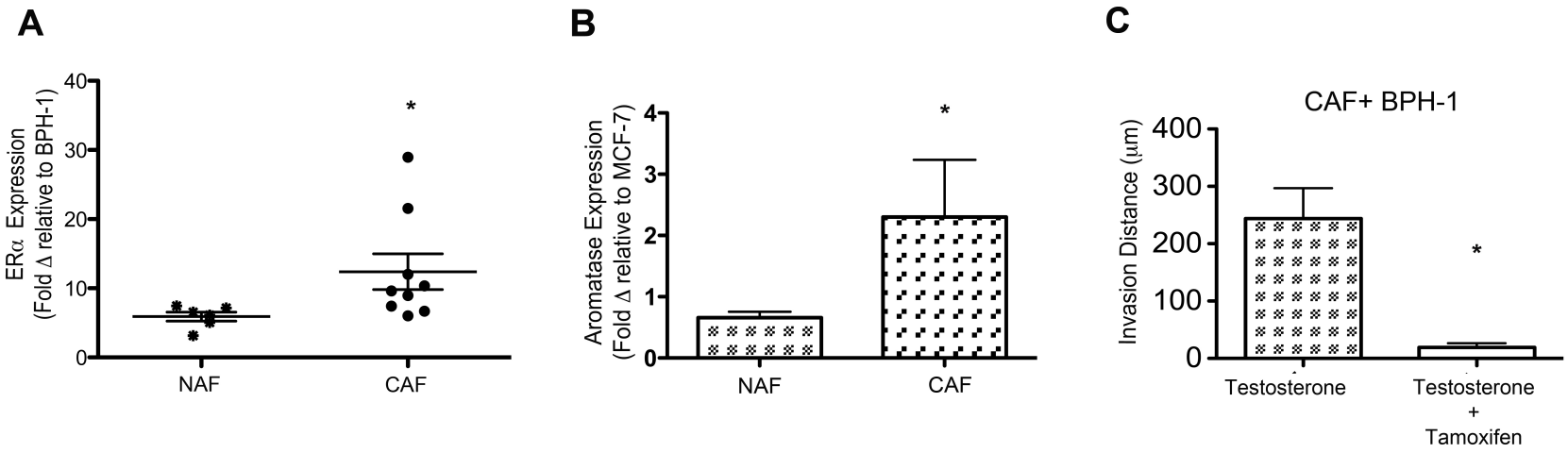

Figure 3.5 ERα and Aromatase expression is upregulated in patient derived CAFs. A) real-time expression analysis of ERα in patient isolated CAFs and NAFs. Expression levels are represented as fold change in comparison BPH-1 cells. **B)** Expression of Aromatase by real-time PCR in NAFs and CAFs. Results presented as fold change in comparison to MCF-7 cells. Significance for expression analysis for ERα and Aromatase determined by ANOVA, p-value ≤0.05. **C)** Measurement of invasion distance in recombinations of BPH-1 cells with CAFs in mice supplemented with testosterone or testosterone with Tamoxifen. Distance measure in micrometers. Statistical significance determined by t-test, p-value ≤0.05.

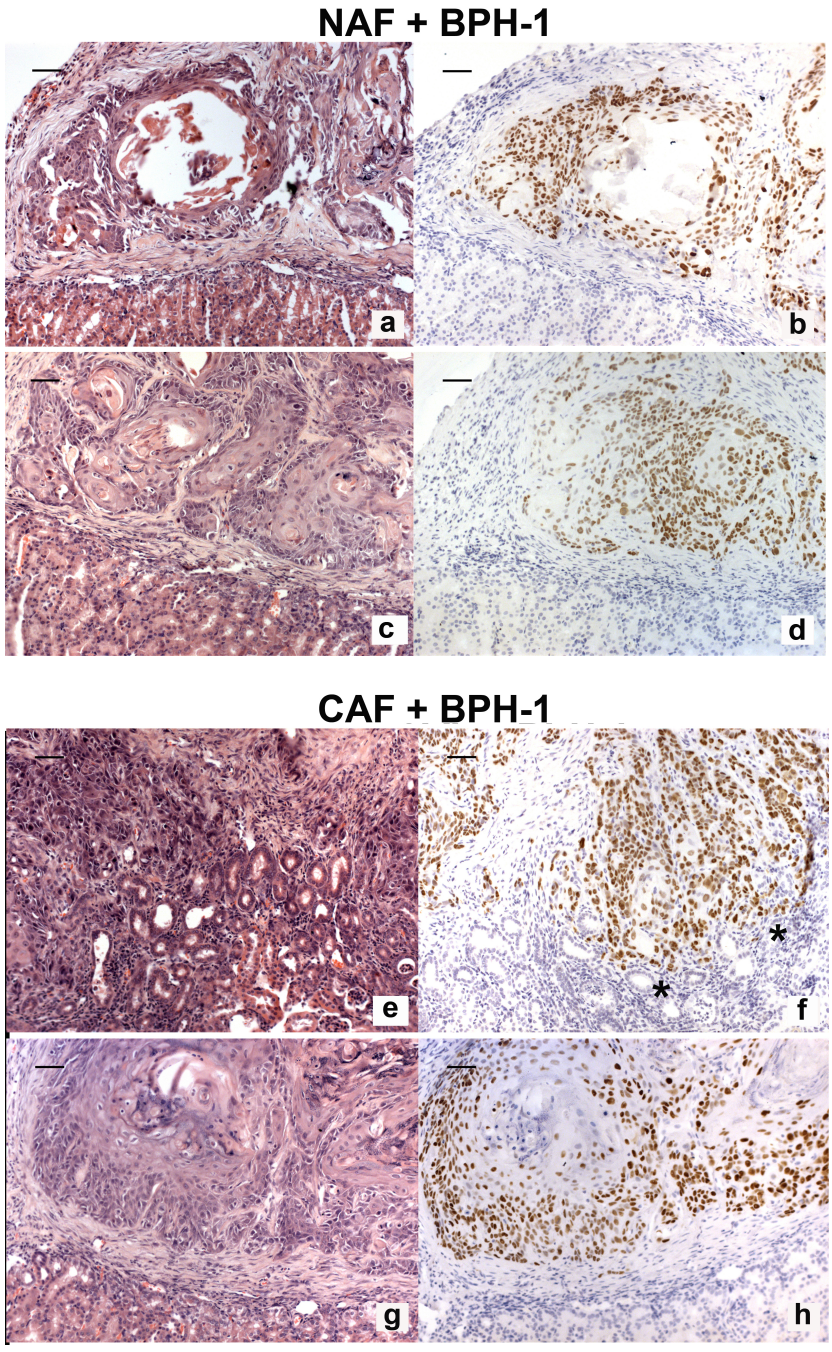

Figure 3.6. Tamoxifen abolishes CAF induced transformation of adjacent epithelium. Tissue recombinations of BPH-1 cells with NAFs (**top**) or CAFs (**bottom**). Mice supplemented with testosterone (a,b,e,f), or testosterone and Tamoxifen (c,d,g,h). SV-40 staining for BPH-1 cells (b,d,f,h). Invasion of BPH-1 into kidney denoted by asterisk (f).

3.4 Discussion

The actions of testicular androgens have been documented to play a role in male biology dating back to the late 16th century. Before the age of 10, prepubertal boys were castrated to preserve their soprano voices. These castrati, who retained their “soprano” voice range, were used to provide complexity to the music in the Roman church (Jenkins, 2000). For more than two centuries we have known that the prostate is a target for products of the testes. Observations made in 1786 of variations in testicular size correlated with the size of dog prostates led to a direct connection between testes and accessory sex organs (Hunter, 1837). As previously stated, androgens and the AR have been shown to have an integral role in mediating normal proliferation, differentiation, and maintenance of the prostate (Cunha et al., 1982). However, studies on AR in PCa show decreased expression in the cancer associated stroma. These studies concluded that AR expression in the cancer associated stroma is not required to support the growth of the malignant tumor *in vivo* (Vander Griend et al., 2010). Charles Huggins and Clarence Hodges were the first to show that estrogens have indirect effects on the prostate. In men, testosterone declines with age, while estrogen levels remain constant, and in some cases increase, during the time aging men develop detectable PCa

(Harman et al., 2001; Vermeule.A et al., 1969). Estrogens have been shown to be key factors in pathobiology of the prostate. Experiments performed by Noble, showed maximal development of adenocarcinoma in the Nb rat occurred after prolonged exposure to estrogens in combination with androgens (Noble, 1977a). These experiments highlighted the hormonal environment as being a critical mediator in prostate carcinogenesis. Similar results were observed when tissue recombination of rUGM and human prostate epithelial cells were grafted in hosts supplemented with estrogen and testosterone pellets (Wang et al., 2001). Results from the later experiments identified stromal steroid hormone receptors as being responsible for the development of carcinogenesis in response to hormones. Further investigations using ER knockout (ERKO) mouse models showed ERβKO mice develop prostatic hyperplasia and dysplasia in response to estrogen and testosterone supplements. However, ERαKO mice fail to develop any atypical phenotype in response to hormone supplementation (Ricke et al., 2008). These studies highlight the importance estrogenic signaling mediated by stromal ERα specifically, in driving prostatic carcinogenesis.

This and other studies, show an increase in the expression for ERα in the stroma of malignant human prostate tissue (Fixemer et al., 2002). We show here that the overexpression of ERα specifically in human stromal cells, provides a growth advantage in comparison

to stromal cells engineered to overexpress the ERβ subtype. This suggests non-redundant actions of the ER subtypes in cells. For example, on the AP-1 site of the promoter for collagenase, ERα induces transcriptional activation, and is repressed by ERβ, through direct binding with the transcription factor AP-1 (Weihua et al., 2003). The cell cycle regulator cyclin D1, (CD1), which is also linked to stromal promotion of carcinogenesis (He et al., 2007) is known to be regulated by estrogens (Eeckhoute, 2006). Estrogen induction of CD1 expression is dependent on the actions of ERα, and is inhibited when ERβ is ectopically expressed (Liu et al., 2002). In prostate stromal cells, the expression of ERα specifically is mitogenic, whereas the expression of ERβ did not change growth rate in comparison to the EV controls. The phenotype exhibited by these stromal cells has similar effects on the adjacent epithelium in tissue recombination experiments. The over expression of ERs in prostate stromal cells also affects the expression of filamentous proteins associated with myofibroblast cells.

One of the most abundant cell types in connective tissues are fibroblasts. Under normal conditions fibroblast's main function is to maintain tissue homeostasis (Donjacour and Cunha, 1991). The prostate stroma is mostly comprised of smooth muscle cells, which are responsible for the contraction necessary to inject prostatic fluid into the urethra during ejaculation (Keast, 1999; McNeal et al.,

1988a). After an injury, fibroblasts and smooth muscle cells respond by becoming activated and differentiate into a common intermediate myofibroblast (Gabbiani, 1996). A myofibroblast is characterized based on the co-expression of vimentin and αSMA (Schmitt-Graff et al., 1994). The switching of the stromal cell phenotype normally seen after an injury, is also observed in cancer. The term reactive stroma describes the generation of a new stromal microenvironment in response to a wound or cancer (Tuxhorn et al., 2001). The overexpression of the ERs in the BHPrS cell line resulted in the co-expression of vimentin and αSMA, whereas the EV control cells solely expressed vimentin. Expression of terminal differentiated markers of smooth muscle cells was examined. We believe the overexpression of the ERα in the BHPrS cell line produces a reactive stromal phenotype based on the co-expression of myofibroblast markers. In PCa, the reactive stroma phenotype has been shown to be an important prognostic indicator of cancer progression and of patient death. Patients with no desmin expressed in the stroma had a mean survival time of approximately 17 months, whereas patients with high stromal expression of desmin had a mean survival time of 73 months. Loss of desmin expression is reported to be the best predictor of progression to CR disease (Ayala et al., 2003).

Increased body fat has been cited as the main contributor for the peripheral conversion of androgen to estrogen by the actions of

the enzyme aromatase (Bosland et al., 1995). However, aromatase expression is not exclusive to fat cells. The prostate stroma itself has been shown to express aromatase (Risbridger et al., 2003). Fibroblasts isolated from malignant prostate tissue display greater relative expression of aromatase in comparison to fibroblast isolated from benign prostate tissue. It appears that the cancer-associated stroma contributes significantly to the promotion of an estrogen-rich microenvironment allowing for the increased availability of ligand for the cognitive receptor. To test this hypothesis we performed tissue recombinations of CAFs with BPH-1 cells and grafted into SCID mice supplemented with testosterone and tamoxifen pellets.

Tamoxifen was clinically introduced in the early 1970s to treat women with hormone-responsive breast cancer. Tamoxifen inhibits the ER activation predominantly by blocking the ability of estrogen to bind with the estrogen receptor (Cole et al., 1971). Tamoxifen has been shown to have anti-proliferative and apoptotic effects in PCa cell lines (El Etreby et al., 2000; Rohlff et al., 1998). We examined whether tamoxifen could inhibit the ability of CAF to induce a malignant conversion in tissue recombination experiments, based upon our observation of increased expression of ERα and aromatase in CAFs in comparison to NAF. Historical tissue recombination experiments of BPH-1 cells with CAFs grafted in mice supplemented with testosterone produces robust tumorigenesis and a malignant

conversion of a nontumorigenic prostate epithelial cell line (Hayward et al., 2001). Unpublished studies have shown that castrated mice grafted with recombinations of CAFs and BPH-1 cells fail to form tumors or exhibit a transformed phenotype. Inhibition of the ERα in the cancer associated stroma abrogates the ability of the CAFs to induce a malignant transformation of the nontumorigenic BPH-1 cells. We have previously published on several molecules found to be aberrantly expressed in the cancer-associated stroma, that induce conversion to malignancy when overexpressed NPF in tissue recombination experiments.

In vivo tissue recombination experiments using BPH-1 cells as a reporter epithelium, with the ERβ overexpressing stromal cells produced significantly smaller grafts in comparison to recombinations with the ERα overexpressing stromal cells when mice were supplemented with a combination of estrogen and testosterone. The tumors that resulted from recombinations with the ERα overexpressing stromal cells produced histologies similar to recombinations of BPH-1 cells with rUGM. It has been previously reported that recombinations of rUGM with BPH-1 in mice supplemented with estrogen and testosterone produces a malignant conversion, however, mice supplemented with testosterone alone produces benign growth (Wang et al., 2001). Our experiments show BPH-1 cells grafted with the ERα overexpressing stromal cells exhibit

a similar malignant conversion by invading into the kidney when grafted to the sub-renal site. Mouse models using wild type and ERβ knock-out mice treated on a regimen of estrogen and testosterone for a period of 4 months develop carcinogenesis of the prostate, whereas ERα knock-out mice remain free from pathologies (Ricke et al., 2008). Taken together, these findings make a strong argument for the actions of the ERα in the stroma as a driving force for the development of malignant disease and an ideal target for therapeutic intervention.

Our study highlights human ERα expression in the cancer associated stroma. The overexpression of the ERα in benign human prostate stromal cells promotes the expression of markers associated with the reactive stromal phenotype. We see that overexpression of ERα specifically in the stroma is sufficient to drive tumorigenesis and induce the malignant conversion of initiated, but non-transformed prostate epithelial cells. We also show CAFs express relatively higher levels of ERα and the enzyme aromatase in comparison to NAFs. We believe the increased expression of aromatase aids in producing a local estrogen rich environment promoting the development of prostate tumorigenesis. Lastly this study identifies stromal ERα as a target for therapeutic intervention by showing pharmacologic inhibition of the ERα in the cancer associated stroma inhibits malignant transformation in the adjacent epithelium.

Chapter 4

General discussion, conclusions, and future directions of this project

Sexual development of the male reproductive tract is strongly dependent on the actions of androgens. During development, androgens are required for the conversion of the Wölffian ducts into epididymis, vasa deferentia, and seminal vesicles (Siiteri and Wilson, 1974; Wilson, 1973). Androgens, specifically DHT, are also responsible for the formation of the prostate and external genitalia (Cunha, 1972a; Imperato-McGinley et al., 1985). The importance of androgens in male sexual development are best illustrated by deregulation or mutations in the enzymes involved androgenic production or in the AR which result in manifestation of a female phenotype (Quigley et al., 1995). The profound actions of androgenic signaling in male biology have captivated most of the attention, specifically in the treatment of PCa for the past 72 years (Denmeade and Isaacs, 2002). Since the 1940's however, we have known that the prostates in developing male fetuses are responsive to estrogens (Brody and Goldman, 1940).

Prostatic squamous metaplasia (PSQM) is the normal benign response to high levels of maternal estrogens which occurs around

week 22 of gestation (Yonemura et al., 1995). PSQM is characterized by the proliferation and multi-layering of basal cells along with the loss of secretory epithelial cells, and is completely reversible once the estrogen exposure is removed (Driscoll and Taylor, 1980; Huggins, 1940) (Huggins and Clark, 1940). Chemical castration with diethylstilbestrol (DES) in adult men induces regression of the prostate due to androgen withdrawal, followed by squamous metaplasia (Risbridger, 2001). PSQM is a reliable and reproducible pathological marker for response to estrogens in numerous animal species (Andersson and Tisell, 1982; Aumuller et al., 1982; Deschamps et al., 1987; Driscoll and Taylor, 1980). The metaplastic response of the prostate to estrogen is mediated by the actions of the ER. Epidemiological studies have highlighted the importance of estrogenic signaling in both normal development and the development of prostatic pathologies (Ho et al., 2006; Horvath et al., 2001; Leav et al., 2001; Srinivasan and Campbell, 1995). Animal models have shown high doses of estrogens can induce prostatic dysplasia, and when combined with androgens induces malignancy (Noble, 1977; Pylkkänen et al., 1996; Risbridger et al., 2001; Wang et al., 2001). The role of the ER in the pathogenesis of PCa warrants further investigation.

A general focus of this dissertation was the role of ERα in human prostate stromal promotion of prostate tumorigenesis,

focusing specifically on how ERα overexpression in stroma drives malignant conversion of adjacent epithelium. To address the focal points of this dissertation, genetically modified human stromal cell lines were employed along with tissue recombination techniques and sub-renal capsule grafting in immunodeficient mice. This dissertation provided advances in the field of PCa biology by 1) identifying expansion of ERα positive cells in PCa reactive stroma and in patient derived primary CAFs. 2) Showing pharmacological inhibition of the ERα abrogates epithelial malignant conversion by the cancer associated stroma. 3) Showing that ER responsive genes are expressed in the reactive stroma of malignant prostate tissue and promotes prostate tumorigenesis. These data further demonstrate stromal ERα role in prostate tumorigenesis and highlight ERα as a therapeutic target for chemoprevention.

1) Identifying expansion of ERα positive cells in PCa reactive stroma and in patient derived primary CAFs.

Historically, the field of cancer biology has primarily been focused on studying the malignant epithelial cells comprising tumors (Weinberg, 2008). The emergence of the tumor microenvironment as a field of active study is providing some much needed insight into how non-malignant cells associated with cancer (cancer associated stroma) can promote or suppress tumorigenesis (Li et al., 2007). The

stromal phenotype has been shown to be a powerful prognostic indicator of cancer progression and of patient death underlining its importance in defining lethal versus indolent phenotypes (Li et al., 2007) (Ayala et al., 2003; Li and Fan, 2007). The most common marker of reactive stroma is the appearance of activated stromal cells with myofibroblastic characteristics like the co-expression of vimentin and smooth muscle α-actin (αSMA) (Owens, 1995; Schmitt-Graff et al., 1994). CAFs cultured from malignant tissue isolated from radical prostatectomy co-express the vimentin and αSMA indicating the strong likelihood these fibroblast are myofibroblast derived from regions of reactive stroma. NAFs isolated from benign prostate tissue do not express vimentin, but do express αSMA, indicating these cells are not derived from reactive stroma. Expression analysis of ERα in our patient derived prostate fibroblast stocks, show an enrichment for ERα in the CAFs. This indicates a significant component of the PCa reactive stroma are ERα positive fibroblasts.

Another characteristic of the PCa reactive stroma is change in the extracellular matrix composition and remodeling (Martin et al., 1996; Noel and Foidart, 1998; Ronnov-Jessen et al., 1996). Specifically, there is increased deposition of collagens type I and III (Tuxhorn et al., 2001). Remodeling of the matrix proteins is controlled by the stromal expression of proteases belonging to the matrix metalloproteinases (MMPs) family, specifically MMP-2 and 9 . The

elevated expression of proteases strongly correlates with increased metastatic potential (MacDougall and Matrisian, 1995; Schmitt et al., 1992). These PCa-stromal derived proteases which are hypothesized to contribute to the progression of PCa, are known estrogen-regulated genes (Wingrove et al., 1998; Zhang et al., 2007).

Findings from immunohistochemical staining show an expansion of ERα expressing stromal cells in the malignant prostate tissue where stromal derived factors associated with PCa disease progression are known to be overexpressed. Taken together with the expression analysis of ERα in CAFs, these data support the hypothesis that ERα expressing prostate stromal cells are the myofibroblast cells which comprise a significant component of the PCa reactive stroma. This hypothesis is supported by animal models that show tamoxifen treatment decreases myofibroblast cells in wound healing experiments (Delle et al., 2012; Siqueira et al., 2013).

Staining of tumor associated normal prostate tissue also displays an expansion of ERα expressing stromal cells in comparison to normal prostate tissue. This data suggest a “field effect” or “field cancerization” of increased ERα expression in histologically normal prostate stroma adjacent to malignant lesions. The term “field effect” or “field cancerization” was originally introduced in oral squamous cell carcinoma in 1953 (Slaughter et al., 1953). Since it’s conception, field effect has been established in other malignancies including colon,

head and neck, and breast (Heaphy et al., 2006; Jothy et al., 1996; Tabor et al., 2004). In comparison to the previously mentioned cancers, field effect in PCa is relatively new (Halin et al., 2011; Nonn et al., 2009).

Reports ranging between 30-50% of men are initially diagnosed with false-negatives because needle biopsies miss small malignant lesions (Levy and Jones, 2011; Patel and Jones, 2009; Rabbani et al., 1998). Field effect in PCa offers potential towards improving the false-negative diagnosis. In the event a needle biopsy misses areas of malignancy, the field effect of increased stromal expression of ERα in histologically normal tissue would indicate the presence of PCa.

2) Showing pharmacological inhibition of the ER abrogates epithelial malignant conversion by the cancer associated stroma.

Historical experiments showing PCa associated stroma promotes the malignant conversion of benign epithelium established that the stroma plays an active role in the carcinoma evolution. These experiments also showed stroma surrounding the nascent tumor undergoes phenotypic alterations that can enhance the invasive potential of the epithelial tumor (Grossfeld, 1998; Olumi et al., 1999b). These experiments were performed in male mice that

required additional supplementation with testosterone to drive this malignant conversion. Studies have shown the stromal AR is essential for the PCa progression, and malignant conversion (Ricke et al., 2012).

Evidence supporting the role of AR in the development of PCa was the basis for clinical trials using 5α-reductase inhibitors as a chemopreventative agent. The first Prostate Cancer Prevention Trial (PCPT) using finastestride started in 1993 and ended a year early in 2003 because a significant reduction (25%) in the incidence of PCa development was observed, however, men who did develop PCa while taking finasteride experienced elevated incidences (60%) of high-grade tumors (Pitts, 2004). The second PCa prevention trial Reduction by Dutasteride of Prostate Cancer Events (REDUCE) was initiated in 2003 and used Dutasteride, a different 5α-reductase inhibitor, which inhibits isoform types I and II of 5α-reductase. Unlike, finasteride which only inhibits 5α-reductase type I (Andriole et al., 2010). Similar to the findings concluded in the PCPT, REDUCE trial showed no favorable risk benefit as a chemopreventative agent for PCa in healthy men (Theoret et al., 2011). The use of 5α-reductase inhibitors for the prevention of PCa development in men, may have failed because the inhibitors were leading to the increased production of estradiol.

In men, testosterone is a more significant precursor for

estradiol then estrone which is synthesized from androstenedione. 20% of estradiol is produced by the Leydig cells, whereas 80% of estradiol is formed by the peripheral aromatization of testosterone directly (Baird et al., 1969; MACDONALD and MADDEN, 1979; MacDonald et al., 1979). Estrogen in combinations with androgen have been shown to act synergistically in the promotion of prostatic hyperplasia in canine models, and carcinoma in rodent models (Noble, 1977; Trachtenberg et al., 1980). Unpublished studies have shown the ability of CAFs to induce malignant conversion in recombination studies requires additional androgens to be supplemented. Result presented in this dissertation show increased expression for aromatase and ERα in patient isolated CAFs in comparison to NAFs. Suggesting CAFs have increased potential for the conversion of testosterone to estrogen, along with increased expression for estrogen responsive genes (i.e. CathD). When the ER was inhibited with tamoxifen in tissue recombination experiments of CAFs with BPH-1 cells, the resulting grafts failed to demonstrate tumorigenesis and malignant conversion even when additional testosterone was supplemented. These findings demonstrate the stromal promotion of prostate tumorigenesis by CAFs is more dependent on the activity of the ER, rather than the AR. There are genetic polymorphisms which result in greater aromatase activity. One in specific is the $TTTA_{7-13}$ short tandem repeat in intron 4 of

CYP19 (Feigelson et al., 1998). CYP19 is the gene which encodes for aromatase. The presence of $TTTA_{7-13}$ repeat has been shown to be an independent risk factor for death and a strong predictor of survival in men with bone metastatic PCa (Cussenot et al., 2007; Tsuchiya et al., 2006).

The AR and its role in the development of PCa is cemented in place, and rightfully so when pertaining to androgen-induced PCa, however, points raised in this document and published findings present the ER and its role in the development of PCa should be considered with equal focus in PCa chemoprevention. Men who have been identified as being at greater risk for the development of PCa, should be stratified by mutations related to the AR/androgen metabolism versus ER/estrogen metabolism. Men who have $TTTA_{7-13}$ polymorphism in the CYP19 gene, or mutations that lead to increased ERα expression may respond more favorably to ER specific antagonist and/or aromatase inhibitors. Whereas men who have AR mutations may respond better to 5α-reductase inhibitor therapy for PCa prevention.

In breast cancer, aromatase inhibitors are actively being evaluated as chemopreventative agents for women who are considered to be at high risk. Published results from a 5 year clinical study with the aromatase inhibitor exemestane showed a 65% reduction in breast cancer incidence (Goss, 2003). This significant

reduction in cancer incidence may have been greater, if 33% of the exemestane group been in compliance and taken the drug as directed (Litton et al., 2012). Some of the women in the exemestane group experienced hot flashes as a side effect and decided to stop taking the drug.

3) ER responsive genes are expressed in the reactive stroma of malignant prostate tissue and promotes prostate tumorigenesis.

As previously stated, numerous factors have been identified to be overexpressed in the cancer associated stroma that promote tumorigenesis and malignant conversion when overexpressed in benign/normal stromal fibroblasts. Findings in this study demonstrate how stromal CD1 overexpression leads to the dysregulation of the lysosomal protease CathD. This study further goes on to highlight stromal derived CathD as another critical factor in the promotion of prostate tumorigenesis *in vivo*. This study also identifies CathD as a potential target for therapeutic intervention.

CathD which is intracellularly confined under normal physiologic conditions, is targetable in malignant conditions because it becomes hypersecreted. Pepstatin A is a potent competitive inhibitor of most aspartic proteases, and went through clinical trials as a treatment for duodenal ulcers (Bonnevie et al., 1979; Svendsen

et al., 1979). These trials showed Pepstatin A did not improve symptoms associated with the ulcers. In vitro experiments have shown Pepstatin A to be cytotoxic to cancer cells (Castino et al., 2002). When combined with other chemotherapeutic drugs, Pepstatin A was shown to reduce metastasis in mouse tumor studies (Tumminello et al., 1993) (Leto et al., 1994). Clinical and experimental observations identify CathD as an attractive therapeutic target for cancer treatment, however, numerous pro-tumorigenic factors have been and will continue to be identified as investigators continue to unravel the mystery of PCa development and disease progression. The emerging problem that presents itself here, is the fact that there are so many factors that are proving to promote malignancy. Which raises the question of, which factor should be the focus for targeting therapeutically?

The majority of anti-cancer therapies induce apoptosis by exploiting the relatively high rate of proliferation malignant cells display in relation to normal cells. Ionizing radiation, anti-mitotic drugs (i.e. Docetaxel), and DNA intercalators (i.e. Cisplatin), are designed to arrest DNA replication. Other therapeutics target cancer cells dependence on growth hormones (i.e. CAB, or tamoxifen). All of these targeted therapeutics come a with a laundry list of systemic side effects and adverse dose limiting toxicities like neuropathy, and immunosuppression, which reduce the therapeutic efficacy. Due to

the dose limiting toxicities associated with various anti-cancer therapies, tumors eventually acquire resistance to therapeutics. Instead of designing new classes of compounds that inhibit specific pathways or growth factors, what if therapies were targeted to be delivered as a pro-drug, that can only be activated at tumor specific sites?

"Molecular grenades" are a novel approach to drug development and delivery that "detonate" restrictively within the extracellular microenvironment at cancer sites (Denmeade and Isaacs, 2002). Part of this novel drug delivery system involves delivering a pro-toxin, that can be cleaved by a defined protease that displays restricted expression to the cancer site (Brennen et al., 2012). Results presented in this dissertation identify the protease CathD, who's expression is restricted to intracellular organelles in normal prostate stroma, but becomes hypersecreted in PCa associated stroma. The results presented here also correlate stromal CathD expression with high grade PCa in comparison to benign prostate tissue. *In vitro* experiments show CathD is secreted as an inactive prozyme, that can be converted extracellularly to an active protease by malignant epithelial cells. Pro-CathD has been shown to be converted extracellularly in the mouse prostate and cleave specific substrates (Piwnica et al., 2006). Results presented here suggest stromal derived CathD liberates TGF-β sequestered in the extracellular

matrix for presentation to epithelial cells leading to tumorigenesis. Published studies have identified TGF-β bound within the inhibitory complex as a substrate for CathD (Lyons et al., 1988). Stromal CathD may be a potential target for “molecular grenades” to deliver their pro-toxin payloads to cancer sites for “detonation” or proteolytic activation of the pro-toxin.

The tumor microenvironment has shown to be an active player in the development and progression of PCa, and has shown to be a powerful prognostic indicator in the identification of aggressive from indolent disease (Grossfeld, 1998; Li and Fan, 2007; Olumi et al., 1999a; Olumi et al., 1999b). This unique environment holds the key to preventing PCa specific mortality, and to a larger extent cancer specific mortality by lending itself to being targeted for therapeutic intervention.

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